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Mitochondria and its role in metabolic regulation and skeletal muscle function in healthy and disease conditions

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Mitochondria and its role in metabolic regulation and skeletal muscle function in healthy and disease conditions

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By

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To my parents

ABSTRACT

Skeletal muscle function is critical for our overall health and to be able to perform daily activities. Skeletal muscle has the ability to adapt to various stimuli and mitochondria are known to play an important role in these adaptation processes. Healthy mitochondria are essential for providing skeletal muscle with energy, which are used for various biochemical reactions including generating force and maintaining muscle mass, whereas dysfunctional mitochondria have been associated with loss of skeletal muscle mass and function.

In **study I**, we investigated the role of nuclear-encoded mitochondrial protein NDUFA4L2 in skeletal muscle. NDUFA4L2 has been shown to decrease oxidative phosphorylation and the production of reactive oxygen species in various tissues and cell lines. We ectopically expressed NDUFA4L2 in mouse skeletal muscles with adenovirus-mediated expression and *in vivo* electroporation. We found that ectopic NDUFA4L2 expression in skeletal muscle reduced mitochondrial respiration and reactive oxygen species production, together with lowered levels of AMP, ADP, ATP, and NAD+, while the overall protein content of mitochondrial remained unchanged. Furthermore, ectopic expression of NDUFA4L2 resulted in smaller muscle mass and hence weaker muscles. The loss of muscle mass was associated with the activation of atrogenes MurF1 and Mul1, and apoptotic genes caspase 3. We used unilateral femoral artery ligation (FAL) as a mouse model of peripheral vascular disease (PVD) to induce muscle ischemia. Our results showed that NDUFA4L2 was induced in skeletal muscle after FAL. The gene expression of Ndufa4l2 correlated with the reduced capacity of the muscle to produce force.

In **study II**, we aim to study the role of mitochondria in PVD-induced muscle dysfunction. PVD lowers blood flow to the lower limbs, causing debilitating skeletal muscle myopathy. Interventions that improve distal arterial pressures (i.e., bypass surgery) generally fail to normalize the functional performance of muscle indicating pathophysiological mechanisms inside the skeletal myofibers that reduce overall muscle function. We performed FAL surgery on mice that were fed either a normal chow diet (ND) or a high-fat diet (HFD) for eight weeks. Our results showed that the muscle weakness induced by FAL was exacerbated in mice fed HFD, together with more serious fibrosis and ectopic fat accumulation in these muscles. Our RNA-sequencing results showed that mitochondrial gene expressions had synchronized reduction in ND-FAL legs, while the reduction was attenuated in HFD-FAL legs. Mitochondrial assembly and cellular respiration were identified as the top suppressed pathway in ND-FAL legs, but not in HFD mice. Fibrosis, fat metabolism, and myosin heavy chain isotypes were amongst the top variable genes in control and FAL muscle from normal and obese mice. Inference of proportions of different cell types with ImmuCC found that HFD has already induced an inflammatory response in skeletal muscle without FAL. Our results suggested that mitochondria content and function may be potential targets to improve muscle function in PVD associated with T2D.

Insulin resistance and defects in mitochondrial oxidative phosphorylation (OXPHOS) have been suggested to play an important role in the metabolic dysfunction and muscle impairments caused by T2D. However, we are currently lacking effective treatment against muscle dysfunction in T2D. **In study III**, we manipulated the mitochondrial electron transport chain (ETC) with our novel NDUFA4L2 genetically knocked-out mouse model. Skeletal muscle lacking NDUFA4L2 appeared stronger, more fatigue resistant, and exhibited higher capillary density and whole-body glucose clearance. NDUFA4L2 knockout mice showed a different metabolic status compared with wild-type litters. Our results indicated that NDUFA4L2 influences skeletal muscle function and hence may be a novel target for T2D-associated muscle dysfunction.

The coactivator PGC-1α1 is pivotal to the regulation of mitochondrial function and content in skeletal muscle. In skeletal muscle after exercise, PGC-1α1 enhanced the expression of kynurenine aminotransferases (Kats), an enzyme that catalyzes the conversion from kynurenine to kynurenic acid. In **study IV**, we observed that PGC-1α1 increased the expression of genes associated with glycolysis and malate-aspartate shuttle (MAS), together with an elevation in aspartate and glutamate levels. These processes promote energy utilization and facilitate the transfer of electrons from the donors to mitochondrial respiration. Thus, trained skeletal muscle can use kynurenine metabolism to increase the bioenergetic efficiency of glucose oxidation through this PGC-1α1-dependent mechanism. Inhibition of Kat with carbidopa resulted in impairments in aspartate biosynthesis, mitochondrial respiration, and skeletal muscle function. After all, the activate MAS and kynurenine catabolism in skeletal muscle after exercise by PGC-1α1 is important for the muscle's adaptation to endurance training.

Taken together, these four studies presented in this thesis highlighted the important role of mitochondria in skeletal muscle and the feasibility of targeting mitochondria for the improvement of skeletal muscle function in both healthy and diseased conditions.

LIST OF SCIENTIFIC PAPERS

I. **Liu Z***, Chaillou T*, Santos Alves E, Mader T, Jude B, Ferreira DMS, Hynynen H, Cheng AJ, Jonsson WO, Pironti G, Andersson DC, Kenne E, Ruas JL, Tavi P, Lanner JT. *Mitochondrial NDUFA4L2 is a novel regulator of skeletal muscle mass and force.* FASEB J. 2021 Dec;35(12):e22010.
* equal contribution

II. **Liu Z**, Chaillou T, Jude B, Mader T, Mous D, Lanner JT. *Skeletal muscle remodeling is response to peripheral artery disease in normal and obese mice*. Manuscript

III. **Liu Z**, Santos Alves E, Borg M, Pettersson AM, Zierath J, Lanner JT. *Role of mitochondrial protein NDUFA4L2 in skeletal muscle under diseased and healthy condition*. Manuscript

IV. Agudelo LZ, Ferreira DMS, Dadvar S, Cervenka I, Ketscher L, Izadi M, **Zhengye L**, Furrer R, Handschin C, Venckunas T, Brazaitis M, Kamandulis S, Lanner JT, Ruas JL. *Skeletal muscle PGC-1α1 reroutes kynurenine metabolism to increase energy efficiency and fatigue-resistance.* Nat Commun. 2019 Jun 24;10(1):2767.

SCIENTIFIC PAPERS NOT INCLUDED IN THIS THESIS

I. Correia JC, Kelahmetoglu Y, Jannig PR, Schweingruber C, Shvaikovskaya D, **Zhengye L**, Cervenka I, Khan N, Stec M, Oliveira M, Nijssen J, Martínez-Redondo V, Ducommun S, Azzolini M, Lanner JT, Kleiner S, Hedlund E, Ruas JL. *Muscle-secreted neurturin couples myofiber oxidative metabolism and slow motor neuron identity*. Cell Metab. 2021 Nov 2;33(11):2215-2230.e8.

II. Gabriel BM, Altıntaş A, Smith JAB, Sardon-Puig L, Zhang X, Basse AL, Laker RC, Gao H, **Liu Z**, Dollet L, Treebak JT, Zorzano A, Huo Z, Rydén M, Lanner JT, Esser KA, Barrès R, Pillon NJ, Krook A, Zierath JR. Disrupted circadian oscillations in type 2 diabetes are linked to altered rhythmic mitochondrial metabolism in skeletal muscle. Sci Adv. 2021 Oct 22;7(43):eabi9654.

CONTENTS

LIST OF ABBREVIATIONS

ATP	adenosine triphosphate
Bax	BCL2-associated X protein
CLI	critical limb ischemia
CoQ	ubiquinone
CAD	coronary artery disease
Casp-3	caspase 3
CS	citrate synthase
CSA	cross-sectional area
DMD	duchenne muscular dystrophy
DMEM	Dulbecco's modified Eagle's medium
EC	endothelial cells
ECM	extracellular matrix
EDL	extensor digitorum longus
ETC	elecron transporter chain
FCSA	fiber cross-sectional area
FAL	femoral artery ligatin
FAPs	fibro/adipogenic progenitors
FBS	fetal bovine serum
FCCP	carbonyl cyanide-p-trifluoromethoxyphenylhydrazone
FMN	flavin mononucleotide
G3PS	glycerol-3-phosphate (G3P) shuttle
Got1/2	Glutamic-oxaloacetic transaminases 1/2
GSEA	Gene Set Enrichment Analysis
HFD	high-fat diet
HGF	hepatocyte growth factor
HIF1a	Hypoxia-inducible factor 1-alpha
IC	intermittent claudication
Kat	kynurenine aminotransferase

KEGG	Kyoto Encyclopedia of Genes and Genomes
Kyn	kynurenine
Kyna	kynurenic acid
MAS	malate-aspartate shuttle
Mul1	mitochondrial ubiquitin ligase activator of NFκB 1
Murf1	muscle RING finger 1
NAD	nicotinamide adenine dinucleotide
NDUFA4L2	NADH dehydrogenase 1 alpha subcomplex subunit 4-like 2 genes
NRF1/2	nuclear factor erythroid 2–related factor 1/2
PGC-1α1	peroxisome proliferator-activated receptor gamma coactivator 1-alpha 1
PLP	pyridoxal-5' -phosphate
PPARγ	peroxisome proliferator-activated receptor gamma
PVD	peripheral vascular disease
QH2	ubiquinol
RER	respiratory exchange ratio
ROS	reactive oxygen species
T2D	type 2 diabetes
TCA	tricarboxylic acid
Tfam	mitochondrial transcriptional factor a
VDAC	VDAC
Vegf	vascular endothelial growth factor
VHL	Von Hippel-Lindau

1 INTRODUCTION

1.1 SKELETAL MUSCLE

Skeletal muscle accounts for approximately 50% of our body mass and is crucial for our ability to move and breathe. Skeletal muscle is attached to bones via tendons which enable our bodies to move (Birbrair et al., 2014). Skeletal muscle consists of long, multinucleated muscle fibers held together by interstitial connective tissues. A myofiber is a syncytium formed during development by the fusion of individual muscle precursors called myoblasts. Each myofiber is a bundle of myofibrils which are filaments with the contractile proteins' myosin and actin as major components (Fig. 1). Thick myosin filaments and thin actin filaments overlap with each other and form sarcomeres, the basic contractile units of skeletal muscle.

The ability of myosin and actin to slide past each other enables the skeletal muscle to contract and relax. The presence of sarcomeres shows a series of dark and light bands due to the overlapping pattern of these filaments. Regularly occurring sarcomeres give the skeletal muscle a striated pattern (Irving, 2017). Skeletal muscle fibers are classified by their contraction speed, enzymatic velocity, and metabolic profile. Three types of muscle fibers can be found in skeletal muscle, and the proportion of each may vary depending on the functional role of the muscle. These fibers are slow oxidative fibers (Type I fibers), fast oxidative glycolytic fibers (Type IIa fibers), and fast glycolytic fibers (Type IIB fibers) (Pette & Staron, 2000). The connective tissue that surrounds the muscle fibers is also an essential part of muscle function. The connective tissue not only continues as tendons that attach the skeletal muscle to bones but also contains a rich supply of blood vessels and nerves that communicate with the muscle.

