THE EFFECTS OF ENDURANCE EXERCISE AND TRAINING ON MITOCHONDRIAL DYNAMICS AND REMODELLING IN HUMAN SKELETAL MUSCLE

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Abstract

Exercise is one of the most effective lifestyle interventions for the prevention of non-communicable diseases. Although exercise is beneficial at multiple levels, skeletal muscle represents the primary adaptive tissue. Despite the increasing understanding of the beneficial effects of exercise, it remains unknown how different types of exercise affect the molecular pathways mediating improvements in health and performance. The aims of this thesis were: i) to explore the effects of different exercise prescriptions on the regulation of early markers of autophagy following exercise and training; ii) the cellular and mitochondrial responses to two very different exercise prescriptions emphasising high-volume (moderate-intensity continuous exercise, MICE) and high-intensity (sprint-interval exercise, SIE); iii) and to study the mitochondrial remodelling following 8 weeks of two types of exercise training (moderate-intensity continuous training, MICT; and sprint-interval training, SIT).

In Chapter 1, the role of mitochondrial characteristics in the context of endurance performance was reviewed. There was convincing evidence that multiple mitochondrial adaptations represent a commonly observed adaptation necessary for training-induced improvements in endurance performance. Despite the large body of evidence suggesting that mitochondrial characteristics do not limit maximal oxygen consumption, the importance of mitochondrial characteristics for endurance performance is often supported. A new framework for the determining physiological and biological factors of endurance performance, in which mitochondrial characteristics are incorporated and play a central role, was proposed.

In Chapter 2, the effects of exercise on the regulation of the multiple layers of mitochondrial dynamics and mitochondrial stress were explored. Given the limited data in humans, a summary of each pathway was established and related to exercise and training studies. Furthermore, and in line with my experimental chapters, a role for the mitochondrial-specific

integrated stress response was discussed in the context of skeletal muscle and exercise. Despite limited evidence on the effects of exercise, there was emerging literature highlighting the importance of mitochondrial-specific stress response in the context of skeletal muscle diseases, where chronic mitochondrial dysfunction is a distinctive feature.

In the first experimental chapter (Chapter 3), I discussed the effects of exercise on the regulation of markers of autophagy. Given that a key step in mitochondrial dynamics is mitochondrial-specific autophagy (mitophagy) it was important to answer a standing question in the field of exercise in humans: is autophagy downregulated following exercise? These conclusions were based on the decreased content of lipidated LC3 (LC3-II) observed in human skeletal muscle but not in rodents. It is shown that the exercise-induced decrease in LC3B-II protein content is a consistent finding in human skeletal muscle, independently of exercise intensity. Furthermore, the divergent response observed in LC3B between rodents and humans were reproduced. Conversely, by using a novel and adapted *ex vivo* autophagy flux assay, this study was able to show that autophagy does not decrease after exercise, and follows a similar pattern as previously shown in rodents. This chapter suggests that making autophagy conclusions based on basal LC3-II protein content can lead to erroneous interpretation. It calls for the use of a more valid approach, with the use of the *ex vivo* autophagy flux assay, as well as including multiple members of the ATG8 family for a more comprehensive analysis.

In the second experimental chapter (Chapter 4), the effects of different exercise prescriptions, focused on high-volume (MICE) or high-intensity (SIE), on the cellular and mitochondrial-related signalling was discussed. Transcriptomic analysis showed that the most enriched pathway divergently activated was the unfolded protein response. This pathway was enriched following SIE and included the main genes linked to the mammalian mitochondrial unfolded protein response. This was corroborated by an enrichment analysis of the ‘Mitostress’ geneset, which was significantly enriched following SIE, but not MICE. In agreement with this, the

TEM analyses showed that mitochondrial morphology and structure were robustly altered following SIE, while it remained unchanged following MICE. Interestingly, this occurred independently changes in the gene expression level of the 'master regulator' of mitochondrial biogenesis PGC-1α. This is the first time such mitochondrial stress and transcriptional upregulation has been reported following exercise in human studies. Given the results, it was important to elucidate the mitochondrial remodelling following exercise training.

In the last experimental chapter (Chapter 5), the physiological adaptations and mitochondrial remodelling following 8 weeks of exercise training with MICT or SIT were discussed. Following MICT there was predominantly an increase in variables associated with mitochondrial content, including mitochondrial volume density ($mito_{VD}$), citrate synthase (CS) activity and total OXPHOS complexes. On the other hand, SIT resulted in a significant improvement in mitochondrial respiratory function and mitochondrial size without concomitant increases in markers of mitochondrial content. Correlation analyses showed that training-induced changes in complex I was the only variable to be correlated with training-induced changes in markers of endurance performance. These results highlight that the exercise-induced mitochondrial stress observed following sprint-interval exercise is adaptive and leads to positive mitochondrial adaptations. Furthermore, it highlights the complexity of mitochondrial adaptations to multiple exercise training prescriptions, and demonstrates the relevance of these findings for optimised exercise prescription.

In conclusion, these findings provide new and novel evidence about signalling pathways divergently activated following distinct exercise sessions, and how this, when repeated over time can lead to divergent mitochondrial remodelling. Improving the understanding of the molecular regulation of skeletal muscle following exercise and training not only provides new and valuable knowledge for the discovery of new pathways involved in the adaptive responses

to exercise, but also aids at improving the individualised exercise prescription in health and performance.

Student Declaration

I, Mr Javier Botella Ruiz, declare that the PhD thesis entitled “Effects of endurance exercise and training on mitochondrial dynamics and remodelling in human skeletal muscle” is no more than 100,000 words in length including quotes and exclusive of tables, figures, appendices, bibliography, references and footnotes. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma. Except where otherwise indicated, this thesis is my own work.

Signature:  Date: 4/11/2020

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List of Publications, Conferences, Awards, and Grants

Below is a list of publications, conference presentations, awards, and grants that were obtained during the PhD. These are the result of research conducted in relation to this thesis and also from contributions to additional collaborative projects during this time.

Publications:

Pillon N, Gabriel B, Dollet L, Smith J, Sardón-Puig L, **Botella J**, Bishop DJ, Krook A, Zierath J. Transcriptomic Profiling of Skeletal Muscle Adaptations to Exercise and Inactivity. Nature Communications. 2020 Jan 24;11(1):470. doi: 10.1038/s41467-019-13869-w.

Diaz-Lara F.J., **Botella J**., Reisman E. Are enhanced muscle adaptations associated with carbohydrate restriction regulated by absolute muscle glycogen concentration? J Physiol. 2020 Jan;598(2):221-223. doi: 10.1113/JP279076.

Bishop DJ, **Botella J**, Granata C. CrossTalk opposing view: Exercise training volume is more important than training intensity to promote increases in mitochondrial content. J Physiol. 2019 Aug; 597(16):4115-4118. doi: 10.1113/JP277634. Epub 2019 Jul 15

Bishop DJ, **Botella J**, Granata C. Rebuttal from David J. Bishop, Javier Botella and Cesare Granata. J Physiol. 2019 Aug;597(16):4121-4122. doi: 10.1113/JP278329.

Bishop DJ, **Botella J**, Genders AJ, Lee M JC, Saner NJ, Kuang J, Yan X, Granata C. High-Intensity Exercise and Mitochondrial Biogenesis: Current Controversies and Future Research Directions. Physiology. 2019 Jan 1;34(1):56-70. doi: 10.1152/physiol.00038.2018.

Botella J, Saner NJ, Granata C. Guardian of mitochondrial function: an expanded role of Parkin in skeletal muscle. Journal of Physiology. 2018 Dec; 596(24):6139-6140. doi: 10.1113/JP276841.

Jamnick NA, **Botella J**, Pyne DB, Bishop DJ. Manipulating graded exercise test variables affects the validity of the lactate threshold and $\dot{V}O_{2max}$. PLoS ONE. 2018 13(7): e0199794. doi: 10.1371/journal.pone.0199794

First author conference abstracts

Botella J., Jamnick N.A., Perri E., Saner N., Diaz-Lara J., Oorschot V., Ramm G., Lazarou M., Bishop D.J. Sprint-interval exercise, but not moderate intensity continuous exercise, disrupts the mitochondrial pool and initiates the regulation of mitophagy. 2020. 25th Annual Congress of the European College of Sport Science. 1st position at Young Investigator Award – **Oral presentation**

Botella J., Perri E., Saner N., Jamnick N.A., Diaz-Lara J., Oorschot V., Ramm G., Lazarou M., Bishop D.J. Exercise- and training-induced skeletal muscle mitochondrial remodelling in healthy males. 2019. Australian Physiological Society Conference. Canberra, Australia - **Oral presentation**

Botella J., Perri E., Saner N., Jamnick N.A., Diaz-Lara J., Oorschot V., Ramm G., Lazarou M., Bishop D.J. Skeletal muscle mitochondrial remodelling following endurance training in healthy males. 2019. Cell Symposia, Exercise Metabolism. Sitges, Spain – **Poster presentation**

Botella J., Perri E., Granata C., Bishop D.J. Autophagy response following a high-intensity interval training session, before and after 3 weeks of training, in humans. 2018. 23rd Annual Congress of the European College of Sport Science. Dublin, Ireland - **Oral presentation**

Co-authored conference abstracts

Bishop, D. J Granata, C., Relijic, B., **Botella J**., Jamnick, N. A., Kuang, J., Janssen, H., Thorburn, D. R., Frazier, A. E., & Stroud, D. A. Analysis of the mitochondrial proteome from human skeletal muscle in response to endurance training reveals volume-dependent remodelling. 2018. AussieMit. Melbourne, Australia.

Jamnick, N. A., **Botella J**., Pyne D. B., & Bishop, D. J. Manipulating graded exercise test variables affects the validity of the lactate threshold and $\dot{V}O_2peak$. 2018. 65th Annual Meeting of the American College of Sports Medicine. Minneapolis, USA.

Jamnick, N. A., **Botella J**., Pyne D. B., & Bishop, D. J. Manipulating graded exercise test variables affects the validity of the lactate threshold and $\dot{V}O_2peak$. 2018. Exercise and Sport Science Australia (ESSA) Research to Practice Conference. Brisbane, Australia.

Bishop D. J., Kuang J., Granata C., **Botella J.,** Jamnick N. A., Janssen H. A. Increased respiratory chain supercomplex formation and mitochondrial respiration in response to exercise training in human skeletal muscle. Cell Symposia, Exercise Metabolism. May 21-23, 2017. Gothenburg, Sweden.

Awards and grants

1st position at Young Investigator Award – 25th Congress of the European College of Sport Science, 2020 - $6700

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Australian Physiological Society (AuPS) PhD research training award, 2019

Secomb Travel grant, 2019 - $895

The Physiological Society travel grant, 2019 - $900

List of Figures

List of Tables

List of Abbreviations

AMPK = AMP-activated protein kinase

ATF4 = activating transcription factor 4

ATP = adenosine triphosphate

A-VO_2 = arterio-venous oxygen difference

BNIP3 = BCL2/adenovirus E1B 19 kDa interacting protein 3

BNIP3L/NIX = BCL2/adenovirus E1B 19 kDa interacting protein 3 like

Ca^{2+} = calcium

CAMKII = Ca^{2+}/Calmodulin-dependent kinases II

CS = citrate synthase

CP = critical power

CHOP = CCAAT/enhancer-binding protein homology protein

CCCP = carbonyl cyanide 3-chlorophenylhydrazone

DELE1 = DAP3 Binding Cell Death Enhancer 1

DRP1 = dynamin-related protein 1

DNM2 = dynamin-2

eIF2α = alpha subunit of the eukaryotic translation initiation factor 2 alpha

ER = endoplasmic reticulum

ETC = electron transport chain

FAM210A = family with sequence similarity 210 Member A

FIS1 = mitochondrial fission 1 protein

FGF21 = fibroblast growth factor 21

FUNDC1 = FUN14 Domain Containing 1

GCN2 = general control nonderepressible 2

GDF15 = growth differentiation factor 15

HRI = heme-regulated eIF2α kinase

HSPD1 = heat Shock Protein Family D (Hsp60) Member 1

HIIE = high-intensity interval exercise

HIIT = high-intensity interval training

IMM = inner mitochondrial membrane

IMS = inter membrane space

ISR = integrated stress response

KO = knockout

La = lactate

LT = lactate threshold

LONP1 = Lon protease homolog 1

LIR = LC3-interacting region

MFN1 = mitofusin 1

MFN2 = mitofusin 2

MICT = moderate-intensity continuous training

MICE = moderate-intensity continuous exercise

$Mito_{VD}$ = mitochondrial volume density

MLSS = maximal lactate steady state

mtDNA = mitochondrial deoxyribonucleic acid

MFF = mitochondrial fission factor

MiD49 = mitochondrial dynamics protein of 49 kDa

MiD51 = mitochondrial dynamics protein of 51 kDa

MTCH1 = mitochondrial carrier homolog 1

MDV = mitochondrial-derived vesicle

MM = mitochondrial myopathy

MSR = mitochondrial stress response

NuGEMPs = nuclear genes encoding mitochondrial proteins

NRF2 = nuclear respiratory factor 2

NRF1 = nuclear respiratory factor 1

NDP52 = nuclear dot protein 52

NBR1 = neighbour of BRCA1 gene 1

Hb = haemoglobin

O_2 = oxygen

OXPHOS = oxidative phosphorylation

OPA1 = dynamin-like 120 kDa protein Optic Atrophy 1

OMM = outer mitochondrial membrane

OPTN = Optineurin

OMA1 = zinc metalloprotease overlapping with m-AA protease

p38 = p38 mitogen-activated protein kinases

PCr = phosphocreatine

PGC-1α = peroxisome proliferator-activated receptor gamma coactivator 1-alpha

p53 = tumour suppressor p53

P_i = inorganic phosphate

PGAM5 = mitochondrial serine-threonine phosphatase PGAM family member 5

PARL = presenlin-associated rhomboid-like protein

PINK1 = PTEN-induced putative kinase 1

PARKIN = E3 ubiquitin-protein ligase parkin

PKR = protein kinase R

PERK = protein kinase R (PKR)-like endoplasmic reticulum kinase

$\dot{Q}_{max}$ = maximal cardiac output

ROS = reactive oxygen species

SFXN3 = sideroflexin 3

SDH = succinate dehydrogenase

SIT = sprint-interval training

SIE = sprint-interval exercise

SQSTM1/p62 = sequestosome 1

TCA = Tricarboxylic Acid Cycle

TEM = transmission electron microscopy

TF = transcription factor

TOMM = translocase of outer mitochondrial membrane

TIMM = translocase of inner mitochondrial membrane

TFAM = mitochondrial transcription factor A

T2D = type 2 diabetes

TAX1BP1 = Tax1-binding protein 1

ULK1 = Unc-51 Like Autophagy Activating Kinase 1

UPR = unfolded protein response

UPR^{ER} = endoplasmic reticulum unfolded protein response

UPR^{mt} = mitochondrial unfolded protein response

uORF = upstream open reading frame

$\dot{W}_{max}$ = peak aerobic power

W’ = finite work capacity above CP

$\dot{V}O_{2max}$ = maximal rate of oxygen consumption

YME1L1 = ATP-dependent protease yeast mitochondrial DNA escape 1-like

β-HAD = beta-hydroxyacyl-CoA dehydrogenase

CHAPTER 1 – THE BIOLOGY AND PHYSIOLOGY OF ENDURANCE PERFORMANCE

This chapter aims to provide a review of the literature regarding the biological and physiological characteristics considered important for endurance performance. Among these components, special emphasis will be placed on the role of skeletal muscle mitochondrial characteristics in determining endurance performance.

1.1 Introduction

Aerobic exercise refers to activities requiring repeated submaximal muscle contractions (e.g, cycling, swimming, rowing and running). Aerobic exercise spans from current guidelines for physical activity (e.g., walking) to major endurance events (e.g., the *Tour de France*, where athletes cycle for 21 consecutive days completing over 3000 km). Endurance performance can be defined as the maximum velocity that an individual is capable of maintaining for a given distance (1). This performance not only relies on the physiological characteristics of the individual but is also influenced by psychological and biomechanical factors, which have been discussed in some excellent reviews (2, 3). For the purpose of this review, I will focus on physiological characteristics important for endurance performance.

During sustained aerobic exercise, oxygen (O_2) needs to be delivered to working muscles so that mitochondria can maintain the rate of ATP resynthesis needed for muscle contraction. A wide range of whole-body adaptations aim at enhancing the capability of the body to transport and extract O_2. Central adaptations are a major component, and increases in stroke and blood volume, as well as other adaptations (e.g., haemoglobin mass), will increase the levels of oxygen delivered to the peripheral working muscles. There are also peripheral adaptations, such as increases in the vascular network surrounding the working muscles and improved mitochondrial characteristics that ultimately increase their ability to extract and utilise O_2.

Each of the steps, from delivery to utilisation of the O_2 in skeletal muscle mitochondria, are possible limiting steps in endurance performance. Due to the central role of mitochondria in oxidative ATP resynthesis, mitochondria have been proposed to be important for endurance performance. However, there is limited empirical evidence to support the hypothesis that mitochondria are important determinants of endurance performance.

1.2 Physiological determinants of endurance performance

For decades, exercise scientists have made efforts to better understand the physiological variables that determine the endurance performance of highly-trained athletes. This would allow scientists to assess and monitor important physiological characteristics and to design training interventions to improve these variables. Nobel laurate Archibald Vivian Hill, with his contributions in the 1920s, first described the concept of the maximal oxygen uptake ($\dot{V}O_{2max}$) and the existence of multiple O_2-dependent and -independent energy systems (4). Exercise physiologist, like Per-Olof Åstrand and Bengt Saltin, expanded on our understanding of muscle characteristics and limiting factors of $\dot{V}O_{2max}$ (5-8). Subsequently, David Costill (9-11) and Edward Coyle (1, 2, 12-15) also made very important contributions to understanding the physiological determinants of endurance performance.

Following decades of research, the current understanding of the physiological and biological variables determining endurance performance was last summarised in 2008 (15). The current understanding is that the combination of three main physiological variables dictates endurance performance: 1) the maximal rate of oxygen consumption ($\dot{V}O_{2max}$); 2) the lactate threshold (or as referred here, submaximal anchors); 3) and efficiency (or economy) of movement. I have modified and extended the previous schematic and it is presented below (Figure 1.1).

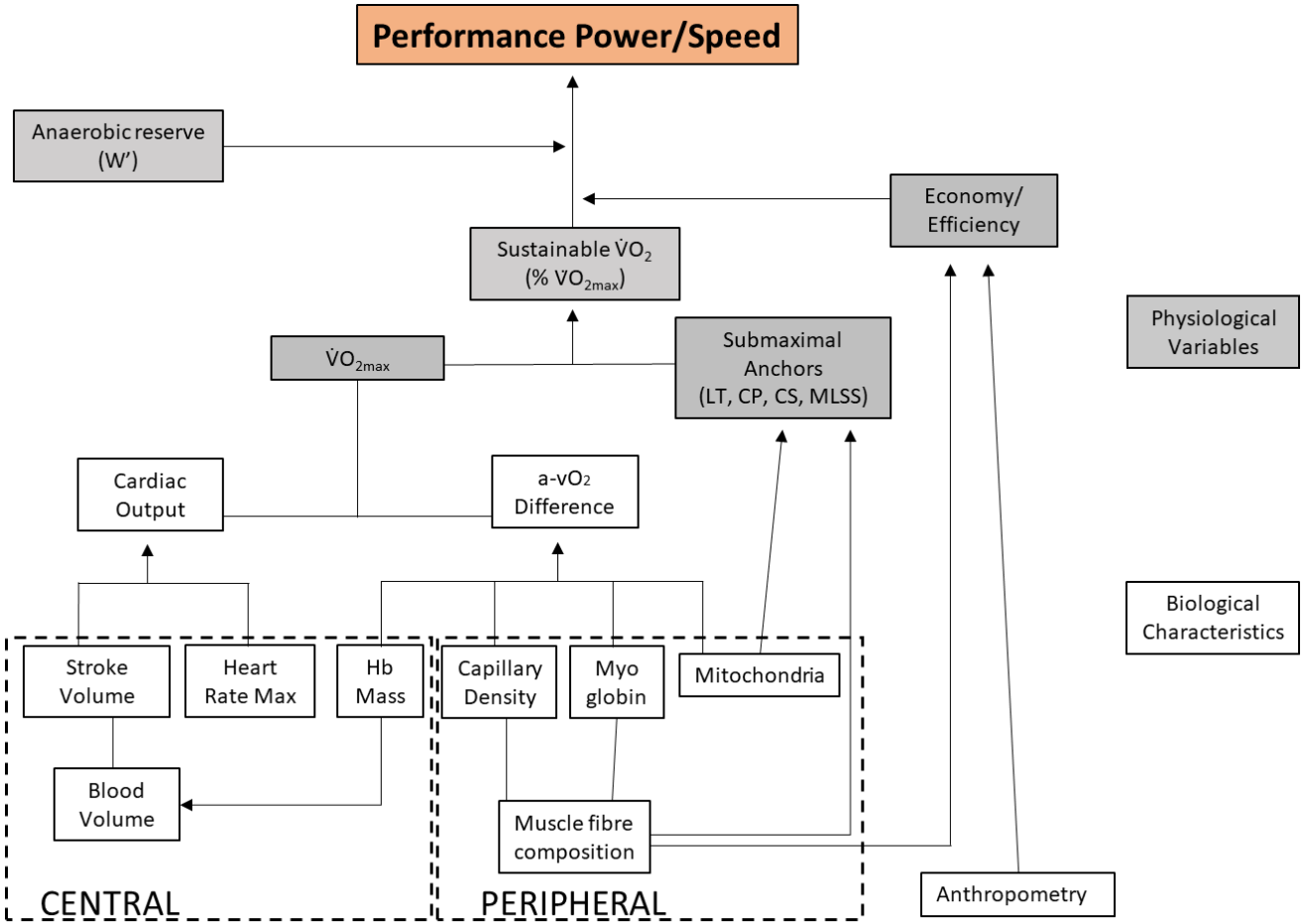

Figure 1.1. Proposed biological and physiological variables determining endurance performance. Hb = haemoglobin; a-vO_2 = arterio-venous oxygen difference; $\dot{V}O_{2max}$ = Maximal rate of oxygen consumption; LT = Lactate threshold; CP = Critical Power; CS = Critical Speed; MLSS = Maximal Lactate Steady State; W' = finite work capacity above CP.

1.2.1 Maximal rate of oxygen consumption ($\dot{V}O_{2max}$)

The $\dot{V}O_{2max}$ represents the upper limit of an athlete's ability to transport and utilise oxygen (16). It is amongst the most important physiological parameters, as it has been linked to both endurance performance (Figure 1.2.A) and life expectancy (9, 17). From a physiological standpoint, Hill and Lupton (16) described the $\dot{V}O_{2max}$ to be the result of the cardiac output (Q; stroke volume x heart rate) and the arterio-venous oxygen difference (a-vO_2 diff). A change in any of these components would alter the $\dot{V}O_{2max}$. Current literature supports the notion that $\dot{V}O_{2max}$ is primarily determined by central factors, such as stroke volume, blood volume, and total haemoglobin mass (18). Readers are referred to excellent reviews on the determinants of $\dot{V}O_{2max}$ (19, 20).

1.2.2 Submaximal anchors

Due to the inability of $\dot{V}O_{2max}$ to differentiate between differing endurance performances in homogeneous trained groups (21, 22), the lactate threshold (LT) was suggested as one of the main physiological determinants of endurance performance (Figure 1.2.B) (2, 9, 12). Briefly, the LT can be defined as the highest submaximal intensity that can be maintained for a prolonged time without a disproportional increase in blood lactate (9). The LT has been linked to endurance performance (Figure 1.2.B), and is also currently used as a means of exercise prescription (9, 23, 24).

1.2.3 Exercise efficiency

While LT determines the fraction of $\dot{V}O_{2max}$ that can be sustained, exercise efficiency determines how much mechanical work can be produced for a given energy expenditure. Exercise efficiency is calculated as the relationship between the energy expended (i.e., oxygen consumption) and the work performed (i.e., Watts produced). Similarly to exercise efficiency, exercise economy refers to the energy expenditure per unit of distance (i.e., $mL \cdot O_2^{-1} \cdot km^{-1}$) and

it is employed in sports such as running where the measurements of work (Joules) is not methodologically possible or reliable (25).

Running economy is strongly correlated with endurance performance (Figure 1.2.C) (26), and has been proposed to be a contributing factor in the success of East-African runners (27). Meanwhile, cycling efficiency does not seem to differ between recreational and world-class cyclists (28), suggesting that it is a less important physiological factor in cycling performance.

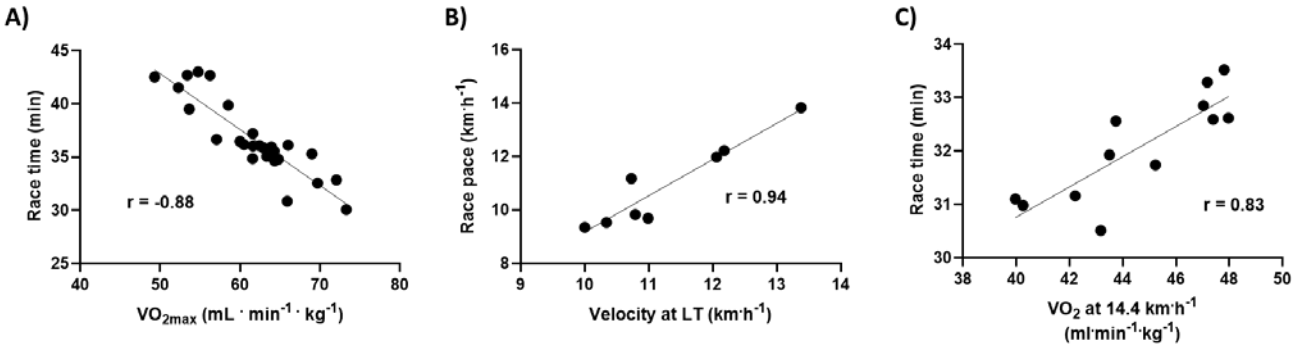

Figure 1.2. Overview of the reported relationships between endurance performance (measured as race time or race speed) and the three main physiological variables [A) $\dot{V}O_{2max}$; B) LT; C) economy]. Adapted from published research (10, 14, 29).

1.2.4 Summary

Endurance performance, from a physiological standpoint, is the result of the combined contribution of $\dot{V}O_{2max}$, submaximal anchors, and efficiency/economy. While there is no single factor that explains all of the variance in endurance performance, the combination of these factors currently provides the most comprehensive information (Figure 1.1 and 1.2). Despite the large increase in research exploring the physiological factors affecting endurance performance, less is known regarding the underlying biological characteristics that determine these physiological variables.

1.3 Biological characteristics contributing to the physiological determinants of endurance performance.

For the purpose of this review, biological characteristics refer to those components of the human organism (e.g., capillary density) that adapt following exercise training and contribute to endurance performance. Training-induced adaptations are usually divided into two components: central and peripheral adaptations. The first refers to adaptations in those areas where the O_2 uptake and transport take place (e.g., the heart and circulation), while peripheral adaptations refers to those areas that impact O_2 extraction (e.g., muscle).

The oxygen cascade is termed after the O_2 path, which starts in the atmosphere and goes through different steps before reaching its end point - the skeletal muscle mitochondria (Figure 1.3). Each step in this oxygen cascade is thought to represent a potential limitation to O_2 difussion, and, as such, a limiting factor for O_2 uptake during exercise. Symmorphosis is the theory that suggests all components of a structure are equally important for its final function (30). In the case of the oxygen cascade, and based on symmorphosis theory, any of the following components could be limiting steps: 1) the pulmonary system, 2) the maximum cardiac output and the O_2 carrying capacity (heart and blood), or 3) O_2 extraction and utilisation in the muscle (capillarisation and mitochondria). However, it is difficult to distinguish and isolate distinct O_2 cascade steps, and to exactly delimit what is central and peripheral. Readers are referred to important reviews in the field for further information on the oxygen cascade and exercise (30-33).

While all these factors are important for endurance performance, whether or not one more strongly determines maximal endurance performance is of great interest for the scientific community. However, it is most likely that all components are critical and it is the combination of them, rather than a single component, that is important for an optimised system.

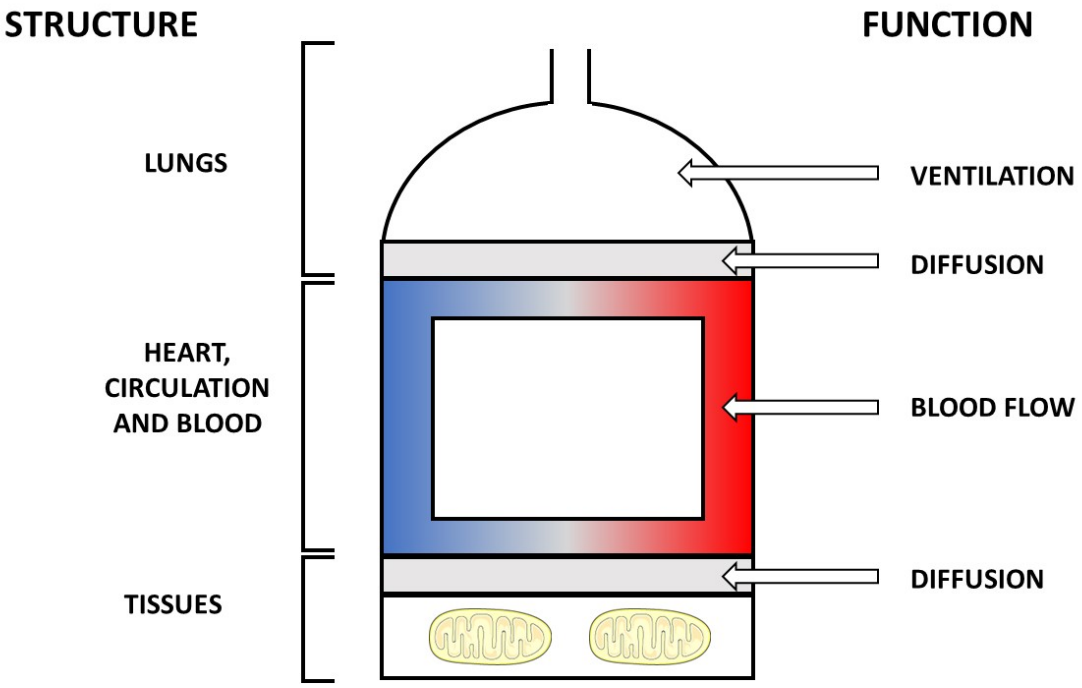

Figure 1.3. Initial O_2 cascade model of central and peripheral steps. Found in tissues and depicted in yellow are mitochondria. Red represents arterial blood and blue represents venous blood. Adapted from Wagner (31).

1.3.1 Central adaptations

The first step in the central component, the pulmonary system, is thought to be overbuilt for its function in healthy, non-athletic populations and to not represent a limitation for blood oxygenation (32). However, some have suggested that in highly trained athletes arterial O_2 desaturation may occur during maximal work (34) and become a limiting step. This is proposed to be due to decreased red blood cell transit time, reducing the time to fully oxygenate the blood before it exits the pulmonary system (34), which can affect endurance performance (33).

The major contributor to the central adaptations is the cardiovascular system, which includes the heart and the vessels through which blood is transported (35). Increased stroke volume, plasma volume, and total blood volume, are the main adaptations observed following exercise training (18, 35). While initially plasma volume is expanded following a single exercise session

(36), red blood cells and total blood volume increases can be observed following weeks of training and will reflect an increased oxygen carrying capacity (37, 38). Following increased blood volume, this adaptation results in increased cardiac preload and increases in stroke volume via the Frank-Starling mechanism, which, when sustained over time, will result in structural cardiac adaptations (39).

The role of central adaptations has been mainly focused around $\dot{V}O_{2max}$ (19, 20, 35, 40). This is based on accumulated correlational data between haematological variables and $\dot{V}O_{2max}$ (37, 40), as well as interventional studies where blood drawn to pre-training values restored $\dot{V}O_{2max}$ (38). This research shows that central factors will affect endurance performance due to their importance on $\dot{V}O_{2max}$.

1.3.2 Peripheral adaptations

Peripheral adaptations refer to those adaptations that occur at or near the muscle level (Figure 1.3). The two most commonly described and reported peripheral adaptations are increased capillary density (the result of a mechanism known as angiogenesis; (41)) and mitochondrial adaptations (42, 43). However, any adaptations from the capillary bed to the muscle is considered a peripheral adaptation.

Adaptations in capillarisation, measured as changes in the total capillary bed surrounding the working muscle, are assessed by capillary-to-fibre ratio or capillary per volume of muscle, which correlates with critical power (44) and time to fatigue (12) in trained participants. These adaptations will translate to an increased total area where gas and metabolite exchange can take place (45). Interestingly, in a rabbit model of chronic low-frequency stimulation, changes in capillarisation in skeletal muscle seem to occur early, and precede changes in mitochondrial enzyme activity (46). However, a similar time-course study has not been performed in humans.

Other peripheral factors remain underexplored in the literature. Myoglobin, the protein responsible for the binding and transporting of O_2 inside the muscle, remains understudied. Myoglobin desaturation is enhanced in trained rodents (47), and the myoglobin protein levels in skeletal muscle can increase following exercise training in a volume-dependant manner (48). Whether myoglobin has an impact on changes in endurance performance remains to be fully elucidated, but warrants future research. In rodents, it is known that myoglobin colocalises with mitochondria in soleus skeletal muscle (49) and may adapt in parallel to match O_2 extraction to mitochondrial respiratory function at the skeletal muscle level.

Mitochondria are the organelles within the cell where O_2 is utilised in the electron transport chain with the final goal of resynthesising ATP. Multiple mitochondrial characteristics are studied in the context of skeletal muscle (43); among them, mitochondrial content and mitochondrial respiratory function are the most frequently used (50-54). Mitochondrial characteristics have been repeatedly linked to endurance performance (12, 31, 55) and have been shown to be the biological characteristic that best reflected the decreases in submaximal endurance performance over a detraining period (13). Therefore, given the central role of mitochondrial characteristics in endurance performance, this topic will be expanded in the following sections of this chapter.

1.4 Evidence for a link between mitochondria and endurance performance

Given that skeletal muscle is exposed to large changes in energy demands during exercise, it is not surprising that mitochondria, the ‘powerhouses’ of the cell, have been suggested to be pivotal for endurance performance (11, 56). Mitochondrial characteristics can be multiple, and do not necessarily correlate with each other. Given this, I will refer to mitochondrial content as any variable that is correlated with the abundance of mitochondria or mitochondrial volume

density. Mitochondrial respiratory function will refer to any *ex vivo* respirometry analysis of skeletal muscle (for further information readers are directed to section 2.4).

While evidence in favour of mitochondrial characteristics as determining factors of endurance performance has been obtained in different settings (see section 1.4.1, 1.4.2, and 1.4.3), much of the work to date has focused on the role of mitochondria as a limiting factor of $\dot{V}O_{2max}$ (6, 57, 58). A long-standing question is, why do mitochondrial characteristics increase more than $\dot{V}O_{2max}$ following training? This has been used as an argument to indirectly suggest that mitochondrial characteristics are not a limiting factor. Seminal work showed that, in untrained participants, the *in vivo* $\dot{V}O_2$ (~4.5 $mmol \cdot kg^{-1} \cdot min^{-1}$) during maximal exercise involving a low muscle mass (arm cranking or single-leg extension) matches the *ex vivo* $\dot{V}O_2$ (6, 57). On the contrary, when a higher muscle mass is engaged (two-leg cycling), the *in vivo* $\dot{V}O_2$ (~5 $mmol \cdot kg^{-1} \cdot min^{-1}$) is lower than the *ex vivo* $\dot{V}O_2$ (~6.9 $mmol \cdot kg^{-1} \cdot min^{-1}$), suggesting a 35% excess in mitochondrial respiratory function. However, it was also shown that the *in vivo* $\dot{V}O_2$ exceeds *ex vivo* $\dot{V}O_2$ by up to 2-fold in low muscle mass exercise (5, 8), and is suggested to be the result of damaged mitochondria from isolation and fibre permeabilisation. Interestingly, in another study in which untrained (~ 38 $mL \cdot min^{-1} \cdot kg^{-1}$) and trained (~59 $mL \cdot min^{-1} \cdot kg^{-1}$) participants were subjected to a maximal knee-extension exercise the results showed that, in contrast to untrained participants, trained individuals had a large mitochondrial respiratory reserve capacity (59). These studies established the concept of mitochondria being 'overbuilt' in skeletal muscle and to not represent a limiting factor in trained participants.

A common assumption in these studies is that the whole musculature is recruited and working maximally during maximal graded exercise, and that mitochondrial characteristics derived from *vastus lateralis* or *triceps brachii* can be extrapolated to the rest of the musculature. Studies have shown that during a maximal run to exhaustion only ~67-73% of the lower-limb musculature is recruited (60). Furthermore, trained cyclists have optimised their neuromuscular

pattern of muscle recruitment characterised by a lower coactivation and shorter muscle activity periods (61). This would suggest that the mitochondrial respiratory demands are not equally shared between, and within, different muscles.

Overall, the literature suggests that, while in untrained and sedentary participants mitochondrial respiratory function may limit *in vivo* $\dot{V}O_2$, the excess in mitochondrial respiratory function and mitochondrial characteristics following training may be to maximise the O_2 extraction when O_2 delivery to the muscle is a known limiting factor as in whole-body exercise or in highly trained participants (6). Furthermore, despite mitochondrial respiratory function being the best current method to assess *ex vivo* $\dot{V}O_2$, it is currently unknown whether it is capable of behaving as *in vivo*, where multiple cellular signals (e.g., Ca^{2+}) converge at the same time, and whether a single reading reflects the *in vivo* mitochondrial respiratory function over a longer exercise time.

While these studies collectively suggest that mitochondrial characteristics are important for endurance performance, but do not limit $\dot{V}O_{2max}$, they also highlight the complexity of whole-body exercise and endurance performance. In the following sections I will examine the multiple layers of evidence provided in the literature that link mitochondrial characteristics with markers of endurance performance.

1.4.1 Cross-sectional studies

Cross-sectional studies, in which heterogeneous groups of participants are studied, are usually the initial evidence to provide support for a potential causal relationship. In human studies, combining groups with differing endurance performance levels (or surrogate markers) has been the initial step for the investigating relationships between mitochondrial characteristics and markers of endurance performance. This allows to interrogate the importance of a given

biological characteristic in the outcome analysed and to provide preliminary evidence and strengthen the need for further research.

Initial findings in 1972 showed that succinate dehydrogenase (SDH) enzyme activity, a correlate of mitochondrial content (62), was increased in the skeletal muscle of trained participants compared to their untrained counterparts (7). This appeared to be due to training as they were able to show an increased SDH activity in the predominant muscle used in the respective sport of the participants (e.g., leg for cycling, arm for canoeing). This suggested that greater mitochondrial content was a specific adaptation of the exercising muscles. Moreover, skeletal muscle mitochondrial ultrastructural characteristics, through the analysis of transmission electron microscopy (TEM) micrographs, provided evidence that trained participants had a greater mitochondrial volume density ($mito_{VD}$; untrained = 3.97%, vs trained = 6.57%) (56). Similarly, citrate synthase (CS) activity, another marker of mitochondrial content (62), was also shown to differ between participants of different training status (63-65).

Mitochondrial respiratory function also differed between sedentary (V_{max} = 3.7 µmol $O_2 \cdot min^{-1} \cdot g$ dry $weight^{-1}$; $\dot{V}O_{2peak}$ = 28.5 $mL \cdot min^{-1} \cdot kg^{-1}$; $\dot{V}O_2$ at LT =15.2 $mL \cdot min^{-1} \cdot kg^{-1}$), active ($V_{max}$ = 5.9 µmol $O_2 \cdot min^{-1} \cdot g$ dry $weight^{-1}$; $\dot{V}O_{2peak}$ = 36.3 $mL \cdot min^{-1} \cdot kg^{-1}$; $\dot{V}O_2$ at LT =19.3 $mL \cdot min^{-1} \cdot kg^{-1}$) and trained ($V_{max}$ = 7.9 µmol $O_2 \cdot min^{-1} \cdot g$ dry $weight^{-1}$; $\dot{V}O_{2peak}$ = 50.1 $mL \cdot min^{-1} \cdot kg^{-1}$; $\dot{V}O_2$ at LT =32.7 $mL \cdot min^{-1} \cdot kg^{-1}$) participants (64). However, contrary to mitochondrial content, which was different between groups, mitochondrial respiratory function was not different between chronic heart-failure patients (V_{max} = 3.55 µmol $O_2 \cdot min^{-1} \cdot g$ dry $weight^{-1}$) and sedentary participants (V_{max} = 3.17 µmol $O_2 \cdot min^{-1} \cdot g$ dry $weight^{-1}$) despite significant differences in physiological markers of endurance performance ($\dot{V}O_{2max}$ = 19.2 $mL \cdot min^{-1} \cdot kg^{-1}$ and $\dot{V}O_2$ at LT = 8.51 $mL \cdot min^{-1} \cdot kg^{-1}$ in heart-failure patients vs $\dot{V}O_{2max}$ = 40.1 $mL \cdot min^{-1} \cdot kg^{-1}$ and $\dot{V}O_2$ at LT = 14.99 $mL \cdot min^{-1} \cdot kg^{-1}$ in sedentary participants) (65).

