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DISSERTATION

High-resolution respirometry for measurement of mitochondrial function in PAD patients before and after revascularisation – a pilot study

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For my grandfather,
who wasn't able to finish this adventure with me.

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LIST OF ABBREVIATIONS

°C	Degree Celsius
31P-MRS	^{31} Phosphorus magnetic resonance spectroscopy
ABI	Ankle brachial systolic pressure index
ACE	Angiotensin converting enzyme
ADP	Adenosine diphosphate
AmA	Antimycin A
ATP	Adenosine triphosphate
BIOPS	Biopsy preservation solution
BMI	Body mass index
BNC	Binuclear reduction centre
CI	Complex I
CII	Complex II
CIII	Complex III
CIV	Complex IV
Ca	Calcium
CFA	Common femoral artery
CIA	Common iliac artery
Cl_2	Chloride
CoA	Coenzyme A
CSA	Citrate synthase activity
CTA	Computed tomography angiography
Cu	Copper
Cu_A	Copper A centre
Cu_B	Copper B centre
Cyt C	Cytochrome C
e^-	Electron
EGTA	Ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid
EIA	External iliac artery
ETC	Electron transfer chain
ETS	Electron transfer state
FAD	Flavin adenine dinucleotide (oxidised form)

$FADH_2$	Flavin adenine dinucleotide (reduced form)
FCCP	Carbonyl cyanide p-(triflouromethoxy) phenylhydrazone
Fe/S	Iron-sulfur cluster
FMN	Flavin mononucleotide (oxidised form)
$FMNH_2$	Flavin mononucleotide (reduced form)
g	Gram
H^+	Proton
H_2	Hydrogen
H_2O	Water
HDL	High-density lipoprotein
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
His	Histidine
HRR	High-resolution respirometry
IC	Intermittent claudication
IMM	Inner mitochondrial membrane
IMS	Intermembrane space
K^+	Potassium
l	Litre
LDL	Low-density lipoprotein
M	Malate
Mg	Magnesium
MIRO6	Mitochondrial respiration medium
mL	Millilitre
µL	Microlitre
mm	Millimetre
mM	Millimolar
µM	Micromolar
MRA	Magnetic resonance angiography
NAD^+	Nicotinamide adenine dinucleotide (oxidise form)
NADH	Nicotinamide adenine dinucleotide (reduced form)
NIRS	Near infrared-spectroscopy
O_2	Oxygen
Oct	Octanoylcarnitine

OMM	Outer mitochondrial membrane
OXPHOS	Oxidative phosphorylation
P	Pyruvate
PAD	Peripheral arterial disease
pAVK	Peripheral arterial disease
PCr	Phosphocreatine
PFA	Profound femoral artery
P_i	Inorganic phosphate
PO_4	Phosphate
PTA	Percutaneous transluminal angioplasty
Q	Ubiquinone
QH_2	Ubiquinole
Q_i	Binding site of ubiquinone at the inner part of the inner mitochondrial membrane
Q_o	Binding site of ubiquinone at the outer part of the inner mitochondrial membrane
QoL	Quality of life
ROS	Reactive oxygen species
Rot	Rotenone
S	Succinate
SFA	Superficial femoral artery
SUIT	Substrate uncoupler inhibitor titration
U	Units

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ABSTRACT

Introduction: Peripheral arterial disease (PAD) is characterized by hemodynamically relevant atherosclerotic lesions with subsequent low blood supply to affected muscle regions. Patients who are suffering from symptomatic PAD report a pain induced by walking in affected muscles with relief of symptoms after resting. This repeating ischemia-reperfusion situation leads to histomorphological and metabolic changes in affected muscle regions. Mitochondria are known to play a crucial role in this pathophysiological mechanism. The aim of this study is the evaluation of mitochondrial content and function in affected muscles of symptomatic PAD patients and the potential of mitochondrial regeneration six weeks after successful revascularisation.

Methods: Ten patients suffering from PAD grade IIB – IV with isolated pathologies of the superficial femoral artery with planned revascularisation have been recruited into the study group. Ten participants known to be vascularly healthy have been included into the control group. Muscle biopsies of the medial belly of the gastrocnemius muscle have been obtained just before and six weeks after successful revascularisation in the study group, whereas within the control group biopsies have been taken at one instance. High-resolution respirometry has been performed to evaluate mitochondrial respiration and citrate synthase activity (CSA) has been measured to determine mitochondrial content. By correlation of values from high-resolution respirometry to values from CSA, mitochondrial function has been investigated.

Results: Demographic data of the study group and the control group showed no statistically significant difference. CSA measurements in the study group showed a statistically significant increase of mitochondrial content after successful revascularisation coming close to those of healthy control subjects. Complex I- and complex I+II-related respiration normalized to CSA showed a statistically significant reduction of mitochondrial function six weeks after successful revascularisation compared to preoperative values. These results show increasing correspondence with the results of healthy control subjects.

Conclusion: This study showed that mitochondria in PAD patients are reduced in number but they compensate with increased function ability. After successful revascularisation, mitochondrial content and function approach that of healthy control subjects. Demonstrating a regeneration of initially impaired mitochondria. High-

resolution respirometry in combination with measurement of CSA seems therefore useful for determining mitochondrial impairments and may be used for investigating pathophysiology of PAD related myopathy.

ZUSAMMENFASSUNG

Einleitung: Bei der peripheren arteriellen Verschlusskrankheit (pAVK) kommt es in Folge von hämodynamisch relevanten atherosklerotisch bedingten Stenosen oder Verschlüssen zu einer Minderversorgung von betroffenen Muskelabschnitten mit Sauerstoff. Als Symptomatik berichten Patienten über belastungsabhängige Schmerzen in den betroffenen Muskeln, welche sich nach einer Ruhepause bessern. Diese sich immer wiederholende Ischämie-Reperfusions-Situation führt zu histomorphologischen und metabolischen Veränderungen des betroffenen Muskels und Mitochondrien spielen bei diesem Mechanismus eine entscheidende Rolle. Ziel der Studie ist es, den Mitochondriengehalt sowie die Mitochondrienfunktion in betroffenen Muskelabschnitten von pAVK Patienten sowie die Regenerationsfähigkeit nach erfolgreicher Revaskularisation von Mitochondrien zu untersuchen.

Methodik: Es wurden 10 Patienten im Stadium IIB – IV einer pAVK, mit isolierten Pathologien der Arteria femoralis superficialis sowie geplanter Revaskularisation in die Studiengruppe und 10 gesunde Probanden in die Kontrollgruppe eingeschlossen. Muskelbiospien wurden aus dem Musculus gastrocnemius entnommen, in der Studiengruppe unmittelbar vor sowie 6 Wochen nach erfolgreicher Revaskularisation, in der Kontrollgruppe erfolgte die Probenentnahme einmalig. Zur Beurteilung des Mitochondriengehaltes erfolgte die Messung der Citrat-Synthase Aktivität (CSA), die mitochondriale Respiration wurde anhand von hochauflösender Respirometrie bestimmt. Durch Korrelation der Werte der Respiration auf die Werte der CSA Messung erfolgte die Bestimmung der Mitochondrienfunktion.

Ergebnisse: Die demographischen Daten der Patienten der Studiengruppe und der Probanden der Kontrollgruppe zeigten keine statistisch signifikanten Unterschiede. Bei der Bestimmung der CSA zeigte sich nach erfolgreicher Revaskularisation ein signifikanter Anstieg der Werte wobei dies einer Adaptierung an die Werte der Kontrollgruppe entsprach. Bei der Komplex I und Komplex I+II abhängigen Respiration normalisiert auf die CSA zeigte sich vor Revaskularisation eine signifikant erhöhte Mitochondrienfunktion mit postoperativer Angleichung der Werte an die Kontrollgruppe.

Schlussfolgerung: Es konnte gezeigt werden, dass es in betroffenen Muskelabschnitten von pAVK Patienten zu einer Reduktion der Mitochondrienzahl und einer kompensatorischen Zunahme der Mitochondrienfunktion kommt. Nach erfolgreicher

Revaskularisation zeigte sich mit der Zunahme der Mitochondrienzahl und der Abnahme der Mitochondrienfunktion eine Angleichung an die untersuchten gesunden Probanden und somit konnte eine Regerationsfähigkeit der Mitochondrien nachgewiesen werden. Die hochauflösende Respirometrie in Kombination mit der Bestimmung der CSA bieten somit eine gute Möglichkeit zur Untersuchung von mitochondrialen Veränderungen bei pAVK Patienten und ermöglichen ein besseres Verständnis der pathophysiologischen Veränderungen der mit dieser Erkrankung verbundenen Myopathie.

1. INTRODUCTION

1.1. Peripheral arterial disease (PAD)

PAD is defined as a narrowing of peripheral arteries caused by atherosclerotic changes of the arterial wall resulting in stenosis and occlusions of affected arteries. PAD most commonly occurs in lower extremities but may also be present in upper extremities. As a result of flow-limiting alterations of affected arteries, reduced blood supply leads to ischemia in subsequent regions. Extensive epidemiological studies showed that the incidence of asymptomatic PAD is much higher than estimated and associated with an increased risk of ischemic cardiac disease and therefore also associated with an increased mortality[1].

The prevalence of asymptomatic PAD can easily be estimated by non-invasive measurement of the ankle brachial systolic pressure index (ABI). The ABI describes the ratio between arterial systolic pressure of the lower extremity and arterial systolic pressure of the upper extremity and is >1.0 in healthy adults. A resting ABI of ≤ 0.9 is caused by hemodynamically significant arterial stenosis and is most often used as a definition of PAD[2]. Another possibility of non-invasive detection of asymptomatic PAD is ultrasound-guided examination to identify atherosclerotic lesions in arteries of the lower extremities.

To evaluate the prevalence of symptomatic PAD, pre-defined questionnaires may be used to detect the presence of intermittent claudication (IC), a walking-induced pain caused by reduced blood supply in affected muscle, mainly located in the lower legs which is relieved by a short rest after which walking can be continued.

1.1.1. Epidemiology

There have been several studies investigating the incidence and prevalence of asymptomatic and symptomatic PAD in defined populations and most of them gave special consideration to the correlation of PAD with a potentially increased risk of ischemic cardiac disease.

The group of Criqui published a study in 1985 that showed a prevalence of large-vessel PAD in 11.7% of the investigated population, whereas IC was present only in 2.2% of men and 1.7% of women. The tools they used were a questionnaire concerning IC, pulse palpation and non-invasive testing such as the measurement of segmental blood

pressure. They concluded that the prevalence of asymptomatic PAD is quite underestimated[3].

In 2004, data were published including 2,174 participants out of the National Health and Nutrition Examination Survey from 1999 to 2000 to assess the prevalence of PAD and the prevalence of cardiovascular risk among patients with PAD in the United States of America. They used the ABI as diagnostic tool and defined PAD as an ABI ≤0.9 in either leg. A prevalence of PAD of 4.3% was found among individuals aged ≥40 years with a dramatic increase of prevalence of up to 14.5% among individuals aged ≥70 years. Smoking status, pre-existent diabetes, impaired renal function and self-reported history of cardiovascular disease were identified as risk factors[4].

The Edinburgh Artery Study used ABI measurement to detect asymptomatic PAD. With an additional duplex ultrasound examination they found that a third of asymptomatic PAD patients had a complete occlusion of a major artery of the leg. They also suggested ABI as a general predictor for atherosclerosis as they described an increased risk of ischemic heart disease in patients with asymptomatic PAD compared with the normal population. These data were confirmed by a study published in 1992 with a study population of 624 men and women that have been investigated by non-invasive testing and standardized questionnaires to identify asymptomatic and symptomatic PAD. An association of large-vessel PAD with elevated mortality from all causes was described but most of the deaths were caused by cardiovascular disease, especially coronary heart disease[5,6].

Newman et al published similar findings as they also investigated ABI as a marker for atherosclerosis using data from the Cardiovascular Health Study. They described a strong relation between a decrease of ABI and subclinical cardiovascular disease as measured by carotid ultrasound, echocardiogram and echocardiography. Patients with an ABI of ≥0.8 to <0.9 were more than twice likely to have local wall motion abnormalities compared with participants with a normal ABI, whereas an ABI <0.8 was more than four times as likely associated with pathological findings in echocardiography[7].

A large multinational registry, the Reduction of Atherothrombosis for Continued Health Registry, is providing data about the spectrum of disease progression, cardiovascular outcomes and options of treatment. Patients aged 45 years or older with established coronary artery disease, cerebrovascular disease, PAD or with at least 3 cardiovascular

risk factors have been enrolled and a total of 67,888 patients have met the inclusion criteria for this registry. Three-year outcome of this registry was published in 2009 and revealed a rate of cardiovascular mortality, myocardial infarction or stroke of 14.8% in patients with PAD. Regarding vascular mortality, patients with PAD had the highest risk compared with the groups of coronary artery disease and cerebrovascular disease[8,9].

1.1.2. Pathogenesis of Atherosclerosis

During the last century, the initial hypothesis of the pathogenesis of atherosclerosis as storage of cholesterol and thrombotic debris has been replaced by the notion that a chronic inflammatory process plays the major role in the development of atherosclerotic plaques[10].

Atherosclerotic changes start with endothelial dysfunction induced by several risk factors such as elevated and modified low density lipoprotein (LDL), free radicals caused by e.g. cigarette smoking, hypertension, diabetes mellitus, genetic disorders and elevated plasma homocysteine concentrations. Followed by a compensatory response, including an increase in adhesiveness for leukocytes or platelets as well as an increase in its permeability changes in affected arteries continue. In combination with an induction of procoagulant properties and the formation of vasoactive molecules, cytokine and growth factor migration as well as proliferation of smooth-muscle cells occurs. All these processes lead to an increased thickness of the arterial wall that may be compensated by local dilatation of the whole artery, resulting in a non-occlusive formation of atherosclerotic changes. The continued inflammatory process results in an increased number of macrophages and lymphocytes that both migrate from the blood itself and then multiply within the atherosclerotic lesion. Consequent activation of these cells induces the release of hydrolytic enzymes, cytokines, chemokines and growth factors that cause further damage and local necrosis. In the end, the formation of fibrous tissue and the enlargement of the initial lesions lead to the formation of a fibrous cap that overlies a core of lipid and necrotic tissue. At this stage of atherosclerosis, the artery is not able to compensate for the transformation and the lesion might penetrate into the arterial lumen and interfere with blood flow[11].