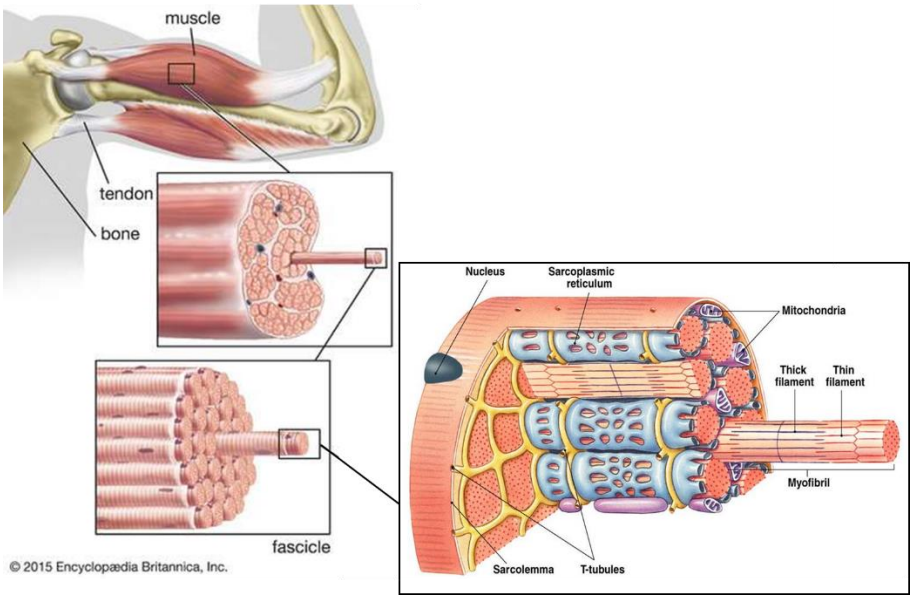

Fig 1. The gross anatomical structure of mature mammalian skeletal muscle. Figure adapted from https://www.britannica.com/science/skeletal-muscle and https://www.austincc.edu/apreview/PhysText/Muscle.html

1.2 MITOCHONDRIA

Mitochondria are important organelles that can be found in almost all eukaryotic cells. They are involved in the various functions of the cells including the adenosine triphosphate (ATP) production, differentiation, Ca^{2+} signaling, and apoptosis (Galluzzi, Kepp, & Kroemer, 2012). Mitochondria generate most of the ATP that the cells consume. They have the ability to adapt to external stimuli in order to meet the increased need for oxygen under anaerobic conditions, through changing their volume, structure, and function (Hood, Memme, Oliveira, & Triolo, 2019). In skeletal muscle, mitochondria play an essential role in regulating the metabolic status of muscle fibers. The adaptation of mitochondria in skeletal muscle can be substantial, where the oxygen consumption rates per gram tissue may increase over tenfold (Hood, Gorski, & Terjung, 1986). During exercise, the enzyme activity and respiration of mitochondria in skeletal muscle increase, which leads to an enhanced oxidative capacity by promoting fatty acid oxidation, glycogen sparing, and less anaerobic glycolysis (Hood, 2001). These changes together, result in an increased mitochondria content in skeletal muscle. The adaptation of mitochondria and mitochondrial biogenesis after exercise stimuli is mediated via a number of different molecular pathways. Among these, signaling cascades initiated by exercise, including increased intracellular Ca^{2+}, higher ATP turnover, and reactive oxygen species (ROS) production has been mostly investigated (Hood et al., 2019). These signaling cascades all lead to the activation of PGC-1α, which has been considered as the major regulator of mitochondrial biogenesis (J. Lin et al., 2002). PGC-1α is a transcriptional coactivator that transcriptionally promotes the expression of nuclear encoded mitochondrial genes by coactivating with various transcriptional factors including PPARγ and NRF1/2, (Finck & Kelly, 2006; Handschin & Spiegelman, 2006). Expressing PGC-1α in skeletal muscle at physiological levels leads to a fiber type conversion, from glycolytic Type II fibers to Type I fibers that are rich in mitochondria (J. Lin et al., 2002). PGC-1α orchestrates the expression of a variety of genes to regulate fuel supply and mitochondrial function, modulating the energy metabolism in skeletal muscle (Correia, Ferreira, & Ruas, 2015). Apart from PGC-1α, Tfam (mitochondrial transcriptional factor a) and p53 also mediate important pathways that regulate mitochondrial biogenesis, by promoting the expression of the mitochondrial genome (Beyfuss, Erlich, Triolo, & Hood, 2018; Theilen, Kunkel, & Tyagi, 2017).

1.3 MITOCHONDRIAL ELECTRON TRANSPORT CHAIN (ETC)

The mitochondrial ETC is a series of protein complexes that catalyze multiple redox reactions to transfer electrons from electron donors to acceptors and generate ATP (Nolfi-Donegan, Braganza, & Shiva, 2020). These reactions also generate low levels of ROS for cellular signaling in the meantime (Theilen et al., 2017). The mitochondrial ETC is comprised of five complexes: Complex I (NADH ubiquinone oxidoreductase), Complex II (succinate dehydrogenase), Complex III (cytochrome bc1 complex), Complex IV (cytochrome c oxidase), and Complex V (ATP synthase). NADH and $FADH_2$ that are generated by the

tricarboxylic acid (TCA) cycle in the mitochondrial matrix, are two electron donors that enter the ETC at either Complex I or Complex II, respectively (Zhao, Jiang, Zhang, & Yu, 2019). The electrons from NADH are transferred to ubiquinone (CoQ) in ETC Complex I through a chain of cofactors. Complex I firstly bind with $NADH+H^+$, and transport 2 H^+ from $NADH+H^+$ to flavin mononucleotide (FMN). Then the electrons are transported to CoQ by iron-sulfur protein (Fe2S2, Fe4S4), reducing it to ubiquinol (QH_2). In this process, the protons (H^+) are pumped from the mitochondrial matrix to the intermembrane space of mitochondria. Subsequently, in Complex III, the reduction of cytochrome c and the oxidation of ubiquinol further transferred protons from the matrix into the intermembrane of mitochondria. Complex IV, which located is in the mitochondrial membrane, receives electrons from cytochrome c and then transfers them to oxygen. During this process, it binds four protons from the inner aqueous phase to make water, also, it translocates four protons across the membrane, helping to establish a transmembrane difference of proton electrochemical potential that the ATP synthase then uses to synthesize ATP.

1.4 NDUFA4L2

NADH dehydrogenase (ubiquinone) 1 alpha subcomplex subunit 4-like 2 genes (NDUFA4L2) is a nuclear-encoded mitochondrial protein. It is homolog to mitochondrial protein NDUFA4. This protein was first identified in mRNA microarray screens in Von Hippel-Lindau (VHL)-deficient cell lines and in cells after hypoxia (Favier et al., 2009; Fredlund, Ovenberger, Borg, & Pahlman, 2008; Papandreou, Cairns, Fontana, Lim, & Denko, 2006). Tello and colleagues described NDUFA4L2 as a downstream target gene of HIF-1α (Tello et al., 2011). The expression of NDUFA4L2 is induced by hypoxia in various tissues. Activated NDUFA4L2 reduced mitochondria Complex I activity in fibroblasts and thus reduced mitochondrial oxygen consumption and ROS production under hypoxia (Tello et al., 2011). Overexpression of NDUFA4L2 in certain tumor cell types is believed to be related to their drug resistance, angiogenesis, proliferation, and malignancy, through controlling their mitochondrial activity and oxygen consumption (Lai et al., 2016; L. Liu et al., 2016; Lucarelli et al., 2018; Lv et al., 2017; Wang et al., 2017). NDUFA4L2 is also involved in the transition of fibroblasts to cancer-associated fibroblasts by mediating its metabolic switch (Zhang et al., 2015). The overall knowledge about NDUFA4L2 is limited and little is known about its exact function in muscle tissues under physiological and pathological conditions.

1.5 PERIPHERAL VASCULAR DISEASE (PVD)

1.5.1 Type 2 diabetes and PVD

Type 2 diabetes (T2D) is characterized by high hyperglycemia and insulin resistance and is known to have a strong correlation with mobility limitation (Bianchi, Zuliani, & Volpato, 2013). Previous studies have shown that muscle strength and muscle quality such as mitochondria function are impaired in T2D patients. (Lauretani et al., 2003; Park et al., 2006; Park et al., 2007; Visser et al., 2002) The progressive loss of skeletal muscle mass and changes in body composition are also proposed to be important factors contributing to the

mortality limitation (Volpato et al., 2012). Complications and comorbidities of T2D are strong determinants of the patients' physical and exercise capacity (Thiruvoipati, Kielhorn, & Armstrong, 2015; Volpato et al., 2002). Among the T2D related comorbidities, peripheral vascular disease (PVD) is of special interest in our laboratory. The presence of T2D greatly increases the risk of PVD, accelerates its progress, and ultimately leads to a serious impairment of patients' functional status (Marso & Hiatt, 2006; M. T. Vogt, Cauley, Kuller, & Nevitt, 1994).

1.5.2 PVD

PVD refers to diseases or disorders affecting vessels outside the heart and brain. PVD most commonly affects the lower extremities, but other vascularized organs such as kidneys can also be affected (Shu & Santulli, 2018). PVD is usually initiated by obstructions or narrowing of the vessels due to atherosclerosis or thrombosis, leading to a lack of blood flow in the diseased organs. In the legs, the subsequent reduction in oxygen and nutrient might cause symptoms like physical and exercise limitations, and less commonly, intermittent claudication, ischemic rest pain, and ulcers (Violi, Basili, Berger, & Hiatt, 2012). However, without proper interventions, there is a considerable risk of developing severe complications including critical limb ischemia (CLI) and associated tissue infection, necrosis, which will ultimately require amputation (Ouriel, 2001). It is commonly reported that patients with PVD, either symptomatic or asymptomatic, have an increased risk of mortality and morbidity (like myocardial infarction and stroke) (Criqui & Aboyans, 2015). it's estimated that ~200 million people worldwide have PVD (Fowkes et al., 2013; Shu & Santulli, 2018). Apart from T2D, other predominant risk factors for developing PVD, include cigarette smoking, kidney disease, aging, hypertension, and lipid disorders (Berger et al., 2013; Fowkes et al., 2013).

1.5.3 Limb symptoms

Patients with PVD can have a diverse range of clinical manifestations. Of all afflicted patients, approximately half are asymptomatic. However, even for those asymptomatic patients, it is common to have a functional decline in their limbs (McDermott, 2015). As the disease progresses and the ischemia of lower extremities worsens, the patients will present atypical limb symptoms or typical intermittent claudication (IC) (McDermott, Mehta, & Greenland, 1999). Atypical limb symptoms are more commonly seen in PVD patients and can be classified into two types: type 1, "leg pain on exertion and rest"; type 2, "leg pain/carry on". The former refers to exertional pain that may start at rest but differs from critical limb ischemia. The latter is exertional leg pain during walking, but the patients can stand it(McDermott, 2015). Intermittent claudication is pain that occurred in the leg after exercising and is relieved during rest. However, as the disease worsens, the pain may occur at rest (Smith, Shipley, & Rose, 1990).

PVD patients with hemodynamic changes consistently show a reduction in their exercise performance and daily physical activities regardless of their limb symptoms (Gardner, 1993). Compared with healthy controls, peak exercise performance in patients with PVD is

decreased by half. In one study enrolling 460 PVD patients, a third of the asymptomatic patients didn't manage to walk over six blocks in one week (which is equivalent to ~ 600-1200 meters) (McDermott et al., 2001).

1.5.4 Treatments

In most conditions, PVD is considered to be a separate and specific disease, with similarities to coronary artery disease (CAD) and cerebrovascular disease (Aronow & Beckman, 2016). However, medical care of patients with PVD is much less emphasized, compared with patients of other atherosclerosis disorders (Pande, Perlstein, Beckman, & Creager, 2011). Besides, the goal of current treatments is more to minimize disability and for cardiovascular prevention, rather than restoration of ambulation and symptom eradication (Sobieszczyk & Beckman, 2015). The prevalence and functional impairments of PVD may be underestimated due to the high percentage of asymptomatic PVD (McDermott, 2015; McDermott et al., 1999). For instance, pathological changes in skeletal muscle including loss of muscle mass, impaired mitochondrial activity, increased reactive oxygen species and increased muscle fat content/fibrosis have been observed even in legs of PVD patients without exertional pain (McDermott, Dayanidhi, et al., 2021; McDermott et al., 2020; McDermott et al., 2001; McDermott et al., 2009). The degree of these pathological changes is strongly associated with the degree of functional impairments, thus skeletal muscles are an important target for the PVD treatment (McDermott et al., 2020; McDermott et al., 2009).

Currently, there are limited medical methods to reduce disability and improve exercise performance in PVD patients. Phosphodiesterase-3 inhibitors, particularly cilostazol, are reported to modestly increase the functional performance of the PVD patients, potentially by vasodilation and inhibition of platelet aggregation (Dawson, Cutler, Meissner, & Strandness, 1998). The pharmacological effects of platelet inhibition or vasodilation are immediate, however, the clinically beneficial effects of cilostazol became more and more evident with time over 24 weeks of treatment indicating a more complex response to the treatment (Brass, 2013). The mechanism of cilostazol treatment in PVD is still unclear but the beneficial effects highlight the importance of further study.

Prescribed treatments for relieving IC include supervised exercise therapy and percutaneous revascularization (Aboyans et al., 2018). A meta-analysis of 30 randomized control trial studies found that exercise therapy significantly increased both walking distance and walking time on the treadmill of patients with IC (Lane, Ellis, Watson, & Leng, 2014). Randomized trials that have included asymptomatic patients suggest that exercise therapy is beneficial even for asymptomatic PVD patients. Even though supervised exercise therapy is shown to be more efficient and cost-effective than unsupervised therapy, it is less used due to reasons such as it is time-consuming and logistically challenging (European Association of Cardiovascular et al., 2010; Fokkenrood et al., 2013; Gommans et al., 2015). Besides, home-based low-intensity exercise was shown to be not significantly different from non-exercise subjects, while high-intensity exercise improved 6-minute walk distance substantially in PVD patients (McDermott, Spring, et al., 2021). The increased nitrate content in plasma after

supervised exercise may improve skeletal muscle perfusion in the lower extremity and lead to a better walking performance in patients with PVD (McDermott, Dayanidhi, et al., 2021).