In a comparison of sedentary and trained participants that differed in both mitochondrial content and respiratory function (measured as ATP_{max} from PCr recovery), they were also shown to differ in cycling efficiency (66). In a different study with trained and untrained participants a between-group difference was observed for CS activity, but not mitochondrial respiratory function or fibre type distribution, and there was a significant difference in gross cycling efficiency at 80% of $\dot{V}O_{2max}$ in favour of the trained participants (21.0% in trained vs 18.8% in untrained) (67); this suggested mitochondrial content to be more important than respiratory function for a greater cycling efficiency.

While markers of mitochondrial content and respiratory function seem to differ between groups of differing fitness, a common limitation is that the mitochondrial respiratory function differences could be solely due to altered mitochondrial content levels. In an effort to address this question, Jacobs and Lundby (63) divided a group of participants ranging from ~ 50 to ~77 $\dot{V}O_{2max}$ ($mL \cdot min^{-1} \cdot kg^{-1}$) in four different groups. They showed that there were both differences in mitochondrial content markers (measured as CS activity) and mitochondrial respiratory function. Even when mitochondrial respiratory function was corrected for unit of CS activity, these differences were still present between the active and endurance-trained participants. This suggested that not only quantitative, but also qualitative, mitochondrial characteristics differ between active and endurance-trained participants.

While cross-sectional studies provide a first layer of evidence to suggest differences in mitochondrial characteristics may contribute to differences in endurance performance, other studies are needed to determine whether these mitochondrial differences are needed for improved endurance performance.

1.4.2 Correlation studies

Correlation studies provide a simple way of linking two variables, which may, or may not be, influencing one another. Furthermore, correlation studies allow researchers to observe if in a homogeneous group of participants (i.e., well-trained cyclists) these relationships still hold true. This technique is widely used in research and is needed as evidence of a relationship between two variables.

Early correlation findings showed that mitochondrial content was highly correlated with relative $\dot{V}O_{2max}$ ($r = 0.82$) in untrained to highly trained participants (56). More recently, SDH enzyme activity was strongly correlated with relative $\dot{V}O_{2max}$ ($r^2=0.81$) in participants ranging from heart failure patients to professional cyclists (68). However, there is no relationship between CS activity and cycling efficiency ($r = 0.13$-0.21, all $p > 0.05$) in a heterogeneous group (69). In a different study, there was a moderate correlation between $mito_{VD}$ and cycling efficiency ($r = 0.34$-0.44; $p < 0.05$) in older adults (66). These results would suggest that mitochondrial content is important for $\dot{V}O_{2max}$ and less important for cycling efficiency.

Mitochondrial respiratory function (maximal ADP-stimulated respiratory function with pyruvate as a substrate) was strongly related to multiple endurance performance variables, such as the oxygen consumption (VO_2) at the LT ($r = 0.94$) and $\dot{V}O_{2max}$ ($r = 0.83$) in a group of 13 active and trained participants (11). Among the main physiological variables, exercise efficiency seems the one that is less correlated with mitochondrial respiratory function. It was shown that respiratory function (measured as ATP_{max} from PCr recovery) was moderately correlated with cycling efficiency ($r = 0.42$-0.50) in sedentary to trained older adults (66). While heterogeneous groups are commonly used in the literature, a study performed by Jacobs et al. (55) aimed to determine the best predictor of a 26-km time-trial performance in an homogeneous group of highly-trained cyclists. The single parameter that best explained time-trial performance was the maximal mitochondrial respiratory function ($r^2 = 0.47$; $p = 0.0035$).

This would suggest that mitochondrial characteristics often seen to correlate with important physiological factors in heterogeneous groups of participants, may also be important and a distinctive factor for endurance performance in trained athletes. Unfortunately, they did not include any marker of mitochondrial content to be able to elucidate their divergent importance in endurance performance.

Correlation studies should be interpreted with caution as these relationships do not prove causation. Once a possible link between two variables has been identified, interventional studies, where one of the variables is manipulated, should be used to untangle any intricate relationship between the variables. In the following section, intervention and longitudinal studies performed in humans will be discussed.

1.4.3 Intervention and longitudinal studies

The following studies will explore how physiological (e.g., exercise training, detraining) and non-physiological (e.g., phlebotomy) interventions can have an effect on mitochondrial variables and endurance performance. Furthermore, their change over time (longitudinal) can be used as evidence of a relationship, and, when possible, compared to other biological characteristics important for endurance performance.

In pioneering work by Coyle et al (13), trained participants underwent a detraining period of 84 days. The time-course of changes in CS activity, which decreased ~20% after only 12 days without training, paralleled the reductions in the $\dot{V}O_2$ at the LT and $\dot{V}O_{2max}$. These changes also occurred concurrently with changes in other enzyme activities, such as SDH and beta-hydroxyacyl-CoA dehydrogenase (β-HAD). While changes in estimated cardiac output ($\dot{Q}_{max}$) also decreased, these changes could not explain the decreases in the VO_2 at the LT or $\dot{V}O_{2max}$, as there were no further reductions in $\dot{Q}_{max}$ following 21 days of detraining (70). Furthermore, changes in the a-vO2 difference were only modest following 84 days (a significant -7%

reduction), which seemed in line with a non-significant decrease in capillarity (3-7% decrease) over a detraining period.

Evidence in favour of the importance of mitochondrial characteristics can also be obtained from three human studies in which the training-induced central adaptations were manipulated (37, 38, 71). In a study of Bonne et al. (38), the effects of 6 weeks of moderate-intensity continuous training (MICT) on improvements in central components and $\dot{V}O_{2max}$ were studied. Despite the increase in blood volume, $\dot{V}O_{2max}$, and $\dot{Q}_{max}$ following the 6-week period, these improvements were abolished when blood volume was re-established to baseline parameters (via phlebotomy). However, despite the reductions in central components and $\dot{V}O_{2max}$, peak aerobic power ($\dot{W}_{max}$), which is strongly correlated with time-trial performance (55), remained increased when compared to pretraining levels (+17%; 64% of the training-induced improvement), which can be suggested to be a product of maintained peripheral adaptations. This study was replicated by Montero et al. (37) and included peripheral measurements ($mito_{VD}$, mitochondrial respiratory capacity, capillary-to-fibre ratio, and a-vO_2 difference). Similar to the previous study, $\dot{V}O_{2peak}$ was back to baseline values when blood parameters were re-established to pre-training levels. However, the peripheral adaptations, which included increased capillary-to-fibre ratio (17.6 ± 16.2%), increased $mito_{VD}$ (42.7 ± 29.5%), unchanged a-vO_2 difference (not-significant 2.2 ± 9.3%), and unchanged mitochondrial respiratory function (not-significant 10.5 ± 28.6% increase), were maintained. As previously, $\dot{W}_{max}$ did not return to baseline values and remained increased (+9%; 62% of the training-induced improvement), probably due to improved mitochondrial content and capillarisation that remained in place. Lastly, in a recent study from Skattebo et al. (71), it was shown that following 10 weeks of endurance exercise, including both high-intensity exercise training and MICT, there were improvements in central (improved blood volume, haemoglobin mass, left-ventricle mass) and peripheral (unchanged capillary-to-fibre ratio (+17%; $p = 0.065$), as well

as increases in protein levels of mitochondrial enzymes (+48-80%)). Re-establishment of blood parameters did not translate into decreased $\dot{V}O_{2peak}$, which was probably due to the small blood withdrawal and structural cardiac adaptations. However, as in the previous studies, $\dot{W}_{max}$ remained increased (+9%; 75% of the training-induced improvement) despite the reduction in blood volume and haemoglobin mass. In conclusion, these three studies suggest that at least two thirds of the improvements in $\dot{W}_{max}$ following a training intervention are due to peripheral adaptations that remain in place when blood volume is reduced to pre-training levels.

On the other hand, the idea of mitochondria not being a limiting factor was reinforced in an intervention study showing decreased mitochondrial respiratory function (-22%) and enzyme activity (-15%) following a 42-day cross-country ski expedition, despite maintained pulmonary and leg $\dot{V}O_{2,}$ and $\dot{W}_{max}$ (non-significant -3.4%) (58). Surprisingly, the fibre-type distribution was also modified following the 42-day expedition with a decreased type I fibre distribution (-12%) and increased type IIx (+85%). This could also suggest that the differences observed may be a result of the fibre-type distribution from the muscle bundles obtained at the different timepoints.

While human studies have shed some light into the biological mechanisms of endurance performance, there is limited capabilities for fully answering some of these fundamental questions. A seminal animal study has also shown the impact of mitochondrial respiratory function on determinants of endurance performance markers (72). However, exogenous alteration of mitochondrial content or function (i.e., iron deficiency) lack ecological validity given the importance of mitochondria in multiple other cellular processes. As an example, some strategies used to study gene gain- or loss-of-function (as those employed to study the importance of a given mitochondrial protein) in animal models have been shown to have off-target effects disturbing mitochondria (73).

1.4.4 Summary

The importance of enhanced mitochondrial characteristics for endurance performance has been demonstrated by the cross-sectional, correlational, and intervention studies that support these premises. More specifically, mitochondrial content variables seem to be a crucial first adaptation, as they differ across non-homogeneous populations in multiple studies, and seems to be the main characteristic by which endurance performance is partially maintained in phlebotomy and detraining studies. On the other hand, while mitochondrial respiratory function may not be as sensitive as mitochondrial content across different populations, it seems to be a crucial adaptation for enhanced endurance performance in homogeneous populations, as observed in highly-trained participants.

1.5. Conclusion

Multiple lines of evidence suggest that mitochondrial characteristics are an important biological determinant of endurance performance. While it seems that mitochondrial characteristics are not limiting the achievement of a high $\dot{V}O_{2max}$, evidence points towards a more important role of mitochondria in endurance performance.

Despite all of the abovementioned research, multiple factors should be still taken into account. Are mitochondria behaving similarly *ex vivo* compared to *in vivo*? If so, are they capable of resynthesising ATP as efficiently throughout an endurance race? A recent study suggests that over a 4-min bout of maximal exercise the ADP-specific ATP resynthesis declines up to 25%, suggesting an increased mitochondrial uncoupling (74). The presumably overbuilt mitochondrial 'capacity' may not take into account that mitochondrial efficiency may not be constant. Whether specific mitochondrial characteristics are more or less important for adaptations in endurance physiological variables (e.g., cycling efficiency) remains to be fully elucidated.

While further research is required, it will be important to understand how mitochondrial senses and responds to distinct exercise sessions, and how they remodel following exercise training so as to improve endurance performance.

CHAPTER 2 – MITOCHONDRIAL DYNAMICS, STRESS, AND REMODELLING IN SKELETAL MUSCLE.

Having established an important role of mitochondria for endurance performance in chapter 1, this chapter provides a review of the literature exploring the role of mitochondrial dynamics and mitochondrial stress in skeletal muscle, and how they are influenced by exercise. Furthermore, the effects of exercise training on the observed mitochondrial remodelling in skeletal muscle will be discussed. This literature review will provide a summary of mitochondrial dynamics and mitochondrial stress pathways, trying to pinpoint the gaps in the literature that should be addressed in future research.

2.1 What are mitochondria?

Mitochondria, from the Greek words *mitos* (‘thread’) and *chondrion* (‘granule’), are named after their shape and are fundamental organelles found in most eukaryotic cells. Mitochondria are descended from α-protobacteria, which were thought to be engulfed by pre-eukaryotic cells 1.5 billion years ago (75). This endosymbiotic event gave eukaryotic cells a competitive advantage as they were able to utilise a wider variety of energy sources. From a structural perspective, mitochondria can be divided into five sub-compartments: Outer Mitochondrial Membrane (OMM), Intermembrane Space (IMS), Inner Mitochondrial Membrane (IMM), Cristae, and the Matrix. In mammals, the mitochondrial proteome is composed of over ~ 1200 proteins (MitoCarta). Mitochondria contain their own genome, the mitochondrial DNA (mtDNA). The size of human mtDNA is 16,569 base pairs and it encodes 37 genes, of which 13 are subunits of the electron transport chain (ETC); the remaining genes consist of 2 ribosomal RNAs (rRNA) and 22 transfer RNAs (tRNA). The small size of the mtDNA genome appears to be due to extensive transfer of genetic information to the nuclear genome.

Given that more than 1100 mitochondrial proteins are nuclear-encoded, and mito-nuclear coordination is indispensable for improvements in mitochondrial characteristics, it will be important to establish how mitochondria dynamically senses and remodels following a physiological stressor (e.g., exercise).

2.2 Mitochondrial dynamics

Mitochondrial dynamics describes the processes that allow mitochondria to selectively interact, reorganise, or be recycled, to ensure a functional and coordinated mitochondrial pool. These processes are termed mitochondrial fusion, mitochondrial fission, and mitochondrial degradation (mitophagy). Depending on the cell-type studied, mitochondrial trafficking or motility may be also involved; however, this does not seem to occur in skeletal muscle (76). In

the current chapter, mitochondrial biogenesis has also been included as part of the mitochondrial dynamics; however this is not widely accepted as a mitochondrial dynamic event. The final characteristics of the mitochondrial pool will be the result of a fine-tuned regulation of mitochondrial dynamics (77, 78).

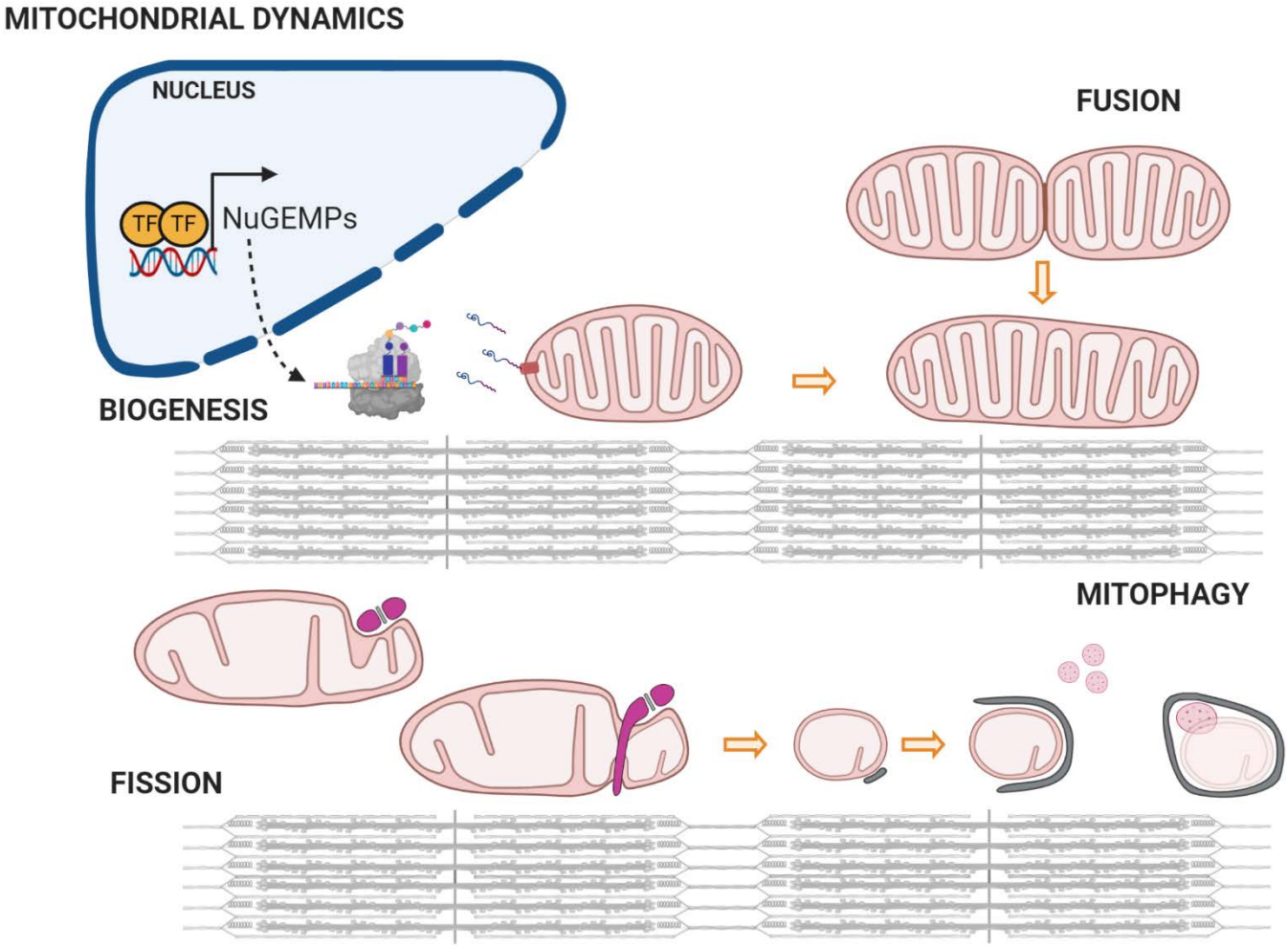

Figure 2.1. Diagram of the different steps involved in mitochondrial dynamics. Mitochondrial biogenesis is represented as increased translation and import of nuclear-encoded mitochondrial proteins; there is also an increased translation of mitochondrial-encoded proteins. Mitochondrial fusion is shown as increased interaction of two adjacent mitochondria, and subsequent merging into a single mitochondrion. Mitochondrial fission is represented with a stressed mitochondrion from which a small part is fissioned with the help of the sarcoplasmic reticulum. Lastly, the fissioned mitochondrion is tagged with the formation of a mitophagosome around it, finalising with the incorporation of a lysosome and subsequent degradation. NuGEMPs = nuclear genes encoding mitochondrial proteins; TF = transcription factor.

2.2.1 Mitochondrial biogenesis

2.2.1.1 Definition

Mitochondria cannot be created *de novo* (79). Mitochondrial biogenesis refers to the generation of new mitochondrial proteins, and relies on the coordinative function of the nuclear and mitochondrial genomes. This requires mitochondria to have an appropriate import system for these newly synthesised, nuclear-encoded, precursor proteins. Translocase of Outer Mitochondrial Membrane (TOMM) is a protein complex found in the OMM that interacts with the N-terminal of mitochondrial precursor proteins (to identify that their destination is the mitochondria). Translocase of Inner Mitochondrial Membrane (TIMM) then helps to import the protein into the matrix where proteins are fully folded by mitochondrial-resident chaperones (80, 81). Upon failure to correctly import and fold mitochondrial proteins (82), a retrograde signalling response is rapidly activated influencing whole-cell protein synthesis in efforts to balance transcription and translation (83, 84). Mitochondrial biogenesis is regulated by transcriptional, translational, and post-translational events requiring both the nuclear and mitochondrial genomes (53). Among the most common transcription factors known to influence mitochondrial biogenesis are peroxisome proliferator-activated receptor gamma coactivator-1α (PGC-1α), nuclear respiratory factor 1 (NRF1) and 2 (NRF2), and the tumour suppressor p53 (52, 85).

2.2.1.2 How is it measured?

Despite many decades of research there is no single, widely-accepted method for measuring mitochondrial biogenesis - especially when working with human tissue samples like skeletal muscle (43, 86). In cell or animal models, stable isotope labelling by amino acids (SILAC) can be used to detect the incorporation of proteins and to allow the monitoring of *de novo* production of proteins of interest through pulsed SILAC (87). In human tissue samples,

numerous techniques have been used in the literature to assess mitochondrial biogenesis. Among those, the most commonly used are: mRNA content of key transcription factors, content of mitochondrial proteins, and rates of mitochondrial protein synthesis (86). Nonetheless, a combination of early events like gene expression (e.g., PGC1α mRNA), mitochondrial protein synthesis, and protein signalling of known targets (e.g., phosphorylation of AMPK), combined with the final outcome of accumulative, increased or decreased, mitochondrial biogenesis will be reflected by changes in mitochondrial respiratory function and/or content (43, 86).

2.2.1.3 Skeletal muscle

In humans, skeletal muscle is the most abundant tissue and represents 40 to 50% of the whole body mass. During exercise, humans can increase their metabolic rate by 20 fold (88). As such, skeletal muscle represent a unique tissue with high mitochondrial content and plasticity needed to adapt to physiological and metabolic challenges (53). In skeletal muscle, mitochondrial biogenesis signalling can be acutely increased following a single exercise session (89, 90), and endurance training seems to positively modify the mitochondrial pool (53).

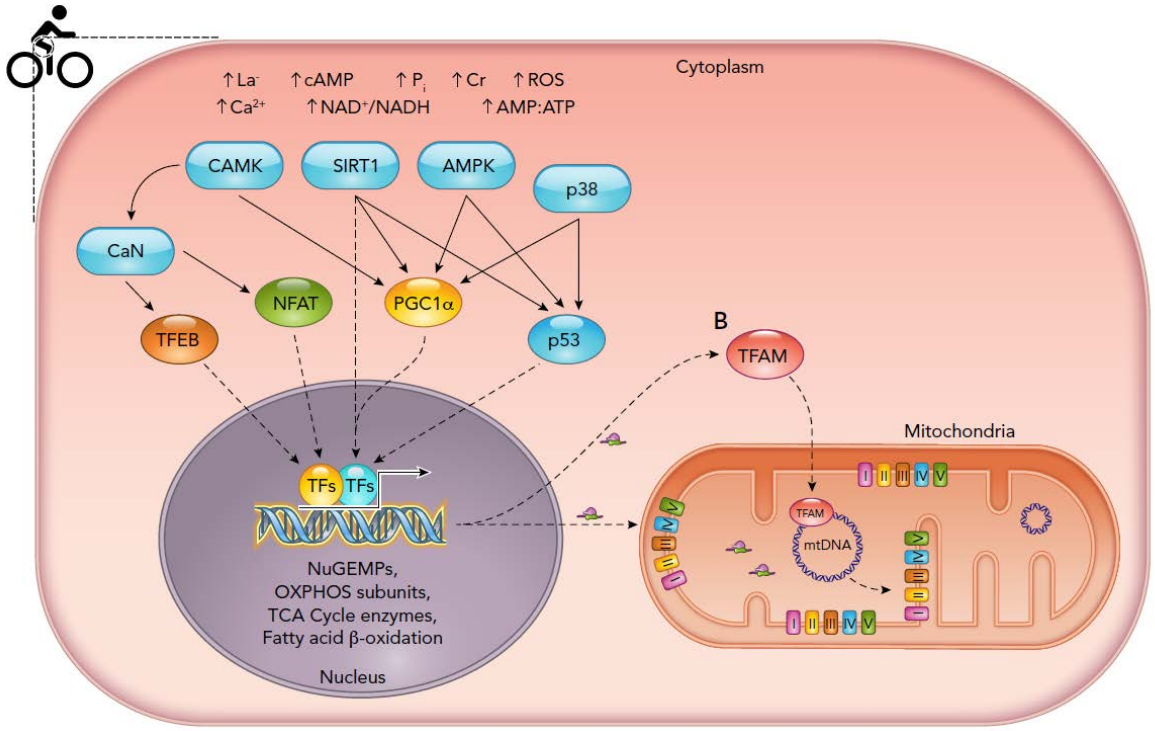

Figure 2.2. Schematic representation of cellular molecular events associated with mitochondrial biogenesis occurring during an endurance exercise session. NuGEMPs = nuclear genes encoding mitochondrial proteins; TFs = transcription factors; La = lactate; cAMP = cytosolic adenosine monophosphate; P_i = inorganic phosphate; Cr = creatine; ROS = reactive oxygen species; Ca^{2+} = calcium; NAD^+/NADH = ratio between oxidised and reduced nicotinamide adenine dinucleotide; AMP/ATP = ratio between adenosine monophosphate and adenosine triphosphate. Reproduced from (51).

2.2.1.4 Effects of exercise

During a single session of exercise, alteration in the concentration of metabolites like adenosine monophosphate (AMP) and calcium (Ca^{2+}) will cause an homeostatic perturbation and trigger specific signalling cascades. Due to the increased ATP turnover, there is an increase in the concentration of ADP and AMP, which will modify the AMP:ATP ratio, stimulating the activation of AMP-activated protein kinase (AMPK) (91), although other mechanisms such as fitness level and mitochondrial damage may also be involved (92, 93). Concurrently, the

increased calcium flux will increase the cytosolic [Ca^{2+}] and will lead to the activation of Ca^{2+}/Calmodulin-dependent kinases II (CaMKII). Furthermore, due to increased cellular respiration, there will be a change in the ratio of [NAD+]/[NADH] and an increase in the production of reactive oxygen species (ROS), activating the SIRT1 and p38 mitogen-activated protein kinases (p38 MAPK) (53).

In human skeletal muscle, following an exercise session there is an increased nuclear accumulation of PGC-1α and p53 protein - two well-known transcription factors associated with mitochondrial biogenesis. This response is larger following sprint-interval exercise (SIE) compared to moderate-intensity continuous exercise (MICE) (89) and is blunted following a training period (50). Concurrently, following exercise there is promoter hypomethylation in regions regulating genes involved in mitochondrial biogenesis (e.g., PGC-1α) – a process that is also dependent on exercise intensity (94). These responses may be partially influenced by changes in AMP:ATP ratio and cytosolic calcium, as reflected by AMPK and CAMKII phosphorylation levels (95). These alterations following an exercise session are early events that would dictate the transcriptional upregulation of genes involved in mitochondrial biogenesis, and, theoretically, subsequent increases in the synthesis of mitochondrial proteins.

2.2.2 Mitochondrial fusion

2.2.2.1 Definition

Mitochondrial fusion is a two-step process, where two or more adjacent mitochondria combine their outer and inner membranes to become one. Mitochondrial fusion is a fast-merging event that can take place in less than two minutes (96). The proteins responsible for the fusion events are mitofusin-1 (MFN1) and mitofusin-2 (MFN2) for the OMM fusion, and dynamin-like 120 kDa protein Optic Atrophy 1 (OPA1) for IMM fusion. These processes are thought to occur individually as a multi-step process, although physical interaction between these proteins has

been found (97). Fusion events are important for oxidative phosphorylation (OXPHOS) activity, to regulate and spread mutations in mtDNA, to dilute and share any damage caused by reactive oxygen species (ROS), and for the transfer and exchange of proteins, lipids, and metabolites between mitochondria (98).

2.2.2.1.1 Outer mitochondrial membrane fusion

Both MFN1 and MFN2 are the proteins responsible for mitochondrial outer membrane fusion, while MFN2 also tethers mitochondria to lipids and adjacent organelles such as the endoplasmic reticulum (99). Interestingly, MFN1 and MFN2 show distinct protein abundances across different tissues, suggested to possibly underlie distinct mitochondrial fusion needs (100).

Both proteins have a first coiled-coiled heptad repeat (HR1) and second (HR2) regions that can oligomerise with themselves and with analogous HR regions (e.g., MFN1 or MFN2) (101). This step helps tether adjacent mitochondria and, possibly through a conformational change in the guanosine triphosphatase (GTPase) domain, facilitate OMM mitochondrial fusion (98). MFN2 is widely considered as the more important protein in mitochondrial fusion (102, 103). Classically considered to have the C and N terminal facing the cytosol, it has been recently suggested that the C-terminal may face the inter membrane space (IMS) and have redox sensing capabilities (104). Other authors suggest MFN1 to be more important for mitochondrial fusion (100).

2.2.2.1.2 Inner mitochondrial membrane fusion

The main protein responsible for the IMM fusion is OPA1. However, the role of OPA1 is multiple as it also controls cristae remodelling (105). The alternative splicing of OPA1 can result in at least 8 different isoforms (106). Upon OPA1 protein arrival to the IMM, it can be cleaved by two distinct proteases: the ATP-dependent protease yeast mitochondrial DNA

escape 1-like (YME1L or YME1L1) and the zinc metalloprotease overlapping with m-AA protease (OMA1). The activity of these protease will dictate the balance between the short and long OPA1 proteins. The correct balance between short-OPA1 and long-OPA1 is crucial, as it will dictate the balance between fusion and fission (107, 108).

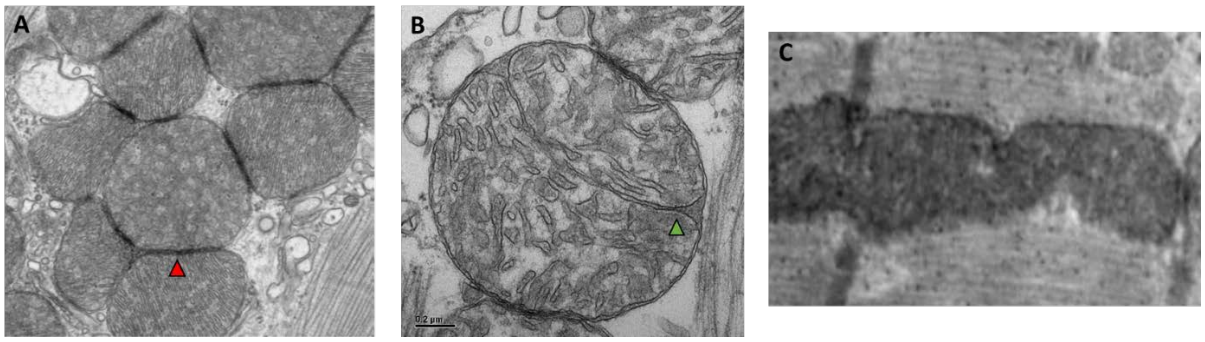

Figure 2.3. An illustration of the different steps of mitochondrial fusion. A) Increased electron density at membrane junctions (red arrow); an event described as a 'kiss' between adjacent mitochondria (mice cardiac muscle; (109)); B) Outer membrane fusion, but not inner membrane (green arrow), incomplete or partial fusion event (*Astyanax mexicanus* skeletal muscle; with permission from Mr. Luke Olsen, University of Kansas Medical Center); C) a whole mitochondrion with completed inner and outer membrane fusion (human skeletal muscle; (110)).

2.2.2.2 How is it measured?

Mitochondrial fusion rates can be measured in the skeletal muscle of animal models (e.g., rodents). This is possible due to the creation of mouse models with a photo-convertible fluorescent protein localised to the mitochondrial domain (e.g., mito-Dendra2) (111). Upon the photo-conversion of a subpopulation (e.g., mitochondria switch to red), the mitochondrial fusion rates can be quantified *in vivo* by tracking the colour changes (upon fusion) of adjacent mitochondria. On the contrary, no method has been employed in human skeletal muscle due to ethical limitations. However, some authors have suggested that by combining different indirect methods it may be possible to infer changes in mitochondrial fusion parameters. Picard, Gentil (112), using electron microscopy micrographs, quantified the number of adjacent

mitochondrial contacts from post-exercise samples and compared them to resting samples. It was suggested that exercise leads to an increased number of physical interactions between mitochondria and this could serve to indicate a pre-fusion event (Figure 2.3.A).

2.2.2.3 Skeletal muscle

Mitochondrial fusion seems to occur at a slower frequency in rat skeletal muscle fibres (0.5 fusion events per minute) when compared to human myoblasts (3.5 fusion events per minute) or rat myotubes (5.8 fusion events per minute) (113), and this has been suggested to be due to the limited space found in muscle fibres (113, 114). Interestingly, silencing of MFN1 led to decreased rates of mitochondrial fusion, which did not occur following silencing of MFN2 (96), suggesting a greater importance of MFN1 in regulating fusion levels in mammalian skeletal muscle. In skeletal muscle, mitochondrial fusion has been reported to be important for excitation-contraction coupling and to prevent dysregulation of calcium (96). Fusion rates are known to be different between type I and IIx skeletal muscle fibres in mice, and to correlate with mitochondrial respiratory rates (basal and maximal mitochondrial oxygen consumption rates), which further supports their role in OXPHOS activity (115).

In human skeletal muscle, mitochondrial fusion rates have not been measured and only changes in mRNA or protein changes in the abovementioned proteins involved in the underlying processes have been reported. Although not a true marker of mitochondrial fusion, mRNA and protein levels can provide a hint as to whether mitochondrial fusion has been upregulated. Multiple studies have assessed the differences in these markers at baseline between physically active, inactive, and metabolically impaired populations (altered glucose and/or insulin levels). The results show that physically active groups have higher levels of MFN1 and MFN2 mRNA (116, 117), MFN2 protein (118, 119), and OPA1 protein (119), when compared to their sedentary counterparts.

2.2.2.4 Effects of exercise

The literature is controversial, as some of the published studies have shown that neither the mRNA, nor the protein levels of MFN1, MFN2, and OPA1, are changed following a single exercise session (85, 120, 121). An initial study showed that both MFN1 and MFN2 mRNA increased 24 hours following a 10-km time trial, but both were unaltered 2 hours post-exercise (122). Another study showed that following SIE, but not high-intensity interval exercise (HIIE), there is an increase in MFN1 and MFN2 mRNA (123), while others have shown increased MFN2 mRNA 3 hours following SIE and MICE (89). Finally, it was shown that MFN2 mRNA levels increase following MICE in healthy participants but not in individuals with type 2 diabetes (T2D) (117).

Concurrently, it has been shown that MFN2 protein levels were increased 3 hours following SIE and HIIE, while OPA1 protein levels were only increased following HIIE (123). However, several methodological limitations were found in this study as the internal standard used for protein levels adjustment was heat shock protein 70 (a stress-sensitive protein; (124)) and the resting muscle biopsy was taken on a different day. Another study has shown that in healthy participants, but not those with T2D, there was an exercise-induced increase in MFN2 protein levels (117).

2.2.2.5 Effects of exercise training

Studies generally support an increased MFN1 mRNA (85), MFN2 mRNA, and protein (125-127), and OPA1 mRNA and protein (116, 128), in response to exercise training. However, others did not observe any changes (52, 129). Metabolically impaired participants have shown a blunted MFN2 protein increase upon exercise training when compared to healthy participants (126), which would align with the exercise-induced response in MFN2 mRNA and protein

(117). Collectively, this suggests the potential to increase the expression of genes regulating mitochondrial fusion, as well as their protein abundance, following exercise training in healthy participants.

Exercise training in rodents has been reported to increase fusion rates in the type IIx fibres, and seems to be essential for protecting against the deleterious effects of mtDNA mutations in skeletal muscle (130). Increased mitochondrial fusion has proven to be an effective strategy to decrease the mtDNA heteroplasmy in rodent skeletal muscle (130). Voluntary exercise in rodents has been shown to reduce the mtDNA mutation load and improve the phenotypic score of mice with mtDNA mutations (131). Whether exercise may positively act in decreasing the mutation load of mtDNA through increases in mitochondrial fusion is an area of interest and warrants future research.

2.2.3 Mitochondrial fission

2.2.3.1 Definition

Mitochondrial fission is the selective division of a mitochondrion into two or more daughter mitochondria. The GTPase dynamin-related protein 1 (DRP1) is the principal protein constricting and dividing a mitochondrion into two. Mitochondrial fission takes place in three steps: a) marking the fission site of the mitochondria with receptors (mitochondrial fission 1 protein [FIS1], mitochondrial fission factor [MFF], mitochondrial dynamics protein of 49 kDa [MiD49], and mitochondrial dynamics protein of 51 kDa [MiD51]) that recruit DRP1; b) formation of a DRP1 oligomer superstructure around the fission site; c) and guanosine triphosphate (GTP) is hydrolysed and the DRP1 helix constricts the site and divides the mitochondrion with the help of dynamin-2 (DNM2) and the endoplasmic reticulum (ER).

Different receptors have been suggested to recruit DRP1 to the mitochondria. The first receptor identified was FIS1. Despite its original name, it is not yet clear what the role of FIS1 is as it

is not required for fission (132). Current research has shown that the mitochondrial fission factor (MFF) and mitochondrial dynamics proteins of 49- and 51-kDa (MiD49, MiD51) are the main proteins responsible for recruiting DRP1 to the fission site (133). Nonetheless, FIS1 still has a role in fission (132), although minor when compared to other adaptor proteins. This has led to the current idea that FIS1 is important for the regulation of mitophagy in skeletal muscle and other tissues (134, 135).

DRP1 is a cytosolic protein that upon post-translational modifications is translocated and recruited to the mitochondria. Increases in DRP1 phosphorylation at serine 616 lead to the translocation of DRP1 to the OMM (136). On the other hand, phosphorylation of DRP1 at serine 637 is thought to prevent the translocation of DRP1 to mitochondria. In fact, dephosphorylation of DRP1 at s637, due to calcium-dependant activation of calcineurin, induces Drp1 translocation to mitochondria and increases fragmentation (137). Activation of the energy-sensing kinase AMPK can also promote mitochondrial fission through phosphorylation of MFF at serine 155 and 172 (138). Models of DRP1 knockout (KO) have allowed researchers to pinpoint the role of DRP1 in dividing dysfunctional, or redundant, mitochondria and 'cutting' them to the 'right' size to be engulfed by mitophagosomes (see below in 2.3.4. Mitochondrial degradation – mitophagy). However, any data obtained concerning DRP1 and MFF need to be interpreted with caution as these proteins are also involved in peroxisome fission (139).

While the traditional view is that OMM and IMM fission occur simultaneously, a recent preprint article suggests these steps may be physically independent and there is a set of proteins controlling IMM fission (140). Four mammalian proteins were presented as candidates: the mitochondrial serine-threonine phosphatase PGAM family member 5 (PGAM5), family with sequence similarity 210 Member A (FAM210A), sideroflexin 3 (SFXN3), and the mitochondrial carrier homolog 1 (MTCH1) (141).

2.2.3.2 How is it measured?

Similarly to mitochondrial fusion, mitochondrial fission is measured with the use of live imaging techniques. Briefly, cells or tissues expressing a mitochondrial matrix fluorescent protein (e.g., DsRed) are imaged over a period of time under control or experimental conditions (e.g., laser photo damage). Retraction of a single mitochondrion, resulting in division into two or more daughter mitochondria, is considered a fission event. By quantifying the occurrence of these events, fission can be calculated.

As with mitochondrial fusion, live imaging of mitochondrial fission cannot currently be employed in human tissue. However, capturing the occurrence of events, like mitochondrial pinching (early phase of mitochondrial fission) or mitochondrial constrictions with TEM micrographs, can provide indirect evidence of fission events (142). Another marker of mitochondrial fission may be quantifying the phosphorylation levels of both DRP1 and MFF, as these events are known to increase the number of mitochondrial fission events (138, 142). Furthermore, DRP1 oligomerises when fissioning a mitochondrion, and, although not widely used, measuring DRP1 protein in its native form could indicate when it forms oligomer structures and be suggestive of fission levels. Whether this technique would be sensitive to changes in mitochondrial fission remains to be elucidated in future research.

2.2.3.3 Skeletal muscle

Fission rates, by measuring the frequency of mitochondrial constrictions, have been estimated to be 1.3% in mice extensor digitorum longus skeletal muscle (mainly type II fibres) (142). To the best of my knowledge, there is no evidence regarding estimates of fission rates in human skeletal muscle. In humans, cross-sectional studies have shown that healthy control participants have lower DRP1 protein levels when compared to their sedentary counterpart (125), as well as lower DRP1 mRNA when compared to metabolically-impaired participants (117). Trained

participants had a higher DRP1 protein and phosphorylation of DRP1 at s616 when compared to untrained controls (118). This may suggest that at baseline healthy participants have a lower fission machinery when compared to diseased participants, while in response to chronic exercise this machinery needs to be increased as a part of the enhanced quality control.

2.2.3.4 Effects of exercise

Work done in mice has shown that exercise increases the phosphorylation levels of DRP1 at s616 (143), and this was paralleled by an increase in the number of mitochondrial constrictions reaching 5.6% (142). Another study in mice showed a similar exercise-induced increase in the phosphorylation of DRP1 at s616, which returned to baseline levels 3 hours post-exercise, whereas phosphorylation of DRP1 at serine 637 (the inhibitory site) increased and remained phosphorylated until 6 hours post-exercise (144). These results suggest an upregulation of mitochondrial fission early in the recovery from an exhaustive endurance exercise, which returns to baseline (~ 3-6 h) when other processes may be more prominent (e.g., mitophagy).