1.1.3. Risk factors

Risk factors play an important role in the pathophysiology of atherosclerosis and the reduction of preventable risk factors is crucial in conservative treatment strategies of cardiovascular diseases.

The risk factor correlated mainly with the onset of PAD is cigarette smoking, as smokers have a 1.7- to 5.6-fold increase in the development of PAD compared with non-smokers. The severity of PAD tends to increase with the number of cigarettes smoked so that heavy smokers have a 4-fold increased risk developing IC compared with non-smokers. Smoking cessation is associated with lower amputation rates and longer survival but followed by poor rates of long-term cessation[2,12,13].

Patients suffering from diabetes mellitus also have an increased risk, as the group of Kannel showed a 3.5-fold increase of IC in men with glycosuria compared to patients without diabetes. These data have been collected by the Farmingham Study, and another group showed within the same population that in both sexes, the presence of both diabetes and symptomatic PAD results in an increased risk of cardiovascular events. As the male sex is known to have a higher risk of atherosclerosis, they concluded that the presence of diabetes mellitus in women eliminates the protection against atherosclerosis compared to males[13-15].

Other risk factors associated with a significant increase of PAD are hypertension, dyslipidaemia and obesity. Whereas in patients with hypertension, odds ratios ranged from 1.50 to 2.20, big index studies also have shown an association of dyslipidaemia and PAD. Elevated levels of total cholesterol, LDL, triglycerides and lipoprotein A cause atherosclerosis, whereas elevated levels of high-density lipoprotein (HDL) have a protective potential to the endothelium[1,7,16,17].

1.1.4. Classification of PAD and clinical symptoms

The most commonly used classifications of PAD were published by Fontaine in 1954 (Table 1-1) and Rutherford in 1997 (Table 1-2)[18,19]. Whereas the Fontaine classification is used mainly in the clinical setting, the classification of Rutherford is more often used in scientific context. Taking a closer look at the classification of Fontaine, there is a differentiation of grade II regarding the distance a patient is able to walk without the need to rest due to IC. This differentiation is mandatory for the relative indication for endovascular or surgical treatment in patients with PAD, as the symptoms reduce the quality of life (QoL) of the patient. Patients with PAD grade III or IV have an absolute indication for endovascular or surgical revascularisation, as these stages have the risk of progressive tissue damage due to low blood supply and therefore are associated with an increased rate of amputations and consequently a higher rate of mortality.

Classification of Fontaine	
Grade	
I	Asymptomatic PAD with incomplete vessel obstruction
II	Intermittent claudication
IIa	Intermittent claudication with a walking distance > 200 meters
IIB	Intermittent claudication with a walking distance < 200 meters
III	Rest pain
IV	Trophic lesions

Table 1-1 – Classification of Fontaine[18]

Classification of Rutherford		
Grade	Category	
0	0	Asymptomatic
I	1	Mild claudication
I	2	Moderate claudication
I	3	Severe claudication
II	4	Rest pain
III	5	Minor tissue loss
IV	6	Major tissue loss

Table 1-2 – Classification of Rutherford[19]

1.1.5. Conservative treatment options for PAD

As described above, patients with PAD have a significantly increased risk of suffering severe cardiovascular events with an overall increased mortality compared to healthy patients. As the risk factors cigarette smoking, diabetes mellitus, hypertension, dyslipidaemia and obesity are known to have effects on both PAD, as coronary artery disease, it is imperative to reduce these risk factors by life-style modification as well as by medical treatment. The modification of atherosclerotic risk factors is described in detail in the Trans-Atlantic Inter-Society Consensus for the Management of Peripheral Arterial Diseases, published by Norgren in 2007[2].

Patients suffering from IC due to PAD should be advised to do an exercise program for the improvement of their walking distance as it is known that muscle function, vascular endothelium function and metabolic adaptations may be improved through exercise. As an increase of the performed walking distance elevates QoL of the patients, most studies defined this parameter as one of their endpoints. In 1995, Gardner et al. published a meta-analysis of exercise rehabilitation programs to evaluate the most effective components to improve IC. They showed that the optimal exercise program to improve the walking distance uses intermittent walking to near-maximal pain during a program with duration of at least six months. An improvement of walking distance of 122% was shown in this meta-analysis, so that the importance of a structured exercise program has been proven and is therefore recommended in the TASC II guidelines[20,21].

Smoking cigarettes is the main risk factor of atherosclerosis as described above. Therefore, it is imperative to achieve smoking cessation in patients with diagnosed PAD. As it is known that spontaneous smoking cessation rates are very low (less than 10%), there are several nicotine replacement therapies. The amphetamine bupropion with its antidepressant effect is also used in achieving smoking cessation. It has been shown that the combination of these two approaches has better results than either of them alone[22]. Another approach is the substance varenicline, a partial agonist at the $\alpha_4\beta_2$ acetylcholine nicotine receptor that showed a 3-fold higher cessation rate compared to a placebo group and it has also proved superiority compared to participants using bupropion has been shown[23,24]. In some patients, smoking cessation is associated with an improvement of walking distances but in others it is not. Therefore, patients should be advised to stop smoking to reduce their risk of cardiovascular events and amputation rates but they should not be promised to have an improved walking performance right after smoking cessation[2].

Regarding the latest published guidelines for the management of high blood pressure in adults, a systolic arterial blood pressure greater than 130 mmHg is considered as arterial hypertension and the treatment of patients with PAD and arterial hypertension should be identical to that of patients with arterial hypertension alone. It is known that besides the lowering of blood pressure, antihypertensive medication reduces the risk of cerebrovascular and cardiovascular events as well as the risk of death. There is no evidence that any class of antihypertensive medication is superior to another but

thiazide diuretics, angiotensin converting enzyme (ACE) inhibitors, angiotensin II receptor blockers and calcium channel blockers are recommended as primary agents[25]. With the enrolment of more than 20,000 patients at high-risk of cardiovascular events, the Heart Protection Study showed in 2002 that an aggressive lowering of LDL is associated with a significant reduction in cardiovascular events, including myocardial infarction, stroke and vascular death. Therefore an application of statins in this collective of patients is recommended[26]. A lowering of the LDL level to <2.59 mmol/dL or < 100 mg/dL is recommended for all symptomatic PAD patients in the TASC II guidelines and statins should be the primary therapeutic agent. Besides pharmacological interventions, patients who are overweight or obese should be advised to lose weight by inducing a negative caloric balance by reducing calorie and carbohydrate intake and increasing exercise[2].

As PAD indicates an elevated risk of coronary and cerebrovascular disorders and as it is known that antiplatelet agents reduce cardiovascular events and mortality in patients with IC, the Peripheral Arterial Diseases Antiplatelet Consensus Group recommended in 2003 to use antiplatelet drugs in all patients with IC or who have had previous vascular intervention. Agents should be either aspirin 75 – 325 mg daily or clopidogrel 75 mg per day[27].

1.1.6. Endovascular and operative treatment options for PAD

In patients with IC due to PAD in which exercise training did not lead to an improvement of the maximal walking distance, endovascular or open surgical revascularisation is recommended in recently published European guidelines[28]. This indication for invasive treatment is relative at this stage of disease, whereas in patients with a PAD stage III or IV of the Fontaine classification, treatment is absolutely indicated because without revascularisation the risk of tissue loss with consequent damage is increased. The decision whether endovascular or open surgical treatment is preferable depends on the type, location and exact morphology of the atherosclerotic stenosis or occlusion. In 2007, the TASC II guidelines were published with the recommendation for endovascular or open approach depending on location and extension of atherosclerotic lesions, by classification into Type A to D lesions. At this time, in both aortoiliac and femoropopliteal Type A lesions were recommended to be treated endovascular, Type D lesions were recommended to be treated by open surgery. In Type B lesions, endovascular treatment was the preferred treatment option, whereas in Type C lesions, open operative

treatment was the preferred treatment option. For these two types of lesions, the patients' co-morbidities, preference given by the fully informed patient and the operator's long-term success rates should be taken into account when choosing between open and endovascular treatment[2]. An update of the TASC II guidelines was published in 2015, the original version of recommendation for the choice of revascularisation strategy has not been revised but it indicated that it must be based on the respective vascular centers' competence and experience. Also the patient's comorbidities and overall prognosis as well as the anatomical complexity should be taken into the account. It also stated that in particular for aortoiliac pathologies in a medically fit patient, open surgery may be favoured as the durability seems to be superior to endovascular approaches[29].

The most current guidelines have been published recently by the European Society of Vascular Surgery. These guidelines have put more emphasis on the patient's condition in regard to the decision between endovascular and open surgery compared to the TASC guidelines. It is recommended that in aortoiliac occlusive lesions, short lesions should be treated by endovascular methods. If the patient is fit for surgery, aorto- (bi-) femoral bypass surgery should also be considered, as the long-term patency is higher compared to endovascular treatment. If the patient suffers from severe comorbidities, endovascular methods should be given preference. If there is a combined iliacofemoral occlusive lesion, hybrid procedures should be considered with stenting of the iliac pathology and endarterectomy or bypass of the femoral lesion. These guidelines recommend that in the case, an endovascular strategy for treating aortoiliac lesions is considered as first-line strategy, only an experienced team should perform these procedures and the procedure should not compromise subsequent surgical options. In femoropopliteal occlusive lesions, short lesions should be treated by an endovascular first strategy, whereas long lesions of the superficial femoral artery (SFA) (≥ 15 cm) should be treated by bypass surgery if an autologous vein is available and the life expectancy of the patient is more than two years. Only if there is no autologous vein available, prosthetic material should be considered in above-the-knee femoropopliteal pathologies. If the patient is unfit for surgery, endovascular methods should be considered for long lesions of the SFA as well. At the below-the-knee segment, invasive therapy is only indicated for limb salvage[28].

Endovascular treatment options include percutaneous transluminal angioplasties (PTA) using balloons that are introduced by wires and catheters. By using contrast-enhanced imaging, the balloon can be placed exactly at the site of stenosis or occlusion to achieve optimal results. If necessary, stenting could be performed in addition to the PTA to maintain longer patency rates in massive atherosclerotic lesions.

Open surgical treatment includes local endarterectomy with removal of short atherosclerotic plaques and reconstruction with patch plasties. For longer stenosis or occlusions, bypass surgery is an option in which the affected artery is bypassed with prosthetic or autologous venous grafts.

1.2. Mitochondria – Power plants of cells

Mitochondria are organelles and exist in almost all eukaryotic cells. They are home to the enzymes of the respiratory chain and are essential to the process of oxidative phosphorylation, which generates most of the animal cell's high-energy metabolite adenosine triphosphate (ATP) and are therefore called the power plants of cells.

Two membranes surround mitochondria. The outer membrane serves as protection to the surrounding structures and the inner membrane, which is highly enlarged by its infolding formation called cristae, houses the enzymes of the respiratory chain. These two membranes divide the mitochondria into different compartments: the intermembrane space, which is located between the outer and the inner membrane, and the matrix, which defines the space within the inner membrane.

For the production of the high-energy metabolite ATP, mitochondria are involved in several catabolic pathways for energy generation, utilizing the energy stored in carbohydrates, fats and proteins. Glucose, for example, is converted into pyruvate and acetyl coenzyme A (acetyl-CoA) in glycolysis and fats are converted into acetyl-CoA during β-oxidation. Afterwards, acetyl-CoA is introduced into the citric acid cycle, which is located in the mitochondrial matrix, while amino acids are directly introduced into this cycle after deamination. Regarding the citric acid cycle, the reducing equivalents nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$) are produced that serve as reductive equivalents in the electron transport chain (ETC). At the end of this pathway, ATP is produced via oxidative phosphorylation (OXPHOS), which is described in detail below[30]. As they are involved in such important pathways, mitochondria play a crucial role in the pathogenesis of several diseases, also including PAD.

1.2.1. Oxidative Phosphorylation (OXPHOS)

Most of the energy of our cells is generated within the mitochondria by OXPHOS, which describes a process in which electrons are passed throughout a series of carrier molecules called the respiratory chain or the ETC. These molecules are all located in a specific order at the inner mitochondrial membrane and electrons needed to run this mechanism are generated from the reductive equivalents NADH and $FADH_2$ that serve as “electron shuttles”. As electrons pass the ETC, energy is released and stored as an electrochemical gradient across the inner membrane of the mitochondria by translocating protons from the matrix to the intermembrane space. This gradient drives the ATP-synthase and the high-energy metabolite ATP is generated from ADP and inorganic phosphate (P_i) at the end of this pathway[31,32] (Figure 1-1).

