MUSCLE MITOCHONDRIAL HEALTH

BART LAGERWAARD

MUSCLE MITOCHONDRIAL HEALTH

Ageing, Physical Activity and Molecular Mechanisms

Bart Lagerwaard

Propositions

1. Physical activity does not prevent muscle mitochondrial ageing.
   (this thesis)

2. Extracellular matrix characterisation should be *embedded* in skeletal muscle ageing research.
   (this thesis)

3. Preprints accelerate science.

4. Updating the Wikipedia page of your research topic is the most effective way of science communication.

5. From a carbon footprint standpoint, *where* your food comes from is negligible compared to *what* you eat.

6. Technological advancements are the driving force behind physical inactivity, the latest being electronic bicycles and scooters.

7. Smartphones distract you from your professional life with personal things as much as they distract you from your personal life with professional things.

Propositions belonging to the thesis, entitled
Muscle Mitochondrial Health; Ageing, Physical activity and Molecular Mechanisms

Bart Lagerwaard

Wageningen, 12 March 2021

Muscle Mitochondrial Health

Ageing, Physical Activity and Molecular Mechanisms

Bart Lagerwaard

Muscle Mitochondrial Health

Ageing, Physical Activity and Molecular Mechanisms

Bart Lagerwaard

Thesis committee

Promotor
Prof. Dr J. Keijer
Professor of Human and Animal Physiology
Wageningen University & Research

Co-promotors
Dr A.G. Nieuwenhuizen
Assistant professor, Human and Animal Physiology
Wageningen University & Research

Dr V.C.J. De Boer
Assistant professor, Human and Animal Physiology
Wageningen University & Research

Other members
Prof. Dr C.P.G.M. de Groot, Wageningen University & Research
Prof. Dr L. Deldicque, Catholic University de Louvain, Belgium
Dr R. Wüst, VU Amsterdam
Dr M.C.E. Bragt - van Wijngaarden, FrieslandCampina, Wageningen

This research was conducted under the auspices of the Graduate School VLAG (advanced studies in Food Technology, Agrobiotechnology, Nutrition and Health sciences).

Thesis

submitted in fulfilment of the requirements for the degree of doctor

at Wageningen University

by the authority of the Rector Magnificus,

Prof. Dr. A.P.J. Mol,

in the presence of the

Thesis Committee appointed by the Academic Board

to be defended in public

on Friday 12 March 2021

at 4 p.m. in the Aula

Bart Lagerwaard

Muscle Mitochondrial Health: Ageing, Physical Activity and Molecular Mechanisms, 226 pages.

PhD thesis, Wageningen University, Wageningen, NL (2021)

With references, with summary in English

ISBN: 978-94-6395-663-5

DOI: https://doi.org/10.18174/537712

Table of Contents

CHAPTER 1

General Introduction

Bart Lagerwaard

1.1. The ageing world population

We have never been as old as we are today. Since the start of the 20th century, life expectancy has increased remarkably. This increase in life expectancy was initially caused by a reduction in child and infant mortality, driven by advancements in public health. Since the 1970s, the increase in life expectancy that has occurred was sustained by a reduction in mortality for diseases, such as cardiovascular disease and cancer (Wilmoth 2000). The number of persons aged 80 years and above has tripled between 1990 and 2019, and this number is expected to triple again by 2050 (United Nations Department of Ecomic and Social Affairs 2019). However, this tremendous achievement of increasing life expectancy comes at a cost, as many diseases become increasingly prevalent at an older age (Prince et al. 2015). Therefore, the challenge for the coming decades is to maintain or even increase people's health during those extra years of life, thus preventing the onset of age-related diseases (Olshansky 2018).

Sarcopenia is an age-related condition that is characterised by a decline in muscle mass and strength (Morley et al. 2001). Due to the vital role of muscle strength in daily life activities, sarcopenia is associated with functional decline, decreased quality of life and increased hospitalisation rates (Tanimoto et al. 2013; Tsekoura et al. 2017). The prevalence of sarcopenia is expected to increase drastically in Europe, over the coming decades. Therefore, the impact of this disease will extend beyond its burden for the patient, as it will impose a great financial burden on society in terms of increased health care costs (Bruyère et al. 2019). Health care costs are expected to double for European countries such as The Netherlands, and it is vitally important to counteract this trend. In this regard, strategies for improving or sustaining muscle mass and strength with advancing age could be effective (European Commission Directorate-General for Economic and Financial Affairs 2015). Furthermore, and arguably more important, loss of skeletal muscle mass and strength are significant predictors for all-cause mortality (Metter et al. 2002; Srikanthan and Karlamangla 2014). Therefore, identifying underlying causes for the loss of muscle strength and mass,

and preventing sarcopenia, will likely have a tremendous impact on patients and society, especially in view of the current demographic trends.

1.2. The ageing muscle

Skeletal muscle is the largest organ in the body, accounting for up to 40% of body mass. The primary role of skeletal muscle is to allow one to maintain posture and, by contraction, to move the skeletal system. Skeletal muscle is one of the most plastic tissues in the human body. It is able to regenerate completely in response to injury (Chargé and Rudnicki 2004) or increase in mass, strength and efficiency following repeated physical exercise stimuli (Hughes et al. 2018). Nevertheless, almost half of the skeletal muscle mass is expected to be lost over a lifetime (Janssen et al. 2002). Skeletal muscle mass peaks in a person's mid-20s, after which the decline is thought to be gradual. However, the first changes become notable after the 5th decade of life (Lexell et al. 1988). Thereafter, the rate of muscle loss starts to accelerate (Janssen et al. 2002), which also becomes apparent from the changes in total energy expenditure (Speakman and Westerterp 2010). Besides the primary role of skeletal muscle in movement, it also has an important role in metabolism, thermoregulation and is the largest store of glucose and amino acids in the body (Shulman et al. 1990). Therefore, losing muscle mass, besides impairing physical functioning, also has metabolic implications, which links it with other age-related diseases, such as type 2 diabetes (Park et al. 2007).

The underlying causes of loss of muscle strength and quality with age are hypothesised to be caused by hallmarks of ageing, such as loss of proteostasis, altered cellular communication and stem cell exhaustion (López-Otín et al. 2013a). This manifests itself as, among other things, decreased innervation (Gonzalez-Freire et al. 2014), decreased muscle regeneration (Carosio et al. 2011), decreased protein synthesis (Balagopal et al. 1997), decreased circulating anabolic hormones (Sakuma and Yamaguchi 2012), low-grade inflammation (Dalle et al. 2017), decreased mitochondrial capacity (Carter et al. 2015) or a

combination of these causes. The loss of muscle strength with age is disproportional to the actual muscle mass lost, such that more muscle strength is lost per unit of muscle mass. This suggests there is a further explanation to the loss of muscle strength, besides the loss of mass. Intrinsic changes in the muscle, often referred to as muscle quality, could explain this excessive loss of muscle strength. (Goodpaster et al. 2006; Mitchell et al. 2012).

Due to the plasticity of muscle in response to exercise and physical activity stimuli, maintaining an active lifestyle has been shown to preserve muscle quality and mass with age (Distefano et al. 2018). However, physical activity has been reported to decline with age, which negatively affects the muscle (Troiano et al. 2008; Hallal et al. 2012). The reasons for the occurring decreased physical activity are multi-factorial, but include changes in lifestyle, physical limitations, comorbidities, hospitalisation and depression (Gomes et al. 2017; Cruz-Jentoft and Sayer 2019). Besides their relationship with physical activity, muscle mass and quality are also associated with dietary habits. For example, muscle loss might accelerate occur due to decreased protein and vitamin D intake (Robinson et al. 2018). In light of this it can be understood that interventions aiming to increase or maintain physical activity and exercise (Haider et al. 2019), or aiming to improve nutritional status (Tieland et al. 2012), have turned out effective in terms of maintaining the muscle mass and quality with age (**Figure 1.1**).

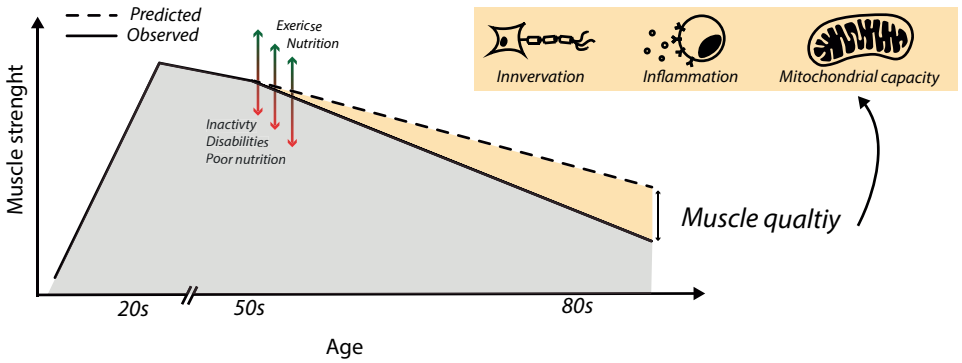

Figure 1.1 Muscle strength declines disproportional to muscle mass with advancing age. The differences between the predicted loss of strength, corresponding to mass, and the observed loss of strength, are thought to be caused by a decline in muscle quality.

Due to the evident interaction between skeletal muscle ageing and lifestyle factors, it is often challenging to attribute the decline in skeletal muscle mass and quality to either changes in lifestyle or to advancing age itself. Therefore, ageing is often further classified as caused by the 'inevitable' loss of cellular function and structure, referred to as primary ageing, while age-related changes in lifestyle and environmental factors are referred to as secondary ageing. Nevertheless, this concept is rather fluid. As increasing understanding of the mechanisms of skeletal muscle ageing might help us disentangle the 'inevitable' from the 'evitable'. This will help us designing and adapting interventions, be these interventions for lifestyle, nutrition, or even pharmacological interventions; all this to ultimately sustain skeletal muscle health with advancing age.

1.3. The Ageing mitochondrion

An important factor that is thought to significantly contribute to loss of muscle quality during ageing, is the decline in mitochondrial capacity (Carter et al. 2015). The mitochondria are cell organelles that utilise breakdown products of glucose, fatty acid and amino acid metabolism to generate energy in the form of adenosine triphosphate (ATP). ATP is the universal energy carrier of the body and is required for all bodily processes, including muscle contraction. In the matrix of the mitochondria, metabolites are oxidised in the tricarboxylic

acid cycle (TCA) and via beta-oxidation, resulting in the production of the electron carriers nicotinamide-adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$). The electrons carried by these carriers are donated to the electron transport complexes of the mitochondrial inner membrane. Electrons from Complex I, Complex II and electron transfer flavoprotein are transferred, via Coenzyme Q, to complex III and then Complex IV, where oxygen is the final electron acceptor. In the process of electron transfer, protons are transported over the mitochondrial inner membrane by complexes I, III and IV, establishing a high concentration of protons on one side of the membrane, also called the proton motive force. This force is used by another inner membrane mitochondrial complex: complex V or ATP synthase, that utilises the hydrogen atoms that flow back into the mitochondrial matrix, to regenerate ATP from adenosine diphosphate (ADP). Due to the role of oxygen as a final electron acceptor in this process, oxygen is indispensable for ATP generation in the mitochondria.

Skeletal muscle mitochondrial capacity represents the capability of the myofibrillar mitochondria to produce ATP. Mitochondrial capacity is positively correlated with physical functioning, for instance in terms of exercise performance, walking speed (Coen et al. 2013) and muscle strength (Zane et al. 2017), and is therefore indispensable for adequate muscle functioning into old age. Nevertheless, with age mitochondrial capacity in *vastus lateralis* (Conley et al. 2000b; Johannsen et al. 2012; Larsen et al. 2012) and *gastrocnemius* (McCully et al. 1993; Waters et al. 2003; Layec et al. 2013) is reduced. This reduction occurs due to, on the one hand, a decrease in the amount of mitochondria (Conley et al. 2000b; Welle et al. 2003; Crane et al. 2010) and, on the other hand, a decrease in the capacity per unit of mitochondria to generate ATP (Trounce et al. 1989; Short et al. 2005; Lanza et al. 2008; Irving et al. 2015). This relative decrease in terms of the ability to generate energy (per volume of mitochondria) supports the notion that a decrease in muscle mitochondrial quality is associated with age. Mechanisms associated with mitochondrial quality decline with ageing will be introduced in section 1.6.

Despite the evidence in support of the claim (Short et al. 2005; Lanza et al. 2008), the decline in mitochondrial capacity with age is still under debate, as a substantial amount of studies report no effect of age on mitochondrial capacity (Rasmussen et al. 2003; Hütter et al. 2007; Gouspillou et al. 2014). The same absence of effect was observed using *in vivo* measurements in the *vastus lateralis* (Layec et al. 2015), *gastrocnemius* (Chilibeck et al. 1998; Wray et al. 2009; Tevald et al. 2014; Hart et al. 2015) and *tibialis anterior* (Kent-Braun and Ng 2000; Lanza et al. 2005, 2007; Christie et al. 2014). Interestingly, a meta-analysis of studies assessing the effect of age on *in vivo* mitochondrial capacity, showed a positive effect of age on mitochondrial capacity, putting into question the central dogma that mitochondrial decline is inherently associated with ageing (Fitzgerald et al. 2016). That same study identified important effect modifiers for mitochondrial decline with ageing, such as sex, muscle group and physical activity. For example, the ageing trajectory might not be the same for every muscle, for instance due to changes in patterns of muscle use (Larsen et al. 2012), and might also differ between males and females (Van der Hoek et al. 2020). In other words, mitochondrial capacity is significantly influenced by these modifiers, which means that a lack of accounting or correcting for these modifiers could explain the discrepancies in the current literature. Regular physical activity and exercise positively affect mitochondrial mass and function (Tonkonogi and Sahlin 2002; Lanza and Nair 2009). This potentially explains why studies that do correct for physical activity often do not find an age-related decrease in mitochondrial capacity, because the positive effect of physical activity masks the age-related decline on mitochondrial capacity (Brierley et al. 1996; Barrientos et al. 1996). Moreover, when comparing physically active elderly to a young control group, physically active older individuals do not display lower mitochondrial capacity (Larsen et al. 2012; Zampieri et al. 2015; Distefano et al. 2018).

Still, there is evidence that primary ageing is an important factor in the process of age-related decline in mitochondrial capacity. For example, although not statically significant, a noticeable decrease in mitochondrial respiration and capacity can be observed in similarly active old individuals, compared to their young individuals in the *vastus lateralis* muscle

(Lanza et al. 2008; Distefano et al. 2018). One can also observe significant differences in the expression of RNA and proteins involved in oxidative metabolism (Lanza et al. 2008). Furthermore, older individuals with low levels of physical activity show lower mitochondrial ATP production (Lanza et al. 2008) and lower *in vivo* mitochondrial capacity in the *vastus lateralis* muscle compared to young controls, when matched for physical activity (Larsen et al. 2012). Therefore, to elucidate this primary ageing effect, additional research on the effect of age on mitochondrial capacity is needed, taking into consideration the important effect modifiers. Uncovering this primary ageing effect will help us understand mitochondrial ageing and will aid research into tailoring adequate interventions, to ameliorate the age-related decline in mitochondrial capacity and its impact on declining muscle mass and strength (**Figure 1.2**).

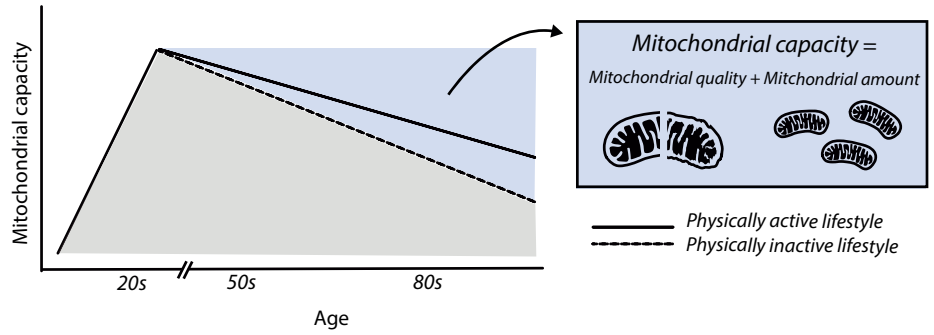

Figure 1.2 Hypothetical effect of a physically active lifestyle on the preservation of muscle mitochondrial capacity.

1.4. Measuring mitochondrial capacity

Differences in respective assessment methods could also explain some of the discrepancies between reports on the effect of age on mitochondrial capacity. For example, *ex vivo* measurements in isolated mitochondria from muscle biopsies have been reported to exaggerate age-related impairments, compared to measurements in permeabilised fibres. This difference possibly stems from the disruption of the mitochondrial morphology during the isolation process (Picard et al. 2010). Along the same line of reasoning, measurements in permeabilised fibres might also not closely resemble the conditions in the muscle, as

measurements are performed at supraphysiological oxygen pressures and substrate concentrations, and do not take factors that influence mitochondrial capacity in *vivo* into consideration, such as oxygen delivery and diffusion. Furthermore, *ex vivo* methods measure in small isolated fibres, which is considered a disadvantage due to the heterogeneity of muscle tissue. *In vivo* methods for assessing mitochondrial capacity offer some solutions to these drawbacks, as measurements on mitochondria are done in a dynamic system and in a larger volume of muscle. Then, besides technical considerations, there are also practical considerations. For example, *ex vivo* methods require the sampling of muscle tissue via a surgical muscle biopsy procedure. Therefore, *in vivo* methods are considered less invasive for research subjects, as these methods do not require surgical procedures and measurements are done topically. However, a disadvantage of *in vivo* methods is that they do not yield mechanistic insights into muscle and mitochondrial quality. Therefore, multiple methodological approaches, both *in vivo* and *ex vivo*, should be combined to measure the mitochondrial capacity in an intact system as well as to obtain mechanical insights. Such a combination will aid efforts to further determine the effects of age on mitochondrial capacity.

The current golden standard for *in vivo* assessment of mitochondrial capacity is 31-phoshorus magnetic resonance spectroscopy (^{31}P-MRS). ^{31}P-MRS uses the nuclear magnetic resonance of phosphorus-containing compounds in the muscle, such as ATP, ADP, phosphocreatine (PCr) and orthophosphate (Pi). ATP storage in the cell is limited and for adequate energy the cell is dependent on rapid regeneration of ATP from ADP. This regeneration requires the energy that is generated via the oxidation of fat and glucose, via glycolysis in the cytosol or aerobic oxidation in the mitochondria. Alternatively, ADP can be regenerated by the donation of Pi from PCr. PCr works as a shuttle for delivering energy where ATP levels are low, but also as a storage of energy that can be released independent from the oxidation of fat and glucose in the mitochondria, using oxygen (Bessman 1987). It is this quality of PCr that makes it an important source of energy at the commencing of exercise, when ATP demand is high, but is not yet met by aerobic ATP generation. When

exercise is ceased, PCr is regenerated from its non-energy counterpart creatine, using ATP generated via aerobic oxidation in the mitochondria. The post-exercise regeneration of readily available energy carriers (i.e., ATP and PCr) is directly linked to aerobic metabolism and faster regeneration of these carriers indirectly reflects mitochondrial metabolism (McMahon and Jenkins 2002). Therefore, the rate of regeneration of PCr after exercise, measured by ^{31}P-MRS, can be used as a proxy for mitochondrial capacity (Meyer 1988), reflecting both the number of mitochondria and the activity per mitochondrion, or mitochondrial quality. Important assumptions for the measurements here are that oxygen availability is not a limiting factor for aerobic oxidation, ensuring adequate vascular oxygen supply and diffusion.

^{31}P-MRS is regarded a valid measurement for mitochondrial capacity if it 1) is correlated to other measurements of mitochondrial capacity and oxidative metabolism both *in vivo* and *ex vivo,* thus underpinning the physiological relevance of the technique and if it 2) detects differences in populations with known differences in mitochondrial capacity, thus underpinning the sensitivity and applicability of the technique. Indeed, mitochondrial capacity measured using ^{31}P-MRS is correlated with *ex vivo* assessment of mitochondrial complex enzymatic activity (McCully et al. 1993; Larson-Meyer et al. 2001), respiration in isolated muscle fibres (Lanza et al. 2011) and *in vivo* measurements of whole-body oxidative capacity, such as whole-body peak oxygen uptake ($\dot{V}O_2$peak) and total daily energy expenditure (Larson-Meyer et al. 2000; Edwards et al. 2013). Additionally, mitochondrial capacity was found to be significantly different between high- and low-fitness individuals (Takahashi et al. 1995), positively affected by endurance training (Larsen et al. 2013), impaired in patients with mitochondrial disease (Taivassalo et al. 1998) and in lower aged individuals (Larsen et al. 2012). Therefore, the general consensus is that ^{31}P-MRS is a robust and valid method for non-invasive, *in vivo* assessment of skeletal muscle mitochondrial capacity. Nonetheless, there are some practical considerations, and possibly shortcomings to this technique. ^{31}P-MRS assessment of mitochondrial capacity requires a magnetic resonance (MR) scanner, which is costly and therefore not widely available. Furthermore,

MR scanners are not portable and therefore on-site measurements are not possible. In the most recent decades, there has emerged a novel method to non-invasively assess muscle mitochondrial capacity *in vivo*, namely near-infrared spectroscopy (NIRS). This method offers solutions for the practical disadvantages inherent to ^{31}P-MRS, such as increased portability and relatively lower costs.

1.5. A novel method to measure mitochondrial capacity non-invasively, using near-infrared spectroscopy

NIRS assessment of mitochondrial capacity makes use of light in a specific region in the near-infrared light spectrum (700-850 nm), of which a significant amount can penetrate biological tissues, where the major absorbing chromophores are haemoglobin and myoglobin. Haemoglobin and myoglobin have oxygen-dependent absorption changes in this spectrum, making it possible to distinguish oxyhaemoglobin/myoglobin (O_2Hb) from deoxyhaemoglobin/myoglobin (HHb) (Jobsis 1977). Haemoglobin is the oxygen binding molecule in the blood, while myoglobin is the oxygen binding molecule specific to the myofibril. The absorption spectra of myoglobin and haemoglobin are identical, and for the sake of simplicity they will in this thesis be referred to as O_2Hb, for the oxygenated form, and HHb,for the non-oxygenated form. When NIRS is used on skeletal muscle in combination with arterial occlusions, it allows for the measurement of muscle oxygen consumption ($m\dot{V}O_2$). When blood flow is occluded, no fresh, oxygenated blood is able to reach the muscle and the change from O_2Hb to HHb reflects the use of oxygen under the NIRS probe (De Blasi 1997). For NIRS assessment of mitochondrial capacity multiple, transient arterial occlusions after a short bout of exercise are used. The temporal measurements of post-exercise $m\dot{V}O_2$ allow for the determination of the rate of recovery of $m\dot{V}O_2$ after exercise (Motobe et al. 2004). Where ^{31}P-MRS uses the rate of PCr recovery, NIRS uses the rate of $m\dot{V}O_2$ recovery as a proxy for mitochondrial capacity, under the assumption that $m\dot{V}O_2$ reflects mitochondrial oxygen consumption in aerobic ATP production, which is directly linked to the recovery of readily available energy carriers used

at the commencement of exercise (**Figure 1.3**). Therefore, a faster return to pre-exercise or basal $m\dot{V}O_2$ is determined by the number of mitochondria and mitochondrial quality, and it hence serves as a measure for mitochondrial capacity (Meyer 1988; Nagasawa et al. 2003).

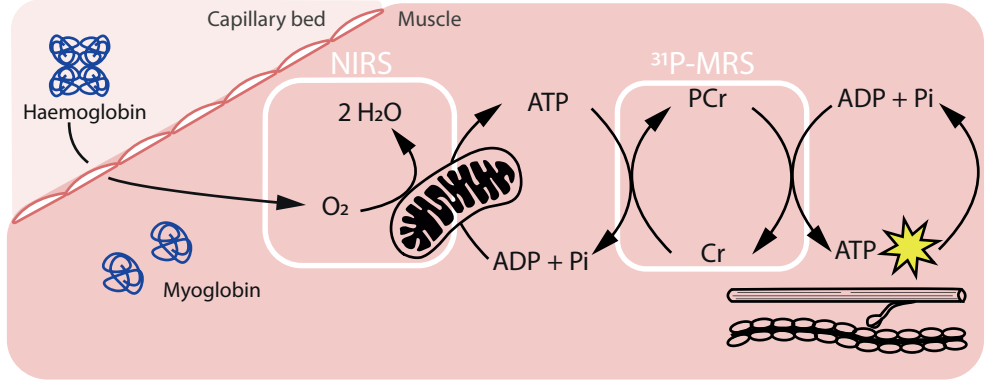

Figure 1.3 NIRS and ^{31}P-MRS make use of recovery of different aspects of recovery of muscle homeostasis after exercise; NIRS uses of the recovery of muscle oxygen consumption, while ^{31}P-MRS uses the recovery of PCr as a proxy for mitochondrial capacity.

Similar to ^{31}P-MRS, NIRS can be regarded as a valid method for the assessment of mitochondrial capacity if it is able to demonstrate physiological relevance, by showing good correlation to other, more established, *in vivo* and *ex vivo* techniques; and by showing adequate sensitivity and applicability to detect differences in populations with evident differences in mitochondrial capacity. Although NIRS is a relatively novel method to asses mitochondrial capacity, considerable efforts to underpin its validity have been undertaken. For example, NIRS has been shown to correlate well with *ex vivo* respiration of isolated muscle fibres (Ryan et al. 2014) and *in vivo* methods for assessment of whole-body oxidative and mitochondrial capacity, including $\dot{V}O_2$peak (Brizendine et al. 2013) and ^{31}P-MRS (Ryan et al. 2013b). Moreover, significant differences in mitochondrial capacity were observed between inactive individuals and endurance athletes (Brizendine et al. 2013), and between patients with spinal cord injury compared to controls (Erickson et al. 2013). However, it is currently unknown whether NIRS is also sensitive enough to detect possible smaller differences in mitochondrial capacity in a normally active, healthy population. Furthermore,

although age-related mitochondrial decline in muscle was measured in the forearm using NIRS (Chung et al. 2018), no dedicated studies looking at the effect of age on mitochondrial capacity that also take sex, physical activity and muscle group into account, have been performed to date. Therefore, to further establish NIRS as a valid method for the assessment of mitochondrial capacity, dedicated studies in more normal and ageing populations need to be performed. Furthermore, with increasing adiposity and subcutaneous adipose tissue thickness (ATT), the amount of NIRS signal from the muscle decreases significantly, making measurements in populations with higher ATT more challenging (van Beekvelt et al. 2001). This also impacts studies in females, who have a different body composition than males and commonly a thicker subcutaneous adipose tissue layer. Therefore, sensitivity studies need to be performed in females, while such studies may also shed light on possible differences in mitochondrial capacity between the sexes.

1.6. Mechanisms of age-related mitochondrial quality decline

The age-related decline in the ability to generate energy per volume of mitochondria, or mitochondrial quality, suggests a malfunctioning of mitochondrial proteins involved in the generation of ATP. That malfunctioning could either be caused by an increased occurrence of damage, or by decreased damage removal at an older age, compromising optimal mitochondrial functioning. The removal of damaged or dysfunctional mitochondrial proteins and mitochondria is called mitophagy and is thought to be a vitally important process for the prevention of ageing associated decline in mitochondrial capacity (Bakula and Scheibye-Knudsen 2020). Mitochondria are mobile, dynamic organelles that can separate (fission) or fuse to form complex networks (fusion). Fusion allows the network to effectively share and use mitochondrial DNA, protein complexes and metabolites (Westermann 2012), while fission is needed for effective removal of damaged or dysfunction mitochondria (Ding and Yin 2012). However, removal of damaged or dysfunctional mitochondria has been reported to decline with age (Ferrington et al. 2005; Ngo and Davies 2007; Drummond et al. 2014). This finding has been supported by observations of mitochondrial morphology being affected by ageing in model species, where increased, fragmented (Iqbal et al. 2013) or enlarged mitochondria (Leduc-Gaudet et al. 2015) have been seen. Therefore, it is possible that an inability to clear dysfunctional proteins and mitochondria causes the accumulation of damaged proteins, compromising mitochondrial quality.

Alternatively, mitochondrial quality could be decreased due to an increase in damage occurrence. An important source of damage to the mitochondria is oxidative damage to proteins, lipids and nucleic acids, by reactive oxygen species (ROS) produced during respiration. It has been hypothesised that cumulative oxidative damage with age would result in malfunctioning mitochondria. These mitochondria would in their turn increase ROS production, which would damage other mitochondrial proteins, entering a harmful and destructive loop (Harman 1972). Indeed, mutations in mitochondrial DNA have been shown to increase with age and are thought to arise from damage by ROS (Van Remmen et al. 2003; Short et al. 2005). Yet, a causal role between ROS and mitochondrial ageing via mitochondrial DNA or other mechanisms remains to be fully established and as such this topic remains controversial.

1.7. The Acylation theory of mitochondrial ageing

A relatively novel and emerging theory in the field of mitochondrial ageing is based on accumulating damage via reversible post-translational modifications (PTM) of proteins by acylation (Wagner and Hirschey 2014). Protein acylation is the covalent binding of an acyl-group to a lysine amino acid on a protein. The addition of the acyl-group neutralises the lysine's positive charge, thereby affecting protein functionality through conformational changes or protein-protein, protein-substrate or protein-cofactor interactions. Acylation is an interesting PTM in light of mitochondrial quality, because 1) almost all enzymes in mitochondrial metabolism can be subject to acylation (Zhao et al. 2010), 2) a variety of

substrates for acylation occur in the mitochondria as intermediates of metabolism and 3) biochemical conditions in the mitochondria favour acylation, also non-enzymatically (Wagner and Payne 2013). Indeed, situations in which acyl-CoAs are elevated through metabolites have been shown to drive mitochondrial protein acylation (Pougovkina et al. 2014a). Therefore, because levels of acyl-CoA drive protein acylation and acylation affects protein functionality, a link between metabolism and cellular signalling via protein acylation is established (Choudhary et al. 2014). Acetyl-CoA, the central metabolite in glucose, fatty acids and amino acid metabolism, is the most well-studied acyl-PTM. Acetylation of proteins in the mitochondria generally compromises their activity (Schwer et al. 2006; Schlicker et al. 2008; Baeza et al. 2016). For example, acylation of mitochondrial complex I decreases its function, possibly demonstrating how increased levels of acetyl-CoA can signal energy availability and regulate metabolism (Ahn et al. 2008).

Acylation is reversible and, in a normal situation, acyl-groups can be removed from proteins by deacylating enzymes, such as sirtuins (SIRTs). Especially SIRT3-SIRT5 are of interest for mitochondria due to their localisation in these organelles. SIRT3, the main mitochondrial deacetylating sirtuin, is upregulated in conditions where increased acetylation is observed, such as fasting, and acetylation is increased significantly in mice lacking this enzyme (Lombard et al. 2007; Hirschey et al. 2010). Similarly, deacetylation of complex I was dependent on SIRT3 and in a SIRT3 knockdown model, complex I activity was decreased (Ahn et al. 2008). Therefore, SIRT3 was crowned caretaker of the mitochondrial acylome and is indispensable for mitochondrial functioning (Lombard et al. 2015). Nevertheless, all sirtuins, including SIRT3, require NAD^+ as a cofactor for their deacylating activity and levels of this cofactor have been reported decrease with age, in rodents (Yoshino et al. 2011; Braidy et al. 2011; Gomes et al. 2013) and some humans tissues (Massudi et al. 2012; Zhu et al. 2015; Zhou et al. 2016; Clement et al. 2019). This forms the basis for the acylation theory of mitochondrial ageing, which assumes the accumulation of acylation damage, or stress, through an inadequate ability to remove this damage, causing a decrease in protein functionality (Wagner and Hirschey 2014). In the mitochondria, the decrease in NAD^+ levels

and increased acylation would decrease mitochondrial protein functionality, such as observed for complex I, resulting in a decreased respiration per mass of mitochondria. Although to date no data that links NAD^+ levels, acylation and mitochondrial quality exists for human skeletal muscle, in older mice skeletal muscle NAD^+ levels are decreased and associated with a decreased mitochondrial capacity (Gomes et al. 2013; Camacho-Pereira et al. 2016). Furthermore, using supplementation with precursors of NAD^+ to increase NAD+ levels at old age in mice, partially ameliorated the age-driven decline in ATP production (Gomes et al. 2013) and physical decline (Zhang et al. 2016). Therefore, the role of acylation in mitochondrial quality requires future research to elucidate the role of acylation in skeletal muscle ageing in humans.

1.8. Protein propionylation as a novel acyl modification

Besides acetyl CoA, multiple intermediates of metabolism and their corresponding acyl-CoA's are able to post-translationally modify proteins. These result in modifications, such as succinylation (Zhang et al. 2011), glutarylation (Tan et al. 2014), malonylation (Peng et al. 2011), crotonylation (Tan et al. 2011), butyrylation and propionylation (Chen et al. 2007). While these modifications are likely to impact the activity of metabolic enzymes, the effect of these modifications on mitochondrial quality and on muscle physiology has hardly been established, especially in relation to ageing. Protein propionylation, the covalent binding of a three carbon propionyl-group to lysine residues of proteins, might be of specific interest to skeletal muscle metabolism due to the metabolic background of propionyl-CoA, which is the substrate for propionylation. Propionyl-CoA can be derived from the breakdown of the amino acids isoleucine, valine, threonine and methionine, cholesterol and odd-chain fatty acids (Sbaï et al. 1994). Additionally, propionyl-CoA can be derived from propionate, produced by the microbiota and taken up in the intestine (Cummings et al. 1987) (**Figure 1.4**). Therefore, in metabolic situations in which propionyl-CoA levels are elevated, for example during muscle protein breakdown, levels of propionylation could increase and be functionally important, or, when present in excess, compromise functionality. For example,

it is known that increased levels of propionyl-CoA were able to propionylate the propionyl-CoA synthetase enzyme in prokaryotes, thereby inactivating it (Garrity et al. 2007). Moreover, analysis of muscle and liver biopsies from patients with inborn errors in propionyl-CoA metabolism that lead to an accumulation of propionyl-CoA, show a defective mitochondrial respiration (Schwab et al. 2006; de Keyzer et al. 2009) and increased protein propionylation (Pougovkina et al. 2014b). This suggests that 1) increased levels of propionyl-CoA can increase cellular propionylation and that 2) increased propionylation can affect protein functionality. Nevertheless, despite its potential importance for metabolic regulation, very little is known about propionylation, providing an evident rationale to further study this PTM.

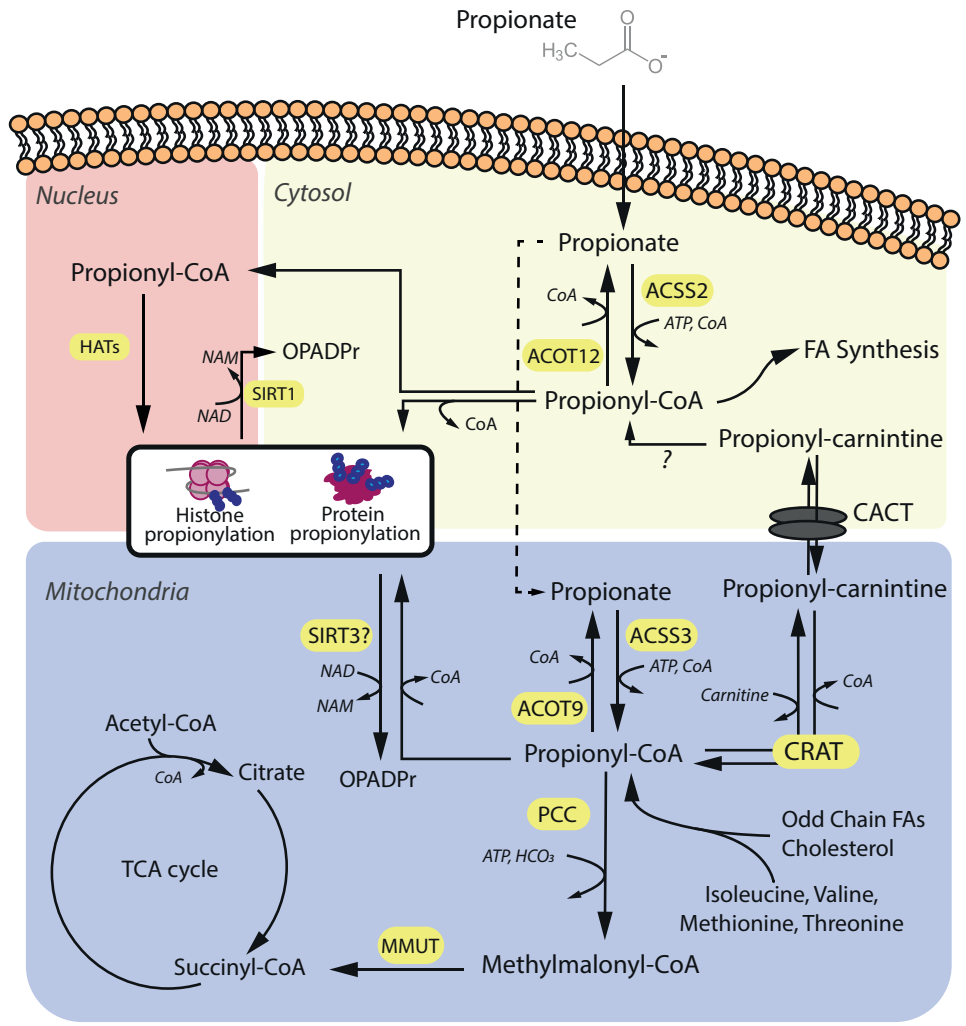

Figure 1.4 Schematic overview of propionate and propionyl-CoA metabolism**.** HATs: Histone Acyltransferases, ACSS2: Acyl-coenzyme A Synthetase Short Chain Family Member 2, ACSS3: Acyl-CoA Synthetase Short Chain Family Member 3, ACOT9: Acyl-CoA Thioesterase 9, ACOT12: Acyl-CoA Thioesterase 12, CRAT: Carnitine Acetyltransferase. PCC: Propionyl-CoA Carboxylase, MMUT: Methylmalonyl-CoA Mutase, CACT: Carnitine-acylcarnitine translocase, HATs: Histone acyltransferases, SIRT3: Sirtuin 3, SIRT1: Sirtuin 1, CoA: Coenzyme A, OPADPr: O-propionyl-ADP-ribose, NAD: Nicotinamide adenine dinucleotide, NAM: Nicotinamide mononucleotide

1.9. Aims and outline of this thesis

To prevent age-related decline in muscle mass and function it is important to understand the ageing process and the individual factors that contribute to it. One important factor is the role of mitochondria in the age-related decline of muscle mass and function. However, although numerous studies have been performed, there appears to be no general consensus on the effect of age on mitochondrial capacity. In part, this is due to a lack of methodology to readily assess mitochondrial capacity *in vivo*, non-invasively. NIRS assessment of mitochondrial capacity could be a valuable tool when further developed and applied to an ageing population. Moreover, due to the positive effect of physical activity and mitochondrial capacity, it is unknown whether the decline in mitochondrial capacity is a secondary, evitable ageing phenomenon (due to a decrease in age-related physical activity) or whether it is a primary, inevitable ageing phenomenon (meaning it declines even when physical activity is maintained). Furthermore, it is known that sex and muscle group are important factors that influence mitochondrial capacity with ageing. Studies that measure mitochondrial capacity should take this into account. Moreover, post-translational protein modifications could be a molecular mechanism involved in mitochondrial quality control. Propionylation is one of these modifications, but the role of propionyl-CoA and propionylation in skeletal muscle metabolism is currently not known.

The overall aim of this thesis is to obtain a better understanding of the role of mitochondria in skeletal muscle ageing.

I aim to:

1) **Further establish NIRS assessment of mitochondrial capacity,** by:
    a. verifying whether NIRS has enough sensitivity to detect differences in mitochondrial capacity in a normal population of high- and low-fitness males and females;

    b. substantiating the physiological relevance of $m\dot{V}O_2$ recovery as a measure of mitochondrial capacity by testing the relationship between $m\dot{V}O_2$ and other measures related to oxidative capacity;
    c. applying NIRS to an ageing population to further substantiate the use of NIRS in skeletal muscle ageing research.
2) **Study the effect of molecular mechanisms of ageing and the effect of ageing on mitochondrial capacity in skeletal muscle, independent of changes in physical activity**, by:
    a. comparing mitochondrial capacity in three different skeletal muscles between young and elderly males with similar physical activity levels;
    b. exploring molecular mechanisms of ageing in *vastus lateralis* muscle biopsies in the aforementioned population using transcriptome sequencing.
3) **Explore the effects of protein propionylation on skeletal muscle and study how this could be linked to mitochondrial quality**, by:
    a. measuring the effect of increased protein propionylation on mitochondrial respiration in a disease model of patients with aberrant propionylation;
    b. investigating functional effects of increased protein propionylation in skeletal muscle cell lines and primary human myotubes.

In **chapter 2** I verify the sensitivity of NIRS assessment of mitochondrial capacity, as well as its ability to detect differences in mitochondrial capacity in a normal population of recreationally active, healthy males, divided into a high- and low-fitness group. Furthermore, I explore the relationship with other parameters related to oxidative capacity to further substantiate the physiological relevance of NIRS assessment of mitochondrial capacity. In **chapter 3** I further verify the sensitivity and applicability of NIRS assessment of mitochondrial capacity in females. Increased adiposity beneath the skin hampers penetration of NIR-light in the muscle. Therefore, testing similar sensitivity to detect

relevant physiological differences in females extends the applicability of NIRS for this population.

In **chapter 4** I study the effect of age in three different skeletal muscles. I apply NIRS to a population of young and older males with similar physical activity patterns. By matching for this physical activity, this allows us to study the effect of age on mitochondrial capacity, independent from differences in physical activity patterns between the age groups. Furthermore, by measuring mitochondrial capacity in three different locomotor muscles, the differences in mitochondrial ageing between muscles will be investigated. Additionally, in **chapter 5** I identify early markers and molecular mechanisms of muscle ageing, using transcriptome sequencing in biopsies taken from the *vastus lateralis,* sampled from the same population of young and older males.

In **chapter 6** I investigate the effect of protein propionylation on mitochondrial quality. To address this, I turn to a disease model of patients with an inborn metabolic error in the PCC gene. As a consequence, these patients accumulate propionyl-CoA and display higher levels of protein propionylation. I use this model to study the effect of propionylation on mitochondrial quality in different cell types, including skeletal muscle. **Chapter 7** builds on the findings of **chapter 6** and addresses the consequences of increased cellular propionylation, specifically in the skeletal muscle. Patients with an inborn error in the PCC gene have a myopathic phenotype and I aim to explore whether the increased protein propionylation could contribute to this phenotype and has functional consequences. For this, I use skeletal muscle cell lines and primary human myotubes.

Finally, the main findings of this thesis, its implications and directions for future research are discussed in **chapter 8**, followed by a short summary of the thesis.

CHAPTER 2

In vivo assessment of muscle mitochondrial function in healthy, young males in relation to parameters of aerobic fitness

Bart Lagerwaard
Jaap Keijer
Kevin K. McCully
Vincent C. J. de Boer
Arie G Nieuwenhuizen

Published in European Journal of Applied Physiology 119, 1799-1808 (2019) Doi: 10.1007/s00421-019-04169-8

Abstract

Objective

The recovery of muscle oxygen consumption (mVO_2) after exercise provides a measure of skeletal muscle mitochondrial capacity, as more and better-functioning mitochondria will be able to restore mVO_2 faster to the pre-exercise state. The aim was to measure muscle mitochondrial capacity using near-infrared spectroscopy (NIRS) within a healthy, normally active population and relate this to parameters of aerobic fitness, investigating the applicability and relevance of using NIRS to assess muscle mitochondrial capacity non-invasively.

Methods

Mitochondrial capacity was vanalysed in the gastrocnemius and flexor digitorum superficialis (FDS) muscles of eight relatively high aerobic fitness ($VO_2peak \geq 57$ mL/kg/min) and eight relatively low aerobic fitness male subjects ($VO_2peak \leq 47$ mL/kg/min). Recovery of whole-body VO_2, i.e. excess post-exercise oxygen consumption (EPOC) was analysed after a cycling protocol.

Results

Mitochondrial capacity, as analysed using NIRS, was significantly higher in high-fitness individuals as compared to low-fitness individuals in the gastrocnemius, but not in the FDS ($p = 0.0036$ and $p = 0.20$, respectively). Mitochondrial capacity in the gastrocnemius was significantly correlated to VO2peak ($R^2 = 0.57$, $p = 0.0019$). Whole-body VO2 recovery was significantly faster in the high-fitness individuals ($p = 0.0048$) and correlated significantly to mitochondrial capacity in the gastrocnemius ($R^2 = 0.34$, $p = 0.028$).

Conclusion

NIRS measurements can be used to assess differences in mitochondrial muscle oxygen consumption within a relatively normal, healthy population. Furthermore, mitochondrial capacity correlated with parameters of aerobic fitness (VO_2peak and EPOC), emphasising the physiological relevance of the NIRS measurements.

2.1. Introduction

Muscle mitochondrial mass and function are positively affected by regular endurance exercise (Tonkonogi and Sahlin 2002). Due to the pivotal role of mitochondria in determining endurance capacity, there is a need for robust and non-invasive measurements of muscle mitochondrial function (Lanza and Nair 2009). Mitochondrial function in skeletal muscle is classically analysed *ex vivo* by measuring oxygen consumption in muscle biopsies. Less-invasive techniques have emerged over the last quarter century, allowing the measurement of mitochondrial function *in vivo*. These techniques are both based on the recovery of muscle homeostasis after exercise (Meyer 1988), assessed by measuring either the regeneration of phosphocreatine (PCr) using magnetic resonance spectroscopy (^{31}P-MRS) or by the return of muscle oxygen consumption ($m\dot{V}O_2$) to basal levels using near-infrared spectroscopy (NIRS). Mitochondrial function analysed by both techniques have been shown to be in good agreement with each other (Ryan et al. 2013b), but NIRS offers advantages over ^{31}P-MRS due to its higher portability and relatively low-costs, making it more suitable for on-site and routine measurements.

NIRS uses the difference in light absorption of oxygenated and deoxygenated haemoglobin and myoglobin in the near-infrared region (Grassi and Quaresima 2016). By emitting light at different wavelengths it is possible to differentiate between the oxygenated and deoxygenated state. When used on muscle and combined with arterial occlusions it allows for measurement of $m\dot{V}O_2$, as the change from oxygenated to deoxygenated haemoglobin and myoglobin reflects the use of oxygen in the tissue underneath the NIRS probe when blood flow is occluded (Van Beekvelt et al. 2001). Multiple, transient arterial occlusions after a short bout of exercise allows for the measurement of post-exercise recovery of $m\dot{V}O_2$, a procedure used to assess mitochondrial capacity (Motobe et al. 2004). The underlying assumption is that post-exercise regeneration of readily available energy carriers (i.e., ATP and PCr) is directly linked to aerobic metabolism and, therefore, a higher mitochondrial capacity will be associated with a faster return of $m\dot{V}O_2$ to the pre-exercise state (McMahon

and Jenkins 2002). Indeed, the NIRS procedure to assess recovery kinetics of $m\dot{V}O_2$ *in* vivo showed a strong correlation to maximal ADP-stimulated respiration of permeabilised muscle fibres *in situ* (Ryan et al. 2014).

On a whole-body level, the regeneration of readily available energy carriers is assumed to contribute to the transient elevation of whole-body oxygen consumption ($m\dot{V}O_2$) above resting values in the immediate post-exercise period, also known as excess post-exercise oxygen consumption (EPOC). EPOC can be divided in a rapid and a prolonged phase, in which the mechanisms that contribute to the elevated $m\dot{V}O_2$ are different (Gaesser and Brooks 1984). In particular, the rapid phase is defined to reflect the myofibrillar consumption of the readily available energy substrates in the beginning of exercise, such as PCr and ATP, as well as the replenishment of tissue and haemoprotein oxygen stores and lactate removal (Chance et al. 1992; Børsheim and Bahr 2003). In accordance with an important role for PCr regeneration in EPOC, Rossiter *et al* showed that whole-body $\dot{V}O_2$ is related to muscle PCr kinetics in the recovery phase (Rossiter et al. 2002). As the latter may be related to skeletal muscle mitochondrial capacity, an inverse relationship between EPOC and NIRS assessment of $m\dot{V}O_2$, reflecting skeletal muscle mitochondrial function, can be hypothesised (Kemp et al. 2015). However, it should be noted that despite clear effects on mitochondrial capacity (Lanza and Nair 2009), the effect of endurance training status on EPOC is controversial, most likely as a result of methodological difficulties (Børsheim and Bahr 2003). When comparing low with high endurance capacity subjects, no research design can control for relative exercise intensity, total work and exercise duration at the same time. Still, by controlling for relative intensity and exercise duration, different $\dot{V}O_2$ recovery dynamics between trained and untrained subjects have been observed (Short and Sedlock 1997). Thus, when using such an approach, post-exercise whole-body $\dot{V}O_2$ recovery dynamics may be a reflection of aerobic fitness and be related skeletal muscle mitochondrial capacity.

NIRS has been used as a non-invasive measure for muscle mitochondrial function in various clinical populations such as COPD and cystic fibrosis patients. In general, NIRS studies

indicate skeletal muscle mitochondrial dysfunction under these pathological conditions (Adami et al. 2017; Willingham and McCully 2017). On the other hand, endurance athletes, characterized by a high whole-body peak oxygen uptake ($\dot{V}O_2$peak), showed a faster post-exercise recovery of $m\dot{V}O_2$ than fully sedentary subjects (Brizendine et al. 2013). Still, the difference in $\dot{V}O_2$peak between the 2 groups was considerable (74 vs 34 ml.kg-1.min-1, respectively). It would be of interest to study whether this technique is also sensitive enough to detect differences within a more normally active, healthy population, as it is as yet unclear to what extent NIRS assessment of skeletal muscle mitochondrial capacity is related to other established measures of oxidative metabolism related to exercise, such as $\dot{V}O_2$peak and EPOC in a normally active, healthy population. This information would further support the applicability and physiological relevance of NIRS assessment of mitochondrial capacity.

The aim of this study is to measure mitochondrial function using NIRS in a recreationally active, healthy population divided in relatively low and relatively high aerobic fitness groups and relate it to parameters of aerobic fitness. The recovery of $m\dot{V}O_2$ in both the frequently activated *gastrocnemius* muscle and in the often undertrained forearm will be measured (Hamner et al. 2010). We hypothesised that the recovery of $m\dot{V}O_2$ and whole-body $\dot{V}O_2$ recovery, i.e. EPOC, is faster in the relatively high-fitness group. Furthermore, we expect muscle and whole-body $\dot{V}O_2$ recoveries to correlate, since post-exercise replenishment of energy stores in the muscle encompasses an important component in the rapid phase of EPOC.

2.2. Material and Methods

2.2.1. Subjects

Healthy males between the age of 18-28 years were recruited from the local university and community population. None of the subjects had a history of cardiovascular, respiratory or metabolic disease. None of the subjects identified as regular smoker (>5 cigarettes per

week), used recreational drugs during the study or reported recent use of performance enhancing drugs or supplements. Subjects were non-anaemic (haemoglobin concentration > 13 g/dL), verified by using HemoCue Hb 201 microcuvette (HemoCue AB, Sweden). Main exercise modalities in high-fitness group were cycling (3x), lacrosse (2x), triathlon (1x), rowing (1x) and running (1x). Main exercise modalities in low-fitness group were sailing (1x), running (1x). weightlifting (1x), volleyball (1x) or no regular exercise at all. Only males were selected in this study, due to the limited penetration depth of the NIRS device used and sex differences in subcutaneous adipose tissue thickness.

2.2.2. Pre-experimental screening protocol

Subjects were selected based on whole-body peak oxygen uptake ($\dot{V}O_2$peak) measured using an incremental exercise test on electrically-braked bicycle ergometer (Corival CPET, Lode, The Netherlands). Subjects were asked to refrain from vigorous exercise for 48 hours and to have consumed their last meal two hours before this test. Oxygen consumption, carbon dioxide production and air flow were measured using MAX-II metabolic cart (AEI technologies, USA). Exhaled air was continuously sampled from a mixing chamber and heart rate was measured with a strap-on chest heart rate monitor (Polar Electro, Finland). After a 3-minute warming-up, the protocol started at a workload of 75W, or 125W for subjects who exercised >3 times a week, and was increased every minute in increments of 25W. Subjects were instructed to maintain a self-selected pedal rate between 70-80 revolutions per minute (RPM). Inability to pedal at a rate above 60 RPM for 15s was considered point of exhaustion, and the end of the test. For the test to be valid, two out of three of the following criteria should have been met: 1) A maximal heart rate within 10 beats of the predicted maximum (220 – age), 2) Attainment of a plateau in $\dot{V}O_2$, i.e. $\dot{V}O_2$ failing to increase with 150 mL/min, despite an increase in work load, 3) Achievement of an RER ≥ 1.1. $\dot{V}O_2$peak was determined by binning data in 15s intervals. Eight relatively high aerobic fitness ($\dot{V}O_2$peak ≥ 57 mL/kg/min) and eight low aerobic fitness subjects ($\dot{V}O_2$peak ≤ 47 mL/kg/min) were selected to take part in the study, based chosen cut offs. A total of 24 subjects were screened to end up with the desired sample size.

2.2.3. Experimental protocol

All measurements were done fasted, i.e. subjects were not allowed to eat after 08:00 PM the night before. The subjects refrained from heavy physical exercise 48 hours prior to testing and from any exercise and consumption of alcohol 24 hours prior to testing. Maximal Voluntary Contraction (MVC) hand grip strength of the dominant hand was measured using a Jamar Hydraulic Hand Dynamometer (Performance Health, IL, USA). Highest value out of three 5s isometric contractions was set as MVC. Body fat percentage was measured according to the four-site method by Durnin-Womersley using the skinfold measurements of the triceps, biceps, sub scapula and supra iliac, measured using a skinfold caliper (Harpenden, UK). Furthermore, skinfold between NIRS receiver and transmitter was measured on the calf and the forearm.

NIRS measurements. Deoxyhaemoglobin (HHb) and oxyhaemoglobin (O_2Hb) were continuously measured using the continuous wave PortaMon wireless, dual-wavelength NIRS system (760 and 850 nm; PortaMon, Artinis Medical Systems, Netherlands). The 40mm channel was used for analysis. Data were collected via bluetooth at 10 Hz using Oxysoft software (Artinis Medical Systems). The NIRS probe was placed longitudinally on the lateral *gastrocnemius* 4 cm distal to the knee joint and on the flexor digitorum superficialis (FDS). To secure the probe and protect it from environmental light, the probe was tightly taped to the skin. To measure oxygen consumption, a blood pressure cuff (Hokanson SC5 and SC12; D.E. Hokanson Inc., Bellevue, WA) was placed proximally of the probe above the knee joint and on the upper arm. The cuff was powered and controlled by a rapid cuff inflator system (Hokanson E20 and AG101 Air source; D.E. Hokanson Inc.) set to a pressure of 230-250 mm Hg. Post-exercise muscle oxygen consumption recovery was assessed similar to previously published protocols (Ryan et al. 2013b). In summary, the protocol consists of three, 30s rest measurements of basal oxygen consumption. To calibrate the signal between individuals, the minimal-oxygenation of the tissue underneath the probe was then determined by 30s maximal hand grip exercise for FDS or by plantar flexion exercise using a rubber resistance band for *gastrocnemius*, followed by an arterial occlusion until baseline or with a maximum

of 4 min total occlusion time. The hyperaemic response after the cuff was released was considered maximal oxygenation. Recovery oxygen consumption after exercise was measured after 30s of intermittent handgrip exercise at 50% of MVC for the FDS or plantar flexion exercise using a rubber resistance band until 50% of maximal oxygenation for *gastrocnemius*. Right after exercise, a series of transient occlusions (5 * 5s on/5s off, 5 * 7s on/7s off, 10 * 10s on/10s off) was used to measure the recovery of muscle oxygen consumption after exercise. Recovery measurements were performed in duplicate with 2 minutes rest in between tests.

Analysis of muscle oxygen consumption data. NIRS data were analysed using Matlab-based (The Mathworks, MA, USA) analysis software (NIRS_UGA, GA, USA). Data were analysed as 100% of maximal oxygenation. $m\dot{V}O_2$ was calculated during every arterial occlusion using the slope of the change in HHb and O_2Hb (Hb difference) for 3s for the 5s occlusions, for 5s for the 7s occlusions, 7s for the 10s occlusions and 15s for the basal measurements. A blood volume correction factor was used for each data point (Ryan et al. 2012) to correct for redistribution of blood distally from the cuff. In short; changes in HHb and O_2Hb should be proportional during arterial occlusions. A blood volume correction factor (β) was calculated to account for possible changes and was used to correct each data point. $m\dot{V}O_2$ recovery measurements post-exercise were fitted to a mono-exponential curve:

$$y\,(t) = End - \Delta * e^{-k\cdot t}$$

with Y representing the $m\dot{V}O_2$ during the arterial occlusions; End being the $m\dot{V}O_2$ immediately after the cessation of exercise; Delta (Δ) being the difference between $m\dot{V}O_2$ after exercise and $m\dot{V}O_2$ during rest; K being the rate constant expressed in time units; t being time. Rate constants of duplicates were averaged. Rate constants calculated from curve fitting with R^2<0.95 were excluded from analysis as a measure of poor data quality.