Despite the lack of high-quality randomized control trials supporting the durability and long term effects of endovascular therapy, it has become the primary treatment for IC, potentially due to factors like less time investment compared to exercise therapy, and immediate alleviation of ischemic symptoms (Fowkes et al., 2013; Lane et al., 2014; Sobieszczyk & Beckman, 2015). According to the 2017 European Society of Cardiology (ESC) guidelines for PVD, endovascular therapies should only be applied when the patients don't benefit from a certain period of exercise or daily activity is greatly affected by the symptoms (Aboyans et al., 2018). Also, there are randomized trials reporting that endovascular revascularization is not advantageous over supervised exercise in improving exercise performance and quality of life of PVD patients (Mazari et al., 2012; T. P. Murphy et al., 2012). Overall, endovascular or surgical revascularization treatments are of limited benefit for the high failure rates of bypass grafts after revascularization and high restenosis rates of endovascular approaches (Conte et al., 2005; Schillinger et al., 2006). Even though the underlying mechanism for the functional deficits in PVD is originally hemodynamic, the reduced blood flow alone does not fully explain the exercise limitations. Even in PVD patients with similar hemodynamic measures (Ankle Brachial Index ratios), symptoms may vary from asymptomatic to intermittent claudication (Ankle Brachial Index et al., 2008).

Additional pathological mechanisms are reported to be involved in the limb symptoms of PVD, including changes in the vasculature and skeletal muscle distal to the narrowing or the obstruction. Limb ischemia is shown to be associated with smaller muscle mass and higher fat content in the limb muscles, and these changes increased rates of motility loss in PVD patients (McDermott, Dayanidhi, et al., 2021; McDermott et al., 2020; McDermott et al., 2009; McDermott et al., 2007). In addition, accumulating evidences point towards mitochondria impairment and increased oxidative stress in the skeletal muscle of PVD patients (Anderson et al., 2009; Kemp et al., 2001; McDermott et al., 2009; Weiss et al., 2013). Besides, the interaction between the vascular system and skeletal muscle, and the stem cell niche (an anatomic location in the tissue that provides a specific microenvironment for stem cells) existing in the muscle, or the muscle-vascular interface also appear to be interesting targets for future studies of PVD treatment. As no effective medical therapies exist so far, it is necessary to think out of the box. Future studies targeting these changes in the lower extremity may potentially provide solutions to the full restoration of limb function for patients with PVD.

1.6 SKELETAL MUSCLE REGENERATION

Skeletal muscle is a highly plastic tissue that can adapt to a variety of physiological or pathological conditions. Upon injury, skeletal muscle can regenerate the lost tissue up to a certain threshold. The high regenerative potential of the skeletal muscle enables it to fully compensate for up to 20% loss of the mass (J. Liu et al., 2018). Muscle regeneration has been described with three main phases; a destructive phase with necrosis and inflammatory

response, a regenerative phase with activation and proliferation of muscle progenitor cells, a remodeling phase of myofiber maturation and scar formation (Laumonier & Menetrey, 2016). Skeletal muscle regeneration is a coordinated action of different populations of muscle resident cells, such as satellite cells, perivascular stem cells, and mesenchymal progenitor cells (M. M. Murphy, Lawson, Mathew, Hutcheson, & Kardon, 2011). Satellite cells have traditionally been implicated as the primary cell population to be responsible for the normal regeneration of skeletal muscles. However, recently published papers have shown the critical role of non-satellite interstitial cells during muscle regeneration, including pericytes and fibro/adipogenic progenitors (FAPs), (Dellavalle et al., 2011; Dellavalle et al., 2007; Giordani et al., 2019; Joe et al., 2010). However, the precise roles of different interstitial cell populations for the regenerative process are still quite unclear, partly due to vague definitions of cell types, and lack of specific molecular markers (Wosczyna & Rando, 2018). Future studies exploring the complex cellular response of different cell types in muscle regeneration may help to understand the physiology and pathology of skeletal muscle regeneration and recovery after injury and damage, and also identify new targets for potential novel treatment to improve muscle function.

1.7 FEMORAL ARTERY LIGATION (FAL)

Small animal models mimicking lower extremity symptoms of PVD are necessary for studying the pathological mechanisms involved in the disease process. ApoE is a protein involved in lipid transport and metabolism, and therefore ApoE knockout (-/-) models were first suggested as a good model for atherosclerosis and the subsequent peripheral vascular disease. However, even though clear atherosclerosis was found in the femoral artery, no blood perfusion loss was detected in the limbs of the mice (Baltgalvis et al., 2014). It is also noted that ApoE−/− mice didn't show any reduction in their capacity to run during an incremental treadmill test in different studies (Baltgalvis et al., 2014; Maxwell et al., 2009; Niebauer et al., 1999). Due to the limited availability of reliable genetic models, surgery-induced arterial occlusion models have been used instead. There are multiple surgical ways of blocking the blood flow to the lower extremities, e.g. a single or double ligation on either the iliac artery proximal to the internal branch or femoral artery proximal to the popliteal artery, with or without removal of a segment of the artery (Fig. 2) (Brenes et al., 2012; Couffinhal et al., 1998; Niiyama, Huang, Rollins, & Cooke, 2009; van Weel et al., 2007). In the case of single knot ligation of either artery, the blood flow to the low extremities is usually restored within 7 days due to the collateral formation and expansion. In comparison, the flow restoration with two knots ligation is significantly delayed with approximately 50% recovery four weeks after surgery (Hellingman et al., 2010). Thus, in our study, we have used double knot ligation together with excision as for studying PVD (Figure 2). Although FAL is an animal model for PVD studies, it to some extent more resembles critical limb ischemia rather than the chronic and gradual process in PVD (J. B. Lin et al., 2015).

1.7.1 Muscle remodeling after FAL

It is reported that the skeletal muscle under ischemia is impaired immediately after the FAL surgery as the muscle fibers get rounder and the extracellular compartment expands (Heemskerk, Strijkers, Drost, van Bochove, & Nicolay, 2007). Almost all fibers turn round and swollen, with almost no nuclei visible on a cross-sectional staining day three after surgery. The recovery of skeletal muscle progress inward, from the peripheral towards the inner region of the muscle within 10 days after FAL surgery (Heemskerk et al., 2007). Muscle remodeling after FAL could be summarized into two phases, the degenerative phase and the regenerative phase (Charge & Rudnicki, 2004). Immediately after the ligation, necrosis of the muscle fibers initiates the degenerative process. Disruption of the sarcolemma increases cell permeability and activates inflammatory and myogenic cells. Activated myogenic cells fused into primary myofibers with centrally located myonuclei. Since the muscle regeneration after FAL followed a centripetal gradient, starting from the outer regions to the central regions. Thus, both degeneration and regeneration areas could appear on the same regenerating muscle (Heemskerk et al., 2007).

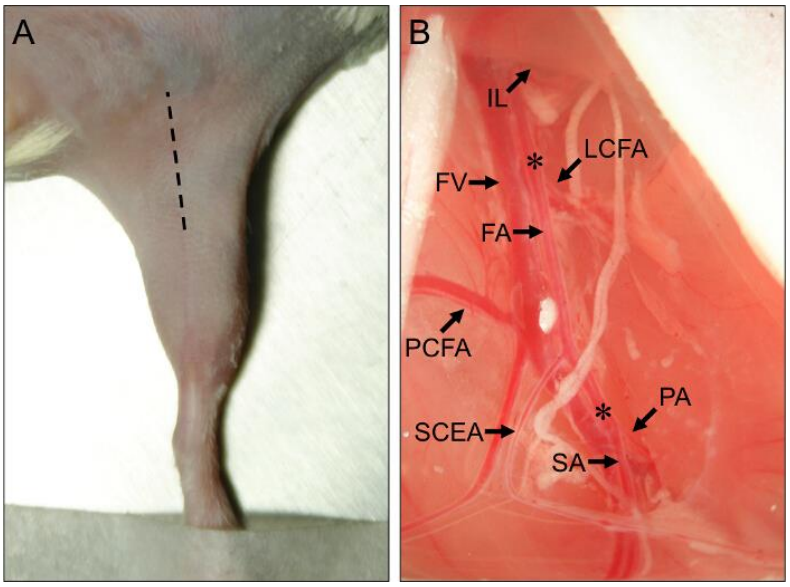

Figure 2. Surgical Site and Vascular anatomy for mouse femoral artery ligation. (A) Hindlimb of a mouse in the supine position. The hatched line indicates the incision of the surgery. (B) View of the proximal mouse hindlimb vasculature. FA: femoral artery; IL: inguinal ligament; PA: popliteal artery; SA: saphenous artery; LCFA: lateral circumflex femoral artery; PCFA: the proximal caudal femoral artery; SCEA: superficial caudal epigastric artery; FV: femoral vein. Asterisks (*) show the proximal and distal sites of ligation. Figure adapted from (Padgett, McCord, McClung, & Kontos, 2016).

This shows that skeletal muscle remodeling plays an important role in the recovery after FAL-induced critical limb ischemia. Indeed, remodeling of skeletal muscle fibers is also noted in PVD patients with a significant modification in their muscle fiber types in the gastrocnemius (McGuigan et al., 2001). Mitochondria dysfunction increased oxidative stress, and a fiber type switch from type II to type I fibers are associated with the myopathy of PVD (Koutakis et al., 2014; Pipinos et al., 2008; Pipinos et al., 2006; Steinacker et al., 2000). The impaired muscle function leads to reduced motility, worse limb function, reduced quality of

life, and eventually higher mortality rates in PVD patients (Evans et al., 2011; Koutakis et al., 2015; McDermott et al., 2012; Thompson et al., 2015).

1.7.2 Inflammatory cells and FAL

In both animal models and human PVD, inflammatory cells play important roles in regulating muscle regeneration and vessel remodeling (Heilmann, Beyersdorf, & Lutter, 2002; Seaman, Cao, Campbell, & Peirce, 2016; Tidball & Villalta, 2010). After muscle injury, inflammatory cells (e.g. neutrophils and macrophages) released from the blood infiltrate the damaged muscles to phagocytose myofiber debris. Besides, cytokines and growth factors such as hepatocyte growth factor (HGF), IL-4, and IL-10 released by inflammatory cells could stimulate myogenesis (Tidball & Villalta, 2010; Villalta, Nguyen, Deng, Gotoh, & Tidball, 2009; Walton et al., 2019). For example, macrophage infiltration after muscle damage supports myogenesis and extracellular matrix (ECM) formation by secreting cytokines like TGFβ or IL-1β. However, dysregulation of cytokine expression or sustained activation of the macrophage may result in aberrant healing such as chronic inflammation and fibrosis, which is, in essence, an excessive accumulation of extracellular matrix components (Grounds, Sorokin, & White, 2005; Kharraz, Guerra, Mann, Serrano, & Munoz-Canoves, 2013). For example, in Duchenne muscular dystrophy (DMD), the perpetual infiltration of macrophages is associated with progressive fibrosis (Desguerre et al., 2009; Serrano & Munoz-Canoves, 2010).

1.7.3 Vascularisation and FAL

The vasculature within the skeletal muscle is a complex network involving capillaries and arterioles includes different cell types such as endothelial cells (EC), pericytes, smooth muscle cells, fibroblasts, and inflammatory cells that interact with the ECM. Neovascularisation post-injury requires delicate interactions between all the factors involved in this complex vascular system, including supporting cells, the extracellular matrix, and circulating blood (Hudlicka, Brown, & Egginton, 1992; Staton, Reed, & Brown, 2009). FAL has been widely used for the mechanistic and therapeutic study of skeletal muscle revascularization. A variety of therapies including cytokine (Becit et al., 2001), gene therapies (Yasumura et al., 2012), nanoparticle-mediated drug delivery (Bickert et al., 2012; Burkhardt et al., 2010; Suzuki et al., 2012), and cell transplantation therapies (Burkhardt et al., 2010) have been studied in this murine model for their angiogenic and arteriogenic potential. However, their clinical potential remains to be concluded.

1.7.4 Mitochondria after FAL

Skeletal muscle is an energy-consuming tissue and mitochondria are an essential energy source for the tissue (Walker, 1991). Besides, mitochondria are the predominant source of ROS production in skeletal muscle (Phaniendra, Jestadi, & Periyasamy, 2015).