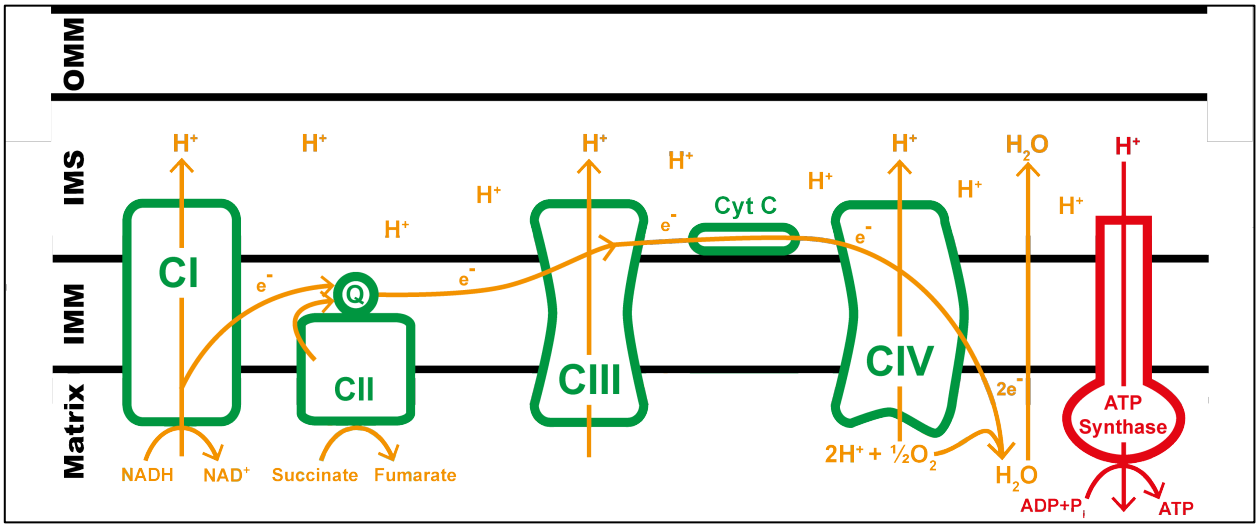

Figure 1-1 - OXPHOS (ADP – adenosine diphosphate, ATP – adenosine triphosphate, CI - Complex I, CII - Complex II, CIII – Complex III, CIV – Complex IV, Cyt C – cytochrome C, e^- - electron, FAD/$FADH_2$ – flavin adenine dinucleotide, H^+ - proton, H_2O – water, IMM – inner mitochondrial membrane, IMS – intermembrane space, O_2 – oxygen, NADH/NAD^+ - nicotinamide adenine dinucleotide, OMM – outer mitochondrial membrane, P_i – inorganic phosphate, Q – ubiquinole);

1.2.1.1. Complex I – NADH:Ubiquinone Oxidoreductase

Complex I or NADH:Ubiquinone Oxidoreductase is the largest complex of the ETC. With a larger hydrophilic part located inside the inner membrane and a smaller hydrophobic part that extends into the mitochondrial matrix, it is L-shaped and receives electrons

from NADH to transfer them to ubiquinone. The primary electron acceptor of complex I is the cofactor flavine mononucleotide (FMN). By receiving two electrons from NADH it is transformed into $FMNH_2$. $FNMH_2$ then releases the electrons to eight different iron-sulfur clusters in which electrons are transferred one by one and in the end, ubiquinone is reduced by one electron to semiquinone and furthermore by a second electron to the fully reduced form ubiquinole. This process might be interrupted by the inhibitor rotenone (Rot), as the transfer of the electrons from the iron-sulfur clusters to ubiquinone is inhibited. Ubiquinone and its fully reduced form ubiquinole are soluble lipophilic molecules situated inside the inner membrane of the mitochondria with the important function of collecting electrons in the ETC and transferring them to complex III. In addition of this reduction mechanism, energy is released to drive several proton pumpers located in the hydrophobic part of complex I. With the transfer of protons into the intermembrane space, an electrochemical gradient required to activate the ATP-Synthase is induced, whereas the exact mechanism is still unknown[33-35] (Figure 1-2).

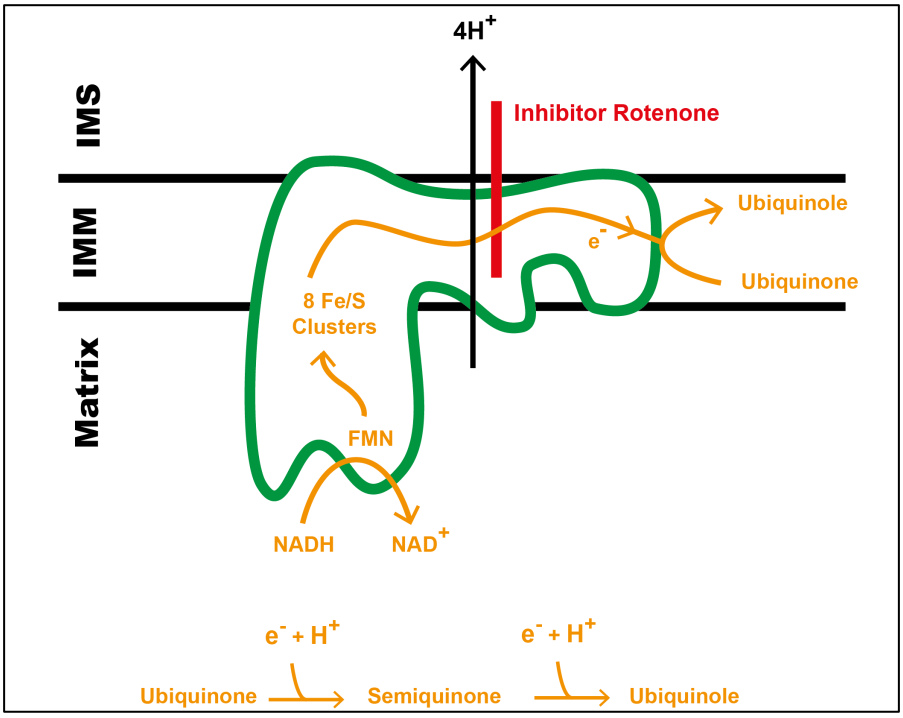

Figure 1-2 - Complex I (e^- - electron, Fe/S – iron-sulfur cluster, FMN – flavin mononucleotide, H^+ - proton, IMM – inner mitochondrial membrane, IMS – intermembrane space, NADH/NAD^+ - nicotinamide adenine dinucleotide);

1.2.1.2. Complex II – Succinate Dehydrogenase or Succinate:Ubiquinone Reductase

Complex II or Succinate Dehydrogenase is much smaller than complex I and is also part of the citric acid cycle. It consists of a hydrophilic part that extends to the mitochondrial matrix, which contains the succinate-binding site, and a membrane-bound part, which is connected to the ubiquinole molecule. Complex II contains flavin adenine dinucleotide (FAD) as covalent bound prosthetic group, an iron-sulfur protein containing three iron-sulfur clusters and a heme prosthetic group. The enzyme catalyses the oxidation of succinate to fumarate in the citric acid cycle by reducing FAD to $FADH_2$, but it is known that this reaction is only possible if FAD is covalently bound to the enzyme. Afterwards, $FADH_2$ transfers both of its electrons to three iron-sulfur clusters and in the end the electrons are used to reduce ubiquinone to ubiquinole, which is located at the membrane-bound part of the enzyme. As the electrons are delivered from the iron-sulfur cluster to ubiquinone, the heme group serves as a tunnel to secure the electron on its way to ubiquinone. With this mechanism, the production of reactive oxygen species (ROS) out of the reaction of intermediates with molecular oxygen is prevented. Complex II might be inhibited by malonate, and this complex does not translocate protons into the intermembrane space; its function is to store electrons in the ubiquione-pool that is then used to transfer the electrons to complex III[32,36,37] (Image 1-3).

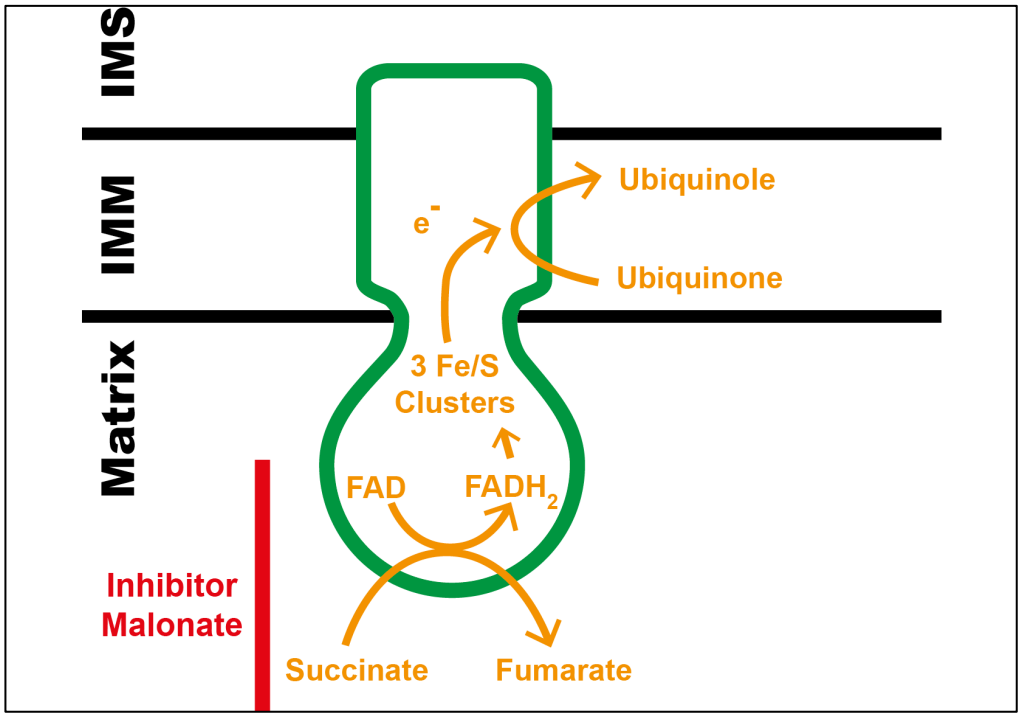

Figure 1-3 - Complex II (e^- - electron, Fe/S – iron sulfur cluster, FAD/$FADH_2$ – flavin adenine dinucleotide, IMM – inner mitochondrial membrane, IMS – intermembrane space);

1.2.1.3. Complex III – Cytochrome bc_1 Complex or Cytochrome C Reductase

Complex III or Cytochrome bc_1 Complex consist of a total of 11 subunits, of which three contain the relevant redox centres to maintain energy conversion. These three relevant subunits include cytochrome b that is located within the membrane, a membrane-coupled iron-sulfur protein containing the Rieske iron-sulfur cluster and the membrane-anchored cytochrome c1. Cytochrome b contains two heme groups as prosthetic groups and cytochrome c1 contains one heme group as prosthetic groups. Also known as coenzyme Q, complex III delivers electrons from ubiquinol to the soluble cytochrome c and this reaction is coupled by the generation of a proton gradient across the inner mitochondrial membrane by the Q-cycle described by Mitchell in 1976[38]. The main issue in the complexity of the Q-cycle is the understanding of two different binding sites for ubiquinone. One is situated at the outer site of the inner mitochondrial membrane (Qo) and can be inhibited by myxothiazol, the other one is located at the inner site of the inner mitochondrial membrane (Q_i) and can be inhibited by antimycin A (AmA). These two inhibitors have been important in the understanding of the structure of complex III, as they made a detailed investigation of this complex possible so that complex III became the best understood complex of the respiratory chain. The Q-cycle describes the transport of protons from the mitochondrial matrix to the intermembrane space to provide a proton gradient that is necessary for the production of ATP at the end of OXPHOS. As described above, ubiquinone has been reduced by the transfer of two electrons by complexes I and II and in addition two protons are collected from the mitochondrial matrix to form the fully reduced ubiquinol. These two protons are then transferred into the intermembrane space and the electrons are transferred from ubiquinole to complex III at the Q_o-binding site. One of the electrons of ubiquinole is then transferred to the Rieske iron-sulfur centre to reach the moveable cytochrome c after a stopover at the membrane bound cytochrome c1. The other electron passes cytochrome b with its two heme-groups and then reaches the Q_i-binding site. There, a further ubiquinone is reduced to ubiquinole by collecting another two protons from the the mitochondrial matrix and translocating them into the intermembrane space. Summarizing, the Q-cycle optimizes the translocation of protons from the mitochondrial matrix to the intermembrane space by duplicating the amount of translocated protons. In

the end of all pathways taking place in complex III, all electrons are transmitted to cytochrome c which is a small moveable molecule localized at the outer part of the inner mitochondrial membrane that transfers the electrons to complex IV[32,39-41] (Figure 1-4).