EPOC measurements. Basal oxygen consumption (method see below) was measured in supine position after an overnight fast. Subject was rested 30 minutes before the facemask was attached. After 20 minutes of basal measurement, the subject cycled for 20 minutes at a work rate adjusted to 55% of $\dot{V}O_2$peak (Maresh et al. 1992). This protocol resulted in equal relative intensity for each subject. Upon cessation of exercise, subjects were placed in supine position for 20 minutes.

Analysis of whole-body oxygen consumption data. Exhaled air was continuously sampled using a strap-on face mask, and binned in 15s intervals. Due to the individualisation of the exercise protocol, absolute oxygen consumption was different between subjects. The recovery of $\dot{V}O_2$ expressed as a percentage of EPOC where the last 10 minutes of exercise were averaged and expressed as 100% EPOC and the values during the last 5 minutes of basal measurements were averaged and expressed as 0% EPOC (Short and Sedlock 1997). The recovery data were plotted using a two phase exponential decay according to the formula:

$$Y = Plateau + YOFast * \exp(-KFast * X) + YOslow * \exp(-KSlow * X), \text{ where}$$
$$Y0Fast = (Y0 - Plateau) * PercentFast * .01$$
$$YOSlow = (Y0 - Plateau) * (100 - PercentFast) * .01$$

In the formula, Plateau represents the basal oxygen consumption, or 0% EPOC. Y0 represents the oxygen consumption during exercise, or 100% EPOC. KFast and KSlow represent the rate constants of recovery as an inverse unit of time. PercentFast is the fraction of the Y that is represented by the fast phase, as percent.

2.2.4. Statistical analyses

Data are presented as mean ± SD. Statistical analyses were performed using GraphPad Prism v.5 (GraphPad Software, CA, USA). Means between the two groups were compared using a Students unpaired t-test. Correlations between variables were compared using regression

analysis. Significance was accepted at $p < 0.05$. Normality was tested using Shapiro-Wilk normality test. Not normal data were compared using Mann-Whitney tests.

2.3. Results

All subjects completed all tests without any contraindications. Physical characteristics are shown in **Table 2.1**. All maximal exercise tests met at least two out of three criteria.

Table 2.1 Physical characteristics of the subjects

	Low-fitness (n=8)	High-fitness (n=8)
Age (yrs)	24.1 ± 2.7	22.6 ± 3.2
Weight (kg)	80.0 ± 8.3	73.3 ± 6.4
Height (m)	1.85 ± 0.05	1.79 ± 0.07
Fat mass (% of weight)	18.7 ± 2.8	12.5 ± 3.2**
$\dot{V}O_2$peak (mL ·Kg $^{-1}$ · min $^{-1}$)	42.5 ± 3.9	62.5 ± 4.1****
Beacke PA score	8.0 ± 0.9	9.5 ± 0.7**
Hemoglobin (g/dL)	15.5 ± 1.1	15.3 ± 1.2
Skinfold forearm	7.2 ± 2.8	5.0 ± 1.6
Skinfold calf	15.0 ± 2.2	9.1 ± 4.1**

*Values are means ± SD. *p <0.05, **p<0.01, ****p<0.0001*

2.3.1. Recovery of m$\dot{V}O_2$ in gastrocnemius and flexor digitorum superficialis

Recovery of oxygen consumption was measured using repeated occlusions after a short exercise protocol in FDS and *gastrocnemius* (**Figure 2.1AB**). Two NIRS data sets were excluded due to $R^2 < 0.95$. Plateau for minimal oxygenation was reached in all individuals. Recovery rate constants were significantly different between high- and low-fitness group for *gastrocnemius* (1.73 ± 0.30 vs. 1.23 ± 0.22, p = 0.0036; **Figure 2.1C**), but not for the flexor digitorum superficialis (1.34 ± 0.35 vs. 1.13 ± 0.28, p = 0.20; **Figure 2.1D**). The recovery constant of the *gastrocnemius* was significantly correlated to $\dot{V}O_2$peak (**Figure 2.2** ;$R^2 = 0.57$, p = 0.0019). In the FDS this correlation was not observed ($R^2 = 0.06$, p = 0.32).

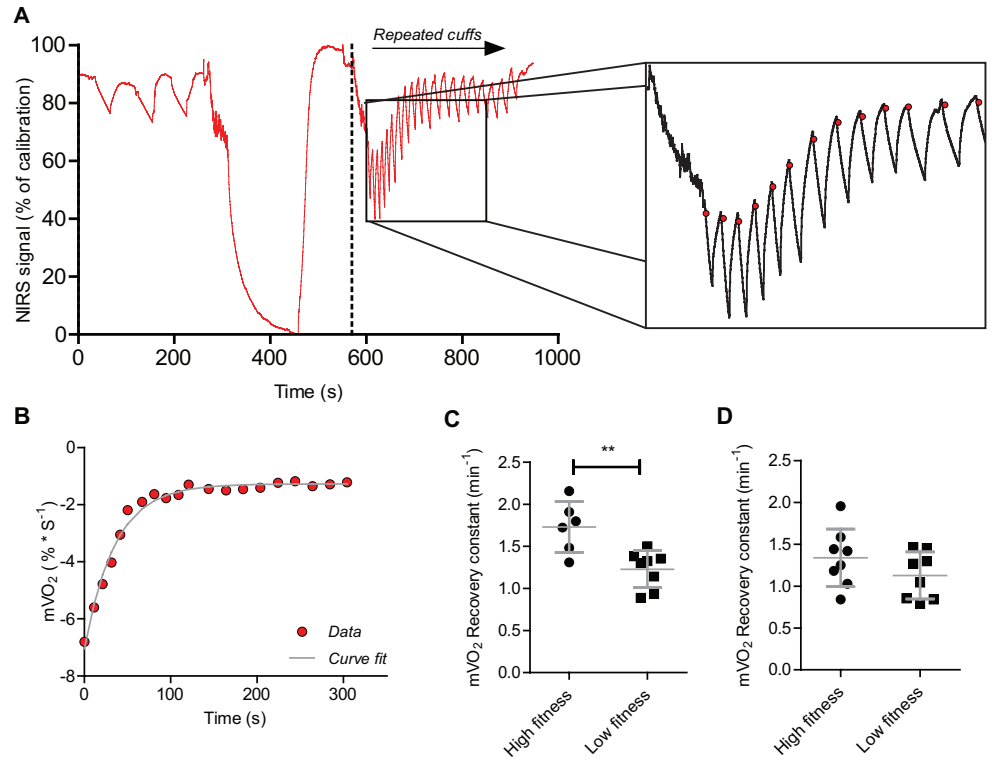

Figure 2.1 (A) Representative plot of NIRS protocol. Red line represents NIRS signal of the Hb difference during protocol in percentage of maximal oxygenation, defined by a calibration procedure. Red dots represent the start of m$\dot{V}O_2$ measurement for each occlusion. **(B)** Curve fitting of m$\dot{V}O_2$ recovery curve, red dots represent a single m$\dot{V}O_2$ measurement. The grey line represents a monoexponential curve fit from which a recovery constant is derived. **(C)** m$\dot{V}O_2$ recovery constants in high-fitness vs low-fitness groups. Recovery constants are derived from monoexponential curve fits of m$\dot{V}O_2$ plots taking from NIRS measurements after 30s of plantar flexion in exercise *gastrocnemius* and 30s of handgrip exercise in **(D)** flexor digitorum superficialis. Values are mean ± SD. **p < 0.005

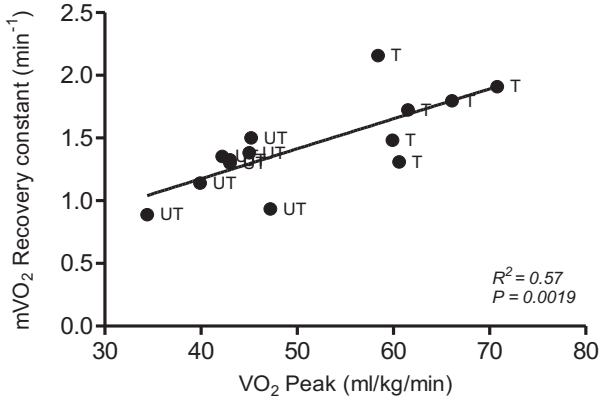

Figure 2.2 Correlation between recovery constants for muscle oxygen recovery in *gastrocnemius* calculated after 30s of plantar flexion exercise using NIRS and maximal oxygen consumption ($\dot{V}O_2$Peak) measured during an incremental exercise test

2.3.2. Recovery of whole-body $\dot{V}O_2$ (EPOC)

The recovery of whole-body $\dot{V}O_2$ was measured after a short cycling protocol in supine position on bed (**Figure 2.3**). Pre-test resting oxygen consumption was not significantly different between the high-fitness (240 ± 37 ml/min) and low-fitness group (229 ± 37 ml/min, p = 0.53). Due to the use of an individualised exercise protocol, oxygen consumption during exercise was significantly larger in the high-fitness group (2409 ± 273 ml/min) compared to the low-fitness group (1710 ± 203 ml/min, P < 0.0001). The relative intensity (as a percentage to one's $\dot{V}O_2$peak was not different between the high-fitness (54 ± 7%) and the low-fitness group (51 ± 6%; p = 0.47). EPOC volume was not significantly different between the high-fitness and low-fitness group (576 ± 241.5 vs 2210 ± 268.8, p = 0.33). Fitting the recovery of $\dot{V}O_2$ expressed as percentage of EPOC to a two phase exponential decay resulted in an good fit (R^2 = 0.95 ± 0.04). The rate constant of the rapid recovery phase was significantly different between the high- and low-fitness group (**Figure 2.4**; p = 0.0048), but not for the rate constant of the prolonged phase (0.34 ± 0.16 min^{-1} vs. 0.18 ± 0.21, p = 0.13). The rate constant of the rapid recovery phase was significantly correlated to $\dot{V}O_2$peak (R^2 = 0.66, p = 0.0004).

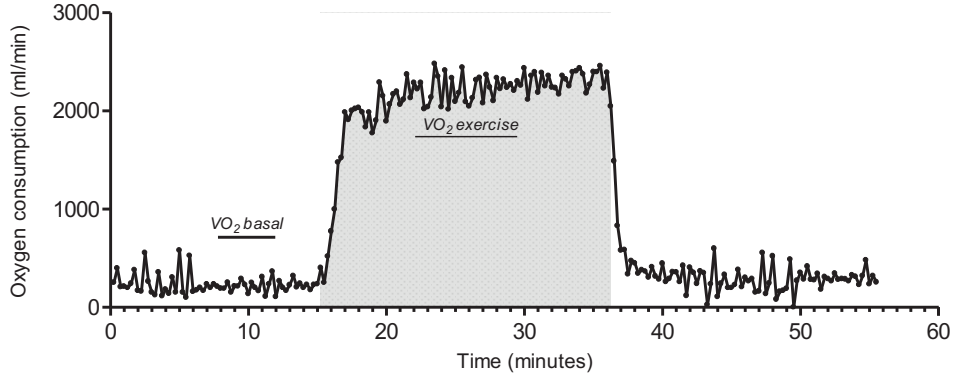

Figure 2.3 Representative measurement of oxygen consumption and recovery during the EPOC protocol. Oxygen consumption during rest and recovery was measured in supine position. Grey area reflects time spent cycling at 55% of $\dot{V}O_2$peak. Oxygen consumption during basal state is indicated with '$\dot{V}O_2$ basal' and during exercise with '$\dot{V}O_2$ exercise'.

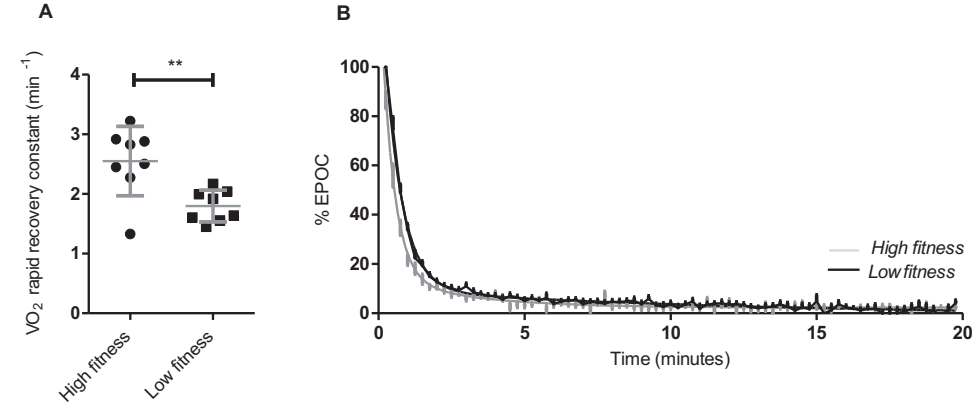

Figure 2.4 (A) Recovery rate constants of the rapid recovery phase of EPOC fitted to two phase exponential decay after 20 min cycling at 55% of $\dot{V}O_2$peak **(B)** Recovery of EPOC presented as percentage of $\dot{V}O_2$ during exercise as an average per group; high-fitness (grey) and low-fitness (dark). Values are mean ± SD. **p < 0.005

2.3.3. Relationship between m$\dot{V}O_2$ and whole-body $\dot{V}O_2$ recovery

In order to test whether there is a relationship between the recovery of oxygen consumption of the muscle after a short exercise and that of the whole-body after an exercise protocol, both conditions were analysed for correlation. Indeed, the recovery

constant of *gastrocnemius* was significantly correlated with the recovery constant of the rapid phase whole-body m$\dot{V}O_2$ (**Figure 2.5**; $R^2 = 0.34$, $p = 0.028$).

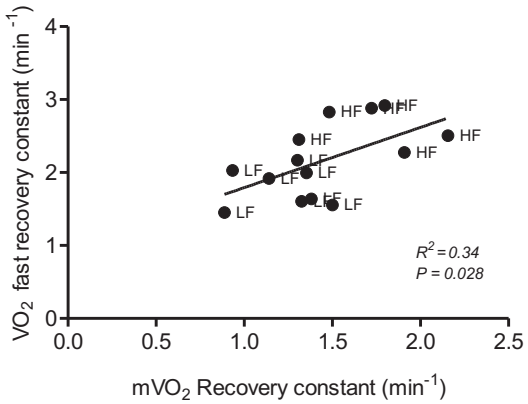

Figure 2.5 Correlation between rate constants of the rapid phase of recovery of whole-body oxygen consumption after 20 minutes cycling protocol at 55% of $\dot{V}O_2$peak and the recovery of muscle oxygen consumption of the *gastrocnemius* after 30s low intensity plantar flexion exercise measured using NIRS. HF = High-fitness, LF = Low-fitness

2.4. Discussion

Our results show that the present NIRS protocol was able to discriminate high-fitness and low-fitness individuals within a relatively homogenous group, i.e., young, healthy and normally active males, based on post-exercise m$\dot{V}O_2$ recovery. Recovery during the rapid phase of the EPOC was faster in the high-fitness group as compared to the low-fitness group. Furthermore, a correlation between whole-body oxygen recovery and m$\dot{V}O_2$ recovery was found in the *gastrocnemius* muscle, pointing out the physiological relevance for NIRS assessment of m$\dot{V}O_2$ recovery.

2.4.1. Relationship between m$\dot{V}O_2$ recovery and aerobic fitness

In the high-fitness group, recovery of oxygen consumption in *gastrocnemius* muscle was faster than in the low-fitness group indicating that mitochondrial capacity of that specific muscle was significantly higher in the high-fitness group compared to the low-fitness group. Our findings are in line with previous assessment of mitochondrial capacity measured using

NIRS in highly trained endurance athletes and inactive individuals, showing an approximate doubling of mitochondrial capacity in the athlete group (Brizendine et al. 2013). However, with an average $\dot{V}O_2$ peak of 34 and 74 mL $\cdot Kg^{-1} \cdot min^{-1}$ for the inactive subjects and endurance athletes, respectively, the difference in endurance capacity, and thus aerobic adaptations, was substantially larger than in the current study. Thus, the present NIRS protocol showed adequate sensitivity to detect the (considerably smaller) difference in mitochondrial capacity between the high- and low-fitness individuals within our relatively homogenous population of healthy, normally active, young males. Our results therefore further widen the application of this non-invasive technique for *in vivo* human metabolic research.

Our results also correlate well with *ex vivo* measurements of skeletal muscle mitochondrial capacity in trained and untrained male subjects, with an average age and $\dot{V}O_2$ peak remarkably similar to our study (Phielix et al. 2012). Maximal mitochondrial respiration of permeabilised *vastus lateralis* muscle fibres from trained subjects was approximately 50% higher when compared to untrained subjects. Endurance training increases aerobic capacity due to, among other mechanisms, an increase in mitochondrial mass and more regular exposure to local exercise stimuli most likely induced mitochondrial biogenesis and increased mitochondrial capacity in the high-fitness group (Ljubicic et al. 2010). This increased mitochondrial capacity may be physiologically relevant since, when taking both groups together, mitochondrial capacity of the *gastrocnemius* was significantly correlated with $\dot{V}O_2$Peak. Our results are in agreement with a study in elderly subjects showing a comparable correlation between *ex vivo* mitochondrial oxidative capacity of permeabilised muscle fibres obtained from a *vastus lateralis* biopsy and $\dot{V}O_2$ peak (Coen et al. 2013). Still, as ageing is generally associated with a decline both in $\dot{V}O_2$ peak and mitochondrial capacity (Conley et al. 2000a), this latter study does not necessarily reflect a correlation between endurance training status and skeletal muscle mitochondrial capacity in young adults. A ^{31}P-MRS study in adult, but not elderly, women revealed a significant correlation between the rate of PCr resynthesis and $\dot{V}O_2$ max (Larson-Meyer et al. 2000), and in young males

cytochrome C oxidase activity of *vastus lateralis* muscle tissue correlated significantly to $\dot{V}O_2$ max (Booth and Narahara 1974). Hence, the results of our NIRS study, showing a relatively strong correlation between *in vivo* oxidative capacity of the muscle and $\dot{V}O_2$ peak, are in line with the aforementioned studies. Therefore, the current study further establishes the application of NIRS to assess skeletal muscle mitochondrial capacity *in vivo*, and provides novel additional data supporting the physiological relevance of skeletal muscle mitochondrial capacity for aerobic performance.

2.4.2. Relationship between gastrocnemius m$\dot{V}O_2$ recovery and whole-body $\dot{V}O_2$ recovery

A faster rapid whole-body oxygen recovery after 20 minutes of cycling at 55% of $\dot{V}O_2$Peak was shown to be correlated with a faster m$\dot{V}O_2$ recovery in the *gastrocnemius*. Although various adaptations to endurance training could underlie the significant difference the rapid recovery phase of the whole-body $\dot{V}O_2$ recovery between the two groups, such as enhanced blood flow and increased vascularisation of the muscle (Egan and Zierath 2013), the significant correlation suggests a role for mitochondrial capacity in post-exercise $\dot{V}O_2$ recovery. Both NIRS and EPOC, at least in part, reflect the ability of the mitochondria to replenish readily available energy substrates post-exercise using oxidative metabolism. It should be noted, however, that the correlation between post-exercise recovery of whole-body oxygen and skeletal muscle mitochondrial capacity as assessed by NIRS was less strong than the correlation between $\dot{V}O_2$peak and mitochondrial capacity, as became apparent from the lower R^2 values. In other words, the contribution of skeletal muscle mitochondrial capacity to the post-exercise $\dot{V}O_2$ recovery dynamics of the current protocol is smaller than its contribution to $\dot{V}O_2$peak, suggesting that other mechanisms, such as lactate removal also play an important role in post-exercise $\dot{V}O_2$ recovery. The ability of NIRS to discriminate between two groups with distinct aerobic fitness levels and the correlation with an established recovery parameter supports the use of measuring m$\dot{V}O_2$ recovery using NIRS as a relevant physiological parameter to reflect mitochondrial capacity.

To assess the effect of increased mitochondrial capacity on recovery of whole-body $\dot{V}O2$ recovery, a short and moderate-intensity exercise protocol was used. Short and moderate exercise protocols have been shown to induce a rapid EPOC component, while limiting an increase in plasma lactate concentration and body temperature (Maresh et al. 1992). Accordingly, while cycling at the same relative intensity, 55% of their $\dot{V}O_2$peak for a duration of 20 minutes, absolute oxygen consumption was higher in high-fitness individuals due to their higher $\dot{V}O_2$peak and higher fat free mass. Possibly, the initial $\dot{V}O_2$ at the end of exercise and the start of the EPOC could affect the rapid phase of recovery. To correct for this, oxygen consumption during exercise was set to 100% and the EPOC expressed as percentage of recovery to baseline values. Alternatively, one could opt for an absolute intensity protocol, in which the magnitude of the oxygen debt at the end of the exercise protocol is equal, regardless of endurance capacity. Yet, the duration of exercise to reach a specific absolute oxygen debt would not be comparable and EPOC is also known to be affected by exercise duration and intensity, this has been extensively reviewed elsewhere (Børsheim and Bahr 2003).

The difference in post-exercise whole-body $\dot{V}O_2$ recovery between the high-fitness and low fitness-group, as observed in the current protocol controlled for relative exercise intensity, is in agreement with a previous study of Short et al., who reported a difference in fast recovery of $\dot{V}O_2$ between trained and untrained subjects when EPOC is expressed as a percentage of recovery to baseline (Short and Sedlock 1997). Likewise, a faster EPOC recovery was reported in trained women compared to untrained women after 300 Kcal cycle test at 65% of $\dot{V}O_2$peak (Frey et al. 1993). On the other hand, several studies using an absolute EPOC protocol found no differences in EPOC in trained and untrained individuals (Brehm and Gutin 1986; Sedlock 1994). Due to these different study outcomes, probably resulting from the different experimental setups, there is no clear consensus on the effects of (endurance) training status on EPOC. However, in agreement with the above-mentioned results, our results, not only show a faster $\dot{V}O2$ recovery in high-fitness group but also show a significant correlation between skeletal muscle mitochondrial capacity and $\dot{V}O_2$ recovery,

provide novel supporting evidence that post-exercise $\dot{V}O_2$ recovery by using a protocol controlling for relative exercise intensity, may reflect aerobic fitness. This notion is further substantiated by the significant correlation between this parameter and $\dot{V}O_2$peak in the present study.

2.4.3. m$\dot{V}O_2$ recovery in FDS and gastrocnemius

Besides local effects of exercise, one-legged cycling exercise can also trigger responses in muscles that are not engaged in exercise, as was shown by Catoire et al. (Catoire et al. 2012). We hypothesised that high-fitness individuals could therefore also have increased mitochondrial capacity in muscle groups that are not directly activated by exercise, such as the FDS muscle in the forearm, but are indirectly activated through systemic effects of exercise. Yet, although mitochondrial capacity was higher in the *gastrocnemius* of high-fitness individuals compared to low-fitness individuals, the difference in mitochondrial capacity in FDS was smaller but not significant with a p-value of 0.2. It could be that the effects of systemic exercise on the indirectly stimulated muscle is too small to be detected by the current sample size, or that those systemic effects on mitochondrial capacity did not occur.

In a previous study, mitochondrial capacity was analysed in the *vastus lateralis* muscle (Brizendine et al. 2013). The current study is the first to analyse differences in mitochondrial capacity comparing endurance capacity using the *gastrocnemius* instead. Measurements in the *vastus lateralis* are done with a cuff placed high up on the leg which is generally considered more uncomfortable for a subject than measurements in the *gastrocnemius*, where the cuff is placed just below or above the knee joint. This measurement technique likely increases the throughput and tolerability of the NIRS measurement, which will ensure reliability of the data and possibly lowers variability between measurements.

2.5. Conclusions

This study provides evidence that NIRS measurements can be used to assess differences in mitochondrial muscle oxygen consumption within a relatively normal, healthy population. In a normally active population, mitochondrial capacity was significantly higher in high-fitness individuals with a relatively high $\dot{V}O_2$peak. Furthermore, mitochondrial capacity correlated with $\dot{V}O_2$peak, but also to post-exercise whole-body $\dot{V}O_2$ recovery, emphasising the physiological relevance of the NIRS measurements. The observed correlation between skeletal muscle mitochondrial capacity and whole-body $\dot{V}O_2$ recovery supports the delineation of this latter measure as a parameter of aerobic fitness. Future research that aims to study mitochondrial capacity could use the non-invasive nature of NIRS, it's relative affordability and increased portability, which allows it to be easily applied to assess mitochondrial functionality to study effects of lifestyle and/or dietary interventions.

Acknowledgements We acknowledge Floor den Ouden and Simone Rentél for assistance with data collection during the study.

Author contributions BL performed all experiments and principal data analysis; KKM methodological advise and data analysis; BL, AGN, VCJB., JK conception and design of research; data analysis and interpretation, drafting of manuscript. All authors edited, revised and approved final version of manuscript.

Compliance with ethical standards The study was approved by the medical ethical committee of Wageningen University with reference number NL60823.081.17. All procedures performed were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards (Fortaleza, Brazil 2013). The study is registered in the Dutch trial register (NTR6470). Informed consent Informed consent was obtained from all individual participants included in the study

CHAPTER 3

Muscle mitochondrial capacity in high- and low-fitness females using near-infrared spectroscopy

Bart Lagerwaard
Joëlle J.E. Janssen
Iris Cuijpers
Jaap Keijer
Vincent C. J. de Boer
Arie G. Nieuwenhuizen

Abstract

Objective
The recovery of muscle oxygen consumption (mVO_2) after exercise measured using near-infrared spectroscopy (NIRS) provides a measure of skeletal muscle mitochondrial capacity. As females generally have a larger subcutaneous adipose tissue thickness and lower intramuscular myoglobin and hemoglobin levels compared to males, both factors that influence signal to noise ratio, it is unknown whether NIRS can detect physiologically relevant differences within an exclusively female population. Therefore, the aim of this study was to measure mitochondrial capacity in females divided in a high- and low-fitness group to further support the use of NIRS assessment of muscle mitochondrial capacity in this population.

Methods
Mitochondrial capacity was analysed using NIRS in the gastrocnemius muscle and the wrist flexors of 32 young female adults, equally divided in relatively high ($VO_2peak \geq 47$ mL/kg/min) and relatively low aerobic fitness group ($VO_2peak \leq 37$ mL/kg/min).

Results
mVO_2 recovery was significantly faster in the high compared to the low-fitness group in the gastrocnemius, but not in the wrist flexors ($p = 0.009$ and $p = 0.0528$, respectively). Furthermore, VO2peak was significantly correlated to mVO_2 recovery in both gastrocnemius ($R^2 = 0.27$, $p = 0.0051$) and wrist flexors ($R^2 = 0.13$, $p = 0.0393$).

Conclusion
NIRS measurements can be used to assess differences in mitochondrial capacity within a healthy population of females and is correlated to VO_2peak. This further underlines the use of NIRS assessment of muscle mitochondrial capacity providing additional support for NIRS as a promising approach to monitor mitochondrial capacity.

3.1. Introduction

Regular endurance exercise increases whole-body peak oxygen uptake ($\dot{V}O_2peak$) due to bodily adaptations that increase oxygen transport, delivery and consumption. At the level of the skeletal muscle, maximal oxygen consumption increases due to an increase in muscle mitochondrial mass and function (Lanza and Nair 2009). The exact contribution of this increased skeletal muscle oxidative capacity to the improved $\dot{V}O_2peak$ after regular endurance exercise remains debated. Nevertheless, there appears to be a strong link between mitochondrial mass and $\dot{V}O_2peak$ (Weibel et al. 1991). Furthermore, skeletal muscle oxidative capacity is suggested to be a determining factor in prolonged strenuous exercise performance (Holloszy and Coyle 1984). Classically, skeletal muscle oxidative or mitochondrial capacity is analysed *ex vivo,* by measuring oxygen consumption in permeabilised muscle fibres from muscle biopsies. The invasive nature of this technique, the isolation of the tissue from its physiological environment, as well as the infringement of cell integrity by the permeabilisation procedure provides a rationale for non-invasive assessment of muscle mitochondrial capacity in an intact system.

A near-infrared spectroscopy (NIRS)-based technique has been developed to assess skeletal muscle mitochondrial capacity *in vivo* (Nagasawa et al. 2003). Using multiple, transient vascular occlusions after a short bout of exercise it allows for the measurement of post-exercise recovery of $m\dot{V}O_2$ (Motobe et al. 2004). The underlying assumption is that post-exercise regeneration of readily available energy carriers, i.e., ATP and phosphocreatine (PCr), is directly linked to aerobic metabolism and, therefore, a higher mitochondrial capacity will be associated with a faster recovery to the pre-exercise state (McMahon and Jenkins 2002). NIRS offers advantages over other non-invasive techniques, such as magnetic resonance spectroscopy (^{31}P-MRS), due to its higher portability and relatively low-costs, making it more suitable for on-site and routine measurements. However, a limitation of the NIRS technique is the limited penetration depth in the tissue, as the greater the distance the NIR light has to travel to reach muscle, the lower the resolution of this signal (van

Beekvelt et al. 2001). Therefore, measurements in individuals with higher subcutaneous adipose tissue thickness (ATT) are often more challenging, as less light can reach the muscle and the intensity of light received at the detector is lower (Craig et al. 2017). Hence, the signal to noise ratio of the technique may be smaller in females, who generally have a higher adiposity and ATT compared to males. This may even be muscle dependent, as females exhibit lower levels of total hemoglobin and myoglobin in the *gastrocnemius* muscle, but not in others, e.g. wrist flexors, compared to males (Craig et al. 2017).

In a normally active male population, we previously showed that NIRS is able to detect differences in mitochondrial capacity in the *gastrocnemius* muscle between relatively high- and low-fitness subjects, which correlated to $\dot{V}O_2$peak (Lagerwaard et al. 2019). It is unsure if these results are easily extrapolated to a female population because of the above-mentioned differences in ATT depth and total hemoglobin and myoglobin, that could affect the signal to noise ratio, but also because of possible sex differences in the relationship between mitochondrial capacity $\dot{V}O_2$peak. For instance, males showed a larger stimulation of mitochondrial biogenesis than females upon sprint interval training (Scalzo et al. 2014). Indeed, a recent NIRS study showed no correlation between mitochondrial capacity in the *gastrocnemius* muscle and $\dot{V}O_2$peak when males and females were combined (Beever et al. 2020), which contrasted our previous findings in males only (Lagerwaard et al. 2019).

Thus, even though studies in mixed population clearly confirm the application of the technique in both sexes (Sako et al. 2001; Hamaoka et al. 2011; Ryan et al. 2013a, 2014; Brizendine et al. 2013), it is unknown whether NIRS is able to detect physiologically relevant differences within an exclusively female population. To date there are no studies using NIRS in an exclusively female population, while females are underrepresented as subjects in sports and exercise research (Costello et al. 2014). Therefore, the aim of this study is to measure skeletal muscle mitochondrial capacity in healthy females at different levels of aerobic fitness to further support the applicability of NIRS assessment of mitochondrial capacity in this population. Mitochondrial capacity was measured in both the frequently

activated *gastrocnemius* muscle and the often-undertrained wrist flexors in 32 recreationally active, healthy females divided into a relatively low and a relatively high-fitness group. We hypothesised that high-fitness females will show a higher mitochondrial capacity compared to low-fitness females in both muscles.

3.2. Material and Methods

3.2.1. Subjects

Healthy females between the age of 18-28 years were recruited from the local university and community population. None of the subjects had a history of cardiovascular, respiratory or metabolic disease. None of the subjects were a regular smoker (>5 cigarettes per week), used recreational drugs during the study or reported recent use of performance enhancing drugs or supplements. Subjects were non-anaemic (haemoglobin concentration > 12 g/dL), verified by using HemoCue Hb 201 microcuvette (HemoCue AB, Sweden). None of the subjects were pregnant or lactating. All participants were measured within the end of the follicular phase until menstruation and did not use any hormonal contraceptives with exception of the birth control pill.

3.2.2. Pre-experimental screening protocol

Subjects were selected based on $\dot{V}O_2$peak, measured using an incremental exercise test on electrically braked bicycle ergometer (Corival CPET, Lode, The Netherlands). Subjects were asked to refrain themselves from vigorous exercise for 48 hours and to have consumed their last meal two hours before this test. Oxygen consumption, carbon dioxide production and air flow were measured using MAX-II metabolic cart (AEI technologies, USA). Exhaled air was continuously sampled from a mixing chamber and heart rate was measured with a strap-on chest heart rate monitor (Polar Electro, Finland). After a 3-minute unloaded cycling warming-up, the protocol started at a workload of 50W for subjects who exercised <3 times a week or 75 W for subjects who exercised >3 times per week and was increased every minute in increments of 15 W. Subjects were instructed to maintain a self-selected pedal

rate between 70-80 revolutions per minute (RPM). Inability to pedal at a rate above 60 RPM for 10s was considered point of exhaustion and the end of the test. For the test to be valid, two out of three of the following criteria should have been met: 1) A maximal heart rate within 10 beats of the predicted maximum (220 – age), 2) Attainment of a plateau in $\dot{V}O_2$, i.e. $\dot{V}O_2$ failing to increase with 150 mL/min, despite an increase in work load, 3) Achievement of an RER ≥ 1.1. $\dot{V}O_2$peak was determined by binning data in 15s intervals. 16 relatively high-fitness ($\dot{V}O_2$peak ≥ 47 mL/kg/min) and 16 low-fitness subjects ($\dot{V}O_2$peak ≤ 37 mL/kg/min) were selected to take part in the study, based on chosen cut offs. Main exercise modalities in the high-fitness group were running/athletics (6x), rowing (3x), kickboxing (2x), hockey (1x), swimming (1x), ice skating (1x), climbing (1x) and weightlifting (1x). Main exercise modalities in low-fitness group were aerobics (2x), horseback riding (1x), weightlifting (1x), climbing (1x), walking (1x), dancing (1x), badminton (1x) or no regular exercise (8x). A total of 111 exercise tests were conducted to end up with the desired sample size.

3.2.3. Experimental protocol

The subjects refrained from heavy physical exercise 48 hours prior to testing and from any exercise and consumption of alcohol 24 hours prior to testing. Maximal Voluntary Contraction (MVC) hand grip strength of the non-dominant and dominant hand was measured using a Jamar Hydraulic Hand Dynamometer (Performance Health, IL, USA). Highest value out of three 5s isometric contractions was set as MVC. Body fat percentage was measured according to the four-site method by Durnin-Womersley using the skinfold measurements of the triceps, biceps, sub scapula and supra iliac, measured using a skinfold caliper (Harpenden, UK). Furthermore, skinfold between NIRS receiver and transmitter was measured on the calf and the forearm.

3.2.4. NIRS measurements

Deoxyhaemoglobin (HHb) and oxyhaemoglobin (O_2Hb) were continuously measured using the continuous wave Oxymon, dual-wavelength NIRS system (760 and 850 nm; Artinis

Medical Systems, Netherlands) at three optode distances 15 mm, 45 mm and 55 mm. Data were collected via bluetooth at 10 Hz using Oxysoft software (Artinis Medical Systems). The NIRS probe was placed longitudinally on the belly of the muscle, identified by palpation by an experienced investigator, on the medial *gastrocnemius* and on the wrist flexors of the non-dominant side. To secure the probe and protect it from environmental light, the probe was tightly taped to the skin. To measure oxygen consumption, a blood pressure cuff (Hokanson SC5 and SC12; D.E. Hokanson Inc., Bellevue, WA) was placed proximally of the probe above the knee joint and on the upper arm. The cuff was powered and controlled by a rapid cuff inflator system (Hokanson E20 and AG101 Air source; D.E. Hokanson Inc.) set to a pressure of 230-250 mm Hg. Post-exercise muscle oxygen consumption recovery was assessed similar to previously published protocols (Ryan et al. 2013b). In summary, the protocol consists of three 30s rest measurements of resting oxygen consumption. To calibrate the signal between individuals, the minimal-oxygenation (0%) of the tissue underneath the probe was determined by 30s maximal hand grip exercise for wrist flexors or by plantar flexion exercise using a rubber resistance band for *gastrocnemius*, followed by an 4-minute arterial occlusion. The hyperemic response after the cuff was released was considered maximal oxygenation (100%). Recovery of muscle oxygen consumption after exercise was measured after 30s of intermittent (approximately 0.5 Hz) handgrip exercise at 40% of MVC for the wrist flexors or plantar flexion exercise using a rubber resistance band until 50% of maximal oxygenation for *gastrocnemius*. Right after exercise, a series of transient occlusions (5 * 5s on/5s off, 5 * 7s on/7s off, 10 * 10s on/10s off) was used to measure the recovery of muscle oxygen consumption after exercise. Recovery measurements were performed in duplicate with 2 minutes rest in between tests.

3.2.5. Analysis of muscle oxygen consumption data.

NIRS data were analysed using Matlab-based (The Mathworks, MA, USA) analysis software (NIRS_UGA, GA, USA). Optode distance of 45 mm or 55 mm was used, based on inspection of data of raw light counts during measurements. Data were analysed as % of maximal oxygenation. m$\dot{V}O_2$ was calculated during every arterial occlusion using the slope of the

change in HHb and O_2Hb (Hb difference) for 3s for the 5s occlusions, for 5s for the 7s occlusions, 7s for the 10s occlusions and 15s for the basal measurements. A blood volume correction factor was used for each data point (Ryan et al. 2012) to correct for redistribution of blood distally from the cuff. In short; changes in HHb and O_2Hb should be proportional during arterial occlusions. A blood volume correction factor (β) was calculated to account for possible changes and was used to correct each data point. $m\dot{V}O_2$ recovery measurements post-exercise were fitted to a mono-exponential curve:

$$y\,(t) = End - \Delta * e^{-k\cdot t}$$

with y representing the $m\dot{V}O_2$ during the arterial occlusions; End being the $m\dot{V}O_2$ immediately after the cessation of exercise; delta (Δ) being the difference between $m\dot{V}O_2$ after exercise and $m\dot{V}O_2$ during rest; k being the rate constant expressed in time units; t being time. Recovery of muscle oxygen consumption follows mono-exponential curve (Meyer 1988), therefore data points outside curve were considered artifacts and omitted from curve fitting. Data were analysed blinded by two researchers. In case of discrepancy between analyses, third researcher analysed data set (blinded) and consensus was reached. Rate constants calculated from curve fitting with $R^2 < 0.95$ were excluded from analysis as a measure of poor data quality. Rate constants of duplicates were averaged.

3.2.6. Statistical analyses

Data are presented as mean ± SD, unless indicated otherwise. Statistical analyses were performed using GraphPad Prism v.5 (GraphPad Software, CA, USA). Means between the two groups were compared using a Students unpaired t-test. Correlations between variables were compared using regression analysis. Significance was accepted at $p < 0.05$. Normality was tested using Shapiro-Wilk normality test. Not-normal data were compared using Mann-Whitney tests.

3.3. Results

All subjects completed all tests without any contraindications. All maximal exercise tests met at least two out of three pre-set criteria. Physical characteristics are shown in **Table 3.1**.

Table 3.1 Physical characteristics of the subjects

	Low-fitness (n=16)	High-fitness (n=16)
Age (years)	24.0 [21.3-25.5]	21.8 [21.5-23.6]
Ethnicity	Caucasian (11), Asian (1), Indo-pacific (4)	All Caucasian
Weight (kg)	59.2 ± 7.2	60.8 ± 6.9
Height (m)	1.63 ± 0.07	1.68 ± 0.04*
Fat mass (% of weight)	28.9 ± 3.9	24.6 ± 4.7**
$\dot{V}O_2$peak (mL ·Kg $^{-1}$ · min $^{-1}$)	35.1 [32.2-35.7]	51.0 [49.2-55.4] ***
MVC dominant arm	30.0 [25.3-33.5]	36.5 [32.0-39.50]*
MVC non-dominant arm	27.5 [24.0-33.5]	33.5 [30.3-37.0]*
Haemoglobin (mmol/L)	8.4 ± 0.6	8.5 ± 0.6
ATT wrist flexors (mm)	5.3 [4.3-6.9]	4.0 [2.3-5.0]*
ATT GAS (mm)	8.6 [6.9-10.6]	6.9 [6.0-7.9]*

*Maximal oxygen consumption ($\dot{V}O_2$peak), maximal voluntary contraction (MVC) handgrip strength, adipose tissue thickness (ATT), gastrocnemius (GAS). Values are mean ± SD for normally distributed data, and median [Inter quartile range] for not normally distributed data. *p < 0.05 **p < 0.01 ***p < 0.001*

3.3.1. Recovery of $m\dot{V}O_2$ in gastrocnemius and wrist flexors.

The NIRS protocol, which was used both for the *gastrocnemius* and wrist flexors, included 3 measurements of basal $m\dot{V}O_2$, assessment of minimal and maximal oxygenation level and the repeated occlusions to assess recovery of oxygen consumption after a short exercise protocol (**Figure 3.1A**). For *gastrocnemius*, two data sets were excluded due to $R^2 < 0.95$, two were excluded due to failed calibration measurement, i.e. no plateau for minimal oxygenation was reached, and one data set was excluded due to technical issues (only had 15mm channel). For all other measurements, plateau for minimal oxygenation was reached. Recovery rate constants were significantly different between the high- and low-fitness group for *gastrocnemius* (1.48 ± 0.47 vs. 2.06 ± 0.57, p = 0.009; **Figure 3.1BC**), but not for the wrist flexors (1.10 ± 0.15 vs. 1.24 ± 0.23, p = 0.0528; **Figure 3.1DE**).

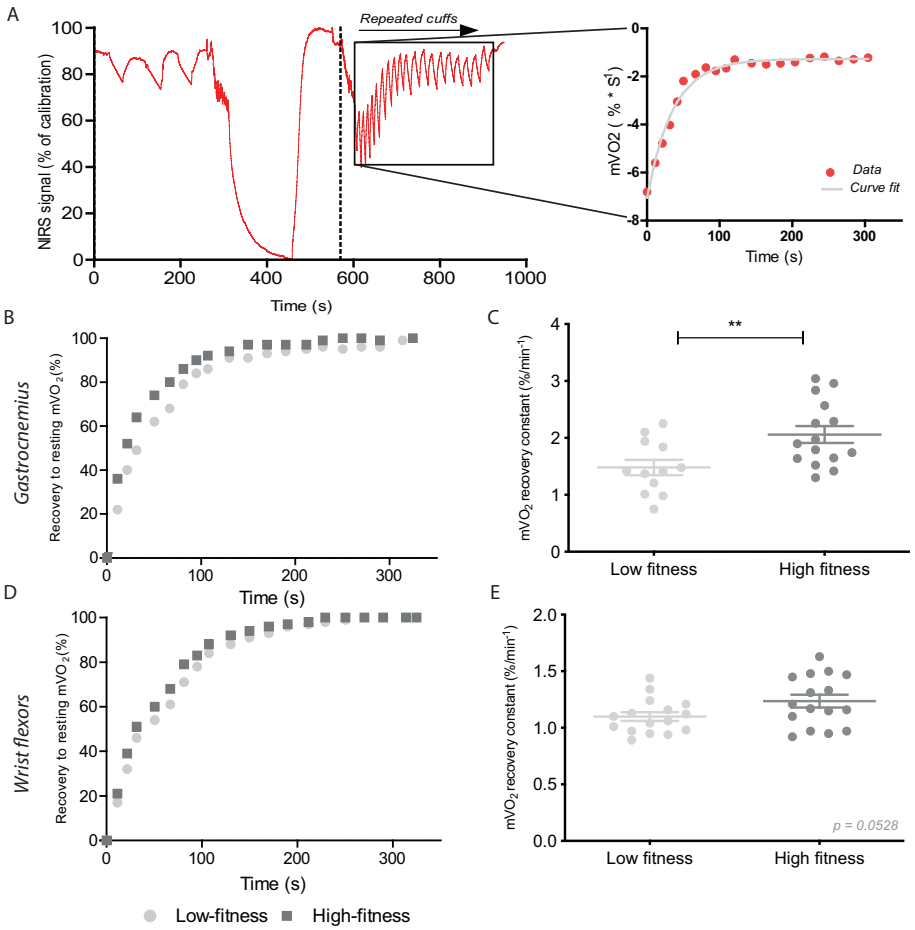

Figure 3.1 (A) Representative plot of NIRS protocol. Red line represents NIRS signal of the Hb difference during protocol as percentage of maximal oxygenation. Repeated measurement m$\dot{V}O_2$ (red dots) are fitted to a monoexponential curve fit (grey line) from which a recovery constant is derived. **(B)** Average curve fits for the low-fitness and high-fitness group for m$\dot{V}O_2$ recovery presented as percentage of basal m$\dot{V}O_2$ after 30s of plantar flexion exercise in *gastrocnemius* and **(D)** after a handgrip exercise in wrist flexors. Recovery constants derived from monoexponential curve fits for *gastrocnemius* **(C)** and wrist flexors **(E)**. For *gastrocnemius* muscle n=12 vs n=15. Values are represented as mean ± SD. **p < 0.01

3.3.2. Relationship between m$\dot{V}O_2$ recovery and whole-body oxygen uptake

In order to test the relationship between endurance capacity, measured as $\dot{V}O_2$peak, and m$\dot{V}O_2$ recovery, measured using NIRS, a correlation analysis was performed. The m$\dot{V}O_2$ recovery constant of the *gastrocnemius* was significantly correlated to $\dot{V}O_2$peak (**Figure 3.2A**; $R^2 = 0.27$, p = 0.0051). Furthermore, in the wrist flexors a significant correlation was observed between m$\dot{V}O_2$ recovery constant and $\dot{V}O_2$peak (**Figure 3.2B**; $R^2 = 0.13$, p = 0.0393).

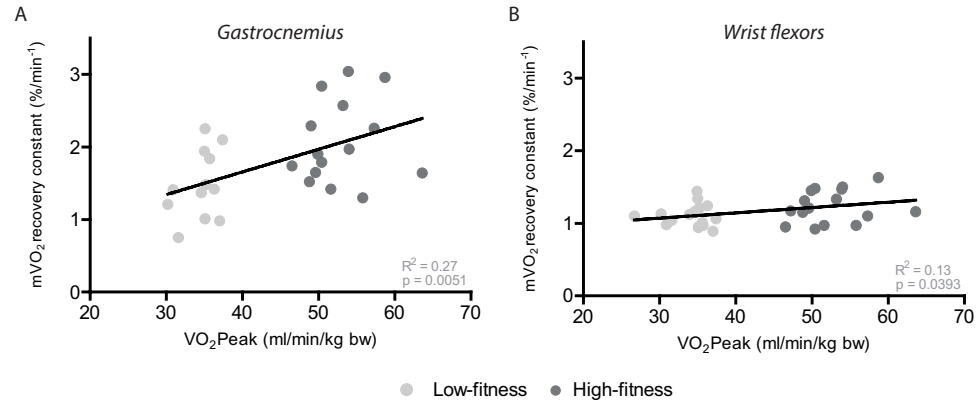

Figure 3.2 Correlation between maximal oxygen consumption ($\dot{V}O_2$Peak) measured during an incremental exercise test and recovery constants for muscle oxygen consumption recovery (m$\dot{V}O_2$) measured using NIRS in *gastrocnemius* **(A)** calculated after 30s of plantar flexion and wrist flexors **(B)** calculated after 30s of handgrip exercise at 50% of MVC in the high-fitness (dark grey) and low-fitness (light grey) group.

3.4. Discussion

The aim of this study was to measure skeletal muscle mitochondrial capacity using NIRS in healthy females in the *gastrocnemius* and wrist flexors muscles to further support NIRS assessment of mitochondrial capacity in this population. We are the first to show that recovery of m$\dot{V}O_2$ after a short bout of exercise as measure for mitochondrial capacity is significantly faster in the *gastrocnemius* muscle of high-fitness compared to low-fitness individuals in an exclusively female population. Recovery of m$\dot{V}O_2$ in the wrist flexors muscle was not statistically different in between two groups. Furthermore, when taking both groups together, we found a significant correlation between $\dot{V}O_2$peak and recovery of m$\dot{V}O_2$ in the *gastrocnemius* and wrist flexors.

3.4.1. m$\dot{V}O2$ recovery in gastrocnemius between high- and low-fitness females

This study shows that NIRS is able to detect physiological relevant differences in mitochondrial capacity in a healthy, recreationally active female population. The differences in mitochondrial capacity likely reflect a higher mitochondrial capacity in high-fitness individuals, i.e., more or more efficient mitochondria were able to reinstate muscle homeostasis faster. In a previous, but unique, study we showed a 40% faster m$\dot{V}O_2$ recovery in the *gastrocnemius* muscle of high-fitness compared to low-fitness males (Lagerwaard et al. 2019). The difference in magnitude of $\dot{V}O_2$peak was comparable with the current study, in which we likewise observed a 40% faster m$\dot{V}O_2$ recovery in high-fitness compared to low-fitness females. Brizendine et al. showed an approximate doubling of mitochondrial capacity in the *vastus lateralis* muscle in endurance athletes compared to inactive individuals (Brizendine et al. 2013). Although this study included both males and females, the vast majority of the endurance athletes were males and with an absolute difference in $\dot{V}O_2$peak of 40 mL/kg/min between the groups, the distinction between the groups was twice as large compared to the current study. Therefore, the present study highlights the sensitivity of NIRS measurements of m$\dot{V}O_2$ recovery to detect smaller differences in mitochondrial capacity and further extends the applicability of the technique, also in an exclusively female population.

The highly comparable results between in m$\dot{V}O_2$ recovery in the *gastrocnemius* muscle between the two sexes indicates the applicability of this NIRS-based technique detect physiological relevant differences also in an exclusively female population. This is an important finding, as sex differences that could affect the NIRS measurement have been identified, such as a lower total hemoglobin and myoglobin in the *gastrocnemius* (Craig et al. 2017) and generally higher ATT in females compared to males. Higher levels of ATT can greatly affect the NIR-signal and consequently the signal to noise ratio of the measurement (van Beekvelt et al. 2001; Craig et al. 2017). Even though in the current experimental protocol the difference in ATT was accounted for by normalising of the signal within each person using a physiological calibration (Hamaoka et al. 1996). Still, interrogation depth of the muscle is decreased with increasing ATT and this can result in a substantial attenuation

of the signal from muscle tissue, such that a doubling of ATT from 4 to 8 mm reduces the contribution of total-[Hb+Mb] to the signal by 50% using a 20 mm source-detector distance (Craig et al. 2017). In the current population, average ATT for the *gastrocnemius* muscle was 8.1 mm, which was expectedly higher than previously observed in males (5.9 mm) (Lagerwaard et al. 2019). Not many studies have measured in these ranges of ATT in females (van Beekvelt et al. 2001; Brizendine et al. 2013; Southern et al. 2014; Adami et al. 2017; Beever et al. 2020). Yet, studies that did measure close to our range in ATT either reported difficulties (Adami et al. 2017), adapted the penetration depth according to the ATT per individual (Ryan et al. 2013b, a) or used a frequency-domain NIRS device that can better quantify the degree of light scattering (Ryan et al. 2014).

To overcome the relatively high ATT, we used a greater source-detector distance of 45mm or 55mm in females, compared to 35mm in males. A greater source-detector distances allows for deeper tissue penetration and consequently increased attribution of muscle to the NIR-signal. Nevertheless, increasing source-detector distance will also cause less light to reach the detector, as more signal is lost due scattering in the tissue. Still, our results showed that with a distance of 45 mm and 55 mm, tissue penetration and signal to noise ratios were sufficiently high to obtain reliable, i.e. $R^2 > 0.95$, m$\dot{V}O_2$ recovery curves and to were able to identify differences in m$\dot{V}O_2$ recovery between two fitness groups in the *gastrocnemius* muscle a healthy, recreationally active female population. Nevertheless, two data sets were excluded from analysis low curve fitting, or R^2. These data sets excluded for low R^2 were among the highest in ATT thickness (10.95 mm and 11.05 mm). Therefore, although other measurements with higher ATT (e.g., 11.05 and 11.35 mm) were successful, and the NIR signal is also affected by other factors such as optode placement, exercise execution and movement artifacts, it could be that the larger contribution of adipose tissue to the NIR signal negatively affected the reliability of the m$\dot{V}O_2$ recovery curves. Therefore, our results suggest that increasing the source-detector distance is an effective, yet limited, approach for the application of NIRS to assess mitochondrial capacity in muscles with a substantial ATT.

3.4.2. The relationship between aerobic fitness and m$\dot{V}O_2$ recovery

Although our primary aim was to find differences in m$\dot{V}O_2$ recovery in high- and low-fitness females, when taking both groups together, we found a significant correlation between $\dot{V}O_2$peak and recovery of m$\dot{V}O_2$ in the *gastrocnemius.* Such a correlation between m$\dot{V}O_2$recovery and $\dot{V}O_2$peak were previously found in the *gastrocnemius* muscle of males (9), and in the *vastus lateralis* of mixed populations (Brizendine et al. 2013; Beever et al. 2020). Besides, the current results are in line with a ^{31}P-MRS study, showing a correlation between the rate of PCr resynthesis in the *gastrocnemius* muscle and $\dot{V}O_2$peak in a female population (Larson-Meyer et al. 2000). Comparable to NIRS, ^{31}P-MRS uses the recovery of muscle homeostasis after exercise, assessed by measuring the regeneration of PCr as a proxy for mitochondrial capacity (Meyer 1988; Nagasawa et al. 2003) and the two techniques show a good agreement (Sako et al. 2001; Ryan et al. 2013b).

On the contrary, in a combined male and female population, m$\dot{V}O_2$recovery in the *gastrocnemius* muscle was not correlated to $\dot{V}O_2$peak corrected for lean body mass (Beever et al. 2020). Possibly, sex differences in the relationship between mitochondrial capacity $\dot{V}O_2$peak might have subverted this correlation when grouping both sexes together, because it has been shown that, for example, men have a larger stimulation of mitochondrial biogenesis in response to exercise training (Scalzo et al. 2014). Nevertheless, the collected NIRS results by Beever et al. do not point towards prominent sexual dimorphism in this respect, as mitochondrial capacity in *gastrocnemius* muscle (but also in *vastus lateralis*) was similar in males and females with comparable $\dot{V}O_2$max corrected for lean body mass (Beever et al. 2020). In the current study, we show that compared with a similar population of males, e.g. similar age, magnitude differences in $\dot{V}O_2$peak and relative range $\dot{V}O_2$peak, high-fitness females have a similar relative increase in m$\dot{V}O_2$ recovery compared to low-fitness females (Lagerwaard et al. 2019). As a result, the slopes of the regression lines of m$\dot{V}O_2$ vs $\dot{V}O_2$peak were not strikingly different for males and females (0.033 ± 0.010 vs. 0.024 ± 0.0060). In other words, for every increase in $\dot{V}O_2$peak, males and females show a similar increase in mitochondrial capacity. Therefore, it seems more likely that differences in methodology are responsible for the observed discrepancies. For example, in the study by Beever et al., exercise intensity was lower than in our studies, which could negatively influence activation of mitochondrial oxidative metabolism and affect recovery kinetics (Wüst et al. 2013). Moreover, differences in the inclusion criteria set for curve fitting (i.e., $R^2 > 0.95$ in the current study versus $R^2 > 0.75$), may have affected the results, as a lower R^2 decreases accuracy of $\dot{V}O_2$ recovery constants. Interestingly, when we combined our current data in females with the data previously obtained in males, there was a significant correlation between mitochondrial capacity in *gastrocnemius* muscle and $\dot{V}O_2$peak ($R^2 = 0.17$, $p = 0.007$).

When comparing the same correlation in males, females have a lower R^2 compared to males (Lagerwaard et al. 2019), suggesting that $\dot{V}O_2$peak is a weaker predictor for m$\dot{V}O_2$ recovery in females, at least in the *gastrocnemius* muscle. However, $\dot{V}O_2$peak does not merely encompass mitochondrial capacity, but rather is an interplay between oxygen uptake, delivery and consumption (Bassett and Howley 2000). In addition, other muscles than solemnly the *gastrocnemius* define maximal oxygen consumption during an incremental cycling test (Wakeling and Horn 2009), likely explaining the rather moderate correlation with m$\dot{V}O_2$ recovery. Despite the moderate correlation, in a recent study looking at predictors of exercise performance on a time-to-completion cycling trial, it was shown that m$\dot{V}O_2$ recovery measured using NIRS best predicted performance on the trial (Batterson et al. 2020). This supports the physiological relevance of NIRS assessment of m$\dot{V}O_2$ recovery assessment as a relevant marker in sports and exercise science. Additionally, NIRS has advantages over established techniques, such as less invasive than a muscle biopsy and more portable and lower cost compared to ^{31}P-MRS. Moreover, recently it has been shown that using a 6-occlusion protocol is a valid and reproducible alternative to protocols using more occlusions, such as the current one (Sumner et al. 2020). Using a shorter protocol reduces testing time or could, if desired, increase replicates to increase precision of the measurement. The strong prediction for exercise performance, relative fast testing and the

portability, appoint $m\dot{V}O_2$ recovery measurements using NIRS as a promising approach to monitor aerobic performance in both laboratory and field-based settings.