Research in humans has shown that a single endurance exercise session is capable of increasing the phosphorylation of DRP1 at serine 616 (117, 120, 121), as well as the mRNA expression of DRP1 and Fis1 (89, 120, 123). This would suggest that exercise is capable of stimulating fission, to selectively isolate mitochondrial parts that are deemed redundant or dysfunctional. Little is known about the effects of a single exercise session on other proteins involved in DRP1 docking on the OMM.

Due to the rapid growth of the field, a search in the MetaMEx (www.metamex.eu) platform was performed to explore if the mRNA content of novel genes, suggested to mediate IMM-fission, were increased following exercise and training. Only FAM210A was upregulated following both aerobic exercise and training. Of the other possible proteins, SFXN3 increased after aerobic training and MTCH1 was decreased after high-intensity training. Future research

will shed some light on the regulation of inner membrane fission and the molecular machinery controlling it.

2.2.3.5 Effects of exercise training

The published literature suggests that DRP1 protein levels are unchanged following exercise training in humans (89, 125, 128) or rodents (143). Only in rodents has it been shown that phosphorylation of serine 637 is increased following 4 weeks of voluntary running (143), suggestive of decreased fission.

2.2.4 Mitochondrial degradation – Mitophagy

2.2.4.1 Definition

Cellular protein quality control is an important pathway by which the cell ensures that proteins meet the required quality (145). Mitochondria have their own protein quality control mechanisms, as well as those shared with other organelles. Distinct pathways are known to exist for the removal of redundant, aged, or damaged mitochondrial proteins, as well as whole organelles (145).

Mitochondrial proteins can be degraded by resident mitochondrial proteases, directed to the proteasome, or can be engulfed and transported to the lysosome by a mitochondrial-derived vesicle (MDV) (146). Mitophagy refers to the degradation of whole organelles through bulk autophagy, and it is estimated to account for approximately one third of the total mitochondrial protein turnover in *Drosophila* (147). The different layers of quality control enables the mitochondria to maintain a population with the highest functional capacity.

To selectively identify which mitochondrion needs to be degraded multiple mechanisms are in place. A key pathway is the PTEN-induced putative kinase 1 (PINK1)-PARKIN pathway (148). PINK1 is a mitochondrial kinase that is constantly imported to the mitochondrial matrix

and cleaved by different mitochondrial proteases (like presenlin-associated rhomboid-like protein (PARL) and Lon protease homolog 1 (LONP1)), and degraded by the proteasome. When the mitochondrial membrane potential is lost, the inability to cleave and degrade PINK1 leads to its accumulation on the OMM. PINK1 then phosphorylates ubiquitin and PARKIN (a cytosolic E3 ubiquitin ligase; gene PARK2) at serine 65. Phosphorylated PARKIN is recruited to mitochondria and conjugates ubiquitin chains on OMM proteins such as MFN1 and MFN2. This leads to increased PINK1-mediated phosphorylation of ubiquitin, amplifying the signalling for autophagosome engulfment (149). The PINK1-PARKIN pathway is responsible for detecting, tagging, and amplifying mitophagy; however, other proteins are responsible for the phagophore recruitment. These proteins bind to the ubiquitin chains formed by PINK1/PARKIN on the OMM, and also contain an LC3-interacting region (LIR) that is capable of binding ATG8 proteins (e.g., LC3B). Among the proteins identified to play this role, Optineurin (OPTN) and nuclear dot protein 52 (NDP52) have been identified as the most important (148, 149). Other proteins include neighbour of BRCA1 gene 1 (NBR1), Tax1-binding protein 1 (TAX1BP1), and sequestosome 1 (SQSTM1/p62). Mitophagy can also occur in the absence of PINK1 or PARKIN, confirming that this is not the only pathway controlling mitophagy (146). Upon mitochondrial damage, different proteins and lipids (e.g., cardiolipin) can accumulate at the OMM and act as mitophagy receptors (146). The different proteins identified are: BCL2/adenovirus E1B 19 kDa interacting protein 3 (BNIP3), BCL2/adenovirus E1B 19 kDa interacting protein 3 like (BNIP3L/NIX), and FUN14 Domain Containing 1 (FUNDC1).

Given the importance of maintaining a healthy mitochondrial pool, the critical role of mitophagy is without question. However, whether the models of mitophagy are reflecting the signalling occurring under physiologically relevant conditions remains questionable. The majority of our knowledge is derived from *in vitro* studies using mitochondrial stressors (e.g.,

oligomycin) that ultimately disrupt the mitochondrial membrane potential of mitochondria. These techniques are important, as they allow researchers to unveil the molecular mechanisms of mitophagy. However, they do not fully explain how mitophagy occurs *in vivo* and in response to common physiological stressors (e.g., nutrient excess, exercise) where only a small fraction of mitochondria are likely to be marked for mitophagy.

2.2.4.2 How is it measured?

Mitophagy can be monitored in cells and animal models through the use of a mitochondrial pH-sensitive fluorescent protein (e.g., mito-Keima, mito- mCherry). These fluorophores allow the detection of mitochondria undergoing lysosome degradation, due to the shift to a lower pH and the resultant fluorescent change. As these fluorophores are resistant to lysosomal degradation, mitopaghic flux can be measured over time.

In mouse skeletal muscle, the use of mitophagy flux has been used for the assessment of mitophagy levels (150). This was done by performing an autophagy flux assay (blockade of autophagosome and lysosome fusion), and quantifying the LC3-II protein levels in mitochondrial fractions. Although this may seem like a valid method, LC3 lipidation takes place in the ER-membranes that are largely associated to mitochondria and co-isolate when fractionating samples (151). This poses the question as to whether the increased LC3-II seen in the mitochondrial fraction is really responsible for mitochondrial-specific autophagosome engulfment or just reflects general autophagy.

In human tissue, there is no single technique to measure changes in mitophagy. However, two techniques have the potential to be used: by using immunofluorescence microscopy to study the multiple steps from mitochondrial engulfment up to mitochondrial degradation in the lysosome; and the quantification of cytosolic proteins that are recruited to mitochondria upon mitophagy intitiation in a mitochondrial fraction.

2.2.4.3 Skeletal muscle

Loss of PINK1 (152) and PARKIN (153) do not affect basal mitophagy in skeletal muscle. However, loss of PARKIN in skeletal muscle leads to decreased force and decreased mitochondrial respiratory function (154). On the other hand, PARKIN overexpression protects skeletal muscle from age-related muscle loss and sarcopenia, while increasing mitochondrial respiratory function (155). The role of mitophagy is thought to be highly relevant in scenarios where elevated levels of mitochondrial damage could upregulate cell death (156) and inflammatory pathways (157), which would worsen the cellular outcome. In mice flexor digitorum brevis muscle (mainly type II fibres), the amount of mitochondria that undergo mitophagy under resting conditions is estimated to be 0.1% (144).

2.2.4.4 Effects of exercise

In rodents, endurance exercise is known to increase the regulation of mitophagy in skeletal (144) and cardiac muscle (157). Using immunofluorescence microscopy, the peak number of co-localised lysosome and mitochondria (mitolysosomes) occurred 6 hours post-exercise, which was preceded by an increased oxidative stress 3 hours post-exercise (144). When AMPK phosphorylation at tyrosine 172 was blunted (due to a muscle-specific transgenic mice of a dominant-negative form of the catalytic α2 subunit of Ampk), the exercise-induced increase in phosphorylation of Unc-51 like autophagy activating kinase 1 (p-ULK1) at s555 was ablated. This happened together with abolished mitolysosome co-localisation, which was also blunted in ULK1 muscle knockout. These results suggested that in skeletal muscle both AMPK and ULK1 are important for the increased exercise-induced mitophagy. This is supported by a recent study using C2C12 cells where AMPK-driven signalling cascade was deemed necessary for increased mitophagy upon a mitochondrial insult (158). *In vivo* studies have shown that following 90 min of continuous exercise mitophagy levels can increase up to 3-fold (144).

Interestingly, a similar exercise protocol did not seem to increase the mitochondrial levels of PINK1 suggesting that exercise-induced mitophagy may be upregulated through another pathway (159). This would seem counterintuitive, as others have shown that PARKIN was required for the increased exercise-induced mitophagy, which would suggest an increased PINK1 accumulation (160). Although these findings may seem conflicting, a key difference may be the intensity of the exercise. In the latter study mice ran until exhaustion (160) and this could be linked to increased exercise-induced mitochondrial disruption (161). This could suggest increased number of mitochondria with disrupted membrane potential (due to rupture of OMM) and possibly increased accumulation of PINK1.

In humans, the main techniques utilised for exploring exercise-induced mitophagy are the measurement of changes in mRNA and protein levels. Two hours of MICE in a fed state, but not in a fasted state, was shown to increase BNIP3 and BNIP3L mRNA without changes in PARK2 mRNA (120). However, 1 hour of MICE did not alter BNIP3 or BNIP3L mRNA content in another study (117). Furthermore, two studies have reported decreased mRNA expression of PARK2 following MICE, HIIE, and SIE (120, 123). Increased PARKIN and BNIP3 protein levels have been reported following 1 hour of MICE (162). Also, different studies have shown an exercise-induced increase in p-ULK1 at s555 (163, 164).

These results probably point towards a lower involvement of PINK1/PARKIN signalling cascade in exercise-induced mitophagy. Multiple exercise phospho-proteomic studies in human and rodent skeletal muscle have shown that OPTN is phosphorylated at multiple sites following exercise (165-167).

2.2.4.5 Effects of exercise training

Regarding exercise training, PINK1 and BNIP3, but not PARKIN, protein levels have been shown to increase following 16 weeks of HIIT in older adults (125), while BNIP3 and PARKIN

protein levels have been shown to increase following 8 weeks of MICT in young adults (162). Others have shown decreased PARKIN protein levels, relative to CS activity, following 12 weeks of MICT training (129). It remains equivocal whether an increased protein levels of these mitophagy proteins actually reflect an increase in the levels of mitophagy.

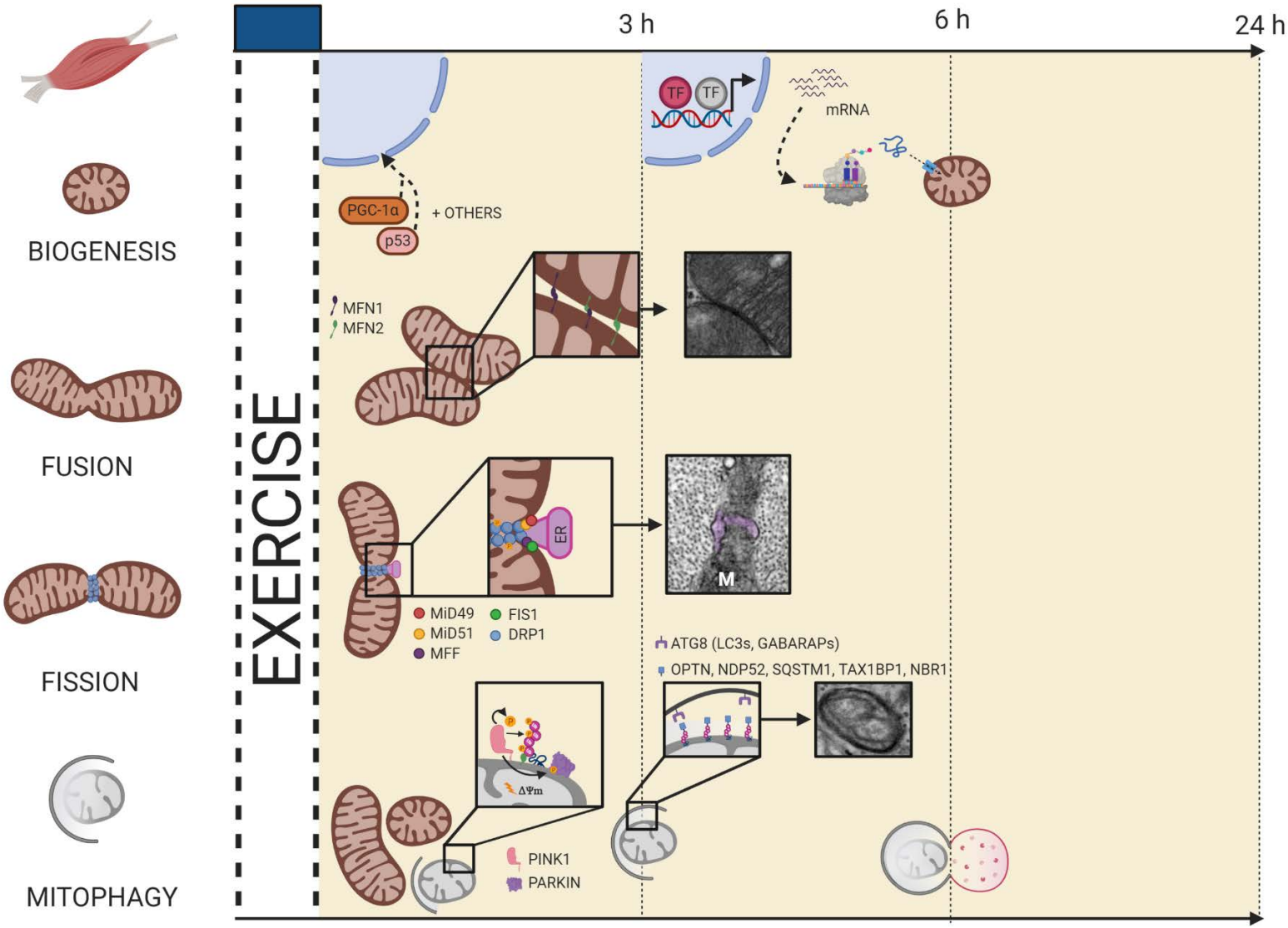

Figure 2.4. Summary of the time course of the different mitochondrial dynamics events occurring up to 24 hours from the end of exercise. TF = transcription

factor; mRNA = messenger ribonucleic acid; PGC-1α = peroxisome proliferator-activated receptor gamma coactivator 1-alpha; p53 = tumour suppressor p53; MFN1 = mitofusin 1; MFN2 = mitofusin 2; MiD49 = mitochondrial dynamics protein MID49; MiD51 = mitochondrial dynamics protein MID51; MFF = mitochondrial fission factor; FIS1 = mitochondrial fission 1 protein; DRP1 = dynamin-related protein 1; ATG8 = autophagy-related protein 8; LC3 = microtubule-associated protein 1A/1B light chain; GABARAP = gamma-aminobutyric acid receptor-associated protein; PINK1 = PTEN-induced kinase 1; PARKIN = E3 ubiquitin-protein ligase PARKIN; OPTN = optineurin; NDP52 = nuclear dot protein 52; SQSTM1 = sequestosome 1; TAX1BP1 = tax1-binding protein 1; NBR1 = next to BRCA1 gene 1 protein. ΔΨm = mitochondrial membrane potential.

2.3 Mitochondrial stress response (MSR).

Multiple stressors can be sensed by mitochondria leading to a stress response aiming at restoring mitochondrial homeostasis. In mammals, the mitochondrial stress response is known to act through the integrated stress response (ISR) pathway (168, 169). The core sensor and activator of the ISR, the alpha subunit of the eukaryotic translation initiation factor 2 alpha (eIF2α), is phosphorylated at serine 51 upon stress. In mammals, four different kinases can sense distinct stressors and phosphorylate eIF2α (170) (Figure 2.5). The heme-regulated eIF2α kinase (HRI/ *EIF2AK1*) reacts to heme depletion and cytosolic unfolded proteins, but recently was also shown to be involved in sensing mitochondrial stress (168, 169, 171, 172); the protein kinase R (PKR/*EIF2AK2*) responds to accumulation of double-stranded RNA in the cytoplasm (e.g., viral infection) (173); the protein kinase R (PKR)-like endoplasmic reticulum kinase (PERK/*EIF2AK3*) responds to accumulation of unfolded proteins in the ER (174); and the general control nonderepressible 2 (GCN2/*EIF2AK4*) is activated by low free amino acid levels and ROS (175). In parallel, two distinct proteins constantly survey and control the ISR levels. The protein PPP1R15B (also known as CReP) is constitutively expressed to regulate the phosphorylation levels of eIF2α, while PPP1R15A (also known as GADD34) is induced during the activation of the ISR.

Initial findings showed that mitochondrial stress (mtDNA depletion and accumulation of truncated proteins) selectively increased the transcription of mitochondrial chaperones (HSPE1 and HSPD1), and CHOP was identified as one transcription factor driving this transcriptional response (176, 177). Extensive studies then began characterising this stress response in lower eukaryotic organisms identifying mitochondrial protein misfolding, or folding stress, as the initiating mechanism and being termed the mitochondrial unfolded protein response (UPR^{mt})

(Figure 2.5). It has been suggested that UPR^{mt} and mitophagy share a common mechanism of regulation and may act in conjunction (145).

In human cells, multiple mitochondrial stressors like blockage of OXPHOS, disruption of membrane potential, inhibition of mitochondrial protein import, and inhibition of mitochondrial protein translation, were found to converge on the activation of an ATF4-mediated transcriptional response (178). Out of the commonly upregulated genes following the different mitochondrial stressors, almost half of the genes (26/59) were ATF4 known targets. However, this study did not detect an upregulation of HSPE1 and HSPD1 in all conditions, which suggested changes in these proteins was not a universal signature induced by all mitochondrial stressors.

More recently, two independent studies performed in human cells found a pathway by which mitochondrial stress is relayed to the cytosol where it signals the ISR through the HRI kinase (168, 169). They elegantly identified that upon treatment with individual mitochondrial stressors – oligomycin, doxycycline, antimycin, rotenone and carbonyl cyanide 3-chlorophenylhydrazone (CCCP) – there was an OMA1-dependant cleavage of DAP3 Binding Cell Death Enhancer 1 (DELE1), leading to an accumulation of cleaved DELE1 protein in the cytosol. Cleaved DELE1 would then interact with HRI, leading to phosphorylated eIF2α and increased ATF4 translation. They showed abolished ATF4 protein synthesis upon mitochondrial stressors (except for doxycycline) with DELE1, HRI, or OMA1 knockdown. Interestingly, doxycycline – a known mitochondrial protein translation inhibitor – has been suggested to act through the GCN2 kinase (179), which could explain its HRI-independent activation of the ISR. It remains to be explored whether the OMA1-DELE1 pathway is a universal signature of mitochondrial stress and occurs in metabolically active tissues like skeletal muscle.

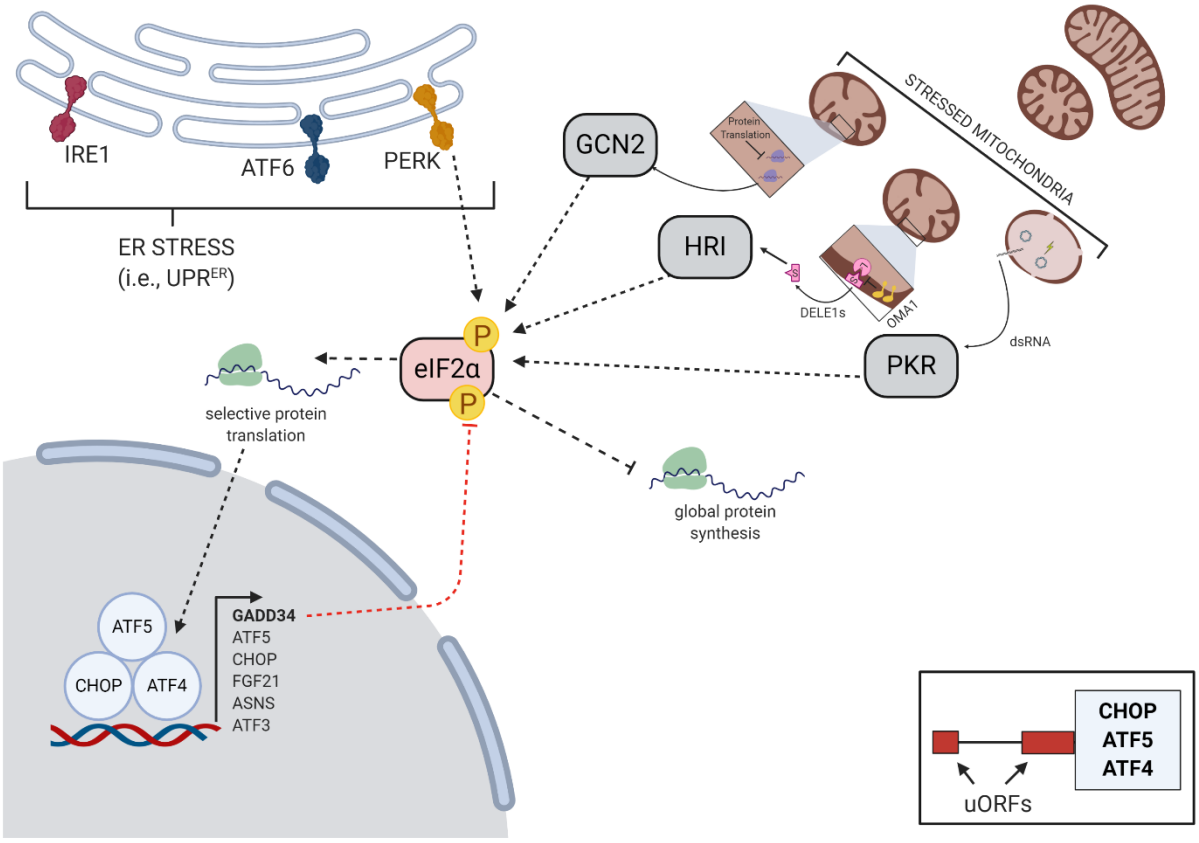

Figure 2.5. Representation of the different kinases that can phosphorylate eukaryotic translation initiation factor 2A (eIF2α) at serine 51, and how they can be related to endoplasmic or mitochondrial stress. uORF = upstream open reading frame; dsRNA = double-stranded RNA; UPR^{ER} = endoplasmic reticulum unfolded protein response. ATF3 = actviating transcription factor 3; ATF4 = activating transcription factor 4;ATF5 = activating transcription factor 5; ATF6 = activating transcription factor 6; GADD34 = growth arrest and DNA damage-inducible protein GADD34; CHOP = CCAAT/enhancer-binding protein homology protein; FGF21 = fibroblast growth factor 21; ASNS = asparagine synthetase; PKR = protein kinase R; HRI = heme-regulated eIF2α kinase; GCN2 = general control nonderepressible 2; IRE1 = inositol-requiring enzyme 1; DELE1 = DAP3 binding cell death enhancer 1; PERK = protein kinase R-like endoplasmic reticulum kinase; OMA1 = OMA1 zinc metallopeptidase.

2.3.1 Measuring the activation of ISR

There is no standardised way for measuring the activation of the integrated stress response. However, as the different kinases converge on the phosphorylation of serine 51 of eIF2α, this can be a direct reading of the ISR status. Furthermore, given that eIF2α phosphorylation increases the translation of ATF4, measuring the translation of ATF4 (168, 169), or the transcriptional levels of ATF4-regulated genes, can also be used as an indirect reading of ISR (180, 181).

On the other hand, as the ISR can be driven by four different kinases, to mechanistically explore which kinase has driven the response the measurement of the protein phosphorylation of these targets can be used as a readout of their activation. However, the literature has not identified what phosphorylation sites are altered upon ISR activation in the different kinases. Furthermore, transcriptional upregulation of these kinases sometimes occurs in response to ISR activation (e.g., PERK), and whether they are a good indicator of their activation remains to be fully elucidated. Currently, there is no single measure to quantify the activation of the MSR.

2.3.2 The MSR in skeletal muscle

In skeletal muscle, the MSR has been studied in the context of mitochondrial myopathies (MM) (178, 180, 181). In humans with MM there is an increased basal mRNA expression of ATF5 and CHOP, but not ATF4 (181). ATF5 and CHOP are known targets regulated by ATF4 (178). Many genes with a conserved amino acid response element (AARE), which is the binding site of activating transcription factors (ATFs) (182), are also upregulated in MM. Among those, fibroblast growth factor 21 (FGF21) and growth differentiation factor 15 (GDF15) are highly upregulated, which is in accordance with the high FGF21 and GDF15 protein levels found in blood serum of MM patients (183, 184). Multiple rodent studies have shown that upon genetic disruption of different component of the mitochondrial dynamics in skeletal muscle (e.g.,

OPA1), there is an increase in muscle FGF21 mRNA expression (185-188). This leads to increased FGF21 protein levels in the blood serum, which can have positive effects in peripheral tissues such as adipose tissue (187).

In mice, ATF4 knockdown in muscle leads to muscle atrophy (189), but when chronically activated can lead to muscle wasting (190) and has been proposed as one of the mediators of inactivity- or aging-induced muscle atrophy (191). On the other hand, it has been suggested that a mild activation of MSR can extend lifespan in lower organisms (192, 193), but evidence for its benefit in mammals remains equivocal (194).

2.3.3 Effects of exercise on the MSR

Little research has explored the effects of exercise on the MSR. Specially, given the multi-factorial stress that skeletal muscle is exposed to during exercise, whether the stressor activating the ISR following different exercises is mitochondria-specific is challenging to elucidate. The majority of studies published to date have focused on PERK, the kinase that is activated upon endoplasmic reticulum stress. The endoplasmic reticulum (ER) – also called sarcoplasmic reticulum in muscle – is the compartment were the majority of proteins are fully folded and where calcium is stored. In skeletal muscle, calcium is a key intracellular ion that is needed for muscle contraction. As such, it is reasonable that during exercise the ER is exposed to stress, which could indeed activate the PERK kinase or any of the other UPR^{ER} kinases (IRE1a, ATF6).

Wu et al. (195) showed that 5 hours after an unaccustomed endurance exercise to exhaustion (total running time not reported), there was an increase in the mRNA expression of UPR^{ER} genes (spliced XBP1, BiP/GRP78, ERdj4), as well as ISR genes (GADD34, ATF3, ATF4, and CHOP), and increased phosphorylation of eIF2α, in the quadriceps of mice. These exercise-induced transcriptional responses were also reported in the gastrocnemius, but not in erector

spinae (a postural muscle) or cardiac muscle, suggesting a transcriptional response specifically elicited by skeletal muscle contraction. When mice were trained for 4 weeks, the exercise-induced increase in ATF4, CHOP, and spliced XBP1 mRNA was blunted, and the extent of GADD34 and ATF3 upregulation was reduced (195). In line with this, mice running for 1 hour at 50% of their peak velocity showed an increased expression of ATF3 mRNA and protein expression, but no detectable change in CHOP or XBP1 ratio mRNA, nor a change in ATF4 protein levels (196). More recently, it was shown that 4 weeks of high-intensity training was capable of increasing the gene expression of UPR^{mt} components in skeletal muscle of aged mice (197). Furthermore, they showed that UPR^{mt} components were correlated with OXPHOS gene transcripts in the skeletal muscle of old humans suggesting a close relationship between the UPR^{mt} and OXPHOS. Lastly, following 4 hours of exercise, mice had an increased phosphorylation level of eIF2α, as well as increased spliced XBP1, ATF3, GADD34, and GRP78 mRNA expression (174). As they hypothesised this transcriptional upregulation was originated from UPR^{ER} stress, they explored the role of PERK kinase in skeletal muscle. The authors generated a transgenic mice that had an increased PERK expression in skeletal muscle and showed that FGF21 mRNA was among the most highly expressed genes. Increased FGF21 mRNA also happened upon treatment with ER stressors (tunicamycin, thapsigargin, brefeldin A and DTT) in C2C12 myotubes, which occurred only in the presence of ATF4. These results suggested that the increased FGF21 mRNA observed following exercise was due to increased ER stress signalling through the PERK kinase. However, similar increases in FGF21 mRNA have been found elsewhere following treatment with oligomycin and antimycin in C2C12 myotubes (184). These findings show that different stressors can signal the ISR in skeletal muscle and increase the transcriptional regulation of FGF21.

In humans, an initial report showed that following 200 km of running there was an increased mRNA expression of short XBP1, total XBP1, and close to a significant increase in ATF4 (p

= 0.051) but not CHOP (198). Furthermore, they reported an increased protein levels of GRP78 - an ER resident chaperone. Ogborn et al (199) studied the effects of one bout of unaccustomed unilateral resistance exercise on the skeletal muscle UPR^{ER} in young and old participants. None of the measured transcripts changed 3 hours from the end of exercise. Their results showed that the mRNA expression of spliced XBP1, ATF6, IRE1α, and eIF2α increased 24 hour after exercise. Some of the transcripts, like XBP1s and XBP1 ratio, remained upregulated 48 hours following the exercise session. Furthermore, there was no change in the phosphorylation of eIF2α at any time-point, but there was an increase in the protein levels of IRE1α at 24 hours and GRP78, IRE-1a, and PERK at 48 hours. However, they did not see any upregulation of ATF4, CHOP, or GADD34 at any time-point. It has also been shown that UPR^{ER} genes are upregulated to a larger extent 18 hours following a resistance exercise session in young when compared to older humans (200).

Both GDF15 and FGF21 are considered mitokines due to their upregulation upon MSR (181). This is supported by increased levels of both GDF15 and FGF21 in serum from patients with MM compared to other myopathies. Both FGF21 and GDF15 are known to be transcriptionally upregulated by ATF4; thus, they can be regulated by any stimulus affecting the ISR. It is known that circulating GDF15 protein levels increase upon exercise (201, 202), and the skeletal muscle GDF15 mRNA content increases after 1 h of exercise at 55% $\dot{V}O_{2peak}$ (202). Intriguingly, exercise-induced elevations in circulating GDF15 protein do not seem to originate, nor be released, from skeletal muscle (201). Similarly, there is an intensity-dependant, exercise-induced increase in circulating levels of FGF21 protein in humans (203); however, no exercise-induced change in FGF21 mRNA or protein expression has been reported in skeletal muscle, which could be due to low FGF21 mRNA transcript levels or due to a muscle-independent release of FGF21 to the blood. Whether distinct exercises are capable of

increasing the expression levels of FGF21 and GDF15 in skeletal muscle remains to be fully elucidated.

2.4 Mitochondrial remodelling

2.4.1. Mitochondrial content

2.4.1.1 Definition

Mitochondrial content refers to the amount of mitochondria found within a given cell (Figure 2.6). Distinct measurement techniques have been used in the literature to assess mitochondrial content (62). The gold standard is direct quantification of mitochondrial volume density ($Mito_{VD}$) from transmission electron microscopy (TEM) micrographs. However, due to the cost and time consuming nature of this technique, indirect measurements, thought to correlate and reflect $Mito_{VD}$, are widely used (62). Among those, the most commonly used are citrate synthase activity and mtDNA copy number (204).

2.4.1.2 How is it measured?

2.4.1.2.1 Transmission Electron Microscopy (TEM)

The use of TEM allows one to obtain direct images of the tissue or cell of interest. These images are of high resolution and allow the direct quantification of the amount of mitochondria in a given space. This technique not only provides quantitative measures of mitochondrial content, but it also allows to examine the structural and morphological characteristics of a given mitochondrion (e.g., cristae density). This technique has the downside of being a method that requires extensive knowledge to perform the experiments, expensive equipment, and it is time consuming to obtain and analyse the images. Furthermore, mitochondria are not homogeneously spread across a given cell or tissue. They are more abundantly found in certain areas where they are most needed (e.g., around the nucleus).

In skeletal muscle, the problem of different fibre types having different mitochondrial content can also alter the results. It is recommended to obtain images of multiple fibres to obtain a true and representative measure (205, 206). Despite this, there are no clear recommendations in the field of exercise and muscle, and these variables are left to the choice of the investigator. A common practice is to classify fibre types by plotting Z-line width and mitochondrial volume density (114, 206). Once classified, the fibres with the highest mitochondrial volume density and Z-line width would be classified as type I and the fibres with lowest mitochondrial volume density and Z-line width would be type II. Usually, intermediate fibres are discarded. Despite this widely used technique (207), it has not been validated against reference methods like immunostaining to detect specific fibre types (208). Unfortunately, this method may skew the data reported on fibre-type differences in mitochondrial characteristics.

This prompts the question of how many images, of what skeletal muscle area, and from how many fibre types need to be quantified to obtain a representative measure of the tissue of interest. This is an often neglected methodological problem that will probably lead to less reproducible results.

2.4.1.2.2 Citrate Synthase (CS) activity

Citrate Synthase is an enzyme involved in the first step of the Krebs' cycle (or Tricarboxylic Acid Cycle; TCA) (62) and is a mitochondrial resident enzyme. The choice of CS activity as a surrogate measure of mitochondrial content is probably due to the notion that TCA cycle enzymes are directly dependant on mitochondrial content. However, to the best of my knowledge, there is no data showing a linear relationship between TCA cycle enzymes and mitochondrial content.

Larsen et al. (62) were able to show that mitochondrial volume density (measured by TEM) correlates well with CS activity at rest. However, they also found nine other measurements that

correlated with mitochondrial volume density (e.g., complex I activity). Secondary analysis of previously published data by our group showed that training-induced changes in CS activity were not related to changes in mitochondrial volume density (51). This would suggest that while CS is a good marker for basal mitochondrial content it may not be useful to determine individual changes in mitochondrial content following exercise training. However, other methodological issues may still be responsible for such a relationship.

2.4.1.2.3 Mitochondrial DNA (mtDNA) copy number

Each mitochondrion is estimated to contain 2 to 10 copies of mtDNA (209). Given this, the abundance of mtDNA in a given tissue is suggested to reflect the mitochondrial content. However, it has been shown that in skeletal muscle that mtDNA copy number did not correlate with $Mito_{VD}$ ($R = 0.35$, $p = 0.23$) (62). A possible explanation for the lack of agreement between measurements is the range of mtDNA copy numbers that each mitochondrion may carry. Whether mtDNA copy number is affected by other factors is currently unknown.

2.4.1.2.4 Cardiolipin content

Cardiolipin is a mitochondrial resident phospholipid and comprises 10 to 15% of the mitochondrial lipidome (210). It is found in membranes generating electrochemical potential, like the mitochondrial membranes, and is involved in the formation and maintenance of the cristae curvature, as well as in the stabilisation of ATP synthase dimers (211). Due to the fact that they are found in the mitochondrial membranes, and especially in the IMM, the content of cardiolipin has been used as a marker of mitochondrial content (62). However, whether cardiolipin content changes are only dependant on mitochondrial content, or are they also dependant on other factors such as the mitochondrial respiratory function (due to their role in cristae and complex V), remains to be elucidated.

2.4.1.2.5 Electron Transport Chain (ETC) protein

The ETC is named after the complexes responsible for electron transfer that result in the generation of the mitochondrial membrane potential. Each complex involved in the ETC is composed of multiple subunits. If the assumption that cristae density is constant is correct (56), it could be agreed that a change in ETC would reflect a change in mitochondrial content. This is a widely used measure of mitochondrial content, given its simplicity of measurement. It has been suggested that complex II and V protein levels are good markers of $mito_{VD}$ (62). However, with the discovery of respiratory supercomplexes, one could hypothesise that increased ETC complex content can occur without concomitant increases in mitochondrial content.

Recent research has shown that the stoichiometry of ETC complexes and subunits is not fixed and can be modified following exercise training in mouse skeletal muscle (212). This would suggest that those assumptions mentioned above may not be useful for skeletal muscle, as a specific adaptations may be driven by the exercise training performed. Probably not all complexes will be modified equally, as it has been suggested that complex I is bypassed during high-intensity exercise (213).

2.4.1.3 Fitness level and exercise training

Initial findings in humans showed that trained participants had a 47% higher $mito_{VD}$, mainly from increased number of SS mitochondria (56). They showed that following 6 weeks of exercise training (30 min at 72 % of $\dot{V}O_{2max}$, 5 times per week), $mito_{VD}$ was increased in both type I (+35%) and IIa (+55%) fibres. When training was continued (n = 2), only further improvements in type I fibres were found (214). Others have shown similar improvements in $mito_{VD}$ (215-217). Unfortunately, few studies have compared changes in $mito_{VD}$ with different exercise prescriptions. To the best of my knowledge, only one study has compared changes in $mito_{VD}$ following moderate-intensity continuous training (MICT) and sprint-interval training

(SIT) in obese individuals (218). There was no training or group effect; however, there was a much larger increase in $mito_{VD}$ following MICT (+90% in MICT vs 46% in SIT). This supports the notion that training volume, rather than training intensity, may be more important for training-induced changes in $mito_{VD}$ (51, 219). However, future studies should further explore the link between exercise variables and training-induced changes in mitochondrial content measured via TEM.

Citrate synthase, a biomarker of $mito_{VD}$ (62), has been extensively used for assessing changes in mitochondrial content (219). It has been shown that changes in CS activity were correlated with changes in $\dot{V}O_{2max}$ in response to an exercise training (220). The ease and extensive use of CS activity makes it possible to accumulate evidence from many different studies. A review of literature reported a linear relationship between total training volume and changes in CS activity (219). However, when utilising studies that had used $mito_{VD}$, this relationship seemed to plateau and there was no further increase in $mito_{VD}$ above 100000 arbitrary units of training volume. It remains a question of interest whether exercise intensity or volume, or a combination of both, is the most important variable for training-induced changes in CS activity (54).

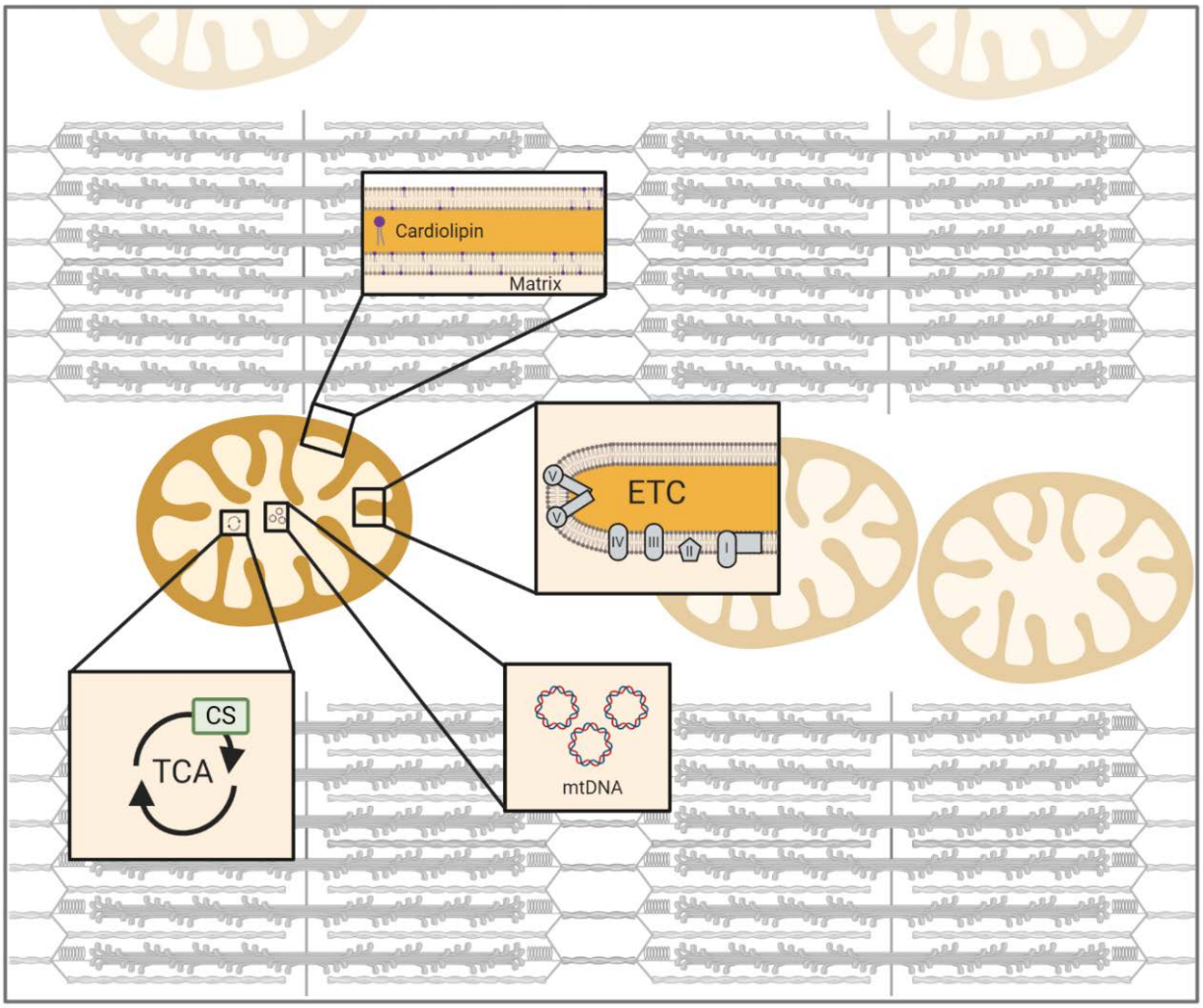

Figure 2.6. Schematic representation of a transmission electron microscopy (TEM) micrograph with a given mitochondrial content. See in each box the different indirect markers of mitochondrial content used in the literature. TCA = Tricarboxylic Acid Cycle; mtDNA = mitochondrial deoxyribonucleic acid; ETC = Electron Transport Chain.