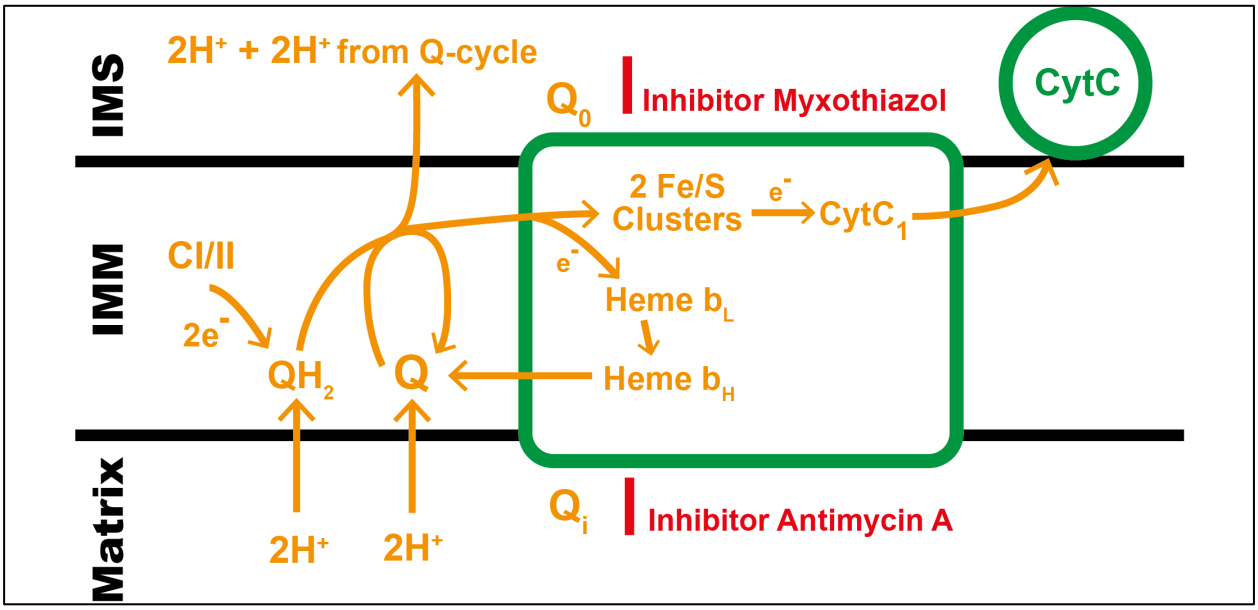

Figure 1-4 - Complex III (CI/II - Complex I and II, Cyt C – cytochrome C, e^- - electron, Fe/S – iron sulfur cluster, H^+ - proton, IMM – inner mitochondrial membrane, IMS – intermembrane space, Q – ubiquinone, QH_2 – ubiquinole, Q_i – binding site of ubiquinone at the inner part of IMM, Q_o – binding site of ubiquinone at the outer part of IMM);

1.2.1.4. Complex IV – Cytochrome C Oxidase

Complex IV or Cytochrome C Oxidase is the last complex of the ETC and its function is to transfer electrons that are used for the reduction of oxygen to water. It consists of 14 subunits of which subunits I and II are the main subunits as they contain the relevant copper centres and heme groups. Subunit I contains the two heme groups, $heme_a$ and $heme_{a3,}$ as well as one of the copper centres Cu_B and subunit II contains the second copper centre Cu_A. The reduction of oxygen to water takes place in between the $heme_{a3}$ and the Cu_B molecules so that these two molecules are called the binuclear oxygen reduction centre (BNC). The moveable cytochrome c reaches complex IV, also called the cytochrome c oxidase, at the outer side of the inner mitochondrial membrane and transfers its electron to Cu_A which is located at the membrane surface in subunit II. Then the electron is transferred to subunit I, namely to $heme_a$ followed by the move over to $heme_{a3}$. Most of the molecular oxygen which has been taken up by respiration, is

located in the BNC of complex IV and as the electron is transferred to $heme_{a3}$ and consequently to Cu_B, four protons are collected from the matrix and used for the reduction of oxygen to water. In addition, another four protons are transferred from the matrix to the intermembrane space. These four protons are than used to maintain an electrochemical gradient to run the ATP-synthase to produce ATP. The exact mechanisms of the transmission of the protons are still unknown, several proton channels and histidine ligands coupled to Cu_B seem to play an important role in this mechanism as well as conformational changes due to the transfer of electrons throughout complex IV[32,42-44] (Figure 1-5).

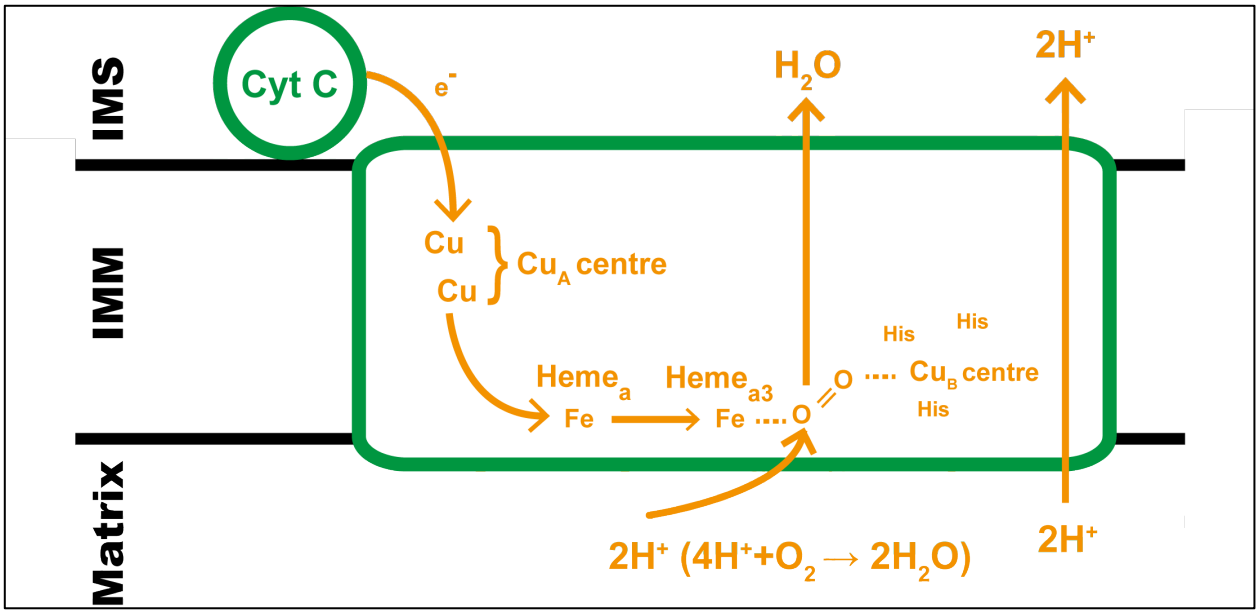

Figure 1-5 - Complex IV (Cu – copper, Cu_A – copper A center, Cu_B – copper B center, Cyt C – cytochrome C, e^- - electron, Fe – iron, H^+ - proton, H_2O – water, His – histidine, IMM – inner mitochondrial membrane, IMS – intermembrane space, O/O_2 – oxygen);

1.2.1.5. ATP-Synthase – F_oF_1 ATPase

The high-energy metabolite ATP is synthetized in three different reactions in different pathways including glycolysis, the citric acid cycle and the respiratory chain, whereas the respiratory chain produces the major portion of ATP. The enzyme complex needed for ATP production is called ATP-Synthase and is located at the inner mitochondrial membrane. The enzyme is a multisubunit protein (at least 16 subunits) that is classified into a membrane-bound F_o-subunit, which is involved in the translocation of protons, and a F_1-subunit that catalyses the reaction of ADP and inorganic phosphate to ATP. The force that drives the ATP-synthase is given by an electrochemical gradient, which is

composed by two components: firstly, a voltage gradient as protons have been translocated from the matrix to the intermembrane space by the complexes of the ETC as described above; secondly, a chemical gradient as the pH value is higher in the matrix compared to the intermembrane space.

The catalytic F_1-subunit consists of six subunits that are located at the matrix side of the inner mitochondrial membrane. By the alternation of α- and β-subunits like segments of an orange, two of each of these subunits form one catalytic centre to maintain the conversion of ADP and inorganic phosphate into ATP. The F_1-subunit is connected to the F_o-subunit by a shaft that is located at the centre between the α- and β-subunits and this shaft contains the γ-subunit. With an additional stator, the b-subunit, the F_1-subunit is stabilized in its position. The F_o-subunit, also called the rotor, consists of eight c-subunits that are arranged in a ring-shaped form and in the middle of this ring the γ-subunit provides the connection to the F_1-subunit. Next to the c-subunits lies the a-subunit and between these two structures protons move from the intermembrane space to the matrix and the electrochemical energy is converted into mechanical energy as the γ-subunit rotates and leads to a conformational change of proteins at the F_1-subunit, precisely at the β-subunit. These conformational changes include a binding of ADP and inorganic phosphate as the binding sides are opened and after closure of the α- and β-subunits, ATP is synthetized and with a reopening of the subunits ATP is released into the mitochondrial matrix so that in total, three different conformational statuses are necessary for the synthesis of ATP[32,45,46] (Image 1-6).

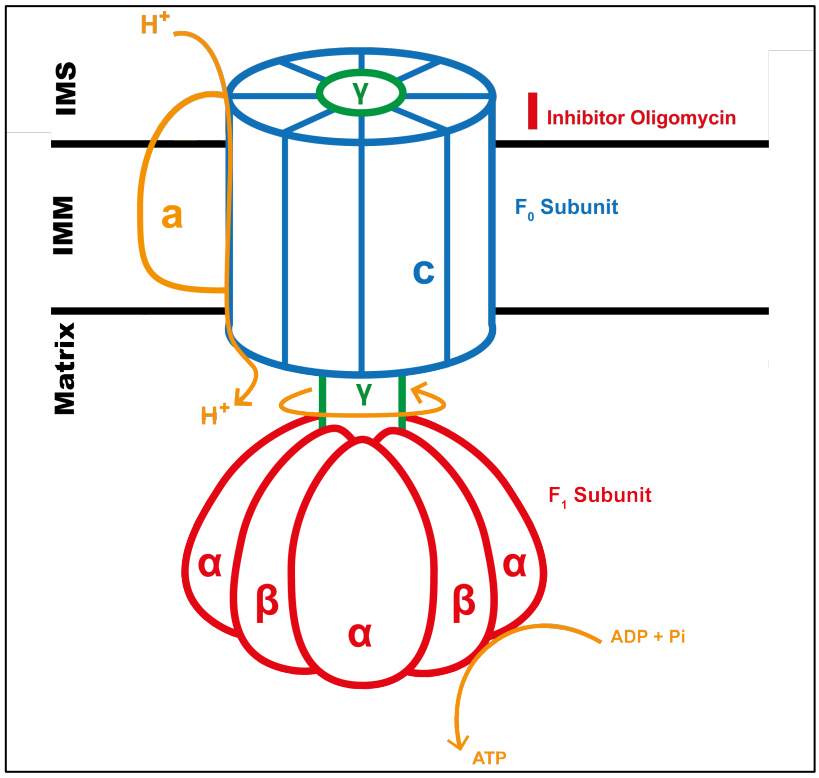

Figure 1-6 - ATP-Synthase (ADP – adenosine diphosphate, ATP – adenosine triphosphate, H^+ - proton, IMM – inner mitochondrial membrane, IMS – intermembrane space, P_i – inorganic phosphate);

1.3. Alterations of affected muscles in PAD patients

As a result of repeated exercise-induced ischemia reperfusion injury in PAD patients, a chronic myopathy is described as one of the leading pathophysiological changes in muscles of PAD patients. These changes include histomorphological as well as metabolic changes and mitochondrial alterations play a crucial role in the pathophysiology of PAD[47].

1.3.1. Histomorphological alterations

Using light and electron microscopy, several morphological studies have revealed quantitative as well as qualitative alterations in affected muscle as a significant part of PAD[47,48]. Evaluating biopsies of the gastrocnemius muscle of 14 male individuals suffering from PAD, the group of Hedberg described morphological abnormalities in all sections such as fibre necrosis, regeneration, angular fibres and abundant connective tissue. These findings confirmed the data published by Sjöström comparing patients with IC to a healthy control group by evaluating biopsies of the anterior tibial muscle.

Compared to the healthy biopsies, which showed well-defined fascicles with a small amount of connective tissue between the fascicles, mostly peripheral nuclei, a contractile material with well-defined myofibrils and structurally normal mitochondria, biopsies of patients suffering from PAD showed numerous morphological changes. These changes included single fibre necrosis, angular fibres, fibre type grouping and an increase of surrounding connective tissue. A centralisation of nuclei, phagocytosis of necrotic muscle fibres and the presence of inflammatory cells have been detected as well[49,50].

At the subcellular level, observed with an electron microscope, abnormalities of the Z-band, bizarrely shaped mitochondria, numerous macrophages and irregularly organized myofibrils as well as a badly organized sarcoplasmic reticulum have been described[49,50]. In special regard of morphological mitochondrial changes, both qualitative and quantitative changes have been shown, including hypertrophy as well as hyperplasia, hypercristae and lipid vacuolization of the organelles[51]. Regensteiner et al. showed in 1992 that milder forms of PAD were not associated with generalized morphological changes in skeletal muscles. They investigated ambulatory patients, suffering from unilateral IC and compared these findings to the asymptomatic leg as well as to a healthy control group. Besides the morphological evaluation, they also investigated enzyme activities (lactate dehydrogenase, citrate synthase and phosphofructokinase) and concluded that these enzymes showed no differences between symptomatic legs, asymptomatic legs or healthy control groups[52].

In summary, there are two important findings in morphological studies: firstly there is a myopathy present in affected legs of PAD patients, correlating with the severity of the occlusive disease, and secondly, mitochondria are organelles that are mainly affected[47].

1.3.2. Metabolic alterations

Metabolic alterations, mainly affecting mitochondrial pathways, have been described by several groups as another pathophysiological mechanisms of PAD[53].

The group of Dahllof described in 1970 an impairment of mitochondrial oxidation of carbohydrates in a small group of PAD patients. After a two-day period of glucose supplementation, glycogen levels were evaluated in percutaneously taken muscle biopsies, and an increase of muscle glycogen concentrations as well as an improvement of walking performance was shown[54]. These findings were underlined by a study published in 2004, in which PAD patients and healthy controls had been given

carbohydrate supplementation for three days. An improvement of the walking distance correlated with an increase of the active fraction of the pyruvate dehydrogenase, which plays a leading role in the oxidation of carbohydrates, was described in this study[55].

Further signs of incomplete oxidation of substrates as a marker for mitochondrial dysregulation is the accumulation of metabolic intermediates[53]. Various metabolic pathways use coenzyme A (CoA) as cofactor and in the case of incomplete oxidation of substrates, CoA-coupled intermediates might accumulate. These acyl-CoAs are linked to the cellular carnitine pool as the enzyme carnitineacyltransferase transfers the CoA to carnitine, forming acylcarnitine. If, due to mitochondrial dysfunction, oxidation of substrates and acyl-CoA is incomplete, remaining acyl-CoAs are “buffered” by the carnitine pool and concentrations of acylcarnitine are elevated[47,56,57]. PAD is associated with an accumulation of acylcarnitine in plasma as well in skeletal muscle, and the concentrations of acylcarnitine show an inverse correlation with the walking performance of PAD patients[53,58,59]. The group of Hiatt also showed that a six-month treatment period with a carnitine derivate (propionyl-L-carnitine) leads to an improvement of walking distance in patients with IC[60].