3.4.3. $m\dot{V}O_2$ recovery in wrist flexors between high- and low-fitness females

Although a significant difference was found in $m\dot{V}O_2$ recovery in the *gastrocnemius* muscle, a significant difference in $m\dot{V}O_2$ recovery was not observed in wrist flexors between high-fitness and low-fitness females. This result is similar to data obtained in males with similar differences in $\dot{V}O_2$peak (Lagerwaard et al. 2019). However, with a p-value near significance and the weak correlation between $m\dot{V}O_2$ recovery and $\dot{V}O_2$peak, one might argue that a slight increase in sample size would have resulted in a statistically significant difference. Nevertheless, not considering statistical significance, the difference $m\dot{V}O_2$ recovery kinetics is rather small and could be less biologically relevant. This discrepancy between the wrist flexor and *gastrocnemius* muscle might be attributed to less frequent activation of the wrist flexors during endurance exercise and consequently less mitochondrial adaptations, such as increased amount and the efficiency of the mitochondria (Hamner et al. 2010). Therefore, although the wrist flexors are a convenient muscle group to measure due to low ATT levels and exercise standardisation, it is likely a poorer reflection of aerobic fitness. Therefore, $m\dot{V}O_2$ recovery kinetics in this muscle should therefore not be used as a predictor for aerobic capacity or exercise performance.

3.5. Conclusions

This study provides evidence for sensitive measurements of mitochondrial capacity using NIRS in a female population. In a population of healthy, recreationally active females, mitochondrial capacity was significantly higher in the *gastrocnemius* of high-fitness compared to low-fitness females. Furthermore, mitochondrial capacity was significantly correlated to $\dot{V}O_2$peak. These results further substantiate the use of $m\dot{V}O_2$ recovery as a measure for mitochondrial capacity measured non-invasively using NIRS as a relevant physiological parameter. Furthermore, these results support the applicability of this

technique to detect relevant physiological differences in a female population with higher ATT by using a physiological calibration and greater source-detector distances. However, increasing source-detector distance comes with limitations, such as decreased signal intensity at the detector due to the scattering of light in the tissue. Furthermore, one has to consider portability, as commercially available portable NIRS often have a smaller maximal source-detector distance compared to wired NIRS optodes (Perrey and Ferrari 2018). Portability, besides relative faster testing and lower costs, is a promising feature of the NIRS assessment of mitochondrial capacity, allowing measurements in an onsite, field-based setting. Therefore, testing different populations should be considered good practice to further increase applicability of the technique.

Acknowledgements The authors greatly acknowledge the commitment of the volunteers who participated in the study. We acknowledge Laura Kessels for assistance with data collection during the study. We acknowledge professor McCully from the University of Georgia USA for providing the software for data analysis.

Author contributions BL, JJJE, IC performed all experiments and principal data analysis; BL, JJJE, AGN, VCJB., JK conception and design of research; data analysis and interpretation. BL drafting of manuscript. All authors edited, revised and approved final version of manuscript.

Compliance with ethical standards The study was approved by the medical ethical committee of Wageningen University with reference number NL70136.081.19. All procedures performed were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards (Fortaleza, Brazil 2013). The study is registered in the Dutch trial register (NL7891). Written informed consent was obtained from all individual participants included in the study.

CHAPTER 4

In vivo assessment of mitochondrial capacity using NIRS in locomotor muscles of young and elderly males with similar physical activity levels

Bart Lagerwaard
Arie G. Nieuwenhuizen
Vincent C. J. de Boer
Jaap Keijer

Published in Geroscience 42, 299-310 (2020)
Doi: 10.1007/s11357-019-00145-4

Abstract

Objective
Mitochondrial capacity is pivotal to skeletal muscle function and is suggested to decline with age. However, there is large heterogeneity in current data, possibly due to effect modifiers such as physical activity, sex and muscle group. Yet, few studies have compared multiple muscle groups in different age groups with comparable physical activity levels.

Methods
Here, we newly used near-infrared spectroscopy (NIRS) to characterise mitochondrial capacity in three different locomotor muscles in young (19-25 year) and older (65-71 year), healthy males with similar physical activity levels. Mitochondrial capacity and reperfusion after arterial occlusion were measured in the vastus lateralis (VL), the gastrocnemius (GA) and the tibialis anterior (TA).

Results
Physical activity was verified using accelerometry and was not different between the age groups (404.3 ± 214.9 vs 494.9 ± 187.0 activity kcal per day, p = 0.16). Mitochondrial capacity was significantly lower in older males in the GA and VL, but not in the TA (p=0.048, p=0.036 and p=0.64, respectively). Reperfusion rate was not significantly different for the GA (p = 0.55), but was significantly faster in the TA and VL in the young group compared to the older group (p = 0.0094 and p = 0.039, respectively).

Conclusion
We identified distinct modes of mitochondrial ageing in different locomotor muscles in a young and older population with similar physical activity patterns. Furthermore, we show that NIRS is suitable for relatively easy application in ageing research and can reveal novel insights into mitochondrial functioning with age.

4.1. Introduction

Ageing is associated with a decline in skeletal muscle mass and strength, also known as sarcopenia (Morley et al. 2001). Sarcopenia is thought to be mediated in part by a decline in skeletal muscle mitochondrial capacity, as both the amount of mitochondria and their capacity to generate energy decrease with age in the muscle, resulting in a reduced endurance capacity (Welle et al. 2003; Short et al. 2005; Marzetti et al. 2013). Improving or sustaining muscle mitochondrial capacity could delay the age-related decline in endurance capacity, ultimately retaining physical function and improving quality of life (Lanza et al. 2008; Coen et al. 2013).

Due to the pivotal function of mitochondria in the process of ageing, it is essential to routinely and robustly assess mitochondrial capacity. *In vivo* 31-phosphorus magnetic resonance spectroscopy (^{31}P-MRS) and near-infrared spectroscopy (NIRS) are existing techniques that can be applied to assess mitochondrial capacity *in vivo*. Typically, for both techniques, assessment of mitochondrial capacity involves the recovery of muscle homeostasis after exercise, however ^{31}P-MRS measures the recovery of phosphocreatine (PCr), whereas NIRS measures the recovery of muscle oxygen consumption ($m\dot{V}O_2$) as a parameter for mitochondrial capacity (Kemp et al. 2015; Grassi and Quaresima 2016). NIRS makes use of the difference in light absorption in the near-infrared region of oxygenated (O_2Hb) and deoxygenated haemoglobin and myoglobin (HHb) and can therefore be used to monitor muscle oxygenation. Combining NIRS with arterial occlusions is used to measure $m\dot{V}O_2$ in the muscle *in vivo* (Hamaoka et al. 1996). The $m\dot{V}O_2$ recovery kinetics after exercise follow a mono-exponential function of which the rate constant is used as a measure for mitochondrial capacity, as better-functioning mitochondria will recover $m\dot{V}O_2$ faster (Motobe et al. 2004). This application of NIRS correlated well to ^{31}P-MRS measurements of PCr recovery and *ex vivo* high resolution respirometry (Ryan et al. 2013b, 2014). Although ^{31}P-MRS is more widely used, NIRS offers advantages over ^{31}P-MRS due to its higher mobility, relatively low costs and higher throughput, making NIRS more suitable for routine

measurements to, for example, study the effect of age on muscle mitochondrial capacity. Despite its easier applicability, NIRS has not been used to assess the effects of age on muscle mitochondrial capacity in locomotor muscles.

Ex vivo respiratory analysis of muscle biopsies taken from the *vastus lateralis* (VL) show a consistent, negative effect of age on muscle oxidative capacity (Short et al. 2005; Irving et al. 2015; Porter et al. 2015; Lalia et al. 2017). Yet, ^{31}P-MRS analysis of different muscle types report a heterogeneous effect of age on PCr-recovery (Fitzgerald et al. 2016). In the VL, most studies showed a negative effect of age on PCr-recovery (Conley et al. 2000b; Johannsen et al. 2012; Larsen et al. 2012; Choi et al. 2016), but on other locomotor muscles, such as the *gastrocnemius* muscle (GA) and *tibialis anterior* (TA), this effect was not observed (Chilibeck et al. 1998; Wray et al. 2009; Larsen et al. 2012; Tevald et al. 2014; Hart et al. 2015). However, some studies do find a negative effect of age on PCr-recovery in the GA (McCully et al. 1993; Waters et al. 2003; Layec et al. 2013) and it has been suggested that the conflicting results could arise from the use of different populations with different physical activity levels. Physical activity has a positive effect on muscle oxidative capacity (Tonkonogi and Sahlin 2002), and is thought to be able to protect from, or at least mitigate, the deteriorating effect of age (Lanza et al. 2008; Larsen et al. 2012). Yet, physical activity is documented to decrease with advancing age (Troiano et al. 2008) and therefore isolating the effect of age on mitochondrial capacity is challenging, as this effect is often entangled with a decrease in physical activity. Therefore, controlling for the confounding effects of physical activity is essential, if not a requisite, in studies looking into the effect of age on mitochondrial capacity.

Since it is unclear how mitochondrial capacity is affected in different muscle types with aging, we aimed to profile mitochondrial capacity using NIRS in three different muscle types, i.e. the GA, TA and VL in young and older healthy males. These muscles serve an important function during locomotion and are accessible by NIRS due to their superficial position. To negate the effect of physical activity, subjects with similar self-reported physical activity

patterns were included in the study and this was verified using accelerometry measurements. We hypothesised a lower mitochondrial capacity in the GA and the VL and an unaffected or higher mitochondrial capacity in the TA.

4.2. Material and Methods

4.2.1. Subjects

Healthy males between the age of 19-25 (young) and 65-71 (older) years were recruited from the local population. Low to moderately physical active individuals were recruited using a self-reported exercise frequency of 1-2 hours of structured physical activity per week or a Baecke habitual physical activity score between 7-10 points (Baecke et al. 1982). Older individuals were not physically impaired as determined using the short performance battery test (SPPB) with a minimum score of 11 (Guralnik et al. 1994). None of the subjects identified as regular smoker, used recreational drugs during the study or reported recent use of performance enhancing drugs or supplements. Subjects were non-anaemic (haemoglobin concentration > 8.0 mmol/L), verified by using HemoCue Hb 201 microcuvette (HemoCue AB, Sweden). None of the subjects had health concerns regarding respiratory or metabolic disease. One elderly subject used cholesterol-lowering medication, one used a diuretic and one a used both cholesterol-lowering medication and a diuretic.

4.2.2. Experimental protocol

The subjects refrained from heavy physical exercise 48 hours prior to testing and from any exercise and alcohol consumption 24 hours prior to testing. Maximal Voluntary Contraction (MVC) hand grip strength was measured using a Jamar Hydraulic Hand Dynamometer (Performance Health, IL, USA). The dominant and the non-dominant arm were assessed three times while seated upright at a table. The subjects performed the measurement while the dynamometer was resting on the table with the elbow at a 90° angle. Highest value out of three 5s isometric contractions was set as MVC. Body fat percentage was determined according to the four-site method by Durnin-Womersley using skinfold caliper (Harpenden,

UK) measurements of the triceps, biceps, sub scapula and supra iliac. Furthermore, skinfold between NIRS receiver and transmitter was measured on the GA, TA and VL.

4.2.3. NIRS measurements.

Deoxyhaemoglobin (HHb) and oxyhaemoglobin (O_2Hb) were continuously measured using a PortaMon wireless, dual-wavelength system (760 and 850 nm; PortaMon, Artinis Medical Systems, Netherlands). The optode distance (distance between emitter and receiver) of 40 mm was used for analysis. Data were collected at 10 Hz via bluetooth using Oxysoft software (Artinis Medical Systems). The NIRS probe was placed longitudinally 10-15 cm distal to the kneecap on the on the GA and TA and 15 cm proximal to the kneecap on the VL. To secure the probe and protect it from environmental light, the probe was tightly taped to the skin. To measure oxygen consumption, a blood pressure cuff (Hokanson SC5 and SC12; D.E. Hokanson Inc., Bellevue, WA) was placed proximally of the probe just below the knee joint or as high up on the thigh as possible. The cuff was powered and controlled by a rapid cuff inflator system (Hokanson E20 and AG101 Air source; D.E. Hokanson Inc.) set to a pressure of 250 mm Hg. Post exercise muscle oxygen consumption recovery was assessed similar to previously published protocols (Ryan et al. 2013b). In summary, the protocol consists of three 30s rest measurements of basal oxygen consumption. To calibrate the signal between individuals, the minimal oxygenation of the tissue underneath the probe was then determined after 30s of exercise followed by a 4-minute occlusion till signal plateaus. The hyperaemic response after the cuff was released was considered maximal oxygenation. Recovery oxygen consumption was measured immediately after 30s exercise until 50% of oxygenation signal, using a series of transient occlusions (5 * 5s on/5s off, 5 * 7s on/7s off, 10 * 10s on/10s off). Recovery measurements were performed twice with 2 minutes rest in between tests. 30s exercise is defined as 30s plantar flexion using a rubber resistance band for GA (until 50% of oxygenation signal), 30s of dorsiflexion using a rubber resistance band for TA (until 50% of oxygenation signal) and 30s of twitch electrical stimulation (biphasic, duration interval 200/50 µs, 4 Hz) was used for the VL. Four electrodes (Compex, USA) were placed on the skin, proximal and distal to the NIRS and connected to a Compex Pro – THETA

electrical stimulator (Chattanooga, USA). Current intensity was adjusted individually to a maximal tolerable level.

4.2.4. Reperfusion measurements.

Reperfusion rate was measured using the recovery of the O_2Hb NIRS signal after the 4-minute arterial occlusion performed during the NIRS protocol and was defined as the half-life in seconds to reach maximal oxygenation. Maximal oxygenation was defined as the plateau in O_2Hb NIRS signal, i.e. O_2Hb-signal did not increase for 10s. The subject was in supine position with slight elevation of the upper body and instructed to sustain from any movement during reperfusion.

4.2.5. Analysis of muscle oxygen consumption data.

NIRS data were blinded and analysed using Matlab-based (The Mathworks, MA, USA) analysis software (NIRS_UGA, GA, USA). Data were analysed as 100% of maximal oxygenation. $m\dot{V}O_2$ was calculated during every arterial occlusion using the slope of the change in HHb and O_2Hb for 3s for the 5s occlusions, for 5s for the 7s occlusions, 7s for the 10s occlusions and 15s for the basal measurements. A blood volume correction factor was used for each data point (Ryan et al. 2012) to correct for retributions of blood distally from the cuff. $m\dot{V}O_2$ recovery measurements post exercise were fitted to a mono-exponential curve:

$$y\,(t) = End - \Delta * e^{-k \cdot t}$$

Where Y represents the $m\dot{V}O_2$ during the arterial occlusions; End being the $m\dot{V}O_2$ immediately after the cessation of exercise; Delta (Δ) being the difference between $m\dot{V}O_2$ after exercise and $m\dot{V}O_2$ during rest; K being the rate constant expressed in time units; t being time. Rate constants of duplicates were averaged. Rate constants calculated from curve fitting with R^2<0.95 were excluded from analysis for GA and TA as a measure of poor data quality. For VL a curve fitting till R^2<0.90 was accepted due to lower muscle activation using electrical stimulation.

4.2.6. Accelerometry

Subjects were instructed to wear triaxial accelerometer (wGT3X-BT, Actigraph,USA) for seven consecutive days using an elastic band at the waist of the non-dominant leg. The accelerometer was worn during all activities, excluding showering, swimming and sleeping. Wear time was manually verified using daily diaries on shower and bed times. Counts were sampled at 30 Hz and stored in 60s epochs to determine counts per minute (CPM). Two weekend and three week days were used for data analysis. Percentage of wear time in sedentary (SPA), light (LPA) and moderate to vigorous physical activity (MVPA) was determined using the cut offs provided by Troiano et al., being 0 - 99 CPM, 100 - 2019 CPM, 2020 - ∞ CPM respectively (Troiano et al. 2008). Activity kcals per day were calculated using the counts from all axis according to the Freedom VM3 equation (Sasaki et al. 2011).

4.2.7. Statistical analyses

Data were presented as mean ± SD. Statistical analyses were performed using GraphPad Prism v.5 (GraphPad Software, CA, USA). Means between the two groups were compared using a Students unpaired t-test. Normality was tested using Shapiro-Wilk normality test. Correlations between variables were calculated using regression analysis. Means between three muscles were compared using one-way ANOVA with Tukey's multiple comparison test. Significance was accepted at $p< 0.05$.

4.3. Results

4.3.1. Subject characteristics

All subjects completed all tests without any contra-indications. Most physical characteristics were similar, yet the older males were significantly heavier than the young individuals and had a significantly higher fat mass (**Table 4.1**). Fat free mass and MVC were not significantly different between the two groups. Skinfolds on measurements sites were similar for VL and TA, but were slightly lower for old compared to young on the GA. All older individuals had a SPPB score of 11 or higher indicating normal physical function in this group.

Table 4.1 Physical characteristics of the subjects

	Young (n=20)	Older (n=20)	p-value
Age (years)	22 ± 2.0	69 ± 1.9	**<0.000**
BMI (kg/m^2)	22.6 ± 1.9	25.4 ± 1.7	**<0.000**
Body Fat (%)	15.3 ± 3.1	25.1 ± 3.9	<0.000
Fat free mass (kg)	63.5 ± 6.9	61.0 ± 5.0	0.200
MVC dominant arm (kg)	54 ± 7	50 ± 7	0.125
Skinfolds (mm)			
Vastus lateralis	10.4 ± 2.7	9.1 ± 2.7	0.157
Gastrocnemius	10.4 ± 2.8	7.6 ± 2.4	0.002
Tibialis anterior	9.3 ± 3.1	8.5 ± 3.7	0.566
Haemoglobin (mmol/L)	9.4 ± 0.4	9.1 ± 0.7	0.163
SPPB score		11.65 ± 0.5	
Ethnicity	Caucasian (18), Asian (1), Indo-Pacific (1)	Caucasian (20)	
Physical Activity			
Baecke questionnaire score	8.3 ± 0.8	8.7 ± 0.9	0.161
SPA (%)	79.1 ± 7.3	75.4 ± 5.8	0.084
LPA (%)	15.5 ± 3.4	19.6 ± 5.4	**0.006**
MVPA (%)	4.4 ± 2.2	5.0 ± 2.2	0.405
Activity kcal (kcal/day)	404.3 ± 214.9	494.9 ± 187.0	0.1629
Wear time (hr/day)	14.6 ± 1.1	15.4 ± 0.8	**0.011**

Data is presented as mean ± standard deviation. MVC is maximum voluntary contraction. Time spent in sedentary physical activity (SP), light physical activity (LPA), and moderate-to-vigorous physical activity (MVPA) are expressed as a percentage of total wear time. MET is metabolic equivalent of task. TEE is total energy expenditure. PAEE is physical activity energy expenditure. PAL is physical activity level. SPPB is short physical performance battery.

4.3.2. Physical activity

Reported physical activity using the Beacke questionnaire was not significantly different between the two groups (**Table 4.1**). Measured PA using accelerometer showed that MVPA was not significantly different between the young and older individuals, however older individuals spent significantly more time in LPA ($p = 0.006$) at the expense of time spent in SPA ($p = 0.084$; **Figure 4.1A**). Activity kcal per day were not different between the groups ($p = 0.1629$; **Figure 4.1B**) and wear time was significantly higher in the older group compared to the young group.

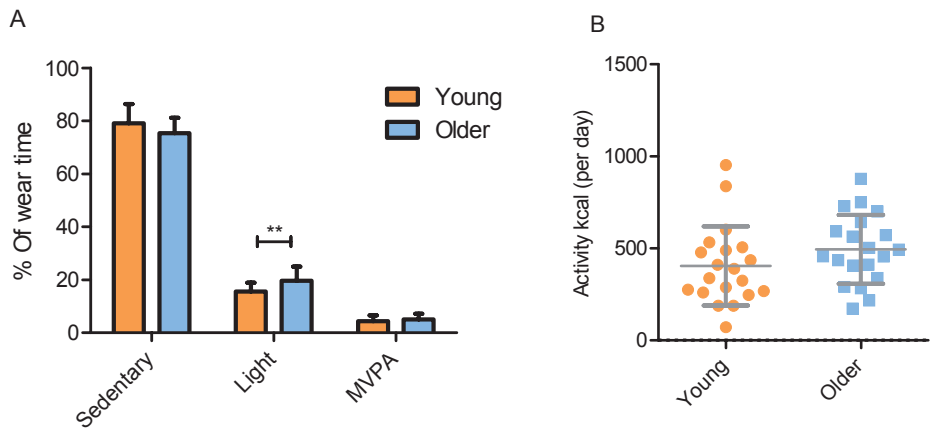

Figure 4.1 Percentage of wear time spent in sedentary, light and moderate-to-vigorous physical activity **(A)** and calculated activity kcal **(B)** for young and older group calculated from accelerometry data. Values are mean ± SD. **p < 0.005.

4.3.3. Mitochondrial capacity in the gastrocnemius, tibialis anterior and vastus lateralis.

Mitochondrial capacity was measured using repeated occlusions after a short exercise protocol in the GA, TA and VL. Mitochondrial capacity was significantly different between young and old group for GA (p = 0.048; **Figure 4.2A**) and VL (p = 0.036; **Figure 4.2C**), but not for the TA (p = 0.64; **Figure 4.2B**). When comparing mitochondrial capacity between the three muscles, there was a significant higher mitochondrial capacity in VL compared to TA (F = 5.33, p = 0.006). Two data sets for TA and six data sets for GA were excluded due to R^2 < 0.95. 24 data sets for the VL were excluded due to insufficient muscle activation using electrostimulation or a R^2 < 0.90.

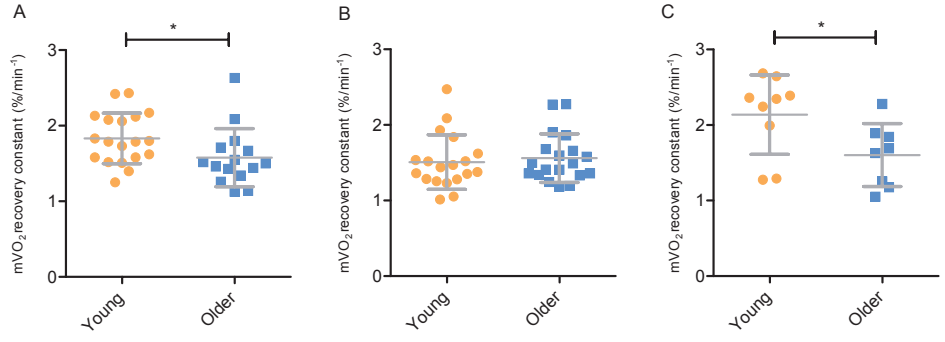

Figure 4.2 Recovery constants derived from monoexponential curve fits of m$\dot{V}O_2$ recovery from NIRS measurements after 30s of plantar flexion exercise in *gastrocnemius* **(A)**, 30s of dorsiflexion exercise in *tibialis anterior* **(B)** and 30s of electrical stimulation in *vastus lateralis* **(C)**. Values are mean ± SD. *p < 0.05

4.3.4. Reperfusion rate in gastrocnemius, tibialis anterior and vastus lateralis.

Reperfusion rate was measured after a 4-minute occlusion and defined using the half life time at 50% maximal oxygenation (**Figure 4.3A**). Reperfusion rate was not significantly different in the GA (p = 0.55 ; **Figure 4.3B**), but was significantly faster in the TA and VL in the young group compared to the older group (p = 0.0094 and p = 0.039 respectively; **Figure 4.3CD**).

4.3.5. Associations between mitochondrial capacity and physical activity

The mitochondrial capacity of the GA was significantly correlated to time spent in MVPA in the older group (R^2 = 0.27, p = 0.048), but this was not the case not for the TA and VL (R^2 = 0.0, p = 0.99 and R^2 = 0.15, p = 0.34 respectively; **Figure 4.4**). In the young group MVPA was not correlated to mitochondrial capacity for the three muscles.

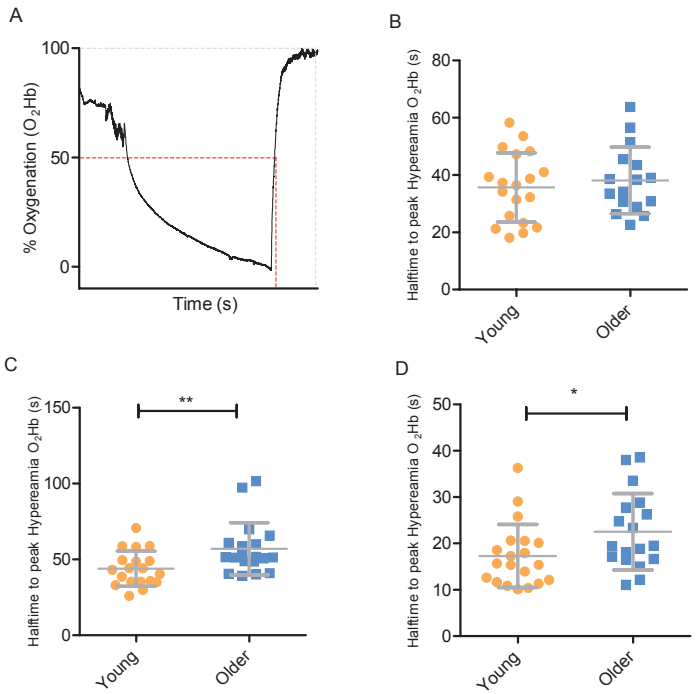

Figure 4.3 Representative plot of NIRS measurements of O_2Hb during 4-minute occlusion and reperfusion measurements. Red dotted line represents time to reach 50% oxygenation (halftime to peak hyperaemia) as a measure for reperfusion rate **(A)**. Reperfusion was measured in the *gastrocnemius* **(B)**, *tibialis anterior* **(C)** and *vastus lateralis* **(D)**. Values are mean ± SD. *p < 0.05 **p < 0.005

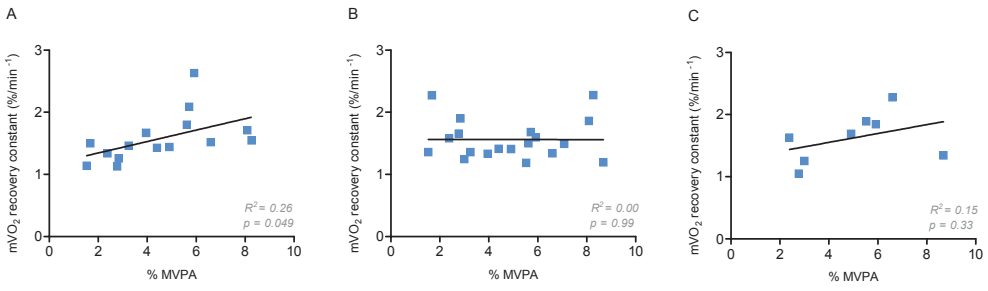

Figure 4.4 Correlation of recovery constants derived from monoexponential curve fits of $m\dot{V}O_2$ recovery from NIRS measurements after 30s of plantar flexion exercise in *gastrocnemius* (**A**, n=15), 30s of dorsiflexion exercise in *tibialis anterior* (**B**, n=19) and 30s of electrical stimulation in *vastus lateralis* (**C**, n=8) and percentage of wear time spent in moderate-to-vigorous physical activity (%MVPA) for the older group.

4.4. Discussion

The primary objective of this study was to determine the effect of age on mitochondrial capacity in young and older healthy males in three different locomotor muscles. Due to the similar moderate-to-vigorous physical activity levels between the older and young individuals in our study, it was possible to assess the effect of age on muscle mitochondrial capacity, independent from the effect of physical activity. Ageing negatively affected $m\dot{V}O_2$ recovery in the GA and VL, but not in the TA, showing that the age-driven decline in mitochondrial capacity is muscle specific. Furthermore, reperfusion rate after a 4-minute occlusion was decreased with age in the VL and TA, but not in the GA, showing that it is important to consider parameters of vascularisation in muscle mitochondrial measurements.

We are the first to measure the effect of age on $m\dot{V}O_2$ recovery in three different locomotor muscle using NIRS. Other research primarily used ^{31}P-MRS to measure mitochondrial capacity *in vivo* and, although ^{31}P-MRS and NIRS are based on the same underlying assumption of post-exercise recovery of metabolism and NIRS is correlated with ^{31}P-MRS (Ryan et al. 2013b), these techniques do measure distinct physiological mechanisms (i.e., recovery of $m\dot{V}O_2$ or PCr, respectively). Since a decrease in coupling of mitochondrial oxygen consumption, or phosphorus to oxygen ratio (P/O), has been reported with ageing (Amara et al. 2007), one could hypothesise that, if uncoupling is an important feature in mitochondrial ageing, more oxygen would be needed for the recovery of the same amount of PCr in old compared to young, directly affecting measurements of $m\dot{V}O_2$ recovery, yet only indirectly affecting PCr-recovery. Since we observed similar ageing effects in the current study compared to existing *in vivo* literature (Kent and Fitzgerald 2016), we conclude that NIRS is applicable in aging research to faithfully measure mitochondrial capacity, which is corroborated by a NIRS study performed in young and old individuals, but was not controlled for physical activity (Chung et al. 2018).

4.4.1. Ageing and muscle mitochondrial capacity

In the GA, a slower m$\dot{V}O_2$ recovery with age was observed and this was negatively correlated with %MVPA in the older group. Additionally, we observed a correlation between time spent in MVPA and m$\dot{V}O_2$recovery. Therefore, it could be that higher levels of physical activity are required to preserve mitochondrial capacity of the GA with age. Yet, the effect of age on mitochondrial capacity in the GA is debated. Some studies observed a negative effect of age on PCr-recovery in the GA (McCully et al. 1993; Waters et al. 2003; Layec et al. 2013), whereas others did not observe this (Waters et al. 2003; Wray et al. 2009; Tevald et al. 2014; Hart et al. 2015). Although it is challenging to compare the literature due to differences in study populations with regard to sex and physical activity level, the results of the current study seem unique in its kind, since a decrease in m$\dot{V}O_2$ recovery with age was observed despite controlling for physical activity. Interestingly, one other study that measured PCr-recovery in older individuals with decreased PA compared to young, did not observe an age effect (Tevald et al. 2014). Yet, the aforementioned study included both males and females and had lower sample size for males only. While not all studies found an effect of age on mitochondrial capacity, according to our data, some degree mitochondrial ageing in the GA seems to be inevitable. Yet, having a higher MVPA is associated with mitigating this unfavourable effect.

In the VL, a 25% slower m$\dot{V}O_2$ recovery in older individuals was observed, reflecting a decrease in mitochondrial capacity with age. This finding is in agreement with studies measuring PCr-recovery in the VL (Conley et al. 2000b; Johannsen et al. 2012; Larsen et al. 2012; Choi et al. 2016; Adelnia et al. 2019) and with studies measuring *ex vivo* oxygen consumption using high resolution respirometry (Porter et al. 2015; Distefano et al. 2018). Specifically, Larsen et al found a 23% decrease in PCr-recovery in older adults compared to younger adults in a similar-aged and physical activity-matched population (Larsen et al. 2012). Therefore, the VL seems to be particularly affected by ageing, even when physical activity levels were maintained. In fact, we did not we did not observe a correlation between %MVPA and m$\dot{V}O_2$ recovery in the VL, suggesting that increased levels of physical activity

do not preserve the VL from an age-driven decline in mitochondrial capacity. However, this result should be interpreted with care, due to the low number of measurements that were included in the correlation. Furthermore, inactive or sedentary older individuals do show a lower mitochondrial capacity than their more active counterparts, indicating that a protective effect of physical activity on the VL may not be excluded (Larsen et al. 2012; Distefano et al. 2018). The lower number of measurements in VL were the result of insufficient activation of the muscle during the NIRS protocol. A previous study reported sufficient muscle activation using a similar electrical stimulation (Brizendine et al. 2013). Yet, for half of the participants we were unable to produce sufficient muscle activation, and thus measure increased muscle oxygen consumption and its recovery. Possibly, a 30s isometric contraction could have been used for muscle activation, as this has been shown to be a good alternative for electrical stimulation (Ryan et al. 2014).

While the GA and VL are affected by age in the current study, the m$\dot{V}O_2$ recovery in the TA is not significantly different between the young and the older individuals. This confirms consensus in literature that age does not affect mitochondrial capacity in this muscle (Kent-Braun and Ng 2000; Lanza et al. 2007; Christie et al. 2014). A possible explanation for this distinct effect could be attributed to intrinsic characteristics of the TA, such as its fibre type composition. The TA has a higher proportion of oxidative type I fibres than the GA and VL (Jakobsson et al. 1988) and it has been suggested that ageing less severely effects mitochondria in predominantly type I muscle fibres due to a mild induction of uncoupling, possibly reducing the production of reactive oxygen species and consequent damage (Amara et al. 2007). This could explain why the TA is protected from the age-driven decline in m$\dot{V}O_2$ recovery. Yet, this hypothesis is not supported by research in mice, where the most oxidative muscle, the soleus, was shown to be most negatively affected by age (Picard et al. 2011). Moreover, it cannot be excluded that extrinsic factors also play a role in the resistance against the age-driven decline in m$\dot{V}O_2$ recovery. The TA is unique in its higher activation during locomotion with advancing age (Jakobsson et al. 1988), whereas the demand on other muscles, such as the VL, is thought to decrease with age (Hortobágyi and

DeVita 2000; Tirosh and Sparrow 2005), perhaps exerting muscle-specific (de)training-like adaptations that can affect mitochondrial capacity. Alternatively, the TA may intrinsically be less susceptible to effects of physical activity. Indeed, the effect of a 5-week bed rest did not affect TA thickness, while the VL and GA thickness were significantly reduced after this period, showing that decreased activity did not induce muscle atrophy in TA as much as in other muscles (de Boer et al. 2008). Moreover, the TA has a lower association with MVPA compared to the VL in young and elderly subjects (Larsen et al. 2009, 2012), further supporting that TA is less affected by the levels of physical activity. Therefore, differences in fibre type, different activation patterns or susceptibility to (de)training-like adaptations could explain why the TA seems to be less susceptible to the age-driven decline in mitochondrial capacity.

Analysing $m\dot{V}O_2$ recovery of both the young and the older group together, there was a significantly higher mitochondrial capacity in the VL compared to the TA. This difference in $m\dot{V}O_2$ recovery between muscles has been previously shown in elderly males and in young adults (Larsen et al. 2009, 2012). Also, it has been reported that the GA has a higher mitochondrial capacity than the TA in elderly women (Tevald et al. 2014), which we also observed in our data when just GA and TA were compared directly. In the TA, the majority of fibres is classified as oxidative type I fibres, whereas the VL and GA have a more even distribution between type I and type II fibres (Edgerton et al. 1975; Jakobsson et al. 1988; Hedges et al. 2019). Therefore, it seems that the percentage of type I fibres does not predispose a higher mitochondrial capacity, at least not measured using these *in vivo* techniques. Besides, in the current study we observed that the muscle with highest reported proportion of type I fibres is also most resistant to ageing. However, this is speculative in its nature because no fibre typing was done in the tissue underneath the NIRS probe and it is unknown what is the effect of fibre type on $m\dot{V}O_2$ recovery measured using NIRS. Yet, it does advocate that the metabolic properties of these muscles are influenced by their intrinsic characteristics and that exploring these muscle-specific effects could help to elucidate ageing mechanisms.

4.4.2. Ageing and muscle reperfusion rate

Ageing has been associated with a decrease in vasodilation and microvascular function in the muscle (Tonson et al. 2017). Recovery of $m\dot{V}O_2$ or recovery of PCr is only a measure for mitochondrial capacity if oxygen availability is not limited during the recovery period. In the current study we measured reperfusion rate as the return to maximal oxygenation after a 4-minute arterial occlusion. This was significantly lower in the TA and VL in the older group compared to the young group. While no difference in reperfusion rate was observed in the GA. Although there was an age-related decline in reperfusion in the TA, this did not result in an age-related decline in $m\dot{V}O_2$ recovery. This suggest that the muscle reperfusion rate was not limiting for $m\dot{V}O_2$ recovery in our study. This notion is further supported by our results in GA, where reperfusion rates are not different between the two age groups, but a difference in $m\dot{V}O_2$ recovery was observed. In contrast, a decrease in end-exercise perfusion rate was reported in a study by Wray et al., which was not accompanied by a decrease PCr-recovery (Wray et al. 2009). However, differences in physical activity were not accounted for in this study. With exercise and physical activity being a known inducer of angiogenesis, this result could partly be explained by differences in physical activity. The effect of age on reperfusion rate was not observed by Hart et all, who did neither observe a decrease in oxygen delivery nor a decrease in PCr-recovery between young and older adults in the GA (Hart et al. 2015).

On the other hand, for the VL both reperfusion rate and mitochondrial capacity were decreased in the older males in the current study. Therefore, we cannot be certain that the $m\dot{V}O_2$recovery was not negatively affected by the decrease reperfusion rate with age in this muscle. However, using the current protocol, muscle oxygenation during testing does not fall below 50% of one's maximal oxygenation. Therefore, it is unlikely that oxygen availability was a limiting factor during $m\dot{V}O_2$ recovery, attributing the age-related decline in $m\dot{V}O_2$ recovery to decreased mitochondrial capacity. Moreover, a decrease in perfusion rate can be compensated by an increase oxygen diffusion to the muscle tissue, ultimately not lowering oxygen availability at the level of mitochondria.

Nevertheless, our data do not show that oxygen availability or reperfusion rate are per definition not a limiting factor for physical functioning with advancing age, as it can be still be limiting during maximal or prolonged periods of exercise. This is supported by recent research, which reported that ageing was associated with a decrease in resting muscle perfusion in middle-aged and elderly adults. Resting muscle perfusion rates were negatively associated with muscle PCr-recovery and whole-body oxidative capacity, suggesting that changes in reperfusion could affect physical functioning with advancing age (Adelnia et al. 2019). However, in that study the population was not matched for physical activity, making it again difficult to draw conclusions from the results. Therefore, measuring reperfusion of the muscle should be considered good practise, because an impaired reperfusion could violate the underlying assumptions of the mitochondrial capacity measurement. To our knowledge we are the first to measure the effect of age on reperfusion rate in three locomotors muscles using NIRS and an advantage of the current NIRS protocol is that it allows assessment of mitochondrial capacity and reperfusion in the same measurement.

4.5. Conclusions and future perspectives

The current study provides evidence for distinct modes of mitochondrial ageing in different locomotor muscles in a relatively large population of physical activity-matched young and older males. We identified that the TA, but not the VL and GA, was resistant to an age-driven decline in mitochondrial ageing. This exemplifies the limitations of generalising results obtained in one type of muscle to other muscles and underlines the importance of controlling for important effect modifiers such as sex, physical activity or even muscle activity assessed using electromyography. Being the first to use NIRS to study the effect of age on mitochondrial capacity, we show that NIRS is suitable for application in ageing research. Application of this non-invasive technique could accelerate research in this field, including studies on the clinical efficacy of interventions aiming to improve either vascular function (Kiss et al. 2019) or mitochondrial function (Nacarelli et al. 2018) in an elderly population.

An advantage of using *in vivo* techniques over *ex vivo* measurements is the possibility to study muscle mitochondrial capacity in the intact system, with a physiological oxygen pressure and the dynamic interplay between oxygen delivery, diffusion and consumption. However, *ex vivo* respirometry measurements on muscle biopsies more directly assess mitochondrial capacity and can provide mechanistic insights into muscle ageing. Since the VL is mostly used for taking biopsies, there is a gap in current literature for the *ex vivo* mitochondrial capacity other muscles than the VL, possibly presenting a biased view on mitochondrial ageing in the muscle. Identifying the molecular signatures of age-unaffected muscles, such as the TA, could shine new lights upon mechanisms underlying muscle ageing. This could provide leads for pharmacological or tailored exercise interventions to counteract the decline mitochondrial capacity with advancing ageing, potentially retaining physical function and improving quality of life.

Acknowledgements The authors greatly acknowledge the commitment of the volunteers who participated in the study and Rick Dijk for help with data collection.

Author contributions BL performed all experiments and principal data analysis; BL, AGN, VCJB, JK. conception and design of research; data analysis and interpretation, drafting of manuscript. All authors edited, revised and approved final version of manuscript.

Compliance with ethical standards The study was approved by the medical ethical committee of Wageningen University with reference number NL65872.081.18. All procedures performed were in in accordance with the principles of the Declaration of Helsinki (Fortaleza, Brazil 2013) and according to national law (WMO, The Hague, 1998). The study is registered in the Dutch trial register (NL7695). Subjects were written and verbally informed on all experimental procedures, including possible risks and discomforts. All subjects provided written informed consent before testing.

CHAPTER 5

Cell adhesion, innervation and mitochondrial gene expression as early muscle ageing signature in older compared to young males with similar physical activity

Bart Lagerwaard
Arie G. Nieuwenhuizen
Annelies Bunschoten
Vincent C. J. de Boer
Jaap Keijer

Abstract

Objective
Due to the interaction between skeletal muscle ageing and lifestyle factors, it is often challenging to attribute the decline in muscle mass and quality to either changes in lifestyle or to advancing age itself. Since many of the physiological factors affecting muscle mass and quality are modulated by physical activity and physical activity declines with age, the aim of this study is to better understand the effects of early ageing on muscle function by using a population of healthy older and young males with similar physical activity patterns.

Methods
18 older (65-71 years) and 20 young (19-25 years) males were recruited based on similar, self-reported physical activity, which was verified using accelerometry measurements. Gene expression profiles of vastus lateralis biopsies obtained by RNA sequencing were compared and key results were validated using qPCR and Western blot.

Results
A clear transcriptional ageing signature was observed despite similar physical activity. Cell-adhesion, the matrisome, innervation and inflammation were the main upregulated processes and oxidative metabolism was the main downregulated process in old compared to young muscle. Furthermore, protein expression of mitochondrial complexes was downregulated in the older muscle, and this expression was correlated with in vivo mitochondrial capacity.

Conclusion
Based on changes in gene expression we appoint early skeletal muscle ageing processes that occur despite similar physical activity. Improved understanding of these processes will be key to design targeted anti-ageing therapies.

5.1. Introduction

Ageing is associated with a decline in skeletal muscle mass and strength, also known as sarcopenia (Morley et al. 2001). Sarcopenia is linked to an increased risk of developing disabilities, negatively impacting self-reliance and quality of life (Janssen et al. 2002; Tsekoura et al. 2017). The loss of muscle strength is disproportional to the loss in muscle mass, suggesting that intrinsic changes in the muscle could explain the loss in muscle functionality, often referred to as muscle quality (Goodpaster et al. 2006; Mitchell et al. 2012). The underlying causes of loss of muscle mass and quality with age have been suggested to be related to, among others, a decline in skeletal muscle mitochondrial capacity (Short et al. 2005), decreased innervation (Gonzalez-Freire et al. 2014) decreased protein synthesis (Balagopal et al. 1997), decreased circulation of anabolic hormones (Sakuma and Yamaguchi 2012), low-grade inflammation (Dalle et al. 2017), decreased muscle repair (Carosio et al. 2011) or a combination of these phenomena.

Many of the factors affecting muscle mass and quality are modulated by physical activity. However, many studies examining age-related changes in skeletal muscle do not explicitly take the effect of physical activity levels into account, while levels of physical activity decrease with advancing age (Troiano et al. 2008; Hallal et al. 2012). The effect of physical activity is especially apparent for mitochondrial capacity, because, although the decline in skeletal muscle mitochondrial capacity is likely due to inherent age-related alterations in mitochondrial synthesis and degradation, it has been reported that active older individuals have a mitochondrial capacity comparable to similarly-active younger individuals (Lanza et al. 2008; Distefano et al. 2018). This exemplifies the challenge to disentangle the effect of age and physical activity, making it difficult to attribute age-related changes to either of these two factors.

Here, we aimed to better understand the effects of early ageing on skeletal muscle function, using a population of healthy older and young males in a narrow age range (65-71 years and

19-27 years, respectively). Furthermore, in efforts to disentangle the effect of age and physical activity, both age groups were selected for similar levels of physical activity based on self-reported physical activity, verified using accelerometery. The age range was chosen because it can be anticipated that changes occurring in earlier phases of the ageing process are the most amenable to intervention and will most likely offer targets for intervention. In addition, many studies investigating the effect of age on muscle function employed a wide range of ages for subject recruitment, which further blurs the effect of age because the loss of muscle mass with age is thought not to be linear (Janssen et al. 2000). Furthermore, the ageing trajectory is different between males and females, demanding separate studies for each of the sexes (Gheller et al. 2016; Van der Hoek et al. 2020). Gene expression profiles of *vastus lateralis* biopsies, obtained by RNA sequencing, were compared between the groups and key results were validated using qPCR and Western blot. Our results show that the cell adhesion, the matrisome, innervation, inflammation and oxidative metabolism are the most significantly regulated processes. These results will be of substantial value to design anti-ageing interventions for a population that is still amendable for improvements in skeletal muscle quality.

5.2. Material and Methods

5.2.1. Study population

Low to moderately physical active healthy males between the age of 19-25 (young) and 65-71 (older) years were recruited using a self-reported exercise frequency of 1-2 hours of structured exercise per week or a Baecke habitual physical activity score between 7-10 points (Baecke et al. 1982). Older males were not physically impaired, as determined using the short performance battery test (SPPB) with a minimum score of 11 (Guralnik et al. 1994). None of the subjects were identified as a regular smoker (>5 cigarettes per week), used recreational drugs during the study or reported recent use of performance enhancing drugs or supplements. Subjects were non-anaemic (haemoglobin concentration > 8.0 mmol/L), verified using HemoCue Hb 201 microcuvette (HemoCue AB, Sweden). None of the subjects

had health concerns regarding respiratory or metabolic disease. One older subject used cholesterol-lowering medication, one used diuretic medication and one used both cholesterol-lowering and diuretic medication. An overview of subject characteristics can be found in **Table 5.1**. *In vivo* measurements of mitochondrial capacity and physical activity using accelerometery, but not of gene or protein expression, were reported earlier (Lagerwaard et al. 2020a). Here, information was used only from those individuals of which high quality RNA was obtained from the *vastus lateralis* (i.e., 2 elderly subjects were excluded).

5.2.2. Accelerometery

Subjects were instructed to wear triaxial accelerometer (wGT3X-BT, Actigraph, USA) for seven consecutive days using an elastic band at the waist of the nondominant leg. The accelerometer was worn during all activities, excluding showering, swimming and sleeping. Wear time was manually verified using daily diaries on shower and bedtimes. Counts were sampled at 30 Hz and stored in 60 s epochs to determine counts per minute (CPM). Two weekend days and three weekdays were used for data analysis. The percentage of wear time in sedentary physical activity (SPA), light physical activity (LPA) and moderate-to-vigorous physical activity (MVPA) was determined using the cut-offs provided by Troiano et al., being 0– 99 CPM, 100–2019 CPM and 2020–∞ CPM respectively (Troiano et al. 2008). Activity kcals per day were calculated using the counts from all axis according to the Freedom VM3 equation (Sasaki et al. 2011).

Table 5.1 Physical characteristics of the subjects

	Young (N = 20)	Older (N = 18)	p-value
Age (years)	22 ± 2.0	69 ± 2.0	**<0.0001**
BMI (kg/m^2)	22.6 ± 1.9	25.4 ± 1.8	**<0.0001**
Body Fat (%)	15.3 ± 3.1	25.0 ± 4.1	**<0.0001**
Fat free mass (kg)	63.5 ± 6.9	61.1 ± 5.3	0.25
MVC dominant arm (kg)	54 ± 7	50 ± 8	0.17
Skinfold v*astus lateralis* (mm)	10.4 ± 2.7	9.2 ± 2.8	0.19
Haemoglobin (mmol/L)	9.4 ± 0.4	9.1 ± 0.7	0.19
SPPB score		11.65 ± 0.5	
Physical Activity			
Baecke questionnaire score	8.3 ± 0.8	8.6 ± 0.9	0.26
Accelerometry			
SPA (%)	79.1 ± 7.3	75.2 ± 6.0	0.0778
LPA (%)	15.5 ± 3.4	19.7 ± 5.6	**0.0074**
MVPA (%)	4.4 ± 2.2	5.1 ± 2.3	0.32
MET (kcal/kg/hour)	1.37 ± 0.12	1.45 ± 0.11	**0.037**
TEE (kcal/day)	2478 ± 375	2851 ± 343	**0.003**
PAEE (kcal/day)	404 ± 215	411 ± 189	0.11
PAL	1.4 ± 0.1	1.4 ± 0.1	0.51
Wear time (hr/day)	14.6 ± 1.1	15.5 ± 0.8	**0.0078**
Mitochondrial capacity *vastus lateralis*	2.13 ± 0.53 (n=9)	1.58 ± 0.49 (n=6)	0.0606

Data is presented as mean ± standard deviation. MVC is maximum voluntary contraction. Time spent in sedentary physical activity (SPA), light physical activity (LPA), and moderate-to-vigorous physical activity (MVPA) are expressed as a percentage of total wear time measured using 5-day accelerometer data. MET is metabolic equivalent of task. TEE is total energy expenditure. PAEE is physical activity energy expenditure. PAL is physical activity level. SPPB is short physical performance battery. Mitochondrial capacity is defined as the rate of recovery of muscle oxygen consumption after exercise determined by near-infrared spectroscopy (Lagerwaard et al. 2020a).

5.2.3. Sample collection and RNA extraction

Subjects were fasted and refrained from exercise and alcohol 24 hours prior to testing. Biopsies from *vastus lateralis* were collected using the suction modified Bergström technique (Bergström et al. 1967) from the non-dominant leg under local anaesthesia using a using a 5-mm Bergström needle. Collected tissue was snap frozen in liquid nitrogen and stored at -80°C until further analysis. Total RNA was extracted by grinding frozen muscle tissue and dissolving approximately 30 mg in 1 mL Trizol reagent in Tissue lyser II (Qiagen) for 2 min at 30 Hz. 240 µL of cold chloroform was added to the lysates, shaken thoroughly

for 30 seconds and incubated on ice for 5 min before centrifugation at 12,000 RPM for 15 min at 4˚C (Mikro 200R, Hettich, Germany). The aqueous layer was transferred to a new tube and 700 µL of ice-cold isopropanol was added, mixed with RLT buffer (Qiagen) and loaded onto RNAeasy spin columns (Qiagen). RNA purification was done according to manufacturers' protocol. RNA concentration was measured using NanoDrop spectrophotometer (Thermofisher). RNA integrity was measured using RNA Screentape on the 2200 Tapestation (Agilent). All RNA samples had an RNA integrity value (RIN) > 7.0 and 260/280 ratio >1.8.

5.2.4. RNA sequencing and differential gene expression analysis

RNA preparation, library construction and sequencing on BGISEQ-500 was performed at Beijing Genomics Institute. Clean reads were obtained in FASTAQ format and quality check was performed using FASTQC (Andrews 2010). Reads were aligned to the human genome (GRch38.p13) using STAR (Dobin et al. 2013) and counts were quantified using HTSeq (Anders et al. 2015). Average sequencing depth was 23M paired end reads, of which at least 92% were uniquely mapped.

Data analysis and statistical testing was performed in R version 3.6 and using appropriate Bioconductor packages. Differentially expressed genes (DEGs) between the groups were identified using DE2seq (Love et al. 2014). Genes with less than 10 reads per row were removed and Benjamini-Hochberg multiple testing correction was used to obtain adjusted p-values; a false discovery rate (FDR) of 5% was accepted. Principal component analysis was done using variance stabilising transformed (VST) data. Associations between gene expression and the continuous variable MVPA were done using the Limma-Voom (Ritchie et al. 2015). Normalisation factors were calculated using calcNormfactors using EdgeR (Robinson et al. 2010) and low-expressed genes were filtered. Voom was used to calculate the mean-variance relationship of the log-counts and linear model was fitted using weighted least squares for each gene. Empirical Bayes was used for smoothing of standard errors (Smyth 2004).

5.2.5. Gene set enrichment analyses

Gene set enrichment analysis was done using clusterprofiler (Yu et al. 2012) for the 'biological process' gene ontology gene sets. Gene sets were considered enriched with a Benjamini-Hochberg adjusted p-value < 0.05 and GO terms were filtered based on 75% overlap in genes. Protein interaction networks were made using STRING v10 (Szklarczyk et al. 2015) with p-value for enrichment < 1.0 E-16. Edges represent molecular action and nodes were coloured manually according to protein function. Nodes with no connection to the network were deleted. The human MitoCarta gene set was used as a reference inventory for mitochondrial genes (Calvo et al. 2016) and the matrisome gene set as a reference inventory for extracellular matrix genes (Shao et al. 2020).

5.2.6. Semi-Quantitative Real-Time Polymerase Chain Reaction (qPCR)

cDNA was synthesised from 300 ng total RNA with ISCRIPT cDNA synthesis kit (Bio-Rad) (5' 25°C, 30' 42°C, 5' 85°C, cooled to 10 °C and stored at -20 °C). Lowly expressed genes were pre-amplified using the SsoAdvanced PreAmp Supermix (Bio-rad). Transcripts were quantified using a CFX96 Touch Real-Time PCR Detection System (Bio-Rad) and SYBR green master mix (Bio-Rad). The cycling program was set as 95℃ for 30s, 60℃ for 30s in 40 cycles. Primers were designed using NCBI primer blast; an overview of primer sequences can be found in **Table 5.2**. Normalised expression was calculated according to the ΔΔCq method, by making use of geometric averaging of multiple reference genes using CFX maestro software (Bio-rad). Data is presented as fold change of expression, which was set to one for the young group.

Table 5.2 Primer sequences

Symbol	RefSeq	Forward primer*	Reverse primer*	bp
ITGA7	NM_002206.3	GAACAGCACCTTTCTGGAGGAGTA	CACTGTGGAGGCATCTCGGA	118
CDH15	NM_004933.3	CTGTTGGCCCAGAGCCT	TGTGGTTCTCGGATACGCTG	145
BDNF	NM_001709.5	GCCAGAATCGGAACCACGAT	CTCACCTGGTGGAACTCGG	79
MUSK	NM_005592.4	ACCTAAAATAACTCGTCCTCCC	TTCGGGAATTTTCCCTGAGAG	140
MYH8	NM_002472.3	TGACCTCAGCAGTAACGCAG	AGGGCGTTCTTGGCTTTAGT	300
NTRK2	NM_001007097.3	ACAGCAACCTGCAGCACATC	ATTGCCCACCAGGATCAGTTC	104
NR4A1	NM_173157.3	TCGGGGACTGGATTGACAGTA	CCCATGCCGGTCGGTGAT	116
NR4A3	NM_173200.2	ATCCAGAAGCTGGGCAGAAAA	TCAGCAGTGTTTGACCTGATGG	129
PRKAG3	NM_017431.3	GATGACGAACTGCGGAAACC	GGCCTTCTTGATCTCCAGC	129
PPARGC1A	NM_013261.5	ACCCCAGAGTCACCAAATGAC	GCAGTTCCAGAGAGTTCCACA	89
TFAM	NM_001270782.1	GCTGAAGACTGTAAAGGAAAACTGG	CCTGTGCCTATCCATTGTGA	240
RPLP0 (reference)	NM_001002.4	GGCAGCATCTACAACCCTGA	GCCTTGACCTTTTCAGCAAGT	215
TBP (reference)	NM_003194.5	AGCCAAGAGTGAAGAACAGTCC	AACTTCACATCACAGCTCCCC	129
RPS15 (reference)	NM_001018.5	AGAAGCCGGAAGTGGTGAAGAC	AGAGGGATGAAGCGGGAGGAG	220
B2M (reference)	NM_004048.4	TGCCGTGTGAACCATGTG	GCGGCATCTTCAAACCTC	92

** from 5' to 3', bp = fragment length*

5.2.7. Protein isolation and Western blotting

Protein extraction was done by adding 500ul of ice-cold lysis buffer (50 mM Tris-HCL, pH 7.4, 1% Triton X100, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 0.25% NaDeoxylate and protease and deacylase inhibitors trichostatin A (1 µM) and nicotinamide (20 mM)) to ± 25 mg frozen grinded muscle tissue and lysed using Qiagen Tissue lyser II for 1 minute at 30 Hz. Lysates were centrifuged for 7 minutes at 10,000 g at 4˚C and protein concentration of supernatants were determined using BCA protein assay kit (Pierce). 25 ug protein was loaded on NuPAGE 4-12% BIS-TRIS and TRIS-acetate gels (Invitrogen), transferred to nitrocellulose or PVDF membrane, blocked in 3% BSA in PBS with 0.1% Tween-20 at room temperature and incubated overnight with antibodies in the same buffer at 4ºC. Primary antibodies used: anti-OXPHOS cocktail (1:1000, #ab110411 Abcam) and anti-TFAM (1:2000, #ab, Abcam). IR-dye based secondary antibodies (LI-COR) were used to detect antibody signals using an Odyssey scanner (LI-COR). Quantification of protein loading was done using

Revert 700 Total Protein Stain (LI-COR) and used for normalisation of target protein after quantification using ImageJ.