2.4.2 Mitochondrial morphology and structure

2.4.2.1 Definition

Mitochondrial morphology refers to the analysis of mitochondrial shape characteristics. Mitochondria are dynamic organelles that continuously undergo morphological and structural changes. These characteristics are affected by acute or sustained exposure to any environmental stressor, such as increased energy demand.

Mitochondrial morphology is tightly controlled by the metabolic and cellular environment, and will be antagonistically influenced by mitochondrial fusion and fission. Fragmentation will occur upon an increase in mitochondrial fission (reduced size and increased roundness) and is considered a sign of stress (221, 222). Enlarged mitochondria will occur upon increased mitochondrial fusion (127) and, if cristae density is maintained, is considered a positive adaptation of the mitochondrial pool. Liesa and Shirihai (103) suggested that nutrient excess induces mitochondrial fragmentation and this phenotypic change was also seen in metabolic diseases (223). Furthermore, skeletal muscle of mtDNA deletor mouse (mitochondrial myopathy) have an increased mitochondrial circularity and lower cristae density (180, 181). An in depth analysis of the different morphological and structural components of mitochondria will allow the pinpointing of positive or negative adaptations to the tissue of interest.

2.4.2.2 How is it measured?

Mitochondrial morphology and structure can be measured by obtaining images of the sample of interest with different levels of resolution, which will be based on the microscopy techniques available. The highest resolution is TEM and allows the measurement of mitochondrial structural characteristics even to the level of individual crista width. With the advancement of technology, super-resolution live imaging, with fluorescent markers targeted to mitochondrial compartments, are also allowing researchers to study cristae dynamics in living cells (224, 225).

2.4.2.3 Fitness level and exercise training

Literature regarding human skeletal muscle mitochondrial morphology and structure was initially termed as ultrastructural mitochondrial characteristics (56). These were first studied by Hans Hoppeler, who reported that trained participants had 12% larger mitochondria than untrained counterparts (56). However, it was widely accepted that the cristae density was

constant and not a factor to take into account (56). No structural differences had been reported, and it was suggested that '*mitochondria in human skeletal muscle are optimized and well balanced units*'. However, Nielsen & Ortenblad (114) challenged this notion and reported that mitochondrial cristae density was different between metabolically unhealthy, healthy active, and highly trained participants. They suggested that, given the reduced space availability found in a given skeletal muscle cell, increasing mitochondrial cristae density was a way to overcome this. The 23% increase in cristae density was a layer of adaptation only found in highly trained athletes. However, they also showed that there was no change in cristae density following 10 weeks of MICT in obese individuals with T2D. Interestingly, a recent paper including some of the previous authors (226) showed that cristae density of mitochondria from the gastrocnemius skeletal muscle was not affected in older adults (~ 65 y) with or without critical limb ischemia (114). When comparing the cristae density values to their previous findings, there was no difference between the older adult cohort and the highly trained athletes. This challenges the notion that cristae density is a layer of adaptation and calls for further research and methodological standardisation of the field.

Exercise training studies examining mitochondrial morphological changes are limited, to the best of my knowledge, to one study. Meinild-Lundby et al. (216) reported that 6 weeks of MICT led to increased size of mitochondria. These findings were indirectly based on their reported increased $mito_{VD}$ but unchanged mitochondrial profiles per area (216), which concluded with the authors suggesting that mitochondria enlarge following exercise training and do not increase their number. In accordance with their findings, they showed increase MFN2 protein levels, which they suggested to be a result of an increased mitochondrial fusion. However, this could have been solely due to the reported increase in mitochondrial content. Furthermore, analyses of mitochondrial morphological variables, such as area and aspect ratio,

should have been included to avoid interpreting these results from the relationship between number of mitochondria and $mito_{VD}$.

2.4.3 Mitochondrial function

2.4.3.1 Definition

Mitochondrial function, based on the etymology of the word function ('to perform' or 'execute'), refers to those measurements that aim at quantifying how well mitochondria are performing their 'tasks'. Mitochondria have multiple tasks within a cell; however, ultimately their oxidative phosphorylation capacity is widely recognised as their most important role. Thus, the performance of their oxidative phosphorylation machinery, the ability to use O_2 and substrates to ultimately resynthesise ADP to ATP can be used as a marker of the mitochondrial respiratory function.

2.4.3.2 How is it measured?

2.4.3.2.1 Mitochondrial respiratory function

Mitochondrial respiratory function relies on the premise that the oxidative phosphorylation of ADP to ATP is coupled to the level of electron transfer capacity by the ETC. Different substrates, uncouplers, and inhibitors are used in different combinations to analyse the involvement of individual complexes of the ETC. The assessment is performed using an oxygen-sensitive electrode, which is able to determine rates of oxygen consumption. A common limitation is the need to perform the experiments on fresh tissue; however, a recent protocol paper proposed a novel approach to utilise frozen tissue (227).

These mitochondrial respiratory function measurements can be done in whole tissue (e.g., skeletal muscle), whole cell (e.g., primary myotubes), or mitochondrial preparations (isolated mitochondria). Different factors will favour, or not, the use of these different preparations.

Whole-tissue or whole-cell preparations are thought to be the most approximate measure of the tissue/cell *in vivo*. In skeletal muscle tissue, the use of whole-tissue preparations requires permeabilisation of the sarcolemma to ensure the different substrates/chemicals can diffuse and reach the entire mitochondrial pool (both SS and IMF). Furthermore, due to the different fibre types within skeletal muscle tissue, a different ratio of type-I to type-II fibres may be found in different tissue preparations and may affect the results (228). The same holds true for tissues where multiple cell types may be found. While permeabilised muscle tissue may represent the best approximate to *in vivo* mitochondrial respiratory function (229), isolated mitochondria may be an interesting approach to quantify the function of mitochondria in isolation. Having both tissue and isolated mitochondria measurements would provide important information in disease research, where dysregulated mitochondria are not always due to inherent mitochondrial changes, but due to other environmental factors in the analysed tissue.

Human skeletal muscle research has shown that the variability between whole-muscle biological replicates can be ~ 15%, and that user-to-user variability may be even higher (230, 231). This may be aggravated by the fact that most researchers perform measurements in duplicate. Our group has unpublished data suggesting that a third, or fourth, measurement is needed to decrease the between-sample variability to ~6%. Ways of minimising technical and biological variability of mitochondrial respiratory function warrant future research.

2.4.3.2.2 Enzyme activity assay

The enzymatic activity of the specific complexes of the ETC have also been considered as mitochondrial function measurement (232). They provide a direct measurement of the different complexes involved in the ETC (233). This allows for comparison between mitochondrial complexes that contain mitochondrial-encoded subunits (e.g., Complex I) versus the fully nuclear-encoded complex II (234). Furthermore, these assays do not rely on an intact

mitochondrial membrane potential, which allows then to be performed on frozen tissue. However, whether they are the most appropriate measures of mitochondrial function remains equivocal.

2.4.3.2.3 Mitochondrial membrane potential

The mitochondrial double membrane provides mitochondria with their own membrane potential (ΔΨm). This is a vital component of mitochondria and mitochondrial membrane potential has been considered to be a measure of mitochondrial function. This is not surprising due to the fact that processes such as the protein import, oxidative phosphorylation, and mito-nuclear signalling, all partially rely on the mitochondrial membrane potential (235). Multiple ways of measuring membrane potential can be found in the literature. The most commonly used is tetramethylrhodamine methyl ester (TMRM) dye, which when taken up by mitochondria (driven by ΔΨm) shifts their excitation and emission fluorescence spectra (236). Research in human skeletal muscle has shown ΔΨm is relatively stable among young and old participants (237). Furthermore, upon maximal ADP-stimulated respiratory function (state 3), there is a decrease in ΔΨm in permeabilised muscle fibres (237).

2.4.3.3 Fitness level and exercise training

In the different studies that have compared work-matched MICT (~ 50-60% $\dot{W}_{max}$) to HIIT (~65-90% $\dot{W}_{max}$), only in the latter was mitochondrial respiratory function increased (238, 239). What drives this positive adaptation in skeletal muscle respiratory function remains a question of interest. In contrast, following MICT there is generally an unchanged mitochondrial respiratory function (52), or small increases (216), which are usually driven by the larger increase in mitochondrial content (52, 216) and are not observed when expressed relative to mitochondrial content markers. Changes in mitochondrial respiratory function are used to assess mitochondrial adaptations to exercise training. While there are discrepancies among the

exercise variables affecting changes in mitochondrial content (51, 54), exercise intensity seems to be more important for positive training-induced changes in mitochondrial respiratory function (219).

Regarding ΔΨm, there is little research regarding skeletal muscle and exercise. It is of interest that although there are no differences between healthy young and old participants, there is a decreased basal ΔΨm in old untrained counterparts (237). Whether this decreased ΔΨm reflects an increased number of dysfunctional mitochondria, and whether this is reversible by exercise training, remains to be elucidated.

2.5 Conclusion

Work from Hearn & Wainio (240), and later Holloszy (241), initiated the research area of mitochondrial adaptations to exercise training and paved the road to our current understanding. It is now widely accepted that endurance training positively affects multiple mitochondrial characteristics in skeletal muscle (Figure 2.7). Among the adaptations reported in the literature, increased CS activity (219, 238, 242), increased $mito_{VD}$ (214, 243, 244), increased mitochondrial size (214, 243), increased mitochondrial respiratory function (52, 239, 245), and increased mitochondrial supercomplex assembly (246), have been reported following endurance training (Figure 2.7).

Despite the large increase in knowledge regarding mitochondrial adaptations to endurance exercise, whether the exercise prescription affects specific mitochondrial adaptations remains a question of debate (51, 54). Granata et al. (219) summarised a vast part of the literature and suggested that increases in CS activity were related to training volume. They further showed that improvements in mitochondrial respiratory function were larger when exercise was performed at a higher intensity. On the other hand, the group led by Professor Gibala (54) proposes that exercise intensity is the main driver of most mitochondrial adaptations. This is

supported by short-term and long-term studies where high-intensity exercise was demonstrated to be equal, or superior (when work-matched), for changes in CS activity (238, 242, 247). Collectively, these findings suggest that training intensity may be a key factor for the *efficiency* of mitochondrial adaptations; however, this does not indicate that it is the most *effective*. Resolving this long-lasting question would represent an important advancement in the field (51, 54).

Similarly, the molecular events dictating the adaptations to a single exercise session remain equivocal. While there is an increased body of knowledge regarding the exercise variables that dictate molecular pathways regulating mitochondrial biogenesis (see section 2.2.1 Mitochondrial biogenesis), there are many other pathways that remain partially unexplored (see section 2.2 Mitochondrial Dynamics; and 2.3 Mitochondrial Stress Response). Given that mitochondria are complex organelles that sense and regulate multiple cellular functions, it seems reasonable that multifactorial mitochondrial events may be responsible for some of the often reported divergent mitochondrial adaptations.

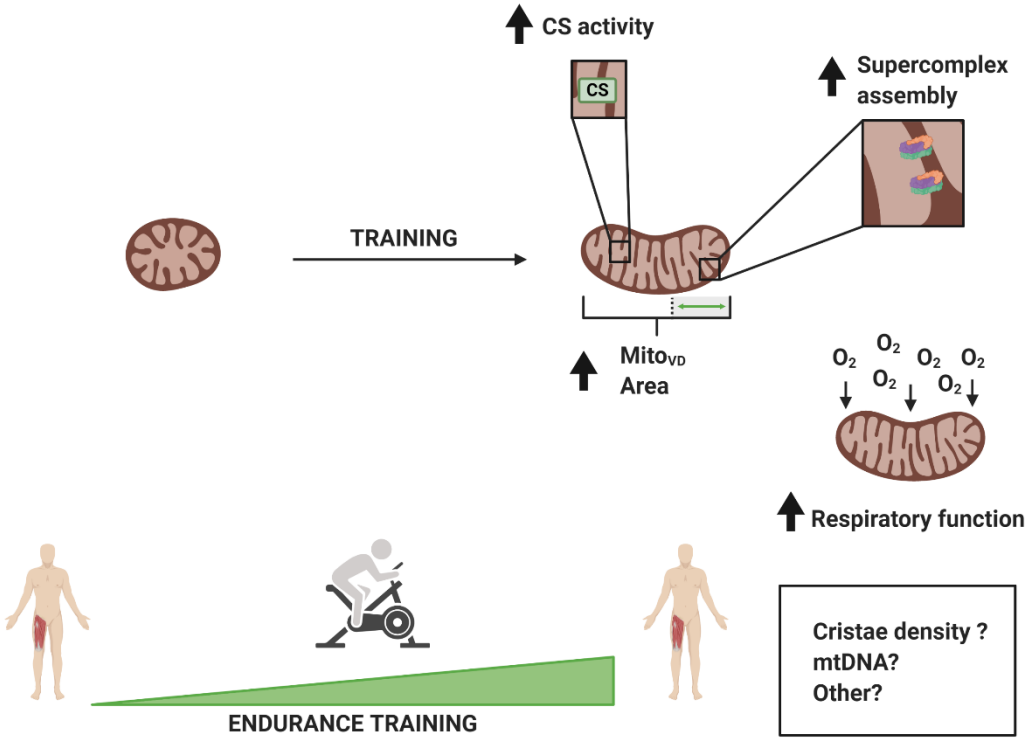

Figure 2.7. Summary of the mitochondrial adaptations reported in the literature. mtDNA = mitochondrial deoxyribonucleic acid; $Mito_{VD}$ = mitochondrial volume density; O_2 = oxygen.

CHAPTER 3 – REGULATION OF AUTOPHAGOSOME CONTENT FOLLOWING ENDURANCE EXERCISE IN THE SKELETAL MUSCLE OF RATS AND MEN.

The aims of this chapter were: 1) to address the question of whether the exercise-induced LC3B regulation differs between rodents and humans; 2) to elucidate in humans whether LC3B changes are dependent on exercise intensity; 3) to explore whether basal LC3B protein levels can be increased following a period of exercise training; and 4) to examine in humans if the exercise-induced decrease in LC3B-II is reflective of decreased autophagy flux. The results from this chapter will help to better understand how general autophagy is regulated following endurance exercise in humans, and how it compares to rodents studies. Due to the importance of selective autophagy in mitochondrial remodelling, this chapter will allow to clarify how autophagosome content is modified following exercise. Data from this work has been presented at the European Congress of Sport Sciences (ECSS) Conference, in Dublin 2018.

3.1 Introduction

Autophagy is the cellular process by which an autophagosome (a double–membrane vesicle) engulfs, and delivers to the lysosome, proteins or organelles that need to be degraded. It is the recycling machinery of the cell and is important for the correct removal of intracellular pathogens or misfolded proteins, among others, which may activate deleterious cellular signalling pathways (e.g., inflammation) (248). In skeletal muscle, autophagy has been shown to be important to prevent mitochondrial damage (249), for positive muscle regeneration (250), for correct glucose metabolism (251), and for training-induced increases in mitochondrial proteins and endurance performance (252). Thus, it is important to better understand the factors that influence autophagy in skeletal muscle.

The autophagy machinery consists of a core set of autophagy-related (ATG) proteins (253). Among these, the ATG8 family (LC3A, LC3B, LC3C, GABARAP, GABARAPL1, and GABARAPL2) promote autophagosome formation and autophagosome-lysosome fusion (254). In skeletal muscle LC3s are abundantly expressed (255), which makes LC3 a widely used marker of autophagosome content. LC3 can be found in two forms; LC3-I is the ‘inactive’ form and when conjugated to the lipid phosphatidylethanolamine it forms LC3-II, which is membrane-bound and helps in autophagosome maturation (255). This allows LC3-II protein levels to be a marker of changes in autophagosome content and autophagy flux (256). LC3-II binds to autophagy receptor proteins through their LC3-interacting region (257). Through this interaction autophagosomes are able to engulf dysfunctional proteins or organelles that have been previously tagged by autophagy receptors, such as p62 (258). Changes in p62 protein levels can also be used to monitor changes in autophagy flux, since it is degraded with the autophagosome following fusion with the lysosome (257).

Decreased energy availability (e.g., starvation) is a strategy to stimulate and increase the levels of autophagy through AMP-activated protein kinase (AMPK) (259) and endurance exercise is also known to activate AMPK (260). In one of the first exercise studies in rodents, endurance exercise acutely increased the levels of LC3-II (and the LC3-II/I ratio) as well as the appearance of LC3 puncta (assessed via immunofluorescence microscopy) in both skeletal (the soleus, tibialis anterior [TA], and extensor digitorum longus [EDL]) and cardiac muscle (251). These increases were significant after 30 min of exercise, and peaked after 80 to 110 minutes of exercise, which occurred concurrently with a decrease in p62 protein levels. These findings pointed towards an increased exercise-induced autophagosome content and lysosomal degradation. Another study also showed that endurance exercise combined with a run to exhaustion increased LC3-II protein levels in the TA, along with a tendency for increased exercise-induced autophagy flux, but there were unchanged p62 protein levels following exercise, which increased 1.5 hours into the recovery (261). In contrast, following a similar exercise session in mice (an incremental run to exhaustion lasting ~ 90 min), there was no significant change in the soleus muscle for LC3-II, the LC3-II/I ratio, or p62 protein levels; but there was an increase in LC3-I protein levels (262). Finally, the only study comparing two different exercise regimes in mice showed that both low-intensity (40 min at 14 $m \cdot min^{-1}$) and moderate-intensity (20 min at 18 $m \cdot min^{-1}$) increased the LC3A/B-II protein levels and the LC3A/B-II/I ratio 3 hours following the end of exercise (263). Interestingly, p62 protein levels were unchanged 3 hours following the exercise, but were decreased in both conditions 6 hours into the recovery; this occurred concurrently with the LC3A/B-II/I ratio returning to baseline. Unfortunately, most studies in rodents did not report what LC3 isoform was used for their analysis, and therefore it is not possible to tease out any LC3 subfamily member-specific regulation. Although findings are inconclusive in regards to exercise-induced p62 protein

changes, the LC3 findings collectively suggest that autophagosome content, and probably autophagy flux, are increased after a moderate intensity endurance exercise session in rodents.

In contrast to rodent studies, human findings have generally shown a distinct pattern. Protein levels of LC3B-II, and the LC3B-II/I ratio, have been shown to decrease 0 to1 h following different types of endurance exercise (cycling 60 min at 50% of $\dot{V}O_{2max}$; cycling 60 min at 70% of $\dot{V}O_{2max}$; 60 min one-legged knee extensor at 80-100% of peak work load; cycling 120 min at 55% and 70% of $\dot{V}O_{2peak}$) and return to baseline values after 3 to 4 h of recovery in human skeletal muscle (163, 164, 264, 265). On the other hand, 60 min of exercise at 60% of $\dot{V}O_{2max}$ has been reported to increase the levels of LC3A/B-II protein levels 2 h after the end of exercise (266). Following most types of endurance exercise, the protein levels of the autophagy receptor p62 remained unchanged (162-164, 264). On the contrary, following 2 h at 70% of $\dot{V}O_{2peak}$, but not at 55% of $\dot{V}O_{2peak}$, p62 protein levels decreased, which could suggest an effect of exercise intensity on the exercise-induced p62 protein changes (265). However, since both the exercise intensity and the total work completed were different, it is difficult to isolate any of these factors. Other differences such as training status, timing of biopsies, antibodies, or sample size may also contribute to the reported discrepancies between human studies. Whether exercise intensity distinctly affects the LC3B and p62 protein levels changes following an exercise session remains to be fully elucidated.

Multiple exercise-induced mRNA and protein changes observed following a single exercise session have been shown to be reduced following training (50, 85). However, exercise-induced autophagy signalling at different time points in a training period has not been reported in the literature. Nonetheless, it is known that the content of autophagy proteins can be increased after training. In mice, four weeks of voluntary wheel running increased the LC3A/B-II and p62 protein levels, but not the LC3A/B-II/I ratio in the soleus muscle (252). In humans, three weeks of one-legged knee extension training led to an increased LC3B-II protein levels, while p62

and the LC3B-II/I ratio remained unchanged (164). On the other hand, 8 weeks of moderate-intensity training increased the LC3A/B-I protein levels, while p62 and LC3A/B-II protein levels remained unchanged (162). Whether the training-induced changes in basal LC3 protein levels may affect the exercise-induced response remains to be explored.

Autophagy flux is the term used for the combined autophagy steps, which includes autophagosome formation, maturation, fusion with lysosomes, and breakdown. Autophagy flux assays are considered the 'gold-standard' to assess autophagy levels (267). One such assay aims to chemically block the fusion of autophagosomes with the lysosome (the end-point of the degradation process) and to monitor the accumulation of LC3-II (267). Performing an *in vivo* autophagy flux is not ethically possible in human tissues and remains a limitation. This means that human studies have relied on markers of autophagosome and autophagy receptor protein levels (162-164, 264, 265). Although not previously used in humans, animal models have also utilised an *ex vivo* autophagy flux analysis (268). Implementing this *ex vivo* LC3-II flux assay could provide a direct assessment of autophagy in human studies and would avoid having to rely on indirect markers (i.e., LC3-II/I ratio). This approach would improve the understanding of skeletal muscle autophagy and perhaps helps explain some of the discrepancies reported in the literature.

Despite the increase in autophagy research in skeletal muscle, there is currently no consensus on the exercise-induced regulation of autophagosome content in skeletal muscle. The aims of the current study are multiple: 1) to address the question of whether the exercise-induced LC3B and p62 protein regulation differs between rodents and humans; 2) to elucidate in humans if LC3B and p62 protein changes are affected by exercise intensity; 3) to explore whether training has an effect on the basal and exercise-induced LC3B and p62 protein levels; and 4) to asses *ex vivo* autophagy flux in humans to better interpret exercise-induced changes in autophagy proteins.

3.2 Methodology and procedures

Four different exercise studies were included in this chapter: a single exercise session in rodents (Study 1), exercise in humans at three different work-matched intensities (Study 2), high-intensity exercise and training in humans (Study 3), and a single exercise session in humans (Study 4). All human participants were deemed healthy, and their characteristics can be found in Table 1. Studies were performed at Victoria University (Melbourne, Australia), and analyses were done under similar conditions in the same laboratory. All studies were approved by the Victoria University Animal Ethics Committee and the Victoria University Human Research Ethics Committee (Study 1, 15-002; Study 2, HRE 017-035; Study 3, HRE 15-126; Study 4, HRE 017-075). Informed consent was obtained from all human participants prior to study participation.

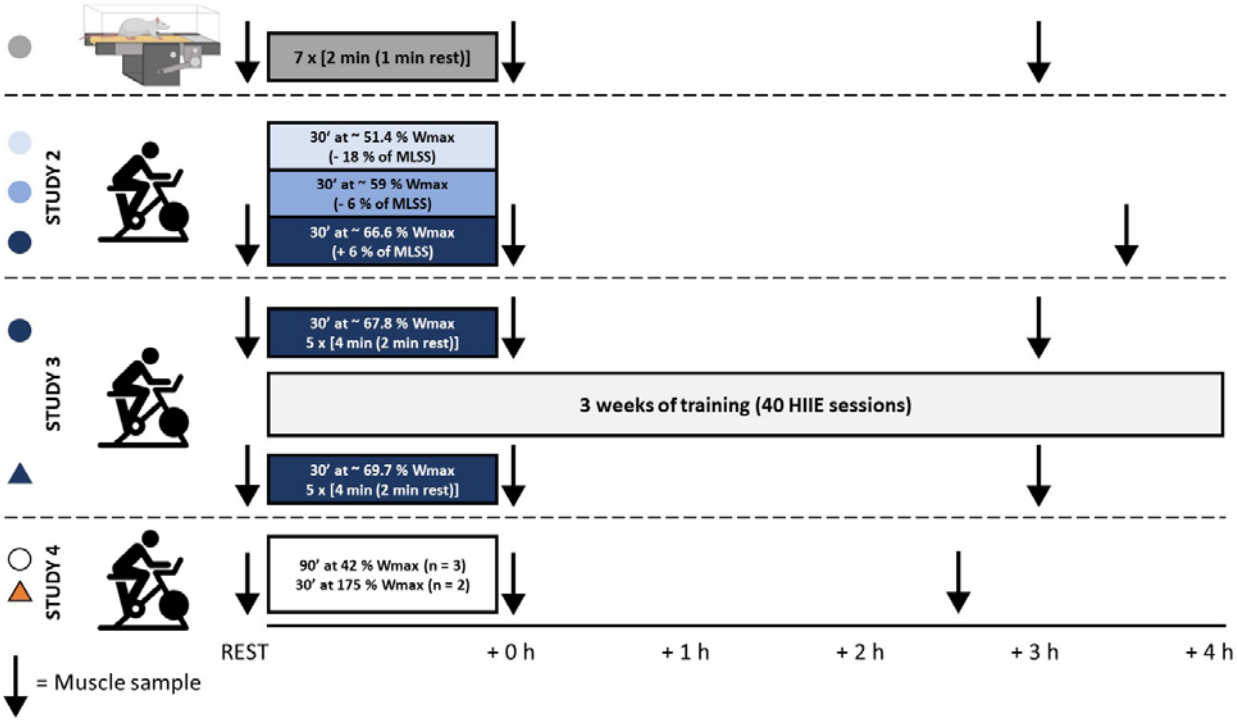

Figure 3.1. Schematic representation of the four different experimental studies with the time-course of the muscle samples collected. MLSS = Maximal lactate steady state; HIIE = High-intensity interval exercise; $\dot{W}_{max}$ = peak aerobic power determined from a graded exercise test.

3.2.1 Study 1 – Exercise in rats

3.2.1.1 Overview

Twenty-eight male Wistar rats (8 weeks old) were obtained from the Animal Resource Centre (Perth, Australia). The animals underwent acclimatisation over three days, using five separate 15'min sessions (ranging from being placed on a non-moving treadmill belt to running at a speed of 0.25 $m \cdot s^{-1}$). At least 48 h before the experimental exercise session, the animals performed an incremental exercise test. The incline of the treadmill was set at 10 degrees and the test was started at 0.16 $m \cdot s^{-1}$. The speed of the treadmill was increased 0.05 $m \cdot s^{-1}$ every three minutes. Animals were removed from the treadmill when they could no longer keep up with the speed despite encouragement (air puff).

3.2.1.2 Experimental session

On the experimental day, animals were exercised at 80% of their top speed achieved in their incremental test (approximately 0.38 $m \cdot s^{-1}$ at a 10 degree incline) for seven 2-minute intervals interspersed with 1 minute of rest. Prior to (REST), immediately after (+0 h), or 3 h after the completion of the exercise protocol, rats were humanely killed using 90 $mg \cdot kg^{-1}$ i.p. pentobarbitone and the soleus was removed and immediately frozen in liquid nitrogen and stored at -80 °C.

3.2.2 Study 2 – Exercise in humans: effects of exercise intensity

3.2.2.1 Overview

Participants were required to attend the laboratory at Victoria University 8 to 11 times. For the first trial, participants underwent a cycling graded exercise test (GXT) with 1-min increments as previously described (24). The following visits were dedicated to determining the maximal lactate steady state (MLSS), which was established by a series of 30-min constant power sessions. After the establishment of the MLSS, participants completed two constant power

exercise sessions to exhaustion at +6% of the MLSS. Following this, they performed three experimental sessions that included skeletal muscle biopsies.

3.2.2.2 Experimental session

The three experimental sessions were performed in a randomised order at -18%, -6% or +6% of the MLSS. The MLSS was selected as the reference point because it is a critical intensity that delineates heavy from severe exercise intensity (269), and three intensities (2 below and 1 above the MLSS) were chosen for the study. Participants were given 48 h of complete rest before each trial, and at least 7 days between the successive experimental trials. They were asked to maintain their normal diet and to replicate it on the day before and during the experimental trials. Biopsies were taken at rest before the start of exercise (REST), immediately upon completion of the exercise session (+ 0 h), and approximately 3.5 h after the end of the exercise (+ 3.5 h). Samples were immediately cleaned of excess blood, fat, or connective tissue, and rapidly frozen in liquid nitrogen. Samples were stored at -80 °C until subsequent analyses.

3.2.3 Study 3 - Exercise in humans: effects of training

3.2.3.1 Overview

Participants underwent two experimental sessions, one before and one after 20 days of twice-a-day high-intensity interval exercise (HIIE), similar to a previously published study (50, 270). In brief, participants performed a GXT with 4-min increments at least 72 h before each experimental session, where maximal aerobic power ($\dot{W}_{max}$), power at the lactate threshold (W_{LT}), and $\dot{V}O_{2peak}$ were determined. HIIE sessions involved 5 bouts of 4 min (at 50% between W_{LT} and $\dot{W}_{max}$) interspersed with 2 min of recovery. This allowed the exercise intensity to be individually prescribed and adjusted. After the initial experimental session, participants started the 20 days of twice-a-day HIIE exercise training.

3.2.3.2 Experimental sessions

Participants were given 48 to 72 h of rest before each trial. All samples were obtained from the *vastus lateralis* muscle and participants were provided standardised meals, as previously described (270, 271). Biopsies were taken at rest before the start of exercise (rest), immediately upon completion of the exercise session (+ 0 h), and following 3 hours of recovery (+ 3 h; with no access to food and access to water *ab libitum*). Samples were immediately cleaned of excess blood, fat, or connective tissue, and rapidly frozen in liquid nitrogen. Samples were stored at -80 °C for subsequent analyses.

3.2.4 Study 4 – Autophagy flux in humans

3.2.4.1 Overview

As recruitment was concluded for studies 2 and 3, when the *ex vivo* autophagy flux assay was ready, only samples from five healthy participants from a larger study (see chapter 4) were able to be analysed. The GXT protocol utilised in this study was the same as in Study 2.

3.2.4.2 Experimental session

Two participants underwent the following exercise: six 30-s 'all-out' cycling bouts against a resistance initially set at 0.075 kg·kg body mass $^{-1}$ (~ 175% $\dot{W}_{max}$), interspersed with a 4-min recovery period. The other three participants performed a session consisting of 90 minutes of continuous cycling at a fixed power equivalent to ~ 42 % of $\dot{W}_{max}$. Participants were given 72 h of rest before the experimental session. All samples were obtained from the *vastus lateralis* muscleand participants were provided standardised meals, as in previous studies (270, 271). Biopsies were taken at rest before the start of exercise (REST), immediately upon completion of the exercise bout (+ 0 h), and 2.5 hours into the recovery (+ 2.5 h). Small muscle portions were immediately immersed into two separate vials with 3 mL of oxygenated DMEM, and the

autophagy flux assay was started (see below in *Ex vivo autophagy flux assay*). Once the protocol was finalised, samples were stored at -80 °C for subsequent analyses

3.2.5 Skeletal Muscle Analyses

Preparation of whole-muscle lysates

Approximately 10 to 20 mg of frozen muscle was homogenised two times for two minutes at a speed of 30 Hz with a TyssueLyser instrument (Qiagen, Canada) in an ice-cold lysis buffer (1:20 w/v) containing 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 5 mM Na4P207, 1 mM Na3V04, 1 % NP-40, with added protease and phosphatase inhibitors at a 1:100 concentration (Cell Signaling Technology). Protein concentration was determined using a commercial colourimetric assay (Bio-Rad Protein Assay kit II, Bio-Rad Laboratories Pty Ltd, Gladesville, NSW, AUS) and lysates were then diluted with an equal volume in 2x Laemmli buffer containing 10% B-mercaptoethanol.

Table 3.1. Descriptive data of the human participants recruited for studies 2, 3, and 4.

	Age (y)	$\dot{V}O_{2peak/max}$ ($mL \cdot min^{-1} \cdot kg^{-1}$)	Trial	Exercise intensity (% $\dot{W}_{max}$)	Absolute intensity (W)
Study 2 (n = 10)	27.5 ± 7.7	55.8 ± 10.0	- 18 %	51 ± 4	181 ± 39
			- 6 %	59 ± 4	207 ± 45
			+ 6 %	67 ± 5	234 ± 51
Study 3 (n = 9)	22.4 ± 5.2	47.0 ± 7.5	PRE	68 ± 2	208 ± 38
			POST	70 ± 1	244 ± 39
Study 4 (n = 5)	30.0 ± 7.3	48.1 ± 4.4	SIE	175 ± 21	444 ±179
			MICE	42 ± 2	142 ± 35

Western blotting

For each protein of interest, a signal linearity test was conducted to determine the ideal loading amount. Muscle lysates were then loaded in equal amounts (10 to 20 μg) and separated by electrophoresis for 1.5 to 2.5 h at 100 V using pre-cast stain-free SDS-PAGE gels (4-20%). Once resolved, the gels were then wet transferred onto PVDF membranes using a Turbo Transfer system (Bio-rad Laboratories Pty Ltd, Gladesville, NSW, AUS). Membranes were blocked at room temperature for 1 h using 5% skim milk or 5% Bovine Serum Albumin (BSA) in Tris Buffer Saline (TBS) 0.1% Tween-20 (TBS-T). After 3 x 5-min washes in TBS-T, membranes were incubated overnight at 4 °C with gentle agitation in primary antibody solutions (1:1000 antibody in 5% BSA, plus 0.02% Na Azide). The antibody for LC3B was purchased from Cell Signalling (#3868S) and the antibody for p62 from Abcam (#ab56416). Immunoblotting was carried out using the desired antibody. The following morning, membranes were washed 3 x 5-min in TBS-T and subsequently incubated under gentle agitation at room temperature with the appropriate host species-specific secondary antibody for 60-90 min in 1-5% skim milk in TBS-T. Membranes were then washed again for 3 x 5-min in TBS-T before being immersed for 5 min under gentle agitation at room temperature in Clarity ECL detection substrate (Bio-rad Laboratories Pty Ltd, Gladesville, NSW, AUS). Protein bands were visualised using a Bio-Rad Versa-Doc imaging system and band densities were determined using Bio-Rad Quantity One image analysis software (Bio-Rad Laboratories Pty Ltd, Gladesville, NSW, AUS). All samples for each participant were loaded on the same gel, along with different concentrations of a mixed-homogenate internal standard (IS), and a calibration curve plotted of density against protein amount. From the subsequent linear regression equation, protein abundance was calculated from the measured band intensity for each lane on the gel. Total protein content of each lane was obtained from the stain-free image of the membrane and was used for normalisation of the results.

Ex vivo autophagy flux assay.

The following protocol was adapted from previous studies performing *ex vivo* autophagy flux in rodents (268, 272). Upon collection of the skeletal muscle sample, two small pieces (~ 10 mg) were placed in 3 mL of oxygenated DMEM media at 37 °C. The tissues were then incubated with continuous oxygenation for 1 h with, or without ('untreated' sample), 60 uL of NH_4CL (20 $uL \cdot mL^{-1}$; 40 mM) and 30 uL Leupeptin (10 $uL \cdot mL^{-1}$; 100 uM) ('treated' sample, with inhibitors). Upon completion of a 1-h incubation, samples were snap-frozen and stored at -80 °C until further analysis. Autophagy flux (Net LC3B-II flux) was obtained by subtraction of the densitometric value of LC3B-II from treated compared to the untreated sample.

3.2.6 Statistical analysis

All values are reported as mean ± standard deviation (SD). All statistical analyses were done on the raw values normalised to the total protein loading and calibration curve. For study 1, one-way repeated-measures of ANOVA with Holm-Sidak post-hoc were used. For studies 2 and 3, two-way repeated measures of ANOVA were used. Main effects and interactions were further analysed using Holm-Sidak post-hoc tests. For study 4, a one-way ANOVA with Holm-Sidak post-hoc tests were utilised. Effect sizes (ES) were quantified and defined as: small (0.2), moderate (0.5), large (0.8), and very large (1.3). Statistical significance was set at $p < 0.05$ for all analyses. GraphPad Prism 8.3 software was used for the statistical analysis.

3.3 Results

3.3.1 Study 1 - Exercise-induced changes in LC3B and p62 protein changes in soleus muscle of Wistar rat.

There was a main effect of time for LC3B-I and LC3B-II protein levels (both $p = 0.01$), as well as the LC3B-II/I ratio ($p = 0.0003$). There was a significant increase in LC3B-I protein from REST to + 0 h (+ 109 ± 103%; ES = 1.1; $p = 0.017$) and from REST to + 3 h (+ 82 ± 62%; ES = 1.1; $p = 0.04$). LC3B-II content did not significantly change from REST to + 0 h (- 20 ± 46%; ES = - 0.32; $p = 0.63$), but significantly increased from REST to + 3 h (+ 97 ± 102 %; ES = 0.95; $p = 0.04$) and from + 0 h to + 3 h (+ 159 ± 129%; ES = 1.2; $p = 0.02$). There was a significant decrease in the LC3B-II/I ratio after exercise (- 65 ± 12 %; ES = - 1.5; $p = 0.001$), followed by a significant increase from + 0 h to + 3 h (+ 164 ± 98%; ES = 1.5; $p = 0.001$). SQSTM1/p62 protein levels did not significantly change at any time point ($p > 0.05$). These findings show, in the soleus muscle of Wistar rats, that following an exercise session there is an increase in LC3B-I protein levels, which is followed 3 h into the recovery by increases in LC3B-II and LC3B-II/I ratio protein levels, while p62 protein levels remained unchanged.

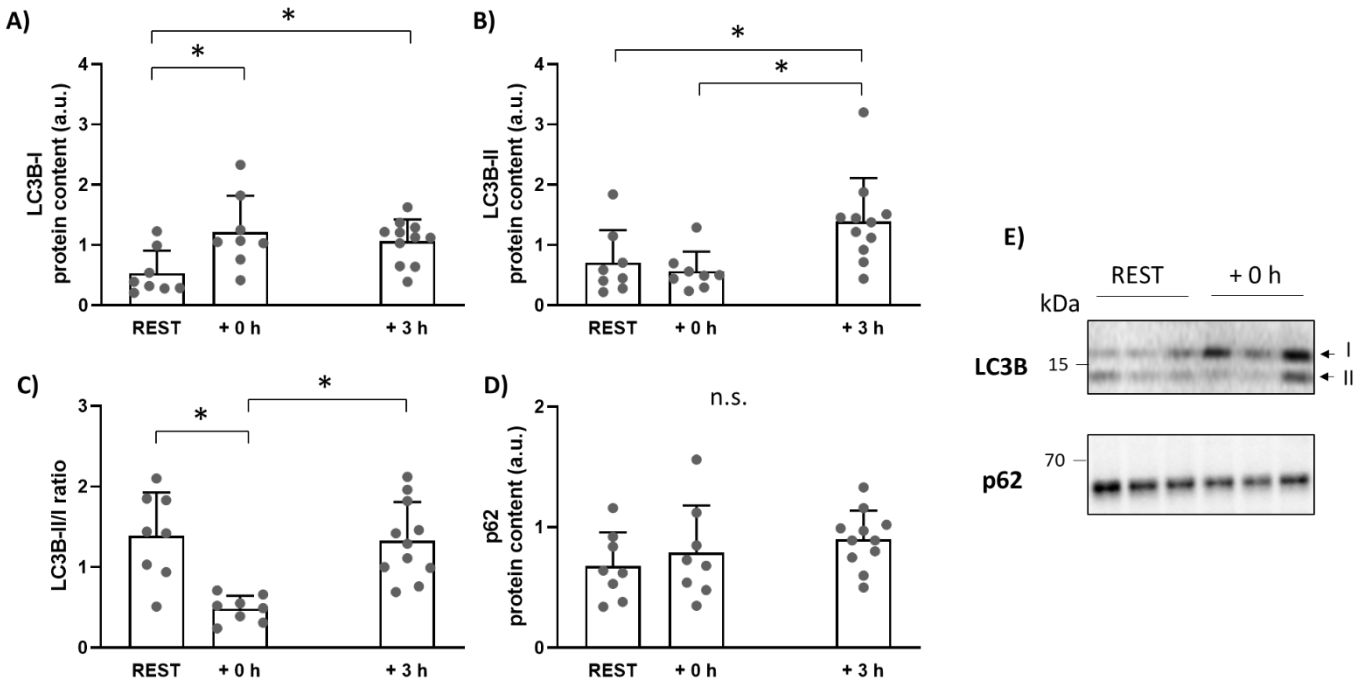

Figure 3.2. Effects of exercise on protein levels of A) LC3B-I and; B) LC3B-II in the soleus muscle of Wistar rats; C) effects of exercise on the LC3BII/I ratio and; D) p62 protein levels in the soleus muscle of Wistar rats; E) representative blots of LC3B and p62 protein. Data were analysed using one-way ANOVA; * = $p < 0.05$. Bars are shown as mean + SD; n.s = not significant.