As a result of ischemia reperfusion injury, oxidative stress leads to tissue damage in PAD. Non-diabetic patients with PAD compared to a healthy control group showed significantly increased levels of malondialdehyde, a metabolite from lipid peroxidation of fatty acids and a marker for oxidative stress[61]. As neutrophils are known to cause tissue damage by microvascular damage through the release of oxygen-derived free radicals as well, Neumann and co-workers investigated neutrophils in arterial and venous blood in patients with PAD at rest, immediately after exercise and after a resting period of ten minutes. They showed an increase in the total count of neutrophils as well as the count of activated neutrophils with reduced deformability and concluded that these alterations may contribute to the pathogenesis of ischemic tissue damage[62].

1.3.3. Alterations of mitochondrial content and respiration

Impairments of respiratory pathways are known to be another relevant mechanism in the pathophysiology of PAD as it has been described by several groups using in-vivo and in-vitro methods for the evaluation of mitochondrial respiration[47,48]. The advantages of in-vivo methods are the possibility of the determination of oxidative capacity under physiologic conditions and the non-invasiveness but these methods are restricted, as it is unclear whether the impairment of mitochondrial respirations is caused by limited

blood flow, reduced mitochondrial volume, an alteration of mitochondrial function, or a combination of these factors. In contrast, in-vitro methods are invasive, as muscle samples are needed for further investigation. Using respirometry for the evaluation of mitochondrial function, specific complexes of the ETC may be investigated under normoxic conditions with the disadvantages that for this method, tissue has to be fresh. Compared to spectrophotometric measurement of mitochondrial volume density by the determination of protein levels or mitochondrial enzyme activities, tissue samples may be stored frozen[63].

1.3.3.1. ^{31}P Magnetic Resonance Spectroscopy (MRS)

Using the non-invasive in-vivo method of ^{31}phosphorus magnetic resonance spectroscopy (31P-MRS), real-time testing of mitochondrial function is possible before, during and after exercise. Already in 1986, Zatina et al. showed that by the measurement of the concentrations of ADP, ATP, phosphocreatine (PCr) and the intracellular pH value, a correlation to mitochondrial respiration is possible[64]. The phosphorylated creatine derivate PCr serves as rapidly available provider of high-energy phosphate in skeletal muscle and brain. With its phosphate, the conversion from ADP to ATP is provided by the mitochondrial ATP synthase during exercise. To keep levels of ATP high, PCr releases its phosphate to ADP so that at the end of exercise, PCr concentrations are low and ADP concentrations are high. During rest, PCr and ADP recover to normal levels and this process is assisted by ATP which is produced by mitochondrial OXPHOS. As OXPHOS exclusively occurs in mitochondria, the recovery rates of PCr and ADP are an indicator for mitochondrial respiration and may be non-invasively measured by 31P-MRS[47,48,64,65]. Using 31P-MRS for the evaluation of mitochondrial dysfunction, the group of Pipinos compared patients with mild or moderate claudication to healthy control subjects. During exercise, the concentrations of PCr decreased, while the concentrations of ADP and inorganic phosphate increased. The levels of ATP remained stable and there were no significant differences of these individual phosphorous metabolites between claudicants and healthy controls. Also, the intracellular pH stayed stable and there were also no differences between the groups. Investigating the recovery rates of PCr and ADP, this study showed prolonged recovery rates in PAD patients compared with healthy volunteers, so that this group suggested an intrinsic mitochondrial defect resulting in impaired OXPHOS in patients with mild or moderate claudication[66]. Another group investigated patients with unilateral PAD during

isometric exercise and showed that in the symptomatic leg of PAD patients, there are prolonged PCr time constants and an increased amount of PCr hydrolysis during exercise and during recovery compared to the asymptomatic leg as well as to healthy volunteers, and a mitochondrial defect has been suggested in this study as well[67]. Another non-invasive method for the evaluation of mitochondrial respiration is the measurement of muscle oxygenation by near infrared-spectroscopy (NIRS) and it has been shown that this method correlates with the results of 31P-MRS as well as with the in-vitro method of high-resolution respirometry (HRR)[68,69].

1.3.3.2. Measurement of citrate synthase activity (CSA) for evaluation of mitochondrial content

Located at the mitochondrial matrix, the enzyme citrate synthase serves as marker enzyme for mitochondrial content. It has been proven that beside cardiolipin, a component of the inner mitochondrial membrane, citrate synthase is highly associated with mitochondrial fractional area and therefore useful for the determination of mitochondrial content[70]. Several groups have been investigating different mitochondrial enzymes to determine mitochondrial content in PAD patients on the one hand and adaptive changes of muscle tissue after exercise training or revascularisation on the other. Bylund and co-workers already reported in 1976 about an increase of several mitochondrial enzymes in tissue of the gastrocnemius muscle of PAD patients. Citrate synthase was described to be significantly increased as well as the enzymes 3-OH-acyl-CoA-dehydrogenase and cytochrome c oxidase. The increase declined with the severity of the disease but remained present[71]. A Swedish group has published similar findings as they investigated patients with unilateral PAD and by taking muscle biopsies from both the symptomatic and the asymptomatic legs. They reported increased activity of citrate synthase and creatine kinase in muscle biopsies of symptomatic legs compared to the healthy leg and concluded that there is a metabolic adaption in affected muscle tissue in response to restricted blood flow[72]. By randomisation of PAD patients into different treatment groups (surgical revascularisation alone, exercise training alone, surgical revascularisation combined with exercise training) and evaluation of mitochondrial content before and 6 – 12 months after the performed treatment, Lundgren et al. noted a decrease of CSA after surgical revascularisation alone, but there were no changes in mitochondrial content after exercise training or a combination of both treatment strategies. Regarding cytochrome c oxidase in this population, there

was a significant decrease of enzyme activity after surgical revascularisation, a significant increase after exercise training and no observable changes after a combination of both treatment options. Compared to control subjects, there were higher enzyme activities, including CSA, before all treatment strategies[73].

1.3.3.3. Measurement of enzyme activity specific for complexes of ETC

By using spectrophotometric methods, the activity of enzymes specific for the different complexes of ETC may be determined. After normalization to CSA as a marker for mitochondrial content (see above), a correlation to respiratory capacity may be observed. Pipinos and co-workers proved in 2006 abnormal ETC complex activities in PAD patients compared to healthy controls. Complex I was determined by the measurement of rotenone-sensitive NADH dehydrogenase (ubiquinone) activity, complex II by the reduction of 2,6-dichlorophenolindophenol, complex III activity by the reduction of cytochrome c and complex IV activity by monitoring cytochrome c oxidation. They identified a significant decrease of enzyme activities in complexes I, III and IV and correlated these findings with results from respirometry that showed alterations of complex-I- and complex-III-supported respiration. They concluded that there is a correlation between injured ETC complexes and impaired mitochondrial oxidative respiration in PAD muscle[74].

1.3.3.4. In-vitro respirometry for evaluation of mitochondrial oxidative phosphorylation

Using the in-vitro method respirometry, isolated cells, permeabilized muscle fibres or even isolated mitochondria may be investigated and normalized to mitochondrial enzyme activity such as citrate synthase activity. The utilization of permeabilized muscle fibres allows an investigation of mitochondria with the organelles remaining in their physiological environment and only about five milligrams of muscle tissue is needed. Compared to the isolation of mitochondria by homogenization and centrifugation, only a fraction of the total pool of mitochondria might be investigated and also the reticular structure of the organelles might be disrupted by the procedure of isolation[63]. As respirometry takes place in a biochemically controlled and normoxic environment, potential oxygen restrictions resulting from arterial stenosis or occlusions are excluded and specific complexes of ETC might be evaluated by the titration of metabolites of the citric acid cycle[47]. Using a Clark electrode for the measurement of mitochondrial respiration, Pipinos and co-workers compared muscle biopsies of the gastrocnemius

muscle of patients with severe PAD or critical limb ischemia with healthy control subjects. After stimulating the ETC with electron donor substrates and ADP, maximal mitochondrial respiration rate was significantly lower in PAD patients compared with the healthy control group[75]. They confirmed their findings three years later by specifically describing the deficits of the respiratory chain in PAD patients to complexes I, III and IV[74]. Using HRR for the investigation of muscle biopsies of PAD patients before and after exercise (calf raises), a Norwegian group published data in 2016 where they showed an impairment of complex I supported mitochondrial respiration before exercise compared with healthy control subjects[76]. These findings underline the combined pathophysiological mechanisms in PAD: on the one hand, an impairment of oxygen and nutrient supply due to stenotic or occluded arteries and secondly, defects of the mitochondrial respiratory system ending in a reduction of producing the high-energy metabolite ATP[48].

1.3.3.5. Reactive oxygen species (ROS) production in mitochondria

It is known that at least 90% of ROS are produced in the mitochondria and complexes I and III[47,48,77-79]. In their work published in 2006, the group of Pipinos evaluated oxidative stress in PAD patients compared with healthy controls. They investigated oxidative stress biomarkers (protein carbonyls, lipid hyperperoxides and 4-hydroxy-2-nonenal) as well as one of the main antioxidant defence enzymes in mitochondria, mitochondrial superoxide dismutase. They were able to prove an increase of levels of oxidative stress biomarkers and a decrease of antioxidant enzymes in PAD muscle compared with healthy control subjects and concluded that a defect of complexes I and III leads to an significant increment of ROS production in muscle of PAD patients as another pathophysiological mechanism in PAD[74].

1.4. Aim of study

As it is known from previously published studies, mitochondrial dysfunction plays a crucial role in pathophysiology of PAD. The aim of this study is to evaluate the reaction of mitochondrial content and function in muscle biopsies of PAD patients to invasive revascularisation and therefore increased blood supply. By comparing the results with values from healthy control subjects, the changes in mitochondrial content and respiration before and after revascularisation should be evaluated with the hypothesis that an increased blood supply should change the content and function of mitochondria in investigated muscle tissue. By selective investigation of patients with isolated

pathologies of the SFA, a homogenous collective of patients is evaluated regarding the morphology of atherosclerotic alterations to obtain comparability within the study group. With this study, a better understanding of mitochondrial pathophysiology in PAD should be provided with special regard to the potential of mitochondrial regeneration after successful revascularisation.

2. MATERIALS AND METHODS

The experimental design and all study protocols was approved by the local ethics committee (Charité's Ethics Committee, Universitätsmedizin Berlin; Number of proposal: EA4/021/16) and all participants gave informed consent. Using an algorithm of numbers and letters, pseudonymization of participants was performed and the list of pseudonymization was accessible only to approved staff. Study documentation was obtained by using case report forms and the information gained was then recorded in an excel file (Excel ®, Microsoft Office 2016, Microsoft Corporation, One Microsoft Way, Redmond, WA 98052-6399, United States of America).

2.1. Participants

2.1.1. Study group

Ten patients suffering from PAD were recruited between May 2016 and May 2017 at the Department of General, Visceral and Vascular Surgery at the University Hospital Berlin, Charité. Inclusion criteria were PAD in a Fontaine stage of IIB to IV with planned intervention (endovascular or open) for revascularisation of isolated pathologies (flow-limiting stenosis or occlusion) of the SFA. Exclusion criteria were flow-limiting pathologies of the aorta, the common iliac artery (CIA), external iliac artery (EIA) or profound femoral artery (PFA), age under 18 years and allergy to local anaesthesia. Patients were screened for eligibility at the out-patient clinic of the Department of General, Visceral and Vascular Surgery at the University Hospital Berlin, Charité. Detailed medical history, including cardiovascular risk factors (diabetes mellitus, arterial hypertension, hyperlipidaemia, current smoking), previous vascular interventions, duration of symptoms and walking distance was obtained as well as demographic and anthropometric data (age, gender, body-mass index). Physical examination included pulse status, measurement of ABI, clinical examination for the presence of trophic lesions at the lower extremities and duplex ultrasound of the arterial tree of the affected leg to exclude hemodynamically relevant pathologies at the CIA, EIA and PFA and to specify the morphology of the pathology affecting the SFA. Further imaging was done by either computed tomography angiography (CTA) or magnetic resonance angiography (MRA) to plan therapeutic options. Every patient and every indication for interventional therapy was discussed within an interdisciplinary board, which included

vascular surgeons as well as interventional radiologists, to plan either endovascular or open surgical treatment.

2.1.2. Control group

Ten healthy subjects, with normal pulse status, normal ABI and without any history of vascular disease were recruited between May 2016 and May 2017 to serve as control group. The participants underwent surgical procedures of the extremities for reasons other than PAD or have participated voluntarily, without any planned surgical procedure.

2.2. Muscle biopsy

Muscle biopsies were collected from the medial belly of the gastrocnemius muscle. As the study group showed isolated atherosclerotic pathologies from the SFA, the gastrocnemius muscle was suspected to be altered due to low oxygen supply.

Patients of the study group underwent muscle biopsies just before the intervention for revascularisation. Depending on the planned intervention, biopsy was performed either under general or local anaesthesia and a second biopsy was performed six weeks after successful revascularisation, this time under local anaesthesia. Control subjects underwent muscle biopsy once, again depending on the planned procedure under general or local anaesthesia or if the participants took part voluntarily under local anaesthesia.