Under ischemic conditions, mitochondria OXPHOS and the ETC are inhibited (Dubowitz, Sewry, & Oldfors, 2013; Lu et al., 2014). The changes in mitochondrial metabolism lead to

the accumulation of NAD^+, lactate, Ca^{2+}, H^+, and inhibition of glycolysis in rat limb muscle (Eliason & Wakefield, 2009; Welsh & Lindinger, 1997). It is also reported that increased succinate accumulation during ischemia and the subsequent oxidation after reperfusion is involved in exacerbated mitochondrial ROS production and ischemia-reperfusion injury in animal ischemia-reperfusion models (Chouchani et al., 2014).

Preclinical studies using the unilateral FAL model showed a decreased oxygen consumption in mitochondria isolated from ischemic muscle fibers compared to the control muscle (Pipinos et al., 2008; Ryan et al., 2016). Surprisingly, mitochondria content was found to be significantly increased in ischemic muscle from the ligated legs in a staged ligation model twelve weeks after the induction (first stage, the femoral artery was ligated proximal to the superficial epigastric artery, second stage, two weeks later, the iliac artery was ligated distal to the aortic bifurcation)(Pipinos et al., 2008). In addition to that, patients with PVD experience impaired muscle function in the lower extremities, they also have an increased muscle mitochondria content which is strongly correlated with PVD mortality rates (Makris et al., 2007; Pipinos et al., 2006; Thompson et al., 2015). The increase of mitochondrial content in muscle of PVD patients is potentially due to a switch of ischemic myofibers from fast-twitch fibers to slow-twitch fibers since fast-twitch fibers are more vulnerable to the reduction of energy supply (Steinacker et al., 2000; Thompson et al., 2015) Mitochondrial dysfunction are associated with decreased oxidative phosphorylation and increased oxidative stress, which eventually could contribute to the PVD claudication (Dubowitz et al., 2013; Makris et al., 2007; Pipinos et al., 2007).

Blunted arteriogenesis and angiogenesis in T2D exacerbated vascular alteration in PVD patients, however, they cannot adequately explain the functional deficits in skeletal muscle (Leenders et al., 2013; Park et al., 2007). T2D is known to be associated with reduced PGC1α expression, ETC activity, altered mitochondrial morphology, and lower mitochondria content (Kelley, He, Menshikova, & Ritov, 2002; Mootha et al., 2003; Patti et al., 2003). Mitochondrial dysfunction and increased mitochondrial oxidative stress are observed in T2D, which in turn is thought to increase the susceptibility of PVD patients with T2D to develop muscle impairments (Ryan et al., 2016). Muscle dysfunction is more prominent in mice fed a high-fat diet (HFD) after FAL, with increased tissue necrosis, impaired muscle regeneration, and exacerbated mitochondrial dysfunction. Overexpressing catalase in mitochondria of muscles following FAL rescued the HFD-induced myopathy (Ryan et al., 2016). These findings demonstrated mitochondria as a potential pathological link between T2D and PVD. Our study exploring the role of mitochondrial protein NDUFA4L2 in muscle remodeling of PVD may reveal further insight into PVD myopathy and may identify novel targets for future treatments of PVD.

Our results have shown an upregulation of mitochondrial protein, NDUFA4L2 in ischemic muscles after FAL, while the activation is blunted in obese mice. The role of mitochondrial protein, NDUFA4L2 in skeletal muscle remodeling induced by PVD is of great interest to us.

Our studies on NDUFA4L2 may clarify how skeletal muscles respond to ischemia and hypoxia induced by FAL and potentially reveal a novel target for PVD treatment.

1.8 KYNURENINE PATHWAY

Over 90% of peripheral tryptophan, an essential amino acid, is known to be metabolized by the kynurenine (Kyn) pathway. Kyn and many of its metabolites have been reported to have neuroactive and/or immunomodulatory properties and may contribute to depression (Claes et al., 2011; Muller & Schwarz, 2007; Myint & Kim, 2014). Previous study has indicated that PGC-1α regulate a muscle to brain crosstalk after aerobic training, which may protect against stress-induced depression (Agudelo et al., 2014). This effect of PGC-1α is mediated via the increased expression of several kynurenine aminotransferases (Kats) in skeletal muscle. Unlike kynurenic acid (Kyna), Kyn can readily cross the blood-brain barrier and contribute to around 60% of Kyn in the brain, which may be associated with several mental disorders (Dadvar, Ferreira, Cervenka, & Ruas, 2018; Fukui, Schwarcz, Rapoport, Takada, & Smith, 1991; Gal & Sherman, 1980). The increased Kats in skeletal muscle converts Kyn into Kyna, which can further increase the energy expenditure of adipose tissue and promotes an anti-inflammatory effect (Agudelo et al., 2018).

2 RESEARCH AIMS

The overall aim of this thesis is to study mitochondrial function and regulation in skeletal muscle during healthy conditions and after FAL, with a special interest in nuclear-encoded mitochondrial protein NDUFA4L2. This thesis is further divided into 4 sub-aims：

1. Clarify the role of NDUFA4L2 in the regulation of skeletal muscle mass and function

2. Define the mechanism behind the skeletal muscle remodeling in response to femoral artery ligation in normal and obese mice

3. Elucidate the effects of genetic deletion of mitochondrial complex I component NDUFA4L2 in skeletal muscle.

4. Illuminate the role of PGC-1α1, the pivotal coactivator of mitochondria regulation, in the adaptation of skeletal muscle to fatigue resistance.

3 MATERIALS AND METHODS

3.1 ANIMAL EXPERIMENTS

3.1.1 Ethical permits

All studies involving animal experiments were performed according to the Swedish Animal Welfare Act, the Swedish Welfare ordinance and applicable regulations and recommendations from the Swedish authorities. The studies were approved by the Stockholm North Ethical Committee on Animal Experiments (see respective ethical numbers in the different papers)

3.1.2 AKO-NDUFA4L2 MICE

We have generated a NDUFA4L2 null mouse line (AKO-NDUFA4L2) by crossing NDUFA4L2fl/fl mice, which carries LoxP sequences surrounding exon two of the NDUFA4L2 gene, with beta-actin-cre transgenic mice. Heterozygous mice were then mated to obtain NDUFA4L2 −/− mice.

3.1.3 FAL

Unilateral FAL was induced in the mice according to a previous publication (Niiyama et al., 2009). Anaesthesia was induced with isoflurane and the mice were kept on a heating blanket throughout the experiment. The proximal and distal part of the femoral artery was occluded with two sets of double knots ligation and the femoral artery was cut off in between the double knots. The incision was sutured, and mice were placed back in their cage to recovery. The mice were sacrificed at different time points (2, 8, 15, 30 day) after the surgery for tissue collection

3.1.4 In vivo electroporation

We performed *in vivo* electroporation in flexor digitorum brevis (FDB) muscle to transfect either pcDNA.3-NDUFA4L2 plasmids or empty vectors into the muscle. In brief, after anesthetizing the mice with isoflurane, we inject 10 µL of hyaluronidase solution (20mg/mL) into both footpads of the mice. After one hour, the plasmid was injected into the footpad. In co-electroporation experiments, the pcDNA.3-NDUFA4L2 plasmids or empty vectors were combined with 20 µg of the DNA plasmid pEGFP-N. 15 min after the injection, two acupuncture needles were placed under the skin of the feet, one in the heel and the other one close to the toes. The muscles were electroplated for 20 pulses with 20 ms duration/pulse at 100V. The mice were sacrificed seven days after the transfection for tissue collection.

3.1.5 Measurement of blood flow

Laser speckle (Moor Instruments) was used to measure blood flow in the lower limbs at day 0 and day 8 after FAL surgery. Three measurements were obtained from each animal, and we calculated their average blood flow ratio (Limbourg et al., 2009).

3.2 *EX VIVO* MUSCLE FORCE MEASUREMENT

Ex vivo force production was measured in intact soleus and extensor digitorum longus (EDL) muscle. The muscles were excised directly after sacrifice and incubated in a Tyrode solution (in mM): 121 NaCl, 5 KCl, 1.8 $CaCl_2$, 0.4 NaH_2PO_4, 0.5 $MgCl_2$, 24 $NaHCO_3$, 0.1 EDTA, and 5.5 glucose) with a pH kept at 7.4 by continuous superfusion with carbogen (95% O_2 /5% CO_2). The muscles were then transferred to a stimulation chamber filled with Tyrode solution, and were mounted between a force transducer with adjustable holders. The muscle length was adjusted to optimal length and the muscles were then stimulated at different frequencies (1Hz to 120 Hz, 1000 ms tetanic duration for the soleus muscles; 1Hz to 150 Hz, 300 ms tetanic duration for the EDL muscles) to determine a force-frequency relationship. The muscle cross-sectional area (CSA) was calculated by dividing muscle mass by the product of muscle length and muscle density (1.06 g/cm3) and then use the CSA was used for muscle specific force (kN/m2) calculations.

3.3 IMMUNOHISTOCHEMISTRY STAINING

Whole EDL muscles were frozen in ice-cold isopentane in a liquid nitrogen tank. The frozen muscles were sectioned with a cryostat set at -20 °C and thickness at 7μm. For wheat germ agglutinin (WGA) staining, the muscle sections were dried at room temperature for over one hour, fixed with 4% paraformaldehyde, and permeabilized. The slides were incubated in WGA (1:50 for 30 min; Alexa Texas Red, #W21405, Invitrogen), and the nuclei were stained with DAPI (ProLong™ Diamond Antifade Mountant; P36961, Thermo Fisher). For oil red O staining, the muscle sections were thawed in 4% paraformaldehyde, rinsed with 60% isopropanol, and stained with freshly prepared oil red O working solution for 15 mins, and rinsed again with 60% isopropanol before mounting. All the figures were quantified with Image J.

3.4 RNA ISOLATION, REVERSE TRANSCRIPTION, AND QPCR

Total RNA was isolated from skeletal muscles using TRIzol reagent (Life Technologies, Stockholm, Sweden) according to the manufacturer's manual. RNA samples were treated with TURBO DNase (Life Technologies) and underwent reverse transcription with oligo(dT) primer and Superscript III reverse transcriptase (Life Technologies) according to the manufacturer's instructions. qPCR was performed by mixing 5 μL iTaq Universal SYBR Green Supermix (2x) (Bio-Rad Laboratories AB, Solna, Sweden), 2 μL of cDNA (1/10 or 1/20 dilution), 0.4 μL forward and reverse primers, and 2.2 μL of water in a 10 μL final volume.

3.5 WESTERN BLOTTING

Frozen muscles or cells were homogenized in lysis buffer (in mM): 20 HEPES, 150 NaCl, 5 EDTA, 25 NaF, 1 Na_3VO_4, 5% glycerol (v/v), 0.5% Triton X (v/v), protease inhibitor cocktail (#11836145001, Roche, 1 tablet per 50 ml), and then centrifuged at 700g for 10 min at 4 °C. After quantifying the protein content of the homogenates using Bio-Rad Protein

Assay (#500-0006), the samples were diluted with loading buffer and heated at 95°C for 5 min. Equal amounts of protein were then loaded onto the electrophoresis gel and run for 60-90 min at 150V. Then the gel was wet transferred to a polyvinylidene fluoride membrane for 3 hours at 100V. After that, the membrane was incubated for 60 min in LI-COR blocking buffer (927-40000, LI-COR). Then the membrane was incubated with primary antibody overnight at 4 °C. The next day, the membrane was washed (3 x 20 min in TBS-T) and incubated with secondary antibody at room temperature (1h). The bands on the membrane were then visualized and analysed with infrared fluorescence. The bands' density was either normalized against total protein content or a certain reference protein.

3.6 SEAHORSE EXPERIMENT

FDB muscles were isolated from mice immediately after sacrifice and kept in a Tyrode solution (in mM): 121 NaCl, 5 KCl, 1.8 $CaCl_2$, 0.4 NaH_2PO_4, 0.5 $MgCl_2$, 24 $NaHCO_3$, 0.1 EDTA, and 5.5 glucose). Each muscle was placed in a six-well plate well containing 3 ml of Dulbecco's modified Eagle's medium (DMEM) supplemented with 3mg/ml collagenase type I and 10% fetal bovine serum (FBS) for approximately 60 to 80 min at 37°C and 5% CO_2. After digestion, each muscle was transferred into a well of a new six-well plate with fresh DMEM supplemented with 10% FBS. Each muscle was pipette ~10 times up and down with a 1000 µl pipette tip to dissociate fibers. Dissociated fibers were transferred to laminin-coated "Seahorse, cell culture microplate" and were allowed to attach for 15-30 min in 37°C, 5% CO_2 incubator. DMEM cell culture media were exchanged with Seahorse-Tyrode solution and fibers were incubated in a CO_2-free incubator for 1h. Oligomycin [5 µM], FCCP [5 µM], and antimycin A [5 µM] were loaded into the seahorse microplate cartridge. The cell plates were loaded in the Seahorse machine and seahorse XF mitochondrial stress assay was performed. The final working concentrations of oligomycin, FCCP, and antimycin A were 1 µM, 1,2 µM, and 1 µM, respectively.