3.3.2 Study 2 - Effects of exercise intensity on exercise-induced LC3B and p62 protein changes in human skeletal muscle.

There was no main or interaction effect for LC3B-I protein levels (Figure 3.3). There was a main effect of time for LC3B-II protein levels ($p < 0.0001$), with a significant difference between REST and + 0 h (- 24 ± 16%; 90 % CI [- 28, -19%]; ES = - 0.82; $p = 0.0001$), no significant difference between REST and + 3 h sample (+ 6 ± 31%; 90 % CI [- 4, 15%]; ES = 0.01; $p > 0.99$), and a significant difference between + 0 h and + 3 h (+ 40 ± 38%; 90 % CI [28, 51%]; ES = 0.85; $p < 0.0001$). There was no time x intensity effect ($p = 0.85$) There was also a main effect of time for LC3B-II/I ratio ($p < 0.0001$), with a significant difference between REST and + 0 h (- 21 ± 17%; 90 % CI [- 26, - 16%]; ES = - 0.65; $p = 0.003$), no significant difference between REST and + 3 h (+ 10 ± 45 %; 90 % CI [- 4, 24%]; ES = 0.18; $p = 0.27$), but significantly different between + 0 h and + 3 h (+ 40 ± 45%; 90 % CI [27, 54%]; ES = 0.67; $p = 0.0001$). There was no time x intensity effect ($p = 0.85$). There was no time ($p = 0.57$) or time x intensity ($p = 0.29$) effect for p62 protein levels. There was no time ($p = 0.76$) or time x intensity ($p = 0.35$) effect for LC3B-I protein levels. These findings indicate that in humans there is a rapid decrease in LC3B-II and the LC3B-II/I ratio following exercise, with a return to baseline levels 3 h post-exercise. These changes are independent of exercise intensity and not accompanied by changes in p62 protein levels.

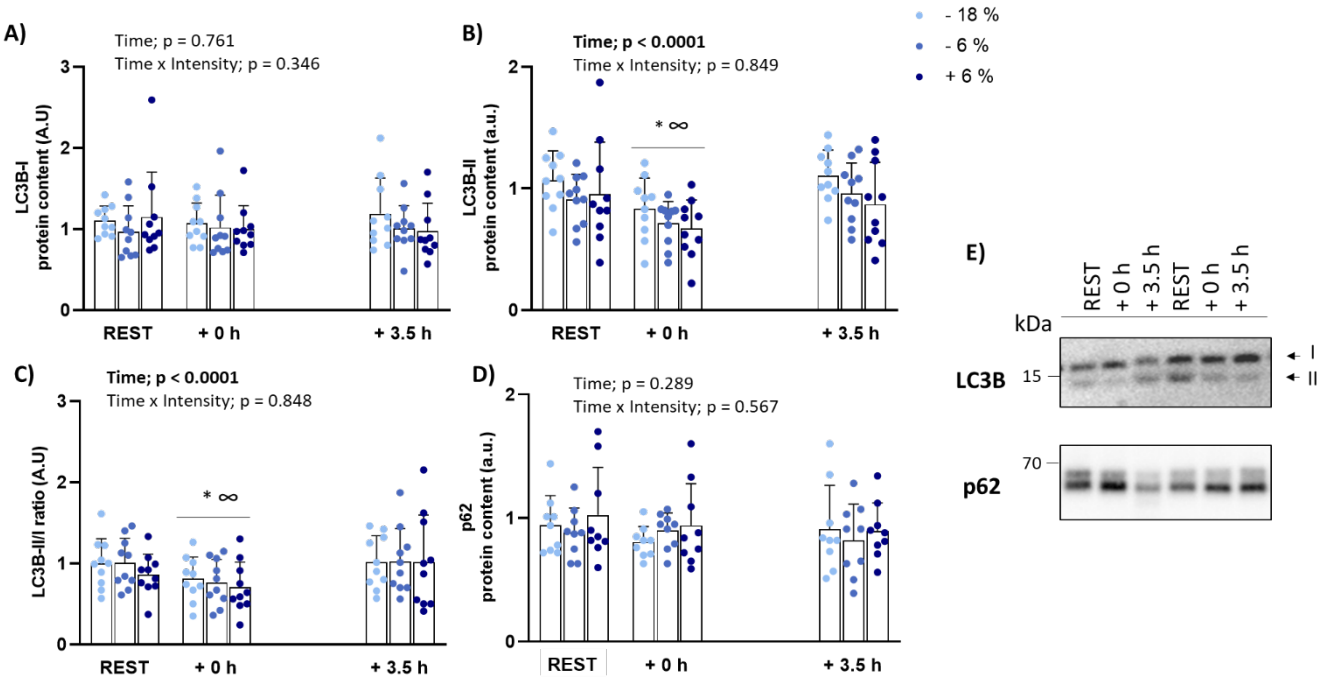

Figure 3.3. A) Protein levels of LC3B-I and LC3B-II at rest, as well as 0 h and 3.5 h after the end of exercise; B) the LC3BII/I ratio and p62 protein levels at rest, 0 h and 3.5 h after the end of exercise; C) representative blots of LC3B and p62 protein. Participants performed the exercise at three different intensities (-18% = light blue, -6 % = normal blue, and +6% = dark blue of the maximal lactate steady state). n = 9 for p62, n = 10 for LC3B. * = different than REST; ∞ = different from + 3.5 h. Bars shown are mean + SD.

3.3.3 Study 3 – Effects of training on exercise-induced changes in LC3B and p62 protein changes in human skeletal muscle.

There was no time x group effect for any variable (all $p > 0.05$). These findings suggest that the exercise-induced response in LC3B and p62 protein levels (+ 0 h and + 3 h) are not different between PRE and POST 3 weeks of exercise training.

3.3.4 Training-induced changes in LC3B and p62 protein levels in human skeletal muscle.

After the training intervention there was a significant increase in resting LC3B-II protein levels (+ 132 ± 140%; 90 % CI [+ 55, 209%]; ES = 0.85; $p = 0.04$), which evidenced an increase in autophagosome content. There was no change following the training intervention in LC3B-I, LC3B-II/I ratio, and p62 protein levels remained unchanged (all $p > 0.05$).

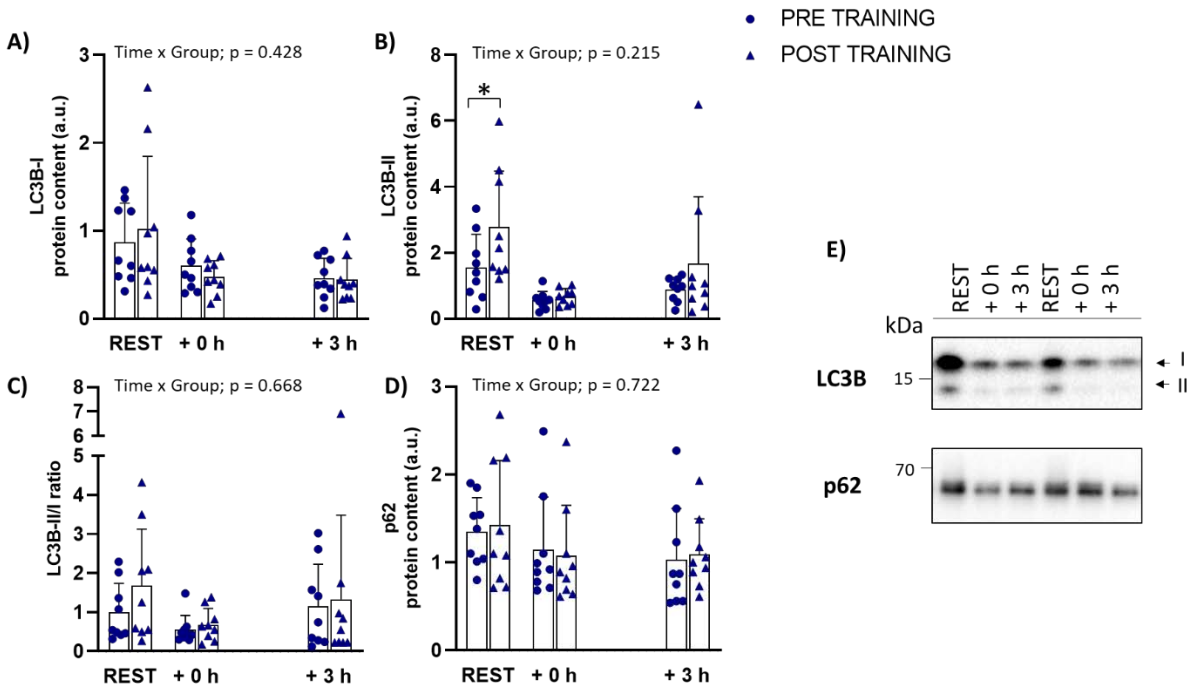

Figure 3.4. Protein levels of A) LC3B-I and B) LC3B-II at rest, 0 h and 3 h after the end of exercise in PRE and POST training samples; C) the LC3BII/I ratio and D) p62 protein levels at rest, 0 h and 3 h after the end of exercise in PRE and POST training samples; E) representative blots of LC3B and p62 proteins. Samples taken before the training period (Pre training) are shown with circles, and samples after the high-volume training period (Post training) are shown with triangles. * = significantly different than PRE training sample ($p < 0.05$). Bars are shown as mean + SD.

3.3.5 Study 4 - Exercise-induced changes in autophagy flux changes in human skeletal muscle.

Given the consistent results from exercise-induced changes in LC3B-II protein levels, which seemed independent of intensity exercise intensity, it was important to establish if autophagy flux was altered following exercise in humans.

There was a main effect for LC3B-II protein levels in untreated samples ($p = 0.014$), but there were no significant changes from REST to + 0 h (- 26 ± 23%; 90 % CI [- 43, - 10%]; ES = - 0.77; $p = 0.068$), or from REST to + 2.5 h (23 ± 27%; 90 % CI [3, 43%]; ES = - 0.64; $p = 0.13$). After 2.5 h of recovery, LC3B-II was not significantly different when compared to the + 0 h sample (+ 84.1 ± 91.3 %; 90 % CI [+ 16.9, + 151.3 %]; ES = 1.08; $p = 0.052$). There was no main effect for Net LC3B-II flux ($p = 0.26$). Effect size analyses showed a large positive change from REST to + 0 h (+ 117 ± 163%; 90 % CI [- 3, + 237%]; ES = 0.82; $p = 0.44$) and from REST to + 2.5 h (+ 113 ± 178%; 90 % CI [- 18, + 244%]; ES = 0.88; $p = 0.37$). These findings suggest that endurance exercise does not lead to decreased autophagy flux; instead, the effect size analyses suggest a moderate increase in autophagy flux, which is in the opposite direction to the changes in post-exercise LC3B-II protein levels.

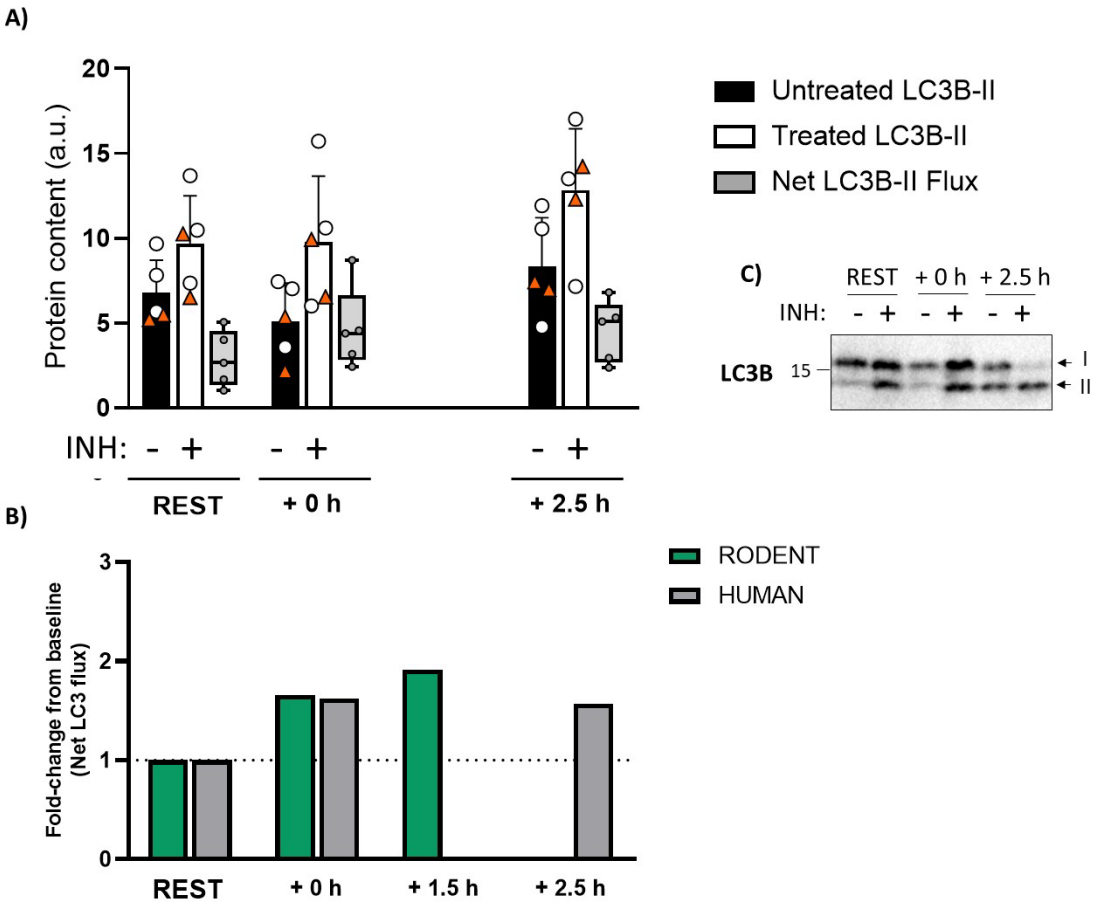

Figure 3.5. A) LC3B-II protein levels from untreated (black bars) and treated samples (white bars), and the net LC3B-II flux (in grey; calculated by subtracting untreated LC3B-II protein levels from treated sample). C) Representative blot from one participant. Orange triangles represent participants performing SIE; white circles represent participants performing MICE. B) Fold-change following exercise in the net LC3-II flux from the present study (LC3B-II, human) and a published rodent study (LC3-II, adapted from (273)). Bars for the treated and untreated samples display the mean + SD. Individual data points along with box and whisker plots are shown for net LC3B-II flux. INH = inhibitors NH_4Cl (40 mM) and Leupeptin (100 µM) added to the treated sample.

3.4 Discussion

The main finding of the present study was that exercise-induced changes in LC3B protein levels differ between rodents and humans. These differences did not appear to be attributable to differences in exercise intensity or training status in human studies. Importantly, the results showed that the exercise-induced decrease in LC3B-II protein levels observed in humans were not reflective of a decreased autophagy flux. Moreover, exercise training was capable of increasing the resting levels of LC3B-II protein levels in humans.

The results of the present study, based on four experiments performed in the same lab, showed that the exercise-induced changes in LC3B protein levels differ between rodents and humans. In the rodent study (study 1), there was an increased LC3B-I protein levels 0 to 3 hours following a single endurance exercise session. This was not observed in any of the three human studies (studies 2, 3, and 4; neither in Chapter 4). An increase of LC3B-I protein levels would suggest an increased LC3B mRNA translation. In fact, LC3B mRNA expression has been shown to increase following exercise to exhaustion in mice (273), and an exercise-induced increase in LC3-I protein levels has also been previously shown (262). However, it is difficult to compare across studies as not many studies report the changes of LC3-I protein levels. In rats, LC3B-II protein levels were unaltered immediately after exercise, but were significantly increased 3 hours into the recovery. This is in line with previous research showing that LC3-II is significantly increased in rodents 80 to 180 min from the start of exercise (164, 251, 274). These previous results suggest that due to the short duration of the exercise session in the current study (30 min), the post-exercise sample may have been unable to capture any increase in LC3B-II protein, which was observed in the sample obtained 3 hours into the recovery (i.e., 210 min from the start of exercise). On the other hand, the protein levels of the autophagy receptor p62 remained unchanged at any time point in the current study. While this is in contrast

to some rodent studies (251, 275), it is in agreement with most of the literature (262, 263, 273, 276). A possible explanation was again the duration of the exercise in the different studies. In fact, the only two studies reporting an exercise-induced decrease in p62 protein levels did exercise for at least 110 min (251, 275), and a decrease in p62 was not seen at earlier time points or in the recovery period (275). On the other hand, p62 protein level was also shown to be decreased 6 hours into the recovery from both low- and moderate-intensity exercise (263), suggesting a delayed lysosomal degradation of autophagosomes, which may have been missed by most studies, including the present study. Unfortunately, due to the incomplete information regarding the antibodies used, it was impossible to recapitulate the LC3 subfamily members used in some of the rodent studies. Future research should address whether the different LC3 subfamily members are similarly modified following exercise in rodents. Furthermore, future studies in skeletal muscle should explore the role of other ATG8 family members in exercise-induced autophagy, as it has been previously suggested that GABARAP subfamily members may play a more important role in autophagy (277).

In contrast to rodent studies (and the results from study 1), the findings from the human study (study 2) support the notion that LC3B-II protein levels and the LC3B-II/I ratio were decreased following exercise, returning to baseline 3.5 hours into the recovery. This was in accordance with most human studies (163, 164, 264, 265), but not all (162). A major difference with the study of Brandt, Gunnarsson (162) was the protein analysed. While the rest of studies, as well as the present study, have utilised an antibody targeting LC3B subfamily (163, 164, 264, 265), Brandt, Gunnarsson (162) used an antibody targeting a combination of LC3A and LC3B. Whether the protein levels of the different the LC3 subfamily members are differentially regulated following exercise remains to be elucidated. Interestingly, an analysis of proteomic studies in human skeletal muscle until 2015 only identified the LC3A isoform (278), which may indicate a greater protein abundance of LC3A when compared to the other isoforms in

skeletal muscle. The present findings also show that LC3B-I protein levels were not altered following exercise, consistent with previous studies in humans (163, 264). This could be due to unchanged mRNA expression of LC3B following exercise (264); however Schwalm, Jamart (265) showed LC3B mRNA expression to be increased following 2 h of exercise at 70% of $\dot{V}O_{2peak}$, and unchanged following 2 h at 55% of $\dot{V}O_{2peak}$. The unchanged LC3B-I protein levels could also be due to a rapid conjugation of LC3-I into LC3-II and increased autophagosome degradation.

The present findings also showed the immediate exercise-induced decrease in LC3B-II protein levels and the LC3B-II/I ratio occurs independently of exercise intensity. This was in accordance with previous studies (163, 164, 264, 265), which collectively suggest that exercise lasting 30 to 120 min and within a range of 50 to 85% of $\dot{V}O_{2max/peak}$ has a similar effect on immediate exercise-induced LC3B-II protein and the LC3B-II/I ratio changes, returning to baseline values 3 to 4 hours into the recovery. While an extreme exercise volume seemed to have a different impact on LC3B-II protein levels, evidenced from a 554% increase following an ultra-endurance running event (279), whether higher intensities (> 100% $\dot{V}O_{2max}$) can lead to different exercise-induced responses in LC3B protein remains to be fully elucidated. Furthermore, the finding of unchanged p62 protein levels following exercise, independent of exercise intensity (study 2), were in accordance with most studies (162-164, 264). It also suggested that the previously reported role of exercise intensity on p62 protein changes (265) may not be due to exercise intensity differences between protocols, but possibly related to other factors such as total work performed.

Differences between rodents and humans in exercise-induced LC3B regulation had been previously raised (164). The present study shows that one clear difference between species is the exercise-induced changes in LC3B-I protein. This could suggest differences in LC3B mRNA transcription levels and translation, which could limit the rate of new LC3B-I protein

formation, and later on LC3B-II changes; this could help explain why LC3B-II protein levels only increased in rodents. Another possible explanation is distinct LC3 isoform expression in the skeletal muscle of rodents and humans, which may be differentially regulated following exercise. Regarding p62 protein levels, the present findings were in accordance with the majority of research reporting unchanged p62 protein levels after exercise in both rodents and humans (164, 251, 264, 265, 273, 274). This could be due to matched sustained level of lysosomal degradation and p62 mRNA translation in the first few hours after exercise, resulting in no detectable differences in p62 protein levels. Whether a higher exercise volume (> 110 min) and intensity was needed, or whether the timing of muscle sampling needs to be delayed (e.g., 6 hours post exercise (263)), to observe p62 protein decreases remains to be explored. It is important to mention that other proteins can also act as autophagy receptors (e.g., NBR1, OPTN) (280), and how these are altered by exercise requires further investigation.

Repeated exercise over time is known to reduce some of the exercise-induced signalling responses (50, 85). It was next investigated whether short-term exercise training would influence the exercise-induced changes in LC3B and p62 protein levels. In the present study there was no significant difference in the exercise-induced protein response between the exercise sessions performed before or after the training period, despite a large increase in aerobic fitness (as measured by Granata et al., *unpublished*). Although not highlighted in the literature, this was in agreement with the notion that most studies, independently of age, health, or training status, have reported a similar exercise-induced regulation of LC3B-II, LC3B-II/I, and p62 protein levels (164, 264, 265). An increase in basal LC3B-II protein level was observed following the 3-week training period, suggesting that a high volume of endurance training is capable of increasing the basal LC3B-II protein levels. This was in line with a previous study where 3 weeks of one-legged knee extensor training led to an increase in LC3B-II protein levels

(164). However, others have not shown any effect of endurance training on LC3A/B-II protein levels, despite an increase in LC3A/B-I protein levels (162).

A limitation of human studies to date is the use of LC3B-II and p62 protein levels to infer changes in autophagy flux. This has led to the idea that a decrease in LC3B-II protein levels following exercise could be reflective of a temporary decrease in autophagy flux (264). In the current study, a protocol adapted from a rodent study (268) was used to examine for the first time the effects of exercise on *ex vivo* autophagy flux in human skeletal muscle. The results showed that autophagy flux (measured as net LC3B-II flux) did not decrease after exercise (+ 0 h) or 2.5 h into the recovery from exercise. Although limited by the low number of participants, the effect size analyses instead suggested an increase in autophagy flux following exercise (+ 113-117%; ES = 0.82-0.88). This finding, in human samples, was in agreement with a rodent study showing a similar exercise-induced fold-change in autophagy flux (273). These findings would suggest that exercise-induced autophagy flux is similar between rodents and humans, despite a different LC3B protein regulation. Although this should be addressed in detail in future research, the use of an *ex vivo* autophagy flux assay in future human studies will allow researchers to overcome the limitation of solely relying on protein markers. Future research should interrogate the skeletal muscle autophagy flux response to different skeletal muscle stimuli (e.g., inactivity) and with larger sample sizes.

In conclusion, the results of the current study showed that exercise-induced LC3B protein changes differ between rodents and humans. Caution must be taken when extrapolating autophagy protein results from rodents to humans. Furthermore, findings from the current study suggested that a reduction in LC3B-II protein levels following exercise in humans was consistent across exercise intensities, and independent of training status, but was not indicative of autophagy flux. This suggests studies should avoid looking at ‘static’ levels of LC3 protein levels and include autophagy flux assays to provide a more valid assessment of changes in

autophagy with exercise. Future studies should also explore the regulation of multiple ATG8 family members to fully elucidate the molecular regulation of autophagy in mammalian skeletal muscle.

CHAPTER 4 – THE EFFECTS OF MODERATE-INTENSITY CONTINUOUS EXERCISE (MICE) AND SPRINT-INTERVAL EXERCISE (SIE) ON MITOCHONDRIAL DYNAMICS AND STRESS RESPONSE.

In the previous chapter, exercise-induced changes in markers of autophagosome content were found to be independent of exercise intensity. However, whether the mitochondrial-specific quality control mechanisms differed between different exercises was not studied. The aim of this chapter was explore the mitochondrial dynamics and stress response to different endurance exercise prescriptions. To address this, and based on the findings of chapter 3, I used two exercise prescriptions emphasising exercise volume (MICE) or exercise intensity (SIE) to polarise the exercise stimulus. The results of the present study will help elucidate cellular pathways that are differentially influenced by high-volume and high-intensity exercise prescriptions. Part of this work has been presented at the annual conference of the Australian Physiological Society (2019) and the annual congress of the European College of Sport Science (2020).

4.1 Introduction

Exercise is one of the most effective lifestyle interventions for the prevention of non-communicable diseases (281). One of the positive events occurring following exercise in skeletal muscle is the upregulation of pathways involved in mitochondrial biogenesis (166, 282). Mitochondria are well-known to be responsible for ATP resynthesis, while also regulating many other critical cellular functions (e.g., metabolism, cell death) (283). Bestowed with such responsibilities, it is not surprising that mitochondrial characteristics, at the skeletal muscle level, have been linked to both endurance performance (63) and health (284). A greater understanding of the cellular signalling responses to different types of exercise would help to better understand and implement targeted exercise prescription to improve both health and performance (285).

During exercise, skeletal muscle oxygen demands can increase up to 20-fold (286). Through changes in bioenergetic and mechanical demands, exercise leads to disturbances of intracellular parameters (e.g., AMP, Ca^{2+}) that activate key protein kinases to initiate cellular and transcriptional responses to restore homeostasis (287). A single exercise session is capable of altering over a thousand protein phosphorylation sites (166), to alter the expression of thousands of genes (282), and to have positive metabolic effects for up to 48 hours (288). These acute transcriptional responses, when repeated over time, are the basis of mitochondrial adaptations (85, 289). Many of the intracellular kinases modulated by exercise partially converge on the regulation of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), an important regulator of mitochondrial biogenesis (53, 287, 290). However, exercise results in a complex molecular signature in skeletal muscle (166, 167, 282), which contributes to the altered mitochondrial characteristics following exercise training.

Understanding how different exercises affect cellular signalling will expand our understanding of the exercise-induced mechanisms underlying mitochondrial adaptations.

In skeletal muscle, mitochondria form a coordinated reticulum (291). Upon regional disturbance mitochondria are depolarised and physically separated from the network, forming isolated spherical mitochondria (292). This is achieved by an orchestrated series of processes called mitochondrial dynamics, which help to maintain a functional mitochondrial pool (103). Fusion rates are linked to the fibre oxidative capacity (increased in type I fibres) (115), but are lower than those seen in isolated myoblasts. This suggests a limited *in vivo* mitochondrial dynamism, probably due to limited space in myofibres (113). In mice, running increases the number of mitochondrial membrane interactions (possibly reflecting an increased mitochondrial fusion) (293), increases the frequency of mitochondrial constrictions (paralleled by an increased phosphorylation of dynamin related protein 1 (DRP1) at serine 616) (294), and upregulates markers of mitophagy (increased colocalisation of lysosome and mitochondria) through an AMPK-ULK1 regulation (144). Human studies have been limited to measures of mitochondrial morphology (123), mRNA expression (90, 122, 271), or protein levels (52, 119, 125, 129), but nonetheless indicate that these effects are also partially observed in human skeletal muscle. However, the role of different exercise prescriptions on signalling associated with mitochondrial dynamics remains equivocal in humans.

Mitochondria, which are highly exposed to cellular stressors, have evolved to communicate their status to the nucleus and to orchestrate a transcriptional response to prevent harmful cellular events (194). Mitochondrial stress can have multiple origins (178) and converges on the activation of the integrated stress-response (ISR) (168, 169, 178). Rodent studies have shown that exercise can lead to phosphorylation of the alpha subunit of eukaryotic translation initiation factor 2 (eIF2α) at serine 51, the core ISR sensor, acting through the protein kinase R-like endoplasmic reticulum kinase (PERK) pathway, which senses endoplasmic reticulum

(ER) stress (174, 195, 295). Activating the ISR leads to decreases in protein synthesis (296), and preferential translation of genes with an upstream open reading frame (uORF), such as activating transcription factor 4 (ATF4) or CCAAT/enhancer binding protein homology protein (CHOP), orchestrating a transcriptional program to recover cellular homeostasis (297). In skeletal muscle, genetically altering mitochondrial dynamics has been shown to activate the ISR (185). Furthermore, human models of mitochondrial dysfunction (mitochondrial myopathies) show increased transcript levels of ISR transcription factors such as CHOP and ATF5 (178, 298, 299), and, fibroblast growth factor 21 (FGF21) - a downstream target of ISR is currently used as a marker for mitochondrial disease (300). Whether exercise is capable of inducing mitochondrial stress and initiating a mitochondrial-specific ISR response remains unexplored.

The aims of the current study are multiple. First, to elucidate any divergent response in the transcriptional response to moderate-intensity continuous exercise (MICE) and sprint-interval exercise (SIE). Secondly, to explore if markers of mitochondrial dynamics and stress are differentially regulated by different exercise prescriptions. It was hypothesised that SIE, due to the larger homeostatic disturbance, will disturb the mitochondrial pool and lead to a mitochondrial-specific stress response.

4.2 Methodology and procedures

4.2.1 Participants

Participants were provided with the information to participants document (Appendix 1) and were informed of the study requirements, benefits, and risks involved, before giving their written informed consent. Twenty-eight healthy males (26.5 ± 5.3 y; 179.1 ± 6.3 cm; 76.8 ± 10.3 kg; 23.9 ± 2.7 BMI) volunteered to take part in this study. Participants were matched for their peak aerobic power ($\dot{W}_{max}$; $W \cdot kg^{-1}$) and 20-km time trial performance (20-kmTT; $W \cdot kg^{-1}$), and assigned in a random counter-balanced order to one of the two exercise groups. Groups were matched for physiological ($\dot{V}O_{2max}$, $\dot{W}_{max}$), biological (age), and anthropometrical (height, body mass, body mass index) characteristics (Table 4.1; all between-group differences are $p > 0.05$).

4.2.2 Study design

Following the initial graded exercise test (GXT), the participants were allocated to their exercise group. During the next two weeks, participants performed one 4-km time trial (4-kmTT), one 20-kmTT, two familiarisations of their assigned exercise session (the first one being 50% of the experimental day load, and the second one being the total exercise session), and a submaximal GXT. Following the familiarisation period, participants underwent another week of testing involving a GXT, a 4-kmTT, and a 20-kmTT. Ethics approval for the study was obtained by the Victoria University Human Research Ethics Committee (HRE17-075), and the study was registered as a clinical trial under ANZCTR (ACTRN12617001105336).

Table 4.1. Characteristics of participants in each exercise group. Data are mean ± SD. GXT = graded exercise test; $\dot{W}_{max}$ = peak power attained during the GXT; BMI = body mass index; MICE = moderate-intensity continuous exercise; SIE = sprint-interval exercise.

			GXT Results		Experimental Trial		
	Age (y)	BMI ($kg \cdot m^2$)	$\dot{V}O_{2max}$ ($mL \cdot min^{-1} \cdot kg^{-1}$)	$\dot{W}_{max}$ ($W \cdot kg^{-1}$)	Mean Power ($W \cdot kg^{-1}$)	% of $\dot{V}O_{2max}$	Total Work (kJ)
MICE (n=14)	26.1 ± 5.7	24.1 ± 2.6	52.2 ± 6.4	4.16 ± 0.58	1.64 ± 0.44	51 ± 5	687 ± 205
SIE (n=14)	26.9 ± 4.9	23.7 ± 2.8	51.9 ± 6.5	4.17 ± 0.76	7.51 ± 0.88	-	133 ± 19

4.2.3 Control procedures

To minimise between-subject variation, participants were provided with standardised caloric and macronutrient intake for the 24 h leading to the experimental exercise trial. During the familiarisation period participants were asked to provide information on their habitual food intake and habits. Based on their habitual diet they were given a dinner in the evening (55 $kJ \cdot kg^{-1}$ body mass, providing 2.1 g carbohydrate$\cdot kg^{-1}$, 0.3 g fat$\cdot kg^{-1}$, and 0.6 g protein$\cdot kg^{-1}$), and a breakfast to be consumed 3 h before the start of the experimental session (41 $kJ \cdot kg^{-1}$, providing 1.8 g carbohydrate$\cdot kg^{-1}$, 0.2 g fat$\cdot kg^{-1}$, and 0.3 g protein$\cdot kg^{-1}$) as in previous studies (50, 52, 271, 301). These two standardised meals were provided before the experimental exercise trial, as well as before the + 24 h biopsy. Participants were also asked to refrain from caffeine during the day of the trial, as well as to avoid any exercise for at least 48 h before the trial.

4.2.4 Exercise groups

4.2.4.1 Sprint Interval Exercise.

Fourteen participants were randomly allocated to this group (26.9 ± 4.9 y; 179.6 ± 5.9 cm; 76.5 ± 9.7 kg; 23.7 ± 2.8 BMI). The exercise session consisted of six, 30-s, 'all-out' cycling bouts against a resistance initially set at 0.075 kg·kg body mass^{-1}, interspersed with a 4-min recovery period (302). During the recovery participants remained on the bikes and were allowed to either rest or cycle against no resistance. During the last 30 s of the recovery period participants were instructed to begin pedalling and to reach a cadence of 90 rpm against no resistance, and in the last 10 seconds they were advised to get ready and the countdown began. They were instructed to begin pedalling as fast and hard as possible when the countdown reached zero. At this time, the load was applied via the ergometer software (Lode Excalibur v2.0, The Netherlands). Participants were verbally encouraged to pedal as fast and hard as possible during the entire duration of the bout.

4.2.4.2 Moderate Intensity Continuous Exercise.

Fourteen participants were randomly allocated to this group (26.1 ± 5.7 y; 178.6 ± 6.5 cm; 77.0 ± 10.7 kg; 24.1 ± 2.6 BMI). The exercise session consisted of continuous cycling at a fixed power equivalent to ~ 90 to 100 % of LT_1 (defined as the first increase in lactate from baseline during a submaximal GXT) (303). The duration of the session was 90 min. Given the inherent differences (~ 4 to 5 fold) in exercise intensity between the selected exercise groups, the MICE exercise session was designed to produce a similar difference for total work (i.e., 4 to 5 fold).

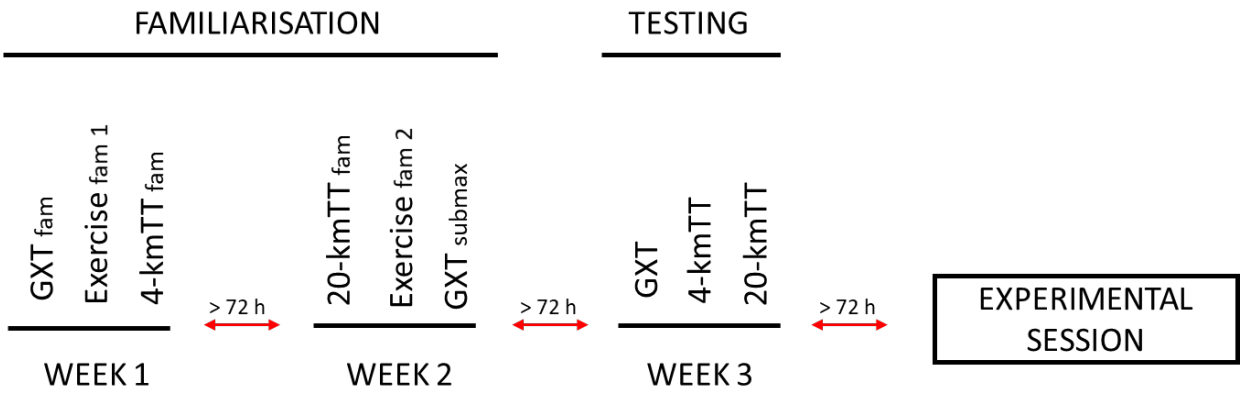

Figure 4.1. Schematic overview of the initial period of familiarisation and testing before the experimental exercise session (figure 4.2.B). GXT = Graded exercise test; fam 1 = familiarisation 1; fam 2 = familiarisation 2; 20-kmTT = 20-kilometre time trial; 4-kmTT = 4-kilometre time trial.

4.2.5 Blood parameters

During the experimental exercise session, 2 mL of antecubital venous blood were drawn at four different time points: resting, + 30 min, + 90 min, and + 180 min (Figure 4.2). Following initial calibration, and according to manufacturer's recommendations, samples were measured in duplicate for lactate and pH (ABL800 FLEX, Radiometer Medical ApS, Denmark).

4.2.6 Physiological testing

4.2.5.1 Graded Exercise Test (GXT).

The GXTs were conducted using an electronically-braked cycle ergometer (Lode Excalibur v2.0, The Netherlands). A metabolic analyser (Quark Cardiopulmonary Exercise Testing, Cosmed, Italy) was used during the testing to assess $\dot{V}O_2$, $\dot{V}CO_2$, $\dot{V}E$, on a breath-by-breath basis, and heart rate was also measured (Garmin, USA). A GXT with 1-min stages was chosen (24), and the increase in Watts between stages was adjusted following the familiarisation trial to attain a total duration of 9 to 11 min upon exhaustion. After reaching exhaustion, 5 min of

rest were provided before a verification bout (time to exhaustion at 90 % of $\dot{W}_{max}$) was applied to verify that $\dot{V}O_{2max}$ was achieved.

4.2.5.2 Submaximal Test.

Following the completion of the GXT, the ventilatory parameters obtained ($\dot{V}O_2$, $\dot{V}CO_2$, $\dot{V}E$) were plotted and visually inspected to estimate VT_1 (304). Accordingly, a power output of 30 to 40 W lower than the estimated VT_1 was selected as the starting point of the submaximal test. After 3 min (stage length) the intensity was increased by 10 W and continued increasing every 3 min until the LT_1 could be identified. Antecubital venous blood was taken in the last 15 s of each stage and was analysed using a blood lactate analyser (YSI 2300 STAT Plus, YSI, USA). Once the blood lactate reached 2 $mmol \cdot L^{-1}$ the test was stopped as this indicated the LT_1 had already been reached.

4.2.7 Muscle analyses

4.2.7.1 Muscle biopsies

All muscle samples were obtained at a similar time of the day (morning), at a constant depth of around 2 to 3 cm, by an experienced medical doctor. Resting muscle biopsies were taken from the *vastus lateralis* muscle using the Bergström biopsy needle technique with suction. Figure 4.2 shows the timing of biopsies in the different training groups. These time-points were selected in an effort to overcome the possible confounding effect of the different exercise prescriptions having a different total exercise time (30 min vs 90 min). As such, the biopsy timing was designed to have at least 3 muscle biopsies with the same between-group timing from both the start of exercise (REST, + 1.5 h, + 2.5 h, + 24 h) and from the end of exercise (REST, + 0 h, + 2.5 h) (Figure 4.2.B). Participants rested in the supine position, and after injection of a local anaesthetic into the skin and fascia (1% xylocaine, Astra Zeneca) a small incision was made for each biopsy. Once the muscle sample was obtained it was processed,

cleaned of excess blood, fat, and connective tissue, and split in two portions. One portion (5 to 10 mg) was immediately fixed in a 0.2 M Na-Cacodylate buffered 2.5% glutaraldehyde and 2% paraformaldehyde for transmission electron microscopy imaging. The remaining portion was immediately frozen in liquid nitrogen and stored at -80 °C for subsequent analyses.