Biopsies were taken using Bergstrom Muscle Biopsy Needles with a diameter of 4.0 mm (Dixon Surgical Instuments, Essex, United Kingdom). Before biopsy, incision sites were shaved and disinfected using povidone iodine (Braunol ®, B.Braun, Melsungen, Germany) and if the biopsy was taken under local anaesthesia, lidocaine without adrenalin (Lidocaine-HCl 2%, B.Braun) was infiltrated into the skin and the subcutaneous tissue without infiltrating lidocaine into the muscle to prevent effects on the muscle's mitochondria. A skin incision of about 5 mm was performed using a size 10 scalpel (Aesculap ® Safety Scalpel, B.Braun) and the muscle fascia was incised as well. The biopsy needle was introduced into the muscle and about 10 – 20 mg of muscle was obtained. The muscle sample was divided into two portions, about 5 mg for permeabilization and further respirometry and the rest was frozen in liquid nitrogen and afterwards stored at -80°C for further evaluation of CSA.

2.3. Permeabilization of skeletal muscle fibres

The muscle tissue was directly transferred into freezing cold biopsy preservation solution (BIOPS) containing 10 mM Ca-EGTA buffer, 0.1 µM free calcium, 20 mM imidazole, 20 mM taurine, 50 mM 2-(N-morpholino)esthane-sulfonic acid hydrate, 0.5 mM dithiothreitol, 6.25 mM $MgCl_2$, 5.77 ATP and 15 mM phosphocreatine (pH 7.1) and stored on ice (80). A small amount of the muscle tissue was transferred into a small petri dish filled with BIOPS and placed on a freezing cold metal plate. Connective tissue was removed from the muscle samples and about 5 mg of muscle tissue was mechanically permeabilized using sharp angular forceps resulting in separated muscle fibres, remaining connected in a mesh-like framework[80]. After that, the fibre bundles were incubated in 2 ml BIOPS containing 50µg/ml saponine for 30 minutes while being gently shaken on ice. Saponine is a glycoside that selectively attacks the cholesterol-rich sarcolemma (myocyte membrane) without affecting the integrity of the mitochondria membrane (low in cholesterol) themselves[75]. After 10 minutes at 4 °C while the muscle fibres were washed again by being gently shaken in a mitochondrial respiration medium (MIRO6) containing 110 mM D-sucrose, 60 mM K^+-lactobionate, 0.5 mM EGTA, 3 mM $MgCl_2$, 20 mM taurine, 10 mM KH_2PO_4, 20 mM HEPES, 1g/l bovine serum albumin and 280 U/ml catalase. After placing the sample on dry filter paper, a wet weight of 1 – 3 mg was determined using a microbalance (Balance XSE105DU, Mettler-Toledo Intl Inc., Giessen, Germany) and afterwards the samples were directly transferred to the respirometry chamber.

2.4. High-resolution respirometry (HRR)

HRR was performed using an Oxygraph-2k (Orobros Instruments, Innsbruck, Austria). Briefly, the Oxygraph-2k consist of two closed chambers in which an investigation of mitochondrial respiration specific to the complexes of the ETC is possible. With this device, isolated mitochondria, permabilized cells or permeabilized fibres might be evaluated, as the chambers are equipped with a polarsographic oxygen sensor. As mitochondria consume oxygen while producing ATP during respiratory chain, oxygen concentration within the chambers drops during mitochondrial respiration. This alteration of oxygen concentration is recorded by the Datlab software (Orobros Instruments). Oxygen flux was determined as picomole per second per milligram wet weight of muscle tissue (pmol/(s*mg)) [80,81]. Chambers were filled with 2 ml MiRO6 and the samples were transferred to the chamber. Samples were continuously stirred within the medium at a defined speed of 750 rounds per minutes and temperature was

adjusted at 37 °C. Content of oxygen was raised to around 400 µM to achieve hyper-oxygenated chambers to prevent oxygen limitation diffusion. Substrates, uncouplers and inhibitors of mitochondrial metabolic pathways were titrated (SUIT-protocol) into the chambers to investigate mitochondrial respiration[80]. In order to obtain the so-called LEAK-state for the measurement of LEAK respiration, 2 mM of malate (M) (a substrate of the enzyme malate dehydrogenase that is oxidized in the citric acid cycle to oxaloacetate) and 0.2 mM of octanoylcarnitine (Oct) (a medium-chain fatty acid that serves as a substrate of fatty acid oxidation) were titrated into the glass chambers. LEAK respiration describes the situation in mitochondrial respiration when substrates of the respiratory chain are available while ADP is absent. A non-phosphorylating resting state is induced, as protons are transferred into the intermembrane space but as in the absence of ADP there is no substrate for the ATP-synthase, the enzyme remains inactive and no ATP is synthetized. Instead, the electrochemical gradient between the mitochondrial matrix and the intermembrane space is increased to a maximum.

After stabilization of oxygen flux, 5 mM of ADP were added to achieve the active state of the ATP-synthase and to evaluate the capacity of OXPHOS. Followed by the titration of 5 mM of pyruvate (P) (catalysed in the mitochondrial cytosol by pyruvate dehydrogenase to acetyl-CoA that is further introduced into the citric acid cycle) and 10 mM of succinate (S) (formed within the citric acid cycle and direct substrate of complex II, also known as succinate dehydrogenase) titration of substrates was completed. By the reaction of succinate to fumarate, electrons are released into the Q-cycle and are therefore important for the activation of complex III as complex II itself is not able to transfer protons into the intermembrane space. After stabilization of oxygen flux the ADP-activated state of OXPHOS was reached.

Afterwards, electron transfer state (ETS) was induced by stepwise titration (0.05 mM steps) of carbonyl cyanide p-(triflouromethoxy) phenylhydrazone (FCCP) which is a protonophore or proton translocator that transports protons across the inner mitochondrial membrane to uncouple the ATP-synthase from the respiratory chain. Followed by the titration of 0.5 µM rotenone (Rot) complex I was inhibited. With the final titration of 2.5 mM Antimycin A (AmA), an inhibitor of the Q_i-binding site of complex III, respiration was terminated. An example of recording oxygen flux for the evaluation of mitochondrial respiration with the described SUIT-protocol is shown in figure 2-1.

Figure 2-1 – Example of recording oxygen flux using the Datlab software (M – malate, Oct – octanoylcarnitine, ADP – adenosindisphosphate, P – pyruvate, S – succinate, FCCP - carbonyl cyanide p-(triflouromethoxy) phenylhydrazone, Rot – rotenone, Ama – antimycin A);

2.5. Citrate-Synthase activity (CSA)

As citrate synthase is a mitochondrial marker enzyme to determine mitochondrial content, CSA of the muscle samples was evaluated and afterwards the result from HRR was normalized to CSA.

First muscle samples that were stored at -80°C were thawed and approximately 10 mg wet weight of each sample was put into a 1.5 ml test tube and 200 µl CellLytic™MT reagent (CellLytic™MT, Sigma-Aldrich, St. Louis, MO, United States of America) containing 2 µl Protease Inhibitor Cocktail (Sigma-Aldrich) per 10 mg wet weight of muscle tissue was added. Samples were homogenized by using a disposable tissue grinder pestile (Capitol Scientific Inc., Austin, TX, United States of America) and afterwards centrifuged for 10 minutes at 13,000 x g using a Thermo Scientific™ Sorvall™ Primo™ R Benchtop Centrifuge (Fisher Scientific, Leicestershire, United Kingdom). The protein-containing supernatant was transferred into a chilled 1.5 ml test tube. Protein concentrations of the lysates were determined using a QunatiPro™ BCA Assay Kit (Sigma-Aldrich). Briefly, protein lysates were diluted in a proportion of 1:200 (5 µl protein lysate + 995 µl H_2O) and 100 µl of each diluted protein lysate was pipetted into a 96-well plate (Sarstedt AG&Co, Nürnbrecht, Germany) in which each well contained 100 µl of a predetermined QuantiPro Working Reagent so that the ratio of the lysate volume and the QuantiPro Working Reagent volume was 1:1. To determine the exact protein concentration, a protein standard solution with a dilution of 1, 2, 4 and 8,

also in a 1:1 ratio with the QuantiPro Working Reagent, was used. The 96-well plate was sealed with a plate sealer (Greiner bio-one, Kremsmünster, Austria) and incubated for 60 minutes at 60°C. The absorbance of the different solutions was measured at 550 nm using a ELx808 Absorbance Reader (BioTek, Bad Friedrichshall, Germany). Protein concentrations were finally determined by using linear regression.

CSA measurements were performed using the Citrate Synthase Assay Kit (Sigma-Aldrich) according to the manufacturer's instructions. Briefly, depending on the previous determined protein concentration of the particular protein lysates, a specific amount of protein lysate was prepared with Assay Puffer (186 µl – specific amount of protein lysate), 10 µl of acetyl-CoA and 10 µl of 5,5`-Dithiobis-(2-nitrobenzoic acid) and pipetted into a 96-well plate (Sarstedt AG&Co, Nürnbrecht, Germany). The absorbance was measured at 412 nm using a ELx808 Absorbance Reader (BioTek, Bad Friedrichshall, Germany). Finally, 10 µl of oxaloacetic acid was added into the wells and the absorbance was measured again at 412 nm. CSA was calculated as described in the manufacturer's instructions and expressed as nmol/min/mg protein.

2.6. Evaluation of mitochondrial function

Complex I-related respiration was defined as absolute respiration after titration of ADP. Complex I+II-related respiration was defined as absolute respiration after titration of succinate. ETS was defined as absolute respiration after titration of FCCP.

2.7. Statistics

Comparisons between preoperative and postoperative values were made by performing a Wilcoxon single-rank test and between postoperative values and values of the control group using a Mann Whitney U-test. A p-value of <0.05 was considered to be statistically significant. Results were expressed as mean ± standard error of the mean. SPSS (SAS Institute GmbH, Heidelberg, Germany) software was used for statistical analysis.

3. RESULTS

3.1. Demographic data

Within the study group, the mean age of patients was 67.8 years (± 2.2 years; range 55.2 – 77.4 years) compared to a mean age of 51.5 years (± 6.6 years; range 22.7 – 83.1 years) within the control group. Eight patients of the study group were male compared to six participants of the control group. The mean body mass index (BMI) was 22.7 m^2/kg (± 2.4 m^2/kg; range 17.2 – 29.0 m^2/kg) within the study group compared to 26.1 m^2/kg (± 1.3 m^2/kg; range 22.4 – 33.8 m^2/kg) within the control group.

3.2. Cardiovascular risk factors

Cardiovascular risk factors included the presence of diabetes mellitus, arterial hypertension, hyperlipidaemia and current smoking. Within the study group, diabetes mellitus was present in five, arterial hypertension in nine, hyperlipidaemia in five and current smoking in nine out of ten patients. Within the control group, none of the participants suffered from diabetes mellitus or arterial hypertension, two suffered from hyperlipidaemia and one participant admitted of being a current smoker.

3.3. Peripheral hemodynamics and maximal walking distance

Regarding peripheral hemodynamics of the patients of the study group, the mean ABI of the symptomatic leg was 0.51 (± 0.31) whereas the asymptomatic leg showed a mean ABI of 0.91 (± 0.18). In two patients the ABI was not detectable due to severity of vascular disease or due to ulcerations at the foot. Participants of the control group showed an ABI of 1.0 on both legs. The mean subjective maximal walking distance was 51 meters (± 31 meters) within the study group, participants of the control group reported an unlimited walking distance with no occurrence of IC.

3.4. Clinical symptoms

Using the classification of Fontaine (see Table 1-2), seven out of ten patients of the study group presented in a Fontaine grade IIB PAD, as they reported a maximal walking distance of less than 200 meters. One patient reported rest pain, meaning a grade III PAD and two patients had ulcerations of the affected leg, so that they were graded as a grade IV PAD.

3.5. Morphology of lesion and type of vascular intervention

In all patients, ultrasound imaging was performed to ensure that there were no flow-limiting pathologies of the iliac arteries or PFA. In nine out of ten patients, further

imaging for evaluation of the pathology of the SFA and for further planning of vascular intervention was performed by CTA, in one patient by MRA. An occlusion of the SFA was detected in five patients, the other half of the patients showed a stenosis of the SFA. The length of the lesion was determined as short lesion in six patients, whereas four patients showed extended pathologies with a length of the lesion greater than 15 centimetres.

In five patients open surgery was performed to treat the detected lesion. Out of these five patients, four were treated by bypass surgery and one were treated by an endarterectomy of the proximal SFA right after its origin from the femoral bifurcation followed by an embolectomy of the femoropopliteal segment. Endovascular methods were used in two patients, whereas one was treated by primary percutaneous stenting and the other by PTA with a drug-coated balloon of the SFA. The remaining three patients were treated by a hybrid procedure with an endarterectomy and patch plasty of the proximal SFA right after its origin from the femoral bifurcation with consequent stenting in two cases and a balloon angioplasty with a drug-coated balloon of the distal SFA in one case.