3.7 MITOSOX LIVE CELL IMAGING

FDB muscles were isolated from mice immediately after sacrifice. The muscles were dissociated in 0.3% collagenase type I (C0130-500MG, Sigma-Aldrich, 30mg in 10 ml DMEM supplemented with 10%FBS) for 2 hours. the dissociated muscwere then transferred to fresh DMEM + 10% FBS to stop the digestion. The muscle fibers were fully separated by pipetting carefully and slowly. Appropriate amount of media containing muscle fibers were transferred to a laminin coated dish with a glass bottom in the middle. Fibers were allowed to attach for 15 mins, and then more DMEM were added to the dish.

The mitoSOX solution was prepared while keeping the agents light protected. 50ug of mitoSOX (M36008, Thermo Fisher) was diluted with 50 µl pluronic F127 and 50 µl DMSO to make a 0.5mM stock solution. The mitoSOX stock solution was diluted with DMEM 1:100 to reach a final concentration of 5 µM and the cells were incubated with 5 µM mitoSOX for 15 min. The mitoSOX solution were then removed and the dishes were perfused with Tyrode solution with continuous superfusion with carbogen. The cells were

perfused with Tyrode solution for 10 min and then switched to 0.25uM FCCP diluted in Tyrode for 20 min, while being scanned with a confocal microscope with a two min interval. All images were quantified with ImageJ.

3.8 MITOCHONDRIAL RESPIRATION IN PERMEABILIZED FDB FIBERS

BIOPS and MIR05 (mitochondrial respiration media) solutions were prepared in advance and kept at 4˚C. The composition of BIOPS solution is as follows (in mM):10 Ca-EGTA buffer, 10-4 free Ca2+, 20 imidazole, 20 taurine, 50 K-MES, 0.5 DTT, 6.56 MgCl2, 5.77 ATP, 15 phosphocreatine, pH 7.1. The MIR05 solution is prepared as follows (in mM): 0.5 EGTA, 3 MgCl2, 60 lactobionate, 20 taurine, 10 KH_2PO_4, 20 HEPES and 110 sucrose, with 1 g/L fatty-acid free BSA, pH 7.1.

After sacrificing the mice, FDB muscles were directly taken and cleaned with ice-cold BIOPS solution. The fiber bundles were separated under microscope and transferred the separated bundles to cold BIOPS with saponin (50 µg/mL). The fiber bundles were gently shaken for 30 min at 4°C to permeabilize the sarcolemma without damaging the mitochondria and intracellular structures (P. K. Vogt, Jiang, & Aoki, 2005). After that, the fiber bundles were washed with cold MIR05 solution for 10 min at 4°C. The buffer was removed from the fiber bundles with filter papers carefully. The muscles were weighed, and immediately placed into the Oxygraph-2k high-resolution respirometer (Oroboros Instruments, Innsbruck, Austria) containing MiR05 at 37°C (Wollenman, Vander Ploeg, Miller, Zhang, & Bazil, 2017).

Oxygen flux in real-time was monitored by measuring changes in the negative time derivative of the chamber oxygen concentration signal following background calibrations using Datlab software (Oroboros Instruments). Oxygen consumptions were measured at basal condition and after adding saturating levels of sodium pyruvate (10 mM), malate (2 mM), glutamate (10 mM) and ADP (5 mM), to determine the basal mitochondrial respiration and the maximal O_2 consumption from complex I.

Saturating levels of succinate was then added (10 mM) to determine the maximal O_2 consumption from complexes I and II. Cytochrome c (10 µM) was then added to confirm mitochondrial integrity. Rotenone (0.5 µM) was then added to inhibit Complex I and evaluate the mitochondrial respiration from complex II. Antimycin (1 µM) was added to inhibit complex III and thus blocking the mitochondrial electron flux. Any residual oxygen consumption cannot be attributed to mitochondrial respiration after oligomycin addition. The respiratory rate of oligomycin state was used for background calculation of O_2 and was subtracted from the previous values in the respiratory analysis. After the assay was finished the fibers were collected, centrifugated at 10,000g, 4˚C to take out the MIR05 buffer and stored at -80˚C for citrate synthase (CS) analysis.

3.9 TRANSCRIPTOMIC ANALYSIS

RNA-seq of skeletal muscle (TA) from mice fed on ND and HFD 15 days after FAL were performed at National Genomics Infrastructure (NGI, https://www.scilifelab.se/units/ngi-stockholm/). DESeq2 (Version 1.34.0) has been used for differential analyses between different group with the count data (Love, Huber, & Anders, 2014). Differentially expressed genes (adjusted p-value [adjusted with Benjamini and Hochberg method] < 0.05) were used for downstream pathway analysis. Gene Set Enrichment Analysis (GSEA) of differential genes was performed by referring to a predefined gene set (Gene Ontology Sets [GO], obtained from MSigDB [Molecular Signatures Database, http://software.broadinstitute.org/gsea/msigdb]) to obtain a list of enriched pathways. Over-Representation Analysis (ORA) were also performed for the pathway analysis of differential genes with predefined gene sets (Kyoto Encyclopedia of Genes and Genomes [KEGG]). The pathway analyses were performed and visualized with ClusterProfiler (version 4.2.2) (Wu et al., 2021).

3.10 CELL PROPORTION INFERRING WITH CIBERSORTX AND IMMUCC

CIBERSORTx and seq-ImmuCC were employed for imputing cell-type specific gene expression profiles and inferring proportion of different cell types in skeletal muscles with and without a FAL insult by deconvoluting our RNA-seq results (Chen et al., 2018; Newman et al., 2019). CIBERSORTx requires a single-cell transcriptomic matrix as input to generate an optimized expression profiles that discriminates different cell types termed as “signature matrix”. Single-cell datasets of skeletal muscle from Tabula Muris and single-nucleus sequencing datasets of 5-month tibialis anterior (TA) from previous publications were used for generating the signature matrix (Petrany et al., 2020; Tabula Muris et al., 2018).

3.11 KINASE ACTIVITY ASSAY WITH PAMCHIP®

Lysates of skeletal muscles were prepared with M-PER buffer supplemented with phosphatase and protease inhibitors. The lysate (5 µg) was applied on the PamChip®. In the meantime, a tyrosine peptide kinase PamChip® (Pamgene) was blocked with 2% bovine serum albumin on PamStation® 12 System (Pamgene). The kinase buffer was prepared as follow and chilled on ice: 1× PK buffer, 1× PTK additive, 10 mM DTT, 0.5 mg/mL bovine serum albumin, 400 µM ATP, and 5 µg/mL PY20-FITC. The kinase buffer was immediately added to 5 µg of lysate and applied to the tyrosine peptide kinase PamChip®. The assay was run with a standard protocol of the PamStation® 12 System. For the serine/threonine peptide kinase PamChip® (Pamgene), the kinase buffer was prepared as follows: 1× PK buffer, 1× STK antibody mix, 0.5 mg/Ml bovine serum albumin, and 400 Mm ATP. Similarly, the buffer ice chilled and immediately added to 5 µg of lysate and applied to the PamChip®. The assay was also run with a standard protocol. After that, detection mix (1× antibody buffer, 5 µg/mL STK antibody FITC-labelled) was prepared and added to the PamChip®. Detection was run on a PamStation® 12 System with standard protocol.

The resulting data were quantified and linked to peptide using BioNavigator®. Each peptide was assigned a score and kinase score was calculated as an average of the scores for each associated peptide in its set. Top 35 kinases based on the score were used for pathway enrichment by referring to KEGG and Reactome pathway databases.

3.12 METABOLOMIC ANALYSIS

Metabolomic data from skeletal muscle of mck-PGC-1α1 mice were analyzed. The enrichment analysis of metabolites was performed with MetaboAnalystR package (Xia, Sinelnikov, Han, & Wishart, 2015; Xia & Wishart, 2016). The same package was also used for integrating transcriptomic and metabolomic data (Hatazawa et al., 2015).

3.13 PROTEIOMIC ANALYSIS

Publicly available skeletal muscle proteomics data from murine and humans obtained after exercise training were analyzed (Egan et al., 2011; Hussey et al., 2013; Kleinert et al., 2018).

3.14 STATISTICAL ANALYSIS

All data are expressed as mean ± S.E.M. Two-tailed unpaired t-tests were used in the case of two-group comparison. To compare significant differences in the muscle force measurement between the groups, two-way repeated-measures ANOVA (for the force measurements) were used. In all analyses, significance was accepted at $P < 0.05$.

4 RESULTS AND DICUSSION

4.1 EFFECT OF MITOCHONDRIAL PROTEIN NDUFA4L2 IN REGULATING SKELETAL MUSCLE MASS AND FORCE

Skeletal muscle has the capacity to adapt to a variety of stimuli such as hypoxia, through altering its size and contractile properties. Physiological intermittent local hypoxia which can be caused by high energy demand during exercise may lead to subsequent glycolytic adaptation. However, the persistency of severe local hypoxia may generate a negative effect on skeletal muscle function (Chaillou & Lanner, 2016; Howald & Hoppeler, 2003; Levett et al., 2015; Murray, 2016).

4.1.1 NDUFA4L2 is induced by hypoxia in myotubes and skeletal muscle fibers

It has been reported that hypoxia induces the expression of NDUFA4L2, a downstream target of HIF-1α, in several different tissues or cell lines (Lai et al., 2016; Tello et al., 2011). However, the expression and role of NDUFA4L2 in skeletal muscle under hypoxia are still under exploration. **In paper I,** murine myotubes and adult isolated muscle fibers were exposed to normoxia (Nx, 20% O_2) or hypoxia (Hx, 1% O_2) for 24 h. We observed a drastic increase in the expression of NDUFA4L2 in myotubes and muscle fibers, together with the increased expression of HIF-1 targets (Paper I: Fig 1A, C), which is in line with the observations from other cell lines (Tello et al., 2011) (Bellot et al., 2009; Guo et al., 2001). Hypoxia also induced the expression of autophagic gene Bnip3 and atrophy-related gene Atrogin1, but no difference was observed in the expression of ubiquitin ligases muscle RING finger 1 (Murf1) or mitochondrial ubiquitin ligase activator of NFκB 1 (Mul1) in myotubes. While in FDB fibers, 24h exposure increased the mRNA expression of Atrogin 1 and Mul1.

4.1.2 Ectopically expressed NDUFA4L2 is localized to mitochondria and leads to reduced mitochondrial respiration and lower levels of intramuscular adenine nucleotides and NAD^+

We used an *in vivo* electroporation method to transfect a plasmid encoding NDUFA4L2 into skeletal muscle (FDB) of female C57BL6jRj mice, and an empty vector (mock) into the contralateral FDB muscle. Mice were sacrificed seven days after the transfection. The successful transfection of NDUFA4L2 into the skeletal muscle was confirmed with qPCR and immunoblotting. The ectopically expressed NDUFA4L2 protein colocalized with the mitochondrial voltage-dependent anion channel (VDAC) in the immunofluorescent staining of FDB fibers, which indicated that NDUFA4L2 is localized to the mitochondria.

Previous publications have suggested that NDUFAL2 expression is associated with a reduced ETC function (Tello et al., 2011). Our high-resolution respirometry with permeabilized FDB muscles showed that there was no difference in proton leak between NDUFA4L2 transfected and control muscle, while the maximal O_2 consumption from mitochondrial Complex I, Complex II, as also from Complex I and Complex II together, were significantly reduced in NDUFA4L2 transfected muscles.

We also used mass spectrometry (LC/MS) to quantify intramuscular metabolites after the transfection, which showed the levels of AMP, ADP and ATP were significantly lower in muscle with ectopically expressed NDUFA4L2 than control muscle, whereas the AMP/ATP and ADP/ATP ratios were not significantly altered.