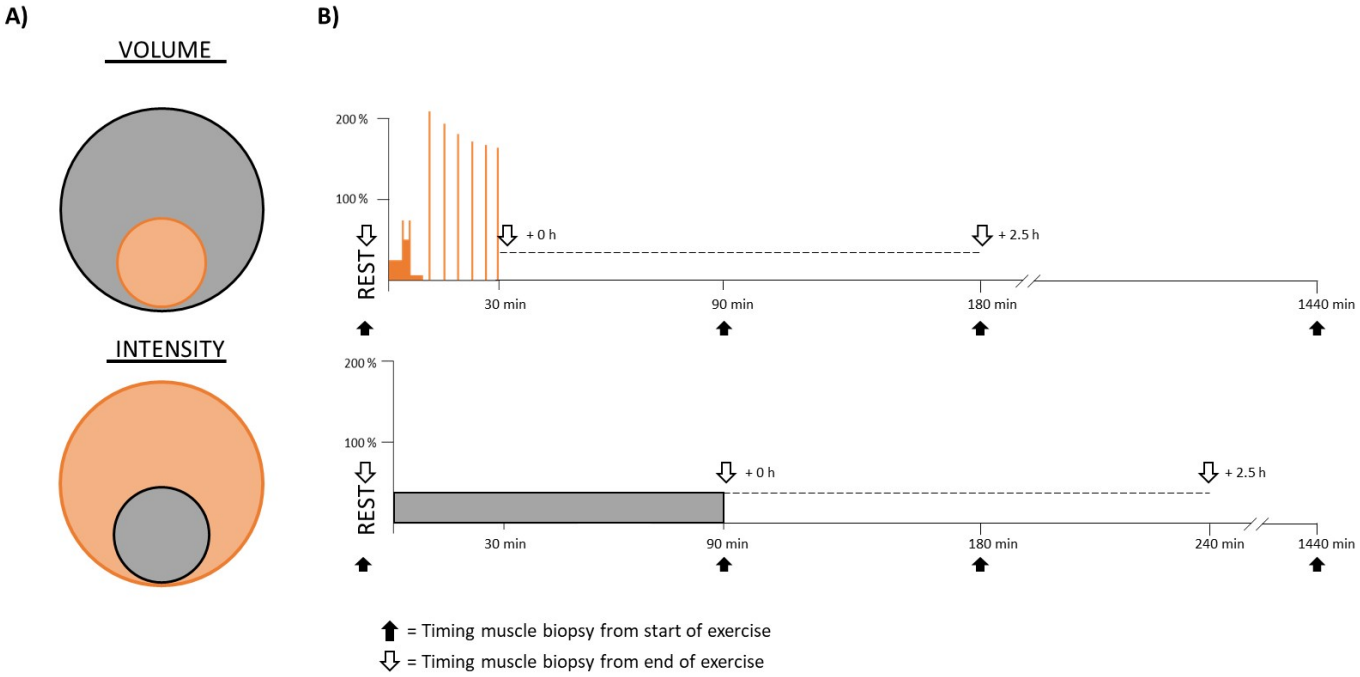

Figure 4.2. A) Volume and intensities relative to each group, during the exercise session. B) Timing of the skeletal muscle biopsies during the experimental session. X axis represents time, Y axis represents intensity of exercise as a percentage of $\dot{W}_{max}$. Solid lines display the timing of biopsies from the start of exercise. Dashed lines display timing of biopsies from the end of exercise. For both figure A and B orange = Sprint-interval exercise and grey = Moderate-intensity continuous exercise.

4.2.7.2 Transmission Electron Microscopy

Skeletal muscle samples were fixed overnight at 4 °C with 0.2 M sodium cacodylate– buffered, 2.5% glutaraldehyde, and 2% paraformaldehyde. Fixed samples were rinsed with 0.1 M sodium cacodylate, and postfixed with ferricyanide-reduced osmium tetroxide (1 % OsO_4, 1.5 % $K_3[Fe(CN)_6]$, and 0.065 M cacodylate buffer) for 2 h at 4 °C. The postfixed samples were rinsed

with distilled water, and then stored overnight in 70% ethanol. Dehydration was performed by graduated ethanol series (80%, 90%, 95%, 100%, and 100%; 10 min each) and propylene oxide (100% and 100%; 5 min each). Samples were infiltrated with Araldite 502/Embed 812 by graduated concentration series in propylene oxide (25% for 1 h, 33% for 1 h, 50% overnight; 66% for 4 h, 75% for 4 h, 100% overnight; and 100% for 5 h) and then polymerised at 60 °C for 48 h. Embedded samples were sectioned using an Ultracut UCT ultramicrotome (Leica Biosystems) equipped with a 45 ° diamond knife (Diatome) to cut 75-nm ultrathin sections. The grids were stained at room temperature using 2% aqueous uranyl acetate (5 min) and Reynolds lead citrate (3 min) before routine imaging. All TEM imaging was performed at 80 kV on a Hitachi H-7500 TEM using a Gatan 791 MultiScan side-mount CCD camera and DigitalMicrograph (Version 1.71.38) acquisition software. All images were obtained from the longitudinal section. Image acquisition was performed by a blinded investigator. Ten images were randomly acquired, from at least three different fibres, in the PRE and + 0 h samples from a subset of participants (n = 4 in MICE, n = 5 in SIE). Mitochondrial contact number was calculated as the frequency that two adjacent mitochondria had membrane contact relative to total number of mitochondria (total number of visualised mitochondria = 4923), obtained from micrographs with enough quality to detect such contacts (n = 184). Mitochondria shape and size descriptors were obtained using ImageJ (NIH, USA), as previously published (205), by manually tracing clear mitochondria (MICE PRE, n = 428; MICE + 0 h, n = 446; SIE PRE, n = 603; SIE + 0 h, n = 551). Frequency distribution plots were generated using the ggplot package in R. Disturbed mitochondria were evaluated by counting the mitochondria from micrographs (micrographs = 207; total number of visualised mitochondria = ~ 5400; disturbed mitochondria = 228). Given the striking morphological and structural difference in mitochondria following SIE, whole tile-sets of at least 4 individual fibres from each post-SIE

sample (n = 8) were imaged and visually inspected to further explore these exercise-induced morphological and structural changes.

4.2.7.3 *Preparation of whole-muscle lysates*

Approximately 10 to 20 mg of frozen muscle was homogenised 2 times for 2 minutes at a speed of 30 Hz with a TyssueLyser instrument (Qiagen, Canada) in an ice-cold lysis buffer (1:20) containing 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 5 mM $Na_4P_2O_7$, 1 mM Na_3VO_4, 1% NP-40, with added protease and phosphatase inhibitors at a 1:100 concentration (Cell Signaling Technology). Protein concentration was determined using a commercial colorimetric assay (Bio-Rad Protein Assay kit II, Bio-Rad Laboratories Pty Ltd, Gladesville, NSW, AUS) and lysates were then diluted with an equal volume in 2x Laemmli buffer containing 10% β-mercaptoethanol.

4.2.7.4 Western blotting.

For each protein of interest a linearity study was conducted to determine the ideal loading amount. Muscle lysates were then loaded in equal amounts (10 to 20 μg according to target protein) and separated by electrophoresis for 1.5 to 2.5 h at 100 V using pre-cast SDS-PAGE gels (4-20%). Once resolved, the gels were then wet transferred onto PVDF LF or nitrocellulose membranes using a Turbo Transfer (Bio-rad Laboratories Pty Ltd, Gladesville, NSW, AUS). Membranes were imaged for total protein, which was used later as a loading control. Membranes were then blocked at room temperature for 1 h using 3% skim milk or 3% Bovine Serum Albumin (BSA) in Tris Buffer Saline (TBS) 0.1% Tween-20 (TBS-T). After 3 x 5-min washes in TBS-T, membranes were incubated overnight at 4°C with gentle agitation in primary antibody solutions (3% BSA plus 0.02% Na Azide). Immunoblotting was carried out using the desired antibody. OMA1 (sc-515788), FIS1 (sc-376469), MFN2 (CST9482), pULK1 s757 (CST14202), pULK1 s556 (CST5869), LC3B (CST3868S), p62 (ab56416), pACC s79 (CST3661), 4HNE (ab46545), pp38 Thr180/Tyr182 (CST9211). The following

morning, membranes were washed 3 x 5 min in TBS-T and subsequently incubated under gentle agitation at room temperature with the appropriate secondary antibody for 60 to 90 min in 5% skim milk in TBS-T. Membranes were then washed again for 3 x 5 min in TBS-T before being immersed for 5 min under gentle agitation at room temperature in Clarity ECL detection substrate (Bio-rad Laboratories Pty Ltd, Gladesville, NSW, AUS). Protein bands were visualised using a Bio-Rad ChemiDoc imaging system and band densities were determined using Bio-Rad Image Lab analysis software (Bio-Rad Laboratories Pty Ltd, Gladesville, NSW, AUS). Finally, all samples for each participant were loaded on the same gel and the different concentrations of a mixed-homogenate internal standard (IS) were also loaded on each gel and a calibration curve plotted of density against protein amount. From the subsequent linear regression equation, protein abundance was calculated from the measured band intensity for each lane on the gel. Total protein content of each lane was obtained from the stain-free image of the membrane and was used for normalisation of the results.

4.2.7.5 RNA extraction

Approximately 10 to 15 mg of frozen muscle was used to isolate RNA using the RNeasy Mini Kit (Qiagen, Canada) according to the manufacturer's instructions. Samples were homogenised using the TissueLyser II (Qiagen, Canada). RNA concentration and purity was determined spectrophotometrically (NanoDrop 2000, Thermo Fisher Scientific, Wilmington, DE) at 260 and 280 nm. RNA integrity was assessed by using Agilent Bioanalyzer according to manufacturer's instructions. The RNA was stored at -80 °C until reverse transcription.

4.2.7.6 Reverse transcription

For each sample, 1 µg of RNA was transcribed into cDNA on a thermal cycler (S1000TM Thermal Cycler, Bio-Rad, USA) using the iScriptTM cDNA Synthesis Kit (Bio-Rad, USA) and the following incubation profile: 5 min at 25 °C, 30 min at 42 °C and 5 min at 85 °C. The transcription was performed with random hexamers and oligo dTs in accordance with the

manufacturer's instructions. A reverse transcriptase (RT) negative control was also generated. cDNA was stored at - 20°C until subsequent analysis.

4.2.7.7 qPCR.

All samples were run together to decrease technical variation. Forward and reverse primers for the target and housekeeping genes were designed based on NCBI RefSeq using NCBI Primer-BLAST (www.ncbi.nlm.nih.gov/BLAST/). Specificity of the amplified product was confirmed by melting point dissociation curves. The mRNA expression was performed by quantitative real-time RT-PCR (Mastercycler® RealPlex2, Eppendorf, Germany), using a 5 µL PCR reaction volume with SYBR Green supermix (SsoAdvanced™ Universal SYBR® Green Supermix, Bio-Rad, USA). All samples were run in duplicate simultaneously with template free controls, using an automated pipetting system (epMotion 5070, Eppendorf, Germany). The following PCR cycling patterns were used: initial denaturation at 95° C (3 min), 40 cycles of 95° C (15 s) and 60° C (60 s). The mRNA expression of five housekeeping genes was quantified, and the three most stable genes were determined using the BestKeeper software (305). 18S ribosomal RNA (18s), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and beta-2-microglobulin (B2M) were classified as most stable and utilised for the analysis. Expression of each target gene was calculated as previously published (305).

4.2.7.8 RNA-sequencing

Transcriptomic analyses were performed by the Beijing Genomic Institute (BGI). Sequencing was performed using 100bp paired-end sequencing on the DNBseq technology platform. A total of 1 µg RNA from each of the 72 samples (24 participants and 3 time-points) were used. From the raw reads obtained, quality control was performed using FastQC. Reads were then aligned against the GRCh38 Human genome assembly using STAR 2.7.3a (306). Reads were mapped to their corresponding genes using RSEM (307). The read counts obtained were then used in R for differential gene expression with limma and DESeq2 package following

previously published guidelines (308). Pathway enrichment analysis was performed using Metascape (309). Geneset enrichment analysis of mitostress geneset (178) was performed as previously published (310).

4.2.8 Statistics

All values are reported as mean ± standard deviation (SD). Normality was assessed with a Shapiro-Wilk test, and datasets were log-transformed if they failed the normality test. To investigate the effect of exercise (5 time-points) and group (MICE or SIE), and the interaction between these, two-way repeated measures of ANOVA were performed. Interactions were followed by Bonferroni post hoc tests to assess the differences between time points (both within and between groups). Main effects of exercise were further analysed with one-way ANOVA pre-planned contrasts to compare the effects of exercise within groups. Due to the extent of data skewness, and inability to normalise mitochondrial morphology variables, these were analysed with non-parametric Mann-Whitney tests. Effect sizes (ES) were defined as: small (0.2), moderate (0.5), large (0.8), and very large (1.3). For RNA-sequencing trasncripts the adjusted p value ($p < 0.05$) was used to set statistical significance. For geneset enrichment analysis the false discovery rate (FDR) q-value was used to set statistical significance (< 0.05). The level of statistical significance was set at $p < 0.05$. GraphPad Prism 8.3 software was used for all statistical analyses.

4.3 Results

4.3.1 Physiological differences

As designed, the total work performed in MICE was 5.2 fold greater than in SIE (687± 205 kJ vs 133 ± 19 kJ; ES = 1.75; $p < 0.0001$; Fig 4.3.A). The intensity of the exercise (excluding warm up) was 4.7-fold greater in SIE than in MICE (183 ± 21 % of $\dot{W}_{max}$ vs 39 ± 6 % of $\dot{W}_{max}$; ES = 1.93; $p < 0.0001$; Fig 4.3.A). The rating of perceived exertion was significantly higher following SIE than following MICE (9.5 ± 0.7 a.u. vs 3.7 ± 0.8 a.u.; $p < 0.0001$).

Blood lactate concentration and pH values from the two experimental trials are displayed in figure 4.3. There was a significant exercise effect ($p < 0.001$), and a group x exercise effect ($p < 0.001$), for blood lactate concentration. Blood lactate was significantly increased from REST to +30 min in the SIE group (from 0.9 ± 0.2 to 10.7 ± 1.9 $mmol \cdot L^{-1}$; ES = 1.90; $p < 0.0001$). In the MICE group, blood lactate increased from REST to +30 min (0.7 ± 0.2 to 1.3 ± 0.3 $mmol \cdot L^{-1}$; ES = 1.50; $p < 0.0001$), but did not increase further at +90 min (1.3 ± 0.3 to 1.2 ± 0.2 $mmol \cdot L^{-1}$; ES = - 0.34; $p = 0.19$). The increase in blood lactate was significantly larger in SIE when compared to MICE (9.8 ± 1.8 $mmol \cdot L^{-1}$ vs 0.5 ± 0.2 $mmol \cdot L^{-1}$; ES = 1.90; $p < 0.0001$). There was a group x exercise interaction for blood pH ($p < 0.0001$), which was significantly decreased from REST to +30 min in the SIE group (7.35 ± 0.03 to 7.02 ± 0.08; ES = - 1.76; $p < 0.0001$), returning back to baseline at +90 min. There were no changes in pH following MICE (all $p > 0.999$).

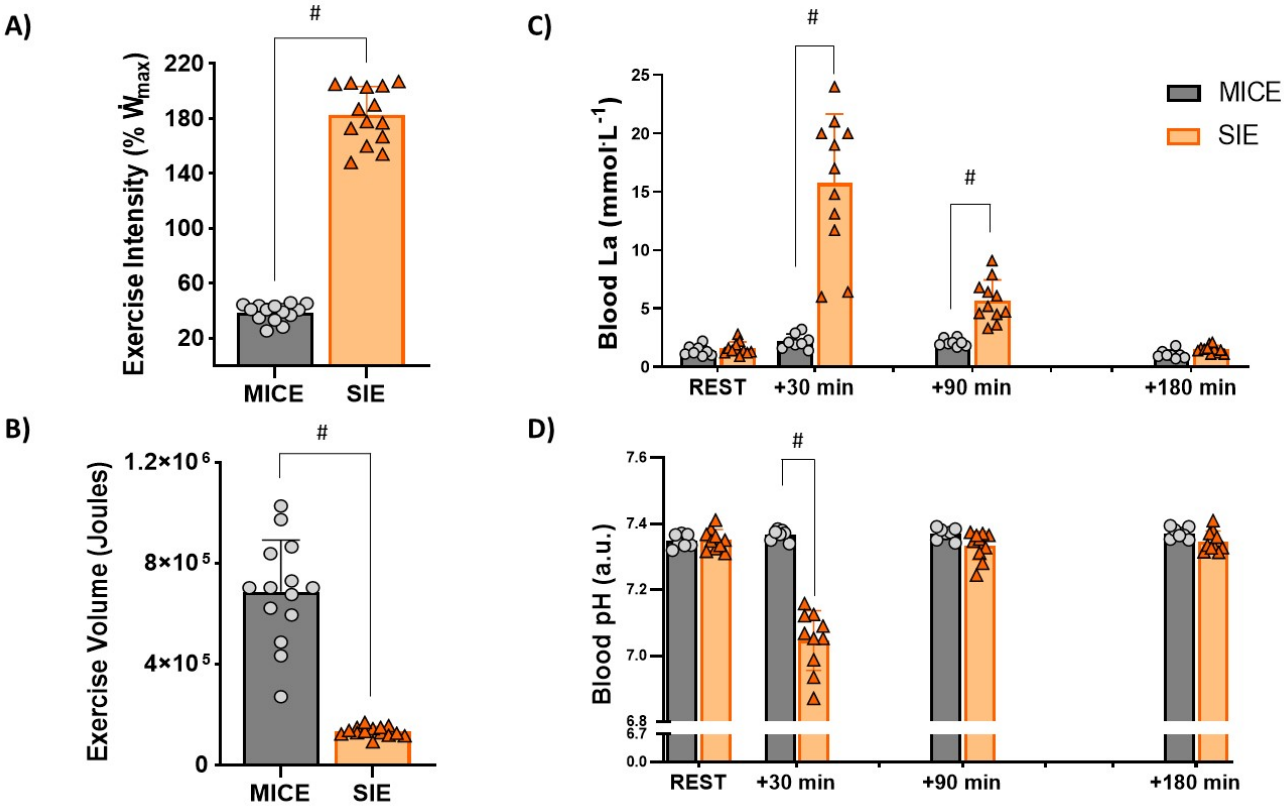

Figure 4.3. A) Relative exercise intensity and B) volume completed during the experimental trial ; C) Blood lactate (La) concentration time course from the start of exercise; D) blood pH levels before and after the exercise session; Orange = Sprint-interval exercise (SIE); Grey = Moderate-intensity continuous exercise (MICE). # = $p < 0.05$ between-group difference. Values shown are mean + SD.

4.3.2 Exercise-induced transcriptional response

Given the very different exercise prescriptions and physiological responses (Figure 4.3), in order to obtain an unbiased analysis of the divergent responses to MICE and SIE, RNA-sequencing was performed on samples obtained at rest, at +180 min, and at +1440 min from the start of exercise.

There were no differences in any transcript at rest between MICE and SIE. The largest differences were found at +180 min, where 205 (90 greater in MICE, 115 greater in SIE) transcripts were differentially expressed between groups (adjusted p-value < 0.05). Pathway-enrichment analysis showed that the largest pathway differentially regulated following SIE, when compared to MICE, was response to unfolded protein (GO:0006986; data not shown). Only 16 genes (1 greater in MICE, 15 greater in SIE) were differentially expressed between groups at +1440 min, with the pathways of protein folding (GO:0006457; data not shown) the most enriched in SIE.

Cluster analyses were then performed on the 205 differentially regulated genes at +180 min (Table S4.1; Figure 4.4.A). This analysis revealed that within the unfolded protein response cluster there was an enrichment for establishment of protein localisation to mitochondrion, suggesting a mitochondrial-specific stress. These results pointed towards the activation of the unfolded protein response pathway as one of the main differences between MICE and SIE. Given that mitochondrial chaperonin 60 (HSPD1) and CCAAT/enhancer binding protein homology protein (CHOP; also known as DNA damage inducible transcript 3, DDIT3) were included in the differentially regulated genes and these two genes were the first described to drive the mitochondrial-specific stress response in mammalian cells (311), it was next explored whether the unfolded protein response had a mitochondrial origin. To test this, a geneset enrichment analysis was performed using the geneset ‘Mitostress’ published by Quiros et al. (178), which included all the commonly upregulated transcripts following treatment of human

cells with four different mitochondrial stressors. In line with the previous results, this geneset was only enriched following SIE (normalised enrichment score [NES] = 1.50; false-discovery rate [FDR] q-value = 0.008; family-wise error rate [FWER] p-value = 0.004; Figure 4.4.B), but not increased following MICE (NES = 1.06; FDR q-value = 0.33; FWER p-value = 0.17). This geneset did not reach statistical significance when the samples at +180 min following SIE were compared to +180 min following MICE (NES = 1.27; FDR q-value = 0.10; FWER p-value = 0.054).

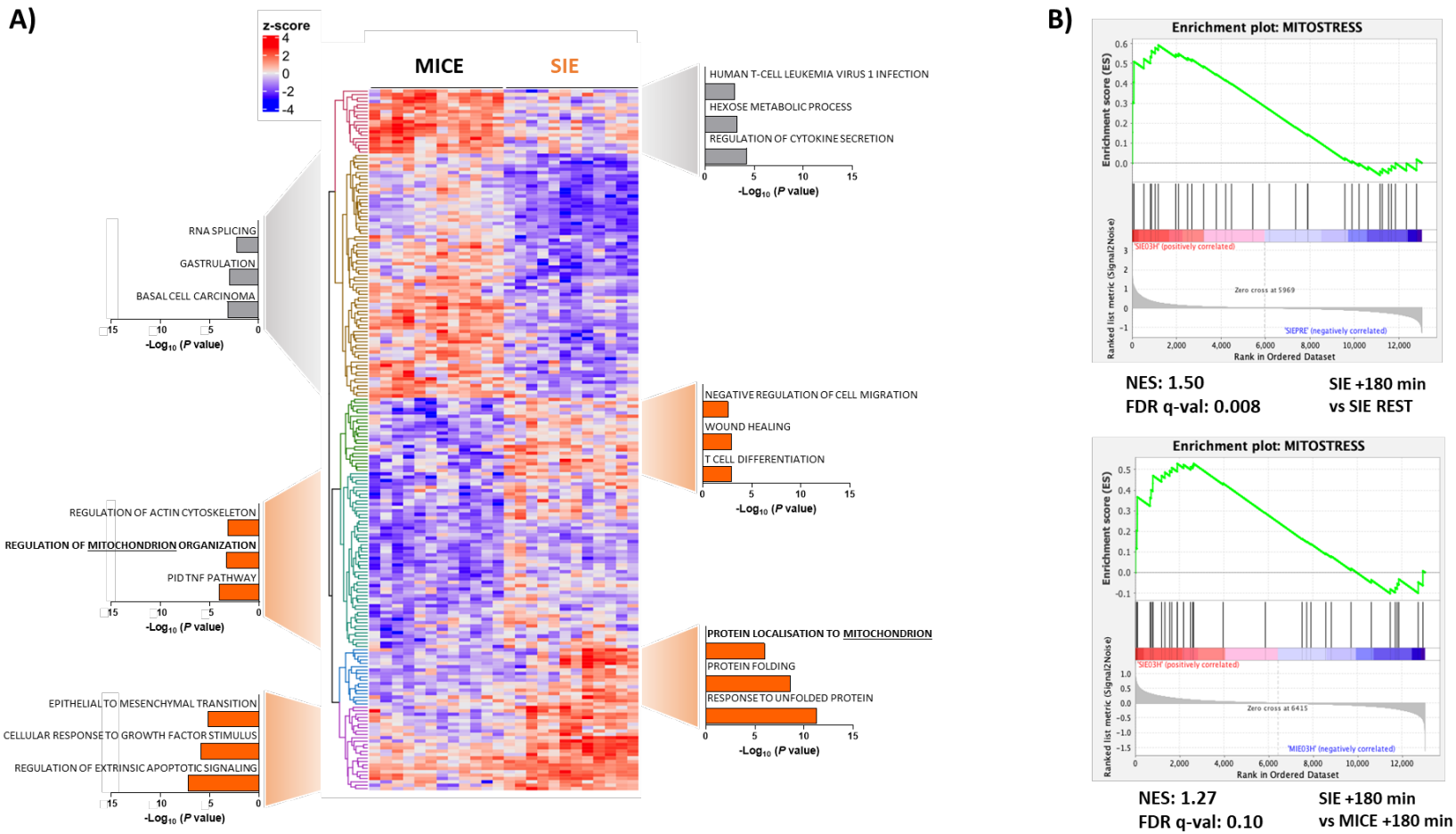

Figure 4.4. A) Hierarchical clustering heatmap of the 205 differentially expressed genes between MICE and SIE at +180 min with their respective top3 enriched pathways within each cluster. In orange those pathways significantly increased following SIE when compared to MICE; in grey those pathways significantly increased following MICE when compared to SIE. B) Gene set enrichment analysis of the 'Mitostress' gene list following SIE, and between MICE and SIE at +180 min. SIE = Sprint-interval exercise; MICE = Moderate-intensity continuous exercise.

4.3.3 Regulation of the integrated stress response (ISR)

Multiple unfolded protein response pathways act through the ISR (312), which is also the case for the mitochondrial unfolded protein response (168, 169). Therefore, transcriptional regulation of key members of ISR were explored via qPCR to validate that this pathway was differentially regulated between MICE and SIE (Figure 4.5).

There was an effect of exercise ($p = 0.01$) for activating transcription factor 4 (ATF4) mRNA content. From the **start of exercise**, there was only an increased mRNA content at +1440 min following both MICE (1.9 ± 1.2 fold; 90 % CI [1.3, 2.4 fold]; ES = 0.84; $p = 0.002$) and SIE (1.7 ± 1.0 fold; 90 % CI [1.2, 2.1 fold]; ES = 0.96; $p = 0.009$). There was an effect of exercise ($p < 0.0001$), and an exercise x group effect ($p = 0.025$), for CHOP mRNA content. From the **start of exercise**, there was only an increased mRNA content at +180 min following SIE (1.5 ± 0.8 fold; 90 % CI [1.2, 1.9 fold]; ES = 0.75; $p = 0.031$), and not MICE. There was a between-group difference at +90 min ($p = 0.0001$) and at +180 min ($p = 0.0003$). From the **end of exercise**, there was a significant increase following SIE at +2.5 h (1.5 ± 0.8 fold; 90 % CI [1.2, 1.9 fold]; ES = 0.75; $p = 0.031$). There was no effect of exercise ($p = 0.603$) or an exercise x group effect (0.070) for activating transcription factor 5 (ATF5) mRNA content. Pre-planned analyses showed that, from the **start of exercise**, there was an increased mRNA content at +90 min (1.5 ± 0.7 fold; 90 % CI [1.2, 1.8 fold]; ES = 0.57; $p = 0.0404$) following SIE. There was an effect of exercise ($p < 0.0001$), and an exercise x group effect ($p = 0.025$), for activating transcription factor 3 (ATF3) mRNA content. From the **start of exercise**, there was an increased mRNA content at +90 min following SIE (33.4 ± 25.6 fold; 90 % CI [22.1, 44.6 fold]; ES = 1.67; $p < 0.0001$); there was an increased mRNA content at +180 min for both MICE (9.3 ± 8.7 fold; 90 % CI [5.3, 12.2 fold]; ES =1.05; $p =0.0053$) and SIE (31.1 ± 25.0 fold; 90 % CI [20.2, 42.1 fold]; ES = 1.61; $p < 0.0001$), without a between-group difference. From the **end of exercise**, there was a significant increase at +0 h following SIE (16.8 ± 30.4

fold; 90 % CI [3.5, 30.2 fold]; ES =0.96; p = 0.026), and at +2.5 h (31.1 ± 25.0 fold; 90 % CI [20.2, 42.1 fold]; ES = 1.61; p < 0.0001). There was also a between group difference at +2.5 h (p = 0.0015). There was an effect of exercise (p < 0.0001), and an exercise x group effect (p = 0.025), for growth arrest and DNA damage-inducible protein GADD34 (GADD34; also known as protein phosphatase 1 regulatory subunit 15A, PPP1R15A) mRNA content. GADD34 is a negative regulator of eukaryotic translation initiation factor 2 alpha (eIF2α), and increased GADD34 is reflective of increased phosphorylation of eIF2α. From the **start of exercise**, there was an increased mRNA content following SIE at +90 min (14.4 ± 13.3 fold; 90 % CI [8.6, 20.2 fold]; ES = 1.12; p < 0.0001); and at +180 min (10.1 ± 13.6 fold; 90 % CI [4.1, 16.0 fold]; ES = 1.05; p = 0.0002). There was a between-group difference at +90 min (p = 0.0003) and at +180 min (p = 0.0005). From the **end of exercise**, there was a significant increase following SIE at +2.5 h (10.1 ± 13.6 fold; 90 % CI [4.1, 16.0 fold]; ES = 1.05; p = 0.0002). There was also a between-group difference at +2.5 h (p = 0.0055). There was an effect of exercise (p < 0.0001), and an exercise x group effect (p = 0.0001), for fibroblast growth factor 21 (FGF21) mRNA content. From the **start of exercise**, there was an increased mRNA content at +90 min following SIE (40.1 ± 51.9 fold; 90 % CI [17.3, 62.9 fold]; ES = 1.18; p < 0.0001); and at +180 min (38.9 ± 49.0 fold; 90 % CI [17.4, 60.5 fold]; ES = 1.06; p < 0.0001) following SIE. There was a between-group difference at +90 min (p = 0.0005), and at + 180 min (p < 0.0001). From the **end of exercise**, there was a significant increase following SIE at +2.5 h (38.9 ± 49.0 fold; 90 % CI [17.4, 60.5 fold]; ES = 1.06; p < 0.0001). There was also a between-group difference at +2.5 h (p < 0.0001).

In mammals, the ISR can be activated through four different kinases. Exercise can lead to endoplasmic reticulum unfolded protein response (UPR^{er}) (174), which activates the ISR through protein kinase R (PKR)-like endoplasmic reticulum kinase (PERK). Genes regulating different UPR^{er} stress response were subsequently analysed. There was an exercise (p < 0.0001)

and an exercise x group ($p = 0.038$) effect for immunoglobulin heavy chain-binding protein (BiP; also called GRP78 and HSPA5). It was upregulated at +1440 min following both MICE (4.2 ± 5.2 fold; 90 % CI [1.8, 6.5 fold]; ES = 1.05; $p = 0.024$) and SIE (2.7 ± 2.0 fold; 90 % CI [1.8, 3.6 fold]; ES =1.11; $p < 0.0001$). There was a between-group difference at +1440 min favouring MICE ($p = 0.002$). There was an exercise ($p < 0.0001$) but no exercise x group ($p = 0.46$) effect for activating transcription factor 6 (ATF6). It was upregulated at +1440 min following both MICE (1.8 ± 0.8 fold; 90 % CI [1.5, 2.2 fold]; ES = 1.04; $p < 0.0001$) and SIE (1.7 ± 0.8 fold; 90 % CI [1.3, 2.0 fold]; ES = 1.02; $p =0.003$). There was an exercise ($p < 0.0001$) but no exercise x group ($p = 0.22$) effect for short X-box binding protein (XBP1) (splicing mediated by inositol-requiring enzyme 1 (IRE1) upon ER stress). It was increased at +1440 min following MICE (4.0 ± 4.2 fold; 90 % CI [2.1, 5.9 fold]; ES = 1.02; $p < 0.0001$) and SIE (3.7 ± 3.5 fold; 90 % CI [2.2, 5.3 fold]; ES = 1.0; $p = 0.002$). These mRNA results suggest that the PERK-ISR signalling pathway was similarly induced following both exercises and did not differ between MICE and SIE (Figure S4.1).

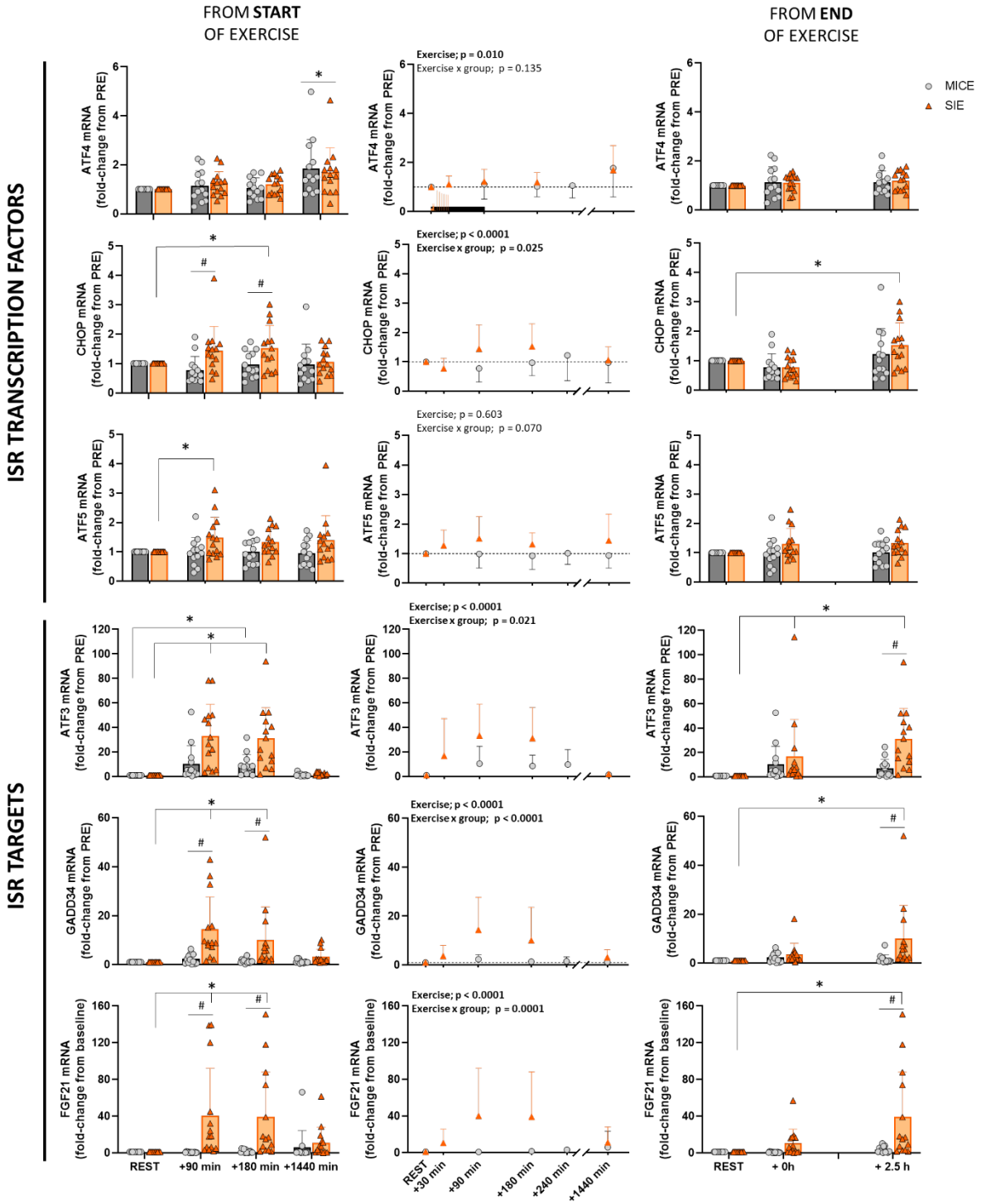

Figure 4.5. Changes in mRNA expression of genes that act as transcription factors, or are targets, of the integrated stress response (ISR). The left column represents biopsy times from the start of exercise, the middle column shows all samples relative to the start of exercise; the right column shows biopsy times from the end of exercise. Orange symbols and bars represent SIE. Grey symbols and bars represent MICE. * = significantly different than REST; # = significantly different between groups. Values are shown as mean ± SD.

4.3.4 Mitochondrial morphological and structural changes following exercise

Given that the transcriptional responses pointed towards a mitochondrial-specific stress, I next explored whether there was any morphological changes to mitochondria following MICE or SIE (Figure 4.6; Table S4.2).

No significant differences were observed in any variable following MICE (all $p > 0.05$; Figure 4.6.A). On the other hand, following SIE there was an increase in mitochondrial roundness (12%; ES = 0.39; $p < 0.0001$; Figure 4.6.A) and circularity (9%; ES = 0.40; $p < 0.0001$); both are signs of stressed mitochondria. There was also an increase in the mitochondrial size following SIE (+48%; ES = 0.35; $p < 0.0001$) suggesting that mitochondria were swollen. Furthermore, there was a decrease in form factor (-10%; ES = - 0.35; $p < 0.0001$) and aspect ratio (-15%; ES = - 0.38; $p < 0.0001$; Figure 4.6.A), which suggested a lower complexity of mitochondria (less branching or interconnected mitochondrial reticulum).

While analysing the morphological profiles, signs of mitochondrial structural disturbance were observed and the frequency of these structural disturbances were quantified. At rest, no mitochondria had any visible disturbance or damage in either group. Following MICE, there were no mitochondria that had any visible structural disturbance. On the other hand, 15.7% of all mitochondria analysed following SIE had signs of moderate or severe structural disturbance (Figure 4.6.C). To further support these results, micrographs from three more SIE participants were obtained (n = 8); all had visibly disturbed mitochondria, confirming these findings. Structural analyses showed that mitochondria classified in the ‘moderate’ disturbance phenotype had a decrease in cristae density (- 63%; $p < 0.0001$), and in cristae junctions (- 68%; $p < 0.0001$), while unchanged crista width. Similarly, ‘severely’ disturbed mitochondria had a greater decrease in cristae density (- 82%; $p < 0.0001$), and cristae junctions (- 87%; $p <$

0.0001), but not in crista width. In addition, only following SIE there were visible signs of mitophagosomes, rupture of outer mitochondrial membrane, and mitoaggregates (Figure 4.6.D), which collectively suggested an increased mitochondrial stress.

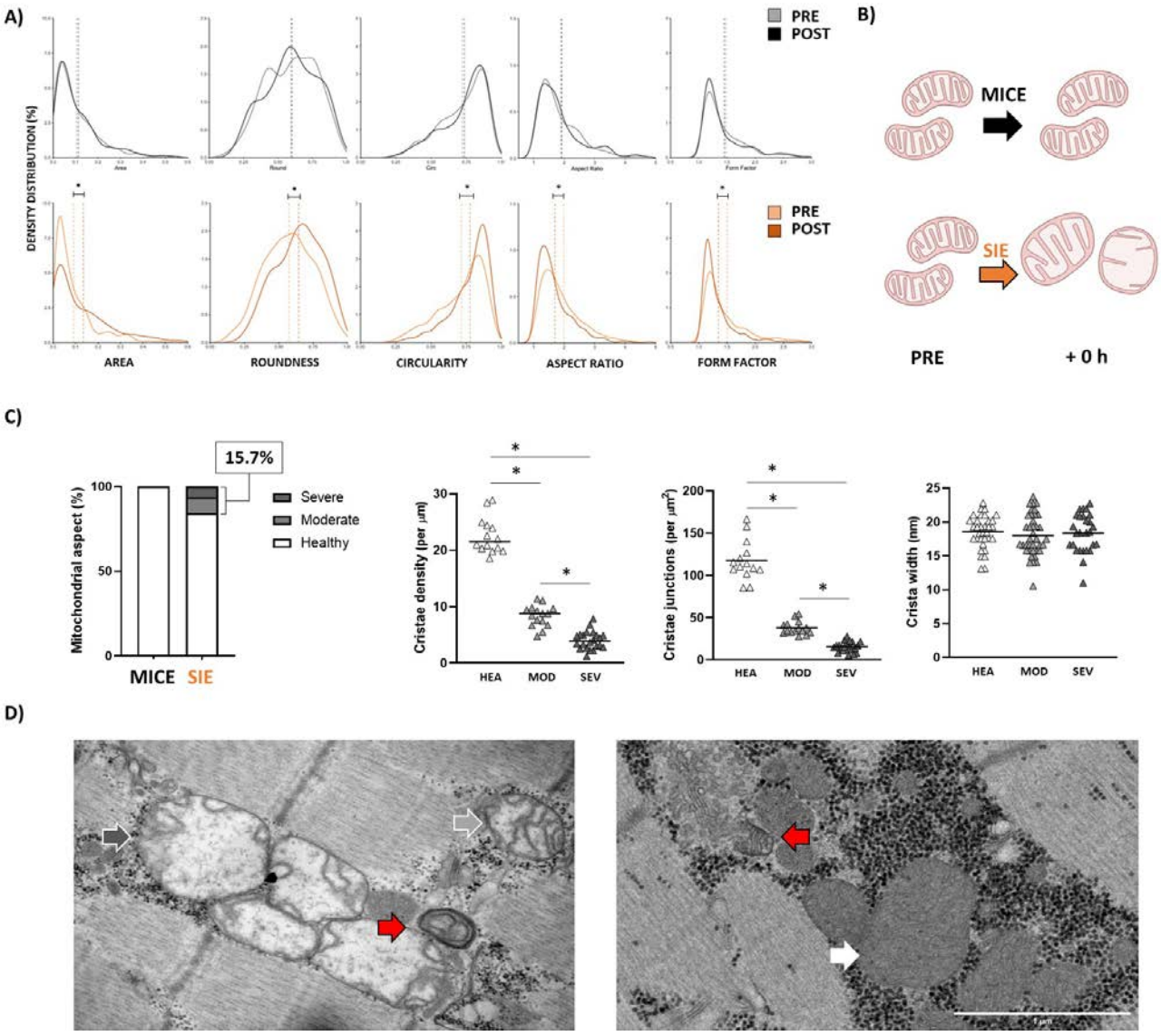

Figure 4.6. A) Frequency distribution of mitochondrial morphology at rest and at + 0 h following MICE (n = 874) and SIE (n = 1154). B) Representation of mitochondrial morphological changes following both exercises. C) Relative levels of moderately and severely disturbed mitochondria following both MICE and SIE, with structural characteristics of each subgroup. D) Micrograph from two different participants following SIE, where the different types of mitochondrial aspects can be observed (dark gray = severely disturbed, light gray = moderately disturbed, white = healthy). Red arrows show mitochondrial degradation processes. HEA = Healthy; MOD = Moderate; SEV = Severe; MICE = Moderate-intensity continuous exercise; SIE = Sprint-interval exercise; * = significant difference between groups ($p < 0.05$).