An overview of the study and control groups regarding demographic data, maximal walking distances and cardiovascular risk factors is listed in table 3-1 and an overview of clinical symptoms, morphology of the lesion of the SFA and the vascular intervention performed in patients from study group is listed in table 3-2.

	study group (n=10)	**control group (n=10)**
Age (years)	67.8 ± 2.2	51.5 ± 6.6
Gender (male/female)	8/2	6/4
Body mass index (m^2/kg)	22.7 ± 2.4	26.1 ± 1.3
Ankle brachial index	0.51 ± 0.31 **	1.00 ± 0
maximal walking distance (m)	51 ± 31	n.a
cardiovascular risk factors	3 **	0.3

Table 3-1 - Demographic data of study and control group. Data are expressed as mean ± standard error of the mean. ** indicates statistically significant differences ($p<0.01$);

	Age	Gender	Fontaine grade	Morphology of SFA pathology	Vascular intervention	Details of vascular intervention
1	55.2	male	IIB	short stenosis	endovascular	stenting SFA
2	73.8	male	III	extended occlusion	open surgery	above the knee femoropopliteal bypass
3	68.3	male	IV	short stenosis	hybrid procedure	endarterectomy and drug coated balloon angioplasty
4	61.4	male	IIB	short stenosis	hybrid procedure	endarterectomy and stenting SFA
5	74.5	female	IIB	extended occlusion	open surgery	endarteiectomy and embolectomy
6	77.4	female	IIB	short occlusion	open surgery *	above the knee femoropopliteal bypass
7	61.6	male	IIB	short stenosis	hybrid procedure	endarterectomy and stenting SFA
8	66.9	male	IIB	short stenosis	endovascular	drug coated balloon angioplasty
9	65.9	male	IV	extended occlusion	open surgery	below the knee femoropopliteal bypass
10	72.2	male	IIB	extended occlusion	open surgery	above the knee femoropopliteal bypass

Table 3-2 - Clinical symptoms, morphology of SFA pathology and intervention performed in study group; extended was defined by >15 cm of length; short was defined by <15 cm of length; * open surgery due to previous failure of endovascular procedures;

3.6. Proof of technical success of vascular intervention

All patients underwent follow-up examination six weeks after revascularisation concerning technical success of the vascular intervention. ABI improved from 0.51 ± 0.31 to 0.81 ± 0.31 (p=0.06). Clinical stage of PAD decreased in all patients who initially presented with a stage IIB PAD. Three out of seven patients reported an improvement of walking distance and four out of seven reported to be asymptomatic. The patient with an initial stage III PAD reported to be symptom-free and without limitation of walking distance any more. One of the two patients with ulcerations had complete wound healing, whereas the other patient who initially presented with extensive ulcerations presented with a remaining but healing ulceration and clear reduction of extent of the ulceration. All patients underwent ultrasound imaging to prove patency of the revascularisation performed and all patients showed patent reconstructions of the SFA without residual hemodynamic relevant stenosis.

3.7. Mitochondrial content and function

3.7.1. Citrate synthase activity (CSA)

In muscle biopsies from PAD patients, a CSA of 303.321 (± 28.206) nmol/min/mg protein before revascularisation compared to 418.895 (± 27.983) nmol/min/mg protein six weeks after revascularisation was determined. Muscle biopsies of control group showed a CSA of 393.751 (± 38.840) nmol/min/mg protein.

3.7.2. Respirometry

Complex I-related respiration in muscle biopsies of PAD patients was 25.731 (± 2.108) pmol/(s*mg) before revascularisation compared to 20.843 (± 1.744) pmol/(s*mg) six weeks after revascularisation. Muscle biopsies of the control group showed a complex I-related respiration of 13.520 (± 2.065) pmol/(s*mg).

Complex I+II-related respiration in muscle biopsies of PAD patients was 69.355 (± 3.560) pmol/(s*mg) before revascularisation compared to 53.075 (± 2.927) pmol/(s*mg) six weeks after revascularisation. Muscle biopsies of the control group showed a complex I+II-related respiration of 46.915 (± 4.766) pmol/(s*mg).

Electron transfer system capacity (ETS) in muscle biopsies of PAD patients before revascularisation was 73.707 (± 4.100) pmol/(s*mg) compared to 56.376 (± 3.262) pmol/(s*mg) six weeks after revascularisation. Muscle biopsies of the control group showed an ETS of 45.548 (± 5.067) pmol/(s*mg).

3.7.3. Respirometry normalized to CSA

Complex I-related respiration normalized to CSA in muscle biopsies of PAD patients was 0.093 (± 0.010) pmol/(s*mg) per CSA before revascularisation compared to 0.051 (± 0.006) pmol/(s*mg) per CSA six weeks after revascularisation. Muscle biopsies of the control group showed a complex I-related respiration normalized to CSA of 0.035 (± 0.008) pmol/(s*mg) per CSA.

Complex I+II-related respiration normalized to CSA in muscle biopsies of PAD patients was 0.250 (± 0.016) pmol/(s*mg) per CSA before revascularisation compared to 0.137 (± 0.013) pmol/(s*mg) per CSA six weeks after revascularisation. Muscle biopsies of the control group showed a complex I+II-related respiration normalized to CSA of 0.126(± 0.021) pmol/(s*mg) per CSA.

ETS normalized to CSA in muscle biopsies of PAD patients was 0.270 (± 0.021) pmol/(s*mg) per CSA before revascularisation compared to 0.146 (± 0.015) pmol/(s*mg) per CSA six weeks after revascularisation. Muscle biopsies of the control group showed an ETS normalized to CSA of 0.124 (± 0.023) pmol/(s*mg) per CSA.

3.7.4. Alteration of mitochondrial content and function in muscle biopsies of PAD patients before and after revascularisation

3.7.4.1. Citrate Synthase Activity (CSA)

A significant increase of CSA and therefore mitochondrial content was seen six weeks after successful revascularisation (303.321 ± 28.206 nmol/min/mg protein preoperatively vs. 418.895 ± 27.983 nmol/min/g protein postoperatively; p=0.006) corresponding an adaptation to values from healthy control subjects (418.895 ± 27.983 nmol/min/mg protein postoperatively vs. 393.751 ± 38.840 nmol/min/mg protein control group, p=0.743) (Figure 3-1)

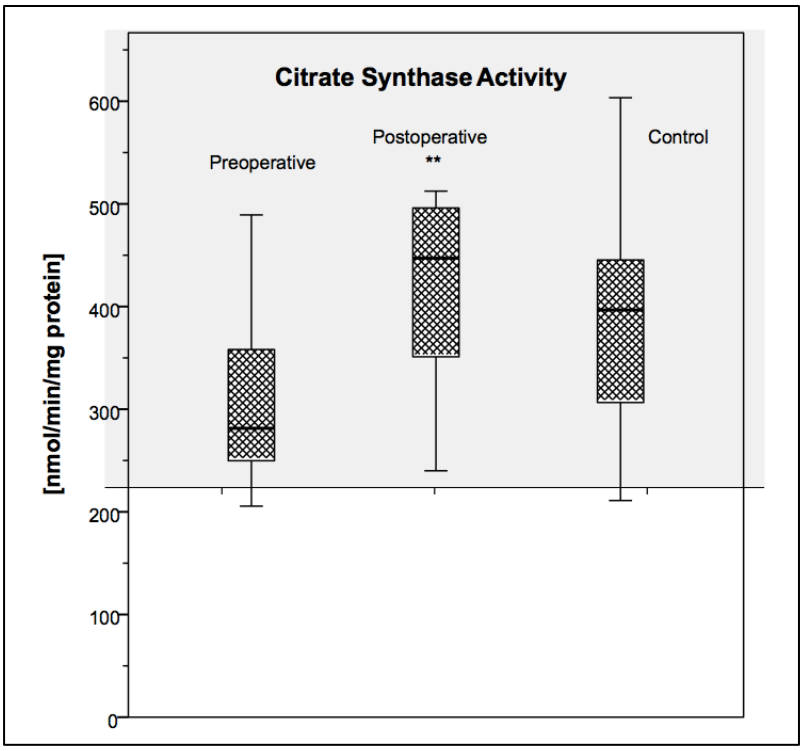

Figure 3-1 - CSA in preoperative, postoperative and control biopsies; ** indicates statistically significant difference between preoperative and postoperative values ($p<0.01$);

3.7.4.2. Respirometry

Results from high-resolution respirometry showed a significant reduction of mitochondrial respiration six weeks after successful revascularisation of complex I+II-related respiration (69.355 ± 3.560 pmol/(s*mg) preoperatively vs. 53.075 ± 2.927 pmol/(s*mg) postoperatively; $p<0.001$) as well as in ETS (73.707 ± 4.100 pmol/(s*mg) preoperatively vs. 56.378 ± 3.262 pmol/(s*mg) postoperatively, $p<0.001$). This postoperative reduction of mitochondrial respiration correlates with an approximation of the results to the results of healthy control subjects (complex I+II-related respiration 53.075 ± 2.927 pmol/(s*mg) postoperatively vs. 46.915 ± 4.766 pmol/(s*mg) of control group, $p=0.481$; ETS 56.378 ± 3.262 pmol/(s*mg) postoperatively vs. 45.548 ± 5.067 pmol/(s*mg) of control group, $p=0.114$). A reduction of complex I-related respiration in muscle biopsies of PAD patients six weeks after successful revascularisation was also noticed, but the differences were not significant (25.731 ± 2.108 pmol/(s*mg) preoperatively vs. 20.843 ± 1.744 pmol/(s*mg) postoperatively, $p=0.099$) and this reduction did not correlate with the values of the healthy control subjects (20.843 ±

1.744 pmol/(s*mg) postoperatively vs. 13.520 ± 2.065 pmol/(s*mg) of control group, p=0.074) (Figure 3-2).

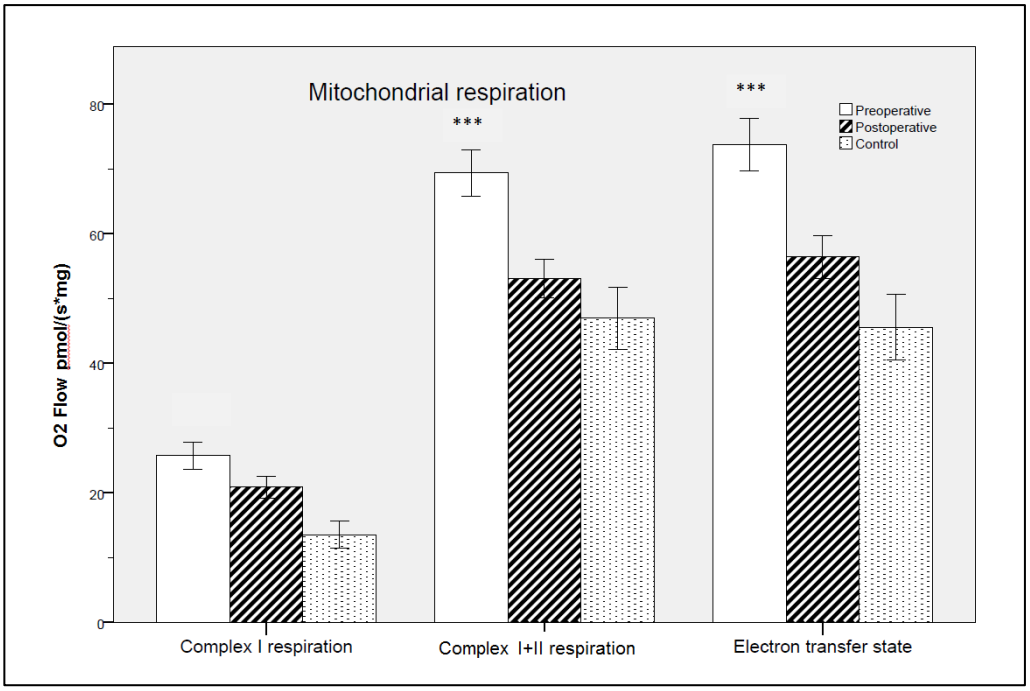

Figure 3-2 - Mitochondrial respiration in preoperative, postoperative and control biopsies; *** indicates statistically significant differences between preoperative and postoperative values (p<0.001);

3.7.4.3. Respirometry normalized to CSA

After normalization of results from respirometry to CSA, the reduction of mitochondrial respiration six weeks after successful revascularisation was significant in complex I related respiration (0.093 ± 0.010 pmol/(s*mg) preoperatively vs. 0.051 ± 0.006 pmol/(s*mg) per CSA postoperatively; p=0.020), complex I+II-related respiration (0.250 ± 0.016 pmol/(s*mg) per CSA preoperatively vs. 0.137 ± 0.013 pmol/(s*mg) per CSA postoperatively; p=0.008) as well as in ETS (0.270 ± 0.021 pmol/(s*mg) per CSA preoperatively vs. 0.146 (± 0.015) pmol/(s*mg) per CSA postoperatively; p=0.008). Through this reduction of mitochondrial respiration, the results approached the results obtained from healthy control subjects (complex I-related respiration 0.051 ± 0.006 pmol/(s*mg) per CSA postoperatively vs. 0.035 (± 0.008) pmol/(s*mg) per CSA of control group, p=0.139; complex I+II-related respiration 0.137 ± 0.013) pmol/(s*mg) per CSA postoperatively vs. 0.126 (± 0.021) pmol/(s*mg) per CSA of control group, p=0.888; ETS 0.146 (±0.015) pmol/(s*mg) per CSA postoperatively vs 0.124 (± 0.023) pmol/(s*mg) per CSA in control group, p=0.481) (Figure 3-3).

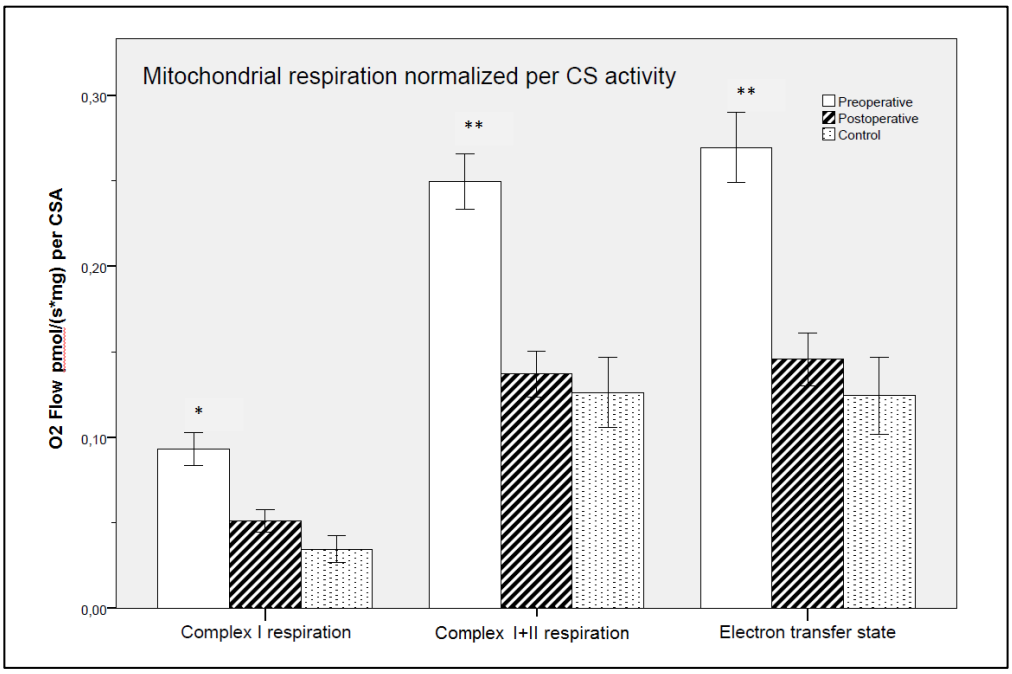

Figure 3-3 - Mitochondrial respiration normalized per CSA in preoperative, postoperative and control biopsies; * indicates statistically significant differences between preoperative and postoperative values ($p<0.05$); ** indicates statistically significant differences between preoperative and postoperative values ($p<0.01$); CS – Citrate Synthase;

Detailed information about results of mitochondrial content and function in preoperative, postoperative and control biopsies is described in table 3-3.