NAD^+ and NADH are molecules that play an important role in mitochondrial respiration. It has been reported that a reduced level of intramuscular NAD+ is associated with mitochondrial dysfunction and age-associated sarcopenia (Migliavacca et al., 2019). We quantified the levels of intramuscular NAD^+ and NADH with LC/MS. We found that muscles with ectopically expressed NDUFA4L2 had significantly less NAD^+ compared with muscle from the contralateral legs, while no significant differences were observed in the level of NADH. We have also measured the protein expression of ETC complexes after ectopically expressing NDUFA4L2 by using a cocktail antibody in immunoblotting (Complex I: NDUFAB8; Complex II: SDHB; Complex III: UQCRC2; Complex IV: MTCO1; Complex V: ATP5A), but no difference was observed (Paper I: Fig 2J). It appears that the reduction of mitochondrial respiration in muscles ectopically expressing NDUFA4L2 is not due to altered ETC protein levels. Instead, these results indicate that NDUFA4L2 acts allosterically as an inhibitory subunit of the ETC complexes in skeletal muscle, which leads to reduced oxygen consumption, and lower adenine nucleotide and NAD^+ levels in skeletal muscles.

4.1.3 Ectopic NDUFA4L2 expression induces muscle atrophy

We also measured the muscle mass and muscle force production in muscle with ectopically expressed NDUFA4L2. We observed a ~20% reduction in skeletal muscle size seven days after NDUFA4L2 electroporation. In NDUFA4L2 ectopically expressed TA muscles, fiber cross-sectional area (FCSA) was reduced and the distribution of FCSA shifted towards the left, i.e., FCSA tends to be smaller. In FDB muscles electroporated with an NDUFA4L2 encoding plasmid, the fiber diameters were found to be smaller. These results suggest that NDUFA4L2 expression generated a negative effect on skeletal muscle size. However, we also noticed that the negative impact of NDUFA4L2 on skeletal muscle is not mediated via the expression of transcription factors involved in muscle fiber regeneration (MyoD and Myogenin) or differentiation (Mef2D). And we didn't observe any signs of increased proliferation as assessed by counting Ki-67+ cells in cross-sectional stainings.

4.1.4 Reduced force production and higher expression levels of atrophy-related genes in muscle with ectopically expressed NDUFA4L2

In Paper I, we further assessed if ectopic expression of NDUFA4L2 has any effect on muscle force production with an *ex vivo* force measurement. As expected, the reduced muscle mass is accompanied by a reduction in the absolute force production of the muscles. However, the specific force production, which is calculated by normalizing the absolute force to the muscle weight, remained unchanged between the groups. This indicated that the reduction of absolute force was due to the smaller muscle size of the NDUFA4L2 ectopically expressed muscle, which was ~20 % smaller than the controls (Paper I: Fig X). Besides, the reduced capacity of force production of the ectopically expressing NDUFA4L2 muscles was also

observed in the fatigue training and recovery experiments, where the force of these muscles remained lower throughout the fatigue stimulation and recovery stage. These fatigue experiments assess intrinsic properties of the FDB muscle and challenge the creatine phosphate system and the glycolytic system and hence not the oxidative aerobic system in which NDUFA4L2 interferes. Since when the muscle size was normalized to the mass, there was no difference in the rate of fatigue or recovery.

Muscle atrophy is a complex process that involves various pathways which control muscle degradation. Activation of ubiquitin-proteasome pathway, autophagy-lysosome pathway (Milan et al., 2015; Sandri, 2013), and E3 ubiquitin ligases including Murf1, Atrogin 1, and Mul1are known to be associated with skeletal muscle remodeling and atrophy (Bodine & Baehr, 2014; Palus, von Haehling, & Springer, 2014). In order to assess the role of these pathways in the loss of skeletal muscle mass after electroporation, we measured the expression of these genes with RT-qPCR. In muscles with ectopically expressed NDUFA4L2, we observed an increased expression of MurF1 and Mul1, but not Atrogin1 (Paper I: Fig 4H). Since Murf1 and Atrogin1were reported to be downstream of Forkhead box Os (FOXOs), we also assessed the expression of genes in the FoxO family including FoxO1, FoxO3 and FoxO4, but no difference was observed (Paper I: Fig 4I). Besides, the gene expression of apoptotic genes caspase 3 (Casp-3) and BCL2-associated X protein (Bax), but not the Casp7 or the anti-apoptotic Bcl2 were significantly induced by ectopic expression of NDUFA4L2. Furthermore, we observed no difference in the expression of autophagic genes (Beclin1, Map1lc3b, and Bnip3) or the conversion of the autophagosome biomarker LC3-I to LC3-II after ectopic NDUFA4L2 expression (Paper I: Fig 4O-P). Even though increased protein degradation reduced anabolic signaling and protein synthesis can also lead to a smaller muscle mass, we observed no difference in protein synthesis rate assessed by puromycin incorporation in primary myocytes, or in the activation of Akt and mTOR signaling. These results indicated that NDUFA4L2 contributed to muscle atrophy through a cascade of reactions including the induction of Murf1, Mul1, Casp-3 and Bax.

4.1.5 Ndufa4l2 mRNA expression is inversely related to force production in skeletal muscle after FAL

In Paper I, we also used a unilateral FAL model to induce muscle ischemia to test if NDUFA4L2 was induced in this process associated with hypoxia. We collected the muscles eight days after the surgery, which is a time point comparable to the overexpression experiments. We observed that the blood perfusion of the ischemic leg was ~50% lower than in the contralateral control leg. The fiber cross-sectional area of the EDL muscle was significantly reduced after the surgery, together with an enhanced presence of fibrosis, which was characteristic of muscle regeneration. Both the gene and protein level of NDUFA4L2 were significantly increased after FAL, together with a dramatic reduction in the force production of both EDL and soleus muscles (Paper I: Fig 1D-G). The gene expression of NDUFA4L2 was inversely related to the specific force production (R^2=0.89). Besides, we also observed an increased expression of Murf1 and Mul1, but not Atrogin1 after FAL, which is consistent with the changes after ectopically expressing NDUFA4L2. FAL induced

expression of the apoptosis-related genes Casp3, Casp7, and Bax, as well as the autophagic genes Bnip3, Beclin1 and Map1lc3b. These results show that together with genes well established for their importance in muscle remodeling, NDUFA4L2 is induced by muscle injury after FAL.

4.2 SKELETAL MUSCLE REMODELING IS A RESPONSE TO FEMORAL ARTERY LIGATION IN NORMAL AND OBESE MICE

T2D is characterized by hyperglycemia and insulin resistance (Bianchi et al., 2013). In addition to not being able to maintain normal glucose levels, patients with obesity and T2D often exhibit poor physical performance, which together limits the individual's ability to move and reduces the quality of life. Previous studies have shown that muscle strength and muscle quality, including mitochondria function are impaired in T2D patients (Lauretani et al., 2003; Park et al., 2006; Park et al., 2007; Visser et al., 2002). Complications and comorbidities of T2D, including PVD, are strong determinants of the patients' exercise capacity (Thiruvoipati et al., 2015; Volpato et al., 2002). It has been reported that functional decline in the lower limbs is commonly seen in patients with PVD, even when they are asymptomatic (McDermott, 2015). **In Paper II,** we used unilateral FAL as a mouse model of PVD on normal C57BL/6 mice and sacrificed them 2, 8, 15, and 30 days after the surgery. Our *ex vivo* force measurement of intact EDL and soleus muscles showed that muscles from the ischemic leg (FAL) generated almost no force two days after the surgery, and then gradually recovered and returned close to normal 30 days after FAL surgery (Paper II: Fig 2A-D, Supplemented Figure S1A-L).

4.2.1 The recovery of muscle force production after FAL was blunted in mice fed on HFD

Since the presence of T2D and obesity greatly increases the risk of PVD, accelerates its progress, and ultimately leads to a serious impairment of patients' functional status (Marso & Hiatt, 2006; M. T. Vogt et al., 1994), we further induced obesity in these C57BL/6 mice by putting them on an HFD (60% of calories comes from fat) for eight weeks. FAL surgery was then performed, and these mice were sacrificed 8 and 15 days after the surgery. In contrast to the ischemic muscles from mice on a normal diet, muscles from the ischemic legs of the obese mice generated almost no force even 8 days post FAL and were weaker also at day 15, indicating that the recovery of the muscle force from FAL surgery is impaired by HFD (Paper II, Fig 1E-H).

We further measured the weight of the muscles to see if the muscle weakness is associated with atrophy. We found that before surgery, muscle weight of soleus, but not EDL, was higher in mice with diet-induced obesity than muscles of mice on ND. This is consistent with previous findings from humans and animal models showing that pre-diabetics can have increased muscle mass (MacDonald et al., 2020; Messa et al., 2020; Park et al., 2006). Nevertheless, muscle weight of both soleus and EDL were lower in mice fed HFD than in

mice fed ND at day 15 post FAL surgery, confirming an impaired muscle recovery in obese mice as we observed with force measurement.

4.2.2 Fibrosis and ectopic fat accumulation induced in ischemic skeletal muscles is strongly exacerbated by 8 weeks of HFD

Previous investigations have demonstrated that hyperglycemia in patients with diabetes can lead to excess extracellular matrix (ECM) accumulation and fibrosis (Kolset, Reinholt, & Jenssen, 2012; Russo & Frangogiannis, 2016). There is also a causal link between insulin resistance in mice fed a high-fat diet and ECM remodeling, a process characterized of increased collagen synthesis and expansion(Kang et al., 2011). For instance, Kang et al have shown that mice fed HFD had insulin resistance and ECM remodeling, while rescuing the muscle insulin resistance by overexpressing mitochondrial-targeted catalase in skeletal muscle or by phosphodiesterase 5a inhibitor, sildenafil, reversed the effect of HFD on ECM remodeling (Kang et al., 2011). To determine whether fibrosis and ECM accumulation were present in the muscle recovering from FAL, we used immunofluorescence imaging and stained connective tissues with WGA and Col1a1 in control and ischemic muscles 15 days post FAL (Paper II, Fig 2A-D). A higher amount of fibrosis was observed in ischemic muscle from mice on HFD than in mice on ND. Moreover, the FCSA tended to be smaller in FAL muscles from HFD mice than mice fed normal diet. Expression of genes that are involved in fibrosis (Postn, Col1a1, Col3a1, Fn, Vim, Acta2, and Col4a1) were all dramatically increased in ischemic muscles 15 days after FAL, but no significant effect of the HFD was observed.

Lipid accumulation, also referred to as fat degradation or steatosis, can occur when the early regeneration processes are altered in skeletal muscle and may lead to muscle weakness (Gumucio et al., 2019; Pagano et al., 2015). In Paper II, lipid accumulation in the ischemic and control muscles 15 days after the surgery was assessed with oil red-O staining. We found that FAL resulted in a significant increase of ectopic fat accumulation in interstitial areas of ischemic muscles of mice fed on ND, which was exacerbated in the ischemic muscles from mice that fed HFD. Thus, our results demonstrate that the fibrosis and ectopic fat accumulation in muscles after FAL are more serious in mice fed HFD, which may ultimately lead to a blunted recovery and worse muscle function (Paper II: Fig 2A-F).

4.2.3 Impaired mitochondrial function in ischemic skeletal muscles from obese mice

Previous studies found that mitochondria dysfunction and excess production of reactive oxygen species (ROS) are closely related to the development of fibrosis (Li et al., 2020; Prado et al., 2018). Furthermore, dysregulated mitochondria and reduced fatty acid oxidation may lead to ectopic lipid accumulation (Gumucio et al., 2019). To examine whether mitochondrial dysfunction is involved in the impaired regeneration in obese mice, we firstly assessed mitochondrial respiration by Seahorse XF in the skeletal muscle fibers isolated from control and ischemic FDB muscles 15 days after surgery from mice fed on HFD or control diet. Muscle fibers from mice on HFD appeared to have a lower basal oxygen consumption

rate and higher spare respiratory capacity than fibers from mice on normal diet, which indicates that muscular mitochondrial function was readily altered by the diet. Proton leak tended to be higher in ischemic muscles irrespectively of diet, which is indicative of serious mitochondrial dysfunction caused by the surgery. However, ischemic muscles from mice fed HFD have reduced capacity to produce ATP compared with the ones from mice on ND (Paper II, Fig 3A-E). Thus, muscle fibers from mice with diet-induced obesity have a reduced mitochondrial function, which is further exacerbated by FAL. This is in line with a previous report showing that both T2D and PVD are associated with impaired muscle mitochondrial function and that patients with both diseases display more serious mitochondrial dysfunction (Pedersen, Baekgaard, & Quistorff, 2009). Blunted arteriogenesis and angiogenesis in T2D exacerbated vascular alteration in PVD patients, however, they could not adequately explain the functional deficits in skeletal muscle (Leenders et al., 2013; Park et al., 2007). Treatments with revascularization have been proven of limited benefits to the PVD patients(Conte et al., 2005; Schillinger et al., 2006). All these facts indicate that mitochondria dysfunction is another important mechanism related to the reduced exercise performance of these patients and should be considered as a target of treatment (Lindegaard Pedersen, Bækgaard, & Quistorff, 2017).