4.3.5 Exercise-induced changes in markers of mitochondrial dynamics

Having established that SIE elicited a mitochondrial stress response, it was next explored whether this occurred in conjunction to any change in markers of key components of mitochondrial dynamics such as fusion or fission.

There was a group x exercise effect ($p = 0.029$) for Optic Atrophy Protein 1 (OPA1) mRNA content (Figure 4.7). From the **end of exercise**, only following SIE there was a significant decrease at +0 h (0.8 ± 0.4 fold; 90 % CI [0.7, 1.0 fold]; ES = - 0.67; $p = 0.019$) following SIE. There was no effect of exercise ($p = 0.153$). There was an exercise effect ($p = 0.022$) for Mitofusin 1 (MFN1) mRNA content. From the **start of exercise**, only following MICE there was a significant increase at +1440 min (1.5 ± 0.6 fold; 90 % CI [1.3, 1.8 fold]; ES = 0.88; $p = 0.002$). There was no group x exercise effect ($p = 0.076$). There were no exercise or group x exercise effects ($p > 0.05$) for Mitofusin 2 (MFN2) mRNA content (data not shown). There was an effect of exercise ($p = 0.0003$) for Dynamin-Related Protein 1 (DRP1) mRNA content. From the **start of exercise**, there was a significant increase at +1440 min following both MICE (1.4 ± 0.5 fold; 90 % CI [1.2, 1.7 fold]; ES = 0.89; $p = 0.006$) and SIE (1.4 ± 0.5 fold; 90 % CI [1.2, 1.6 fold]; ES =0.91; $p = 0.011$). There was no group x exercise effect ($p = 0.541$). There was an effect of exercise ($p = 0.034$), and a group x exercise effect ($p = 0.0004$), for Mitochondrial Elongation Factor 2 (MIEF2; also called MiD49) mRNA content. From the **start of exercise**, following SIE there was a significant increase at +90 min (1.8 ± 0.7 fold; 90 % CI [1.5, 2.1 fold]; ES =0.99; $p = 0.004$), +180 min (2.1 ± 1.0 fold; 90 % CI [1.7, 2.6 fold]; ES = 1.22; $p < 0.0001$), and +1440 min (1.7 ± 0.8 fold; 90 % CI [1.4, 2.1 fold]; ES = 0.95; $p = 0.009$). There was a between-group difference at +180 min ($p = 0.0025$). From the **end of exercise**, it was increased at +2.5 h only following SIE (2.1 ± 1.0 fold; 90 % CI [1.7, 2.6 fold]; ES = 1.22; $p < 0.0001$). There were no exercise or group x exercise effects ($p > 0.05$) for either Mitochondrial Fission Factor (MFF) or PTEN Induced Kinase 1 (PINK1) mRNA content (data

not shown). There was an effect of group x exercise (p = 0.0304) for E3 Ubiquitin-Protein Ligase Parkin (PARK2) mRNA content. From the **start of exercise**, there was a decrease following MICE at +1440 min (0.8 ± 0.4 fold; 90 % CI [0.6, 1.0 fold]; ES = - 0.78; p = 0.028). There was no exercise effect (p = 0.126). There was an effect of exercise (p = 0.017) for Ubiquitin-Binding protein p62 (p62) mRNA content (Figure 4.7). From the **start of exercise**, there was an increase following both SIE at +180 min (1.9 ± 0.9 fold; 90 % CI [1.5, 2.3 fold]; ES = 1.03; p =0.007) and at +1440 min (1.9 ± 1.0 fold; 90 % CI [1.5, 2.4 fold]; ES = 1.12; p =0.012). There was no group x exercise effect (p = 0.092).

Gene expression results showed a subtle difference between exercise groups, suggesting that SIE upregulated mitochondrial fission and some components of mitophagy. Based on that, I explored the protein levels of three proteins involved in mitochondrial dynamics, some of which had been previously shown to increase following SIE (123). In the present study, neither MICE nor SIE altered the protein levels of MFN2, mitochondrial fission protein 1 (FIS1), and metalloendopeptidase OMA1 (OMA1) (all p > 0.05; Figure 4.8.H).

Lastly, micrographs obtained from a subset of participants were used to quantify the frequency of mitochondrial contacts (as an index of pre-fusion events), and mitochondrial pinching by the endoplasmic reticulum (ER) (an early step of mitochondrial fission) (Figure 4.8). Both the frequency of mitochondrial contacts (+74 ± 66%; 90% CI [38, 110 %]; ES = 0.94; p = 0.012), and the frequency of mitochondrial pinching (+188 ± 310%; 90% CI [18, 359 %]; ES = 0.94; p = 0.038) were increased following exercise without between groups differences (Figure 4.8).

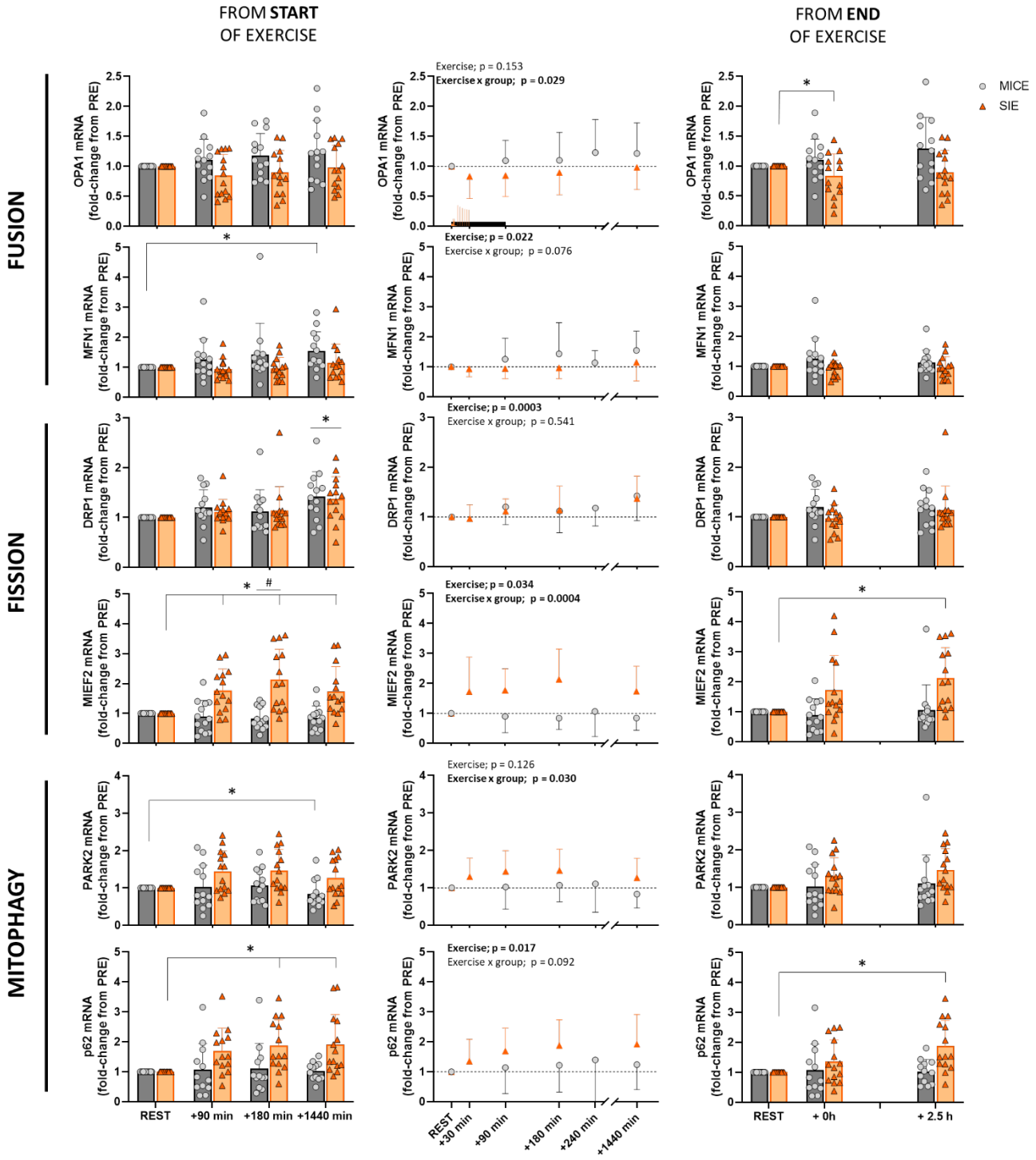

Figure 4.7. Overview of the changes in mRNA expression of selected genes involved in different branches of mitochondrial dynamics (fusion, fission, and mitophagy). The left column represents biopsy times from the start of exercise, the middle column shows all samples relative to the start of exercise; the right column shows biopsy times from the end of exercise. Orange symbols and bars represent SIE. Black symbols and bars represent MICE. * = significantly different than REST; # = significantly different between groups. Values are shown as mean ± SD.

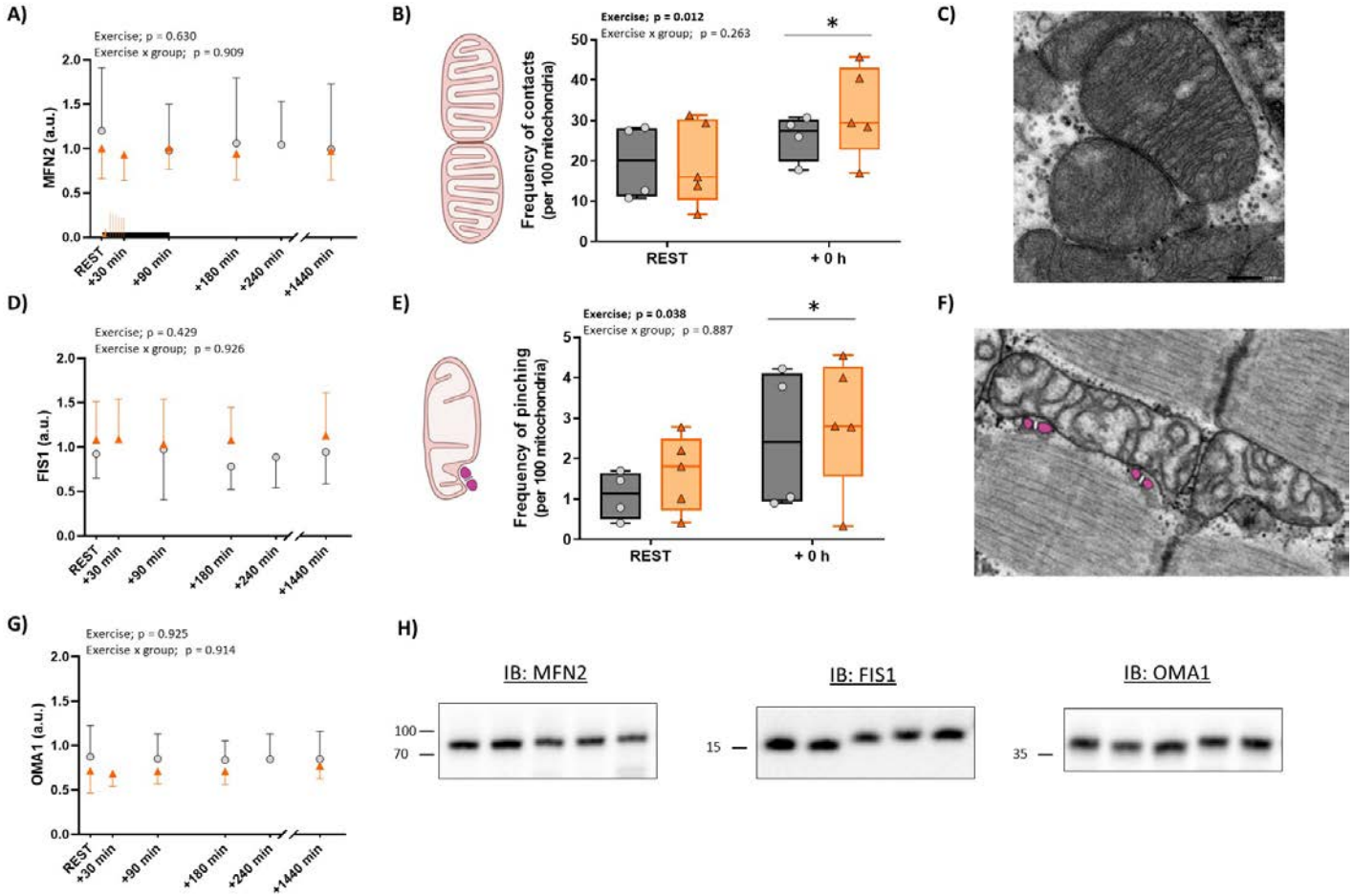

Figure 4.8. A) Protein levels of MFN2; B) Frequency of mitochondrial contacts pre and post MICE and SIE; C) representative micrograph of a mitochondrial contact. D) Protein levels of FIS1; E) frequency of mitochondrial pinching events pre and post MICE and SIE. F) Representative micrographs of a mitochondrial pinching event. Please note that two of the three visible pinching events in F are coloured pink to denote the endoplasmic reticulum G) Protein levels of OMA1; H) representative immunoblots of MFN2, FIS1, and OMA1. IB = immunoblotting; * = significantly different than REST; Values are shown as mean ± SD.

4.3.6 Exercise-induced changes in cellular signalling

Given the divergent responses between MICE and SIE for mitochondrial stress, I next assessed the regulation of key protein kinases classically induced following exercise (95). Unable to measure phosphorylation of AMPK (not detected), phosphorylation of Acetyl-CoA Carboxylase (p-ACC) at serine 79 was utilised as a marker of the energy sensing pathway status (313). There was an exercise ($p < 0.0001$) and a group x exercise effect ($p = 0.030$) for p-ACC (Figure 4.9.A). From the **start of exercise**, both MICE (2.2 ± 1.5 fold; 90 % CI [1.5, 2.9 fold]; ES = 0.91; $p = 0.018$) and SIE (2.1 ± 1.2 fold; 90 % CI [1.5, 2.6 fold]; ES = 0.86; $p = 0.017$) significantly increased the p-ACC levels at +90 min. From the **end of exercise**, at +0 h both MICE (2.2 ± 1.5 fold; 90 % CI [1.5, 2.9 fold]; ES = 0.91; $p = 0.018$) and SIE (3.2 ± 1.9 fold; 90 % CI [2.4, 4.0 fold]; ES = 1.42; $p < 0.0001$) were significantly increased. However, there was a between-group difference at +0 h in p-ACC levels ($p = 0.015$) favouring the SIE group. There was an exercise effect ($p = 0.001$) for phosphorylation of p38 mitogen-activated kinase (p-p38 MAPK) at threonine180 and tyrosine 182 (Figure 4.9.B). From the **start of exercise**, there was a significant increase in the phosphorylation levels of p38 at +90 min only for SIE (6.0 ± 8.5 fold; 90 % CI [2.1, 9.9 fold]; ES = 0.82; $p = 0.041$), and at +180 min only for MICE (4.9 ± 4.8 fold; 90 % CI [2.8, 7.1 fold]; ES = 0.91; $p = 0.015$). From the **end of exercise**, only SIE significantly increased p-p38 at +0 h (7.7 ± 9.0 fold; 90 % CI [3.6, 11.8 fold]; ES = 1.42; $p = 0.007$). There was no group x exercise effect ($p = 0.140$). Next, a global marker of oxidative stress, 4-hydroxynonenal (4HNE), was quantified (Figure 4.9.B). There was no difference between groups and no effect of exercise (all $p > 0.05$). Lastly, the mRNA content of regulator of calcineurin 1 (RCAN1; data not shown) and myogenic regulatory factor 4 (MRF4; Figure 4.9.C) were used as markers of calcineurin activation (314). There was an effect of exercise for RCAN1. From the start of exercise, there was an increase at +180 min for MICE only (1.5 ± 0.5 fold; 90 % CI [1.3, 1.7 fold]; ES = 0.62; $p = 0.032$), while there was an increase at +1440

min for both MICE (2.0 ± 0.8 fold; 90 % CI [1.6, 2.3 fold]; ES = 1.04; $p < 0.0001$) and SIE (2.4± 1.4 fold; 90 % CI [1.8, 3.0 fold]; ES = 1.42; $p = 0.001$). For MRF4, there was an exercise ($p < 0.0001$) and an exercise x group effect ($p = 0.003$), with increased levels at + 180 min (2.7 ± 1.7 fold; 90 % CI [2.0, 3.5 fold]; ES = 0.92; $p = 0.001$), + 2.5 h (2.7 ± 1.5 fold; 90 % CI [2.0, 3.4 fold]; ES = 1.07; $p = 0.001$) and +1440 min (2.6 ± 1.6 fold; 90 % CI [1.9, 3.3 fold]; ES = 0.96; $p < 0.0001$) following MICE only. The differences were significant between groups at all three timepoints (all $p < 0.05$).

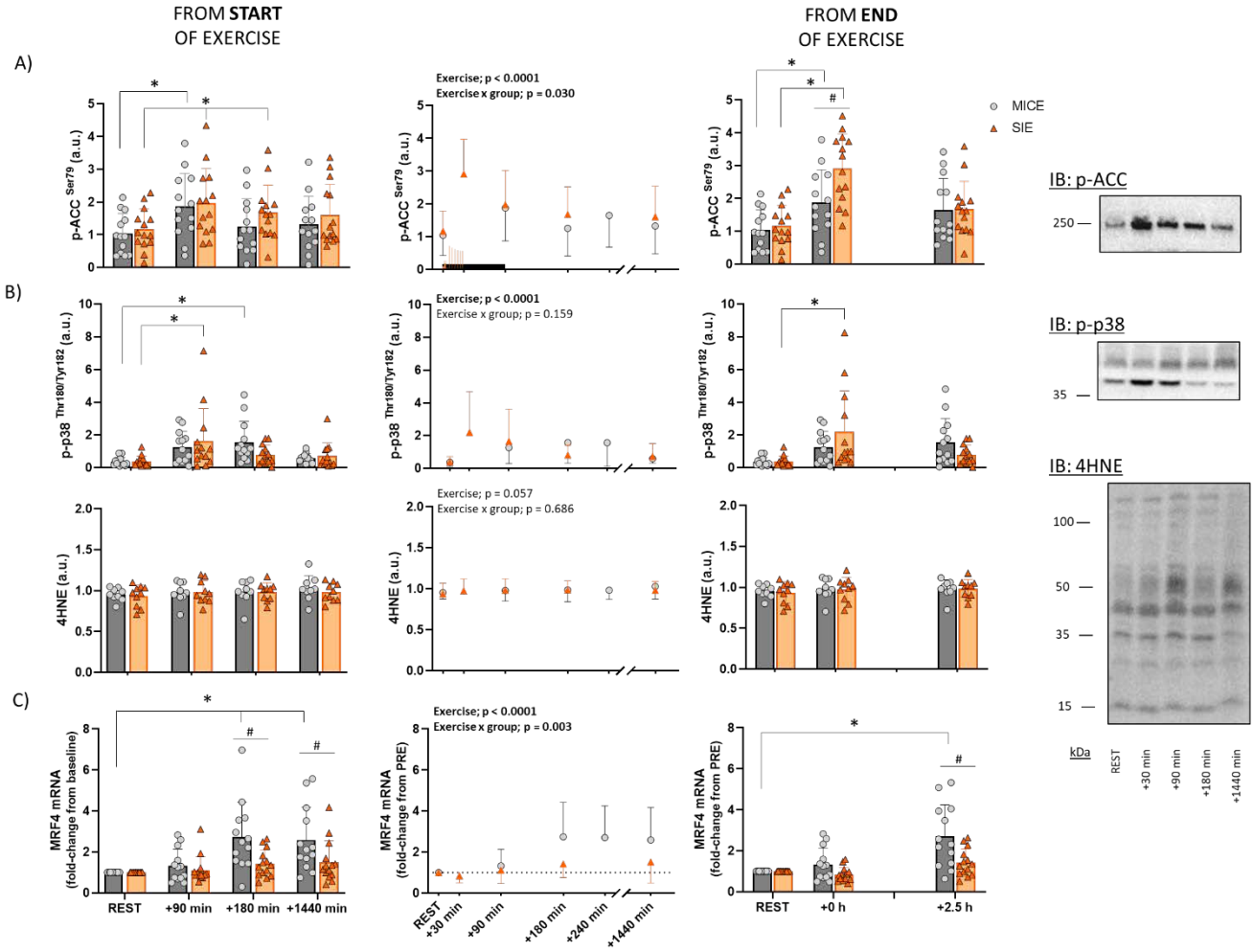

Figure 4.9. A) Changes in the phosphorylation levels of Acetyl-CoA carboxylase (p-ACC); B) changes in p38 mitogen-activated protein kinase (p38) and the oxidative stress marker 4-hydroxynonenal (4HNE); C) mRNA levels of MRF4. The left column represents biopsy times from the start of exercise, the middle column shows all samples relative to the start of exercise; the right column shows biopsy times from the end of exercise. Representative western blots are found on the right side of the figure. Orange symbols and bars represent SIE. Grey symbols and bars represent MICE. IB = immunoblotting; * = significantly different from REST. # = significantly different between groups. Values shown are mean ± SD.

4.3.7 Exercise-induced changes in autophagy signalling.

Following the the between groups differences in the energy sensing pathway (an important regulator of exercise-induced mitophagy (144)) and the mitochondrial stress phenotype observed following SIE, I next investigated if there was a differential regulation of Unc-51 like autophagy activating kinase 1 (ULK1) (Figure 4.10).

Phosphorylation of ULK1 at serine 757 (p-ULK1 s757) is under control of mammalian target of rapamycin 1 (mTORC1), and its phosphorylation inhibits ULK1 interaction with AMPK. There was an exercise ($p = 0.0016$), and group x exercise effect ($p = 0.0014$). From the **end of exercise**, there was a significant decrease following SIE at +0 h (0.6 ± 0.2 fold; 90 % CI [0.5, 0.7 fold]; ES = - 0.86; $p = 0.02$). There were no other differences in any group (all $p > 0.05$). Phosphorylation of ULK1 at serine 556 (p-ULK1 s556; homolog of s555 in mice) is controlled by AMPK, and is needed for exercise-induced increased mitophagy (144). There was no exercise effect ($p = 0.2153$), but there was a significant group x exercise interaction ($p = 0.0012$). From the **start of exercise**, there was a significant increase following SIE at +90 min (1.7 ± 0.7 fold; 90 % CI [1.4, 2.1 fold]; ES = 0.79; $p = 0.001$). There were no significant changes following MICE. ULK1 phosphorylation at serine 556 following SIE would suggest an increased autophagy, and possibly mitophagy. A marker for autophagosome content, microtubule-associated proteins 1A/1B light chain 3B (LC3B) protein and ratio were used. For LC3B-II protein and LC3B-II/I ratio there was an effect of exercise ($p < 0.0001$). No within-group changes were found from the **start of exercise**. From **the end of exercise**, there was a significant increase at +2.5 h in both MICE (3.3 ± 4.4 fold; 90 % CI [1.3, 5.3 fold]; ES = 0.87; $p < 0.0001$) and SIE (2.5 ± 2.1 fold; 90 % CI [1.6, 3.4 fold]; ES = 1.06; $p = 0.003$). Similar changes were found for LC3B-II protein levels (data not shown). No significant differences were found for p62 and LC3B-I protein levels at any time point (data not shown; all $p > 0.05$).

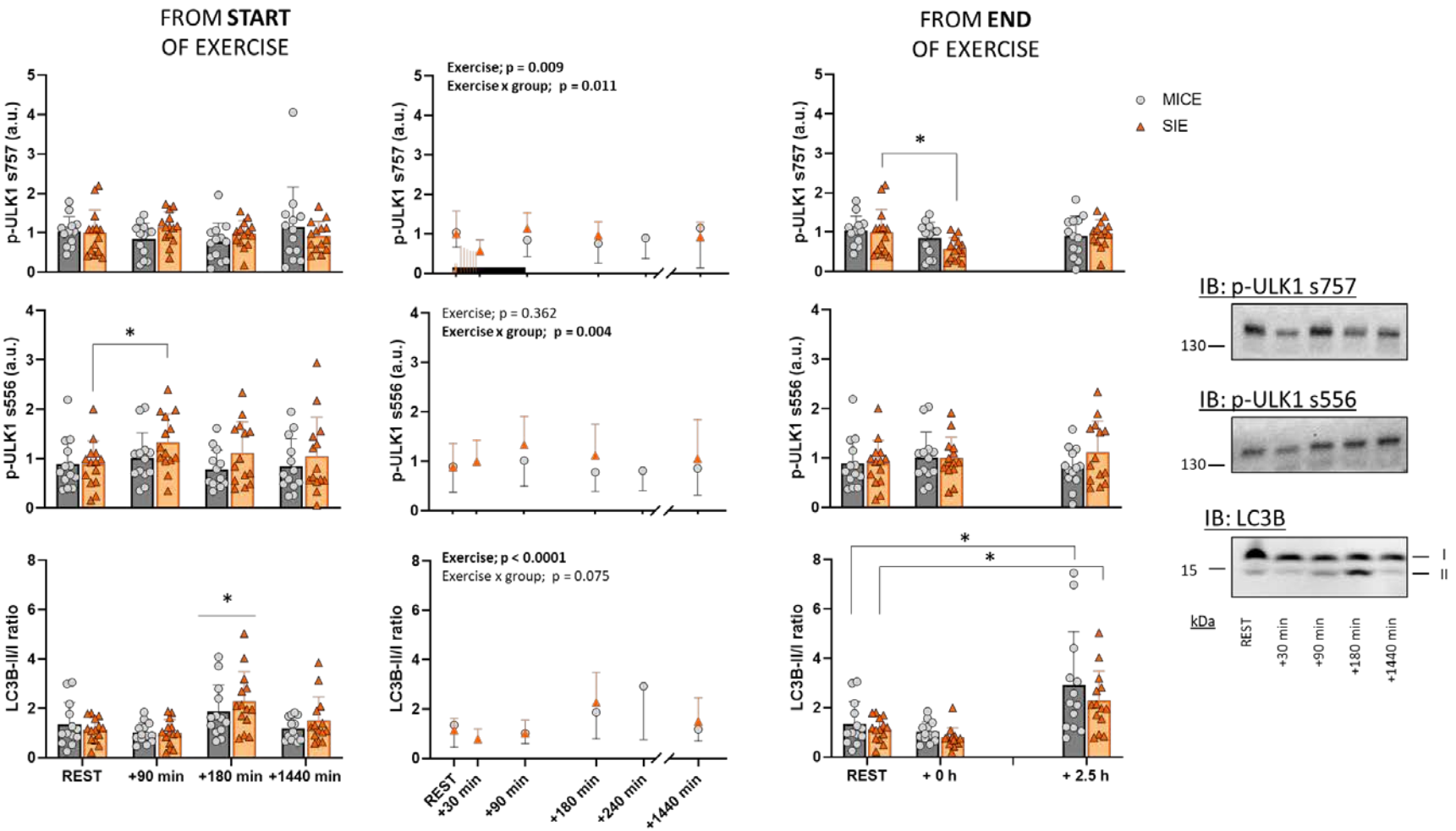

Figure 4.10. Time-course of changes in phosphorylation of ULK1 at s556, and s757, and the LC3B-II/I ratio. The left column represents biopsy times from the start of exercise, the middle column shows all samples relative to the start of exercise; the right column shows biopsy times from the end of exercise. Orange symbols and bars represent SIE. Black symbols and bars represent MICE.* = significantly different from REST. Values shown are mean ± SD.

4.3.8 Exercise-induced response of metabolic and mitochondrial biogenesis genes

Finally, having established a differential regulation of mitochondrial stress between MICE and SIE, and given the importance of mitochondrial biogenesis in the adaptation to exercise, I next assessed whether genes associated with mitochondrial biogenesis and metabolism were differentially regulated following either of the exercises (Figure 4.11). There was an effect of exercise ($p < 0.0001$), and a group x exercise effect ($p = 0.0335$), for peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) mRNA content. From the **start of exercise**, it was increased at +90 min following both MICE (2.4 ± 1.0 fold; 90 % CI [1.9, 2.8 fold]; ES = 1.41; $p = 0.043$) and SIE (4.0 ± 1.3 fold; 90 % CI [3.4, 4.6 fold]; ES =1.59; $p < 0.0001$). There was a between-group difference at +90 min favouring SIE ($p < 0.0001$). Similarly, at +180 min following both MICE (6.3 ± 4.3 fold; 90 % CI [4.3, 8.3 fold]; ES =1.36; $p < 0.0001$) and SIE (4.8 ± 2.2 fold; 90 % CI [3.9, 5.8 fold]; ES =1.67; $p <0.0001$) there was a significant increase. From the **end of exercise**, only following MICE there was a significant increase at +0 h (2.4 ± 1.0 fold; 90 % CI [1.9, 2.8 fold]; ES = 1.41; $p = 0.043$). There was a between-group difference at +0 h favouring MICE ($p = 0.038$). At +2.5 h following both MICE (4.7 ± 2.1 fold; 90 % CI [3.7, 5.6 fold]; ES =1.60; $p <0.0001$) and SIE (4.8 ± 2.2 fold; 90 % CI [3.9, 5.8 fold]; ES =1.67; $p <0.0001$) there was a significant increase. For PGC-1α isoform 4 (PGC1-α4) mRNA content, there was an effect of exercise ($p < 0.0001$) and a group x exercise effect ($p < 0.0001$). From the **start of exercise**, it was increased at +90 min following for MICE (1.6 ± 0.6 fold; 90 % CI [1.3, 1.9 fold]; ES = 0.80; $p = 0.002$) but not SIE ($p > 0.05$). At +180 min following both MICE (11.9 ± 9.5 fold; 90 % CI [7.6, 16.3 fold]; ES =1.24; $p < 0.0001$) and SIE (9.1 ± 4.5 fold; 90 % CI [7.2, 11.1 fold]; ES =1.69; $p < 0.0001$) there was a significant increase. From the **end of exercise**, only following MICE there was a significant increase at +0 h (1.6 ± 0.6 fold; 90 % CI [1.3, 1.9 fold]; ES = 0.80; $p = 0.002$). At +2.5 h following both

MICE (9.4 ± 5.8 fold; 90 % CI [6.8, 12.1 fold]; ES =1.54; p < 0.0001) and SIE (9.1 ± 4.5 fold; 90 % CI [7.2, 11.1 fold]; ES =1.69; p < 0.0001) there was a significant increase. For pyruvate dehydrogenase kinase 4 (PDK4) mRNA content, there was an effect of exercise (p < 0.0001), and a group x exercise effect (p < 0.0001). From the **start of exercise**, mRNA content was increased at +90 min following MICE (9.8 ± 12.6 fold; 90 % CI [4.7, 15.6 fold]; ES = 1.02; p = 0.049), but not following SIE. At +180 min following both MICE (49.3 ± 39.0 fold; 90 % CI [31.5, 67.1 fold]; ES = 1.47; p < 0.0001) and SIE (15.9 ± 13.1 fold; 90 % CI [10.1, 21.7 fold]; ES = 1.42; p = 0.0005) there was a significant increase. At +1440 min, only following MICE there was an increase (32.4 ± 74.2 fold; 90 % CI [- 1.4, 66.3 fold]; ES =1.09; p = 0.002). There was a between-group difference at +180 min favouring MICE (p = 0.0071). From the **end of exercise**, there was a significant increase at +0 h only following MICE (9.8 ± 12.6 fold; 90 % CI [4.7, 15.6 fold]; ES = 1.02; p = 0.049) and not SIE. At +2.5 h following both MICE (58.0 ± 46.6 fold; 90 % CI [36.7, 79.3 fold]; ES = 1.63; p < 0.0001) and SIE (15.9 ± 13.1 fold; 90 % CI [10.1, 21.7 fold]; ES = 1.42; p = 0.0005) there was a significant increase. There was a between-group difference at +2.5 h favouring MICE (p < 0.0001). For peroxisome proliferator-activated receptor alpha (PPARα) mRNA content, there was an effect of exercise (p < 0.0001), and a group x exercise effect (p < 0.0001). From the **start of exercise**, mRNA content was increased at +180 min following both MICE (5.0 ± 3.6 fold; 90 % CI [3.3, 6.6 fold]; ES = 1.18; p < 0.0001) and SIE (5.1 ± 2.1 fold; 90 % CI [4.2, 6.0 fold]; ES = 1.60; p < 0.0001). From the **end of exercise**, there was a significant increase at +2.5 h following both MICE (6.4 ± 3.0 fold; 90 % CI [5.0, 7.8 fold]; ES = 1.58; p < 0.0001) and SIE (5.1 ± 2.1 fold; 90 % CI [4.2, 6.0 fold]; ES = 1.60; p < 0.0001). There were no differences between groups (all p > 0.05). For peroxisome proliferator-activated receptor delta (PPARδ) mRNA content, there was an effect of exercise (p < 0.0001) and a group x exercise effect (p = 0.0217). From the **start of exercise**, mRNA content was increased at +180 min (2.6 ± 1.2 fold; 90 % CI [2.1, 3.1 fold]; ES = 1.36;

$p < 0.0001$) and at +1440 min (2.3 ± 1.6 fold; 90 % CI [1.6, 3.0 fold]; ES = 1.29; $p = 0.0064$) only following SIE. There was a between-group difference at +180 min favouring SIE ($p = 0.0003$). From the **end of exercise**, there was a significant increase at +2.5 h only following SIE (2.6 ± 1.2 fold; 90 % CI [2.1, 3.1 fold]; ES = 1.36; $p < 0.0001$). There were differences between groups at +2.5 h ($p < 0.0001$) favouring SIE. There were no difference at any time point in PPARγ mRNA content (all $p > 0.05$).

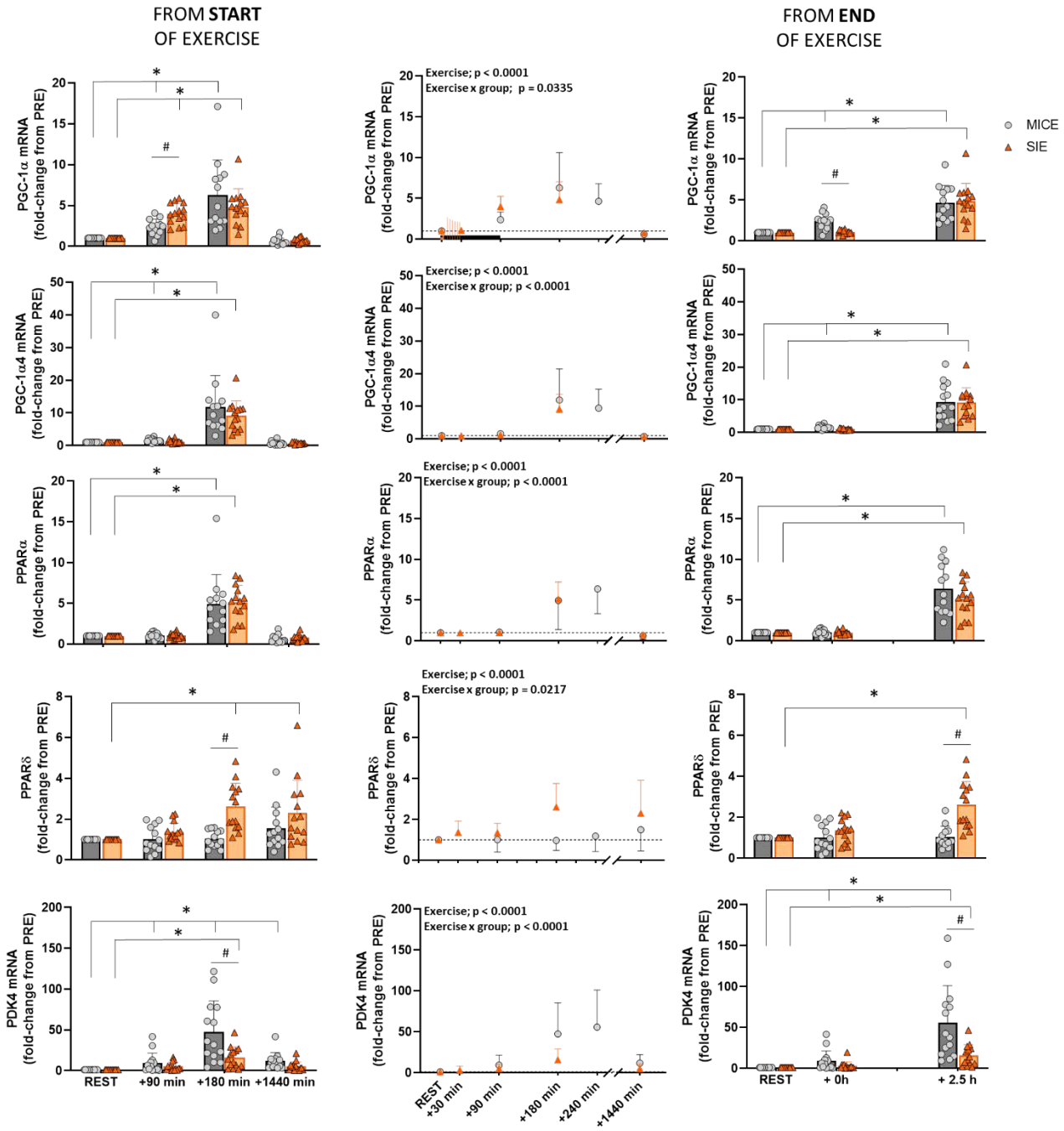

Figure 4.11. PGC-1α, PGC-1α4, PPARα, PPARδ, and PDK4 transcriptional time course. The left column represents biopsy times from the start of exercise, the middle column shows all samples relative to the start of exercise, and the right column shows biopsy times from the end of exercise. Orange symbols and bars represent SIE. Black symbols and bars represent MICE. * = significantly different than REST; # = significantly different between groups. Values shown are mean ± SD.

4.4 Discussion

The main findings of the current study show that large physiological differences in the exercise stimulus between MICE and SIE led to a divergent transcriptional response. Of note, SIE resulted in an upregulation of the unfolded protein response (UPR) with a large mitochondrial component as observed by the enriched geneset 'mitostress'. For the first time, it is shown that SIE causes a robust change in the mitochondrial morphology (towards less complex and more rounded mitochondria), together with an increased structural disturbance (altered cristae characteristics) of the mitochondrial pool. In line with this, genes involved in the integrated stress response (ISR) were strongly upregulated following SIE, but not MICE. In contrast, no differences between MICE and SIE were found in classic markers of mitochondrial biogenesis such as PGC-1α.

The present study utilised two very different exercise prescriptions from the endurance exercise spectrum. By design, ~5 times more work was completed during MICE when compared to SIE, while exercise intensity was ~5 times greater during SIE than MICE. This lead to marked physiological differences; there were greater exercise-induced changes in blood lactate, blood pH, and perceived exertion, following SIE when compared to MICE, which is consistent with previous research (90, 315). These divergent exercise characteristics were associated with distinct transcriptional responses. Analysis of the differential gene expression between MICE and SIE showed the 'unfolded protein response (UPR)' as the primary represented term. Cluster analysis of the differentially expressed genes identified 'establishment of protein localisation to mitochondria' as an enriched term, which was confirmed by showing that both CHOP and HSPD1, the first described members of the mitochondrial UPR (UPR^{mt}) (311), were present among the differentially regulated genes. The transcriptomic results were subsequently interrogated against the geneset 'Mitostress', a mitochondrial-specific stress transcriptional

signature obtained in human cells treated with four different known mitochondrial stressors (178), which was only increased following SIE. This is the first time a mitochondrial-stress transcriptional response has been reported to be induced by a physiological stimulus in human skeletal muscle.

In mammals the UPR^{mt} signals through the ISR (312). Therefore, the gene expression level of GADD34, the negative regulator of the phosphorylation status of the ISR sensor eIF2α (170), was explored and was revealed to be strongly increased following SIE. Similarly, two target genes of the ISR (FGF21 and ATF3) had a large increase +2.5 h following SIE but not MICE. The ISR can be differently activated following different stressors. Genes involved in the endoplasmic reticulum UPR (UPR^{ER}), such as BiP, XBP1s, ATF6, some of which have been shown to increase following exercise (195), showed similar increases following both types of exercise in the present study suggesting that UPR^{ER} may not explain the greater signalling observed following SIE. While multiple mitochondrial stressors can trigger the ISR, the specific mitochondrial stress occurring following SIE remains to be elucidated. It has previously been shown that mitochondrial respiratory function is not affected by either SIE or MICE (316); however, other mitochondrial stressors like reduced import capacity, increased ROS production, or decreased mitochondrial translation, are still possible candidates. Studies in lower organisms have shown that import efficiency regulates the UPR^{mt} (317), and that 30 min of mitochondrial import stress leads to a strong upregulation of cytosolic chaperones and heat-shock proteins (318), which are in line with the present differential expression analyses.