5.2.8. Data availability

The RNAseq data of this study has been deposited under accession number GSE159217

5.2.9. Statistical analyses

Data were presented as mean ± SD. Statistical analyses, other than RNA sequencing analysis and differential gene expression analysis (see above), were performed using GraphPad Prism v.8 (GraphPad Software, CA, USA). Means between the two groups were compared using a Students unpaired t-test. Normality was tested using D-Agostino and Pearson normality test. Not normal distributed data was compared using Mann-Whitney tests. Correlations between variables were calculated using regression analysis. Significance was accepted at $p < 0.05$.

5.3. Results

5.3.1. Distinct transcriptional signature in older muscle despite similar physical activity levels

Total physical activity energy expenditure and physical activity level, as defined by accelerometery, was similar between the groups, although older males spent higher proportion of time in light physical activity compared to young males (**Table 5.1**). PCA on transformed counts RNAseq data using a variance stabilising transformation, showed that the samples from young and older subjects separate in distinct groups based on principle component 1, explaining 20% of the variation in the data (**Figure 5.1A**). This showed that in an early ageing population with similar physical activity, a clear distinction in the transcriptional signature was observed. When comparing the gene counts of the young and older group, 4327 DEGs were identified using an adjusted p-value cut-off of 0.05. 3797 of those genes were identified coding genes, of which 1891 were higher expressed, while 1906

were lower expressed in the older individuals (**Figure 5.1B**). Gene ontology gene set enrichment analysis revealed an enrichment in gene sets related to oxidative metabolism (e.g. oxidative phosphorylation, cellular respiration), cell adhesion (e.g. cell adhesion, cell-cell adhesion), inflammation (e.g. defence response, inflammatory response) and innervation (e.g. synapse organisation and regulation of the synapse structure or activity). Most of these enriched gene sets were upregulated, except for the gene sets related to oxidative metabolism, which were downregulated in the older muscle (**Figure 5.1C**).

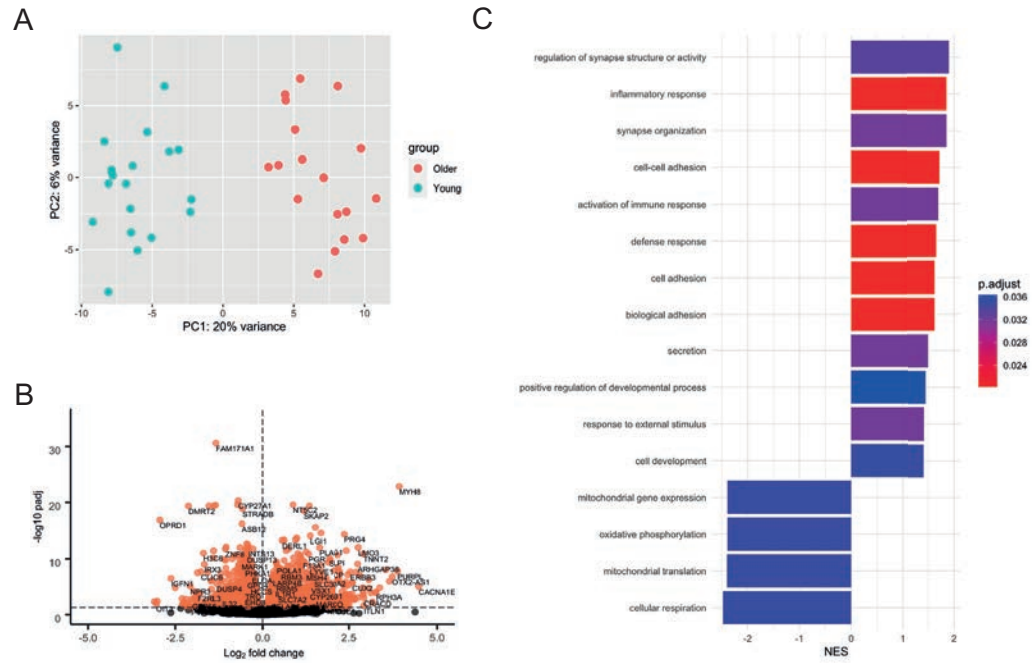

Figure 5.1 Distinct transcriptional signature in the ageing muscle (A) PCA blot of Euclidean distance between samples on variance stabilising transformed data. Samples were coloured based on group. **(B)** Volcano plot displaying the DEGs. 3797 genes with entrez annotation were differentially regulated between the young and older *vastus lateralis* muscle using an adjusted p-value of 0.05 (adjusted according to Benjamini-Hochberg). **(C)** Gene set enrichment analysis of older vs young using GO biological process gene sets (C5 BP MSigdb) using an adjusted p-value of 0.05. GO gene sets were filtered based on 75% overlap in genes.

5.3.2. Cell adhesion and the matrisome belong to most significantly regulated processes with ageing

Cell-adhesion was the most significantly regulated process in older compared to young muscle based on adjusted p-value. Furthermore, using the curated C2 canonical gene sets Molecular Signatures Database (Msigdb), a database of gene sets that are canonical representations of a biological process compiled by domain experts, the matrisome was the most significant enriched gene set in the older group (**Figure S5.1**). We therefore compared the expression of genes between the groups according to the classification of the Matrisome project, a research effort devoted to characterise and classify genes in the extracellular matrix (ECM) (Shao et al. 2020). This classification divides the matrisome into the core matrisome, subdivided in collagens, proteoglycans and ECM glycoproteins, and in matrisome-associated proteins, subdivided in ECM-affiliated proteins, ECM regulators and secreted factors. Out of the 44 genes that encode the 28 different collagen proteins, 20 genes were differentially regulated between the groups. Collagens form a network with which glycoproteins and proteoglycans in the ECM associate. Out of 195 genes encoding ECM glycoproteins, 44 were differentially regulated and out of the 35 genes that encode proteoglycans, 8 were differentially regulated between the young and older muscle. Of the 171 genes that encode for ECM-affiliated proteins and the 238 genes that encode for ECM regulators, respectively 28 and 48 genes were differentially expressed between the groups. Out of 344 genes that encode for secreted factors, 51 genes were differentially regulated between the young and older muscle. Secreted factors encompass all secreted proteins that can be secreted by the cells that reside in the ECM and play a role in tuning communication between cells, such as growth factors. Moreover, it encompasses interleukins that regulate inflammatory processes, for example interleukin 15 (*IL15*; FC = 2.1, padj = 0.002), interleukin 25 (*IL25*; FC = 6.75, padj = 0.001) and its receptor interleukin 17 Receptor B (*IL17R*; FC = 1.24 and padj = 0.037) (**Table S5.1**). Activation of the immune response could be involved in tissue remodelling and fibrosis, as was observed in muscle of old mice (Stearns-Reider et al. 2017). Overall, a substantial number of matrisome components were differentially regulated with age (**Figure 5.2A**).

The ECM forms a framework for muscle fibres and other cells that reside in the ECM, playing an important role in development (Thorsteinsdóttir et al. 2011), growth (Fritz et al. 2013a), repair (Calve et al. 2010) and contractile force transmission (Street 1983). Collagen is the most abundant ECM protein and makes up the intramuscular connective tissue (IMCT), organised in the endo-, peri- and epimysium. The sarcolemma associates with the ECM through a specialised IMCT membrane that contains mostly collagen IV and the glycoprotein laminin. Here, laminin serves as a ligand for the transmembrane receptors dystrophin-associated glycoprotein complex (DGC) and integrins in the sarcolemma, which in their turn bind actin proteins in the sarcoplasm (Wessner et al. 2019). The interaction between the ECM and the sarcolemma establishes cell adhesion and is important for muscle contraction, as it is necessary for lateral transfer of contractile force (Street 1983). Dysregulation in cell adhesion is hypothesised to contribute to the decrease in muscle strength observed in older rats (Hughes et al. 2016). Therefore, we specifically examined expression of genes involved in cell-ECM adhesion. Various genes that encode for the DGC complex and 11 out of 27 genes that encode for integrins, classified according to the HUGO Gene Nomenclature Committee (Braschi et al. 2018), were found to be differentially regulated in older muscle (**Figure 5.2B**). Alpha-7 integrin (ITGA7), part of the alpha7beta1 integrin dimer, is important in mechanical adhesion and force transduction in the muscle (Boppart et al. 2006). Therefore, we examined expression of *ITGA7* by qPCR and verified its differential expression with age (FC = 0.86 p = 0.037) (**Figure 5.2C**).

Besides force transmission and mechanical adhesion, the ECM also plays an important role in muscle hypertrophy and regeneration, by interaction with muscle stem cells, or satellite cells, that reside in the ECM (Thomas et al. 2015). These cell-cell interactions are primarily regulated via cadherins, a group of transmembrane proteins that play a role in regulating myogenesis and differentiation via interaction with catenins, which, in their turn, link with the actin cytoskeleton (Goel et al. 2017). Of the 125 cadherins and catenins, classified according to the HUGO Gene Nomenclature Committee (Braschi et al. 2018), 12 catenins and 30 cadherins were differentially regulated between young and older muscle, of which

were 26 upregulated (**Figure 5.2B**). Using qPCR, we examined gene expression of muscle cadherin (*CDH15* or M-cadherin), which is important for cell adhesion between muscle fibres and satellite cells (Goel et al. 2017; Boers et al. 2017). Indeed, gene expression of *CDH15* was confirmed to be significantly higher in older compared to young muscle (FC = 1.22, p = 0.014) (**Figure 5.2D**). This suggested that not only cell-ECM adhesion was altered with ageing, but also cell-cell adhesion within the ECM.

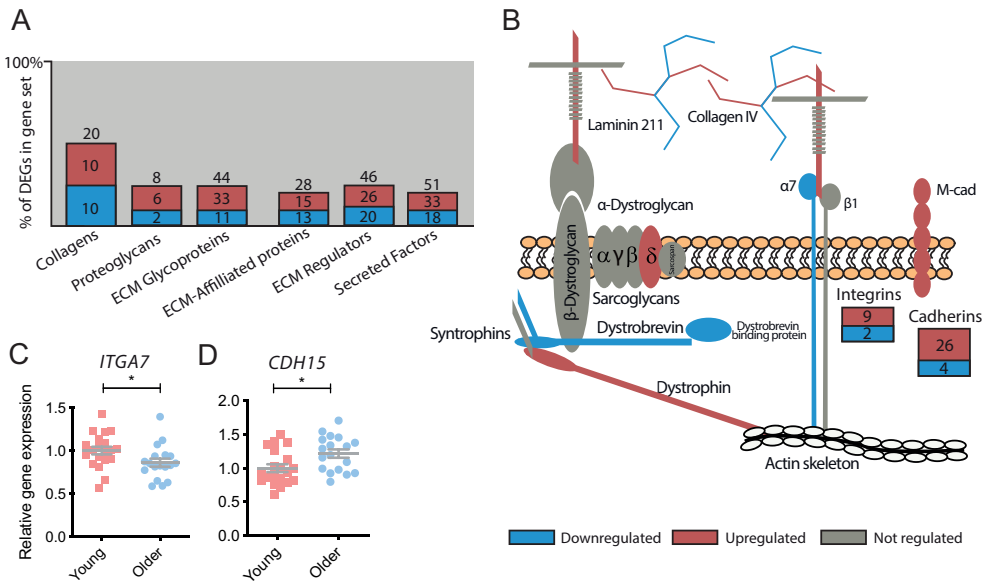

Figure 5.2 Cell adhesion and the matrisome belong to most significantly regulated processes in older muscle (A) Percentage of DEGs for each extra cellular matrix (ECM) component according to 'Matrisome 'classification. **(B)** Schematic presentation of sarcolemma-ECM adhesion complexes. Downregulated (blue) and upregulated (red) are indicated. **(C)** qPCR analysis of integrin 7a (*ITGA7*) and **(D)** Muscle cadherin (*CDH15*). Values are mean ±SD *p < 0.05.

5.3.3. Higher expression of genes related to synaptogenesis at neuromuscular junctions

Another cadherin that was significantly upregulated in the older group was neural cadherin (*CDH2* or N-cadherin; FC = 2.5, padj < 0.001). *CDH2* is upregulated during muscle regeneration and induces neurite outgrowth (Goel et al. 2017). Both CDH2 and CDH15 play an important role in synaptogenesis, the formation of synapses, and synapse plasticity, the

ability of the synapse to change (Arikkath and Reichardt 2008). These processes that were also identified among the most significant upregulated gene sets in the older muscle, namely 'synapse organisation' and 'regulation of the synapse structure or activity' (**Figure 5.1C**). Therefore, we examined the expression of genes related synaptogenesis. We used a selection of genes based on a selection of genes involved in synaptogenesis in older compared to young mice muscle (Aare et al. 2016). Many of the genes related to synaptogenesis were upregulated in older muscle, such as genes that encode for neutrophins, proteins that stimulate nerve and synapse growth, and genes that encode for proteins at the neuromuscular junction (NMJ), the synapse between a myofiber and a motor neuron (**Figure 5.3A**). We verified the expression of four genes by qPCR and confirmed their differential gene expression between the groups, namely: Brain derived neurotrophic factor (*BDNF*; FC = 2.9, p <0.001), a growth factor for neurons and synapses in the central and peripheral nervous system; Neurotrophic tyrosine kinase receptor type 2 (*NTRK2*; FC = 1.35, p = 0.0058), a gene that encodes for the BDNF receptor; Muscle-specific receptor kinase (*MUSK*; FC = 1.38, p <0.013), a key player in the formation of NMJs, and Myosin heavy chain 8 (*MYH8*; FC = 10.8, p <0.001), encoding a perinatal myosin that is only expressed during development and is associated with denervated muscle (Doppler et al. 2008; Schiaffino et al. 2015) (**Figure 5.3B**).

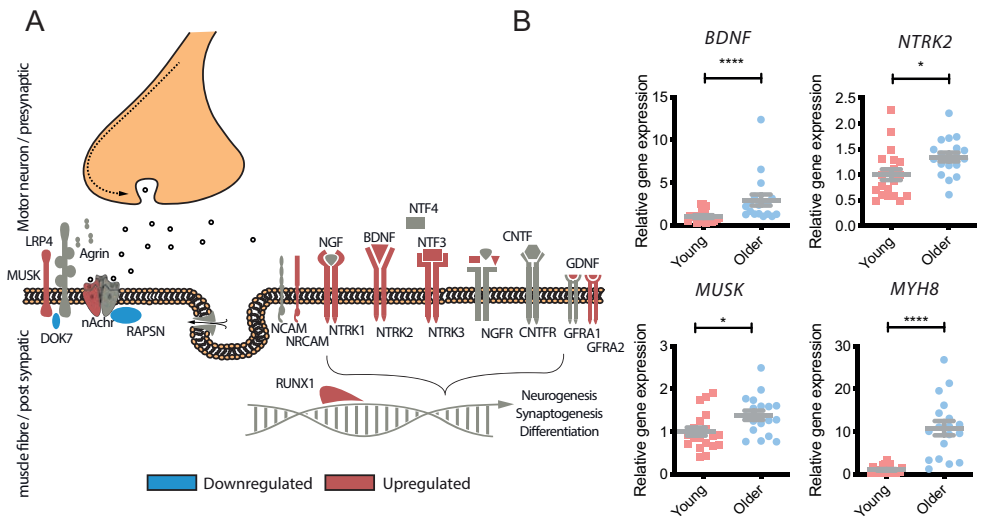

Figure 5.3 Higher expression of genes related to synaptogenesis in older muscle (A) Schematic presentation of neuromuscular junction and proteins involved in synaptogenesis **(B)** qPCR analysis of Brain Derived Neurotrophic Factor (*BDNF*), Neurotrophic Receptor Tyrosine Kinase 2 (*NTRK2*), Muscle Associated Receptor Tyrosine Kinase (*MUSK*) and Myosin Heavy Chain (*MYH8*). Values are mean ± SD *p < 0.05, ****p < 0.0001.

5.3.4. Oxidative respiration is the main downregulated process during ageing

Gene sets related to oxidative metabolism were significantly downregulated in the older group. Therefore, we further explored mitochondrial annotation of the DEGs using the MitoCarta2.0 gene set, a set of 1158 genes with strong support for mitochondrial localisation. 10% of all DEGs were identified as mitochondrial genes and of the downregulated DEGs specifically, 17% of the genes were mitochondrial. Protein interaction network analysis of all downregulated mitochondrial genes showed that many genes were associated with mitochondrial respiratory complexes, mitochondrial ribosomal proteins and membrane associated proteins (**Figure 5.4A**). In particular, many genes that encode subunits that make up mitochondrial respiratory Complex I, or NADH dehydrogenase, and Complex IV, or Cytochrome C oxidase, were lower expressed in old muscle. Semi-quantitative protein levels were determined using Western blot in the protein lysates of the *vastus lateralis* biopsies of 7 older and 7 young individuals. These individuals were selected

because enough material was available and data on *in vivo* mitochondrial capacity in the *vastus lateralis* was previously collected (Lagerwaard et al. 2020a). The transcriptional differences translated into significant lower protein expression of Complex IV and Complex II, or Succinate dehydrogenase (p = 0.011 and p = 0.0009, respectively) (**Figure 5.4B**), whereas a trend could be observed for Complex I, in older compared to young muscle (p = 0.062) (**Figure 5.4C**). The lower protein expression could explain, at least in part, the lower mitochondrial capacity observed in the older compared to the young group, as protein expression of complex I and IV was significantly correlated to mitochondrial capacity in the *vastus lateralis* as measured in vivo using NIRS (p = 0.017, R^2 = 0.42 and p =0.030, R^2 = 0.36) (**Figure 5.4D**).

Mitochondrial mass is regulated by Proliferator-activated receptor gamma coactivator 1-alpha (*PPARGC1A* or *PGC1α*) and gene expression of this transcriptional cofactor was lower in the older group (FC = 0.8, padj = 0.02). Furthermore, when identifying transcription factors that are significantly associated with the DEGs, four transcription factors gene sets were significantly regulated with an FDR q value < 0.2, among which the gene set associated to *PGC1α* (**Figure 5.5A**). Mitochondrial transcription factor A (*TFAM*) is a major target for *PGC1α* and was also downregulated in the older group (FC = 0.85, padj = 0.03). We examined mRNA and protein levels of TFAM using qPCR and Western blot. While differential *TFAM* gene expression was not confirmed (**Figure 5.5D**), TFAM protein levels were significantly lower in the older group, which is in accordance with RNAseq data (**Figure 5.5BC**). The lower expression of *PGC1α* and TFAM could be an explanation for the lower expression of mitochondrial complexes and the lower *in vivo* mitochondrial capacity in the older group.

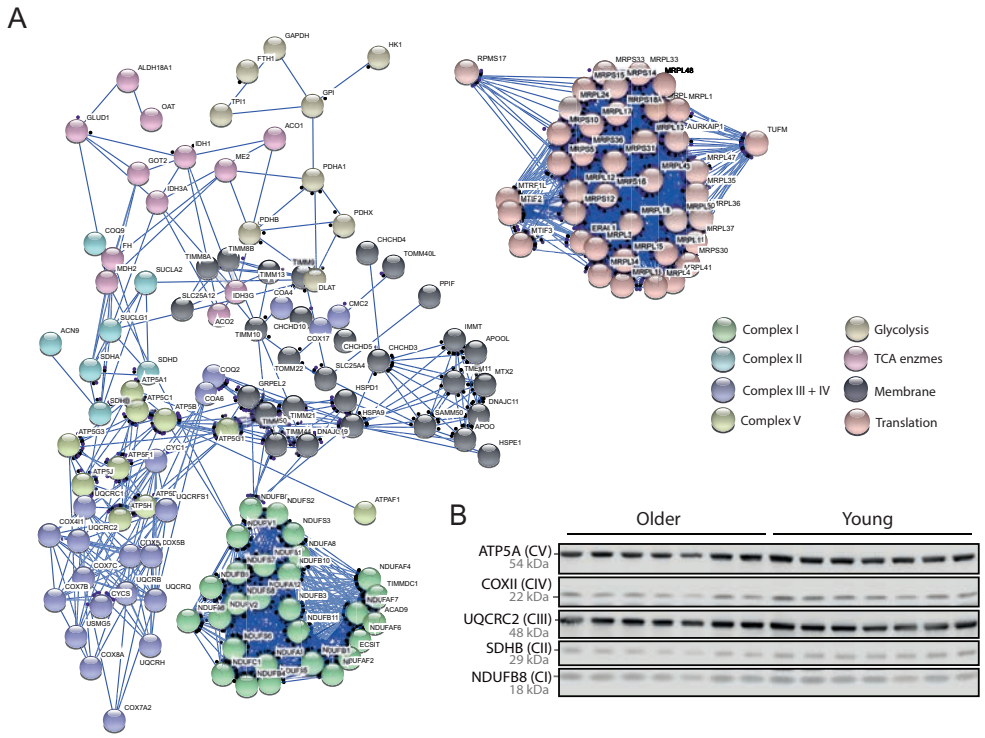

Figure 5.4. Oxidative respiration is the main downregulated process in older muscle (A) Differentially expressed genes were compared to genes in Mitocarta genes set. Protein-protein interaction network for all mitochondrial downregulated genes with no FC cut-off. Nodes and edges with a molecular action are represented and nodes without any connections are removed. Proteins were manually grouped based on function in mitochondria. **(B)** Western blot of protein expression of OXPHOS complexes of 7 older and 7 young *vastus lateralis* muscle on nitrocellulose membrane. **(C)** Quantification of protein expression corrected for total protein. **(D)** Correlation between protein expression and mitochondrial capacity in the *vastus lateralis* measured using NIRS.

In attempt to further explain the lower expression of *PGC1α* and its targets genes in the older muscle, we explored mechanisms upstream from PGC1α. Following muscle contraction, PGC1α is activated by the energy sensing AMP-Activated protein kinase (AMPK) protein complex (Jager et al. 2007). During exercise, the α2/β2/γ3 heterotrimer of this complex is mostly activated and activity of the AMPK complex was mainly associated with the phosphorylation of the skeletal muscle specific subunit of this complex, namely AMP-Activated protein kinase non-catalytic subunit gamma 3 (*PRKAG3*) (Birk and Wojtaszewski 2006). The expression of *PRKAG3* was downregulated in older compared to young muscle (FC = 0.58, padj < 0.001), suggesting that the activation of PGC1α in response to exercise could be lower. When looking at other exercise-responsive genes, Nuclear receptor subfamily 4 group A member 1 (*NR4A1*), Nuclear receptor subfamily 4 group A member 3 (*NR4A3*) and the long non-coding RNA NR4A1 antisense RNA (*NR4A1AS*) were downregulated (FC = 0.45, padj < 0.001, FC = 0.42, padj = 0.04 and FC = 0.34, padj = 0.007 respectively). *NR4A1* and *NR4A3* are known as potent exercise response genes and are involved in regulation of mitochondrial biogenesis and oxidative metabolism, possibly in part via AMPK (Mahoney et al. 2005; Kawasaki et al. 2009; Chao et al. 2012). *NR4A1AS* upregulates *NR4A1* expression via stabilisation of its mRNA (Xie et al. 2019). We verified the lower gene expression of *PGC1α* (FC = 0.77, p = 0.0015), *PRKAG3* (FC = 0.65, p = 0.0005), *NR4A1* (FC = 0.5, p = 0.012) and *NR4A3* (FC = 0.37, p = 0.0017) using qPCR older compared to young muscle. (**Figure 5.5E**). This shows that on molecular level there are differences in expression of important genes that regulate mitochondrial capacity, also associated with the response to exercise and physical activity.

Since mitochondrial protein expression was lower in older compared to younger muscle, it is of interest to study whether a higher physical activity among individuals in the older group associates with a relative higher mitochondrial gene expression, providing a molecular, mechanistic basis for a beneficial effect of physical activity on the ageing muscle. For this, the association between gene expression and the continuous variable 'time spent in MVPA' was calculated for the older group only. Indeed, 662 genes with an Entrez annotation were significantly associated with time spent in MVPA using an unadjusted p-value cut-off of 0.05 (**Figure 5.5D**). 10% of those genes were mitochondrial genes according to the Mitocarta gene set and included 11 out of the 13 genes encoded in the mitochondrial DNA, which all encode for subunits of mitochondrial respiratory complexes. GO enrichment analysis of the significantly associated genes with MVPA showed a clear mitochondrial signature. For the genes that were differentially expressed in the mitochondrial processes, such as 'ATP metabolic process' and 'oxidative phosphorylation', a higher MVPA was correlated to a higher expression of those genes. Whereas for the genes involved in catabolic processes, such as 'positive regulation of catabolic process' and 'regulation of protein catabolic process', the opposite relationship was observed (**Figure 5.5F**). This showed that despite a negative effect of age on mitochondrial capacity, still a higher level of physical activity attenuated this effect.

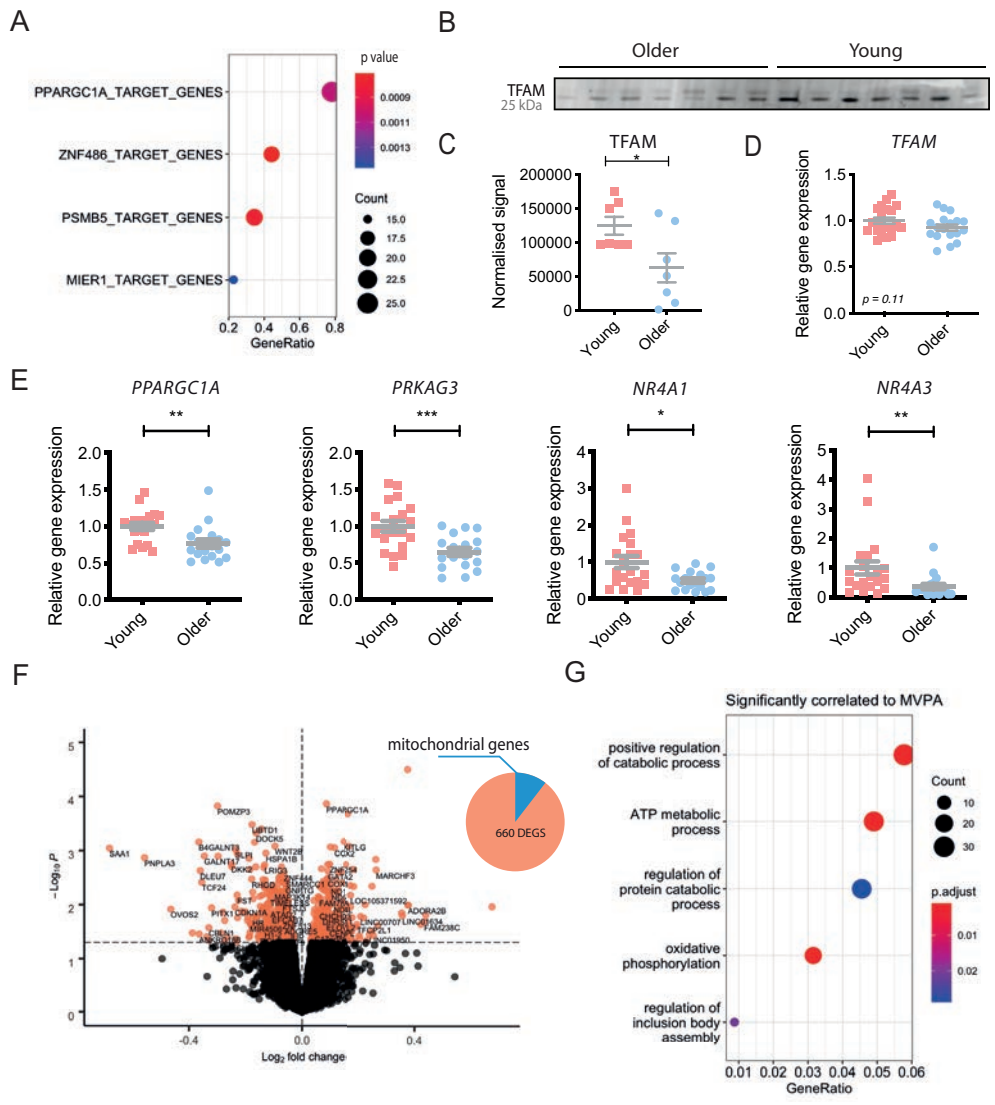

Figure 5.5 Lower expression of genes related to mitochondrial biogenesis in older muscle, yet the expression is correlated to levels of physical activity (A) Transcription factor enrichment analysis for all differentially expressed genes using C3 'TFT:GTRD' from MSigDB was used at FDR <0.2. **(B)** Western blot analysis of Mitochondrial transcription factor A (TFAM) on PVDF membrane **(C)** Quantification of TFAM protein expression normalised for total protein. **(D)** qPCR analysis of *TFAM* expression **(E)** qPCR analysis of Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (*PPARGC1A*), Protein Kinase AMP-Activated Non-Catalytic Subunit Gamma 3 (*PRKAG3*), nuclear receptor subfamily 4 group A member 1 (*NR4A1*) and 3 (*NR4A3*) **(F)** Volcano plot displaying the DEGs. 662 genes with

entrez annotation were significantly correlated with time spent in moderate-to-vigorous physical activity (MVPA) between in older *vastus lateralis* muscle using an p-value of 0.05 and no FDR correction. 10% of those genes were mitochondrial genes. **(G)** GO enrichment analysis of genes significantly correlated to MVPA. The p-value was adjusted using the Benjamini-Hochberg procedure and GO terms were filtered based on 50% overlap in genes. GeneRatio represents the ratio of enriched genes to total number of genes in the specific GO term

5.4. Discussion

The aim of this study was to better understand the effects of early ageing on muscle function by disentangling factors that affect the ageing process, such as sex and physical activity, and advancing age itself. We showed clear differences in the transcriptomic signatures of the *vastus lateralis* muscle of healthy older and young males with similar physical activity. Therefore, we were able to appoint relatively early ageing processes that occur independent from the age-related decline in physical activity. The processes identified were cell adhesion, the matrisome, innervation and inflammation, which were largely upregulated, and oxidative metabolism, which was downregulated, and key genes in these processes were validated using qPCR. Our study encompasses a relatively homogenous age group of older, but not yet old, individuals and this therefore suggest that a decline in mitochondrial function is not a late phenomenon in muscle ageing. Moreover, the lower mitochondrial function is robust, as a large number (± 10% of all DEGs) of mitochondrial genes and processes are affected, including expression of the primary and essential processes of mitochondrial ATP generation, the mitochondrial respiratory complexes. Moreover, we show that the protein expression of these complexes is lower in older compared to young males and is correlated with *in vivo* mitochondrial capacity in the older group. Since we previously measured lower *in vivo* mitochondrial capacity in the *vastus lateralis* muscle of older compared to young males using near infrared spectroscopy (Lagerwaard et al. 2020a), we now strengthen our previous functional observations and show that the observed lower *in vivo* mitochondrial capacity could be caused by a lower expression of mitochondrial proteins.

Our data newly show that the observed decrease in mitochondrial capacity with age cannot be entirely prevented by maintaining physical activity levels, at least with the amount and types of physical activity measured in the current study. This is in contrast with previous studies that reported similar mitochondrial ATP production rates in older compared to young individuals with similar physical activity (Lanza et al. 2008; Distefano et al. 2018). Nevertheless, in the aforementioned studies, a clear trend can be observed for a lower mitochondrial ATP production between the age groups and an absence of effect might be influenced by heterogeneity in the studied populations due to inclusion of wider age-ranges and both sexes. Sex is known to be an important effect modifier in age-related mitochondrial decline (Fitzgerald et al. 2016) and possibly the ageing trajectory is sex dependent (Van der Hoek et al. 2020). Furthermore, in accordance with the current study, a lower mitochondrial complex abundance, *TFAM* expression and *PGC1α* expression was observed, even when comparing similarly active, trained older and young individuals (Lanza et al. 2008), likewise suggesting that physical activity alone is not able to completely halt the age-related lower mitochondrial capacity. Nonetheless, we do show that spending a higher amount of time in moderate-to-vigorous physical activity was associated with higher expression of mitochondrial genes and lower expression of genes involved in catabolic processes. Therefore, we support that increasing or maintaining physical activity is associated with higher mitochondrial capacity and is still a useful strategy to attenuate the age-driven decline in mitochondrial capacity (Lanza et al. 2008; Irving et al. 2015; Balan et al. 2019).

Why mitochondrial protein and gene expression is not similar despite similar physical activity is currently unknown. However, we did find a lower of expression of important genes that regulate mitochondrial biogenesis in the older muscle, such as *TFAM* and *PGC1α*, and genes involved in energy regulation, such as *PRKAG3*. The γ3 subunit of the AMPK complex encoded by *PRKAG3* gene has been shown to be most important in the AMPK activation by phosphorylation following physical activity (Birk and Wojtaszewski 2006) and AMPK activation is essential for the activation of PGC1α, which is its turn regulates

mitochondrial biogenesis via TFAM (Handschin and Spiegelman 2006; Jager et al. 2007). Besides, we show a lower expression of *NR4A1* and *NR4A3,* which are potent exercise response genes that enhance oxidative capacity (Chao et al. 2012) and are regulated in response to local contractile stimuli, possibly in part via AMPK activation (Kawasaki et al. 2009). This suggests that the lower mitochondrial gene and protein expression we observed in the older males might be explained by a decreased sensitivity to exercise. Indeed, an age-related decrease in AMPK activation and mitochondrial biogenesis was previously observed in old compared to young mice (Reznick et al. 2007). Moreover, the response of PGC1α to an exercise intervention after a period of bed rest was higher in young compared to old individuals, failing to restore expression of some mitochondrial complexes in the older individuals (Buso et al. 2019). Likewise, although some of the beneficial effects of exercise interventions are evident and age-independent (Fritzen et al. 2020), there is evidence that a similar intervention increases mitochondrial capacity more in young than older individuals (Irving et al. 2015). However, although PRKAG3 is important for AMPK activity following exercise (Birk and Wojtaszewski 2006), paradoxically its expression is downregulated following an exercise intervention (Pillon et al. 2020). Additionally, an alternative explanation for lower expression of exercise response genes despite similar physical activity, could be due differences in patterns of muscle use. For example, the *vastus lateralis* is thought to be less active during locomotion with advancing age (Hortobágyi and DeVita 2000; Tirosh and Sparrow 2005; Schmitz et al. 2009), which could lead to a local detraining-like molecular response, while whole-body physical activity levels are similar. Therefore, this muscle might not be representative for ageing in all skeletal muscles. Yet muscle biopsies are almost exclusively sampled from the *vastus lateralis* muscle, potentially giving a biased view of skeletal muscle ageing, Therefore, it would be interesting to study the transcriptomic signature in muscles that do not show a lower mitochondrial capacity with age, such as the *tibialis anterior* (Lanza et al. 2007; Christie et al. 2014; Lagerwaard et al. 2020a).

Besides oxidative metabolism, we showed and verified the differential expression of genes that regulate innervation between young and older muscle. The observed gene expression pattern largely agrees with the observations in the *vastus lateralis* muscle of older mice, which was associated with a smaller fibre size and denervation (Rowan et al. 2012; Aare et al. 2016). Moreover, we observed higher expression of the neonatal myosin *MYH8,* which is associated with smaller and denervated muscle fibres during ageing (Soendenbroe et al. 2020). Denervation and remodelling events are a normal neuro-muscular processes. Orphan muscle fibres send signals that stimulate sprouting of neighbouring motor neurons to be re-innervated (Hall and Sanes 1993). However, when denervation wins over innervation, some fibres will lose innervation and this will affect muscle strength (McNeil et al. 2005), possibly due to atrophy of denervated fibres (Rowan et al. 2012). Denervation events are thought to be caused by degradation at the NMJ, but the underlying mechanisms are still unclear (Valdez et al. 2010; Punga and Ruegg 2012). Distortion of NMJs due to accumulative muscle injury (Li et al. 2011) or age-related changes in mitochondria close to the NMJ or in the motor neurons themselves, have been suggested as mechanisms that could play a role in age-related denervation (Rygiel et al. 2014; Anagnostou and Hepple 2020). However, what is cause and what is effect is currently unknown. As, for example, denervation can also directly affect, and by some is thought to precede, mitochondrial dysfunction (Spendiff et al. 2016; Sonjak et al. 2019). Nevertheless, we show that expression of genes encoding for essential parts of the NMJ and synaptogenesis are upregulated in older individuals. We therefore consider mechanistic understanding of NMJ degradation as a critical aspect in the understanding of denervation and muscle ageing, also when physical activity is similar.

Although many anti-ageing interventions are aimed at improving muscle fibre functionality, it is often overlooked that the fibres are embedded in an ECM that is known to regulate development (Thorsteinsdóttir et al. 2011), growth (Fritz et al. 2013a), repair (Calve et al. 2010) and establish contractile force transmission (Street 1983). We show that cell adhesion and the matrisome are most significant regulated processes in the ageing muscle and

therefore argue that the dysregulation of the ECM is an important mechanism in early muscle ageing. Although this process has not been identified as a signature ageing process in a large reannotation of 3000 muscle samples (Su et al. 2015), it was previously observed in a large population of muscle samples from humans of varying ages (Zahn et al. 2006). Here, we now show that a large percentage of genes that encode for proteins involved in the ECM are differentially regulated even with similar physical activity. By interrogation of the current dataset with the matrisome gene set (Shao et al. 2020), we show that this especially includes genes that encode for collagens, approximately 50% of all the genes encoding for collagens. Increased collagen disposition, at least in rats (Gosselin et al. 1998), and higher collagen crosslinking (Haugen et al. 2010) have been observed with age. The higher collagen deposition and collagen crosslinking increases the rigidity of the ECM, which has been put forward as a cause for the increased muscle stiffness with age (Ewald 2020). The increased muscle stiffness might negatively affect muscle regeneration, as it was shown that satellite cells cultured on a rigid membrane displayed decreased survival and increased differentiation (Gilbert et al. 2010; Blau et al. 2015).

Besides an increased collagen deposition and crosslinking, the change in rigidity could also be caused by a lower ECM protein turnover regulated by ECM remodelling factors, such as matrix metalloproteases (MMPs). Lower MMPs were observed in the circulation of old compared to young mice (de Sousa Neto et al., 2018). Nevertheless, our data suggests that remodelling of the ECM by metallopeptidases might not play a major role in early muscle ageing, because only matrix metallopeptidase 15 was downregulated in older muscle (FC = 0.8, padj = 0.002). Nevertheless, some disintegrin and metallopeptidase domain (ADAM) and disintegrin-like metallopeptidases with thrombospondin type 1 motif (ADAMTSs) were differentially upregulated between older and young muscle. These ECM remodelling genes are implicated in muscle development (Jørgensen et al. 2007; Stupka et al. 2013) and could therefore play a role in muscle ageing.

Besides the components and regulators of the ECM, we also show that genes that encode for adhesion between the cells and the ECM were significantly regulated with age. We hypothesised that dysregulation of cell-ECM adhesion could contribute to the loss of force transmission, which was previously observed in rats (Hughes et al. 2016). Indeed, many genes encoding for proteins in adhesion complexes were differentially regulated. We verified the expression of two key genes by qPCR, namely *ITGA7* and *CHD15*. *ITGA7* and *CDH15* encode for major adhesion proteins expressed in skeletal muscle fibres and in satellite cells (Irintchev et al. 1994; Boppart and Mahmassani 2019). For example, *ITGA7* encodes for part of the α7β1 integrin that is thought to be a major contributor to force transmission (Boppart et al. 2006), supporting that dysregulation of force transmission via adhesion complexes could be an important mechanism in early muscle ageing. Moreover, lower expression of *ITGA7* has been previously observed in satellite cells of old mice (Boers et al. 2017). Therefore, these adhesion molecules now also provide an interesting target in human muscle ageing.

Nonetheless, while the ECM is clearly implicated in muscle ageing, the gene expression changes include both up and downregulations, which are difficult to interpret without dedicated studies. For example, dystrophin, the main component of the DGC cellular adhesion complex, was previously shown to be lower expressed in old rats, which was associated with lower force transmission. Interestingly, other components of the DGC were upregulated, possibly in attempt to compensate for loss adhesion (Ramaswamy et al. 2011; Hughes et al. 2016). Likewise, in a mouse model for Duchene's muscular dystrophy, a muscular dystrophy that is caused by a dysfunctional dystrophin protein, cell adhesion proteins such as integrins are upregulated (Boppart and Mahmassani 2019), suggesting a similar compensatory mechanism. Moreover, although mRNA expression seems to be a good indicator for expression of ECM proteins (Makhnovskii et al. 2020), mRNAs encoding for ECM proteins must to be excreted and assembled in complex networks in the ECM, and can be subjected to post translational modifications and degradation (Mouw et al. 2014).

Hence, this limits straightforward mechanistic interpretation of transcriptomics data and requires further mechanistic studies on the identified targets.

Another limitation of the current design is that, although we controlled for time spent in exercise and exercise intensity with accelerometery measurements, type of exercise was not standardised between the groups. Therefore, it could be that differences types of exercise, such as resistance training, could have affected gene expression between the groups. For example, higher expression of adhesion genes, in particular protocadherin gamma genes, was previously observed in a population of older and frail older individuals compared to a young control group (Hangelbroek et al. 2016). Although the latter study did not control for physical activity between the groups, they showed that expression of this gene cluster decreased after training and was correlated with muscle strength. Henceforth, differences in resistance type exercise between the young and older individuals could have affected the expression of these genes, as in the current study we likewise observe an upregulation of protocadherin gamma genes in the older group. Moreover, the effect of resistance exercise is not limited to adhesion genes, as ECM-remodelling factors such as metallopeptidases, were also regulated in an age-dependent manner after resistance training (Wessner et al. 2019). Therefore, future cross-sectional research should aim to not only control for exercise time and intensity, but additionally standardise for exercise type between the age groups. Besides, physical activity was assessed over a five-day period using accelerometery and over period of the preceding year using the Baecke questionnaire, while physical activity over the entire lifetime is likely to be a more important driver for muscle ageing. Therefore, because longitudinal studies over the lifetime present obvious challenges, research tools to asses physical activity retrospectively, such as data from wearable physical activity monitors, should be further developed.

5.5. Conclusion

We showed a clear ageing signature in the gene expression profile of the *vastus lateralis* muscle between young and older males and identified cell adhesion, the matrisome, innervation and inflammation as the main upregulated processes associated with muscle ageing despite similar physical activity levels. Additionally, a strong downregulation of mitochondrial genes and proteins was observed, suggesting an impaired ability for energy generation. These data underpin the previously observed lower *in vivo* mitochondrial capacity at a molecular level. Yet, whether these results can be extended to other skeletal muscles needs to be examined, since we previously reported differences in the effect of age on mitochondrial capacity between muscles (Lagerwaard et al. 2020a). Why similar physical activity was not associated with similar mitochondrial respiratory complex expression needs to be further investigated. Possibly, the downregulation in upstream activators of mitochondrial biogenesis hints at a lower molecular sensitivity to cues for mitochondrial biogenesis, which needs to be confirmed. Nevertheless, our study highlights that mitochondrial function is lower in the older muscle and this strongly associates with impaired sensitivity to physical activity, possibly due to structural changes involving, cell adhesion, the matrisome and innervation. Since, the older males in our study consisted of older, but not old individuals, the identified processes can be considered as a relatively early signature. Henceforth, an improved understanding of the of the processes identified here, will be key to design targeted anti-ageing therapies to ultimately sustain muscle health with advancing age.

Acknowledgements The authors greatly acknowledge the commitment of the volunteers who participated in the study and Paul Nijhuis for help with data collection.

Compliance with ethical standards The study was approved by the medical ethical committee of Wageningen University with reference number NL65872.081.18. All procedures performed were in in accordance with the principles of the Declaration of

Helsinki (Fortaleza, Brazil 2013) and according to national law (WMO, The Hague, 1998). The study is registered in the Dutch trial register (NL7695). Subjects were written and verbally informed on all experimental procedures, including possible risks and discomforts. All subjects provided written informed consent before testing.

Author contributions BL: data acquisition, principal data analysis, drafting the manuscript; AB: qPCR and data interpretation; BL, AGN, VCJB, JK: conception and design of research; data analysis and interpretation. All authors edited, revised and approved final version of manuscript.

Supplementary materials

Table S5.1 Differently regulated matrisome genes between young and older males

Group	Symbol	FC	Padj
collagens	COL4A5	1.39	4.4E-07
collagens	COL6A6	0.36	9.3E-07
collagens	COL28A1	2.06	8.2E-06
collagens	COL19A1	4.12	2.1E-05
collagens	COL5A3	0.68	3.4E-05
collagens	COL4A6	1.69	5.9E-05
collagens	COL3A1	0.45	8.7E-05
collagens	COL5A1	0.59	1.1E-04
collagens	COL4A1	0.53	1.8E-04
collagens	COL4A2	0.58	4.3E-04
collagens	COL8A1	1.5	4.5E-04
collagens	COL21A1	1.91	6.2E-04
collagens	COL12A1	1.48	8.5E-04
collagens	COL1A1	0.64	2.8E-03
collagens	COL25A1	2.61	2.9E-03
collagens	COL13A1	1.58	9.6E-03
collagens	COL15A1	0.74	1.1E-02
collagens	COL8A2	1.65	1.7E-02
collagens	COL5A2	0.76	2.9E-02
collagens	COL1A2	0.72	4.3E-02
ECMglycoproteins	LGI1	2.57	8.8E-15
ECMglycoproteins	EFEMP1	2.06	5.7E-10
ECMglycoproteins	PXDNL	2.83	4.9E-09
ECMglycoproteins	PCOLCE2	1.97	3.8E-08
ECMglycoproteins	SLIT2	1.84	1.6E-07
ECMglycoproteins	NPNT	1.97	8.1E-06
ECMglycoproteins	MFAP5	1.83	1.9E-05
ECMglycoproteins	CRIM1	1.61	9.4E-05
ECMglycoproteins	LAMC2	1.77	4.0E-04
ECMglycoproteins	EFEMP2	0.76	6.3E-04
ECMglycoproteins	MFAP4	1.4	1.0E-03
ECMglycoproteins	THBS2	1.96	1.2E-03
ECMglycoproteins	LAMA2	1.32	1.2E-03
ECMglycoproteins	VWA3B	1.92	1.3E-03
ECMglycoproteins	SPARC	0.71	1.3E-03
ECMglycoproteins	PXDN	0.68	1.4E-03
ECMglycoproteins	VWCE	1.58	1.9E-03
ECMglycoproteins	FBN1	1.54	2.4E-03
ECMglycoproteins	EDIL3	2.16	2.5E-03
ECMglycoproteins	VWA7	1.3	2.7E-03
ECMglycoproteins	EGFLAM	0.84	3.3E-03
ECMglycoproteins	SRPX2	1.44	5.0E-03
ECMglycoproteins	EMILIN2	1.72	6.3E-03
ECMglycoproteins	HMCN2	0.74	7.1E-03
ECMglycoproteins	FBLN1	1.3	7.8E-03
ECMglycoproteins	VWA5B1	5.45	7.9E-03
ECMglycoproteins	FBLN5	1.25	9.8E-03
ECMglycoproteins	OIT3	0.12	1.0E-02
ECMglycoproteins	SLIT3	1.31	1.1E-02
ECMglycoproteins	MXRA5	0.49	1.3E-02
ECMglycoproteins	LTBP2	1.33	1.6E-02
ECMglycoproteins	IGFALS	2.25	2.1E-02
ECMglycoproteins	VWA5A	1.23	2.1E-02
ECMglycoproteins	IGFBP5	0.82	2.5E-02
ECMglycoproteins	IGFBP3	1.33	2.7E-02
ECMglycoproteins	MMRN1	1.82	3.5E-02
ECMglycoproteins	LAMB3	0.71	3.8E-02
ECMglycoproteins	VIT	1.24	3.8E-02
ECMglycoproteins	ELN	1.4	3.8E-02
ECMglycoproteins	IGFBP6	1.32	3.9E-02

ECMglycoproteins	LTBP4	1.16	4.1E-02	SecretedFactors	CRLF1	0.75	3.6E-03
ECMglycoproteins	RELN	1.64	4.5E-02	SecretedFactors	FGF18	2.13	3.7E-03
ECMglycoproteins	NID2	0.74	4.7E-02	SecretedFactors	TNF	0.44	4.2E-03
ECMglycoproteins	FGL2	0.79	4.7E-02	SecretedFactors	VEGFB	0.81	4.5E-03
proteoglycans	PRG4	5.18	4.7E-15	SecretedFactors	PGF	0.68	5.5E-03
proteoglycans	CHAD	1.81	6.9E-05	SecretedFactors	WNT10B	3.28	6.5E-03
proteoglycans	HAPLN3	1.66	6.8E-04	SecretedFactors	CXCL14	1.24	7.4E-03
proteoglycans	DCN	1.31	1.1E-03	SecretedFactors	BMP6	1.41	9.1E-03
proteoglycans	IMPG2	2.13	6.7E-03	SecretedFactors	FGFBP2	2.53	9.2E-03
proteoglycans	HSPG2	0.74	7.5E-03	SecretedFactors	FGF22	2.25	9.9E-03
proteoglycans	PRELP	1.26	2.4E-02	SecretedFactors	CCL28	1.98	1.0E-02
proteoglycans	ASPN	0.76	4.3E-02	SecretedFactors	CCL13	4.85	1.2E-02
SecretedFactors	IL15	1.67	7.8E-07	SecretedFactors	PDGFA	0.82	1.2E-02
SecretedFactors	SCUBE2	0.55	8.5E-06	SecretedFactors	CSF1	1.28	1.3E-02
SecretedFactors	FST	2.26	2.8E-05	SecretedFactors	GDF15	3.47	1.5E-02
SecretedFactors	HGF	2.15	7.2E-05	SecretedFactors	S100A16	0.76	1.6E-02
SecretedFactors	BDNF	4.1	1.2E-04	SecretedFactors	INHBC	3.1	1.9E-02
SecretedFactors	WNT9A	1.88	1.8E-04	SecretedFactors	PRL	5.22	2.0E-02
SecretedFactors	INHBA	1.5	2.3E-04	SecretedFactors	CHRDL2	1.45	2.2E-02
SecretedFactors	THPO	0.55	3.2E-04	SecretedFactors	BMP2	0.63	2.3E-02
SecretedFactors	IL17D	1.5	3.3E-04	SecretedFactors	S100A4	1.24	2.3E-02
SecretedFactors	ANGPT2	1.48	4.4E-04	SecretedFactors	MSTN	0.71	2.4E-02
SecretedFactors	CHRD	0.69	6.0E-04	SecretedFactors	BMP8A	1.38	2.4E-02
SecretedFactors	NTF3	1.67	8.8E-04	SecretedFactors	CXCL2	0.46	2.5E-02
SecretedFactors	FGF13	0.83	1.0E-03	SecretedFactors	S100A10	1.27	2.7E-02
SecretedFactors	IL25	6.75	1.1E-03	SecretedFactors	HBEGF	0.78	3.1E-02
SecretedFactors	SFRP1	2.1	1.1E-03	SecretedFactors	HCFC2	1.18	3.4E-02
SecretedFactors	EGFL7	0.75	1.5E-03	SecretedFactors	CX3CL1	0.61	3.7E-02
SecretedFactors	VEGFC	1.46	1.7E-03	SecretedFactors	WNT2B	1.19	4.1E-02
SecretedFactors	MEGF8	0.83	2.0E-03	SecretedFactors	IL7	1.83	4.4E-02
SecretedFactors	BMP8B	1.69	2.1E-03	SecretedFactors	ZFP91	0.92	4.6E-02
SecretedFactors	GDNF	1.92	2.2E-03	ECMaffiliatedProteins	C1QA	2.07	1.7E-08
ECMaffiliatedProteins	CLEC11A	0.44	3.9E-08	ECMregulators	SERPINF1	0.76	6.4E-08
ECMaffiliatedProteins	GPC4	0.74	6.9E-07	ECMregulators	ADAMTSL4	1.92	7.3E-08
ECMaffiliatedProteins	C1QB	2.46	1.1E-05	ECMregulators	EGLN3	1.8	1.9E-07
ECMaffiliatedProteins	C1QC	2.18	6.2E-05	ECMregulators	MASP2	1.6	3.8E-07
ECMaffiliatedProteins	PLXNC1	1.68	1.2E-04	ECMregulators	MASP1	0.49	9.0E-06
ECMaffiliatedProteins	CD209	1.98	1.2E-04	ECMregulators	CST3	0.75	2.0E-05
ECMaffiliatedProteins	CLEC2B	1.41	1.6E-04	ECMregulators	ADAMTS4	0.36	2.7E-05
ECMaffiliatedProteins	ANXA6	0.87	3.2E-04	ECMregulators	SULF1	1.59	6.9E-05
ECMaffiliatedProteins	GREM1	0.63	6.2E-04	ECMregulators	CTSL	0.83	8.8E-05
ECMaffiliatedProteins	SEMA3E	3.49	7.3E-04	ECMregulators	SERPINB6	0.82	9.5E-05
ECMaffiliatedProteins	SEMA4C	0.69	9.7E-04	ECMregulators	FAM20A	1.58	1.1E-04
ECMaffiliatedProteins	CLEC2L	0.52	1.4E-03	ECMregulators	FAM20B	0.85	1.3E-04
ECMaffiliatedProteins	SDC2	1.3	3.2E-03	ECMregulators	NGLY1	0.82	2.0E-04
ECMaffiliatedProteins	CLEC4F	0.48	7.4E-03	ECMregulators	BMP1	0.72	3.2E-04
ECMaffiliatedProteins	PLXNB2	1.31	8.6E-03	ECMregulators	HYAL2	0.76	4.2E-04
ECMaffiliatedProteins	C1QTNF1	0.85	1.0E-02	ECMregulators	SERPINH1	0.65	6.5E-04
ECMaffiliatedProteins	CLEC7A	1.6	1.0E-02	ECMregulators	CTSG	2.21	1.1E-03
ECMaffiliatedProteins	GPC6	1.48	1.1E-02	ECMregulators	SERPINE2	0.67	1.2E-03
ECMaffiliatedProteins	FREM1	1.43	1.3E-02	ECMregulators	PLAT	1.43	1.2E-03
ECMaffiliatedProteins	COLEC12	0.74	1.4E-02	ECMregulators	SERPING1	1.29	1.8E-03
ECMaffiliatedProteins	C1QTNF6	1.38	1.9E-02	ECMregulators	CTSS	1.44	2.6E-03
ECMaffiliatedProteins	ITLN1	7.58	2.4E-02	ECMregulators	ADAM10	1.15	2.7E-03
ECMaffiliatedProteins	SEMA5B	0.55	2.5E-02	ECMregulators	SPAM1	1.94	4.6E-03
ECMaffiliatedProteins	SEMA6A	0.78	2.5E-02	ECMregulators	SERPINE1	0.49	5.0E-03
ECMaffiliatedProteins	FCN1	1.6	2.9E-02	ECMregulators	FAM20C	0.82	5.6E-03
ECMaffiliatedProteins	SDC3	0.84	3.9E-02	ECMregulators	HPSE	3.51	6.0E-03
ECMaffiliatedProteins	PLXND1	0.86	4.9E-02	ECMregulators	PLOD1	0.85	8.6E-03
ECMregulators	KAZALD1	0.39	3.1E-20	ECMregulators	PAMR1	1.67	1.0E-02
ECMregulators	ADAMTS5	1.96	2.3E-14	ECMregulators	LOXL2	0.64	1.1E-02
ECMregulators	SLPI	3.75	7.8E-11	ECMregulators	CTSV	2.29	1.1E-02
ECMregulators	F13A1	2.23	2.1E-10	ECMregulators	C17orf58	1.29	1.2E-02
ECMregulators	LOXL4	2.15	7.3E-09	ECMregulators	EGLN1	1.25	1.3E-02

ECMregulators	P4HA1	1.27	1.5E-02
ECMregulators	P4HA2	0.8	1.5E-02
ECMregulators	MMP15	0.8	1.7E-02
ECMregulators	ITIH2	1.39	2.1E-02
ECMregulators	SERPINA1	1.71	3.1E-02
ECMregulators	TIMP2	1.22	3.3E-02
ECMregulators	CTSB	1.11	3.4E-02
ECMregulators	CTSF	1.16	4.0E-02
ECMregulators	ADAMTS14	0.48	4.8E-02

FC = Fold change regulation or mean expression older vs. young. Adj. p-value: adjusted p-values using Benjamini-Hochberg method.