We analyzed protein levels of mitochondrial subunits 15 days post-surgery. At the protein level, mitochondrial complexes (assessed with immunoblotting and a mitochondrial antibody cocktail) were decreased in muscles 15 days after FAL surgery, but with no apparent effect of the diet (Paper II, Fig 2G). Moreover, intact mitochondrial complexes were decreased at different time points after the surgery when checked with blue native gel, which indicates impaired integrity of mitochondria in the ischemic muscles.

4.2.4 RNA-seq, pathway enrichment, and the PamGene assay

In Paper II, we performed an RNA sequencing of skeletal muscle from ischemic and control legs of mice fed both diets and plotted a heatmap for the expression of genes that are reported as fibrosis-related. Most of the genes have an increased expression after FAL, with no effect of the diet treatment.

We also performed a Pamgene kinase activity assay to assess kinase activities in skeletal muscles to explore the molecular mechanisms involved in the process. We observed a difference in activities of several kinases in muscles 15 days after FAL in mice from ND and HFD. These kinases are involved in pathways critical for muscle remodeling, including VEGF signaling, PI3K-Akt signaling, and MAPK signaling pathways.

Among the top variable genes between all groups from RNA-seq, there are genes involved in fibrosis (Postn), fat metabolism (Plin1, Adipoq), and myosin heavy chain isotypes (Myh13, Myh3, Myh8), indicating a critical role of these changes after FAL. We compared differentially expressed genes between muscles from FAL to muscles from control legs in mice fed both diets. However, we found that these differentially expressed genes only partially overlap in mice fed both diets. Pathway analyses of the differential genes from

RNA-seq with GSEA analyses using GO gene sets as reference revealed that mitochondria respiratory chain complex assembly and cellular reparation are among the top suppressed biological processes in ischemic muscle from ND mice, however, didn't appear in the mice fed HFD (Mootha et al., 2003; Subramanian et al., 2005). On the other hand, Fig 3A-H (Paper II) showed that both mitochondrial content and mitochondrial function in the obese mice were changed already before the FAL surgery. Overrepresentation analysis (ORA) with the KEGG database of differential genes from RNA-seq between control muscles from mice fed both diets identified similar pathways as we saw in the Pamgene kinase activity assay, which further indicates that these pathways are already altered by the HFD (Kanehisa & Goto, 2000).

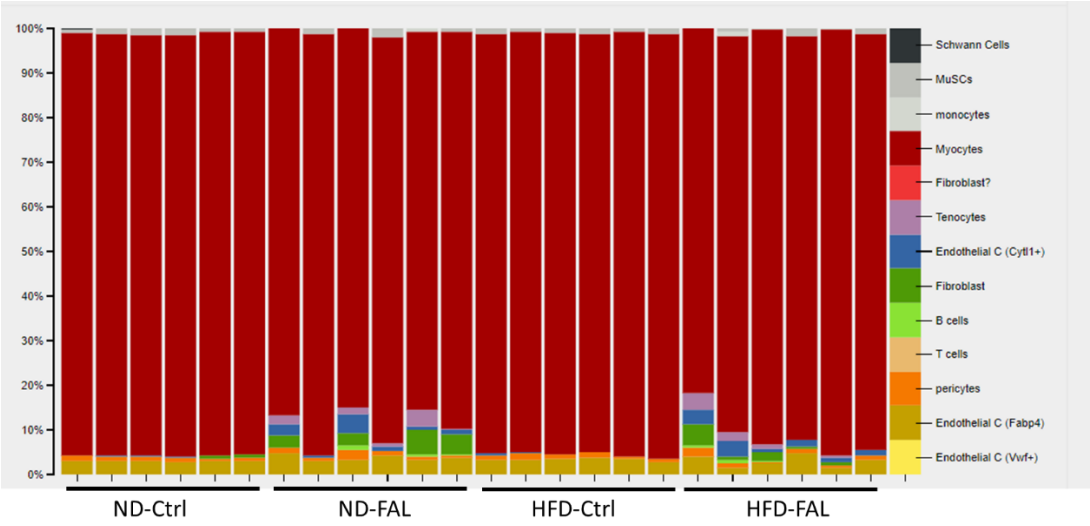

Figure 3. Proportion of different cell types in skeletal muscle from control or FAL muscle of mice fed either ND or HFD, by deconvolution of our RNA-seq results taking single-cell sequencing datasets from Tabula Muris as a signature matrix (Tabula Muris et al., 2018).

4.2.5 Deconvolution of RNA-seq results with CIBERSORTx

Skeletal muscle remodeling after the damage is a complex process involving the joint effects of multiple cell lines. In order to assess the changes in cell type abundance after FAL in mice fed on both diets, we have employed a machine learning method called CIBERSORTx in Paper II, which infers cell type specific gene expression, thus enabling us to estimate the abundance of different cell types in skeletal muscle by deconvolution of our bulk RNA-sequencing results (Newman et al., 2019). By referring to the skeletal muscle single-cell sequencing datasets from Tabula Muris as a signature matrix, we observed an obvious difference in the proportion of cell types between the muscles with or without FAL (Tabula Muris et al., 2018)(**Figure 3**). Myocytes were identified as the major cell type in control muscles, but other cell types were present, e.g. endothelial cells. In skeletal muscles 15 days after femoral artery ligation, the proportion of other supporting cells increased, including Cytl1+ endothelial cells, fibroblasts, tenocytes. Interestingly, in mice fed ND, endothelial

cells in control muscles were mostly Fabp4+ cells, while 15 days after FAL, a new endothelial cell population positive for Cytl1 emerged, highlighting the role of these cells in muscle remodeling after FAL. The proportion of Fabp4+ endothelial cells was not increased after FAL in mice fed HFD, this is because these cells were already increased in control muscles of mice fed HFD. No difference was observed in the proportion of Cytl1+ endothelial cells between mice fed two diets.

Furthermore, we also used a previously published single-nucleus dataset of TA muscles from 5-month-old mice as a reference matrix for CIBERSORTx deconvolution (Petrany et al., 2020). Similarly, interstitial muscle cells including FAPs, endothelial cells, satellite cells, myotendinous junction cells were increased after FAL. We also employed ImmuCC as a tool for inferring immune cell proportion in skeletal muscle, which used an atlas of immune cell composition in various tissues as reference (Chen et al., 2018). We observed different patterns of immune cell composition not only in muscles with or without FAL, but also in mice fed ND or HFD. For instance, the CD4+ T cell proportion was significantly increased 15 days after FAL in mice fed ND, but not in mice fed HFD. On the other hand, there was already a higher CD4+ T cell proportion in control muscles from mice fed HFD compared to the muscle from mice on ND. This indicates that HFD alone induces an inflammatory response in skeletal muscle, which is consistent with the theory that obesity is associated with a low-grade systemic inflammation(Ellulu, Patimah, Khaza'ai, Rahmat, & Abed, 2017).

4.3 ENHANCED CAPILLARY DENISTY AND SKELETAL MUSCLE PERFORMANCE IMPROVEMENT IN MICE LACKING MITOCHONDRIAL SUBUNIT NDUFA4L2

We showed in **Paper I** that mitochondrial protein NDUFA4L2 plays an important role in regulating the mass and force of the skeletal muscle. Moreover, in **Paper II** we showed with a mouse model of FAL that mitochondrial function and content are associated with skeletal muscle's capacity to recover from FAL surgery and appear to be a potential target of intervention. To further clarify the role of mitochondria and NDUFA4L2 in skeletal muscle, in **Paper III** we generated a Ndufa4l2 knockout mouse line (AKO-NDUFA4L2) by breeding a floxed-NDUFA4L2 mouse line, which carries LoxP sequences surrounding exon two of the NDUFA4L2 gene, with the β-actin-cre recombinase-expressing mouse line. Our qPCR confirmed the successful knockout of Ndufa4l2, while no difference was observed in the expression of its homolog Ndufa4 (**Figure 4A**). We observed a modest increase in oxygen consumption without large alterations in the expression of ETC genes, which implies an increased mitochondrial function. Our immunofluorescent staining demonstrated an increased proportion of capillary density after knocking out NDUFA4L2 in skeletal muscle by staining for the endothelial cell marker CD31. In line with this, we also observed higher expression of the vascular endothelial growth factor A (Vegfa) which is known to be involved in promoting the growth of new blood vessels (Shibuya, 2011)(Paper III: Fig 1F).

Skeletal muscle function is dependent on the ability of capillaries to exchange nutrients and oxygen between blood and the tissue. Skeletal muscle is the major organ that is responsible

for glucose uptake in our body (Thiebaud et al., 1982). Maintaining adequate muscle capillary density is also necessary for enhancing muscle glucose uptake (Sove, Goldman, & Fraser, 2017). In **Paper III**, we observed that glucose clearance after an intraperitoneally injected glucose bolus was enhanced in AKO-NDUFA4L2 mice as compared with wild-type littermates (Figure 4B). This shows that altering NDUFA4L2 influences the microvascular structure in skeletal muscle as well as whole-body glucose clearance and hence appears beneficial for the overall metabolism.

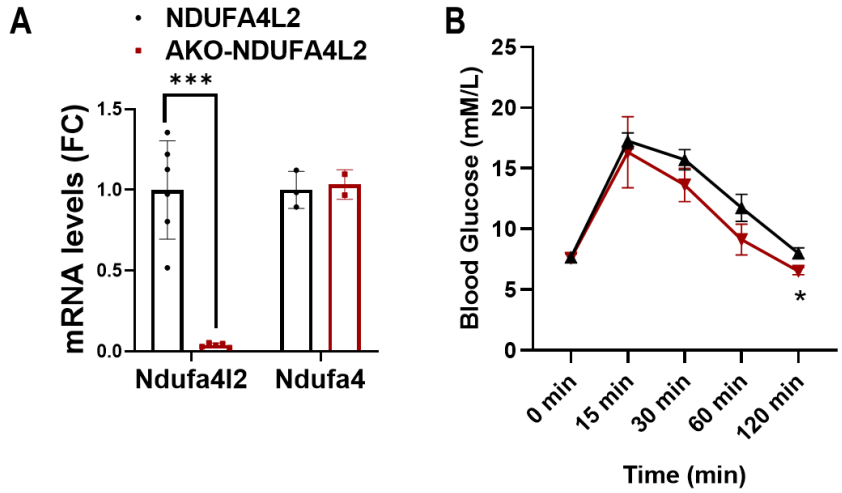

Figure 4. Knocking out of NDUFA4L2 improved glucose clearance after an intraperitoneally injected glucose bolus. (A) Ndufa4l2 and Ndufa4 mRNA expression (mean data ±SEM, n=3-5) in skeletal TA muscle from NDUFA4L2 and AKO-NDUFA4L2 mice; (B) Blood glucose level of NDUFA4L2 and AKO-NDUFA4L2 mice in IPGTT (mean data ±SEM, n=3-6).

Our *ex vivo* force measurement has shown that skeletal muscle from NDUFA4L2 knockout mice tend to be stronger, more fatigue-resistant, and exhibited faster recovery than their littermate controls (Paper III: Fig 2A-F). The absolute force of soleus muscles was significantly stronger than wild-type mice and was more resistant to fatigue. EDL muscles from AKO-NDUFA4L2 mice showed higher absolute and specific force and recovered significantly faster from fatigue. However, the muscle weight of both soleus and EDL were not significantly changed.

Altogether, the AKO-NDUFA4L2 mice showed an exercise phenotype, which is consistent with our previous findings that ectopic expression of NDUFA4L2 is associated with smaller and weaker muscle (Z. Liu et al., 2021).

AKO-NDUFA4L2 mice and wild-type littermates have been placed into TSE or CLAMPS metabolic cages for five days for assessing the differences between their whole-body metabolisms. We observed significant decreases in the overall oxygen consumption (VO_2), carbon dioxide production (VCO_2), respiratory exchange ratio (RER), and ambulation in AKO-NDUFA4L2 mice, but not in overall food consumption or water consumption. Even though food consumption was not different between the two groups in either dark or light

conditions, we observed that AKO-NDUFA4L2 mice ate significantly less food in the refeeding period after fasting. Consistently, they also drank less water in this period. Furthermore, these AKO-NDUFA4L2 mice were less active during the nights as their counts of movements were significantly lower in dark conditions but not in light conditions (Paper III, Fig 3A-F). However, even though average ambulatory measurement at night decreased from 472.55 (se=132.29) to 283.40(se=80.99), by 40.0%, the average VO2 at night only decreased by 10.7%, from 4004.46 (se=743.57) to 3575.94 (se=489.81), and the average VCO2 at night decreased by 14.8%, from 3853.22 (se=872.85). The decrease of ambulatory measurement was much more than the decrease of VO2 and VCO2 at night when the mice were most active.