	Preoperative	Postoperative	Pre- vs. postoperative p-value	Control group	Postoperative vs. control p-value
CSA [nmol/min/mg protein]	303.321 ± 28.206	418.895 ± 27.983	0.006**	393.751 ± 38.840	0.743
Complex I-related respiration [pmol/(s*mg)]	25.731 ± 2.108	20.843 ± 1.744	0.099	13.520 ± 2.065	0.074
Complex I+II-related respiration [pmol/(s*mg)]	69.355 ± 3.560	53.075 ± 2.927	<0.001***	46.915 ± 4.766	0.481
Electron transfer state [pmol/(s*mg)]	73.707 ± 4.100	56.378 ± 3.262	<0.001***	45.548 ± 5.067	0.114
Complex I-related respiration [pmol/(s*mg)] per CSA	0.093 ± 0.010	0.051 ± 0.006	0.020*	0.035 ± 0.008	0.139
Complex I+II-related respiration [pmol/(s*mg)] per CSA	0.250 ± 0.016	0.137 ± 0.013	0.008**	0.126 ± 0.021	0.888
Electron transfer state [pmol/(s*mg)] per CSA	0.270 ± 0.021	0.146 ± 0.015	0.008**	0.124 ± 0.023	0.481

Table 3-3 – Detailed information about results of mitochondrial content and function in preoperative, postoperative and control biopsies; Data are expressed as mean ± standard error of the mean; * Indicates statistically significant differences ($p<0.05$); ** Indicates statistically significant differences ($p<0.01$); *** Indicates statistically significant differences ($p<0.001$);

4. DISCUSSION

Atherosclerotic plaques resulting in hemodynamically relevant stenosis or even occlusions of affected arteries cause PAD. During physical strain, affected muscles do not receive enough blood due to flow-limiting stenosis and therefore oxygen supply is limited, resulting in pain during physical activity such as walking. After taking a short rest, the pain disappears and walking can be resumed. This leads to repeated cycles of ischemia and reperfusion in affected muscle, and it is known that this pathomechanism results in histomorphological as well as metabolic alterations of affected muscles[47,48].

As mitochondrial alterations have been described to be crucial in the pathophysiology of the myopathy associated with PAD, several groups have investigated mitochondrial dysfunction in PAD patients using different methodical approaches[47,48]. The advantage of in-vitro respirometry lies in the possibility of investigating muscle tissue and its mitochondria ex vivo in a normoxic environment with biochemically controlled conditions. Therefore, potentially restricted blood and oxygen supply due to atherosclerotic changes in arteries is avoided. By using SUIT-protocols, HRR allows the investigation of single complexes of ETC. Compared with the in-vivo method 31P-MRS, only a conclusion of the overall mitochondrial function is possible with the above-mentioned limitation of potentially limited oxygen supply by restricted blood flow due to atherosclerosis. The advantages of 31P-MRS compared with HRR are the non-invasiveness and the possibility of investigating mitochondrial respiration before, during and after exercise, as patients are asked to perform calf exercises before and during the examination[64].

With the existing knowledge about mitochondrial dysfunction in PAD patients, the potential of mitochondrial regeneration in PAD patients six weeks after successful revascularisation by using HRR and spectrophotometric measurement of CSA has been investigated in this study.

4.1. Citrate-Synthase activity (CSA) and mitochondrial content

In this study population, an increase of CSA and therefore mitochondrial content of the gastrocnemius muscle was observed six weeks after successful revascularisation. This postoperative increase represents an approximation of values to results from the control group meaning a normalization of CSA after an improvement of blood supply due to successful revascularisation. Regarding literature, it is known for years, that

mitochondrial content is able to increase or decrease, depending on the metabolic demand[81], and it also has been described that an increase of mitochondrial content is associated with an increase of oxidative exercise capacity[82,83]. Mitochondrial enzyme activities, including CSA, decrease in the case of detraining and increase in the case of training[84]. IC as the leading symptom of PAD, a walking-induced pain results in a reduced walking performance of patients, a decrease of mitochondrial content comparable to before mentioned observations after detraining might be expected[53]. Jansson and co-workers investigated patients with unilateral PAD by taking muscle biopsies from the symptomatic as well as from the asymptomatic leg and evaluated different enzyme activities. Surprisingly, they described increased activities of citrate synthase in symptomatic compared to asymptomatic legs[72]. The group of Lundgren described similar findings in 1989 as they reported about significant higher activity of citrate synthase in PAD patients before different treatments compared with control subjects[73]. Interestingly, the findings of this study do not agree with former published results, as a reduced activity of citrate synthase was observed in PAD patients before revascularisation with a significant increase after successful revascularisation i.e. adaptation to values of the control group. These findings are in line with the hypothesis postulated by Brass in 2000[53], that a reduced physical activity due to intermittent claudication in PAD patients occurs with detraining and therefore a reduction in mitochondrial enzyme activities as an adaptation to a reduced metabolic demand seems plausible.

4.2. Mitochondrial respiration and oxidative capacity

Mitochondrial function in PAD patients has been evaluated in several studies before. As different groups have used different methodical approaches, a comparison between results is difficult. As stated above, mitochondria are known to be able to adapt function and oxidative capacity to the metabolic demand[82,83]. Published in 1984, the group of Elander reported about polarographically measured mitochondrial function in PAD patients by using an oxygen electrode. The chambers in which mitochondria were evaluated were sealed to achieve stable oxygen concentration and substrates were added through a capillary-like opening. This method seems quite similar to HRR, as it was used in this study and therefore might be comparable. This study is limited, as the authors did not normalize their results to values from CSA measurements despite having evaluated several enzyme activities including citrate synthase. An increased

mitochondrial content was reported as well as an increase of oxidative enzyme capacity in gastrocnemius muscle of PAD patients, i.e. comparable results to ours when examining mitochondrial respiration, but there was no information given about clinical presentation of patients or morphology of the atherosclerotic lesion, so that a clear comparison with our results is difficult[85]. The group of Pipinos investigated mitochondrial function using a Clark electrode for respirometry. In 2003, they described a significant decrease of overall mitochondrial function in PAD patients compared to healthy control subjects as they evaluated the ratio between maximal mitochondrial stimulation after addition of ADP and maximal mitochondrial inhibition by adding atractyloside, a selective inhibitor of the ATP synthase[75]. In 2006, they had a selective look at specific complexes of ETC and normalized their results to values of CSA. They reported significant decreases of complex I, III and IV related respiration compared with control subjects. Complex II related respiration was decreased as well but with no significance. Results of both of these studies differ from our results, as we have seen increased complex I as well as complex I+II related mitochondrial respiration. The clinical presentation of patients included by Pipinos and co-workers did differ from our patients. In 2003 mainly stage IV patients and in 2006 patients with “advanced PAD” with an ABI of 0.34 (± 0.05) were included[74,75]. As our patients presented with milder forms of PAD with a mean ABI of 0.51 (± 0.31), we suggest that patients with a stage IIB disease are physically more active compared to patients with a stage IV disease as they present with ulcerations or gangrene. The observed increase of CSA and mitochondrial respiration is therefore interpreted as a compensation of mitochondrial metabolism to the decreased blood supply in PAD patients with IC. Recently, a Norwegian study was published with a similar methodical approach (HRR and CSA) regarding evaluation of mitochondrial respiration compared to ours. They investigated mitochondrial function in PAD patients at baseline and eight weeks after randomization in different exercise groups, where one group performed calf exercises and the other group performed walking exercises. By evaluating the maximal walking distance at baseline and eight weeks after randomization, they further divided patients into a negative responder group with no improvement of walking distance and a positive responder group with improvement of walking distances. Regarding CSA, they described an increase in values eight weeks after training in the group of positive responders, whereas mitochondrial respiration did not change within the group of positive responders but

decrease within the group of negative responders. The limitation of this study is that they did not compare their findings to healthy control subjects and therefore, a comparison to our results from preoperative biopsies is not possible as it is not known how the Norwegian results should be interpreted in comparison to healthy values. Another difference to our study is the chosen treatment strategy, as it is known that there are different effects on mitochondrial respiration depending on different treatment[73]. Therefore, results from van Schaardenburg are not comparable to our findings as we investigated patients after interventional revascularisation[76].

4.3. Regeneration of mitochondrial content and function

There are only limited numbers of studies investigating the potential of regeneration of mitochondria in PAD patients after successful revascularisation. In 1986 a group from the United States performed a large-number study to determine the feasibility of using the non-invasive method of 31P-MRS for evaluation of mitochondrial function in PAD patients. They investigated patients at rest and after a short period of exercise and repeated their investigations after successful revascularisation. Patients who have been undergoing endovascular revascularisation have been re-investigated shortly after the procedure (within 24 hours) whereas patients who have been treated by bypass surgery have been investigated after a period of more than a month without clarified time interval in detail. This study concluded that despite the expected improvement of hemodynamic parameters and reduction of clinical symptoms, after revascularisation there was no difference in results of 31P-MRS and therefore no improvement of mitochondrial function. This data are limited as there was no clear time interval between the vascular procedure and the re-evaluation of mitochondrial function and in special in patients undergoing endovascular revascularisation the time interval of 24 hours seems to be too short to detect any potential of regeneration of mitochondria. Also a comparison between different treatment strategies (endovascular vs. open surgery) is not possible as the time intervals between intervention an re-investigation is not equal in these groups[64]. Another group investigated the changes in mitochondrial oxidative capacity after different treatment strategies as they investigated several enzyme activities (3-hydroxyacyl-CoA dehydrogenase and cytochrome-c oxidase) representing mitochondrial function as well as CSA as marker for mitochondrial content. Firstly they compared values from PAD patients to healthy control subjects and secondly they investigated changes in mitochondrial function and contents after randomisation of

patients in different treatment groups. They reported significant higher enzyme activities before revascularisation and after exercise training as treatment strategy as an increase of cytochrome-c oxidase was observed. In contrast, cytochrome-c oxidase and CSA decreased after bypass surgery[73]. These results are in some parts similar to our findings as we have detected an increased mitochondrial respiration and therefore oxidative capacity before treatment of PAD as well but results from CSA do not correlate with our findings. Regarding the development of mitochondrial function and content after successful revascularisation, we observed a reduction of mitochondrial respiration normalized to CSA six weeks after treatment of atherosclerotic lesions with an adaption to values from control subjects and therefore a regeneration of mitochondrial function. This seems to correlate to findings in the study from Lundgren regarding the decrease of cytochrome-c oxidase after bypass surgery but results from CSA and therefore mitochondrial content do not correlate with our findings. To the best of our knowledge, this study is the first that verifies the potential of mitochondrial regeneration both in quantitative (mitochondrial content) and in qualitative (mitochondrial respiration) manners.

4.4. Limitations of our study

The low number of study subjects and the heterogeneity of clinical presentation of patients limited this study. With a number of 10 participants in each group, no further subgroup analysis is possible to get meaningful results about mitochondrial content and function of stage IIB patients compared to stage IV patients. Especially, as it is known that exercise has a positive effect on mitochondrial content and function[81,86] and regarding other studies on PAD patients using respirometry[74,75] with different findings compared to our results, it would be of great interest to continue our investigations with a larger study cohort. We have included patients with isolated SFA pathologies only in order to provide homogeneity within the study group regarding morphology of atherosclerotic pathology, but subgroup analysis regarding different localizations of pathologies in PAD of the lower extremities would be of interest as well.

4.5. Summary and Conclusion

This study showed that in patients with PAD, mitochondrial content is reduced with increased mitochondrial respiration normalized to CSA compared to healthy control subjects. Six weeks after successful revascularisation, an increase of mitochondrial number and a decrease of mitochondrial respiration normalized to CSA with results

approaching those of healthy control subjects indicates a regeneration of mitochondrial content and function after successful revascularisation to normal values. Limited by the heterogeneity of our study cohort but in regard of published literature, an adaptation of mitochondrial metabolism to metabolic demand in physical active PAD patients seems to be an explanation of our results. Therefore, identification of patients with potential for mitochondrial regeneration might be desirable to choose the optimal treatment in the future. With the knowledge of this study, further investigations about detailed mechanisms of mitochondrial damage and regeneration (e.g. role of reactive oxygen species) as well as large-study groups for further subgroup analysis for differentiation between clinical stages and morphologies of PAD as well as localization of atherosclerotic lesions might be promising.

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EIDESSTAATLICHE VERSICHERUNG

„Ich, Dr. med. univ. Alexandra Gratl, versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema: „High-resolution respirometry for measurement of mitochondrial fucntion in PAD patients before and after revascularisation – a pilot study“ selbstständig und ohne nicht offengelegte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel genutzt habe.
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CURRICULUM VITAE

Due to privacy policy, my curriculum vitae is not published in the electronic version of my doctoral thesis.

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