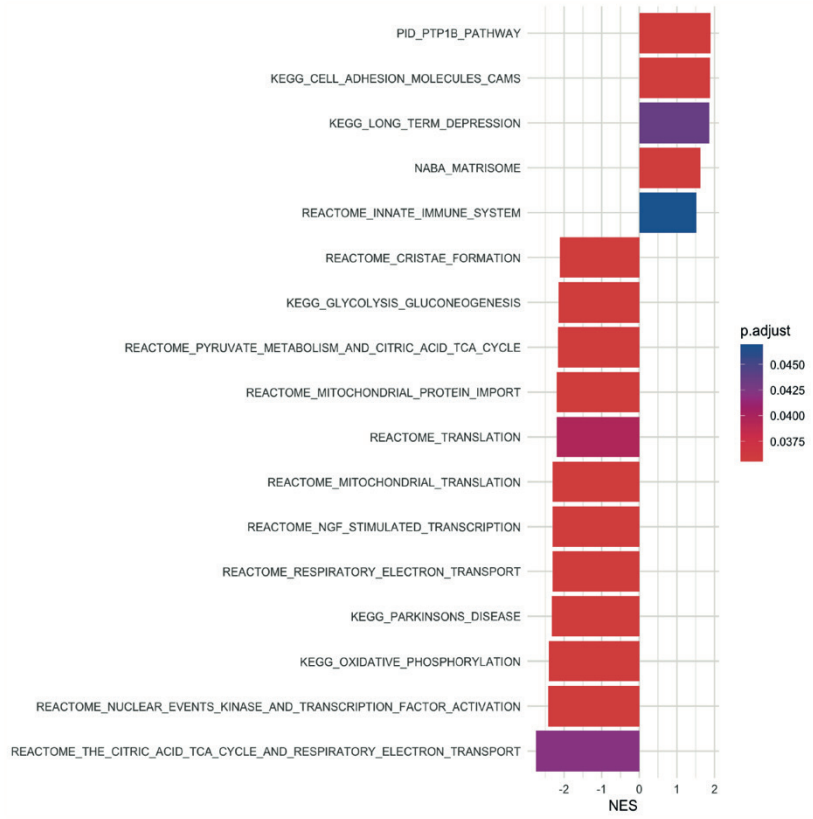

Figure S5.1 Gene set enrichment analysis of older vs young using curated C2 canonical gene sets Msigdb. Gene sets were considered enriched with a Benjamini-Hochberg adjusted p-value < 0.05

CHAPTER 6

Increased protein propionylation contributes to mitochondrial dysfunction in liver cells and fibroblasts, but not in myotubes

Bart Lagerwaard
Olga Pougovkina
Anna F. Bekebrede
Heleen te Brinke
Ronald J.A. Wanders
Arie G. Nieuwenhuizen
Jaap Keijer
Vincent C. J. de Boer

Published in Journal of Inherited Metabolic Disease (2020)
Doi: 10.1002/jimd.12296

Abstract

Objective
Translational protein modifications derived from metabolic intermediates, such as acyl-CoAs, have been shown to regulate mitochondrial function. Patients with a genetic defect in the propionyl-CoA carboxylase (PCC) gene clinically present symptoms related to mitochondrial disorders and are characterised by decreased mitochondrial respiration. Since propionyl-CoA accumulates in PCC deficient patients and protein propionylation can be driven by the level of propionyl-CoA, we hypothesised that protein propionylation could play a role in the pathology of the disease.

Methods
To mimic pathological protein propionylation levels, we exposed cultured fibroblasts, Fao liver cells and C2C12 muscle myotubes to propionate levels that are typically found in these patients.

Results
We identified increased protein propionylation due to pathologic propionyl-CoA accumulation in patient-derived fibroblasts and this was accompanied by defective mitochondrial respiration, as was shown by a decrease in complex I-driven respiration. This induced a global increase in protein propionylation and histone protein propionylation and was also accompanied by a decrease in mitochondrial respiration in liver and fibroblasts. However, in C2C12 myotubes propionate exposure did not decrease mitochondrial respiration, possibly due to differences in propionyl-CoA metabolism as compared to the liver.

Conclusion
Protein propionylation could contribute to the pathology in PCC patients, especially in the liver, and could therefore be an interesting target to pursue in the treatment of this metabolic disease.

6.1. Introduction

Post-translational protein modifications (PTMs) are an important regulatory mechanism for protein functionality and localisation. Modification of proteins offers the cell a rapid and reversible mechanism to respond to changes in the environment, such as changes in metabolite availability. Protein acylation involves the covalent binding of acyl-groups to lysine residues of a protein and directly links metabolism and protein functionality (Choudhary et al. 2014). For example, intermediates of metabolism, such as acetyl-CoA, drive protein acetylation (Pougovkina et al. 2014a) and can serve as a regulatory mechanism in fatty acid oxidation by acetylation of enzymes involved in the breakdown of fatty acids (Hirschey et al. 2010).

Acetylation is only one type of protein acylation and other acyl-lysine PTMs have been identified, such as succinylation (Zhang et al. 2011), glutarylation (Tan et al. 2014), malonylation (Peng et al. 2011), crotonylation (Tan et al. 2011), butyrylation and propionylation (Chen et al. 2007). Propionylation is the covalent binding of a propionyl-group to lysine residues of proteins and although being structurally fairly similar to acetylation, the propionyl group is slightly larger and may well be functionally different. Moreover, propionyl-CoA, the substrate for propionylation, is of special interest, since it is a breakdown product of cholesterol, odd-chain fatty acids and the amino acids isoleucine, valine, threonine and methionine (Sbaï et al. 1994). Additionally, propionate is produced by the microbiota and taken up in the intestine (Cummings et al. 1987). Propionylation was first identified on histones, where it was later characterised as a transcriptionally active PTM in an *in vitro* system (Chen et al. 2007; Kebede et al. 2017). Besides histones, propionylation also occurs on non-histone proteins (Cheng et al. 2009) and increased propionyl-CoA levels were able to propionylate the propionyl-CoA synthetase enzyme in prokaryotes, thereby inactivating it (Garrity et al. 2007). This suggests that propionylation of proteins could have a role in metabolic regulation, for example during catabolism and fasting.

Inborn errors in propionyl-CoA metabolism, such as caused by bi-allelic mutations in one of the two propionyl-CoA carboxylase genes (*PCC*), leads to an accumulation of propionyl-CoA, a condition known as propionic acidemia (PA). PCC catalyses the carboxylation of propionyl-CoA into methylmalonyl-CoA. Methylmalonyl-CoA can be converted into succinyl-CoA, which can be used for anaplerosis of the TCA-cycle (Davis et al. 1980; Wongkittichote et al. 2017). Clinically, the severe neonatal-onset form of this disease often presents within the first days or weeks in life with encephalopathy, metabolic acidosis and hyperammonaemia and, when left untreated, progresses to coma or death. When treated, the prognosis remains rather poor, with patients showing developmental delay, neurological complications, liver abnormalities, myopathic features and cardiomyopathy (Grünert et al. 2013; Haijes et al. 2019). Therefore, more knowledge is required to amend current treatment strategies to improve the disease outcome.

Muscle and liver biopsies from PCC patients show defective mitochondrial respiration, suggesting that mitochondrial dysfunction contributes to the pathology (Schwab et al. 2006; de Keyzer et al. 2009). Interestingly, it is not known how increased levels of propionyl-CoA can contribute to this. Fibroblasts derived from patients display increased protein propionylation, showing that pathological build-up of intermediates of propionyl-CoA metabolism can alter the protein acylome (Pougovkina et al. 2014b). We hypothesised that the increased propionylation disrupts normal mitochondrial function and contributes to the mitochondrial phenotype. Here, we use control and patient-derived fibroblasts and cultured cells to study the mitochondrial effects of increased propionylation to explore a possible role of this PTM in health and disease.

6.2. Material and Methods

6.2.1. Cell culture

Human dermal fibroblasts and Fao hepatoma cells were cultured in Dulbecco's Modified Eagle medium (DMEM) supplemented with 10% (v/v) fetal calf serum (FCS), 2 mM

glutamine and 1% (v/v/v) pen/strep/fungizone. C2C12 myoblasts were cultured in DMEM supplemented with 10% (v/v) FCS, 2 mM glutamine, 25 mM HEPES pH 7.2 and 1% (v/v/v) pen/strep/amphotericin B. Differentiation was induced by replacing medium with DMEM supplemented with 2% (v/v) horse serum (HS) upon confluency. Medium was replaced every other day for 5-7 days. Propionyl-CoA carboxylase deficient fibroblasts were obtained from the Gaslini Biobank and C2C12 and Fao cells were obtained from ATCC. Propionate exposure was induced by culturing cells in growth medium containing 4 mM propionic acid from a 400 mM pH-balanced stock solution. Medium pH remained within the normal range for culture medium (pH 7.3 – 7.5).

6.2.2. SDS –PAGE and Western blotting

Cells were harvested and lysed in TRIS-HCL pH 7.4 with 1% triton X-100 containing protease inhibitors and deacylase inhibitors (1 µM trichostatin A and 20 mM nicotinamide). Lysates were sonicated 5 times 2s at 40 kHz amplitude on ice. Protein concentrations were determined using Pierce BCA protein assay kit (Thermofisher) and equal protein amounts were loaded on NuPAGE 4-12% gels (Invitrogen), transferred to nitrocellulose membrane, blocked in 3% BSA in PBS with 0.1% Tween-20 at room temperature and incubated overnight with antibodies in the same buffer at 4ºC. Primary antibodies used: β-actin (#A5441, Sigma-Aldrich), propionyllysine (#201, PTM biolabs), succinyllysine (#401, PTM biolabs), acetyllysine (#9441, Cell Signalling), histone 3 propionyllysine 23 (#613987, Active Motif), histone 3 acetyllysine 23 (#07-355, Millipore). IR-dye based secondary antibodies (LICOR) were used to detect antibody signals using Odyssey scanner (LICOR).

6.2.3. Seahorse mitochondrial respiratory flux analysis.

Seahorse XFe96 analysis was performed according to manufacturer's instructions. On the day prior to the analysis, cells were plated at 10,000 cells per well for fibroblasts, 30,000 for Fao, 10,000 for C2C12. For myotubes, 20,000 myoblasts were seeded per well and differentiated in the seahorse plate for 7 days prior to exposure. Before the assay, medium was replaced by XF base medium (Agilent) supplemented with 25 mM glucose and 2 mM

glutamine. For analysis of mitochondrial respiration in permeabilised cells, medium was replaced with MAS buffer (220 mM mannitol, 70 mM sucrose, 10 mM KH_2PO_4, 5 mM $MgCl_2$, 2 mM HEPES pH 7.2, 1 mM EGTA and 0.6% BSA-fatty acid free) shortly before the assay. Cells were permeabilised by injection of digitonin with concentration of 30 µg/mL for Fao, 37.5 µg/mL for C2C12 and fibroblasts. Oxygen consumption rates (OCR) were analysed following a single injection of pyruvate/malate/ADP (complex I), succinate/rotenone/ADP (complex II), TMPD, ascorbate/ADP (complex IV). Data were normalised to protein concentration or as ratio of nuclei area over background area using 4',6-diamidino-2-phenylindole (DAPI) staining. For staining: Cells were fixed in 4% formaldehyde for 15 minutes, washed with PBS and stained with 1 µg/mL DAPI for 5 minutes. The BD Pathway 855 microscope (Becton Dickinson; Franklin Lakes, New Jersey, USA) at 10x magnification and an exposure of 0.02. Calculation DAPI pixels over total area in the Seahorse well was done using Adobe Photoshop (Adobe Systems; San Jose, California, USA).

6.2.4. Propionyl-CoA carboxylase activity

Pellets were resuspended in PBS and sonicated. A volume of 10 µl of protein lysate (1mg/ml) was added to 40 µl of the reaction mixture (100 mM TRIS-HCl pH 8.0, 200 mM $KHCO_3$, 10 mM $MgCl_2$, 10 mM ATP, 1 mM propionyl-CoA) to a final protein concentration of 0.2 mg/ml. After 15-minute incubation at 37° C the reaction was terminated with 10 µl of 2 M HCl. The sample was neutralised with 2 M KOH/0.6 M MES buffer after which 30 µl of methanol HPLC grade was added. After centrifugation at 20000 g for 5 minutes at 4° C the supernatant was injected on reversed phase HPLC to analyse the formation of methylmalonyl-CoA.

6.2.5. Propionylcarnitine measurement

Cells were harvested by trypsinisation and pellets were resuspended in 0.5 ml demineralised water to 1 mg of protein homogenate. Internal standard (50 pmol $^2H_3$-propionylcarnitine) was added to the homogenate, followed by 500 µl of acetonitrile. The samples were vortex-mixed and centrifuged at 14000 rpm 4° C for 10 minutes. The supernatant was transferred to a glass vial and the solvent was evaporated at 42° C under a

stream of nitrogen. A 100 µl volume of propylation reagent, a 4:1 (v/v) mixture of propan-2-ol and acetylchloride, was added to the residue, vortex-mixed and incubated for 10 minutes at 65° C. The propylation reagent was evaporated at 42° C under a stream of nitrogen and the residue was taken up in 100 µl of acetonitrile. Propionylcarnitine was quantified by Electrospray Ionization Tandem Mass Spectrometry (ESI-MS/MS) as described previously (Vreken et al. 2002).

6.2.6. Data analysis and statistical testing

Data are presented as mean ± SD. Statistical analyses were performed using GraphPad Prism v.5 (GraphPad Software, CA, USA). Means between groups were compared using a Students unpaired t-test. Significance was accepted at $p < 0.05$.

6.3. Results

6.3.1. Defective mitochondrial respiration in propionyl-CoA carboxylase deficient cells

To evaluate propionyl-CoA accumulation and its functional consequences, we used three independent control cell lines and three independent PCC deficient fibroblast cell lines, in which PCC activity was reduced to undetectable levels (**Figure 6.1A**). All three patient cell lines had a lower mitochondrial spare capacity compared to controls (**Figure 6.1B**). To determine if the decreased OCR was also observed when individual mitochondrial complexes were analysed, we measured respiration in digitonin-permeabilised cells. The use of an optimised amount of digitonin allows permeabilisation of the cell membrane, yet leaving the mitochondrial membrane intact (**Figure 6.1C**). We measured OCR following injection of digitonin, ADP and the substrates pyruvate and malate, that are linked to the generation of NADH and complex 1 respiration. Complex I linked respiration was lower in all the three patient cell lines compared to three control cell lines expressed as increase in OCR after injection of digitonin, ADP and complex I linked substrates (**Figure 6.1D**). When comparing normalised maximal complex I linked respiration, there was a significant

difference between control and patient cell lines, demonstrating the defective mitochondrial respiration in these PCC deficient cells (**Figure 6.1E**).

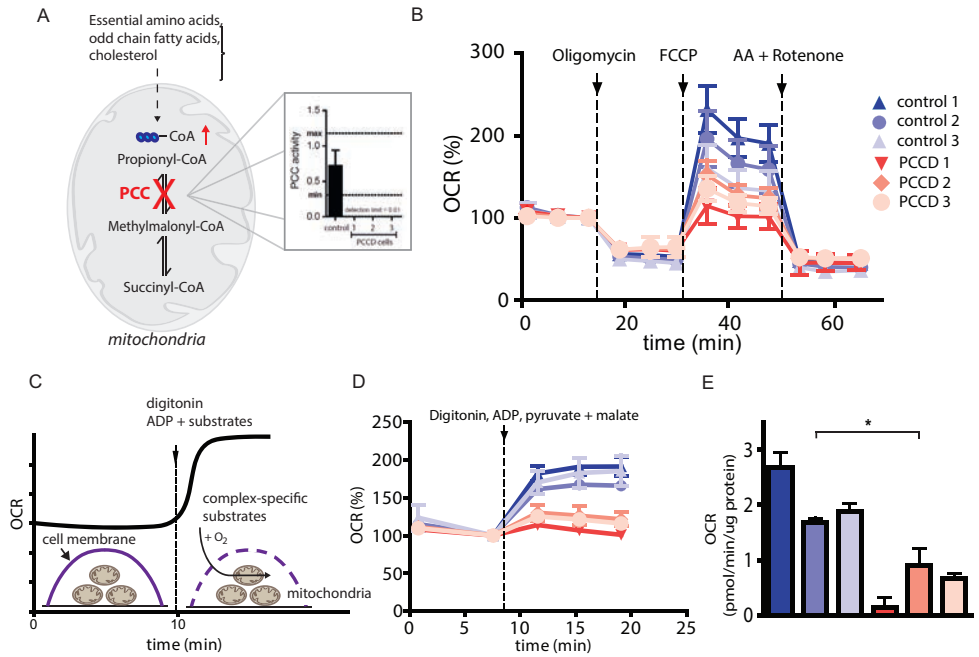

Figure 6.1 Propionyl-CoA carboxylase deficient cells have defective mitochondrial respiration. (A) Schematic representation of propionyl-CoA metabolism and its accumulation in propionyl-CoA carboxylase (PCC) deficient cells and PCC activity in PCC deficient cells. **(B)** Mitochondrial respiration in PCC deficient and control fibroblasts using consecutive injections of oligomycin, FCCP and Rotenone with Antimycin A (AA). **(C)** Schematic representation of the mitochondrial respiration analysis in permeabilised cells. Digitonin was injected together with ADP and complex specific substrates. **(D)** OCR in permeabilised PCC deficient and control fibroblasts with complex I specific substrates, pyruvate and malate. **(E)** bar graph of complex I linked respiration in PCC deficient and control fibroblasts in the (mean ± SD, * indicates $p < 0.05$).

6.3.2. Exposure to propionate provokes a mitochondrial defect in control fibroblasts

During PA, propionate accumulates in plasma to concentrations as high as 5.4 mmol/L, a thousand fold higher than in healthy individuals (Hommes et al. 1968). To mimic this *in vitro*, we exposed control cells to long-term extracellular propionate. Control and patient fibroblasts were cultured in medium containing 4 mM of propionate for three weeks (**Figure 6.2A**). We previously showed that a state of metabolic PA significantly induces protein propionylation (Pougovkina et al. 2014b). Indeed, Protein propionylation was increased in patient as compared to control cells and after exposure to propionate, propionylation in both the patient and control cells was increased significantly (**Figure 6.2B**). Furthermore, profiling of mitochondrial respiration showed that exposure to propionate significantly reduced complex I-driven respiration in five fibroblast control cell lines (**Figure 6.2CD**). This suggests that long-term propionate exposure could contribute to mitochondrial pathophysiology in PCC deficient cells, possibly via aberrant protein propionylation.

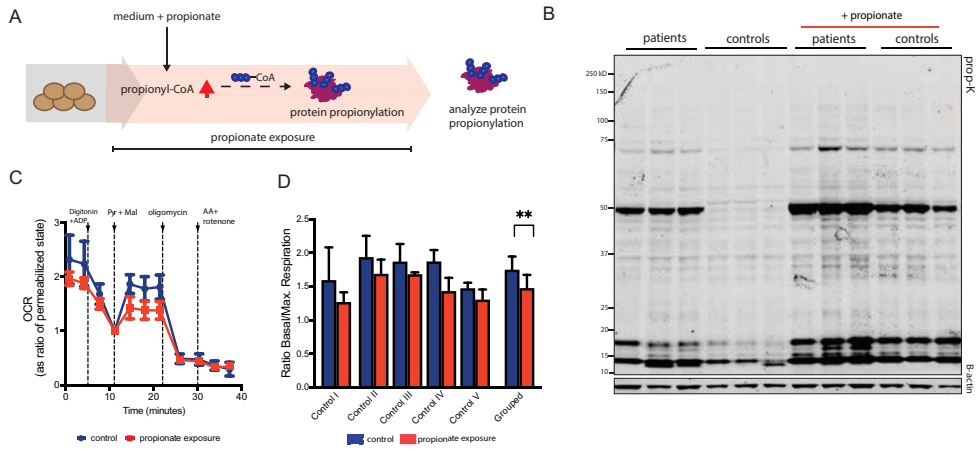

Figure 6.2 Inducing propionylation imitates mitochondrial respiratory defects observed in PCC deficient cells. (A) Schematic representation of the experimental set-up. **(B)** Anti-propionyllysine western blot analysis of three control and patient fibroblasts in control medium and exposed to 4 mM propionate for 3 weeks. **(C)** Representative measurement of mitochondrial respiration of control cell line exposed to propionate or control medium for 10 days. Medium was replaced with control medium day before analysis. Cells were permeabilised with digitonin and ADP, pyruvate and malate were added to induce complex I-driven respiration. Normalised OCR data is expressed as a ratio from basal respiration, determined as OCR after digitonin injection **(D)** Ratio basal to maximal complex I respiration for five control cell lines exposed to control or propionate medium (mean ± SD, ** indicates $p<0.01$).

6.3.3. Exposing Fao cells to propionate induces protein propionylation and causes decreased mitochondrial respiration

The liver is exposed to propionate that is produced and taken up from the colon. Levels in the portal vein can reach concentrations between 17 – 194 µmol/L (Cummings et al. 1987). To test whether we can induce protein propionylation in the liver cells, we exposed Fao rat hepatoma liver cells to 4 mM propionate for five days. We included one day of recovery in regular medium after the exposure, before harvesting the cells (**Figure 6.3**A), to ensure that propionyl-CoA and propionate levels in the cells were not elevated, and the effects of increased protein propionylation rather than increased propionate and/or propionyl-CoA levels would be analysed. Because propionylcarnitine profiles accurately reflect cellular propionyl-CoA levels (Wikoff et al. 2007) and our propionyl-CoA analysis was not sensitive enough to determine propionyl-CoA levels in cultured cells, we monitored propionylcarnitine levels. After 5-day propionate exposure, propionylcarnitine levels were increased. Notably, after one day of recovery, propionylcarnitine levels decreased and normalised to the same levels as in the cells that were not exposed to propionate (**Figure 6.3B**). This implies that one day of culturing on regular medium is sufficient to remove excess cellular propionate. Interestingly, one day recovery on propionate-free medium did not lower protein propionylation levels compared to cells exposed for five days without a recovery day (**Figure 6.3C**). Thus, our experimental set-up yields cells with increased protein propionylation and baseline propionyl-CoA levels and eliminates any confounding effects of propionate, or its derived metabolites, on inhibition of mitochondrial enzymes. Functionally, propionate exposure and increased propionylation was accompanied by a decrease in mitochondrial respiration in permeabilised Fao cells looking at complex I, complex II and complex IV driven respiration (**Figure 6.3DE**).

Liver perfused with 5 mM propionate show significant changes in other acyl-CoA species, such as succinyl-CoA (Wilson et al. 2017). Therefore, we tested whether exposure to propionate increases other protein acylations, such as acetylation or succinylation. We observed an increase in succinylation and acetylation in response to propionate exposure, yet this increase was limited to histone proteins (**Figure 6.3F**), Additionally, we measured acetylation and propionylation of histone 3 lysine 23 (H3K23) to verify if indeed these changes altered acylation on these specific sites. Propionylation and acetylation of H3K23 was increased after exposure to propionate (**Figure 6.3F**). This shows that exposure to propionate can alter other acylations in the cell, although these acylations are not similar in every cell compartment.

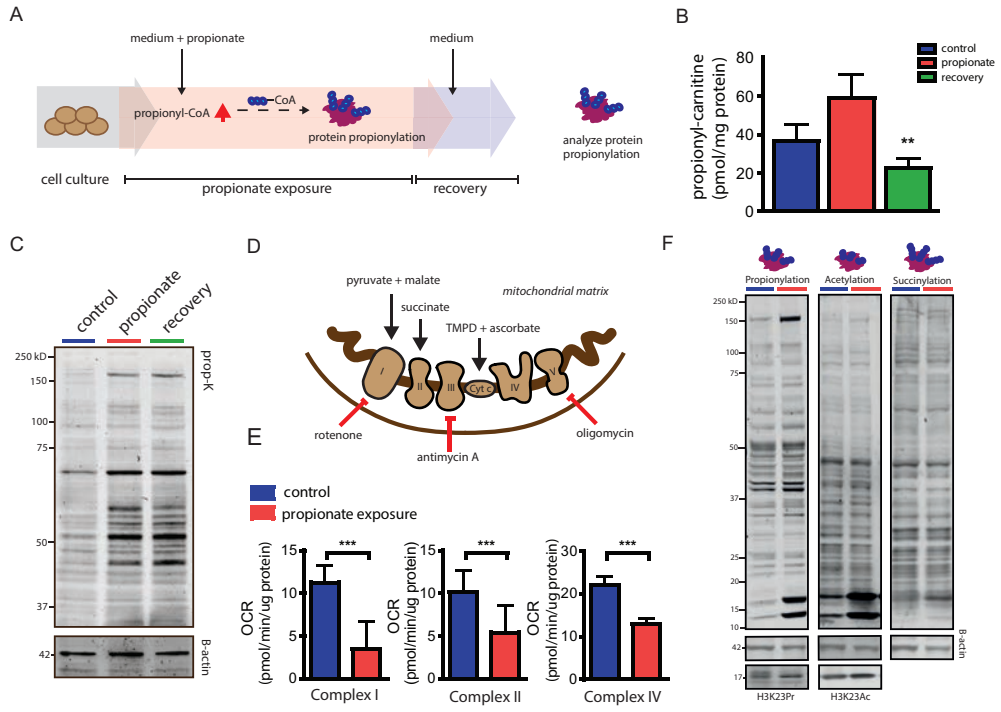

Figure 6.3 Exposure to propionate induces protein propionylation and decreases activity of mitochondrial respiratory complexes in Fao liver cells. (A) Schematic representation of the experimental set-up. Fao cells were exposed to 4 mM propionate for 5 days. This was followed by 1-day recovery step to ensure that propionate and propionyl-CoA levels would decline to normal in the cells. **(B)** Propionylcarnitine levels in the cells after propionate exposure and after the recovery. **(C)** Anti-propionyllysine western blot analysis of cell lysates cultured in regular medium, after propionate exposure and after recovery. **(D)** Schematic representation of mitochondrial complexes with specific substrates and inhibitors that were used. To measure the activity of respiratory complexes pyruvate and malate (complex I), succinate and rotenone (complex II), TMPD + ascorbate (complex IV) were injected together with digitonin and ADP in permeabilised control cells and cells exposed to propionate. **(E)** Quantification of complex respiration control cells and cells exposed to propionate. **(F)** Antipropionyl, -acetyl, -succinyl, histone 3 lysine 23 propionyl (H3K23Pr) and histone 3 lysine 23 acetyl (H3K23Ac) western blot analysis of cell lysates exposed to control or propionate medium for 7 days. (mean ± SD, ** indicates $p < 0.01$, *** indicates $p < 0.001$).

6.3.4. C2C12 myotubes exposed to propionate show increased propionylation, but do not display defective mitochondrial respiration.

Apart from neurological, haematological and hepatic complications, patients with PA may also display myopathic features, including hypotonia and exercise intolerance (Saudubray et al. 2016; Haijes et al. 2019). Therefore, we used a murine C2C12 muscle cell model combined with propionate exposure to test whether protein propionylation could contribute to these features. Interestingly, both C2C12 myoblasts and myotubes exposed for 3 or 5 days to propionate did not show defective mitochondrial respiration (**Figure 6.4AB**). Also, complex I (**Figure 6.4C**), Complex II (**Figure 6.4D**) and complex IV (**Figure 6.4E**) driven respiration were not reduced. Neither, when accounting basal respiration by calculating the ratio between basal over maximal respiration (**Figure 6.4F**). Myotubes exposed to propionate did show increased propionylation levels, but to a lesser extent than we observed in Fao cells. Moreover, a 24-hour recovery period reduced propionylation levels significantly (**Figure 6.4G**), indicating that propionate handling in muscle can be distinct from liver and fibroblasts. Indeed, propionate and propionyl-CoA handling genes were differently expressed between muscle and liver tissue, as was analysed using the Genotype-Tissue Expression (GTEx) portal (**Figure 6.4H**).

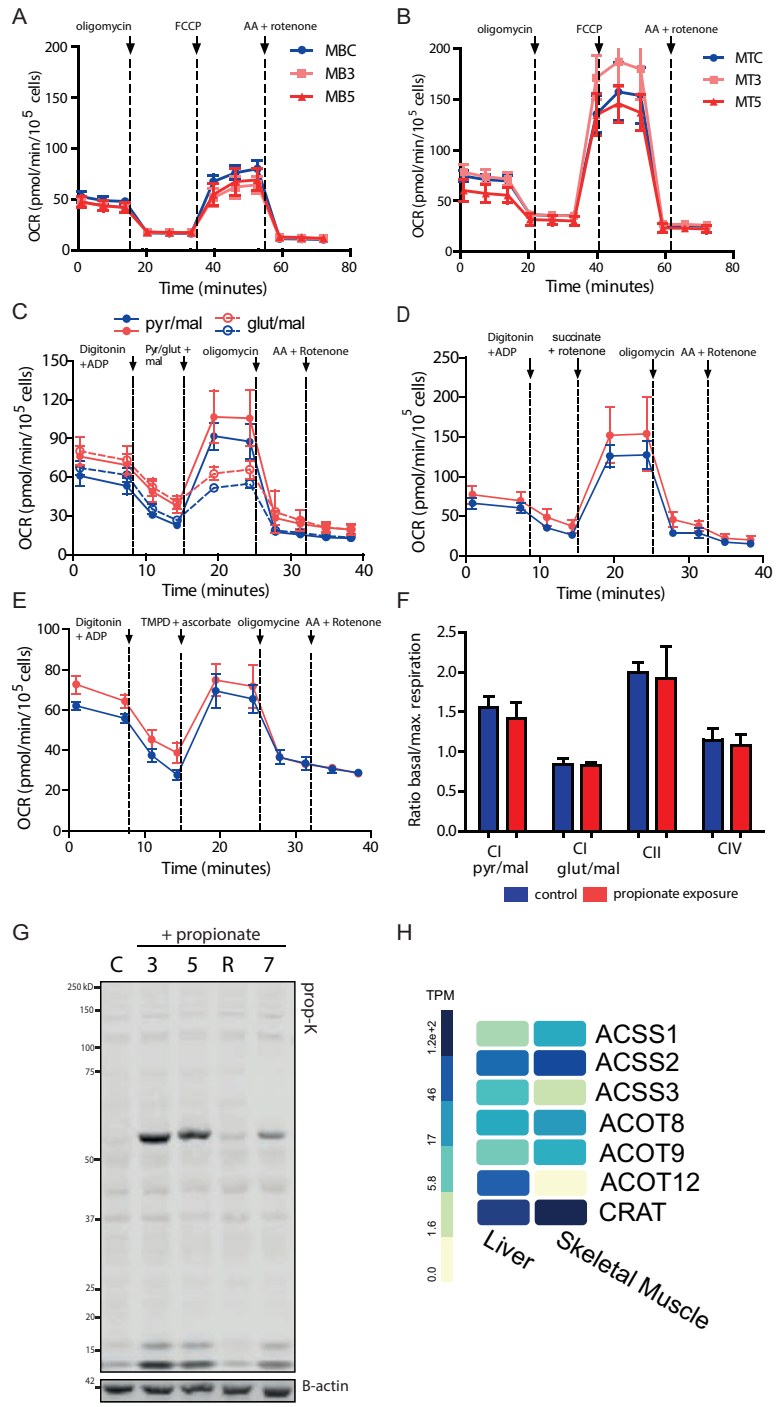

Figure 6.4 C2C12 muscle cells exposed to propionate show increased propionylation, but do not display defective mitochondrial respiration. Mitochondrial respiration in myoblasts **(A)** and myotubes **(B)** in control (MBC/MTC), after 3 days (MB3/MT3) and after 5 days of propionate exposure. Respiration was measured using consecutive injections of oligomycin, FCCP and Rotenone with Antimycin A (AA). Respiration of individual complexes was measured in permeabilised myoblasts after 5 day of propionate exposure and overnight exposure to control medium day before assay for complex I **(C)**, Complex II **(D)** and complex IV **(E)**. **(F)** Quantification of complex respiration control cells and cells exposed to propionate (mean ± SD). **(G)** Anti-propionyllysine western blot analysis of cell lysates cultured in regular medium and after 3, 5- and 7-day propionate exposure and after 1 day of recovery in regular medium. **(H)** Expression of selected genes in propionyl-CoA metabolism for skeletal muscle and liver. Expression data obtained from Genotype-Tissue Expression (GTEx) Portal (Lonsdale et al. 2013). Gene and transcript expression are shown in Transcripts Per Million (TPM). Acyl-CoA synthetase short chain family member 1 (*ASSC1*), acyl-CoA synthetase short chain family member 2 (*ASCC2*), acyl-CoA synthetase short chain family member 3 (*ASCC3*), acyl-CoA synthetase medium chain family member 1 (*ACSM1*), acyl-CoA synthetase medium chain family member 3 (*ACSM 3*), acyl-CoA thioesterase 8 (*ACOT8*), acyl-CoA thioesterase 9 (*ACOT9*), acyl-CoA thioesterase 12 (*ACOT12*), carnitine acetyltransferase (*CRAT*).

6.4. Discussion

The aim of this study was to assess the role of protein propionylation in the aetiology of PA and in cultured cells. We show that fibroblasts of PCC deficient patients displayed increased propionylation of the of proteins throughout the cell, including histones. PCC deficient fibroblasts as well as propionate-exposed control fibroblasts and Fao cells showed a decreased mitochondrial respiration. Since propionylation could impact protein function directly in the mitochondria or indirectly via histone modification, the observed mitochondrial dysfunction could be attributed to elevated protein propionylation in PCC deficient patient cells and could thus play a role in the pathology of PA. In contrast, propionate exposure in C2C12 myotubes did not affect respiration. C2C12 myotubes showed propionylation, although to a lesser extent. Furthermore, there was faster de-propionylation after propionate exposure was removed in C2C12 myotubes compared to Fao cells.

The observed differences in the effects of propionate exposure on mitochondrial respiration between our liver and muscle model may be explained by the intrinsic differences in propionate handling. The liver is exposed to higher physiological concentrations of

propionate compared to the rest of the body (Cummings et al. 1987). While in muscle, propionyl-CoA is not primarily derived from propionate, but instead comes mainly from the breakdown of amino acids (Davis et al. 1980). This is in line with differences in transcriptional regulation of enzymes involved in acyl-CoA metabolism in muscle and liver. The only known specific propionyl-CoA synthetase, acyl-CoA synthetase short chain family member 3 (*ACSS3*), was lower expressed in muscle than in liver, suggesting a higher activity to convert propionate into propionyl-CoA (Ellis et al. 2015; Yoshimura et al. 2016). Although ACSS3 is specific for propionate, at higher concentrations the mitochondrial acetyl-coenzyme A synthetase 2-like (*ACSS1*) also has some affinity for propionate (Fujino et al. 2001). On the other hand, the major mitochondrial propionyl-CoA degrading enzyme, acyl-CoA thioesterase 9 (*ACOT9*) (Tillander et al. 2014; Ellis et al. 2015), is higher expressed in muscle than in liver, whereas the cytosolic acyl-CoA thioesterase 12 (ACOT12) that primarily hydrolyses acetyl-CoA, but also has some activity towards propionyl-CoA (Prass et al. 1980), is expressed lower in muscle compared to in liver. Finally, carnitine acetyltransferase (*CRAT*) has the highest affinity for propionyl-CoA (Violante et al. 2013) and is higher expressed in muscle than in liver. This could indicate that the muscle is likely more capable of lowering mitochondrial propionyl-CoA levels by conjugation with carnitine and is possibly geared towards propionyl-CoA elimination. Indeed, Matsuishi et al showed that exposure of isolated liver mitochondria to 5 mM propionate increased the levels of propionyl-CoA, which concomitantly decreased the respiratory control ratio, whereas exposure of muscle mitochondria to propionate did not increase propionyl-CoA and did not affect respiration (Matsuishi et al. 1991). These differences in propionyl-CoA handling could explain that C2C12 myotube mitochondrial respiration is not affected by propionate exposure, while liver cell mitochondria are more sensitive to propionate exposure, possibly due increased propionyl-CoA levels and consequent aberrant propionylation.

De Keyzer et al (de Keyzer et al. 2009) showed multiple mitochondrial defects in muscle, liver and heart tissues from PA patients and Schwab et al (Schwab et al. 2006) observed mitochondrial defects in muscle biopsies of two young PA patients, showing a decreased

enzyme activity of all mitochondrial complexes. Since we did not observe propionylation-related defects in mitochondrial respiration in myotubes *in vitro*, the mitochondrial defects in PA patients *in vivo* could be unrelated to propionyl-CoA accumulation and propionylation in muscle mitochondria. Alternatively, it could be that the mitochondrial defects observed in PA patients could be secondary, due to, for example, hampered muscle innervation, leading to muscular hypotonia and physical inactivity (Brismar and Ozand 1994; Haijes et al. 2019).

Additionally, it must be noted that our liver and muscle PA model do not truly reflect the metabolic situation in PCC patients. Firstly, using the current model, propionyl-CoA does not build up inside the mitochondria specifically, as is the case in PCC patients. Secondly, exposure to propionate in an intact cell system will result in anaplerosis of the TCA cycle at succinyl-CoA (Wilson et al. 2017; Wang et al. 2018). Therefore, the consequent increase in downstream metabolites might exert its effects on respiration beyond the increase in protein propionylation. For example, exposure to propionate increases levels of methylcitrate, which can inhibit TCA enzymes such as isocitrate dehydrogenase, possibly affecting respiration measurements (Cheema-Dhadli et al. 1975). Yet, after infusion of rat liver with 5 mM propionate, levels of methylcitrate did not reach levels close to the inhibition constant, possibly minimising the inhibitory role of this metabolite on cellular respiration (Wilson et al. 2017). Other metabolites that are known to accumulate in PA patients, such as 3-hydroxypropionic acid, have been shown to directly cause respiratory defects in isolated heart mitochondria (Roginski et al. 2020). Therefore, although it is currently unknown if these metabolites also accumulate in the current PA model used in liver and muscle, these metabolites could have directly influenced respiration in our models.

Another effect of exposure to propionate is the depletion of free cellular CoA due to the formation of propionyl-CoA and methylmalonyl-CoA, also known as CoA trapping (Wilson et al. 2017). Incubation with propionate and carnitine increased free cellular CoA and propionylcarnitine, resulting in less accumulation of propionyl-CoA in heart (Wang et al.

2018) and partially reversed mitochondrial defect in liver (Matsuishi et al. 1991). Less accumulation of propionyl-CoA increases the cellular CoA pool but at the same time could likewise result in lower propionylation of the proteome. Nevertheless, in order to minimise the direct effects CoA trapping and inhibitory effects of products of propionate metabolism on respiration, we incorporated a washout period in our experimental set-up. This washout allowed propionylcarnitine levels, as a proxy for propionyl-CoA, to return to control values, while propionylation remained elevated, presumably allowing for the measurement of respiration with little interferences of these metabolites and CoA trapping.

The PCC enzyme is a mitochondrial enzyme and therefore propionyl-CoA was expected to primarily effect the mitochondrial proteome. However, we show that propionylation in fibroblasts of PCC patients also occurs outside of the mitochondria, since we see increased propionylation on histone proteins. This is accordance with an *in vivo* pcc-/- mouse model, that showed increased histone propionylation (Kebede et al. 2017). Moreover, in Fao cells increased histone propionylation, as well as increase in histone acetylation was observed when exposed to extracellular propionate. Histone proteins can be subjected to various acyl-modifications, hereby distinctly regulating chromatin structure and transcription (Sabari et al. 2016). This could possibly contribute to the pathology of PCC patients and to the phenotype observed in our PA models. Yet, increased histone propionylation does not seem to cause respiratory defects *per se*, as histone propionylation was increased in myotubes in absence of decreased respiration. Nonetheless, the effect of propionylation on histone proteins is not yet fully understood. Acetyltransferases, previously identified to primarily acetylate histones, also show propionylation activity (Kaczmarska et al. 2017; Han et al. 2018) and histone acetylation and propionylation appear to be functionally similar as both marks are associated with transcriptional activation (Kebede et al. 2017). However, whether histone propionylation is merely an additional transcriptional activator or if there are physiological and pathological situations, such as in PA, in which histone propionylation may have distinct regulatory roles on gene expression is yet to be explored.

We show that increased global protein propionylation and specific histone protein propionylation in liver might contribute to the respiratory defects observed in PA and therefore therapies that aim to reduce or reverse propionylation could be beneficial in the treatment of PA. Liver complications, including hepatomegaly and hyperechoic liver, belong to the most common complications in PA (Haijes et al. 2019) and mitochondrial defects are thought to contribute to these pathologies, at least in part. In current treatment strategies, PA patients may benefit from liver transplantation, which may even reverse frequent complications, such as cardiomyopathy (Romano et al. 2010). However, the transplantation is associated with high mortality due to complications (Charbit-Henrion et al. 2015). Other treatment strategies include: carnitine supplementation to decrease intracellular propionyl-CoA levels (Roe et al. 1984; Matsuishi et al. 1991), restriction of dietary factors that end up as propionyl-CoA, such as propiogenic amino acids, and the use of an antibiotics to reduce propionate production by gut bacteria. Despite these strategies, the outcome for PCC patients remains poor with significant clinical impairment, such as delayed mental development and episodes of acute metabolic decompensations (De Baulny et al. 2005; Touati et al. 2006; Grünert et al. 2012).

One explanation for the poor outcome could be that propionyl-CoA levels are still elevated or elevate acutely due to the endogenous production of propionyl-CoA (Sbaï et al. 1994). Therefore, it is of interest to explore alternative strategies that might alleviate the underlying causes that could contribute to the liver pathology, such as aberrant protein propionylation. However, targeting the enzymatic propionylation by acyltransferases would possibly be an ineffective strategy, as much of the propionylation is likely non-enzymatic (Pougovkina et al. 2014b). Hence, to decrease aberrant propionylation we propose to increase de-propionylation activity in PA patients. Although it is not clear which enzymes regulate de-propionylation, possibly sirtuins 1-3 hold some de-propionylation activity, at least *in vitro* (Smith and Denu 2007; Feldman et al. 2013). In particular activation of sirtuin 3 (SIRT3) could be an interesting strategy due to its cellular localisation within the mitochondria and multiple metabolic targets, including mitochondrial complexes (van de

Ven et al. 2017). SIRT1 could be of interest as a potential strategy to remove aberrant propionylation on histone proteins, as this enzyme has been shown to de-propionylate proteins, at least *in vitro* (Cheng et al. 2009). Therefore, efforts are required to identify how proteins are de-propionylated to alleviate aberrant propionylation and its consequences. These efforts could help to improve the treatment of PCC patients and hereby improve the health outcome and quality of life.

Acknowledgements We would like to acknowledge Laura de Smalen for her help in data collection and the Gaslini Biobank in Italy for providing the propionyl-CoA carboxylase deficient fibroblasts.

Author contributions BL, OP, AFB, HB performed experiments, principal data analysis and reporting of results. BL, OP, AFB, HB, RJAW, AGN, JK, VCJB: planning and conduct of the project, conception and design of research, data analysis and interpretation. All authors edited, revised and approved the final version of the manuscript.

CHAPTER 7

Propionate hampers differentiation and modifies histone propionylation and acetylation in skeletal muscle cells

Bart Lagerwaard
Joris Hoeks
Lotte Grevendonk
Arie G. Nieuwenhuizen
Jaap Keijer
Vincent C. J. de Boer

Abstract

Objective
Protein acylation via metabolic acyl-CoA intermediates provides a link between cellular metabolism and protein functionality. For example, a process in which acetyl-CoA and acetylation are fine-tuned is during myogenic differentiation. However, the roles of other protein acylations in this process remain unknown. Protein propionylation, the covalent binding of the three carbon propionyl-group to lysine residues of proteins, could be functionally relevant because propionyl-CoA can be derived from the catabolism of amino acids and fatty acids and protein propionylation has been shown to decrease during muscle differentiation. Therefore, the aim of this paper was to explore the potential role of this post-translational modification in muscle differentiation.

Methods
The effect of increased propionylation will be studied on cultured C2C12 and primary human myotubes by mimicking a pathophysiological situation with high extracellular propionate which was observed to increase levels of propionyl-CoA and protein propionylation, rendering it a model to study increased protein propionylation in the cell.

Results
We show that exposure to extracellular propionate, but not acetate, impairs myogenic differentiation in C2C12 myotubes and propionate exposure impairs myogenic differentiation primary human myotubes. We show that the effect of propionate exposure on differentiation is accompanied by an increase in histone propionylation as well as histone acetylation. Furthermore, we use chromatin immunoprecipitation to show increased histone propionylation at specific regulatory myogenic differentiation sites of the Myod gene.

Conclusion
Combined, our findings suggest there is a role for propionylation and propionyl-CoA in regulation of muscle cell differentiation, possibly via alterations in histone acylation

7.1. Introduction

Post translational modifications of proteins by intermediates of metabolism offers the cell a rapid and integrated mechanism to respond to changes in nutrient availability and adjust consequent cellular decisions. Acyl-CoAs are metabolic intermediates that have an acyl-group that can react with lysine residues on target proteins resulting in protein acylation, thereby regulating protein functionality (Choudhary et al. 2014). For example, acetyl-CoA is a central metabolite in glucose, fatty acid and amino acid metabolism, and levels of this metabolite drive protein acetylation (Pougovkina et al. 2014a). Acetylation of histone proteins serves an important cause as it regulates gene transcription by remodelling of chromatin structure (Sabari et al. 2016). In this way, protein acetylation functions as a metabolic sensor, as it was shown that acetyl-CoA levels are directly linked to acetylation of histone proteins associated with cell growth and proliferation genes (Wellen et al. 2009; Cai et al. 2011).

A process in which acetyl-CoA and acetylation are fine-tuned is during myogenic differentiation. For example, the master regulator of myogenic differentiation, myoblast determination protein 1 (MYOD1 or MYOD), is present in an inactive form in proliferating myoblasts, but following the right cues for differentiation, expression of MYOD is enhanced through acetylation of enhancer regions on the MYOD loci (Hamed et al. 2013). Depleting acetyl-CoA as substrate for acetylation by silencing of the ATP-citrate lyase (ACL) enzyme, resulted in a failed response to upregulate MYOD expression and impaired myogenic differentiation, demonstrating the central role of acetylation in this process (Das et al. 2017).

Similar to histone acetylation, histone propionylation may play an important role in differentiation, as propionylation levels have been reported to decrease following monocytic and myogenic differentiation (Liu et al. 2009; Simithy et al. 2017). Propionylation is the covalent binding of a three carbon propionyl-group to lysine residues of proteins and

propionyl-CoA, the substrate for propionylation, can be derived from the breakdown of cholesterol, odd-chain fatty acids and the amino acids isoleucine, valine, threonine and methionine (Sbaï et al. 1994). Due to the role of propionyl-CoA in metabolism and because propionylation is likely driven by levels of propionyl-CoA, situations in which propionyl-CoA levels are altered, protein propionylation could be functionally important, for example during protein breakdown (Trefely et al. 2020).

Therefore, the aim of this paper was to explore the potential role of this post-translational modification in muscle differentiation. To study this, we used a pathophysiological metabolic situation similar to patients with an inborn defect in the propionyl-CoA carboxylase gene (*PCC*). This defect leads to an accumulation of propionyl-CoA and associated metabolites in plasma, urine and other body fluids and tissues, a condition known as propionic acidaemia. Mimicking this pathophysiological situation by exposing the muscle cells to a similar extracellular concentration of propionate (Hommes et al. 1968), increases levels of propionyl-CoA and protein propionylation, rendering it a model to study increased protein propionylation in the cell (Pougovkina et al. 2014b; Lagerwaard et al. 2020b). PCC patients, apart from neurological, haematological and hepatic complications, can display myopathic features, including hypotonia and exercise intolerance (Saudubray et al. 2016; Haijes et al. 2019), further suggesting a possible role for propionylation in the skeletal muscle. Therefore, we hypothesised that increased propionylation will compromise normal cellular function. Here, we show that extracellular propionate exposure and consequent increase in propionylation hampers myogenic differentiation in C2C12 myotubes and primary human muscle cells, possibly due to perturbations in histone propionylation as well as acetylation. These findings contribute to the understanding of protein propionylation and its link with propionyl-CoA levels in muscle development.

7.2. Material and Methods

7.2.1. Cell culture

Murine C2C12 myoblasts were routinely cultured at 37 °C with 5% CO_2 in high glucose Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 2 mM glutamine, 25 mM HEPES and 1% (v/v/v) Penicillin/Streptomycin/Amphotericin B. Differentiation was induced by replacing culture medium with high glucose DMEM supplemented with 2% (v/v) horse serum, 2 mM glutamine, 25 mM HEPES and 1% (v/v/v) Penicillin/Streptomycin/Amphotericin B when culture was 90-100% confluent. Medium was replaced every other day for 5-7 days. Propionate and acetate exposure were induced in culture medium containing 4 mM propionic or acetic acid from a 400 mM pH-balanced stock solution. Medium pH remained within the normal range for culture medium (pH 7.3 – 7.5)

7.2.2. Muscle biopsy collection and primary cell culture

A muscle biopsy was taken from the m. *vastus lateralis* under local anesthesia (1.0% lidocaine without epinephrine) according to the Bergström method modified with suction (Bergström et al. 1967). Human myoblasts were isolated from the *vastus lateralis* and satellite cells were selected with a CD56 monoclonal antibody (5.1H11; Developmental Studies Hybridoma Bank, Iowa City, USA) using magnetic cell sorting. Satellite cells were grown to 90% confluency and then initiated to differentiate according to previously published methods (Sparks et al. 2011). Myoblasts were grown in low glucose DMEM containing 0.5 mg/mL bovine serum albumin (BSA), 1 µM dexamethasone, 10% (v/v) FBS, 1% (v/v) fetuin, 1% (v/v/v) Penicillin/Streptomycin/Amphotericin B, 50 ug/mL gentamycin and 10 ng/mL Human epidermal growth factor and switched to Minimum Essential Medium (α-MEM) differentiation medium upon 90% confluency containing 2 % (v/v) FBS, 1% (v/v) fetuin and 2 % (v/v) pen/strep for 5 days, changed every other day.

7.2.3. SDS –PAGE and Western blotting

Cells were harvested and lysed in TRIS-HCL pH 7.4 with 1% triton X-100 containing protease inhibitors and deacylase inhibitors (1 µM trichostatin A and 20 mM nicotinamide). Lysates were sonicated 5 times 2s at 40 kHz amplitude on ice. Protein concentrations were determined using Pierce BCA protein assay kit (Thermofisher) and equal protein amounts were loaded on NuPAGE 4-12% gels (Invitrogen), transferred to nitrocellulose membrane in a transfer tank filled with transfer buffer containing 10% (v/v) methanol. Membrane was blocked in 3% BSA in PBS with 0.1% Tween-20 at room temperature and incubated overnight with antibodies in the same buffer at 4ºC. Primary antibodies used: β-actin (#ab8227, 1:5000, Abcam), propionyllysine (#201, 1:1000, PTM biolabs), histone 3 propionyllysine 23 (#613987, 1:2000, Active Motif), histone 3 acetyllysine 23 (#07-355, 1:2000, Millipore), histone 3 acetyllysine 9 (#ab4441, 1:1000, Abcam), histone 4 acetyllysine 8 (#2584, 1:1000, Cell Signalling), murine myosin heavy chain (MF-20, 1:200, DSHB), human myosin heavy chain (ab91506, 1:1000, Abcam). IR-dye based secondary antibodies (LICOR) were used to detect antibody signals using Odyssey scanner (LICOR)

7.2.4. Immunohistochemistry

C2C12 cells were seeded on glass coverslips. Coverslips were harvested, fixed in 4% formaldehyde and permeabilised with 0.5% triton. Slips were incubated in blocking buffer Phosphate buffered saline (PBS) with 2%, BSA, 0.02% HS, 0.1% Triton X-100, 0.05% Tween-20, 100 mM glycine), incubated for 1hr at RT in anti-myosin heavy chain (MHC; Developmental Studies Hybridoma Bank MF-20, 1:200). Coverslips were washed with 0,05% tween-20 in PBS and incubated with secondary anti-body used was Alexa-488 H+L IgG anti-mouse. Nuclear staining was performed by incubating coverslips in a 1 µg/ml 4′,6-diamidino-2-phenylindole (DAPI) staining solution for 5 min. Coverslips were washed in PBS, followed by a PBS:Milli-Q (1:1) wash. Fluormount-G (Southern Biotech) was used to mount coverslips onto glass microscope slides. Representative pictures were taken using a Leica DM6B microscope at 5x magnification

7.2.5. RNA extraction and Semi-Quantitative Real-Time Polymerase Chain Reaction (qPCR)

Cells were washed with 2 mL cold Hanks' Balanced Salt Solution (HBSS) and then directly scraped in 350 µl RLT buffer with 3.5 µl β-mercaptoethanol (β-ME). The cell lysates were homogenised by passing through 21-gauge needle (0.8 mm). RNA was isolated Rneasy Mini Kit (Qiagen, German) according to manufacturer's protocol. The quantity and quality of purified RNA was examined by NanoDrop spectrophotometer (ND-1000). cDNA was synthesised in Eppendorf-Master cycler (5' 25°C, 30' 42°C, 5' 85°C, 10 °C ∞) with ISCRIPT cDNA synthesis kit (Bio-Rad). Measurements of qPCR quantified with CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad) and SYBR green master mix (BioRad, USA). The cycling program was set as 95℃ for 30s, 60℃ for 30s in 40 cycles. Primers were designed using NCBI primer blast; an overview of primer sequences can be found in **Table 7.1**. Normalised expression was calculated according to the ΔΔCq method, by making use of geometric averaging of multiple reference genes using CFX maestro software (Bio-rad). Two housekeeping genes, ribosomal protein S12 (*rps12*) and ribosomal protein S15 (*rps15*) were used as reference genes

Table 7.1 Primer sequences

Gene	Accession number	Forward primer sequence*	Reverse primer sequence*
Myh1	NM_030679.1	TCCCTAAAGGCAGGCTCTCTC	AAGGCTTGTTCTGAGCCTCG
Myf5	NM_008656.5	TGACGGCATGCCTGAATGTA	GCTCGGATGGCTCTGTAGAC
Myod	NM_010866.2	TGCTCTGATGGCATGATGGATT	AGATGCGCTCCACTATGCTG
Myog	NM_031189.2	TCCCAACCCAGGAGATCATTTG	TCAGTTGGGCATGGTTTCGT
Rps12	NM_011295.6	AAGGCATAGCTGCTGGAGGTGTAA	AGTTGGATGCGAGCACACACAGAT
Rps15	NM_009091	CAACGGCAAGACCTTCAACC	TGCTTCACGGGTTTGTAGGT
Myod_CER	NC_000073.6	GGGCATTTATGGGTCTTCCT	CTCATGCCTGGTGTTTAGGG
Myod_DRR	NC_000073.6	TCAGGACCAGGACCATGTCT	CTGGACCTGTGGCCTCTTAC
Myod_PRR	NC_000073.6	GAGTAGACACTGGAGAGGCTTGG	GAAAGCAGTCGTGTCCTGGG
Myod_CD1	NC_000073.6	CATCTGACACTGGAGTCGCTTTG	CAAGCAACACTCCTTGTCATCAC
Myod_−15 Kb	Non-coding region	TGCCCAGAGCCTAGAATCAT	TCATGCATCCTTGCTGGATA
Rpl30	#7014 Cell signalling		

** from 5' to 3', bp = fragment length*

7.2.6. Chromatin Immunoprecipitation

C2C12 were seeded in 15 cm dishes, two dishes of cells were combined for each condition in one batch. Cells were harvested at day 0, 1 and 5 after differentiation. ChIP assay was performed according to manufacturer's instructions from SimpleChIP Plus Enzymatic Chromatin IP kit Magnetic Beads (#9005, Cell Signalling). In short, cells were crosslinked by adding 540 µl of 37% formaldehyde to each dish with 20 mL medium (final concentration 1%) for 10 min at RT with constant agitation. Crosslinking was stopped by adding 2 mL 2.5 M glycine and incubation for 5 min at RT. Cells were washed twice with ice-cold PBS. After that, the cells were scraped in 10 mL ice-cold PBS + 1 tablet EDTA-free Protease Inhibitor Cocktail (Roche, Switzerland) and transferred into 15 mL conical tubes. The cell pellets were collected by centrifuging at 2,000 g for 5 min at 4℃ and chromatin was isolated and digested by adding 0.5 µl micrococcal nuclease (#10011, Cell Signalling) per sample and incubated for 20 min at 37℃ to digest the chromatin to lengths between 150bp-400bp. Nuclei were pelleted and suspended in ChIP buffer and sonicated 3 x 30s on ice with 1 min breaks. Chromatin digestion and shearing conditions were optimised and verified in on gel. DNA was purified using SimpleChIP DNA purification and spin columns (#14209, Cell Signalling) according to manufacturer's protocol and was quantified using NanoDrop spectrophotometer (ND-1000). 10 µg chromatin and 2 µl target antibody or 2 µl normal rabbit antibody IgG (# 2729, Cell Signalling Technology, USA) were incubated at 4℃ for 4 hours with rotation. Before incubation input samples were collected and stored at -20℃ for later use. 30 µl of protein G magnetic beads (#9006, Cell Signalling) were added to each chromatin-antibody solution and incubated for 2 hours at 4℃ with rotation. Antibody-bead-chromatin complexes were washed 3 times with low salt and 1 time with a high salt wash (#9005, Cell Signalling). Chromatin was eluded from antibody-beads for 30 minutes at 65℃ with agitation in 150 µl elution buffer (#9005, Cell Signalling) and antibody-beads were removed. Immunoprecipitated chromatin and input chromatin were reverse cross-linked by adding 6 µl 5 M NaCl and 2 µl 20 mg/ml Proteinase K and incubated overnight at 65℃. DNA was purified using spin columns and quantified using qPCR with SYBR green supermix (Bio-Rad) using Myod locus primers (Yang et al. 2011)(**Table 7.1**). The results are shown as percentage of total input, or enrichment, which calculated by:

$$Percent\ Input\ =\ 2\% \bullet 2^{(C[T]\ 2\%Input\ Sample\ -\ C[T]\ IP\ Sample)}$$

where C[T] is the threshold cycle of the qPCR reaction.