Overall, our results thus far show that AKO-NDUFA4L2 mice have improved skeletal muscle function, with higher capillarization and altered whole-body metabolism, which mimic an exercise phenotype. Future research on NDUFA4L2 may contribute to the development of novel treatments to improve muscle function, glucose handling, and ultimately health and quality of life for patients with obesity or T2D.

4.4 SKELETAL MUSCLE PGC-1α1 REROUTES KYNURENINE METABOLISM TO INCREASE ENERGY EFFICIENCY AND FATIGUE-RESISTANCE

In skeletal muscle, PGC-1α is an exercise-inducible transcriptional co-activator linked to mitochondrial function, numbers and volume, and improved exercise endurance in humans (Correia et al., 2015). PGC-1α has gained great interest by us and others being a node for different pathways that regulate mitochondrial function.

Ppargc1a, the gene encoding PGC-1α, can be expressed from different promoters, sometimes coupled to alternative splicing events. For that reason, in addition to the canonical Pgc-1a1 (Puigserver et al., 1998), Ppargc1a gives rise to other variants such as PGC-1α-b, PGC-1α-c which are from novel promotors, or NT-PGC-1α from alternative splicing, or PGC-1α2, PGC-1α3, PGC-1α4 from both (Martinez-Redondo, Pettersson, & Ruas, 2015). These isoforms are known to play a role in controlling energy metabolism in different tissues with specific biological functions (Martinez-Redondo et al., 2015). PGC-1α4 is the only one so far that doesn't seem to be linked to energy metabolism, but to muscle mass regulation (Ruas et al., 2012), or to liver apoptosis and inflammation (Leveille et al., 2020). Among these isoforms, PGC-1α1 has been reported to be involved in the adaptation of skeletal muscle to aerobic training by coupling fuel supply, oxygen transport, and energy metabolism to the increased exercise performance and fatigue-resistance (Correia et al., 2015). The effect of PGC-1α1 in skeletal muscle has been attributed to the mediation of increasing mitochondrial biogenesis and fat oxidation, as supported by the observation that sustained expression of PGC-1α1 in skeletal muscle (mck-PGC-1α1 transgenics) led to various adaptations to exercise without training (J. Lin et al., 2002). The elevation of PGC-1α1 levels in skeletal muscle of mck-PGC-1α1 mice vs wild-type ranges from 2- to 10-fold higher in oxidative and

glycolytic muscles respectively. The range is consistent to the elevation of PGC-1α1after endurance training in humans (Baar et al., 2002; Mathai, Bonen, Benton, Robinson, & Graham, 2008). In addition, PGC-1α1also regulates other pathways associated with muscle performance, including a muscle to brain crosstalk which is activated by aerobic training, with a protective effect on stress-induced depression (Agudelo et al., 2014). This effect is mediated via the activation of several Kats in skeletal muscle, which clears neurotoxic Kyn from circulation thus preventing its accumulation in the brain.

The renewal of the cytosolic NAD^+ pool is important for maintaining glycolytic flux. Transferring glycolysis generated NADH reducing equivalents to lactate or into the ETC renews the cytosolic NAD^+ pool and maintains glycolytic flux. Since the inner membrane of the mitochondria is not permeable to NADH, in skeletal muscle, glycerol-3-phosphate (G3P) shuttle (G3PS) is used to transport electrons to Coenzyme Q, generating 1.5 ATP per NADH, while in other tissues, malate-aspartate shuttle (MAS) is used to transfer electrons from NADH to mitochondrial Complex I. The glutamate and aspartate are generated by cytosolic and mitochondrial transamination reactions respectively. The generated glutamate and aspartate are then exchanged between compartments. Glutamic-oxaloacetic transaminases 1 and 2 (Got1 and 2) catalyze these reactions (**Figure 5**).

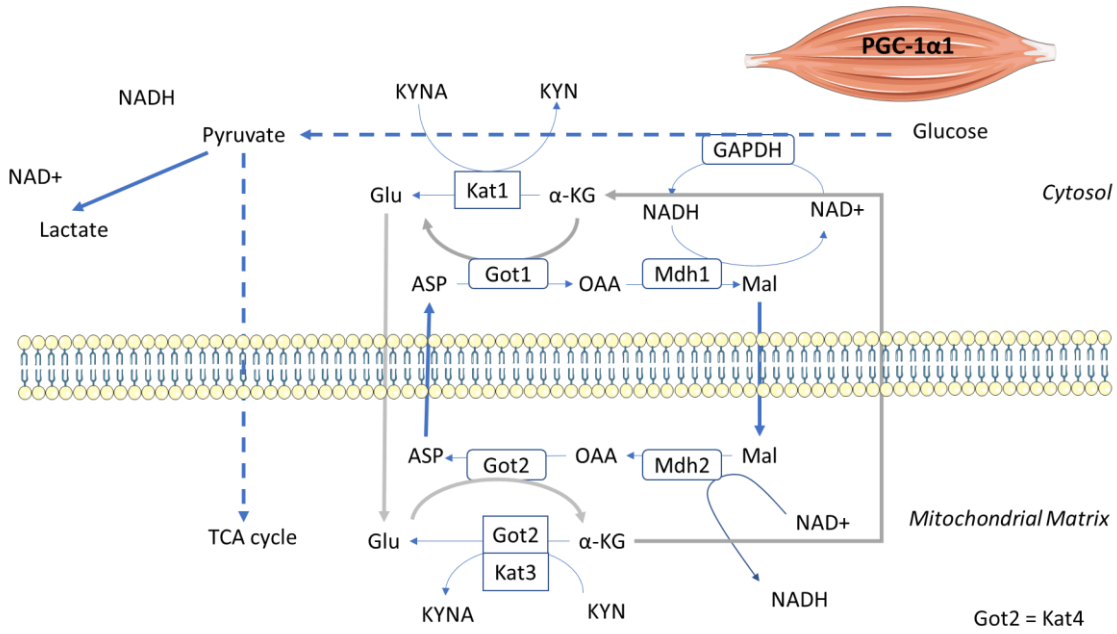

Figure 5. Schematic view of how skeletal muscle PGC-1α1 integrates Kynurenine metabolism into malate-aspartate shuttle. Figure adapted from (Agudelo et al., 2019).

In **Paper IV**, by analyzing the transcriptomics data obtained from skeletal muscle of mck-PGC-1α1 mice, we observed that PGC-1α1 increases the expression of genes involved in the biosynthesis and metabolism of aspartate in skeletal muscle. It was also confirmed with metabolomic data that aspartate and glutamate levels were elevated in the skeletal muscle of

mck-PGC-1α1 mice. PGC-1α1 has been previously shown to increase the expression of Got and Kat transaminases that catalyse important reactions involved in the metabolic pathways including aspartate biosynthesis and Kyn degradation (Agudelo et al., 2014; Birsoy et al., 2015; Han, Robinson, Cai, Tagle, & Li, 2009). We have used RNA-seq data from isogenic BDX mice (archived in http://www.GeneNetwork.org) to assess the KATs-associated network, and we observed that Kat1 and Got2/Kat4 were strongly associated with genes regulating the malate-aspartate shuttle (MAS). The levels of malate were also observed to be elevated in skeletal muscle with sustained expression of PGC-1α1 (mck-PGC-1α1), in strong correlation with the levels of aspartate in skeletal muscle. In line to this, the expression of genes in the MAS was found to be increased in skeletal muscle of mck-PGC-1α1 mice.

In **Paper IV**, we also showed that overexpressing PGC-1α1in myotubes increased glutamate levels and the expression of MAS genes. Supplementing Kyn to myotubes further increased the levels of glutamate and aspartate, expression of MAS genes, several transcriptional factors associated with PGC-1α1 function and of mitochondrial genes, and consequently cellular respiration, in the presence of PGC-1α1. The increased respiration is completely coupled to ATP synthesis, as it can be completely abolished by the addition of oligomycin, an ATP-synthase inhibitor. A single dose treatment with Kyn increased expression of genes in MAS, only in mck-PGC-1α1 mice. The skeletal muscle of the transgenic mice showed higher levels of glutamate, aspartate, and malate, which can be further increased by the treatment of Kyn.

Mice deleted of Pgc-1α in skeletal muscle (MKO-PGC-1α mice) were shown to be unable to use Kyn to support bioenergetics (Handschin et al., 2007). Treating wild-type myotubes or myotubes lacking PGC1a1 (MKO-PGC-1α1) with Kyn reduced the level of respiration in both conditions, indicating that Kyn-accumulation above a physiological threshold is detrimental to aspartate biosynthesis and mitochondrial respiration. However, myotubes transduced with a recombinant adenovirus expressing Pgc-1α1 can withstand the effect of Kyn accumulation (10 μM) on maximal respiration. The results indicate PGC-1α1 promotes ATP synthesis and energy production from glycolysis by allowing the muscle to use Kyn. Interfering with the MAS at different points can have different outcomes including reduction in oxygen consumption, enhanced production of glycolysis-derived lactate, or both.

Carbidopa that irreversibly inactivates the Kat cofactor pyridoxal-5' -phosphate (PLP), has been used in **Paper IV** to inhibit Kat. Treating myotubes with carbidopa reduced the maximal respiration, together with a reduction in aspartate and malate levels. While the reduction of perspiration was exacerbated in myotubes incubated with only pyruvates. Treating myotubes in pyruvates with both carbidopa and etomoxir, a carnitine palmitoyltransferase-1b chemical inhibitor that can inhibit fatty acid oxidation, leading to a great reduction in basal and maximal respiration, while these effects were tempered in myotubes overexpressing PGC-1α1. Mice treated with carbidopa resulted in that they run less in the exercise performance test, while the effect was rescued with aspartate treatment.

Pretreatment of FDB muscles with carbidopa decreased the contractile force production in a fatigue-induction protocol.

Endurance training in mice has been shown to increase the expression of genes related to glycolysis and MAS, together with the levels of malate and aspartate. While the effects of both acute and chronic exercise on MAS-related genes were not seen in mice lacking PGC-1α (MKO-PGC-1α mice). These effects were also found to be elevated in the skeletal muscle of humans. This indicates that the activation of MAS by PGC-1α is part of the adaptation of skeletal muscle to exercise

5 CONCLUSIONS

In conclusion, this thesis:

1) Showed for the first time that mitochondrial protein NDUFA4L2 is a novel regulator of skeletal muscle mass and force. Ectopic expression of NDUFA4L2 inhibited mitochondrial respiration, leading to reduced levels of important intramuscular metabolites, including adenine nucleotides and NAD^+ which were indicative of reduced mitochondrial function. Moreover, ectopic expression of NDUFA4L2 resulted in smaller and weaker muscles. In a mouse model of PVD, NDUFA4L2 was induced in skeletal muscle after ischemic injury, together with muscular atrophy and weakness. Further studies are needed to clarify the causal role of NDUFA4L2 in skeletal muscle wasting after ischemic injury. Nevertheless, our study suggests that NDUFA4L2 acts as a nodal integrator of environmental queries in muscle function, with implications to the mechanisms of muscle weakness in peripheral vascular disease.

2) Demonstrated that FAL, a mouse model of PVD, leads to altered mitochondrial function and ROS production, but also a synchronized downregulation of mitochondrial assembly, which however was blunted in mice with obesity. This is most likely the result that their expression is already altered by the HFD before the FAL surgery. Further studies are needed to address to what extent the dysregulated mitochondrial expression and function contribute to muscle dysfunction in FAL and the blunted recovery in HFD mice after FAL surgery. Above all, mitochondria appear to play a critical role in skeletal muscle function in PVD and may be a potential target for future treatment.

3) Presented that AKO-NDUFA4L2 mice exhibit higher muscular capillarization and altered whole-body metabolism, mimicking an exercise phenotype. Future research on NDUFA4L2 may contribute to the discovery and development of novel treatments to improve muscle function, glucose handling, and ultimately health and quality of life for patients with obesity or T2D.

4) Revealed that PGC-1α1 regulates MAS in skeletal muscle, allowing the trained skeletal muscle to use Kyn to promote aspartate biosynthesis and mitochondrial function, and adapt to endurance training. These findings not only benefit our understanding of muscle adaptation to aerobic training but may also have important clinical implications.

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