7.2.7. Data analysis and statistical testing

Data are presented as mean ± SD. Statistical analyses were performed using GraphPad Prism v.5 (GraphPad Software, CA, USA). Means were compared using one-way ANOVA. Post-hoc testing was performed using least significant differences (LSD) or Tukey's test for more than 2 treatments. Significance was accepted at $p < 0.05$.

7.3. Results

7.3.1. Exposure to propionate, but not acetate impairs C2C12 myoblast differentiation

To analyse whether propionyl-CoA-derived protein propionylation could play a role in muscle physiology, we first exposed C2C12 myoblasts to 4 mM extracellular propionate, an exposure that is comparable to patients in propionic acidaemia, which was previously shown to induce intracellular protein propionylation (Hommes et al. 1968; Lagerwaard et al. 2020b). Indeed, addition of propionate to growth medium increased levels of protein and histone propionylation in C2C12 cells after 5 days exposure (**Figure 7.1A**). However, protein propionylation levels did not significantly increase globally, yet specific increases in protein propionylation were observed for a protein band at ± 50 kDa and for the histone bands at 17 and 14 kDa. This indicated that extracellular propionate exposure is able to induce specific intracellular lysine propionylation.

Next, myoblasts were grown to confluence and differentiation was induced by replacement of culture medium by differentiation medium, either containing 4 mM propionate, 4 mM acetate or control medium (**Figure 7.1B**). An equimolar concentrations acetate was used as a control for, for example, addition of extra substrate and changes in free CoA levels. Moreover, it was previously shown that global histone acetylation decrease during differentiation (Yucel et al. 2019), just as global levels of histone propionylation (Simithy et al. 2017). Immunofluorescence analysis of skeletal myosin heavy chain (MHC), a protein marker exclusively expressed in differentiated myotubes, demonstrated that MHC signals were almost absent in the propionate condition compared to the control and the acetate condition (**Figure 7.1C**), indicating that propionate, but not acetate, inhibited differentiation of myoblasts into myotubes.

We determined the expression of four myogenic regulatory factors using real-time PCR at four different time points after initiation of differentiation, namely day 1, 3, 5 and 7. Myogenic factor 5 (*Myf5*), a marker for proliferating myoblasts which is expected to decrease following the commitment to differentiation (Tomczak et al. 2004), was decreased upon differentiation in control and acetate conditions, but did not decrease in propionate conditions (**Figure 7.1D**). *Myod* expression is increased upon differentiation in all conditions but did not further increase in propionate conditions compared to control and acetate conditions (**Figure 7.1E**). MYOD binds to regulatory sites on myogenin, increasing its expression (Deato et al. 2008). Expression of myogenin increased significantly in all conditions following the induction of differentiation, yet the increase in the propionate condition is significantly lower than in the control and the acetate condition (**Figure 7.1F**). Myogenin and Myod work in synergy, inducing transcription of terminal differentiation genes, such as such as *Myh1* (Cao et al. 2006). Expression of *Myh1* was significantly increased upon differentiation in control and acetate conditions, yet in the propionate condition *Myh1* expression was not upregulated (**Figure 7.1G**). Together, these data confirm that exposure to extracellular propionate hampers normal temporal expression of genes responsible for differentiation in a C2C12 muscle model.

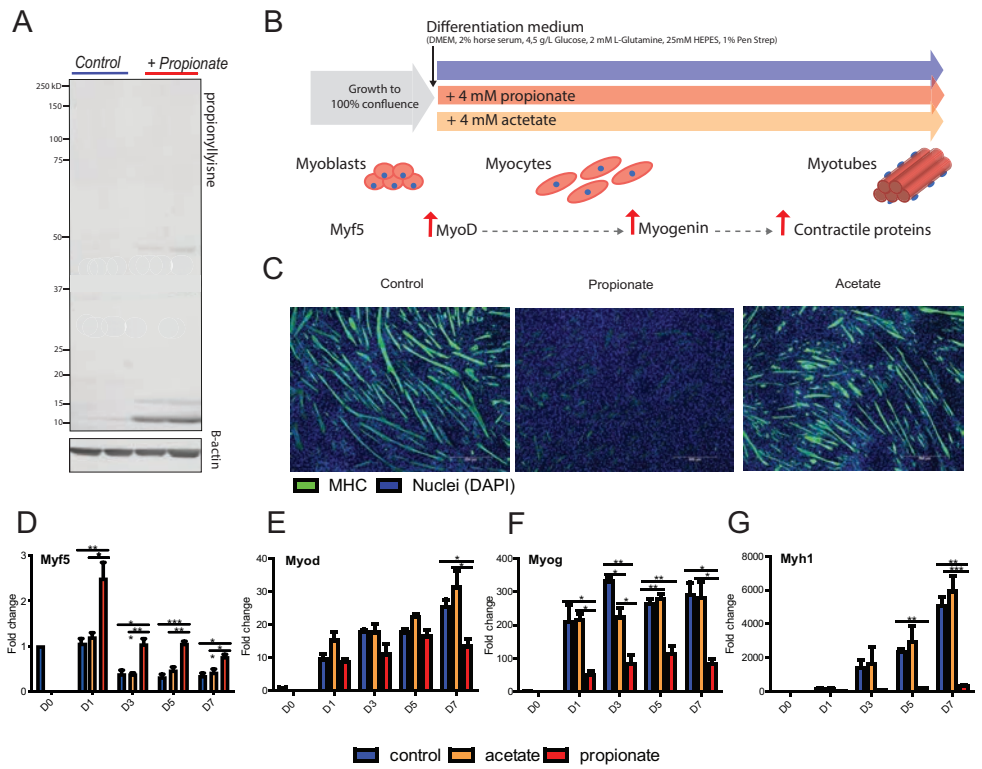

Figure 7.1 Exposure to propionate, but not acetate, impairs C2C12 myoblast differentiation **(A)** Western blot analysis of propionyllysine in C2C12 myoblasts exposed to control medium or medium with 4 mM propionate **(B)** Schematic representation of experimental set up. myoblasts were grown to confluence and differentiation medium was added with +4 mM propionate or Acetate **(C)** Immufluoresence staining after 7 days of differentiation of control, acetate and propionate exposed cells of myosin heavy chain (MHC). Expression of genes involved in myogenic differentiation measured using *q*PCR for Myf5 **(D)**, Myod **(E)**, Myog **(F)**, and Myh1 **(G)** (mean ± SD, * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$).

7.3.2. Exposure to propionate causes aberrant acylation patterns on histone proteins

To further elucidate how propionylation impacts C2C12 differentiation, we analysed the effect of propionate exposure on histones protein acylation during differentiation. Since histone propionylation can dynamically interact with histone acetylation, and we have previously shown that histone acetylation increases in rat Fao liver cells exposed to

propionate (Lagerwaard et al. 2020b). We hypothesised that exposure to propionate would affect acylation levels on histone proteins. To test this, we differentiated C2C12 myoblasts in either control medium or in medium containing 4 mM propionate or 4 mM acetate and determined histone protein acylation using Western blot. In control medium, global histone propionylation and propionylation of histone 3 lysine 23 (H3K23) decreased during myogenic differentiation. On the contrary, in the propionate condition, both global and specific H3K23 propionylation were increased as compared to control (**Figure 7.2A**). Protein propionylation in acetate medium was unaltered and followed the same temporal pattern as in control medium. Indeed, exposure to propionate increased global histone acetylation compared to control, while no clear differences in acetylation of H3K23 were observed (**Figure 7.2A**). This showed that besides an increase in protein propionylation, levels of protein acetylation were also affected in muscle by propionate exposure.

Histone acetyl transferases (HATs) use mainly acetyl-CoA as substrate, but can also use propionyl-CoA as a substrate, albeit to a lesser extent (Kaczmarska et al. 2017; Kebede et al. 2017). Therefore, we explored if our model primarily used enzymatic-driven propionylation or whether the increased propionylation primarily has a non-enzymatic origin. For this, we induced differentiation in C2C12 myoblasts in control medium or medium containing either only propionate, only 10 µM C646, a selective HAT P300 inhibitor (Bowers et al. 2010), or both propionate and C646. Indeed, exposure to C646 decreased global histone propionylation and acetylation (**Figure 7.2B**). Additionally, C646 decreased overall myogenic differentiation, as was shown by the lower expression of MYH1. Acylation of histones using P300 is thus necessary for differentiation. Exposure to both propionate and C646 increased histone propionylation and acetylation, yet this increase was lower than with propionate alone (**Figure 7.2B**). This implies indeed that P300 enzymatic activity was, at least partly, used for propionylation of histones, and that the remaining propionylation happens either non-enzymatically or uses other HATs, such as CBP (Chen et al. 2007), PCAF (Leemhuis et al. 2008) or MOF (Han et al. 2018).

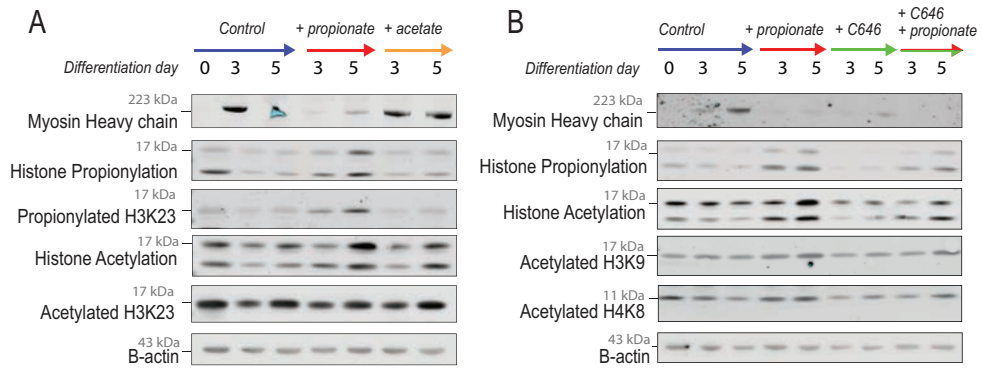

Figure 7.2 Exposure to propionate causes aberrant acylation pattern on histone proteins (A) Western blot analysis of myosin heavy chain, histone propionyllysine (pan-propionyllysine antibody), propionylated histone lysine 23 (H3K23), histone acetylation (pan-acetyllysine antibody) and acetylated histone 3 lysine (H3K23) in C2C12 myoblasts exposed to control medium or medium with 4 mM propionate or acetate on 3 and 5 days after initiation of differentiation. **(B)** Western blot analysis of myosin heavy chain, histone propionyllysine, histone acetylation, acetylated histone 3 lysine 9 (H3K9) and acetylated histone 4 lysine 8 (H4K9) in C2C12 myoblasts exposed to control medium or medium with 4 mM propionate with or without P300 inhibitor C646 on 3 and 5 days after initiation of differentiation.

7.3.3. Propionate exposure increases propionylation and acetylation on regulatory promotor regions of MYOD

We showed that exposure to propionate failed to increase expression of the MYOD target myogenin, suggesting that either MYOD transcription or MYOD activation was impaired. Transcription of MYOD is regulated by three regulatory regions that enhance its transcription, namely a core enhancer region (CER) (Goldhamer et al. 1995), a distal regulatory region (DRR) and a proximal regulatory region (PRR) (Tapscott et al. 1992; Chen et al. 2001, 2002). Early differentiation is associated with recruitment of the HAT P300 to these enhancer regions, with subsequent acetylation and consequential increased expression (Hamed et al. 2013). Therefore, we hypothesised that increased propionylation of MYOD regulatory regions could interfere with normal temporal acylation patterns and consequent expression of MYOD, hereby dysregulating differentiation. In order to test if these regions were indeed propionylated, we performed chromatin immunoprecipitation

using an antibody against propionylated H3K23 and quantified propionylation in C2C12 myoblasts exposed to either control or propionate differentiation medium on two different time points during differentiation on three MYOD regulatory sites. As controls, we additionally quantified two sites that have not been identified as being regulated for MYOD activation, namely one site 15Kb upstream of the transcription initiation site and one site within the coding region of the Myod gene (**Figure 7.3A**).

Propionate exposure significantly increased or had the tendency to increase ($p < 0.1$) propionylation on all sites on both days, expect for the CER after 1 day of differentiation (**Figure 7.3B**). This confirmed that there were indeed increased, albeit low, absolute levels of propionylation of H3K23 on MYOD regulatory sites in the propionate conditions. Still, this increase did not seem to be limited to regulatory sites as it also occurred on the selected non-regulatory MYOD sites. To substantiate this hypothesis, we measured H3K23 propionylation on a gene unrelated to myogenic differentiation, ribosomal protein L30 (Rpl30), on day five of differentiation. In three independent experiments, propionylation was on average 7-fold higher (0.023 ± 0.023 vs. 0.16 ± 0.13 % enrichment of input, $p = 0.134$, data not shown), suggesting that indeed propionylation seems to occur on various loci and is not limited to regulatory regions of MYOD.

Acetylation on MYOD regulatory regions is important for early initiation of differentiation and MYOD expression (Hamed et al. 2013). Moreover, we showed that propionylation uses part of the acetylation machinery and that propionylation was increased on regulatory regions of Myod. Hence, we hypothesised that the increase in histone propionylation could be at the expense of acetylation, hereby dysregulating normal transcriptional activation patterns. We therefore quantified levels of H3K9 histone acetylation on the same regulatory sites and non-regulatory sites, as this acylation mark was shown to increase on MYOD regulatory regions during early differentiation (Yang et al. 2011; Hamed et al. 2013). As was previously observed, levels of H3K9 acetylation increased at the start of differentiation in all regulatory regions. Unexpectedly, propionate exposure augmented this increase in H3K9

acetylation on the regulatory sites, as compared to control (**Figure 7.3C**). Rather than substituting of propionylation for acetylation, propionylation enhanced acetylation on these sites.

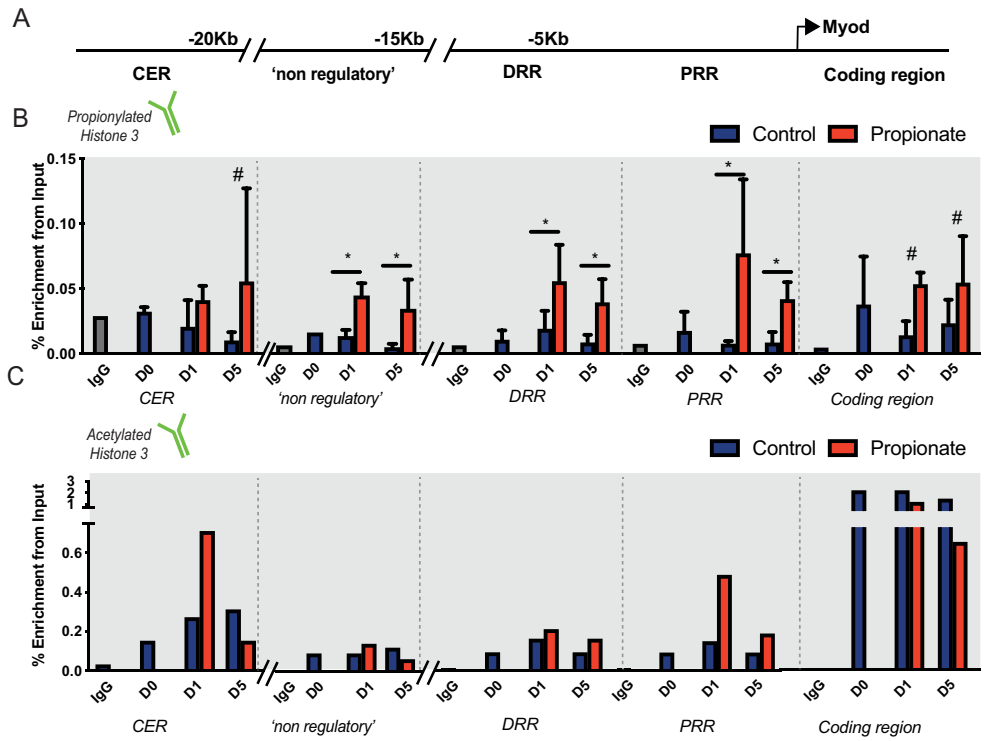

Figure 7.3 Propionate exposure increases propionylation and acetylation on regulatory promotor regions of Myod (A) Schematic presentation of regulatory regions on Myod, namely core enhancer region (CER) a distal regulatory region (DRR) and a proximal regulatory region (PRR). **(B)** Chromatin immunoprecipitation with antibody against propionylated histone 3 lysine 23 (H3K23) and quantified propionylation in C2C12 myoblasts exposed to either control or propionate differentiation medium on day 0 (D0), day 1 (D1) and day 5 (D5) after initiation of differentiation on Myod regulatory sites plus to one site 15Kb upstream and one site on the coding region of the Myod gene (3 independent experiments for D0 and D1, 5 independent experiments for D5). **(C)** Chromatin immunoprecipitation with antibody against acetylated histone lysine 9 (H3K9) in C2C12 myoblasts exposed to either control or propionate differentiation medium on day 0 (D0), day 1 (D1) and day 5 (D5) after initiation of differentiation on Myod regulatory sites plus to one site 15Kb upstream and one site on the coding region of the Myod gene (N = 1). IgG is used as a negative control. (mean ± SD, * indicates $p<0.05$, # indicates $p < 0.1$)

7.3.4. Exposure to propionate hampers differentiation and affects protein acylation in primary human myocytes

To further validate the observed effect of propionate exposure on myogenic differentiation and acylation in humans, we differentiated primary myocytes isolated from *vastus lateralis* biopsies from five healthy human donors in differentiation medium with and without propionate. Propionate exposure increased protein and histone propionylation in all donor cell lines (**Figure 7.4**), showing that also in human derived myoblasts, propionate was able to induce propionylation. Moreover, as was observed in our C2C12 muscle model, MHC protein expression was absent or markedly lower in all five donors in the propionate compared to the control condition, verifying that exposure to propionate also hampered myogenic differentiation in primary human muscle cells. Furthermore, exposure to propionate during differentiation increased global propionylation as well as propionylation of histones, and specific propionylation of H3K23, and increased acetylation on histones, and specific acetylation of H3K23, compared to differentiation in control medium **(Figure 7.4**). The observation that differentiation is also impaired in human-derived myoblasts upon propionate exposure further advocates for a role of propionate, propionyl-CoA and propionylation in muscle differentiation.

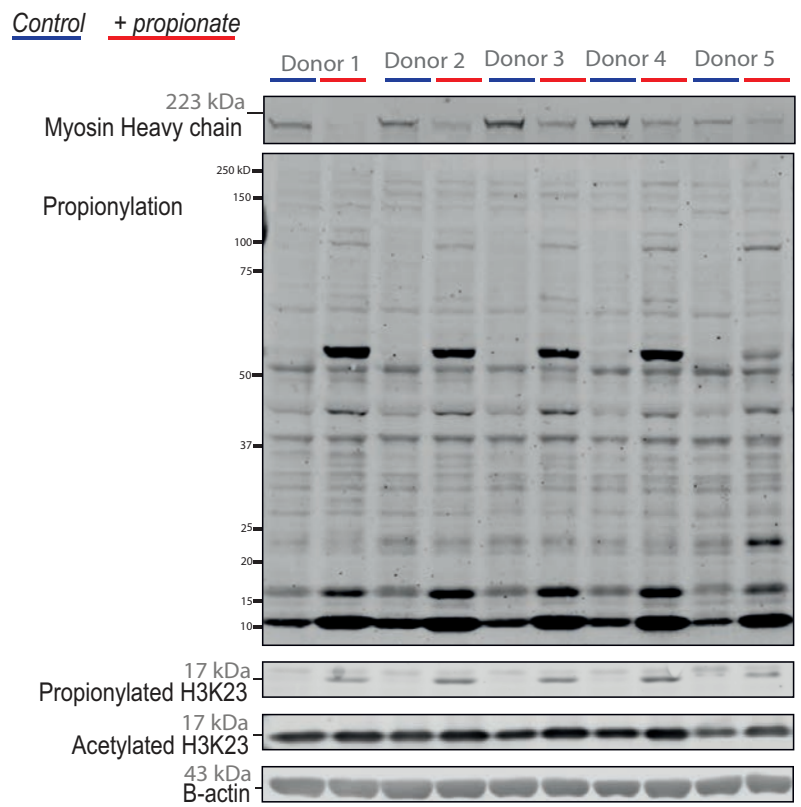

Figure 7.4 Exposure to propionate hampers differentiation and affects protein acylation in primary human myocytes Western blot analysis for propionyllyisne, myosin heavy chain, propionylated histone 3 lysine 23 (H3K23) and acetylated histone 3 lysine 23 (H3K23) primary human myotubes isolated from *vastus lateralis* biopsy of 5 different donors. Cells were differentiated for 5 days on medium with or without 4 mM propionate.

7.4. Discussion

The aim of this study was to explore propionate exposure and subsequent alterations in protein and histone protein propionylation in muscle to elucidate the potential role of this post-translational modification in cellular muscle differentiation. We showed that exposure to extracellular propionate, but not acetate, impaired myogenic differentiation in C2C12 myotubes and that exposure to extracellular propionate impaired myogenic differentiation in primary human myotubes isolated from *vastus lateralis* muscle biopsies. The effect of

propionate exposure on differentiation was accompanied by an increase in histone propionylation and acetylation, also specifically at regulatory myogenic differentiation sites. Regulation of acylation through metabolism is critical for myogenic differentiation (Bracha et al. 2010; Das et al. 2017; Simithy et al. 2017) and because adult myogenic differentiation from satellite cells is an important mechanism in tissue repair and turnover (Blau et al. 2015), these observations could indicate, besides a role for acetyl-CoA and acetylation, a role for propionylation and propionyl-CoA in muscle physiology.

Changes in protein propionylation have been observed before during myogenic differentiation and ageing (Simithy et al. 2017; Baldensperger et al. 2020). We confirm the changes in protein propionylation during myogenic differentiation and newly suggest that increasing these propionylation levels by extracellular exposure to propionate has cellular consequences. Levels of propionyl-CoA drive propionylation and increased cellular levels of propionyl-CoA have been observed in conditions of serum starvation, likely by the catabolism of amino acids, such as isoleucine (Trefely et al. 2020). Therefore, we hypothesise that metabolic events that increase propionyl-CoA levels, such as increased protein catabolism, could have unique signalling roles, possibly via protein and histone propionylation. However, although propionyl-CoA is a distinct metabolite with a distinct metabolic background and despite the observation that only propionate, but not acetate, impaired myogenic differentiation, to date there are no mechanisms on how histone propionylation could be functionally different from histone acetylation. Propionyl-CoA and acetyl-CoA are structurally very similar, likely explaining that propionyl-CoA can use much of the same enzymatic machinery allotted to acetyl-CoA (Kaczmarska et al. 2017; Han et al. 2018) and both acylation neutralise the lysine's positive charge, loosening DNA-histone interactions and facilitate gene transcription (Gorisch 2005; Bannister and Kouzarides 2011; Kebede et al. 2017). Additionally, bromodomains, that are known to recognise acetyllysines and transduce the acylation signal by recruitment of transcription factors, also recognise propionyllysine (Flynn et al. 2015), negating a differential mechanistic effects of acetylation and propionylation.

Nevertheless, there are some indications that propionylation and acetylation differ in how they are cellularly localised. For example, the relative abundance of H3K14 and H3K9 propionylation and acetylation was shown to be reasonably similar in basal conditions (Simithy et al. 2017). This is remarkable, as the affinity of HATs for acetyl-CoA is higher than for propionyl-CoA and acetyl-CoA is 8-fold more abundant compared to propionyl-CoA (Han et al. 2018). This suggests that the concentration of propionyl-CoA must be higher in the nucleus, possibly due to propionyl-CoA compartmentalisation. Indeed, it was shown that the acetyl-CoA:propionyl-CoA ratio was approximately 4 in whole-cell lysates, while in the nucleus the two acyl-CoAs were found in equimolar concentrations (Trefely et al. 2020). In this way, small changes in cellular propionyl-CoA levels can be amplified due to the compartmentalisation of propionyl-CoA in the nucleus. Hence, the increase in histone propionylation could have impaired differentiation by dysregulation of normal acylation patterns, as myogenic differentiation is characterised by an overall reduction in histone acetylation (Yucel et al. 2019).

The effect of propionate exposure was not limited to an increase in propionylation, as we show that histone acetylation also increases in response to propionate exposure, also at regulatory sites of MYOD. Interestingly, the increase in acetylation together with propionylation is in contrast with previous research that showed increased propionylation of H3K14 occurred at the expense of acetylation of H3K14 in isolated nuclei following propionyl-CoA exposure (Simithy et al. 2017). One mechanism by which propionate exposure could increase acetylation is via increasing cellular acetyl-CoA levels. Propionyl-CoA is converted into methylmalonyl-CoA, by propionyl-CoA carboxylase, which in turn can be converted into succinyl-CoA, that can used for anaplerosis of the TCA-cycle (Davis et al. 1980; Wongkittichote et al. 2017). Anaplerosis is matched by cataplerosis, for example via cataplerosis of citrate, which can be converted into cytosolic acetyl-CoA via ACL. Although no data exist for skeletal muscle, an intraperitoneal injection with propionate was shown to increase levels of acetylcarnintine in the heart (Wang et al. 2018). These or similar alterations in homeostatic metabolic mechanisms could have interfered with normal

control of transcriptional regulation by acetylation and therefore could have contributed to the observed phenotype. Nevertheless, this does not explain why acetate exposure, that also increases cytosolic acetyl-CoA levels (Trefely et al. 2020), did not impair myogenic differentiation. Of course, propionic acid and acetic acid are not identical, as differences exist in, for example density, viscosity, ional potential and PKa, which may result in functional differences propionylation and acetylation. Determination of nuclear concentrations of various AcylCoAs in response to acetate, propionate or combined exposures at various concentrations may provide additional insights, especially when related to specific acylation levels. Additionally, another mechanism by which propionyl-CoA could have increased normal cellular acylation levels, is through a direct effect of propionate as a HDAC inhibitor. However, although propionate does possess HDAC activity, it is 10-fold less potent as butyrate in whole-cell assays (Waldecker et al. 2008), suggesting that the role of propionate as an HDAC inhibitor might be limited.

The findings that propionate exposure impairs myogenic differentiation in human myoblasts is of particular interest for patients with similar elevated plasma propionate concentrations, such as patients with a defect in the propionyl-CoA carboxylase gene (Hommes et al. 1968). These patients, apart from neurological, haematological and hepatic complications, may display myopathic features, including hypotonia and exercise intolerance (Saudubray et al. 2016; Haijes et al. 2019). Analysis of muscle biopsies from these patients showed a defective mitochondrial respiration, suggesting that mitochondrial dysfunction could contribute to the myopathic pathology (Schwab et al. 2006; de Keyzer et al. 2009). However, exposure to propionate and consequent cellular elevations in propionyl-CoA and propionylation did not induce mitochondrial defects in cultured myotubes or isolated muscle mitochondria, whereas this was induced in cultured liver cells and isolated liver mitochondria (Matsuishi et al. 1991; Lagerwaard et al. 2020b). This suggests that in skeletal muscle other mechanisms could contribute to the mitochondrial defects observed in *ex vivo* measurements in muscle biopsies. Therefore, the current data on the effect of propionate exposure on myogenic differentiation could play a role, as differentiated

myotubes are more oxidative compared to undifferentiated myoblasts (Remels et al. 2010; Sin et al. 2016), perhaps explaining the mitochondrial defects in patients. Since we show that aberrant acylation might contribute to the differentiation defect, it is of interest to explore strategies that might alleviate the underlying causes. Hence, to decrease the aberrant propionylation we propose to aim for increasing de-propionylation activities. For this, the activation of sirtuin 1 could be of interest, as this enzyme has been shown to de-propionylate proteins in the nucleus, at least *in vitro* (Cheng et al. 2009).

In conclusion, we showed that exposure to extracellular propionate, but not acetate, and the associated increase in propionylation impaired myogenic differentiation. Although the role of propionylation and the mechanism by which increased propionylation hampers myogenic differentiation remains to be elucidated. We show that alterations in histone propionylation and acetylation, also at regulatory myogenic differentiation sites, might play a role. Propionyl-CoA levels drive propionylation and levels of propionyl-CoA have been observed to be altered during starvation, ageing and myogenic differentiation. Therefore, these observations provide additional evidence for a role of propionylation and propionyl-CoA in the regulation of metabolism, which requires further investigation.

Acknowledgements We would like to thank Wei Wu for her help in data collection.

Author Contributions BL performed experiments, principal data analysis, reporting of results and drafted the manuscript. JH, LG collection of human material. BL, AGN, JK, VCJB planning and conduct of the project, conception and design of research, data analysis and interpretation. All authors edited, revised and approved the final version of the manuscript.

Compliance with ethical standards Sampling of human material was part of a study that was approved by the medical ethical committee of Maastricht University (METC azM/UM; NL59895.068.17) and conducted in accordance with the principles of the Declaration of Helsinki (Fortaleza, Brazil 2013) and according to national law (WMO, The Hague, 1998).

The study is registered clinicaltrials.gov under the identifier NCT03666013. Subjects were written and verbally informed on all experimental procedures, including possible risks and discomforts. All subjects provided written informed consent before testing.

CHAPTER 8

General Discussion

Bart Lagerwaard

8.1. Main findings

The overall aim of this thesis was to obtain a better understanding of the role of the mitochondria in age-related loss of muscle mass and strength. To address this aim, I further established the NIRS assessment of mitochondrial capacity by showing that NIRS is able to detect differences in a homogenous population of high- and low-fitness males and females (**chapter 2 and 3**) and I substantiated the physiological relevance of m$\dot{V}O_2$ recovery as relevant marker of mitochondrial capacity by showing a correlation with other parameters of oxidative capacity (**chapter 2**). Additionally, I used NIRS for the first time to assess the mitochondrial capacity of three locomotor muscles in a well-controlled, ageing population (**chapter 4)**. Furthermore, I identified molecular mechanisms of ageing in *vastus lateralis* muscle biopsies in the same population using transcriptome sequencing which were independent of age-related changes in physical activity (**chapter 5**). Moreover, I explored the effects of protein propionylation on mitochondrial respiration in various cell types and investigated the functional effects of increased protein propionylation in skeletal muscle cell lines and primary human myotubes **(chapter 6 and 7)**.

NIRS was shown to be a valuable tool to study muscle mitochondrial capacity in an ageing population (**chapter 2, 3 and 4**) and that despite similar physical activity, muscle mitochondrial capacity and mitochondrial gene and protein expression is still lower in older compared to young males (**chapter 4 and 5**). Furthermore, protein propionylation could be an interesting post translational modification in skeletal muscle, as increased propionylation is associated with impaired differentiation and associated with lower mitochondrial respiration in other cell types (**chapter 6 and 7**).

8.2. The way forward for NIRS assessment of muscle mitochondrial capacity

Nutritional and physical human intervention studies mostly rely on methods that are readily available and can be easily incorporated in current assessment protocols. Furthermore,

often multiple measurements over a longer time period are used in large populations and therefore invasive and time-consuming methods are often less desirable. Due to the increased portability and accessibility, and lower burden for the research subject, NIRS has the potential to be a valid, non-invasive tool to study mitochondrial capacity in human intervention studies. However, for NIRS to live up to this promise, some challenges and pitfalls needed to be addressed. Therefore, one of the aims of this thesis was to further establish the NIRS assessment of muscle mitochondrial capacity. For example, it is was not known whether the method was adequately sensitive to pick up more subtle differences in mitochondrial capacity. NIRS was previously used to detect differences in mitochondrial capacity between inactive individuals and endurance trained athletes (Brizendine et al. 2013). This was likely the result of training adaptations in mitochondrial amount and quality, reflected by the difference in $\dot{V}O_2$peak between the groups (Tonkonogi and Sahlin 2002). Yet, although mitochondrial capacity is not the only determinant for $\dot{V}O_2$peak (Weibel et al. 1991), the difference in $\dot{V}O_2$peak was considerably large between the inactive individuals and endurance trained athletes, about ± 40 mL/kg/min. When comparing an older and young population, differences in $\dot{V}O_2$peak are expected to be more subtle, about ± 15 mL/kg/min, even when physical activity is maintained (Lanza et al. 2008; Distefano et al. 2018). Therefore, in **chapter 2** we applied NIRS to measure differences in mitochondrial capacity in a more normal population, i.e. $\dot{V}O_2$peak values more common in the general population, of high- and low-fitness males with a smaller difference in $\dot{V}O_2$peak between the groups. We show that with an absolute difference of ± 15 mL/kg/min in $\dot{V}O_2$peak between the high- and low-fitness males, we were able to detect the anticipated difference in mitochondrial capacity in the *gastrocnemius* muscle. Likewise, in **chapter 3** we were able to detect similar differences in a female population with a similar difference in $\dot{V}O_2$peak. Therefore, the findings in **chapter 2 and 3** expand the sensitivity of NIRS to detect smaller differences in mitochondrial capacity in a more normal population.

Henceforth, in **chapter 4** we applied NIRS in an ageing population in three different locomotor muscles. We show that NIRS was able to detect differences mitochondrial

capacity in the *gastrocnemius* and *vastus lateralis* between similarly active young and older males. The absolute mean difference in mitochondrial capacity was much smaller in the *gastrocnemius* muscle comparing young and old males than when comparing high- and low-fitness individuals (0.25 vs 0.59 in $m\dot{V}O_2$ recovery constant (min^{-1}), respectively; **Figure 8.1**). However, it must be noted that we did not measure $\dot{V}O_2$peak in the similarly active young and older males and therefore the difference in $\dot{V}O_2$peak is unknown. Nevertheless, these data provide strong evidence for the use of NIRS to assess mitochondrial capacity, also to study the effect of age on mitochondrial capacity. Because NIRS is a more portable, more accessible and a relatively cheaper method to assessment of mitochondrial capacity non-invasively, the use of NIRS can accelerate research in the field of mitochondrial muscle ageing and can be more easily applied to assess the effectiveness of exercise and lifestyle interventions to retain mitochondrial capacity with age.

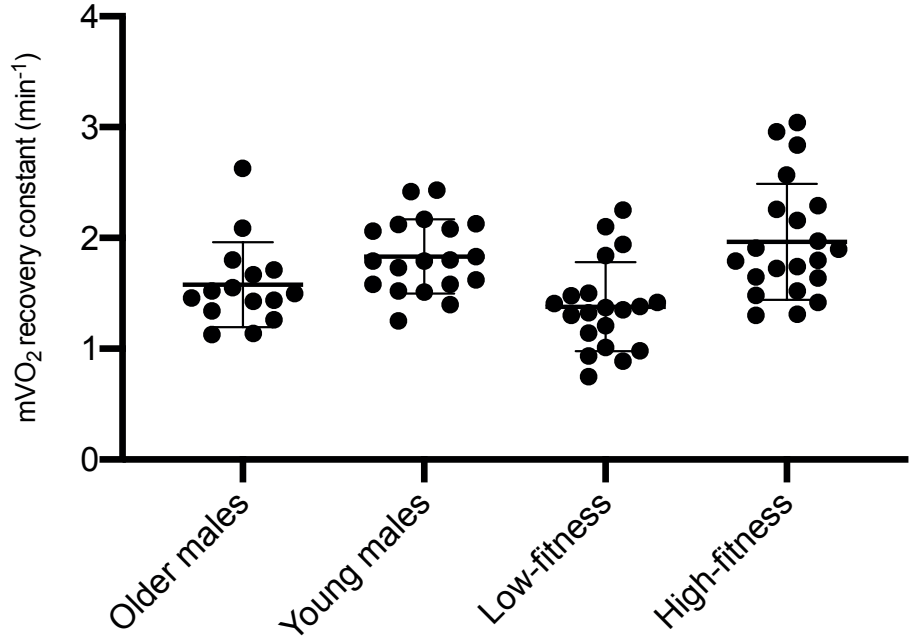

Figure 8.1 $m\dot{V}O_2$ recovery constant derived from monoexponential curve fits of $m\dot{V}O_2$ recovery in *gastrocnemius* after after 30 s of plantar flexion exercise in older males, young males, high-fitness and low-fitness males and females

A technical drawback of the NIRS assessment of mitochondrial capacity, is that without inferring the amount of light scattering using mathematical assumptions, one cannot determine absolute oxygen concentrations in the tissue underneath the probe, at least using the continuous NIRS system that was used in this thesis. This is because differences in subcutaneous adipose tissue thickness (ATT), but also skin thickness, skin pigmentation and blood flow, influence the light scattering (van Beekvelt et al. 2001; Wassenaar and Van den Brand 2005; Craig et al. 2017). Therefore, because these factors differ between subjects, the NIRS signal is normalised for each individual subject using a physiological calibration. This physiological calibration allows for the conversion of the units of the measurement to a percentage of one's maximal muscle oxygenation. This percentage is established after a four-minute arterial occlusion to ensure complete deoxygenation of the tissue (0%) and the corresponding hyperaemic response after caseation of the occlusion (100%) (Hamaoka et al. 1996). In this way, the signal is standardised between subjects or within subjects at different time points.

NIR light is thought to travel in a banana-like shape in the tissue from emitter to receiver and the distance between the receiver and emitter, the optode distance, is thought to be equal to half the interrogation depth of the NIR light in the tissue. If more light is scattered, less light will reach the muscle and the interrogation depth will be lower. Therefore, although the physiological calibration is an effective strategy to standardise the signal between subjects, still, differences in measurement depth can influence the measurement. For example, muscle fibre type distribution can be different at different depths (Johnson et al. 1973; Koga et al. 2015) and when ATT is high and the optode distance is small, the interrogation depth can be insufficient to reach muscle tissue (van Beekvelt et al. 2001). Therefore, the findings in **chapter 3** are important as females generally have higher adiposity and thicker ATT and highlight that NIRS can detect similar differences in mitochondrial capacity in a female population compared to a male population. To overcome the expected larger ATT in the female population, we used a greater optode distance to ensure enough light would reach the muscle. Yet, although we were able to detect similar

differences in a population with higher ATT, some measurements in participants among the highest ATT thickness failed due to low quality data. Therefore, we hypothesise that despite having theoretically sufficient interrogation depth, with increasing ATT the amount of signal contributed to muscle (signal) decreases and the amount of signal from adipose tissue increases (noise), which significantly affects the quality of the measurement. A solution for these limitations can be to use time-resolved NIRS. Time-resolved NIRS makes use of short light pulses and is able to extrapolate absolute oxygen concentrations from tissue optical properties, making use of attenuations in shape and delay light pulses at the receiver (Re et al. 2018). Additionally, increasing the power of the light source will increase the amount of light received at the receiver and allows for measurement in deeper tissues (Koga et al. 2015). Nevertheless, the aforementioned systems are not widely available, require more expertise and are not portable, making them less desirable for routine and onsite measurements.

Another concern for standardisation is the exercise that precedes the $m\dot{V}O_2$ recovery measurement. The assumptions are that mitochondrial oxidative enzymes are maximally activated by the short exercise protocol and that the recovery of readily available energy substrates is directly linked to mitochondrial activity following first-order kinetics between PCr dynamics and mitochondrial oxidative metabolism. However, it was shown that following low-intensity contractions, the recovery of isolated muscle fibres is slower compared to the recovery following medium and high intensity contractions, at least in high oxidative fibres (Wüst et al. 2013). This suggests that mitochondria are not maximally activated at lower exercise intensities which violates first-order kinetics assumptions. This is supported by studies looking at oxygen kinetics at the onset of exercise, where a higher-order kinetic models fits these kinetics better (Wüst et al. 2011; Korzeniewski and Rossiter 2015) possibly due to allosteric control of mitochondrial enzymes by, for example, Ca^{2+} (Glancy and Balaban 2012). This implies that the exercise preceding the measurement should reach a certain minimum exercise intensity to not violate the assumption of maximal activation of mitochondrial oxidative enzymes.

On the contrary, when the exercise intensity is too high, this might also violate assumptions of a valid measurements of $m\dot{V}O_2$ recovery (Ryan et al. 2013a). For example, it has been suggested that following high-intensity exercise, there is a component of PCr recovery that can be contributed to ATP production by glycolysis (Forbes et al. 2009). Moreover, when exercise intensity is high and oxygen consumption is high, oxygen can become a limiting factor and recovery of $m\dot{V}O_2$ only reflects mitochondrial function when oxygen is not a limited factor during recovery (Haseler et al. 2007). Hence, standardisation, i.e. similar intensity, of the exercise preceding $m\dot{V}O_2$ recovery measurements could be essential for a valid measure of mitochondrial capacity. Standardisation of the exercise can be done by standardising force output during the exercise protocol to a percentage of one's maximal force production. This would require equipment to measure one's maximal force prior to the measurement and execute the exercise at a set percentage of that maximal force. However, just as for time-resolved NIRS, although adaptations to the method can be incorporated to increase the validity of the measurement, many of these adaptations compromise the simplicity of the method. This method was developed as simple, more accessible and, if desired, portable tool. Increasing complexity by adapting equipment for exercise standardisation or increasing measurement depth compromises this simplicity. Besides, there is evidence that, despite aforementioned concerns, mitochondrial capacity is independent from exercise intensity and exercise type (Ryan et al. 2013a; Southern et al. 2014). This suggests that in an *in vivo* situation small differences in the exercise preceding the $m\dot{V}O_2$ recovery measurement might be less important or less apparent. Nevertheless, improvements in methodology could be important when small effects sizes are expected. Therefore, I suggest that two methodologies of NIRS assessment of mitochondrial capacity can coexist: a field-based, portable, simpler protocol and a more elaborate laboratory-based, stationary protocol. Nonetheless, with advancements in technology, features of the laboratory NIRS can be implemented in portable devices, which can further advance the portable protocol.

8.3. Physical activity and skeletal muscle mitochondrial ageing

Although mitochondrial dysfunction is referred to as a hallmark of ageing (López-Otín et al. 2013b) and is often implied to be indispensably associated with muscle ageing (Carter et al. 2015), the role of mitochondria in muscle ageing is not yet fully understood. When using an *in vivo* ^{31}P-MRS approach, combining data from 22 studies, mitochondrial capacity was not shown to be negatively affected by age. On the contrary, the effect of age on mitochondrial capacity was positive (Fitzgerald et al. 2016). This would mean that with advancing age, mitochondrial capacity increases and thus either mitochondrial quality improves or the mitochondrial amount increases. This is not in agreement with data from isolated muscle fibres and muscle mitochondria, which show a lower mitochondrial quality and decreased mitochondrial amount with age (Short et al. 2005; Lanza et al. 2008). Nonetheless, the effects of age on mitochondrial capacity are very heterogenous and when dissecting the data, three main factors that modify muscle mitochondrial ageing were identified, namely sex, muscle group and physical activity (Fitzgerald et al. 2016). Therefore, when designing the ageing study in this thesis, we have put considerable effort in either standardising, in case for sex and physical activity, or addressing, in case for muscle group, these effect modifiers. We were therefore able to conclude in **chapter 4**, that mitochondrial capacity was lower in a population of older compared to young males despite similar physical activity levels, and that this effect was indeed dependent on muscle group. More specifically, mitochondrial capacity was lower in the *vastus lateralis* and *gastrocnemius* muscle of older compared to young males, but was not different in the *tibialis anterior*. This shows that in a homogenous population, e.g. same sex, narrow age range and similar physical activity, the effect of age is negative, at least in the *vastus lateralis* and the *gastrocnemius*. Moreover, in **chapter 5** we showed that this lower mitochondrial capacity, at least in *vastus lateralis*, was likely caused by a lower expression of mitochondrial genes and a lower expression of mitochondrial complex proteins. In **chapter 4** we also show that despite a component in muscle ageing, which is independent from physical activity, higher levels of moderate-to-vigorous physical activity were associated with higher mitochondrial capacity, at least in the

gastrocnemius muscle. Likewise, in **chapter 5** we showed that in the *vastus lateralis* muscle, higher levels of moderate-to-vigorous physical activity were associated with increased expression of mitochondrial genes. This supports that maintaining higher levels physical activity at an older age still remains an effective strategy to at least mitigate mitochondrial ageing (Lanza et al. 2008; Irving et al. 2015; Balan et al. 2019).

Although we conclude that muscle mitochondrial capacity is lower in older compared to young males despite similar physical activity levels in the *vastus lateralis* and *gastrocnemius* muscle, there could be other explanations underlying the observed effect that should be taken into consideration:

1) Similar physical activity (intensity, amount or type) is not sufficient to elicit the same molecular response in gene and protein expression in older individuals and therefore is not sufficient to sustain mitochondrial capacity.

2) Whole-body physical activity might not reflect physical activity in every muscle group and this might be age-dependent.

3) Mitochondrial capacity is secondary to other age-related processes that affect muscle ageing that might be less affected by physical activity, such as dysregulation of cell adhesion, increased inflammation or denervation.

4) Physical activity was not maintained over the lifetime, but rather was similar in the recent weeks or months preceding the mitochondrial capacity measurements, i.e. the physical activity measurements are not representative.

8.3.1. Similar physical activity is not sufficient to elicit the same molecular response in gene and protein expression in older individuals

Despite engaging in similar physical activity, there could be a lower sensitivity towards physical activity as the stressor and the adaptive, molecular signalling pathways in response to this stressor in older muscle. Although muscle biopsies in our studies were taken at rest and at least 32 hours after the last exercise session, and therefore do not reflect acute exercise effects, the expression data showed that on a molecular level, there are differences in expression of important genes that regulate mitochondrial capacity in response to physical activity between old and young muscle. For example, the expression of PGC1α and the gamma 3 subunit of the AMPK complex (*PRKAG3*) were lower expressed in the older compared to young males. *PRKAG3* has been shown to be most important in the AMPK activation by phosphorylation following physical activity (Birk and Wojtaszewski 2006) and AMPK activation is essential in the activation of PGC1α, which in turn, regulates mitochondrial biogenesis (Handschin and Spiegelman 2006). Moreover, the expression of the acute exercise-responsive genes NR4A1 and NR4A3 (Kawasaki et al. 2009; Pillon et al. 2020) was lower in the older compared to young males. Expression of these genes enhances oxidative metabolism (Chao et al. 2012) and their expression is, at least in part, controlled by AMPK activation (Kawasaki et al. 2009). Previous work showed an attenuated AMPK response in old compared to young mice, who displayed an age-related decrease in AMPK activation upon exercise (Reznick et al., 2007). Furthermore, the response of PGC1α to an exercise intervention after a period of bed rest was higher in young compared to old individuals, failing to restore expression of some mitochondrial complexes in the older individuals (Buso et al., 2019). Therefore, it could be that, for example, the lower expression of *PRKAG3* limits AMPK activation and hence, the activation of downstream regulators of mitochondrial capacity. This could explain why similar physical activity in older individuals is not able to elicit a similar adaptive molecular response as in young individuals, perhaps explaining the lower expression of mitochondrial complexes and lower *in vivo* mitochondrial capacity. However, this has not yet been established and further research on the acute adaptive response to exercise in old compared young muscle is necessary.

Based on data acquired from the accelerometery measurements, the older subjects spent on average 5% of their wear time in moderate-to-vigorous physical activity. On a weekly basis, this adds up to ± 315 minutes a week. This amount of moderate-to-vigorous physical activity meets the guidelines for physical activity in The Netherlands, which advice 150 minutes of moderate-to-vigorous physical activity per week (Gezondheidsraad 2017). Nevertheless, according to our data, this amount of physical activity is not enough to sustain mitochondrial capacity, questioning the adequacy of the current physical activity guidelines, at least to sustain mitochondrial capacity. Nevertheless, in the current design, only the amount of time and intensity of physical activity were standardised between the two age groups, while type of exercise was not specifically standardised. The current guidelines also advice to engage in resistance exercise for two times a week. Yet, not all older males included in the study met those guidelines. Combined training, including both resistance and endurance training, has been shown to induce the most robust changes in mitochondrial capacity in elderly compared to resistance or endurance training alone (Irving et al. 2015). Therefore, future studies should, besides standardising for equal time and intensity of physical activity, also standardise for type of between the two groups. In this way, differences in resistance physical activities between the age groups could be prevented.

8.3.2. Whole-body physical activity might not reflect physical activity per muscle group and this might be age-dependent

Different patterns of muscle use between the young and older individuals could have affected activation and the consequent molecular response in specific muscles, while on a whole-body level physical activity was similar. This might be especially true for the *vastus lateralis* muscle, as this muscle has been shown to be less activated in old compared to young males during locomotion (Hortobágyi and DeVita 2000; Tirosh and Sparrow 2005; Schmitz et al. 2009). The differences in muscle use with age are substantiated by the differential effect of age on mitochondrial capacity in the *tibialis anterior* muscle, which showed no significant difference between older and young individuals (Lanza et al. 2007;

Christie et al. 2014; Lagerwaard et al. 2020a) and activation during locomotion was shown to be higher in older compared to young individuals in this muscle (Schmitz et al. 2009). Therefore, although the *vastus lateralis* is a muscle that is often sampled for biopsies due to its relatively easy accessibility and larger mass, it might not be representative for muscle ageing in all muscle groups. Muscle biopsies from the *tibialis anterior* have been shown to be safe and well tolerated (Lilleker et al. 2016) and analysing the molecular signature of this muscle might give additional insights in muscle ageing, independent from changes in activity. Additionally, it could be valuable to, besides measuring physical activity, also measure muscle-specific activity, at least in the muscle from which the biopsy is taken or in which mitochondrial capacity is assessed. Hence, I encourage future research to explore the possibilities to combine accelerometry measurements with specific muscle activity measurements, such as surface electromyography (Boccia et al. 2015), which will allow for integration of physical activity measurements, muscle activity measurements and mitochondrial capacity.

8.3.3. Mitochondrial capacity is secondary to other age-related processes that affect muscle ageing that might be less affected by physical activity

Another explanation for the decrease in mitochondrial capacity despite similar physical activity, could be that other age-related processes that affect muscle ageing are the cause of the decline in mitochondrial capacity with age, i.e. a decreased mitochondrial capacity is a secondary ageing phenomenon. In **chapter 5**, we identified cell adhesion, inflammation and muscle innervation as significantly (dys)regulated pathways with age. Therefore, it could be that these processes precede mitochondrial decline. However, ageing studies often use a cross-sectional rather than longitudinal study design. Therefore, appointing chronological cause and effect relationships is not possible, as is the case in this thesis. Yet, some have hypothesised that, for example, age-associated denervation of the muscle fibres precedes mitochondrial dysfunction in the muscle fibre (McNeil et al. 2005), as similar a mitochondrial phenotype has been observed in denervated muscle fibres (Wicks and Hood 1991) as in aged muscle fibres (Spendiff et al. 2016; Sonjak et al. 2019). The denervation

could be caused by ageing processes that occur in the motor neurons and not in the muscle fibres, such as decreased axonal transport of mitochondria in the motor neurons (Takihara et al. 2015). Others hypothesised that extracellular matrix integrity decreases due to an accumulation of damage with age (Ewald 2020). The decreased integrity could influence muscle satellite cell function and muscle repair (Blau et al. 2015), which could in turn increase inflammation leading to tissue fibrosis and further damage the extracellular matrix (Mann et al. 2011). These changes in the extracellular matrix could negatively affect force transmission (Street 1983). Taken together, decreased muscle contractibility due to decreased force transmission, decreased innervation or decreased extracellular matrix integrity, could impair the adaptive molecular response following physical activity. In this way, the decline in muscle mitochondrial capacity might be secondary to these processes, which we identified to occur in the older muscle independent of physical activity. Therefore, it is of interest to explore mechanisms by which these processes are regulated with age and to explore interventions that might ameliorate these changes. For example, muscle stretching interventions in rat muscles have been found to affect the extracellular matrix (Zotz et al. 2016) and enhance muscle blood flow (Hotta et al. 2018), suggesting that interventions should not be limited to increasing or maintaining physical activity alone.

8.3.4. The physical activity measurements were not representative of long-term and habitual physical activity

Lastly, another explanation for the decrease in mitochondrial capacity, despite similar physical activity, could be attributed to the representativeness of the physical activity measurement. Physical activity was measured using three weekdays and two weekend days out of a 7-day, waist-worn, triaxial accelerometry measurement. Triaxial accelerometry measurements provide modest-to-good approximations of total energy expenditure when compared to double-labelled water, the golden standard for energy expenditure measurements (Chomistek et al. 2017). Although double labelled water can provide a more accurate measure for energy expenditure, accelerometry can provide a more in depth analysis of the movement patterns by measuring frequency, duration and intensity of

physical activity (Plasqui et al. 2013). Nevertheless, these measurements only reflect physical activity over the recent past and therefore, might not be a good indicator for the effect of physical activity on mitochondrial capacity over longer periods of time. Although, habitual physical activity was assessed using the Baecke questionnaire, which approximates habitual physical activity over the last year (Baecke et al. 1982) and correlates well with total daily energy expenditure measured using double labelled water (Philippaerts et al. 1999), still, less subjective and measurements of physical activity over even longer periods of time, e.g. years, could have been more determinant with regards to mitochondrial ageing. Therefore, physical activity data collected over very long periods of time (e.g. years) can therefore be useful in mitochondrial ageing research, such as those collected from commercial physical activity trackers. Recent trends in health and physical activity tracking have increased the use of commercially available wrist-worn devices, also in biomedical research (Wright et al. 2017). While wrist-worn devices are inferior to waist-worn alternatives (Ellis et al. 2016) and not all commercially available devices are equally accurate in predicting energy expenditure compared to research-grade accelerometers (O'Driscoll et al. 2018), the data collected over the lifetime can still be of significant value. Furthermore, many of these devices have additional biological sensors, such as heart rate sensors, which increase estimates of energy expenditure in some activities (O'Driscoll et al. 2018). Therefore, measuring mitochondrial capacity in subjects who have been wearing these commercial sensors over an extended period of time could provide robust and relatively easy standardisation of physical activity over longer periods of time in muscle ageing research.

8.4. A role for propionylation in ageing?

Apart from analysing how mitochondrial function is regulated in muscle during aging on the gene and protein level, we also studied the role of novel post-translational modifications in muscle mitochondria (**chapter 6** and **7).** Acylation, the modification of proteins with acyl-groups, has been proposed to play a role in regulating mitochondrial as well as other cellular

functions (Choudhary et al. 2014). We studied specifically how one of these modifications, protein propionylation, contributes to regulation of mitochondrial function. In c**hapter 6**, we used a metabolic disease model with elevated protein propionylation levels to study the effects of increased protein propionylation on mitochondrial respiration. We showed that patients with this metabolic disease have increased levels of propionylation in fibroblasts obtained from skin biopsies. This increase was driven by the accumulation of propionyl-CoA due to a genetic defect in the propionyl-CoA carboxylase genes (Saudubray et al. 2016). The increased propionylation was accompanied by a decreased mitochondrial respiration in fibroblasts of these patients. Furthermore, when inducing increased protein propionylation by exposing control cells to a similar concentration of propionate that can be found in patients, we observed decreased mitochondrial respiration in cultured human fibroblasts and in a liver cell line. This suggested that increased protein propionylation can affect protein functionality, thereby hampering mitochondrial respiration. This is in accordance with our initial hypothesis that protein propionylation would mainly affect mitochondria because the mitochondria are the site where propionyl-CoA is formed and metabolised, and due to favourable biochemical environment for acylation in the mitochondria (Wagner and Payne 2013). Indeed, in mice, many mitochondrial proteins can be subjected to propionylation, among which proteins that are directly involved in ATP generation, such as ATP synthase (Fritz et al. 2013b). Because acylated proteins typically show decreased functionality, thereby affecting cellular respiration (Schwer et al. 2006; Garrity et al. 2007; Ahn et al. 2008; Schlicker et al. 2008; Baeza et al. 2016), in **chapter 6**, we henceforth propose that the increased protein propionylation is the cause of the reduced mitochondrial respiration.

Nonetheless, when we induced propionylation in cultured muscle cells, we did not observe the same mitochondrial defects. Moreover, we observed a lower amount of global protein propionylation and a faster decrease in propionylation after a recovery period in medium that did not contain propionate. In **chapter 6**, we hypothesised how differences in expression of certain enzymes might explain the different effects of propionate exposure

between muscle and liver and argue that the muscle might be geared towards propionyl-CoA elimination. Since propionate exposure not only affects mitochondrial function, we also studied the effects of propionate and propionylation on other cellular functions in muscle cells. In **chapter 7**, we further explored the role of protein propionylation in the muscle and showed that exposure to propionate during differentiation impaired skeletal muscle differentiation in both primary human and cultured muscle cells. Because the increase in protein propionylation mainly occurred on histone proteins and regulation of gene expression is especially important during differentiation, the effect of protein propionylation on histones was investigated. We showed that the increase in propionylation was also observed on histone proteins of regulatory genes of muscle differentiation, providing a possible transcriptional mechanism on how propionate can impair muscle differentiation. In **chapter 7**, we discuss possible mechanisms by which histone propionylation might affect gene transcription, which remain to be elucidated. Nonetheless, the findings that propionate exposure and the consequent increase in protein propionylation is associated with impaired mitochondrial respiration in some cell types and impaired differentiation in skeletal muscle, provide the first indications for a role of protein propionylation in cellular physiology.

Patients with a genetic defect in the propionyl-CoA carboxylase gene do display, apart from neurological, haematological and hepatic complications, a myopathic phenotype, including hypotonia and exercise intolerance (Haijes et al., 2019; Saudubray et al., 2016), suggesting that there might be a role for protein propionylation in the skeletal muscle. However, because propionate exposure did not affect mitochondrial respiration in muscle cells, it could be that this phenotype is regulated through increased propionylation of histone proteins, rather than through an increased mitochondrial protein propionylation. Therefore, our findings additionally suggest that besides our initial hypothesis on the role of propionyl-CoA and protein propionylation on the mitochondrial acylome, this now can be extended to a role for propionyl-CoA in the regulation of gene transcription via histone acylation. In this manner, levels of propionyl-CoA can regulate a wide array of cellular

processes. Interestingly, acetyl-CoA and propionyl-CoA were found in equimolar concentrations in the nucleus (Trefely et al. 2020), while acetyl-CoA is 8-fold more abundant compared to propionyl-CoA in cell lysates (Han et al. 2018), suggesting a compartmentalisation of propionyl-CoA in the nucleus. However, to date, the mechanism how mitochondrial propionyl-CoA derived from the breakdown of metabolites can contribute to nuclear propionyl-CoA levels that can be utilised for histone propionylation is unclear. Since propionyl-CoA cannot be transported over the inner mitochondrial membrane, one mechanism by which this could occur is via mitochondrial acyl-CoA thioesterase, an enzyme that can convert propionyl-CoA into propionate (Tillander et al. 2014; Ellis et al. 2015). Subsequently, in the cytoplasm propionate can be converted back into propionyl-CoA by acyl-CoA synthetase short chain family member 2 (ACSS2) (Ellis et al. 2015; Yoshimura et al. 2016). Another mechanism by which mitochondrial propionyl-CoA can contribute to histone propionylation is via carnitine acetyltransferase (CRAT). CRAT has the highest affinity for propionyl-CoA (Violante et al. 2013) and propionyl-carnitine can be transported out of the mitochondria into cytosol. Although for propionyl-carnitine this has not been investigated, mitochondrial acetyl-carnitine has been suggested to provide nuclear acetyl-CoA for histone acetylation (Madiraju et al. 2009) and therefore, this mechanism likely also exist for propionyl-carnitine.

Whether increased protein propionylation as a cause for defective mitochondrial respiration and impaired myogenic differentiation also contributes to decline in the quality of muscle and muscle mitochondria with age, is yet to be explored. Nevertheless, there are some indications that protein propionylation could play a role. First, levels of protein propionylation are increased in the nuclear and cytosolic fraction of livers of old compared to young mice (Baldensperger et al. 2020). Second, it could be that levels of propionyl-CoA are higher in older individuals, thereby increasing protein propionylation. For example, increased cellular levels of propionyl-CoA have been observed *in vitro* in conditions of serum starvation, derived from the catabolism of the amino acid isoleucine (Trefely et al., 2020). Therefore, as muscle breakdown might increase with age, the catabolism of amino acids

that increase levels of propionyl-CoA could be expected to increase as well. Third, it could be that protein propionylation increases due to lower activity of enzymes that remove propionyl groups from proteins, i.e. depropionylate proteins. Although it is not yet clear which enzymes have depropionylation activity, it is likely that sirtuins are responsible for this, as these enzymes have been shown to regulate other types of acylations on many protein targets (Wagner and Hirschey 2014). Sirtuin enzymes require NAD^+ as their cofactor, yet levels of this cofactor were observed to decrease with age in various tissues, including skeletal muscle in rodents (Yoshino et al. 2011; Braidy et al. 2011; Gomes et al. 2013) and in skin (Massudi et al. 2012), brain (Zhu et al. 2015), liver (Zhou et al. 2016) and plasma (Clement et al. 2019) in humans. Furthermore, NAD^+ levels have been shown to be lower in sarcopenic muscle compared to aged-matched controls (Migliavacca et al. 2019). Noted must be that this decrease has not yet been observed in healthy aged skeletal muscle.

To get insight whether protein propionylation increases in older individuals, we semi-quantified protein propionylation in muscle biopsies analysed in **chapter 5** of seven young and seven older males using Western blot. Protein propionylation had the tendency to be higher in older males. However, this effect was not statistically significant (**Figure 8.2**). Furthermore, we were unable to measure any propionylation on histone proteins, possibly due to the method of protein isolation. Moreover, quality of Western blotting is highly dependent on antibody quality and the appropriate loading controls. Furthermore, the range of quantification is somewhat limited and altogether, the results obtained by Western blotting are considered as semi quantitative. Therefore, a more quantitative approach to measure differences in protein propionylation would be to measure the amount of propionylation using liquid chromatography–mass spectrometry (Baldensperger et al. 2020). Nevertheless, the current preliminary results together with the aforementioned indications of higher protein propionylation with age, provide insight and rationale to further investigate the role of protein propionylation with ageing.

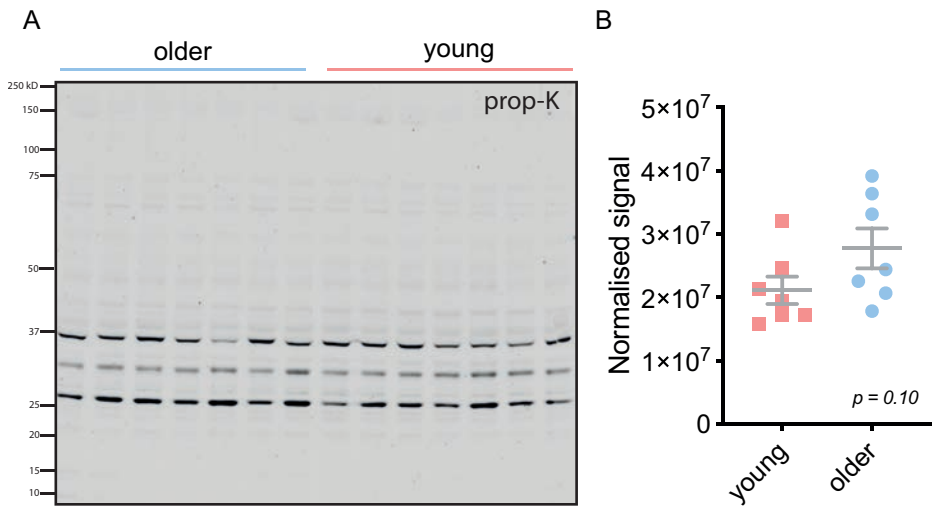

Figure 8.2 (A) Western blot analysis of propionyllysine (prop-K) in *vastus lateralis* muscle of 7 older and 7 young males. **(B)** Quantification of prop-K protein expression corrected for total protein using total protein staining.

If protein propionylation plays a role in muscle ageing, one can speculate on strategies to alleviate the increased propionylation. The removal of these acylation is regulated by sirtuins and therefore, activation of these enzymes is likely an effective strategy to lower cellular propionylation levels. One strategy that is postulated to increase the activity of sirtuins is by increasing the levels of the cofactor of the sirtuin enzymes, NAD^+. Strategies to increase NAD^+ levels via supplementation often make use of NAD^+ precursors, such as nicotinamide riboside (NR) and nicotinamide mononucleotide (NMN), that can be converted into intracellular NAD^+ via the so called 'NAD^+ salvage pathway (Bogan and Brenner 2008). The effects of NAD^+ precursors have been studied on a variety of tissues, organisms and in various conditions (Fang et al. 2017). With regards to muscle, there are indications that supplementation of NMN and NR has been shown to mitigate the age-related decline in mitochondrial capacity (Gomes et al. 2013; Zhang et al. 2016; Mills et al. 2016), increased endurance capacity (Cantó et al. 2012) and prevented muscle satellite cell senescence in mice (Zhang et al. 2016). Nevertheless, in humans, the few trials that

investigated supplementation of NAD^+ precursors did not show a beneficial effect on muscle health (Elhassan et al. 2019; Dollerup et al. 2020; Remie et al. 2020). However, supplementation was not able to greatly increase NAD^+ concentrations in the skeletal muscle in none of the aforementioned studies. Furthermore, it is not clear if levels of NAD^+ were deficient to start with, as no young or healthy control group were included. Translating rodent studies to the human situation is not straightforward, as some animal studies used an intraperitoneal supplementation of NAD^+ precursors, which was shown to increase skeletal muscle NAD^+ more potently compared to oral supplementation (Liu et al. 2018). In conclusion, while supplementation of NAD^+ precursors has shown beneficial effects on mitochondrial capacity in mice, so far studies in humans failed to show similar effects. Besides supplementation, there are other strategies to activate the sirtuins enzymes, such as caloric restriction, physical activity and pharmacological sirtuin activators (Bonkowski and Sinclair 2016). To what extend these strategies can be effective in modifying protein acylation in humans is yet to be explored.

8.5. Conclusions and further perspectives

In this thesis, I contribute to the understanding of the role of mitochondria in age-related loss of muscle mass and strength on the molecular and physiologic level. I show that NIRS assessment of mitochondrial capacity can be a valuable tool for research on muscle mitochondrial ageing and applied it in a well-controlled population of similarly active young and old males. In the same population, I show that muscle mitochondrial capacity is lower in older compared to young males in the *vastus lateralis* and *gastrocnemius* muscle, suggesting that, although increased physical activity was associated with better mitochondrial capacity, similar physical activity alone is not adequate to sustain mitochondrial capacity in all skeletal muscles at old age. Nevertheless, as mitochondrial capacity in the *tibialis anterior* was not significantly different between older and young males, the effect of age on mitochondrial capacity is expected to be muscle specific, be it due to differences in muscle use or intrinsic differences in muscle characteristics. This shows

that besides investigating and promoting physical activity interventions to sustain muscle function in elderly, at the same time, there should be ongoing research on the ageing mechanisms that occur independent of physical activity and how it affects different muscle groups. To more accurately and longitudinally measure physical activity in ageing studies, data from wearable health and activity sensors can be used in future studied investigating the effect of physical activity on muscle mitochondrial ageing.

Possible, yet to be identified, ageing mechanisms might be related to protein acylation and in specific protein propionylation. I show that protein propionylation is associated with mitochondrial dysfunction and impaired skeletal muscle differentiation. Whether protein propionylation also plays a role in ageing is not yet known, but data on compartmentalisation of propionyl-CoA in the nucleus and the role of sirtuins as anti-ageing genes that de-acylate targets provide promising clues. Nevertheless, pieces in this puzzle are still missing, such as what happens to the levels of propionyl-CoA when we age, whether the decrease in NAD^+ is important in muscle ageing, how increased mitochondrial propionyl-CoA can contribute to histone propionylation and what are the functional consequences? Therefore, besides research on the role of propionylation in ageing, more fundamental research on protein propionylation and propionyl-CoA metabolism is required. Elucidating these mechanisms will increase our understanding of muscle ageing and will drive discoveries how to modulate these mechanisms through pharmacological or lifestyle interventions.

REFERENCES

Aare S, Spendiff S, Vuda M, et al (2016) Failed reinnervation in aging skeletal muscle. Skelet Muscle 6:29. https://doi.org/10.1186/s13395-016-0101-y

Adami A, Cao R, Porszasz J, et al (2017) Reproducibility of NIRS assessment of muscle oxidative capacity in smokers with and without COPD. Respir Physiol Neurobiol 235:18–26. https://doi.org/10.1016/j.resp.2016.09.008

Adelnia F, Cameron D, Bergeron CM, et al (2019) The Role of Muscle Perfusion in the Age-Associated Decline of Mitochondrial Function in Healthy Individuals. Front Physiol 10:427. https://doi.org/10.3389/fphys.2019.00427

Ahn B-H, Kim H-S, Song S, et al (2008) A role for the mitochondrial deacetylase Sirt3 in regulating energy homeostasis. Proc Natl Acad Sci 105:14447–14452. https://doi.org/10.1073/pnas.0803790105

Amara CE, Shankland EG, Jubrias SA, et al (2007) Mild mitochondrial uncoupling impacts cellular aging in human muscles in vivo. Proc Natl Acad Sci U S A 104:1057–62. https://doi.org/10.1073/pnas.0610131104

Anagnostou M-E, Hepple RT (2020) Mitochondrial Mechanisms of Neuromuscular Junction Degeneration with Aging. Cells 9:197. https://doi.org/10.3390/cells9010197

Anders S, Pyl PT, Huber W (2015) HTSeq-A Python framework to work with high-throughput sequencing data. Bioinformatics 31:166–169. https://doi.org/10.1093/bioinformatics/btu638

Andrews S (2010) FastQC: A Quality Control Tool for High Throughput Sequence Data. http://www.bioinformatics.babraham.ac.uk/projects/fastqc/

Arikkath J, Reichardt LF (2008) Cadherins and catenins at synapses: roles in synaptogenesis and synaptic plasticity. Trends Neurosci 31:487–494. https://doi.org/10.1016/j.tins.2008.07.001

Baecke JA, Burema J, Frijters JE (1982) A short questionnaire for the measurement of habitual physical activity in epidemiological studies. Am J Clin Nutr 36:936–942. https://doi.org/10.1093/ajcn/36.5.936

Baeza J, Smallegan MJ, Denu JM (2016) Mechanisms and Dynamics of Protein Acetylation in Mitochondria. Trends Biochem Sci 41:231–244. https://doi.org/10.1016/j.tibs.2015.12.006

Bakula D, Scheibye-Knudsen M (2020) MitophAging: Mitophagy in Aging and Disease. Front. Cell Dev. Biol.

Balagopal P, Rooyackers OE, Adey DB, et al (1997) Effects of aging on in vivo synthesis of skeletal muscle myosin heavy-chain and sarcoplasmic protein in humans. Am J Physiol Metab 273:E790–E800. https://doi.org/10.1152/ajpendo.1997.273.4.E790

Balan E, Schwalm C, Naslain D, et al (2019) Regular Endurance Exercise Promotes Fission,

Mitophagy, and Oxidative Phosphorylation in Human Skeletal Muscle Independently of Age. Front Physiol 10:. https://doi.org/10.3389/fphys.2019.01088

Baldensperger T, Eggen M, Kappen J, et al (2020) Comprehensive analysis of posttranslational protein modifications in aging of subcellular compartments. Sci Rep 10:7596. https://doi.org/10.1038/s41598-020-64265-0

Bannister AJ, Kouzarides T (2011) Regulation of chromatin by histone modifications. Cell Res 21:381–395. https://doi.org/10.1038/cr.2011.22

Barrientos A, Casademont J, Rötig A, et al (1996) Absence of Relationship between the Level of Electron Transport Chain Activities and Aging in Human Skeletal Muscle. Biochem Biophys Res Commun 229:536–539. https://doi.org/10.1006/bbrc.1996.1839

Bassett DR, Howley ET (2000) Limiting factors for maximum oxygen uptake and determinants of endurance performance. Med Sci Sports Exerc 32:70–84. https://doi.org/10.1097/00005768-200001000-00012

Batterson PM, Norton MR, Hetz SE, et al (2020) Improving biologic predictors of cycling endurance performance with near-infrared spectroscopy derived measures of skeletal muscle respiration: E pluribus unum. Physiol Rep 8:e14342. https://doi.org/10.14814/phy2.14342

Beever AT, Tripp TR, Zhang J, MacInnis MJ (2020) NIRS-derived skeletal muscle oxidative capacity is correlated with aerobic fitness and independent of sex. J Appl Physiol 129:558–568. https://doi.org/10.1152/japplphysiol.00017.2020

Bergström J, Hermansen L, Hultman E, Saltin B (1967) Diet, Muscle Glycogen and Physical Performance. Acta Physiol Scand 71:140–150. https://doi.org/10.1111/j.1748-1716.1967.tb03720.x

Bessman SP (1987) The creatine phosphate energy shuttle—The molecular asymmetry of a "pool." Anal Biochem 161:519–523. https://doi.org/10.1016/0003-2697(87)90483-0

Birk JB, Wojtaszewski JFP (2006) Predominant α2/β2/γ3 AMPK activation during exercise in human skeletal muscle. J Physiol 577:1021–1032. https://doi.org/10.1113/jphysiol.2006.120972

Blau HM, Cosgrove BD, Ho AT V (2015) The central role of muscle stem cells in regenerative failure with aging. Nat Med 21:854–862. https://doi.org/10.1038/nm.3918

Boccia G, Dardanello D, Rosso V, et al (2015) The Application of sEMG in Aging: A Mini Review. Gerontology 61:477–484. https://doi.org/10.1159/000368655

Boers HE, Haroon M, Le Grand F, et al (2017) Mechanosensitiviy of aged muscle stem cells. J Orthop Res 36:632–642. https://doi.org/10.1002/jor.23797

Bogan KL, Brenner C (2008) Nicotinic Acid, Nicotinamide, and Nicotinamide Riboside: A Molecular Evaluation of NAD + Precursor Vitamins in Human Nutrition. Annu Rev Nutr 28:115–130. https://doi.org/10.1146/annurev.nutr.28.061807.155443

Bonkowski MS, Sinclair DA (2016) Slowing ageing by design: the rise of NAD+ and sirtuin-activating compounds. Nat Rev Mol Cell Biol 17:679–690. https://doi.org/10.1038/nrm.2016.93

Booth FW, Narahara KA (1974) Vastus lateralis cytochrome oxidase activity and its relationship to maximal oxygen consumption in man. Pflügers Arch Eur J Physiol 349:319–324. https://doi.org/10.1007/BF00588417

Boppart MD, Burkin DJ, Kaufman SJ (2006) α 7 β 1 -Integrin regulates mechanotransduction and prevents skeletal muscle injury. Am J Physiol Physiol 290:C1660–C1665. https://doi.org/10.1152/ajpcell.00317.2005

Boppart MD, Mahmassani ZS (2019) Integrin signaling: linking mechanical stimulation to skeletal muscle hypertrophy. Am J Physiol Physiol 317:C629–C641. https://doi.org/10.1152/ajpcell.00009.2019

Børsheim E, Bahr R (2003) Effect of Exercise Intensity, Duration and Mode on Post-Exercise Oxygen Consumption. Sport Med 33:1037–1060. https://doi.org/10.2165/00007256-200333140-00002

Bowers EM, Yan G, Mukherjee C, et al (2010) Virtual Ligand Screening of the p300/CBP Histone Acetyltransferase: Identification of a Selective Small Molecule Inhibitor. Chem Biol 17:471–482. https://doi.org/10.1016/j.chembiol.2010.03.006

Bracha AL, Ramanathan A, Huang S, et al (2010) Carbon metabolism–mediated myogenic differentiation. Nat Chem Biol 6:202–204. https://doi.org/10.1038/nchembio.301

Braidy N, Guillemin GJ, Mansour H, et al (2011) Age related changes in NAD+ metabolism oxidative stress and Sirt1 activity in wistar rats. PLoS One 6:e19194–e19194. https://doi.org/10.1371/journal.pone.0019194

Braschi B, Denny P, Gray K, et al (2018) Genenames.org: the HGNC and VGNC resources in 2019. Nucleic Acids Res 47:D786–D792. https://doi.org/10.1093/nar/gky930

Brehm BA, Gutin B (1986) Recovery energy expenditure for steady state exercise in runners and nonexercisers. Med Sci Sport Exerc 18:205–210. https://doi.org/10.1249/00005768-198604000-00010

Brierley EJ, Johnson MA, James OFW, Turnbull DM (1996) Effects of physical activity and age on mitochondrial function. QJM 89:251–258. https://doi.org/10.1093/qjmed/89.4.251

Brismar J, Ozand PT (1994) CT and MR of the brain in disorders of the propionate and methylmalonate metabolism. AJNR Am J Neuroradiol 15:1459–73

Brizendine JT, Ryan TE, Larson RD, McCully KK (2013) Skeletal muscle metabolism in endurance athletes with near-infrared spectroscopy. Med Sci Sports Exerc 45:869–

875. https://doi.org/10.1249/MSS.0b013e31827e0eb6

Bruyère O, Beaudart C, Ethgen O, et al (2019) The health economics burden of sarcopenia: a systematic review. Maturitas 119:61–69. https://doi.org/10.1016/j.maturitas.2018.11.003

Buso A, Comelli M, Picco R, et al (2019) Mitochondrial Adaptations in Elderly and Young Men Skeletal Muscle Following 2 Weeks of Bed Rest and Rehabilitation. Front Physiol 10:. https://doi.org/10.3389/fphys.2019.00474

Cai L, Sutter BM, Li B, Tu BP (2011) Acetyl-CoA Induces Cell Growth and Proliferation by Promoting the Acetylation of Histones at Growth Genes. Mol Cell 42:426–437. https://doi.org/10.1016/j.molcel.2011.05.004

Calve S, Odelberg SJ, Simon H-G (2010) A transitional extracellular matrix instructs cell behavior during muscle regeneration. Dev Biol 344:259–271. https://doi.org/10.1016/j.ydbio.2010.05.007

Calvo SE, Clauser KR, Mootha VK (2016) MitoCarta2.0: an updated inventory of mammalian mitochondrial proteins. Nucleic Acids Res 44:D1251–D1257. https://doi.org/10.1093/nar/gkv1003

Camacho-Pereira J, Tarragó MG, Chini CCS, et al (2016) CD38 Dictates Age-Related NAD Decline and Mitochondrial Dysfunction through an SIRT3-Dependent Mechanism. Cell Metab 23:1127–1139. https://doi.org/https://doi.org/10.1016/j.cmet.2016.05.006

Cantó C, Houtkooper RH, Pirinen E, et al (2012) The NAD+ Precursor Nicotinamide Riboside Enhances Oxidative Metabolism and Protects against High-Fat Diet-Induced Obesity. Cell Metab 15:838–847. https://doi.org/10.1016/j.cmet.2012.04.022

Cao Y, Kumar RM, Penn BH, et al (2006) Global and gene-specific analyses show distinct roles for Myod and Myog at a common set of promoters. EMBO J 25:502–511. https://doi.org/10.1038/sj.emboj.7600958

Carosio S, Berardinelli MG, Aucello M, Musarò A (2011) Impact of ageing on muscle cell regeneration. Ageing Res Rev 10:35–42. https://doi.org/10.1016/j.arr.2009.08.001

Carter HN, Chen CCW, Hood DA (2015) Mitochondria, Muscle Health, and Exercise with Advancing Age. Physiology 30:208–223. https://doi.org/10.1152/physiol.00039.2014

Catoire M, Mensink M, Boekschoten M V., et al (2012) Pronounced Effects of Acute Endurance Exercise on Gene Expression in Resting and Exercising Human Skeletal Muscle. PLoS One 7:e51066. https://doi.org/10.1371/journal.pone.0051066

Chance B, Dait MT, Zhang C, et al (1992) Recovery from exercise-induced desaturation in the quadriceps muscles of elite competitive rowers. Am J Physiol 262:C766-75. https://doi.org/10.1152/ajpcell.1992.262.3.C766

Chao LC, Wroblewski K, Ilkayeva OR, et al (2012) Skeletal muscle Nur77 expression enhances oxidative metabolism and substrate utilization. J Lipid Res 53:2610–2619. https://doi.org/10.1194/jlr.M029355

Charbit-Henrion F, Lacaille F, McKiernan P, et al (2015) Early and late complications after liver transplantation for propionic acidemia in children: A two centers study. Am J Transplant 15:786–791. https://doi.org/10.1111/ajt.13027

Chargé SBP, Rudnicki MA (2004) Cellular and Molecular Regulation of Muscle Regeneration. Physiol Rev 84:209–238. https://doi.org/10.1152/physrev.00019.2003

Cheema-Dhadli S, Leznoff CC, Halperin ML (1975) Effect of 2-Methylcitrate on Citrate Metabolism: Implications for the Management of Patients with Propionic acidemia and Methylmalonic aciduria. Pediatr Res 9:905–908. https://doi.org/10.1203/00006450-197512000-00008

Chen JCJ, Love CM, Goldhamer DJ (2001) Two upstream enhancers collaborate to regulate the spatial patterning and timing ofMyoD transcription during mouse development. Dev Dyn 221:274–288. https://doi.org/10.1002/dvdy.1138

Chen JCJ, Ramachandran R, Goldhamer DJ (2002) Essential and Redundant Functions of the MyoD Distal Regulatory Region Revealed by Targeted Mutagenesis. Dev Biol 245:213–223. https://doi.org/10.1006/dbio.2002.0638

Chen Y, Sprung R, Tang Y, et al (2007) Lysine propionylation and butyrylation are novel post-translational modifications in histones. Mol Cell Proteomics 6:812–819. https://doi.org/10.1074/mcp.M700021-MCP200

Cheng Z, Tang Y, Chen Y, et al (2009) Molecular Characterization of Propionyllysines in Non-histone Proteins. Mol Cell Proteomics 8:45–52. https://doi.org/10.1074/mcp.M800224-MCP200

Chilibeck P, Paterson D, McCreary C, et al (1998) The effects of age on kinetics of oxygen uptake and phosphocreatine in humans during exercise. Exp Physiol 83:107–117. https://doi.org/10.1113/expphysiol.1998.sp004087

Choi S, Reiter DA, Shardell M, et al (2016) ^{31} P Magnetic Resonance Spectroscopy Assessment of Muscle Bioenergetics as a Predictor of Gait Speed in the Baltimore Longitudinal Study of Aging. Journals Gerontol Ser A Biol Sci Med Sci 71:1638–1645. https://doi.org/10.1093/gerona/glw059

Chomistek AK, Yuan C, Matthews CE, et al (2017) Physical Activity Assessment with the ActiGraph GT3X and Doubly Labeled Water. Med Sci Sports Exerc 49:1935–1944. https://doi.org/10.1249/MSS.0000000000001299

Choudhary C, Weinert BT, Nishida Y, et al (2014) The growing landscape of lysine acetylation links metabolism and cell signalling. Nat Rev Mol Cell Biol 15:536–550. https://doi.org/10.1038/nrm3841

Christie AD, Tonson A, Larsen RG, et al (2014) Human skeletal muscle metabolic economy

in vivo: effects of contraction intensity, age, and mobility impairment. Am J Physiol Integr Comp Physiol 307:R1124–R1135. https://doi.org/10.1152/ajpregu.00083.2014

Chung S, Rosenberry R, Ryan TE, et al (2018) Near-infrared spectroscopy detects age-related differences in skeletal muscle oxidative function: promising implications for geroscience. Physiol Rep 6:e13588. https://doi.org/10.14814/phy2.13588

Clement J, Wong M, Poljak A, et al (2019) The Plasma NAD + Metabolome Is Dysregulated in "Normal" Aging. Rejuvenation Res 22:121–130. https://doi.org/10.1089/rej.2018.2077

Coen PM, Jubrias SA, Distefano G, et al (2013) Skeletal Muscle Mitochondrial Energetics Are Associated With Maximal Aerobic Capacity and Walking Speed in Older Adults. Journals Gerontol Ser A 68:447–455. https://doi.org/10.1093/gerona/gls196

Conley KE, Esselman PC, Jubrias SA, et al (2000a) Ageing, muscle properties and maximal O 2 uptake rate in humans. J Physiol 526:211–217. https://doi.org/10.1111/j.1469-7793.2000.00211.x

Conley KE, Jubrias SA, Esselman PC (2000b) Oxidative capacity and ageing in human muscle. J Physiol 526:203–210. https://doi.org/10.1111/j.1469-7793.2000.t01-1-00203.x

Costello JT, Bieuzen F, Bleakley CM (2014) Where are all the female participants in Sports and Exercise Medicine research? Eur J Sport Sci 14:847–851. https://doi.org/10.1080/17461391.2014.911354

Craig JC, Broxterman RM, Wilcox SL, et al (2017) Effect of adipose tissue thickness, muscle site, and sex on near-infrared spectroscopy derived total-[hemoglobin + myoglobin]. J Appl Physiol 123:1571–1578. https://doi.org/10.1152/japplphysiol.00207.2017

Crane JD, Devries MC, Safdar A, et al (2010) The Effect of Aging on Human Skeletal Muscle Mitochondrial and Intramyocellular Lipid Ultrastructure. Journals Gerontol Ser A Biol Sci Med Sci 65A:119–128. https://doi.org/10.1093/gerona/glp179

Cruz-Jentoft AJ, Sayer AA (2019) Sarcopenia. Lancet 393:2636–2646. https://doi.org/10.1016/S0140-6736(19)31138-9

Cummings JH, Pomare EW, Branch HWJ, et al (1987) Short chain fatty acids in human large intestine, portal, hepatic and venous blood. 28:122–123. https://doi.org/10.1136/gut.28.10.1221

Dalle S, Rossmeislova L, Koppo K (2017) The Role of Inflammation in Age-Related Sarcopenia. Front Physiol 8:10357–10361. https://doi.org/10.3389/fphys.2017.01045

Das S, Morvan F, Morozzi G, et al (2017) ATP Citrate Lyase Regulates Myofiber Differentiation and Increases Regeneration by Altering Histone Acetylation. Cell Rep 21:3003–3011. https://doi.org/10.1016/j.celrep.2017.11.038

Davis EJ, Spydevold O, Bremer J (1980) Pyruvate Carboxylase and Propionyl-CoA Carboxylase as Anaplerotic Enzymes in Skeletal Muscle Mitochondria. Eur J Biochem 110:255–262. https://doi.org/10.1111/j.1432-1033.1980.tb04863.x

De Baulny HO, Benoist JF, Rigal O, et al (2005) Methylmalonic and propionic acidaemias: Management and outcome. J Inherit Metab Dis 28:415–423. https://doi.org/10.1007/s10545-005-7056-1

De Blasi RA (1997) Comparison of two methods of measuring forearm oxygen consumption (VO2) by near infrared spectroscopy. J Biomed Opt 2:171. https://doi.org/10.1117/12.269893

de Boer MD, Seynnes OR, di Prampero PE, et al (2008) Effect of 5 weeks horizontal bed rest on human muscle thickness and architecture of weight bearing and non-weight bearing muscles. Eur J Appl Physiol 104:401–407. https://doi.org/10.1007/s00421-008-0703-0

de Keyzer Y, Valayannopoulos V, Benoist J-F, et al (2009) Multiple OXPHOS Deficiency in the Liver, Kidney, Heart, and Skeletal Muscle of Patients With Methylmalonic Aciduria and Propionic Aciduria. Pediatr Res 66:91–95. https://doi.org/10.1203/PDR.0b013e3181a7c270

Deato MDE, Marr MT, Sottero T, et al (2008) MyoD Targets TAF3/TRF3 to Activate Myogenin Transcription. Mol Cell 32:96–105. https://doi.org/10.1016/j.molcel.2008.09.009

Ding W-X, Yin X-M (2012) Mitophagy: mechanisms, pathophysiological roles, and analysis. Biol Chem 393:547–564. https://doi.org/https://doi.org/10.1515/hsz-2012-0119

Distefano G, Standley RA, Zhang X, et al (2018) Physical activity unveils the relationship between mitochondrial energetics, muscle quality, and physical function in older adults. J Cachexia Sarcopenia Muscle 9:279–294. https://doi.org/10.1002/jcsm.12272

Dobin A, Davis CA, Schlesinger F, et al (2013) STAR: Ultrafast universal RNA-seq aligner. Bioinformatics 29:15–21. https://doi.org/10.1093/bioinformatics/bts635

Dollerup OL, Chubanava S, Agerholm M, et al (2020) Nicotinamide riboside does not alter mitochondrial respiration, content or morphology in skeletal muscle from obese and insulin-resistant men. J Physiol. https://doi.org/10.1113/JP278752

Doppler K, Mittelbronn M, Bornemann A (2008) Myogenesis in human denervated muscle biopsies. Muscle Nerve 37:79–83. https://doi.org/10.1002/mus.20902

Drummond MJ, Addison O, Brunker L, et al (2014) Downregulation of E3 Ubiquitin Ligases and Mitophagy-Related Genes in Skeletal Muscle of Physically Inactive, Frail Older Women: A Cross-Sectional Comparison. Journals Gerontol Ser A Biol Sci Med Sci 69:1040–1048. https://doi.org/10.1093/gerona/glu004

Edgerton VR, Smith JL, Simpson DR (1975) Muscle fibre type populations of human leg muscles. 259–266

Edwards LM, Kemp GJ, Dwyer RM, et al (2013) Integrating muscle cell biochemistry and whole-body physiology in humans:31P-MRS data from the InSight trial. Sci Rep 3:1182. https://doi.org/10.1038/srep01182

Egan B, Zierath JR (2013) Exercise Metabolism and the Molecular Regulation of Skeletal Muscle Adaptation. Cell Metab 17:162–184. https://doi.org/10.1016/j.cmet.2012.12.012

Elhassan YS, Kluckova K, Fletcher RS, et al (2019) Nicotinamide Riboside Augments the Aged Human Skeletal Muscle NAD+ Metabolome and Induces Transcriptomic and Anti-inflammatory Signatures. Cell Rep 28:1717-1728.e6. https://doi.org/10.1016/j.celrep.2019.07.043

Ellis JM, Bowman CE, Wolfgang MJ (2015) Metabolic and Tissue-Specific Regulation of Acyl-CoA Metabolism. PLoS One 10:e0116587. https://doi.org/10.1371/journal.pone.0116587

Ellis K, Kerr J, Godbole S, et al (2016) Hip and Wrist Accelerometer Algorithms for Free-Living Behavior Classification. Med Sci Sports Exerc 48:933–40. https://doi.org/10.1249/MSS.0000000000000840

Erickson ML, Ryan TE, Young H-J, McCully KK (2013) Near-infrared assessments of skeletal muscle oxidative capacity in persons with spinal cord injury. Eur J Appl Physiol 113:2275–83. https://doi.org/10.1007/s00421-013-2657-0

European Commission Directorate-General for Economic and Financial Affairs (2015) The 2015 Ageing Report Economic and budgetary projections for the 28 EU Member States (2013-2060)

Ewald CY (2020) The Matrisome during Aging and Longevity: A Systems-Level Approach toward Defining Matreotypes Promoting Healthy Aging. Gerontology 66:266–274. https://doi.org/10.1159/000504295

Fang EF, Lautrup S, Hou Y, et al (2017) NAD+ in Aging: Molecular Mechanisms and Translational Implications. Trends Mol. Med.

Feldman JL, Baeza J, Denu JM (2013) Activation of the Protein Deacetylase SIRT6 by Long-chain Fatty Acids and Widespread Deacylation by Mammalian Sirtuins. J Biol Chem 288:31350–31356. https://doi.org/10.1074/jbc.C113.511261

Ferrington DA, Husom AD, Thompson L V (2005) Altered proteasome structure, function, and oxidation in aged muscle. FASEB J 19:1–24. https://doi.org/10.1096/fj.04-2578fje

Fitzgerald LF, Christie AD, Kent JA (2016) Heterogeneous effects of old age on human muscle oxidative capacity in vivo: a systematic review and meta-analysis. Appl Physiol Nutr Metab 41:1137–1145. https://doi.org/10.1139/apnm-2016-0195

Flynn EMM, Huang OWW, Poy F, et al (2015) A Subset of Human Bromodomains Recognizes Butyryllysine and Crotonyllysine Histone Peptide Modifications. Structure 23:1801–1814. https://doi.org/10.1016/j.str.2015.08.004

Forbes SC, Paganini AT, Slade JM, et al (2009) Phosphocreatine recovery kinetics following low- and high-intensity exercise in human triceps surae and rat posterior hindlimb muscles. Am J Physiol Integr Comp Physiol 296:R161–R170. https://doi.org/10.1152/ajpregu.90704.2008

Frey GC, Byrnes WC, Mazzeo RS (1993) Factors influencing excess postexercise oxygen consumption in trained and untrained women. Metabolism 42:822–828. https://doi.org/10.1016/0026-0495(93)90053-Q

Fritz KS, Green MF, Petersen DR, Hirschey MD (2013a) Ethanol Metabolism Modifies Hepatic Protein Acylation in Mice. PLoS One 8:e75868. https://doi.org/10.1371/journal.pone.0075868

Fritz KS, Green MF, Petersen DR, Hirschey MD (2013b) Ethanol Metabolism Modifies Hepatic Protein Acylation in Mice. PLoS One. https://doi.org/10.1371/journal.pone.0075868

Fritzen AM, Andersen SP, Qadri KAN, et al (2020) Effect of Aerobic Exercise Training and Deconditioning on Oxidative Capacity and Muscle Mitochondrial Enzyme Machinery in Young and Elderly Individuals. J Clin Med 9:3113. https://doi.org/10.3390/jcm9103113

Fujino T, Kondo J, Ishikawa M, et al (2001) Acetyl-CoA Synthetase 2, a Mitochondrial Matrix Enzyme Involved in the Oxidation of Acetate. J Biol Chem 276:11420–11426. https://doi.org/10.1074/jbc.M008782200

Gaesser GA, Brooks GA (1984) Metabolic bases of excess post-exercise oxygen consumption: a review. Med Sci Sports Exerc 16:29–43. https://doi.org/6369064

Garrity J, Gardner JG, Hawse W, et al (2007) N-lysine propionylation controls the activity of propionyl-CoA synthetase. J Biol Chem 282:30239–45. https://doi.org/10.1074/jbc.M704409200

Gezondheidsraad (2017) Beweegrichtlijnen 2017

Gheller BJF, Riddle ES, Lem MR, Thalacker-Mercer AE (2016) Understanding Age-Related Changes in Skeletal Muscle Metabolism: Differences Between Females and Males. Annu Rev Nutr 36:129–156. https://doi.org/10.1146/annurev-nutr-071715-050901

Gilbert PM, Havenstrite KL, Magnusson KEG, et al (2010) Substrate Elasticity Regulates Skeletal Muscle Stem Cell Self-Renewal in Culture. Science (80-) 329:1078–1081. https://doi.org/10.1126/science.1191035

Glancy B, Balaban RS (2012) Role of Mitochondrial Ca 2+ in the Regulation of Cellular Energetics. Biochemistry 51:2959–2973. https://doi.org/10.1021/bi2018909

Goel AJ, Rieder M-K, Arnold H-H, et al (2017) Niche Cadherins Control the Quiescence-to-

Activation Transition in Muscle Stem Cells. Cell Rep 21:2236–2250. https://doi.org/10.1016/j.celrep.2017.10.102

Goldhamer DJ, Brunk BP, Faerman A, et al (1995) Embryonic activation of the myoD gene is regulated by a highly conserved distal control element. Development 121:637–649

Gomes AP, Price NL, Ling AJYY, et al (2013) Declining NAD(+) induces a pseudohypoxic state disrupting nuclear-mitochondrial communication during aging. Cell 155:1624–1638. https://doi.org/10.1016/j.cell.2013.11.037

Gomes M, Figueiredo D, Teixeira L, et al (2017) Physical inactivity among older adults across Europe based on the SHARE database. Age Ageing. https://doi.org/10.1093/ageing/afw165

Gonzalez-Freire M, de Cabo R, Studenski SA, Ferrucci L (2014) The Neuromuscular Junction: Aging at the Crossroad between Nerves and Muscle. Front Aging Neurosci 6:71–77. https://doi.org/10.3389/fnagi.2014.00208

Goodpaster BH, Park SW, Harris TB, et al (2006) The Loss of Skeletal Muscle Strength, Mass, and Quality in Older Adults: The Health, Aging and Body Composition Study. Journals Gerontol Ser A Biol Sci Med Sci 61:1059–1064. https://doi.org/10.1093/gerona/61.10.1059

Gorisch SM (2005) Histone acetylation increases chromatin accessibility. J Cell Sci 118:5825–5834. https://doi.org/10.1242/jcs.02689

Gosselin LE, Adams C, Cotter TA, et al (1998) Effect of exercise training on passive stiffness in locomotor skeletal muscle: role of extracellular matrix. J Appl Physiol 85:1011–1016. https://doi.org/10.1152/jappl.1998.85.3.1011

Gouspillou G, Sgarioto N, Kapchinsky S, et al (2014) Increased sensitivity to mitochondrial permeability transition and myonuclear translocation of endonuclease G in atrophied muscle of physically active older humans. FASEB J 28:1621–1633. https://doi.org/10.1096/fj.13-242750

Grassi B, Quaresima V (2016) Near-infrared spectroscopy and skeletal muscle oxidative function in vivo in health and disease: a review from an exercise physiology perspective. J Biomed Opt 21:91313. https://doi.org/10.1117/1.JBO.21.9.091313

Grünert SC, Müllerleile S, De Silva L, et al (2012) Propionic acidemia: Neonatal versus selective metabolic screening. J Inherit Metab Dis 35:41–49. https://doi.org/10.1007/s10545-011-9419-0

Grünert SC, Müllerleile S, De Silva L, et al (2013) Propionic acidemia: Clinical course and outcome in 55 pediatric and adolescent patients. Orphanet J Rare Dis 8:. https://doi.org/10.1186/1750-1172-8-6

Guralnik JM, Simonsick EM, Ferrucci L, et al (1994) A short physical performance battery assessing lower extremity function: association with self-reported disability and prediction of mortality and nursing home admission. J Gerontol 49:M85-94

Haider S, Grabovac I, Dorner TE (2019) Effects of physical activity interventions in frail and prefrail community-dwelling people on frailty status, muscle strength, physical performance and muscle mass—a narrative review. Wien. Klin. Wochenschr.

Haijes HA, Jans JJM, Tas SY, et al (2019) Pathophysiology of propionic and methylmalonic acidemias. Part 1: Complications. J Inherit Metab Dis 42:730–744. https://doi.org/10.1002/jimd.12129

Hall ZW, Sanes JR (1993) Synaptic structure and development: The neuromuscular junction. Cell 72:99–121. https://doi.org/10.1016/S0092-8674(05)80031-5

Hallal PC, Andersen LB, Bull FC, et al (2012) Global physical activity levels: surveillance progress, pitfalls, and prospects. Lancet 380:247–257. https://doi.org/10.1016/S0140-6736(12)60646-1

Hamaoka T, Iwane H, Shimomitsu T, et al (1996) Noninvasive measures of oxidative metabolism on working human muscles by near-infrared spectroscopy. J Appl Physiol 81:1410–1417. https://doi.org/10.1152/jappl.1996.81.3.1410

Hamaoka T, McCully KK, Niwayama M, Chance B (2011) The use of muscle near-infrared spectroscopy in sport, health and medical sciences: recent developments. Philos Trans R Soc A Math Phys Eng Sci 369:4591–4604. https://doi.org/10.1098/rsta.2011.0298

Hamed M, Khilji S, Chen J, Li Q (2013) Stepwise acetyltransferase association and histone acetylation at the Myod1 locus during myogenic differentiation. Sci Rep 3:2390. https://doi.org/10.1038/srep02390

Hamner SR, Seth A, Delp SL (2010) Muscle contributions to propulsion and support during running. J Biomech 43:2709–16. https://doi.org/10.1016/j.jbiomech.2010.06.025

Han Z, Wu H, Kim S, et al (2018) Revealing the protein propionylation activity of the histone acetyltransferase MOF (males absent on the first). J Biol Chem 293:3410–3420. https://doi.org/10.1074/jbc.RA117.000529

Handschin C, Spiegelman BM (2006) Peroxisome Proliferator-Activated Receptor γ Coactivator 1 Coactivators, Energy Homeostasis, and Metabolism. Endocr Rev 27:728–735. https://doi.org/10.1210/er.2006-0037

Hangelbroek RWJ, Fazelzadeh P, Tieland M, et al (2016) Expression of protocadherin gamma in skeletal muscle tissue is associated with age and muscle weakness. J Cachexia Sarcopenia Muscle 7:604–614. https://doi.org/10.1002/jcsm.12099

Harman D (1972) The Biologic Clock: The Mitochondria? J Am Geriatr Soc 20:145–147. https://doi.org/10.1111/j.1532-5415.1972.tb00787.x

Hart CR, Layec G, Trinity JD, et al (2015) Evidence of Preserved Oxidative Capacity and

Oxygen Delivery in the Plantar Flexor Muscles With Age. Journals Gerontol Ser A Biol Sci Med Sci 70:1067–1076. https://doi.org/10.1093/gerona/glu139

Haseler LJ, Lin A, Hoff J, Richardson RS (2007) Oxygen availability and PCr recovery rate in untrained human calf muscle: evidence of metabolic limitation in normoxia. Am J Physiol Integr Comp Physiol 293:R2046–R2051. https://doi.org/10.1152/ajpregu.00039.2007

Haugen F, Norheim F, Lian H, et al (2010) IL-7 is expressed and secreted by human skeletal muscle cells. Am J Physiol Physiol 298:C807–C816. https://doi.org/10.1152/ajpcell.00094.2009

Hedges CP, Woodhead JST, Wang HW, et al (2019) Peripheral blood mononuclear cells do not reflect skeletal muscle mitochondrial function or adaptation to high-intensity interval training in healthy young men. J Appl Physiol 126:454–461. https://doi.org/10.1152/japplphysiol.00777.2018

Hirschey MD, Shimazu T, Goetzman E, et al (2010) SIRT3 regulates mitochondrial fatty-acid oxidation by reversible enzyme deacetylation. Nature 464:121–125. https://doi.org/10.1038/nature08778

Holloszy JO, Coyle EF (1984) Adaptations of skeletal muscle to endurance exercise and their metabolic consequences. J Appl Physiol 56:831–8. https://doi.org/10.1152/jappl.1984.56.4.831

Hommes FA, Kuipers JRG, Elema JD, et al (1968) Acidosis propionic acid hereditary disease propionyl coenzyme liver, fatty A carboxylase metabolic disease Propionicacidemia, a New Inborn Error of Metabolism

Hortobágyi T, DeVita P (2000) Muscle pre- and coactivity during downward stepping are associated with leg stiffness in aging. J Electromyogr Kinesiol 10:117–126. https://doi.org/10.1016/S1050-6411(99)00026-7

Hotta K, Behnke BJ, Arjmandi B, et al (2018) Daily muscle stretching enhances blood flow, endothelial function, capillarity, vascular volume and connectivity in aged skeletal muscle. J Physiol 596:1903–1917. https://doi.org/10.1113/JP275459

Hughes DC, Ellefsen S, Baar K (2018) Adaptations to Endurance and Strength Training. Cold Spring Harb Perspect Med 8:a029769. https://doi.org/10.1101/cshperspect.a029769

Hughes DC, Marcotte GR, Marshall AG, et al (2016) Age-related Differences in Dystrophin: Impact on Force Transfer Proteins, Membrane Integrity, and Neuromuscular Junction Stability. Journals Gerontol Ser A Biol Sci Med Sci 72:640–648. https://doi.org/10.1093/gerona/glw109

Hütter E, Skovbro M, Lener B, et al (2007) Oxidative stress and mitochondrial impairment can be separated from lipofuscin accumulation in aged human skeletal muscle. Aging Cell 6:245–256. https://doi.org/10.1111/j.1474-9726.2007.00282.x

Iqbal S, Ostojic O, Singh K, et al (2013) Expression of mitochondrial fission and fusion regulatory proteins in skeletal muscle during chronic use and disuse. Muscle Nerve 48:963–970. https://doi.org/10.1002/mus.23838

Irintchev A, Zeschnigk M, Starzinski-Powitz A, Wernig A (1994) Expression pattern of M-cadherin in normal, denervated, and regenerating mouse muscles. Dev Dyn 199:326–337. https://doi.org/10.1002/aja.1001990407

Irving BA, Lanza IR, Henderson GC, et al (2015) Combined Training Enhances Skeletal Muscle Mitochondrial Oxidative Capacity Independent of Age. J Clin Endocrinol Metab 100:1654–1663. https://doi.org/10.1210/jc.2014-3081

Jager S, Handschin C, St.-Pierre J, Spiegelman BM (2007) AMP-activated protein kinase (AMPK) action in skeletal muscle via direct phosphorylation of PGC-1. Proc Natl Acad Sci 104:12017–12022. https://doi.org/10.1073/pnas.0705070104

Jakobsson F, Borg K, Edström L, Grimby L (1988) Use of motor units in relation to muscle fiber type and size in man. Muscle Nerve 11:1211–1218. https://doi.org/10.1002/mus.880111205

Janssen I, Heymsfield SB, Ross R (2002) Low Relative Skeletal Muscle Mass (Sarcopenia) in Older Persons Is Associated with Functional Impairment and Physical Disability. J Am Geriatr Soc 50:889–896. https://doi.org/10.1046/j.1532-5415.2002.50216.x

Janssen I, Heymsfield SB, Wang Z, Ross R (2000) Skeletal muscle mass and distribution in 468 men and women aged 18–88 yr. J Appl Physiol 89:81–88. https://doi.org/10.1152/jappl.2000.89.1.81

Jobsis F (1977) Noninvasive, infrared monitoring of cerebral and myocardial oxygen sufficiency and circulatory parameters. Science (80-) 198:1264–1267. https://doi.org/10.1126/science.929199

Johannsen DL, Conley KE, Bajpeyi S, et al (2012) Ectopic Lipid Accumulation and Reduced Glucose Tolerance in Elderly Adults Are Accompanied by Altered Skeletal Muscle Mitochondrial Activity. J Clin Endocrinol Metab 97:242–250. https://doi.org/10.1210/jc.2011-1798

Johnson MA, Polgar J, Weightman D, Appleton D (1973) Data on the distribution of fibre types in thirty-six human muscles. J Neurol Sci 18:111–129. https://doi.org/10.1016/0022-510X(73)90023-3

Jørgensen LH, Jensen CH, Wewer UM, Schrøder HD (2007) Transgenic Overexpression of ADAM12 Suppresses Muscle Regeneration and Aggravates Dystrophy in Aged mdx Mice. Am J Pathol 171:1599–1607. https://doi.org/10.2353/ajpath.2007.070435

Kaczmarska Z, Ortega E, Goudarzi A, et al (2017) Structure of p300 in complex with acyl-CoA variants. Nat Chem Biol 13:21–29. https://doi.org/10.1038/nchembio.2217

Kawasaki E, Hokari F, Sasaki M, et al (2009) Role of local muscle contractile activity in the

exercise-induced increase in NR4A receptor mRNA expression. J Appl Physiol 106:1826–1831. https://doi.org/10.1152/japplphysiol.90923.2008

Kebede AF, Nieborak A, Shahidian LZ, et al (2017) Histone propionylation is a mark of active chromatin. Nat Struct Mol Biol 24:1048–1056. https://doi.org/10.1038/nsmb.3490

Kemp GJ, Ahmad RE, Nicolay K, Prompers JJ (2015) Quantification of skeletal muscle mitochondrial function by 31 P magnetic resonance spectroscopy techniques: a quantitative review. Acta Physiol 213:107–144. https://doi.org/10.1111/apha.12307

Kent-Braun JA, Ng A V. (2000) Skeletal muscle oxidative capacity in young and older women and men. J Appl Physiol 89:1072–1078. https://doi.org/10.1152/jappl.2000.89.3.1072

Kent JA, Fitzgerald LF (2016) In vivo mitochondrial function in aging skeletal muscle: capacity, flux, and patterns of use. J Appl Physiol 121:996–1003

Kiss T, Balasubramanian P, Valcarcel-Ares MN, et al (2019) Nicotinamide mononucleotide (NMN) treatment attenuates oxidative stress and rescues angiogenic capacity in aged cerebromicrovascular endothelial cells: a potential mechanism for the prevention of vascular cognitive impairment. GeroScience 41:619–630. https://doi.org/10.1007/s11357-019-00074-2

Koga S, Barstow TJ, Okushima D, et al (2015) Validation of a high-power, time-resolved, near-infrared spectroscopy system for measurement of superficial and deep muscle deoxygenation during exercise. J Appl Physiol 118:1435–1442

Korzeniewski B, Rossiter HB (2015) Each-step activation of oxidative phosphorylation is necessary to explain muscle metabolic kinetic responses to exercise and recovery in humans. J Physiol 593:5255–5268. https://doi.org/10.1113/JP271299

Lagerwaard B, Keijer J, McCully KK, et al (2019) In vivo assessment of muscle mitochondrial function in healthy, young males in relation to parameters of aerobic fitness. Eur J Appl Physiol 119:1799–1808. https://doi.org/10.1007/s00421-019-04169-8

Lagerwaard B, Nieuwenhuizen AG, de Boer VCJ, Keijer J (2020a) In vivo assessment of mitochondrial capacity using NIRS in locomotor muscles of young and elderly males with similar physical activity levels. GeroScience 42:299–310. https://doi.org/10.1007/s11357-019-00145-4

Lagerwaard B, Pougovkina O, Bekebrede AF, et al (2020b) Increased protein propionylation contributes to mitochondrial dysfunction in liver cells and fibroblasts, but not in myotubes. J Inherit Metab Dis jimd.12296. https://doi.org/10.1002/jimd.12296

Lalia AZ, Dasari S, Robinson MM, et al (2017) Influence of omega-3 fatty acids on skeletal muscle protein metabolism and mitochondrial bioenergetics in older adults. Aging (Albany NY) 9:1096–1129. https://doi.org/10.18632/aging.101210

Lanza IR, Befroy DE, Kent-Braun JA (2005) Age-related changes in ATP-producing pathways in human skeletal muscle in vivo. J Appl Physiol 99:1736–1744. https://doi.org/10.1152/japplphysiol.00566.2005

Lanza IR, Bhagra S, Nair KS, Port JD (2011) Measurement of human skeletal muscle oxidative capacity by 31P-MR spectroscopy: a cross-validation with in vitro measurements. J Magn Reson Imaging 34:1143–50. https://doi.org/10.1002/jmri.22733

Lanza IR, Larsen RG, Kent-Braun JA (2007) Effects of old age on human skeletal muscle energetics during fatiguing contractions with and without blood flow. J Physiol 583:1093–1105. https://doi.org/10.1113/jphysiol.2007.138362

Lanza IR, Nair KS (2009) Muscle mitochondrial changes with aging and exercise. Am J Clin Nutr 89:467S-471S. https://doi.org/10.3945/ajcn.2008.26717D

Lanza IR, Short DK, Short KR, et al (2008) Endurance exercise as a countermeasure for aging. Diabetes 57:2933–2942. https://doi.org/10.2337/db08-0349

Larsen RG, Befroy DE, Kent-Braun JA (2013) High-intensity interval training increases in vivo oxidative capacity with no effect on P(i)→ATP rate in resting human muscle. Am J Physiol Regul Integr Comp Physiol 304:R333–R342. https://doi.org/10.1152/ajpregu.00409.2012

Larsen RG, Callahan DM, Foulis SA, Kent-Braun JA (2012) Age-related changes in oxidative capacity differ between locomotory muscles and are associated with physical activity behavior. Appl Physiol Nutr Metab 37:88–99. https://doi.org/10.1139/h11-135

Larsen RG, Callahan DM, Foulis SA, Kent-Braun JA (2009) In vivo oxidative capacity varies with muscle and training status in young adults. J Appl Physiol 107:873–879. https://doi.org/10.1152/japplphysiol.00260.2009

Larson-Meyer DE, Newcomer BR, Hunter GR, et al (2001) Relation between in vivo and in vitro measurements of skeletal muscle oxidative metabolism. Muscle Nerve 24:1665–1676. https://doi.org/10.1002/mus.1202

Larson-Meyer DE, Newcomer BR, Hunter GR, et al (2000) 31P MRS measurement of mitochondrial function in skeletal muscle: reliability, force-level sensitivity and relation to whole body maximal oxygen uptake. NMR Biomed 13:14–27. https://doi.org/10.1002/(SICI)1099-1492(200002)13:1<14::AID-NBM605>3.0.CO;2-0

Layec G, Hart CR, Trinity JD, et al (2015) Skeletal muscle work efficiency with age: the role of non-contractile processes. Clin Sci 128:213–223. https://doi.org/10.1042/CS20140274

Layec G, Haseler LJ, Richardson RS (2013) Reduced muscle oxidative capacity is independent of O2 availability in elderly people. Age (Omaha) 35:1183–1192. https://doi.org/10.1007/s11357-012-9442-6

Leduc-Gaudet J-P, Picard M, Pelletier FS-J, et al (2015) Mitochondrial morphology is altered in atrophied skeletal muscle of aged mice. Oncotarget 6:17923–17937. https://doi.org/10.18632/oncotarget.4235

Leemhuis H, Packman LC, Nightingale KP, Hollfelder F (2008) The Human Histone Acetyltransferase P/CAF is a Promiscuous Histone Propionyltransferase. ChemBioChem 9:499–503. https://doi.org/10.1002/cbic.200700556

Lexell J, Taylor CC, Sjöström M (1988) What is the cause of the ageing atrophy? J Neurol Sci 84:275–294. https://doi.org/10.1016/0022-510X(88)90132-3

Li Y, Lee Y Il, Thompson WJ (2011) Changes in Aging Mouse Neuromuscular Junctions Are Explained by Degeneration and Regeneration of Muscle Fiber Segments at the Synapse. J Neurosci 31:14910–14919. https://doi.org/10.1523/JNEUROSCI.3590-11.2011

Lilleker J, Woodall A, Roberts M, Chinoy H (2016) Tibialis anterior muscle biopsy - a patient reported outcome study. J Neurol Neurosurg Psychiatry 87:e1.92-e1. https://doi.org/10.1136/jnnp-2016-315106.181

Liu B, Lin Y, Darwanto A, et al (2009) Identification and Characterization of Propionylation at Histone H3 Lysine 23 in Mammalian Cells. J Biol Chem 284:32288–32295. https://doi.org/10.1074/jbc.M109.045856

Liu L, Su X, Quinn WJ, et al (2018) Quantitative Analysis of NAD Synthesis-Breakdown Fluxes. Cell Metab 27:1067-1080.e5. https://doi.org/10.1016/j.cmet.2018.03.018

Ljubicic V, Joseph A-M, Saleem A, et al (2010) Transcriptional and post-transcriptional regulation of mitochondrial biogenesis in skeletal muscle: Effects of exercise and aging. Biochim Biophys Acta - Gen Subj 1800:223–234. https://doi.org/10.1016/j.bbagen.2009.07.031

Lombard DB, Alt FW, Cheng H-L, et al (2007) Mammalian Sir2 Homolog SIRT3 Regulates Global Mitochondrial Lysine Acetylation. Mol Cell Biol. https://doi.org/10.1128/mcb.01636-07

Lombard DB, Dash BP, Kumar S (2015) Acetyl-ed question in mitochondrial biology? EMBO J 34:2597–600. https://doi.org/10.15252/embj.201592927

Lonsdale J, Thomas J, Salvatore M, et al (2013) The Genotype-Tissue Expression (GTEx) project. Nat. Genet.

López-Otín C, Blasco MA, Partridge L, et al (2013a) The Hallmarks of Aging. Cell 153:1194–1217. https://doi.org/10.1016/j.cell.2013.05.039

López-Otín C, Blasco MA, Partridge L, et al (2013b) The Hallmarks of Aging Europe PMC Funders Group. Cell June 6:1194–121705. https://doi.org/10.1016/j.cell.2013.05.039

Love MI, Huber W, Anders S (2014) Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol 15:550. https://doi.org/10.1186/s13059-014-0550-8

Madiraju P, Pande S V., Prentki M, Madiraju SRM (2009) Mitochondrial acetylcarnitine provides acetyl groups for nuclear histone acetylation. Epigenetics 4:399–403. https://doi.org/10.4161/epi.4.6.9767

Mahoney DJ, Parise G, Melov S, et al (2005) Analysis of global mRNA expression in human skeletal muscle during recovery from endurance exercise. FASEB J 19:1498–1500. https://doi.org/10.1096/fj.04-3149fje

Makhnovskii PA, Zgoda VG, Bokov RO, et al (2020) Regulation of Proteins in Human Skeletal Muscle: The Role of Transcription. Sci Rep 10:3514. https://doi.org/10.1038/s41598-020-60578-2

Mann CJ, Perdiguero E, Kharraz Y, et al (2011) Aberrant repair and fibrosis development in skeletal muscle. Skelet Muscle 1:21. https://doi.org/10.1186/2044-5040-1-21

Maresh CM, Abraham A, De Souza MJ, et al (1992) Oxygen consumption following exercise of moderate intensity and duration. Eur J Appl Physiol Occup Physiol 65:421–426. https://doi.org/10.1007/BF00243508

Marzetti E, Calvani R, Cesari M, et al (2013) Mitochondrial dysfunction and sarcopenia of aging: From signaling pathways to clinical trials. Int J Biochem Cell Biol 45:2288–2301. https://doi.org/10.1016/J.BIOCEL.2013.06.024

Massudi H, Grant R, Braidy N, et al (2012) Age-associated changes in oxidative stress and NAD+ metabolism in human tissue. PLoS One 7:. https://doi.org/10.1371/journal.pone.0042357

Matsuishi T, Stumpf DA, Seliem M, et al (1991) Propionate mitochondrial toxicity in liver and skeletal muscle: acyl CoA levels. Biochem Med Metab Biol 45:244–53

McCully KK, Fielding RA, Evans WJ, et al (1993) Relationships between in vivo and in vitro measurements of metabolism in young and old human calf muscles. 75:813–81. https://doi.org/8226486

McMahon S, Jenkins D (2002) Factors Affecting the Rate of Phosphocreatine Resynthesis Following Intense Exercise. Sport Med 32:761–784. https://doi.org/10.2165/00007256-200232120-00002

McNeil CJ, Doherty TJ, Stashuk DW, Rice CL (2005) Motor unit number estimates in the tibialis anterior muscle of young, old, and very old men. Muscle Nerve 31:461–467. https://doi.org/10.1002/mus.20276

Metter EJ, Talbot LA, Schrager M, Conwit R (2002) Skeletal Muscle Strength as a Predictor of All-Cause Mortality in Healthy Men. Journals Gerontol Ser A Biol Sci Med Sci 57:B359–B365. https://doi.org/10.1093/gerona/57.10.B359

Meyer R a (1988) A linear model of muscle respiration explains monoexponential phosphocreatine changes. Am J Physiol Physiol 254:C548–C553. https://doi.org/10.1152/ajpcell.1988.254.4.C548

Migliavacca E, Tay SKH, Patel HP, et al (2019) Mitochondrial oxidative capacity and NAD+ biosynthesis are reduced in human sarcopenia across ethnicities. Nat Commun 10:5808. https://doi.org/10.1038/s41467-019-13694-1

Mills KF, Yoshida S, Stein LR, et al (2016) Long-Term Administration of Nicotinamide Mononucleotide Mitigates Age-Associated Physiological Decline in Mice. Cell Metab 24:795–806. https://doi.org/10.1016/j.cmet.2016.09.013

Mitchell WK, Williams J, Atherton P, et al (2012) Sarcopenia, Dynapenia, and the Impact of Advancing Age on Human Skeletal Muscle Size and Strength; a Quantitative Review. Front Physiol 3:. https://doi.org/10.3389/fphys.2012.00260

Morley JE, Baumgartner RN, Roubenoff R, et al (2001) Sarcopenia. J Lab Clin Med 137:231–243. https://doi.org/10.1067/MLC.2001.113504

Motobe M, Murase N, Osada T, et al (2004) Noninvasive monitoring of deterioration in skeletal muscle function with forearm cast immobilization and the prevention of deterioration. Dyn Med 3:2. https://doi.org/10.1186/1476-5918-3-2

Mouw JK, Ou G, Weaver VM (2014) Extracellular matrix assembly: a multiscale deconstruction. Nat Rev Mol Cell Biol 15:771–785. https://doi.org/10.1038/nrm3902

Nacarelli T, Azar A, Altinok O, et al (2018) Rapamycin increases oxidative metabolism and enhances metabolic flexibility in human cardiac fibroblasts. GeroScience 40:243–256. https://doi.org/10.1007/s11357-018-0030-2

Nagasawa T, Hamaoka T, Sako T, et al (2003) A practical indicator of muscle oxidative capacity determined by recovery of muscle O2 consumption using NIR spectroscopy. Eur J Sport Sci 3:1–10. https://doi.org/10.1080/17461390300073207

Ngo JK, Davies KJA (2007) Importance of the Lon Protease in Mitochondrial Maintenance and the Significance of Declining Lon in Aging. Ann N Y Acad Sci 1119:78–87. https://doi.org/10.1196/annals.1404.015

O'Driscoll R, Turicchi J, Beaulieu K, et al (2018) How well do activity monitors estimate energy expenditure? A systematic review and meta-analysis of the validity of current technologies. Br J Sports Med bjsports-2018-099643. https://doi.org/10.1136/bjsports-2018-099643

Olshansky SJ (2018) From Lifespan to Healthspan. JAMA 320:1323. https://doi.org/10.1001/jama.2018.12621

Park SW, Goodpaster BH, Strotmeyer ES, et al (2007) Accelerated Loss of Skeletal Muscle Strength in Older Adults With Type 2 Diabetes: The Health, Aging, and Body Composition Study. Diabetes Care 30:1507–1512. https://doi.org/10.2337/dc06-2537

Peng C, Lu Z, Xie Z, et al (2011) The First Identification of Lysine Malonylation Substrates and Its Regulatory Enzyme. Mol Cell Proteomics 10:M111.012658. https://doi.org/10.1074/mcp.M111.012658

Perrey S, Ferrari M (2018) Muscle Oximetry in Sports Science: A Systematic Review. Sport Med 48:597–616. https://doi.org/10.1007/s40279-017-0820-1

Phielix E, Meex R, Ouwens DM, et al (2012) High Oxidative Capacity Due to Chronic Exercise Training Attenuates Lipid-Induced Insulin Resistance. Diabetes 61:2472–2478. https://doi.org/10.2337/db11-1832

Philippaerts R, Westerterp K, Lefevre J (1999) Doubly Labeled Water Validation of three Physical Activity Questionnaires. Int J Sports Med 20:284–289. https://doi.org/10.1055/s-2007-971132

Picard M, Ritchie D, Thomas MM, et al (2011) Alterations in intrinsic mitochondrial function with aging are fiber type-specific and do not explain differential atrophy between muscles. Aging Cell 10:1047–1055. https://doi.org/10.1111/j.1474-9726.2011.00745.x

Picard M, Ritchie D, Wright KJ, et al (2010) Mitochondrial functional impairment with aging is exaggerated in isolated mitochondria compared to permeabilized myofibers. Aging Cell 9:1032–1046. https://doi.org/10.1111/j.1474-9726.2010.00628.x

Pillon NJ, Gabriel BM, Dollet L, et al (2020) Transcriptomic profiling of skeletal muscle adaptations to exercise and inactivity. Nat Commun 11:470. https://doi.org/10.1038/s41467-019-13869-w

Plasqui G, Bonomi AG, Westerterp KR (2013) Daily physical activity assessment with accelerometers: new insights and validation studies. Obes Rev 14:451–462. https://doi.org/10.1111/obr.12021

Porter C, Hurren NM, Cotter M V., et al (2015) Mitochondrial respiratory capacity and coupling control decline with age in human skeletal muscle. Am J Physiol Metab 309:E224–E232. https://doi.org/10.1152/ajpendo.00125.2015

Pougovkina O, te Brinke H, Ofman R, et al (2014a) Mitochondrial protein acetylation is driven by acetyl-CoA from fatty acid oxidation. Hum Mol Genet 23:3513–3522. https://doi.org/10.1093/hmg/ddu059

Pougovkina O, te Brinke H, Wanders RJA, et al (2014b) Aberrant protein acylation is a common observation in inborn errors of acyl-CoA metabolism. J Inherit Metab Dis 37:709–714. https://doi.org/10.1007/s10545-014-9684-9

Prass RL, Isohashi F, Utter MF (1980) Purification and characterization of an extramitochondrial acetyl coenzyme A hydrolase from rat liver. J Biol Chem 11:5215–1223

Prince MJ, Wu F, Guo Y, et al (2015) The burden of disease in older people and implications for health policy and practice. Lancet 385:549–562. https://doi.org/10.1016/S0140-6736(14)61347-7

Punga AR, Ruegg MA (2012) Signaling and aging at the neuromuscular synapse: lessons learnt from neuromuscular diseases. Curr Opin Pharmacol 12:340–346. https://doi.org/10.1016/j.coph.2012.02.002

Ramaswamy KS, Palmer ML, van der Meulen JH, et al (2011) Lateral transmission of force is impaired in skeletal muscles of dystrophic mice and very old rats. J Physiol 589:1195–1208. https://doi.org/10.1113/jphysiol.2010.201921

Rasmussen UF, Krustrup P, Kjær M, Rasmussen HN (2003) Experimental evidence against the mitochondrial theory of aging A study of isolated human skeletal muscle mitochondria. Exp Gerontol 38:877–886. https://doi.org/10.1016/S0531-5565(03)00092-5

Re R, Pirovano I, Contini D, et al (2018) Time Domain Near Infrared Spectroscopy Device for Monitoring Muscle Oxidative Metabolism: Custom Probe and In Vivo Applications. Sensors 18:264. https://doi.org/10.3390/s18010264

Remels AHV, Langen RCJ, Schrauwen P, et al (2010) Regulation of mitochondrial biogenesis during myogenesis. Mol Cell Endocrinol 315:113–120. https://doi.org/10.1016/j.mce.2009.09.029

Remie CME, Roumans KHM, Moonen MPB, et al (2020) Nicotinamide riboside supplementation alters body composition and skeletal muscle acetylcarnitine concentrations in healthy obese humans. Am J Clin Nutr. https://doi.org/10.1093/ajcn/nqaa072

Reznick RM, Zong H, Li J, et al (2007) Aging-Associated Reductions in AMP-Activated Protein Kinase Activity and Mitochondrial Biogenesis. Cell Metab 5:151–156. https://doi.org/10.1016/j.cmet.2007.01.008

Ritchie ME, Phipson B, Wu D, et al (2015) limma powers differential expression analyses for RNA-sequencing and microarray studies. Nucleic Acids Res 43:e47–e47. https://doi.org/10.1093/nar/gkv007

Robinson MD, McCarthy DJ, Smyth GK (2010) edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics 26:139–140. https://doi.org/10.1093/bioinformatics/btp616

Robinson SM, Reginster JY, Rizzoli R, et al (2018) Does nutrition play a role in the prevention and management of sarcopenia? Clin Nutr 37:1121–1132. https://doi.org/10.1016/j.clnu.2017.08.016

Roe CR, Millington DS, Maltby DA, et al (1984) L-carnitine enhances excretion of propionyl coenzyme A as propionylcarnitine in propionic acidemia. J Clin Invest 73:1785–1788. https://doi.org/10.1172/JCI111387

Roginski AC, Wajner A, Cecatto C, et al (2020) Disturbance of bioenergetics and calcium homeostasis provoked by metabolites accumulating in propionic acidemia in heart mitochondria of developing rats. Biochim Biophys Acta - Mol Basis Dis 1866:165682. https://doi.org/10.1016/j.bbadis.2020.165682

Romano S, Valayannopoulos V, Touati G, et al (2010) Cardiomyopathies in Propionic Aciduria are Reversible After Liver Transplantation. J Pediatr 156:128–134. https://doi.org/10.1016/j.jpeds.2009.07.002

Rossiter HB, Ward SA, Kowalchuk JM, et al (2002) Dynamic asymmetry of phosphocreatine concentration and O 2 uptake between the on- and off-transients of moderate- and high-intensity exercise in humans. J Physiol 541:991–1002. https://doi.org/10.1113/jphysiol.2001.012910

Rowan SL, Rygiel K, Purves-Smith FM, et al (2012) Denervation Causes Fiber Atrophy and Myosin Heavy Chain Co-Expression in Senescent Skeletal Muscle. PLoS One 7:e29082. https://doi.org/10.1371/journal.pone.0029082

Ryan TE, Brizendine JT, McCully KK (2013a) A comparison of exercise type and intensity on the noninvasive assessment of skeletal muscle mitochondrial function using near-infrared spectroscopy. J Appl Physiol 114:230–7. https://doi.org/10.1152/japplphysiol.01043.2012

Ryan TE, Brophy P, Lin C-T, et al (2014) Assessment of in vivo skeletal muscle mitochondrial respiratory capacity in humans by near-infrared spectroscopy: a comparison with in situ measurements. J Physiol 592:3231–3241. https://doi.org/10.1113/jphysiol.2014.274456

Ryan TE, Erickson ML, Brizendine JT, et al (2012) Noninvasive evaluation of skeletal muscle mitochondrial capacity with near-infrared spectroscopy: correcting for blood volume changes. J Appl Physiol 113:175–83. https://doi.org/10.1152/japplphysiol.00319.2012

Ryan TE, Southern WM, Reynolds MA, McCully KK (2013b) A cross-validation of near-infrared spectroscopy measurements of skeletal muscle oxidative capacity with phosphorus magnetic resonance spectroscopy. J Appl Physiol 115:1757–1766

Rygiel KA, Grady JP, Turnbull DM (2014) Respiratory chain deficiency in aged spinal motor neurons. Neurobiol Aging 35:2230–2238. https://doi.org/10.1016/j.neurobiolaging.2014.02.027

Sabari BR, Zhang D, Allis CD, Zhao Y (2016) Metabolic regulation of gene expression through histone acylations. Nat Rev Mol Cell Biol 18:90–101. https://doi.org/10.1038/nrm.2016.140

Sako T, Hamaoka T, Higuchi H, et al (2001) Validity of NIR spectroscopy for quantitatively measuring muscle oxidative metabolic rate in exercise. J Appl Physiol 90:338–344. https://doi.org/10.1152/jappl.2001.90.1.338

Sakuma K, Yamaguchi A (2012) Sarcopenia and Age-Related Endocrine Function. Int J

Endocrinol 2012:1–10. https://doi.org/10.1155/2012/127362

Sasaki JE, John D, Freedson PS (2011) Validation and comparison of ActiGraph activity monitors. J Sci Med Sport 14:411–416. https://doi.org/10.1016/j.jsams.2011.04.003

Saudubray J-M, Baumgartner MR, Walter J (2016) Inborn Metabolic Diseases. In: Saudubray J-M, Baumgartner MR, Walter J (eds). Springer Berlin Heidelberg, Berlin, Heidelberg, p 658

Sbaï D, Narcy C, Thompson GN, et al (1994) Contribution of odd-chain fatty acid oxidation to propionate production in disorders of propionate metabolism. Am J Clin Nutr 59:1332–1337. https://doi.org/10.1093/ajcn/59.6.1332

Scalzo RL, Peltonen GL, Binns SE, et al (2014) Greater muscle protein synthesis and mitochondrial biogenesis in males compared with females during sprint interval training. FASEB J 28:2705–2714. https://doi.org/10.1096/fj.13-246595

Schiaffino S, Rossi AC, Smerdu V, et al (2015) Developmental myosins: expression patterns and functional significance. Skelet Muscle 5:22. https://doi.org/10.1186/s13395-015-0046-6

Schlicker C, Gertz M, Papatheodorou P, et al (2008) Substrates and Regulation Mechanisms for the Human Mitochondrial Sirtuins Sirt3 and Sirt5. J Mol Biol 382:790–801. https://doi.org/10.1016/j.jmb.2008.07.048

Schmitz A, Silder A, Heiderscheit B, et al (2009) Differences in lower-extremity muscular activation during walking between healthy older and young adults. J Electromyogr Kinesiol 19:1085–1091. https://doi.org/10.1016/j.jelekin.2008.10.008

Schwab MA, Sauer SW, Okun JG, et al (2006) Secondary mitochondrial dysfunction in propionic aciduria: a pathogenic role for endogenous mitochondrial toxins. Biochem J 398:107–112. https://doi.org/10.1042/BJ20060221

Schwer B, Bunkenborg J, Verdin RO, et al (2006) Reversible lysine acetylation controls the activity of the mitochondrial enzyme acetyl-CoA synthetase 2. Proc Natl Acad Sci 103:10224–10229. https://doi.org/10.1073/pnas.0603968103

Sedlock DA (1994) Fitness level and postexercise energy expenditure. J Sports Med Phys Fitness 34:336–42

Shao X, Taha IN, Clauser KR, et al (2020) MatrisomeDB: the ECM-protein knowledge database. Nucleic Acids Res 48:D1136–D1144. https://doi.org/10.1093/nar/gkz849

Short KR, Bigelow ML, Kahl J, et al (2005) Decline in skeletal muscle mitochondrial function with aging in humans. Proc Natl Acad Sci 102:5618–5623. https://doi.org/10.1073/pnas.0501559102

Short KR, Sedlock DA (1997) Excess postexercise oxygen consumption and recovery rate in trained and untrained subjects. J Appl Physiol 83:153–159. https://doi.org/10.1152/jappl.1997.83.1.153

Shulman GI, Rothman DL, Jue T, et al (1990) Quantitation of Muscle Glycogen Synthesis in Normal Subjects and Subjects with Non-Insulin-Dependent Diabetes by 13 C Nuclear Magnetic Resonance Spectroscopy. N Engl J Med 322:223–228. https://doi.org/10.1056/NEJM199001253220403

Simithy J, Sidoli S, Yuan Z-F, et al (2017) Characterization of histone acylations links chromatin modifications with metabolism. Nat Commun 8:1141. https://doi.org/10.1038/s41467-017-01384-9

Sin J, Andres AM, Taylor DJR, et al (2016) Mitophagy is required for mitochondrial biogenesis and myogenic differentiation of C2C12 myoblasts. Autophagy 12:369–380. https://doi.org/10.1080/15548627.2015.1115172

Smith BC, Denu JM (2007) Sir2 Deacetylases Exhibit Nucleophilic Participation of Acetyl-Lysine in NAD + Cleavage. J Am Chem Soc 129:5802–5803. https://doi.org/10.1021/ja070162w

Smyth GK (2004) Linear Models and Empirical Bayes Methods for Assessing Differential Expression in Microarray Experiments. Stat Appl Genet Mol Biol 3:1–25. https://doi.org/10.2202/1544-6115.1027

Soendenbroe C, Bechshøft CJL, Heisterberg MF, et al (2020) Key Components of Human Myofibre Denervation and Neuromuscular Junction Stability are Modulated by Age and Exercise. Cells 9:893. https://doi.org/10.3390/cells9040893

Sonjak V, Jacob KJ, Spendiff S, et al (2019) Reduced Mitochondrial Content, Elevated Reactive Oxygen Species, and Modulation by Denervation in Skeletal Muscle of Prefrail or Frail Elderly Women. Journals Gerontol Ser A 74:1887–1895. https://doi.org/10.1093/gerona/glz066

Southern WM, Ryan TE, Reynolds MA, McCully K (2014) Reproducibility of near-infrared spectroscopy measurements of oxidative function and postexercise recovery kinetics in the medial gastrocnemius muscle. Appl Physiol Nutr Metab 39:521–529. https://doi.org/10.1139/apnm-2013-0347

Sparks LM, Moro C, Ukropcova B, et al (2011) Remodeling Lipid Metabolism and Improving Insulin Responsiveness in Human Primary Myotubes. PLoS One 6:e21068. https://doi.org/10.1371/journal.pone.0021068

Speakman JR, Westerterp KR (2010) Associations between energy demands, physical activity, and body composition in adult humans between 18 and 96 y of age. Am J Clin Nutr 92:826–834. https://doi.org/10.3945/ajcn.2009.28540

Spendiff S, Vuda M, Gouspillou G, et al (2016) Denervation drives mitochondrial dysfunction in skeletal muscle of octogenarians. J Physiol 594:7361–7379. https://doi.org/10.1113/JP272487

Srikanthan P, Karlamangla AS (2014) Muscle Mass Index As a Predictor of Longevity in Older Adults. Am J Med 127:547–553. https://doi.org/10.1016/j.amjmed.2014.02.007

Stearns-Reider KM, D'Amore A, Beezhold K, et al (2017) Aging of the skeletal muscle extracellular matrix drives a stem cell fibrogenic conversion. Aging Cell 16:518–528. https://doi.org/10.1111/acel.12578

Street SF (1983) Lateral transmission of tension in frog myofibers: A myofibrillar network and transverse cytoskeletal connections are possible transmitters. J Cell Physiol 114:346–364. https://doi.org/10.1002/jcp.1041140314

Stupka N, Kintakas C, White JD, et al (2013) Versican Processing by a Disintegrin-like and Metalloproteinase Domain with Thrombospondin-1 Repeats Proteinases-5 and -15 Facilitates Myoblast Fusion. J Biol Chem 288:1907–1917. https://doi.org/10.1074/jbc.M112.429647

Su J, Ekman C, Oskolkov N, et al (2015) A novel atlas of gene expression in human skeletal muscle reveals molecular changes associated with aging. Skelet Muscle 5:35. https://doi.org/10.1186/s13395-015-0059-1

Sumner MD, Beard S, Pryor EK, et al (2020) Near Infrared Spectroscopy Measurements of Mitochondrial Capacity Using Partial Recovery Curves. Front Physiol 11:111. https://doi.org/10.3389/fphys.2020.00111

Szklarczyk D, Franceschini A, Wyder S, et al (2015) STRING v10: protein–protein interaction networks, integrated over the tree of life. Nucleic Acids Res 43:D447–D452. https://doi.org/10.1093/nar/gku1003

Taivassalo T, De Stefano N, Argov Z, et al (1998) Effects of aerobic training in patients with mitochondrial myopathies. Neurology 50:1055–1060. https://doi.org/10.1212/WNL.50.4.1055

Takahashi H, Inaki M, Fujimoto K, et al (1995) Control of the rate of phosphocreatine resynthesis after exercise in trained and untrained human quadriceps muscles. Eur J Appl Physiol Occup Physiol 71:396–404. https://doi.org/10.1007/BF00635872

Takihara Y, Inatani M, Eto K, et al (2015) In vivo imaging of axonal transport of mitochondria in the diseased and aged mammalian CNS. Proc Natl Acad Sci 112:10515–10520. https://doi.org/10.1073/pnas.1509879112

Tan M, Luo H, Lee S, et al (2011) Identification of 67 histone marks and histone lysine crotonylation as a new type of histone modification. Cell 146:1016–1028. https://doi.org/10.1016/j.cell.2011.08.008

Tan M, Peng C, Anderson KA, et al (2014) Lysine Glutarylation Is a Protein Post-Translational Modification Regulated by SIRT5. Cell Metab 19:605–617. https://doi.org/10.1016/j.cmet.2014.03.014

Tanimoto Y, Watanabe M, Sun W, et al (2013) Association of sarcopenia with functional decline in community-dwelling elderly subjects in Japan. Geriatr Gerontol Int 13:958–963. https://doi.org/10.1111/ggi.12037

Tapscott SJ, Lassar AB, Weintraub H (1992) A novel myoblast enhancer element mediates MyoD transcription. Mol Cell Biol 12:4994–5003. https://doi.org/10.1128/MCB.12.11.4994

Tevald MA, Foulis SA, Kent JA (2014) Effect of age on in vivo oxidative capacity in two locomotory muscles of the leg. Age (Omaha) 36:9713. https://doi.org/10.1007/s11357-014-9713-5

Thomas K, Engler AJ, Meyer GA (2015) Extracellular matrix regulation in the muscle satellite cell niche. Connect Tissue Res 56:1–8. https://doi.org/10.3109/03008207.2014.947369

Thorsteinsdóttir S, Deries M, Cachaço AS, Bajanca F (2011) The extracellular matrix dimension of skeletal muscle development. Dev Biol 354:191–207. https://doi.org/10.1016/j.ydbio.2011.03.015

Tieland M, Dirks ML, van der Zwaluw N, et al (2012) Protein Supplementation Increases Muscle Mass Gain During Prolonged Resistance-Type Exercise Training in Frail Elderly People: A Randomized, Double-Blind, Placebo-Controlled Trial. J Am Med Dir Assoc 13:713–719. https://doi.org/10.1016/j.jamda.2012.05.020

Tillander V, Nordström EA, Reilly J, et al (2014) Acyl-CoA thioesterase 9 (ACOT9) in mouse may provide a novel link between fatty acid and amino acid metabolism in mitochondria. Cell Mol Life Sci 71:933–948. https://doi.org/10.1007/s00018-013-1422-1

Tirosh O, Sparrow WA (2005) Age and walking speed effects on muscle recruitment in gait termination. Gait Posture 21:279–288. https://doi.org/10.1016/j.gaitpost.2004.03.002

Tomczak KK, Marinescu VD, Ramoni MF, et al (2004) Expression profiling and identification of novel genes involved in myogenic differentiation. FASEB J 18:1–23. https://doi.org/10.1096/fj.03-0568fje

Tonkonogi M, Sahlin K (2002) Physical exercise and mitochondrial function in human skeletal muscle. Exerc Sport Sci Rev 30:129–37. https://doi.org/12150572

Tonson A, Noble KE, Meyer RA, et al (2017) Age Reduces Microvascular Function in the Leg Independent of Physical Activity. Med Sci Sports Exerc 49:1623–1630. https://doi.org/10.1249/MSS.0000000000001281

Touati G, Valayannopoulos V, Mention K, et al (2006) Methylmalonic and propionic acidurias: Management without or with a few supplements of specific amino acid mixture. J Inherit Metab Dis 29:288–298. https://doi.org/10.1007/s10545-006-0351-7

Trefely S, Huber K, Liu J, et al (2020) Quantitative sub-cellular acyl-CoA analysis reveals distinct nuclear regulation. bioRxiv. https://doi.org/10.1101/2020.07.30.229468

Troiano RP, Berrigan D, Dodd KW, et al (2008) Physical activity in the United States measured by accelerometer. Med Sci Sports Exerc 40:181–8. https://doi.org/10.1249/mss.0b013e31815a51b3

Trounce I, Byrne E, Marzuki S (1989) DECLINE IN SKELETAL MUSCLE MITOCHONDRIAL RESPIRATORY CHAIN FUNCTION: POSSIBLE FACTOR IN AGEING. Lancet 333:637–639. https://doi.org/10.1016/S0140-6736(89)92143-0

Tsekoura M, Kastrinis A, Katsoulaki M, et al (2017) Sarcopenia and Its Impact on Quality of Life. In: Advances in Experimental Medicine and Biology. pp 213–218

United Nations Department of Ecomic and Social Affairs (2019) World Population Ageing 2019 Highlights. UN

Valdez G, Tapia JC, Kang H, et al (2010) Attenuation of age-related changes in mouse neuromuscular synapses by caloric restriction and exercise. Proc Natl Acad Sci 107:14863–14868. https://doi.org/10.1073/pnas.1002220107

van Beekvelt MC, Borghuis MS, van Engelen BG, et al (2001) Adipose tissue thickness affects in vivo quantitative near-IR spectroscopy in human skeletal muscle. Clin Sci (Lond) 101:21–8. https://doi.org/10.1042/cs20000247

Van Beekvelt MCP, Colier WNJM, Wevers RA, Van Engelen BGM (2001) Performance of near-infrared spectroscopy in measuring local O 2 consumption and blood flow in skeletal muscle. J Appl Physiol 90:511–519. https://doi.org/10.1152/jappl.2001.90.2.511

van de Ven RAH, Santos D, Haigis MC (2017) Mitochondrial Sirtuins and Molecular Mechanisms of Aging. Trends Mol. Med. 23:320–331

Van der Hoek MD, Nieuwenhuizen AG, Kuda O, et al (2020) Intramuscular short-chain acylcarnitines in elderly people are decreased in (pre-)frail females, but not in males. FASEB J 34:11658–11671. https://doi.org/10.1096/fj.202000493R

Van Remmen H, Hamilton ML, Richardson A (2003) Oxidative Damage to DNA and Aging. Exerc Sport Sci Rev 31:

Violante S, IJlst L, Ruiter J, et al (2013) Substrate specificity of human carnitine acetyltransferase: Implications for fatty acid and branched-chain amino acid metabolism. Biochim Biophys Acta - Mol Basis Dis 1832:773–779. https://doi.org/10.1016/j.bbadis.2013.02.012

Vreken P, van Lint AEM, Bootsma AH, et al (2002) Rapid Diagnosis of Organic Acidemias and Fatty-acid Oxidation Defects by Quantitative Electrospray Tandem-MS Acyl-Carnitine Analysis in Plasma. In: Advances in experimental medicine and biology. pp 327–337

Wagner GR, Hirschey MD (2014) Nonenzymatic Protein Acylation as a Carbon Stress Regulated by Sirtuin Deacylases. Mol Cell 54:5–16. https://doi.org/10.1016/j.molcel.2014.03.027

Wagner GR, Payne RM (2013) Widespread and Enzyme-independent N ϵ -Acetylation and N ϵ -Succinylation of Proteins in the Chemical Conditions of the Mitochondrial Matrix. J Biol Chem 288:29036–29045. https://doi.org/10.1074/jbc.M113.486753

Wakeling JM, Horn T (2009) Neuromechanics of Muscle Synergies During Cycling. J Neurophysiol 101:843–854. https://doi.org/10.1152/jn.90679.2008

Waldecker M, Kautenburger T, Daumann H, et al (2008) Inhibition of histone-deacetylase activity by short-chain fatty acids and some polyphenol metabolites formed in the colon. J Nutr Biochem 19:587–593. https://doi.org/10.1016/j.jnutbio.2007.08.002

Wang Y, Christopher BA, Wilson KA, et al (2018) Propionate-induced changes in cardiac metabolism, notably CoA trapping, are not altered by L-carnitine. Am J Physiol Metab 315:E622–E633. https://doi.org/10.1152/ajpendo.00081.2018

Wassenaar EB, Van den Brand JGH (2005) Reliability of Near-Infrared Spectroscopy in People With Dark Skin Pigmentation. J Clin Monit Comput 19:195–199. https://doi.org/10.1007/s10877-005-1655-0

Waters DL, Brooks WM, Qualls CR, Baumgartner RN (2003) Skeletal muscle mitochondrial function and lean body mass in healthy exercising elderly. Mech Ageing Dev 124:301–309. https://doi.org/10.1016/S0047-6374(02)00197-5

Weibel ER, Taylor CR, Hoppeler H (1991) The concept of symmorphosis: a testable hypothesis of structure-function relationship. Proc Natl Acad Sci 88:10357–10361. https://doi.org/10.1073/pnas.88.22.10357

Welle S, Bhatt K, Shah B, et al (2003) Reduced amount of mitochondrial DNA in aged human muscle. J Appl Physiol 94:1479–1484. https://doi.org/10.1152/japplphysiol.01061.2002

Wellen KE, Hatzivassiliou G, Sachdeva UM, et al (2009) ATP-Citrate Lyase Links Cellular Metabolism to Histone Acetylation. Science (80-) 324:1076–1080. https://doi.org/10.1126/science.1164097

Wessner B, Liebensteiner M, Nachbauer W, Csapo R (2019) Age-specific response of skeletal muscle extracellular matrix to acute resistance exercise: A pilot study. Eur J Sport Sci 19:354–364. https://doi.org/10.1080/17461391.2018.1526974

Westermann B (2012) Bioenergetic role of mitochondrial fusion and fission. Biochim Biophys Acta - Bioenerg 1817:1833–1838. https://doi.org/10.1016/j.bbabio.2012.02.033

Wicks KL, Hood DA (1991) Mitochondrial adaptations in denervated muscle: relationship to muscle performance. Am J Physiol Physiol 260:C841–C850.

https://doi.org/10.1152/ajpcell.1991.260.4.C841

Wikoff WR, Gangoiti JA, Barshop BA, Siuzdak G (2007) Metabolomics Identifies Perturbations in Human Disorders of Propionate Metabolism. Clin Chem 53:2169–2176. https://doi.org/10.1373/clinchem.2007.089011

Willingham TB, McCully KK (2017) In Vivo Assessment of Mitochondrial Dysfunction in Clinical Populations Using Near-Infrared Spectroscopy. Front Physiol 8:689. https://doi.org/10.3389/fphys.2017.00689

Wilmoth JR (2000) Demography of longevity: past, present, and future trends. Exp Gerontol 35:1111–1129. https://doi.org/10.1016/S0531-5565(00)00194-7

Wilson KA, Han Y, Zhang M, et al (2017) Inter-relations between 3-hydroxypropionate and propionate metabolism in rat liver: relevance to disorders of propionyl-CoA metabolism. Am J Physiol Metab 313:E413–E428. https://doi.org/10.1152/ajpendo.00105.2017

Wongkittichote P, Ah Mew N, Chapman KA (2017) Propionyl-CoA carboxylase – A review. Mol Genet Metab 122:145–152. https://doi.org/10.1016/j.ymgme.2017.10.002

Wray DW, Nishiyama SK, Monnet A, et al (2009) Multiparametric NMR-Based Assessment of Skeletal Muscle Perfusion and Metabolism During Exercise in Elderly Persons: Preliminary Findings. Journals Gerontol Ser A Biol Sci Med Sci 64A:968–974. https://doi.org/10.1093/gerona/glp044

Wright SP, Hall Brown TS, Collier SR, Sandberg K (2017) How consumer physical activity monitors could transform human physiology research. Am J Physiol Integr Comp Physiol 312:R358–R367. https://doi.org/10.1152/ajpregu.00349.2016

Wüst RCI, Grassi B, Hogan MC, et al (2011) Kinetic control of oxygen consumption during contractions in self-perfused skeletal muscle. J Physiol 589:3995–4009. https://doi.org/10.1113/jphysiol.2010.203422

Wüst RCI, van der Laarse WJ, Rossiter HB (2013) On-off asymmetries in oxygen consumption kinetics of single Xenopus laevis skeletal muscle fibres suggest higher-order control. J Physiol 591:731–744. https://doi.org/10.1113/jphysiol.2012.241992

Xie X, Lin J, Liu J, et al (2019) A novel lncRNA NR4A1AS up-regulates orphan nuclear receptor NR4A1 expression by blocking UPF1-mediated mRNA destabilization in colorectal cancer. Clin Sci 133:1457–1473. https://doi.org/10.1042/CS20181061

Yang J-H, Song Y, Seol J-H, et al (2011) Myogenic transcriptional activation of MyoD mediated by replication-independent histone deposition. Proc Natl Acad Sci 108:85–90. https://doi.org/10.1073/pnas.1009830108

Yoshimura Y, Araki A, Maruta H, et al (2016) Molecular cloning of rat acss3 and characterization of mammalian propionyl-CoA synthetase in the liver mitochondrial matrix. J Biochem mvw067. https://doi.org/10.1093/jb/mvw067

Yoshino J, Mills KF, Yoon MJ, Imai SI (2011) Nicotinamide mononucleotide, a key NAD + intermediate, treats the pathophysiology of diet- and age-induced diabetes in mice. Cell Metab. https://doi.org/10.1016/j.cmet.2011.08.014

Yu G, Wang LG, Han Y, He QY (2012) ClusterProfiler: An R package for comparing biological themes among gene clusters. Omi A J Integr Biol 16:284–287. https://doi.org/10.1089/omi.2011.0118

Yucel N, Wang YX, Mai T, et al (2019) Glucose Metabolism Drives Histone Acetylation Landscape Transitions that Dictate Muscle Stem Cell Function. Cell Rep 27:3939-3955.e6. https://doi.org/10.1016/j.celrep.2019.05.092

Zahn JM, Sonu R, Vogel H, et al (2006) Transcriptional Profiling of Aging in Human Muscle Reveals a Common Aging Signature. PLoS Genet 2:e115. https://doi.org/10.1371/journal.pgen.0020115

Zampieri S, Pietrangelo L, Loefler S, et al (2015) Lifelong Physical Exercise Delays Age-Associated Skeletal Muscle Decline. Journals Gerontol Ser A Biol Sci Med Sci 70:163–173. https://doi.org/10.1093/gerona/glu006

Zane AC, Reiter DA, Shardell M, et al (2017) Muscle strength mediates the relationship between mitochondrial energetics and walking performance. Aging Cell 16:461–468. https://doi.org/10.1111/acel.12568

Zhang H, Ryu D, Wu Y, et al (2016) NAD+ repletion improves mitochondrial and stem cell function and enhances life span in mice. Science (80-) 352:1436–1443. https://doi.org/10.1126/science.aaf2693

Zhang Z, Tan M, Xie Z, et al (2011) Identification of lysine succinylation as a new post-translational modification. Nat Chem Biol 7:58–63. https://doi.org/10.1038/nchembio.495

Zhao S, Xu W, Jiang W, et al (2010) Regulation of Cellular Metabolism by Protein Lysine Acetylation. Science (80-) 327:1000 LP – 1004. https://doi.org/10.1126/science.1179689

Zhou C-C, Yang X, Hua X, et al (2016) Hepatic NAD + deficiency as a therapeutic target for non-alcoholic fatty liver disease in ageing. Br J Pharmacol 173:2352–2368. https://doi.org/10.1111/bph.13513

Zhu X-H, Lu M, Lee B-Y, et al (2015) In vivo NAD assay reveals the intracellular NAD contents and redox state in healthy human brain and their age dependences. Proc Natl Acad Sci 112:2876–2881. https://doi.org/10.1073/pnas.1417921112

Zotz TG, Capriglione LGA, Zotz R, et al (2016) Acute effects of stretching exercise on the soleus muscle of female aged rats. Acta Histochem 118:1–9. https://doi.org/10.1016/j.acthis.2015.10.004

SUMMARY

Muscle mass and strength are reported to decline with age. Due to the vital role the muscle in daily life activities, increased loss of muscle mass and strength is associated with functional decline, decreased quality of life and increased hospitalisation rates. Therefore, maintaining skeletal muscle mass and strength with age is a key component in healthy ageing. One of the postulated causes of the decline in mass and strength is the decline in the amount and the quality of the muscle mitochondria, collectively referred to as mitochondrial capacity. Nevertheless, the exact contribution of the mitochondria is still under debate. Therefore, this thesis aims to obtain a better understanding of the role of skeletal muscle mitochondria during ageing. Understanding the role of the contributors to skeletal muscle ageing on the physiologic and molecular level could focus intervention strategies to ultimately to sustain muscle mass and strength with age.

Current methods to assess mitochondrial capacity in humans are either invasive, such as the sampling of muscle tissue via a muscle biopsy, or less accessible, due to expensive and specialised equipment, such as ^{31}P-MRS. In recent years, the assessment of mitochondrial capacity using near-infrared spectroscopy (NIRS) has offered relief to these limitations. Yet, additional effort is needed to extend the use of NIRS to also study the effect of age on mitochondrial capacity. For example, previous studies compared groups of subjects with large expected differences in mitochondrial capacity, and therefore it was unknown if NIRS is able to detect relatively smaller differences in mitochondrial capacity, such as might be expected between a young and older population. In **chapter 2** we demonstrated that NIRS was able to detect differences in mitochondrial capacity in the *gastrocnemius* muscle in a homogenous population of high- and low-fitness males, with a smaller expected difference in mitochondrial capacity than was previously assessed. In **chapter 3** we additionally showed that NIRS is able to detect differences in mitochondrial capacity the *gastrocnemius* muscle in a population of a high- and low-fitness females. Furthermore, we show that NIRS correlates with other measures of oxidative capacity, underlining the physiological relevance of NIRS assessment of mitochondrial capacity. This demonstrates that NIRS could be a valuable tool to study muscle mitochondrial capacity in an ageing population

In **chapter 4** we used NIRS to assess the effect of age on mitochondrial capacity in a population of older (65-71 years) and young (19-25 years) males. Due to the interaction between physical activity, mitochondrial capacity and age, the two age groups were selected based on self-reported, similar physical activity, which was verified using a 5-day accelerometry measurement. We showed that NIRS was able to detect differences in mitochondrial capacity between the two age groups in the *gastrocnemius* and *vastus lateralis*, but not *tibialis anterior*. This showed that not all muscle groups display similar mitochondrial ageing and, because we observed these effects despite similar physical activity, the lower mitochondrial capacity is likely a direct effect of ageing and cannot be completely prevented by physical activity. Nevertheless, a higher mitochondrial capacity was correlated with spending more time in moderate-to-vigorous physical activity, suggesting that physical activity might ameliorate part of the age-related decline in mitochondrial capacity. In **chapter 5** we used transcriptome sequencing to identify molecular mechanisms of ageing in *vastus lateralis* muscle biopsies in the aforementioned population. The significant regulated processes in older compared to young muscle included: cell-adhesion, the matrisome, innervation and inflammation, which were largely upregulated, and oxidative metabolism, which was downregulated. In accordance with the transcriptome results, the protein expression of some mitochondrial respiratory complexes was lower in older compared to young muscle. Moreover, the expression of these complexes in the older group was correlated with *in vivo* mitochondrial capacity in the *vastus lateralis*. This showed that the observed lower mitochondrial capacity could be explained by a lower expression of mitochondrial complex proteins and further substantiated the use of NIRS to measure mitochondrial capacity *in vivo.*

In **chapter 6 and 7** we explored the role of protein propionylation as regulatory factor of mitochondrial and muscle function in aging. Post-translational protein modifications are an important regulatory mechanism for protein functionality and offers the cell a rapid and reversible mechanism to respond to changes in the environment. Protein propionylation might be an important post-translational modification in muscle physiology and ageing. In **chapter 6** we turned to a pathophysiological human model in which levels of propionyl-CoA were elevated and protein propionylation was increased. We showed that fibroblasts from patients in this pathological state have impaired mitochondrial function compared to healthy donor cells, possibly due to aberrant propionylation of proteins involved in mitochondrial respiration. Furthermore, increasing propionylation by exposure to pathophysiological concentrations of propionate induced impaired mitochondrial function in cultured fibroblasts and liver cells. Yet, this effect was not observed in cultured muscle cells, possibly due to differences in metabolic handling of propionyl-CoA. Despite an absence of the effect of propionate exposure and increased propionylation on mitochondrial function in muscle cells, in **chapter 7** we showed that exposure to propionate impairs skeletal muscle differentiation. Concomitant with this observation, we observed an increase in histone protein propionylation and acetylation. The increase in propionylation and acetylation occurred on regions of the genome that regulate muscle differentiation, possibly revealing an additional mechanism by which propionyl-CoA and propionylation can influence muscle cellular fates.

In **chapter 8** the conclusions are presented and the main findings of this thesis are further discussed. For example, we further discuss the way forward for NIRS assessment of mitochondrial capacity. Additionally, we discuss the limitations of the study design used in **chapter 4** and **5**. Lastly, the plausible role of protein propionylation in skeletal muscle ageing is discussed.

In conclusion, this thesis aimed to obtain a better understanding of the role of mitochondria in skeletal muscle ageing. We obtained better understanding on the non-invasive assessment of skeletal muscle mitochondrial capacity using NIRS and newly applied this method to study the effect of age on mitochondrial capacity in locomotor muscles. Furthermore, we obtained better understanding on the molecular mechanisms of ageing and identified that the age-related decline in mitochondrial capacity in skeletal muscle occurs despite similar physical activity, although we demonstrated this effect is muscle

dependent. Lastly, we explored the effect of protein propionylation on skeletal muscle cells. Although we did not find direct effects of increased propionylation on mitochondrial function in muscle, we observed that increased propionylation was associated with impaired muscle differentiation and propionylation could therefore play a role in muscle physiology and ageing.

APPENDICES

ACKNOWLEDGEMENTS

TRAINING ACTIVITIES

ABOUT THE AUTHOR

Acknowledgements

Wow. That was pretty hard. Without the help and support of so many colleagues, friends and family, I could certainly not have done this. In this section I would like to highlight a selection of the people that played an important role in the completion of this thesis.

First and foremost, I would like to thank **Jaap** for the opportunity to conduct a PhD in the group of Human and Animal Physiology. I have never doubted a second that, besides doing good science, you had my personal and professional development high on the priority list. Furthermore, I would like to thank you for the continued support in my career and the opportunity to continue to do research and to teach in the group. **Arie and Vincent**, I am also thankful to you for this opportunity and thankful that you wrote a project that was, if I may say so, completely up my alley. Thank you for the supervision over the last four years. **Arie**, thank you specifically for the support on my personal development and that you, from time-to-time, called me out on insecurities. That was very helpful! **Vincent**, thank you for the introduction into the laboratory techniques and for the knowledge you shared with me. I'm also thankful for the opportunity you gave me to continue working on the propionylation data. I would like to thank **Dr. McCully** for introducing me mitochondrial testing using NIRS and the possibility to receive training in his lab.

I would also like to take the opportunity to thank all financial support from TiFN and NWO that allowed me to conduct this research. I consider working within a TiFN-NWO project as an enrichment of my PhD journey. I would like to thank all the partners: FrieslandCampina, Danone, Division of Human Nutrition at Wageningen University, Groningen University and Maastricht University. I appreciated the critical discussions and the sharing of expertise. I hope to work together in the future. **Pol, Niels, Lotte and Carolien**, I enjoyed being together in this project with you as fellow-PhDs.

A great part of work would not have been realised if it wasn't for the MSc students that I supervised and that assisted me in this project. **Raymond, Vivian, Esther** thank you for being there at the very start of this journey. You have helped me a lot with setting up the NIRS method. **Simone, Saskia and Floor** you were of so much help during my first human trial and we had a lot of fun working together. **Floor**, I will never forget that 'one' participant that you had a very awkward Netflix session with, haha! **Saskia**, special thanks to you for the many Seahorse assays you performed and the trips with the blood from Nijmegen. **Laura and Wei**, you have helped me a lot with all the propionylation experiments and it was an honour (and a lot of fun!) to introduce you to the lab. **Wei**, in return, thanks for introducing me to bioinformatics! **Rick**, you were really my hero in the MCAGE study. We worked so much together that my colleagues couldn't even tell us apart! **Paul**, thanks your mega hard work and for your endless enthusiasm for my project. This enthusiasm helped me a lot, especially in the last stretch of my PhD. **Iris, Laura** you guys worked so hard in the BMCORE study. I think no one has ever done so many VO_2max tests and has counted so many menstrual cycles. **Sophie**, you were a pioneer in our lab working with exosomes and I enjoyed working with you a lot. **Maud, Camiel and Rosanne**, although you were supervised by Joëlle, together we were the **BMCORE team** and I enjoyed working with you a lot!

And now…. the best group there is: Human and Animal Physiology! **Annika**, **Annet**, **Irene** and **Corrine**, thank you for all the support with planning, ordering and of course chatting. **Irene** thank you for the extra kilos that I now have to carry with me thanks to the 'snoeppot' and the leftover beers. **Sander**, thank you for those extra points on my VO_2max because of our lunch runs. Although it has been a while and we should start again if you want to be 'fit at forty'! **Katja**, thank you for ±2 hours per week of pure fun and relaxation solving puzzles! **Annelies**, **Marcel**, **Hans**, **Inge**, **Melissa** and **Jur**, thank you all for the support and problem solving in the lab. **Annelies**, thank you so much for setting up the human physiology lab together. Best thing that happened in my life haha ;). **Silvie**, thank you for the listening ear and advice. **Evert**, thank you for the input on my project and of course all the help with the puzzles. **Claudia**, thanks for supporting my singing career ;)! **Sandra and Deli**, thank you for your input during the Tuesday morning presentations. Looking forward to work with all of you in the future. I would also like to acknowledge all the support I got from all other personnel at Zodiac. **The reception** that received all my participants, **services** that received all my packages and **the cleaning team** that was always there to say good morning and (more often than I would have liked) good night. Moreover, I would like to thank the **VLAG office**, **VLAG council** and the **Wageningen PhD council** for the discussions on PhD affairs.

And then all the HAPpy PhD colleagues: **Lonneke**, **Li**, **Wenbiao**, **Jing**, **Jeske**, **Marjanne**, **Maria**, **José**, **Lianne**, **Natasja**, **Taolin**, **Joëlle**, **Anna**, **Dingyi**, **Miranda**, **Jelle**, **Liangyu** and **Cresci-Anne**. Thank you for all the HAPpy dinners and HAPpy activities, but most of all thank you for helping me solving all the drama, which – of course – there was plenty of. You were all an immense and strong support structure. Everybody deserves colleagues like that! **Taolin, Joëlle and Anna**, special shout out to you, our own Mito-team! **Joëlle**, working together with you on the BMCORE study, I realised I am not as organised as I thought I was compared to you, haha. I am NOT going to miss us texting and calling about menstrual cycles on Friday nights, ok maybe just a little. **Anna**, thanks for all the coffees, walks and for all the singing in the lab. I know I introduced you to the 'hot country' play list, you don't have to thank me ;). **Taolin**, you are the best cell culturerer and I will miss our times in the cell culture lab! **Natasja**, you have received the most questions and complaining from me. Thanks for putting up with me, but then again, I had to put up with all your papers smelling like pigs. **Jeske**, thank you for bringing the true 'Brabantse gezelligheid' in the PhD corner, for all your crazy costumes to use for movies and for being my go-to person for all the human work. We have to take a picture at 'The Doctor' together now! **Lianne,** Thank you for all the nice poems during our Sinterklaas activities. **José,** Thanks for making sure we always had enough drinks :P!

Science is fun, but it's not as fun as my friends. A healthy work-life balance is vital to my survival in this world and you have helped me to take my mind off of work and put it to things that matter more: Having fun! Big special thanks to **all my friends** for all the good

times and a big shout-out to **Loesoeee (BLB!), Charon, Friso, Eline, Ynze, Nyima, Michiel, Carola, Javier, Amanda, Rummie, Remco, Vinnie, Billy, Meurco, Christiåån, Flix, Mollo, Jeroen, Arianne, Paolo, Antonella, Kimberly, Cam, Maria (queeeeeee?), Beba, Rodrigo, Hill, Alwin, Daniel, Ali, Tom, Marco, Francisco, Fernando, Fernando, Frank and Bas** because you are in most of my dinner and party pics.

Dit alles had natuurlijk nooit mogelijk geweest zonder de beste familie. **Pap, Mam** bedankt voor alle support de afgelopen jaren van BSc naar MSc naar PhD. Bedankt dat jullie altijd achter mijn keuzes hebben gestaan. **Tante Nel**, ook bedankt voor uw steun door de jaren heen! **Hil, Maaik, Josh, Chiel** bedankt voor alle adviezen en dat ik soms nog het kleine broertje mag zijn. Obrigado pelo apoio da minha **família Trololó** no Brasil. **Carlos**, you know that I don't like cheesy things and feel like anything I would write here would be exactly that. Of course, we both could have done this PhD journey alone, but it was so much easier and a lot more fun to be on this journey together. Thanks for lifting me up whenever I was down! I'm really excited for our next chapters, te amo!

Overview of completed trainings activities

Discipline specific activities

2016	Near infrared spectroscopy training	Athens, USA
2017	Summerschool Mitochondrial Physiology - From Organelle to Organism	Copenhagen, DK
2016	NWO Nutrition Days	Heeze, NL
2018	Energy metabolism and body composition course	Wageningen, NL
2018	NuGO week mitochondria, nutrition and health	Newcastle, UK
2019	Reversible protein acetylation in health and disease conference	Lisbon, PT
2019	ChIP-seq (wet-lab) and basic functional animal genome analysis training	Wageningen, NL

General courses

2016	VLAG PhD week	Baarlo, NL
2016	PhD Competence assessment	Wageningen, NL
2018	Teaching and supervising thesis students	Wageningen, NL
2018	Orientation on teaching for PhD candidates	Wageningen, NL
2017	Introduction to R	Wageningen, NL
2017	Applied statistics for VLAG PhDs	Wageningen, NL
2019	Career perspectives	Wageningen, NL

Other Activities

2016-2020	Weekly HAP group meetings	Wageningen, NL
2016-2018	Lab meetings	Wageningen, NL
2016-2020	Meetings Mitohealth project group	Wageningen, NL
2016-2019	VLAG PhD Council	Wageningen, NL
2018-2019	Wageningen PhD Council	Wageningen, NL
2016-2018	HAP journal clubs	Wageningen, NL

Teaching Activities

Principles of human physiology (HAP10606)
Integrated Human Physiology (HAP21303)
Human and Animal Biology II (HAP20306)
Research Methodology (HNE-25806)
Molecular regulation of health and disease (HAP31806)
Supervision of MSc thesis students (x 13)

About the Author

Bart Lagerwaard was born on the 8th of June 1992 in Spijkenisse, The Netherlands. From a young age, the ways the human body worked was intriguing to Bart. He decided to enroll in the BSc program Human Nutrition and Health at Wageningen University. During his BSc he undertook a minor in Exercise physiology at the Free University in Amsterdam. Bart wrote his thesis on the role of NR4A receptors during exercise. After obtaining his BSc degree, Bart enrolled in the MSc program Molecular Nutrition and Toxicology at Wageningen University. During his MSc he undertook an internship at the Human Nutrition Research Centre at Newcastle University in the United Kingdom. Bart investigated the effects of dietary fiber on microRNA expression in the colon. He concluded his degree with a thesis at the chair group of Human and Animal Physiology. Here, he conducted a study investigating the effects of creatine monohydrate supplementation on anaerobic metabolism in the muscle using a novel technique to monitor muscle oxygenation, namely near-infrared spectroscopy (NIRS). It was at this same chair group that Bart was appointed as a PhD candidate in 2016. During his PhD research he used the aforementioned technique to study the effect of age on mitochondrial function. Furthermore, during his PhD project, he investigated the molecular changes that occur in the muscle during ageing and explored potential ageing mechanisms. Besides primary research activities Bart was actively involved in teaching and supervision of BSc and MSc students. Furthermore, Bart was involved in PhD matters as chair of the Wageningen PhD council. In 2020, Bart was appointed as a postdoctoral researcher at the chair group of Human and Animal physiology to further investigate the molecular effects of muscle ageing.

Colophon

The studies presented in this thesis were performed within the framework of TiFN. This work is part of the research programme Applied and Engineering Sciences (AES) with project number ALWTF.2015.5, which is (partly) financed by the Netherlands Organisation for Scientific Research (NWO).

No conflicts of interest, financial or otherwise, are declared by the authors. FrieslandCampina and Danone Research B.V are sponsors of the TiFN program and partly financed the project. They had no role in data collection and analysis, decision to publish, or preparation of the manuscripts, but commented the study design.

Financial support from Human and Animal Physiology, TiFN, Artinis Medical Systems and NWO for printing this thesis is gratefully acknowledged.

Lay-out by Vincent Peters, Christiaan Smits and Bart Lagerwaard
Cover design by Vincent Peters, Christiaan Smits and Bart Lagerwaard
Printed by proefschriftmaken.nl