A common sign of mitochondrial stress is the morphological and structural alteration of the mitochondrial pool towards a less interconnected mitochondria with disorganised cristae (222). The present findings show that mitochondrial morphological variables were not altered following MICE, which is in line with previous research in mice (112). However, for the first time, this study shows that SIE causes an increase in mitochondrial roundness, circularity, and

average size, while decreasing the aspect ratio and form factor (less complex mitochondrial pool), suggestive of a mitochondrial stress phenotype. Strikingly, ~16% of mitochondria following SIE showed signs of moderate and severe structural damage. These signs were most clear by a decreased cristae density (-63% to -82%) and number of cristae junctions per mitochondrion (- 68% to -87%), which were not observed following MICE. Animal studies have shown that exercise at high-intensity, but not moderate-intensity, could lead to ultrastructural defects in post-exercise skeletal muscle samples (161, 319, 320). However, this was not found in human skeletal muscle (161). Similar structural changes in skeletal muscle had been reported in the literature upon genetic disruption of mitochondrial dynamics (185, 188), or in mitochondrial myopathies (298, 321), and were also associated with the activation of the integrated stress response (ISR) (312).

Mitochondrial dynamics are important events orchestrating the mitochondrial remodelling in response to stress. In the present study, gene expression of proteins involved in mitochondrial fusion showed minor between-group differences (Figure 4.6). It was shown for the first time that the number of mitochondrial contacts, as a sign of pre-fusion events, was increased following exercise without group differences, in line with previous rodent findings (293). Regarding mitochondrial fission, the gene coding for protein mitochondrial dynamics 49 (encoded by MIEF2) had a large increase only following SIE throughout the recovery period. This is the first time exercise has been shown to increase the gene expression of MIEF2 and may suggest that following mitochondrial stress there is an increased need of fission receptors to ultimately tag defective mitochondria and increase fission. In line with this, ER-mitochondrion pinching events were also shown to increase following exercise but without between-group difference, suggestive of increased overall fission. On the other hand, genes involved in mitophagy, like p62 mRNA, increased at 2.5 and 24 hours only following SIE, while PARK2 mRNA content was decreased 24 hours following MICE (without between-

group differences). Previous research has shown exercise-induced p62 mRNA expression to be intensity-dependant (265). Whether this suggests differential regulation of mitophagy following MICE and SIE remains to be fully elucidated. The results of the present study show that ULK1 phosphorylation at serine 757 (an inhibitory site controlled by mTOR) was decreased ~40%, and phosphorylation at serine 556 (controlled by AMPK) was increased ~70% only following SIE. Rodent studies have shown that p-ULK1 s555 is needed for *in vivo* exercise-induced mitophagy (144), and for *in vitro* C2C12 mitophagy (158). However, both groups showed a similar increase in autophagosome content (measured as LC3B-II/I ratio) and unchanged p62 protein levels, suggesting similar exercise-induced increases in autophagosome content. These results collectively suggested a transcriptional upregulation of the mitochondrial fission receptor MIEF2 following SIE, which remained unchanged following MICE, and an increase in the signalling driving mitophagy following SIE.

Analysis of skeletal muscle showed that p38 MAPK, a major ROS-sensitive stress kinase, increased following exercise but did not differ between exercise groups. In agreement with this, there were no differences in exercise-induced changes in 4-Hydroxynonenal (4HNE) protein levels, a marker of global oxidative stress (Figure 4.4). This would suggest that the differences observed between exercises may not be due to differences in ROS levels. However, the energy-sensing pathway (assessed in the present study via p-ACC) was differentially regulated in favour of SIE. This is in line with previous research suggesting that the energy-sensing pathway is dependent on exercise intensity (95) and may be due to increased type II fibre recruitment (322), which occurs during SIE. In contrast, genes regulated by calcineurin (RCAN1, MRF4; (314)) were either unchanged or only upregulated following MICE, which was in line with the transcriptomic analysis showing increased expression of RCAN3, an inhibitor of calcineurin activity (323), following SIE.

An important signalling event following exercise is the increased gene expression of markers of mitochondrial biogenesis (324), with PGC-1α known as the 'master' regulator. In the present study, the exercise-induced levels of PGC-1α mRNA content were similar between groups. Interestingly, due to the multiple samples obtained at different time-points, the biopsy timing was an important factor affecting the interpretation of the results. If comparing the mRNA content of PGC-1α at the end of both exercises (+0 h), there was a between-group difference favouring MICE; if compared from the start of exercise (+90 min), PGC-1α mRNA was greater following SIE. However, if compared later during the recovery (+180 min from the start of exercise, or +2.5 h from the end of exercise) there were no differences between groups. From these results, it can be concluded that timing of biopsies, when differences in exercise duration exist, is a factor that should be taken into account when interpreting and discussing the results of PGC-1α gene expression. Other genes linked to mitochondrial biogenesis and mitochondrial metabolism, such as PGC-1α4, PPARα and PPARγ, showed no between-group differences. On the other hand, PDK4, a gene associated with the switch from glucose to fatty acids as energy source (325), was increased in both groups, but to a larger extent following MICE. Moreover, PPARδ, a gene also associated with mitochondrial fatty acid oxidation (326), and involved in attenuating ER and oxidative stress (327), was only increased following SIE.

In conclusion, the findings of the present study show that the large physiological differences between MICE and SIE were associated with a divergent transcriptional response with SIE inducing an UPR response and ISR activation. For the first time it was shown that this differential transcriptomic response was preceded by a mitochondrial structural and morphological disturbance, which would suggest that a divergent stimuli between MICE and SIE is mitochondrial stress. Furthermore, while exercise was shown to increase mitochondrial contacts and pinching events (both signs of fusion and fission), it was shown that SIE led to a greater upregulation of fission receptor gene expression (MIEF2) and mitophagy signalling (p-

ULK1 s556). On the other hand, no major differences were found in gene expression of markers of mitochondrial biogenesis suggesting a common regulation following exercise. Whether this differential response to exercise, when repeated over time, leads to divergent phenotypical adaptations remains to be elucidated and will be addressed in the next chapter.

4.5. Supplemental information

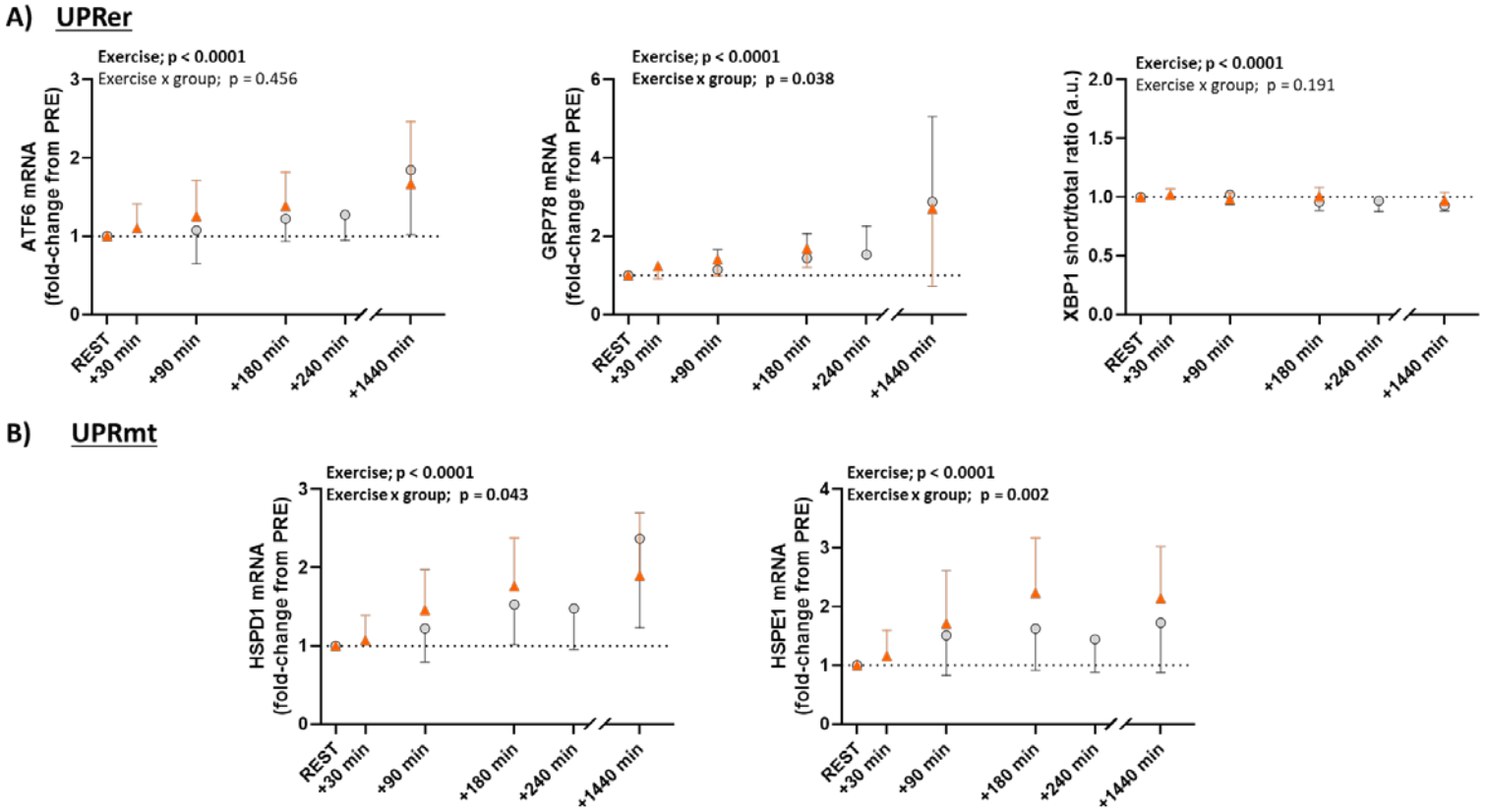

Figure S4.1. A) Gene expression of markers of the endoplasmic reticulum unfolded protein response (UPR^{ER}). B) Gene expression of markers of the mitochondrial unfolded protein response (UPR^{mt}). Statistical analyses were performed with raw values. Data shown as fold-change from REST. Data shown as mean ± SD.

Table S4.1. List of differentially expressed genes between MICE and SIE at +180 min. MICE = moderate-intensity continuous exercise; SIE = sprint-interval exercise. Statistical significance was set at adjusted p-value < 0.05.

MICE	SIE
TBC1D20, GOLGA7, DVL2, TAB1, SETD2, SLC4A2, DPH5, KHDC4, CREM, ULK3, TUBGCP3, CHRAC1, ACIN1, MPLKIP, IP6K1, ZNF263, SURF6, NFKBIL1, SFXN5, RNF44, JADE1, KRCC1, EXT1, RFX7, HSPBAP1, ZFP3, FAM200A, IRF2, LOC105371763, ARSK, TSEN54, ZBTB38, MIDEAS, TBCK, PCF11, UMPS, CENPX, ZNF550, RSKR, KLHL11, PARP16, PPP1R3E, CYREN, ZNF514, ZNF862, FRAT2, FZD5, CREB5, AFAP1L1, KDR, FAM189A2, LOC389765, ZNF844, CREBRF, LINC00205, PER1, KLHL17, SF3A3, ARRDC2, MED26, LOC107985544, PLEKHG1, LRRC32, LOC202181, HOXA11, MRPL23-AS1, APOL6, CNN2, LCP2, WBP1L, SLC25A22, SLC7A8, IQCC, PIK3IP1, ZFP2, SEMA7A, DEPP1, YJEFN3, FLJ46875, ADAMTS9, TMEM140, FLNB, AKAP12, GNA14, GADD45G, PTCH2, ADORA2A, CEBPD, ZIC4, PFKFB3, ATCAY	FBXL14, FLNC-AS1, HSPA1B, HSPA7, HSPA1A, NR4A3, CBARP, NR4A1, TNFRSF12A, DUSP2, GEM, ZNF169, NEXN-AS1, ZFAND2A, PPP1R3C, BAIAP2, LFNG, NAB2, REM1, DNAJA4, PLLP, CTH, FAM102A, FAM117B, AGAP11, DUSP10, ZNF774, FAM124A, SPSB2, ITPKB, ZNF280C, DNAJB1, SLC16A12, SOX9, ITGB4, CCDC88C, HEYL, CMKLR1, HSPA4L, PRICKLE2, PDE9A,RCAN3, UNC5B, NKRF, PPFIA4, PIK3C2B, BCL9, VASN, HSP90AA1, LSR, ARMCX4, ZBTB43, BAG3, HID1, LOC284454, FGD5, NOP14-AS1, TET3, AUTS2, MAP10, NYNRIN, TNFSF10, TMEM30B, DNAJB4, PALM, CD34, SREBF1, BCAS2, RCL1, EXOG, RAPGEF3, DDIT3, MCF2L, STX3, USHBP1, ZNF10, PEAR1, PLXND1, PRKG1, SNRK, PDGFRB, MAP4K2, DEDD2, ITM2C, ZEB2, HSPD1, TRMT10A, CLEC14A, MGMT, TBX3, ACHE, MAP3K1, MYL12A, ENPP1, ELK3, MAP2K3, FKBP4, EBF3, VAT1, FGFR1, AP4B1, CIRBP, PLEKHF2, AKIRIN2, MFSD14B, TNRC18, TMEM184C, HSPA4, UBR7, USPL1, SLC8B1, RSBN1L, GCLC, WASF2, LAMP1, FBXO42

Table S4.2. Mitochondrial morphological changes following moderate-intensity continuous exercise (MICE) and sprint-interval exercise (SIE). Data are presented as mean ± SD. N = number of manually traced individual mitochondria. MICE = moderate-intensity continuous exercise; SIE = sprint-interval exercise.

	MICE REST (n = 428)	MICE +0 h (n = 446)	Effect Size	SIE REST (n = 603)	SIE +0 h (n = 551)	Effect Size
Area (μm^2)	0.104 ± 0.099	0.112 ± 0.109	0.08	0.091 ± 0.092	0.135 ± 0.150*	0.35
Roundness	0.594 ± 0.185	0.600 ± 0.189	0.03	0.574 ± 0.180	0.644 ± 0.172*	0.39
Circularity	0.721 ± 0.158	0.735 ± 0.158	0.09	0.715 ± 0.161	0.776 ± 0.136*	0.40
Feret's diameter (μm)	0.480 ± 0.275	0.496 ± 0.285	0.06	0.456 ± 0.279	0.492 ± 0.308	0.13
Perimeter (μm)	1.282 ± 0.729	1.317 ± 0.738	0.05	1.208 ± 0.744	1.337 ± 0.872	0.16
Form factor	1.482 ± 0.467	1.453 ± 0.457	-0.06	1.502 ± 0.494	1.347 ± 0.362*	-0.35
Aspect ratio	1.923 ± 0.903	1.897 ± 0.805	-0.03	1.989 ± 0.903	1.695 ± 0.581*	-0.38

CHAPTER 5 – MITOCHONDRIAL REMODELLING FOLLOWING EIGHT WEEKS OF MODERATE-CONTINUOUS TRAINING (MICT) AND SPRINT-INTERVAL TRAINING (SIT)

The aim of this chapter was explore the mitochondrial remodelling following eight weeks of either moderate-intensity continuous training (MICT) or sprint-interval training (SIT). In the previous chapter, exercise-induced changes in the mitochondrial stress response were shown to differ between these two divergent exercise prescriptions. These acute differences are usually transient and may not directly reflect subsequent chronic adaptations to exercise training (i.e., repeated exercise sessions). The aim of this chapter was to elucidate how skeletal muscle mitochondria remodel following eight weeks of MICT or SIT. Part of this work has been presented at the Cell Symposia – Exercise Metabolism conference in Spain, 2019.

5.1 Introduction

Mitochondria play a pivotal role in health and endurance performance (53, 328). Mitochondria are plastic organelles known to respond to changes in energy demands such as during exercise training (43, 219) and following periods of inactivity (329, 330). Despite this, little is known about how mitochondria respond to different exercise training prescriptions (43). Improving the understanding of the exercise variables that influence mitochondrial adaptations will result in optimised training interventions.

Pioneering work performed in rodents over 60 years ago showed that markers of mitochondrial content and mitochondrial respiratory function in skeletal muscle were improved following 12 weeks of high-volume, high-intensity exercise training (240, 241). Following this work, multiple studies in humans have tried to elucidate whether the intensity or volume of exercise was the most important variable to improve markers of mitochondrial content and mitochondrial respiratory function (242, 247, 271, 331). While this question remains to be fully elucidated (42, 54, 242), previous reports suggest that training volume is more important for changes in markers of mitochondrial content (219, 301), while a high exercise intensity is necessary for improvements in mitochondrial respiratory function (52, 239, 331). Sprint interval training (SIT) has emerged as a time-efficient strategy to improve cardiorespiratory fitness (332). While SIT elicits large, but intermittent, increases in energy demands, moderate-intensity continuous training (MICT) elicits a sustained energy demand for a longer time, which explains the final difference in exercise intensity and volume often observed between these exercise training prescriptions (242, 247).

Mitochondrial volume density ($Mito_{VD}$), quantified using the gold standard measure of mitochondrial content of transmission electron microscopy (TEM), has been shown to increase following 6 weeks of MICT (214, 216). However, the majority of studies usually rely on citrate

synthase (CS) activity, a common biomarker of mitochondrial content (62), to investigate training-induced changes in mitochondrial content (52, 238, 242, 301). While CS activity has proven to be a valid biomarker of mitochondrial content in a cross-sectional study (62), training-induced changes in CS activity have shown poor correlations with changes in $mito_{VD}$ (51, 216, 333). These poor correlations have been used to argue against the use of CS activity as a marker of training-induced changes in mitochondrial content (243). However, this may also be due to methodological variables in the quantification $mito_{VD}$, such as the number of fibres or the area analysed (62, 243). Collectively this would suggest that a combination of multiple measures of mitochondrial content, rather than the reliance on a single parameter, might provide the best approach to elucidate training-induced changes in mitochondrial content.

Mitochondrial content and function are thought to be intimately related (334); however, this is not always the case in skeletal muscle (271). Training-induced adaptations in mitochondrial respiratory function have been shown to be dissociated from mitochondrial content (89), which suggests a different training stimulus may be required to improve mitochondrial respiratory function. In studies comparing two different exercise prescriptions, mitochondrial respiratory function has been shown to improve only at the higher intensities of exercise (> 65% of $\dot{W}_{max}$) (219, 239, 331). Even when expressed relative to mitochondrial content (i.e., normalised to either $mito_{VD}$ or a mitochondrial content marker) high-intensity interval training (HIIT) and SIT have been found to be superior to MICT (52, 219). However, there are no studies comparing the effects of MICT and SIT on mitochondrial-specific respiratory function utilising $mito_{VD}$ as the normalising variable.

The complexity of mitochondria includes multiple layers of adaptation other than mitochondrial content and respiratory function. Among those adaptations, little attention has been directed to morphological changes (56). Mitochondrial size was suggested to be the

primary adaptation by which the mitochondrial pool adapts to 6 weeks of MICT (243). However, the authors found a correlation between increased number of mitochondria per profile with increased training-induced mitochondrial content, making their conclusions unclear (243). Whether different exercise prescriptions elicit divergent mitochondrial morphological responses remain to be elucidated. Similarly, multiple training studies have investigated protein levels of the oxidative phosphorylation (OXPHOS) complexes (52, 238), but it is unknown whether different exercise trainings will result in divergent adaptations in OXPHOS protein levels.

Although exercise training leads to improvements in mitochondrial characteristics, whether mitochondrial adaptations relate to improvements in endurance performance remains equivocal (55, 335). In cross-sectional studies, both mitochondrial content and respiratory function are increased in highly-trained athletes (63). Combining two very distinct exercise training prescriptions, which have been suggested to elicit divergent mitochondrial adaptations (42, 219, 336), may facilitate the discovery of mitochondrial adaptations important for improvements in endurance performance.

Despite the large increase in mitochondrial research, whether the multiple layers of mitochondria are divergently remodelled following exercise training remains conflicting. The aim of this study was to elucidate how mitochondrial characteristics are modified using two very different exercise prescriptions focusing on exercise volume (MICT) or exercise intensity (SIT). Furthermore, this study will be able to elucidate previously unknown relationships between endurance performance and mitochondrial characteristics.

5.2 Methodology and procedures

5.2.1 Participants

Participants were provided with the information to participants' document and were informed of the study requirements, benefits, and risks before giving their written informed consent. Ethics approval for the study was obtained from the Victoria University Human Research Ethics Committee (HRE17-075), and the study was registered as a clinical trial under ANZCTR (ACTRN12617001105336). Twenty-four healthy males (27.2 ± 5.5 y; 178 ± 6 cm; 75.2 ± 9.9 kg; 23.6 ± 2.7 BMI) volunteered to take part in this study. Participants were assigned in a random counter-balanced order to one of the two exercise groups based on their $\dot{W}_{max}$ ($W \cdot kg^{-1}$). Following allocation participants' characteristics (age, BMI, $\dot{V}O_{2max}$, $\dot{W}_{max}$, 20-kmTT, and 4-kmTT) were matched between groups (all $p > 0.05$).

5.2.2 Study design and training

Following the initial graded exercise test (GXT), the participants were allocated to a training group. During the first two weeks, participants performed one maximal 4-kmTT, one maximal 20-kmTT, and two exercise familiarisation sessions (Figure 5.1). Following the familiarisation, participants underwent another week of testing involving a graded exercise test (GXT), a 4-kmTT, and a 20-kmTT. After the testing week, participants underwent 8 weeks of training (Figure 5.1), before the last muscle sample was obtained and the final testing sessions were performed. Figure 5.1 illustrates the training study timeline for both training groups. These participants also had four muscle biopsies taken following the first exercise training session (analysed in chapter 4); however, this information is not included in figure 5.1 as the analysis of these samples is not included in the current chapter.

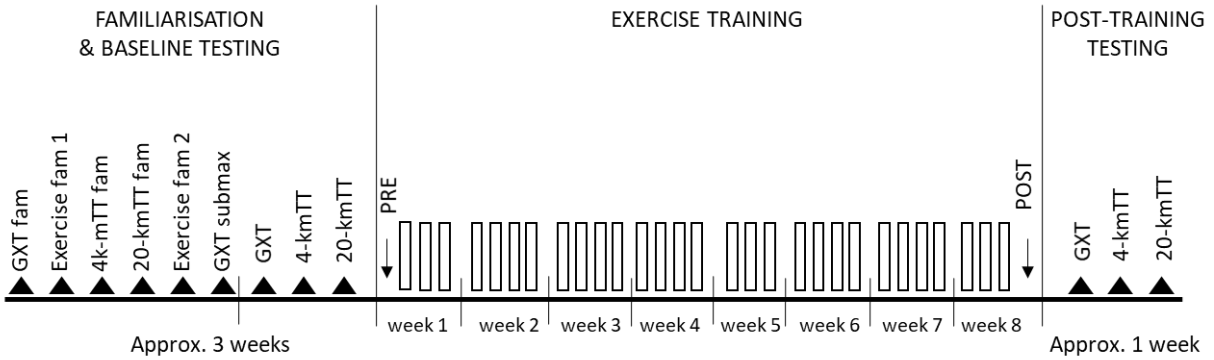

Figure 5.1. Schematic overview of the timeline for the testing and exercise training session before and after the 8-week period. GXT = Graded exercise test; fam = familiarisation session; 20-kmTT = 20-kilometre time trial; 4-kmTT = 4-kilometre time trial; submax = submaximal.

5.2.2.1 Sprint-Interval Training (SIT)

Twelve participants were allocated to this group (27.5 ± 5.1 y; 179 ± 6 cm; 75.5 ± 9.3 kg; 23.5 ± 2.7 BMI). Training sessions consisted of 30-s "all-out" cycling bouts against a resistance initially set at 0.075 $kg \cdot kg^{-1}$ body mass (BM), interspersed with a 4-min recovery period. This load was increased to 0.080 $kg \cdot kg^{-1}$ BM in week 3, to 0.085 $kg \cdot kg^{-1}$ BM in week 5 and to 0.090 $kg \cdot kg^{-1}$ BM in week 7. During the recovery participants remained on the bike and were allowed to either rest or cycle against no resistance. During the last 30 s of the recovery period participants were instructed to pedal and to reach a cadence of 90 rpm against no resistance; in the last 10 seconds they were advised to get ready and the countdown began. They were instructed to begin pedalling as fast and hard as possible when the countdown reached zero. At this time the load was applied via a software (Velotron Wingate Software, Velotron, RacerMate, Seattle, WA, USA) loaded on a computer interfaced with the cycle ergometer. Participants were verbally encouraged to pedal as fast and hard as possible during the entire duration of the bout. Participants started the training with 4 sprints per session and this was increased to 8 sprints per session in week 7.

5.2.2.2 Moderate-Intensity Continuous Training (MICT)

Twelve participants were allocated to this group (26.9 ± 5.6 y; 178 ± 6 cm; 74.9 ± 10.2 kg; 23.7 ± 2.6 BMI). Training sessions consisted of continuous cycling at a fixed power equivalent to ~ 90-100% of LT1 (defined as the first increase in lactate that was ≥ 0.3 $mmol \cdot L^{-1}$ than the previous stage during the submaximal test), and was reassessed in the first training session of week 3, 5 and 7, and exercise intensity was adjusted accordingly. Participants started with 60 min per session and this was increased to 120 min per session in week 7.

5.2.3 Physiological testing

5.2.3.1 4-km & 20-km Time Trial

Cycling time trials were used as a marker of endurance performance and were done on an electronically-braked cycle ergometer (Velotron, RacerMate, Seattle, WA, USA). Prior to each time trial participants completed a 5-min warm-up at a self-selected intensity followed by 5 min of rest. During the time trial, participants could only see the bike gearing and speed, and were constantly provided with encouragement throughout the trial. Performance data were expressed as absolute mean power output (W), and relative mean power output ($W \cdot kg^{-1}$)

5.2.3.2 Graded Exercise Test

GXT's were conducted using an electronically-braked cycle ergometer (Lode Excalibur v2.0, The Netherlands). A metabolic analyser (Quark Cardiopulmonary Exercise Testing, Cosmed, Italy) was used during the testing to assess $\dot{V}O_2$, $\dot{V}CO_2$, and $\dot{V}E$ on a breath-by-breath basis, and heart rate was measured (Garmin, USA). A GXT with 1-min stages was chosen, and the increase in power between stages was adjusted following the familiarisation trial to attain a total duration of 9 to 11 min upon exhaustion. After reaching exhaustion, 5 min of rest were provided before a verification bout was applied (time to exhaustion at 90 % of $\dot{W}_{max}$) to verify that $\dot{V}O_{2max}$ had been achieved.

5.2.3.3 Submaximal Test

Following the completion of GXT, the ventilatory parameters obtained ($\dot{V}O2$, $\dot{V}CO2$, $\dot{V}E$) were plotted and visually inspected to estimate VT1. Accordingly, a power output of 40 W lower than the estimated VT1 was selected as the starting point of the submaximal test, and after 3 min (stage length) the intensity was increased 10 W, and continued increasing by 10 W every 3 min until the LT1 could be identified. Once the blood lactate reached 2 $mmol \cdot L^{-1}$ the test was stopped, as the LT1 had been already reached. Antecubital venous blood was taken in the last 15 s of each stage and was analysed using a blood lactate analyser (YSI 2300 STAT Plus, YSI, USA).

5.2.4 Biochemical analyses

5.2.4.1 Muscle biopsies

All muscle samples were obtained at a similar time of the day (mornings), at a constant depth of around 2 to 3 cm, by an experienced medical doctor. Resting muscle biopsies were taken from the *vastus lateralis* muscle using the Bergström biopsy needle technique modified with suction. Participants rested in the supine position and after injection of a local anaesthetic into the skin and fascia (1% xylocaine, Astra Zeneca) a small incision was made. Pre- and post-training biopsies were done in the same leg, to minimise any possible between-leg variability. Once the muscle sample was obtained, muscle samples were processed, cleaned of excess blood, fat, and connective tissue, and split in three portions. One portion (10 mg) was immediately immersed in a tube containing biopsy preserving solution (BIOPS) kept on ice, and then used for *in situ* measurements of mitochondrial respiratory function. The other portion (5 mg) was immediately fixed in a 0.2 M sodium cacodylate buffered 2.5% glutaraldehyde and 2% paraformaldehyde for electron microscopy imaging. The remaining portion was immediately frozen in liquid nitrogen and stored at -80 °C for subsequent analyses.

5.2.4.2 Preparation of whole-muscle lysates

Approximately 10 to 20 mg of frozen muscle was homogenised 2 times for 2 minutes at a speed of 30 Hz with a TyssueLyser instrument (Qiagen, Canada) in an ice-cold lysis buffer (1:20 w/v) containing 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 5 mM Na4P207, 1 mM Na3V04, 1 % NP-40, with added protease and phosphatase inhibitors at a 1:100 concentration (Cell Signaling Technology). Protein concentration was determined using a commercial colorimetric assay (Bio-Rad Protein Assay kit II, Bio-Rad Laboratories Pty Ltd, Gladesville, NSW, AUS) and lysates were then diluted with an equal volume in 2x Laemmli buffer containing 10% β-mercaptoethanol.

5.2.4.3 Citrate Synthase (CS) activity assay

Using the whole-muscle lysates, CS activity was determined in triplicate on a microtiter plate by adding: 5 µL of a 2 $mg \cdot mL^{-1}$ muscle homogenate, 40 µL of 3 mM acetyl CoA in Tris buffer and 25 µL of 1 mM 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) in Tris buffer to 165 µL of 100 mM Tris buffer (pH 8.3) kept at 30°C. At this point 15 µL of 10 mM oxaloacetic acid were added to the cocktail and the plate was immediately placed in a spectrophotometer kept at 30°C (xMark Microplate Spectrophotometer, Bio-Rad Laboratories Pty Ltd, Gladesville, NSW, AUS). Following 30 s of linear agitation, absorbance at 412 nm was recorded every 15 s for 3 min. CS activity was calculated and reported as $mol \cdot kg\ protein^{-1} \cdot h^{-1}$.

5.2.4.4 Preparation of permeabilised skeletal muscle fibres

A 10 mg fresh muscle sample was placed in ice-cold BIOPS, a biopsy preserving solution containing 2.77 mM CaK_2EGTA, 7.23 mM K_2EGTA, 5.77 mM Na_2ATP, mM 6.56 $MgCl_2$, 20 mM taurine, 50 mM MES, 15 mM Na_2 phosphocreatine, 20 mM imidazole, and 0.5 mM DTT adjusted to pH 7.1. Samples were mechanically separated using pointed forceps while kept on ice. Fibres were subsequently permeabilised by gentle agitation for 30 min at 4°C in BIOPS

containing 50 $\mu g \cdot mL^{-1}$ of saponin. Samples were then washed for 3 x 7 min at 4°C by gentle agitation in MiR05, a respiration medium containing 0.5 mM EGTA, 3 mM $MgCl_2$, 60 mM K-lactobionate, 20 mM taurine, 10 mM KH_2PO_4, 20 mM Hepes, 110 mM sucrose and 1 $g \cdot L^{-1}$ BSA essentially fatty acid-free adjusted to pH 7.1 at 37°C. This method selectively permeabilises the cellular membrane leaving the mitochondria intact and allows for *in situ* measurements of mitochondrial respiration.

5.2.4.5 High-resolution respirometry

After washing, 2 to 4 mg wet weight of muscle fibres were assayed in triplicate in a high-resolution respirometer (Oxygraph-2k, Oroboros Instruments, Innsbruck, Austria) containing 2 mL of MiR05. Mitochondrial respiration was measured at 37°C. Oxygen concentration ($nmol \cdot mL^{-1}$) and oxygen flux ($pmol \cdot s^{-1} \cdot mg^{-1}$) were recorded using DatLab software (Oroboros Instruments, Innsbruck, Austria), and instrumental background oxygen flux, accounting for sensor oxygen consumption and oxygen diffusion between the medium and the chamber boundaries, was corrected online. Re-oxygenation by direct syringe injection of O_2 in the chamber was necessary to maintain O_2 levels between 275 and 450 $nmol \cdot mL^{-1}$, so as to avoid a potential oxygen diffusion limitation. The following substrates and inhibitors were added as following: octanyolcarnitine (0.2 mM) and malate (2 mM) were added to measure the LEAK respiration from electron-transferring flavoprotein (ETF_L); this was followed by addition of $MgCl_2$ (3 mM) and ADP (5 mM) for the measurement of phosphorylation capacity (P) through ETF (ETF_P); the subsequent addition of pyruvate (5 mM) allowed the measurement of complex I and ETF respiration (ETF + CI_P); this was followed by addition of succinate (10 mM), which provided the measurement of P through complex I and complex II ($CI+CII_P$); after these steps cytochrome c (10 mM) was added to test for the outer membrane integrity, followed by a stepwise carbonyl cyanide 4-phenylhydrazone (FCCP) titrations (0.7-1.5 mM) to obtain the electron transport system capacity (E) through CI+CII ($CI+CII_E$). Rotenone (0.5 mM), an

inhibitor of CI, was added to determine E through CII (CII_E). Antimycin A (2.5 mM), an inhibitor of complex II, was then added for the measurement and correction of residual O_2 consumption as a measure of non-mitochondrial O_2 consumption.

5.2.4.6 Transmission Electron Microscopy

Skeletal muscle samples were fixed overnight at 4 °C with 0.2 M sodium cacodylate– buffered, 2.5% glutaraldehyde and 2% paraformaldehyde. Fixed samples were rinsed with 0.1 M sodium cacodylate, and postfixed with ferricyanide-reduced osmium tetroxide (1% OsO4, 1.5% K3 [Fe(CN)6], and 0.065 M cacodylate buffer) for 2 h at 4 °C. The postfixed samples were rinsed with distilled water, and then stored overnight in 70% ethanol. Dehydration was performed by graduated ethanol series (80%, 90%, 95%, 100%, and 100%; 10 min each) and propylene oxide (100% and 100%; 5 min each). Samples were infiltrated with Araldite 502/Embed 812 by graduated concentration series in propylene oxide (25% for 1 h, 33% for 1 h, 50% overnight; 66% for 4 h, 75% for 4 h, 100% overnight; and 100% for 5 h) and then polymerised at 60°C for 48 h. Embedded samples were sectioned using an Ultracut UCT ultramicrotome (Leica Biosystems) equipped with a 45° diamond knife (Diatome) to cut 75 nm ultrathin sections. The grids were stained at room temperature using 2% aqueous uranyl acetate (5 min) and Reynolds lead citrate (3 min) before routine imaging. All TEM imaging was performed at 80 kV on a Hitachi H-7500 TEM using a Gatan 791 MultiScan side-mount CCD camera and DigitalMicrograph (Version 1.71.38) acquisition software. Image acquisition was performed by an experienced blinded investigator. Twenty images were randomly acquired, from at least five different fibres, and of the intermyofibrillar region. Mitochondrial morphological values were obtained by manually tracing at least 100 mitochondria from each participant at each timepoint. An experienced investigator traced a total of 5724 mitochondria, distributed as follows: PRE MICT (1291), POST MICT (1538), PRE SIT (1598), and POST SIT (1297). Images were quantified for mitochondrial morphological variables using the open access

software ImageJ (NIH, USA), following previously published guidelines (112, 205). Morphological distribution analyses were performed with ggplot in R software.

5.2.4.7 Western blotting.

For each protein of interest a linearity study was conducted to determine the ideal loading amount. Muscle lysates were then loaded in equal amounts (10 to 40 μg according to target protein) and separated by electrophoresis for 1.5-2.5 h at 100 V using pre-cast SDS-PAGE gels (4-20% or other). Once resolved, the gels were then wet transferred onto PVDF membranes using a Turbo Transfer (Bio-rad Laboratories Pty Ltd, Gladesville, NSW, AUS). Once transfer was confirmed, membranes were blocked at room temperature for 1 h using 5% skim milk in Tris Buffer Saline (TBS) 0.1% Tween-20 (TBS-T). After 3 x 5-min washes in TBS-T, membranes were incubated overnight at 4 °C with gentle agitation in primary antibody solutions (3-5% BSA or 5% skim milk, plus 0.02% Na Azide). The following antibodies were used: UQCRC2 (ab14745), COX IV (ab14744), SDHA (ab14715), NDUFA9 (ab14713), ATP5A (ab14748), OPA1 (R&D MAB9506), DRP1 (cell signalling #8570), MFN2 (cell signalling #9482). Immunoblotting was carried out using the desired antibody. The following morning, membranes were washed for 3 x 5-min in TBS-T and subsequently incubated under gentle agitation at room temperature with the appropriate host species-specific secondary antibody for 90 min in 1-5% skim milk in TBS-T. Membranes were then washed again for 3 x 5-min in TBS-T before being immersed for 5 min under gentle agitation at room temperature in Clarity ECL detection substrate (Bio-rad Laboratories Pty Ltd, Gladesville, NSW, AUS). Protein bands were visualised using a Bio-Rad ChemiDoc imaging system and band densities were determined using Bio-Rad Image Lab analysis software (Bio-Rad Laboratories Pty Ltd, Gladesville, NSW, AUS). Finally, all samples for each participant were loaded on the same gel and the different concentrations of a mixed-homogenate internal standard (IS) were also loaded on each gel and a calibration curve plotted of density against protein amount. From the

subsequent linear regression equation, protein abundance was calculated from the measured band intensity for each lane on the gel. Total protein level of each lane was obtained from the stain-free image of the membrane and was used for normalisation of the results.

5.2.5 Statistics

All values are reported as mean ± standard deviation (SD). Two-tailed unpaired t-tests were used to assess differences between groups at baseline. Two-way repeated measures of ANOVA were used to investigate the influence of training, group, and the interaction between these. Post-hoc Bonferroni analyses were performed when there was a training or group x training effect. In addition, pre-planned paired t-test contrasts were used to compare pre and post MICT and SIT for the main outcomes analysed in the current study (e.g., mitochondrial respiratory function). Where training and group effect was found, unpaired t-test was performed to test any significant difference in relative changes between groups. Due to the extent of data skewness, and the inability to normalise mitochondrial morphology variables, these were analysed with non-parametric Mann-Whitney tests. Correlation coefficients were calculated according to Pearson's product moment (r) and statistical significance was set at $p < 0.05$. Correlation analyses were performed using corr package in R. Agreement between two variables were assessed using Bland-Altman plots. Effect sizes (ES) were quantified and defined as: small (0.2), moderate (0.5), large (0.8), and very large (1.3). The level of statistical significance was set at $p < 0.05$. GraphPad Prism 8.3 software was used for all statistical analyses.

5.3 Results

5.3.1 Mitochondrial content

Given that mitochondrial volume density ($mito_{VD}$) obtained from TEM is often described as the gold standard for measuring mitochondrial content (62), the effects of training on $mito_{VD}$ were first assessed. There was a training effect for $mito_{VD}$ ($p = 0.045$), but no interaction between groups. Pre-planned analysis showed that following MICT (19 ± 25%; 90% CI [6, 32%]; ES = 0.84; $p = 0.048$), but not SIT (16 ± 41%; 90% CI [-3, 35%]; ES = 0.31; $p = 0.35$), there was an increase in $mito_{VD}$ (Figure 5.2.A).

It was next explored if the most commonly used marker for mitochondrial content, CS activity (62), was altered following training. There was a training effect ($p = 0.0002$), with no interaction between groups. Pre-planned analysis revealed that CS activity significantly increased following MICT (20 ± 16%; 90% CI [12, 28%]; ES = 0.79; $p = 0.001$) but not following SIT (11 ± 15%; 90% CI [4, 18%]; ES = 0.33; $p = 0.076$) (Figure 5.2.B).

As $mito_{VD}$ and CS activity have been shown to correlate at rest (62), but not when training-induced changes were compared (51, 333), the relationship between $mito_{VD}$ and CS activity was also investigated. There was a significant positive correlation between CS activity and $mito_{VD}$ at rest ($r = 0.415$ [0.135, 0.634]; $p = 0.005$). Furthermore, for the first time it is reported that the training-induced changes in both variables were significantly correlated, both in absolute ($r = 0.456$ [0.043, 0.736]; $p = 0.033$; Figure 5.2.C) and relative changes ($r = 0.452$ [0.037, 0.734]; $p = 0.035$).

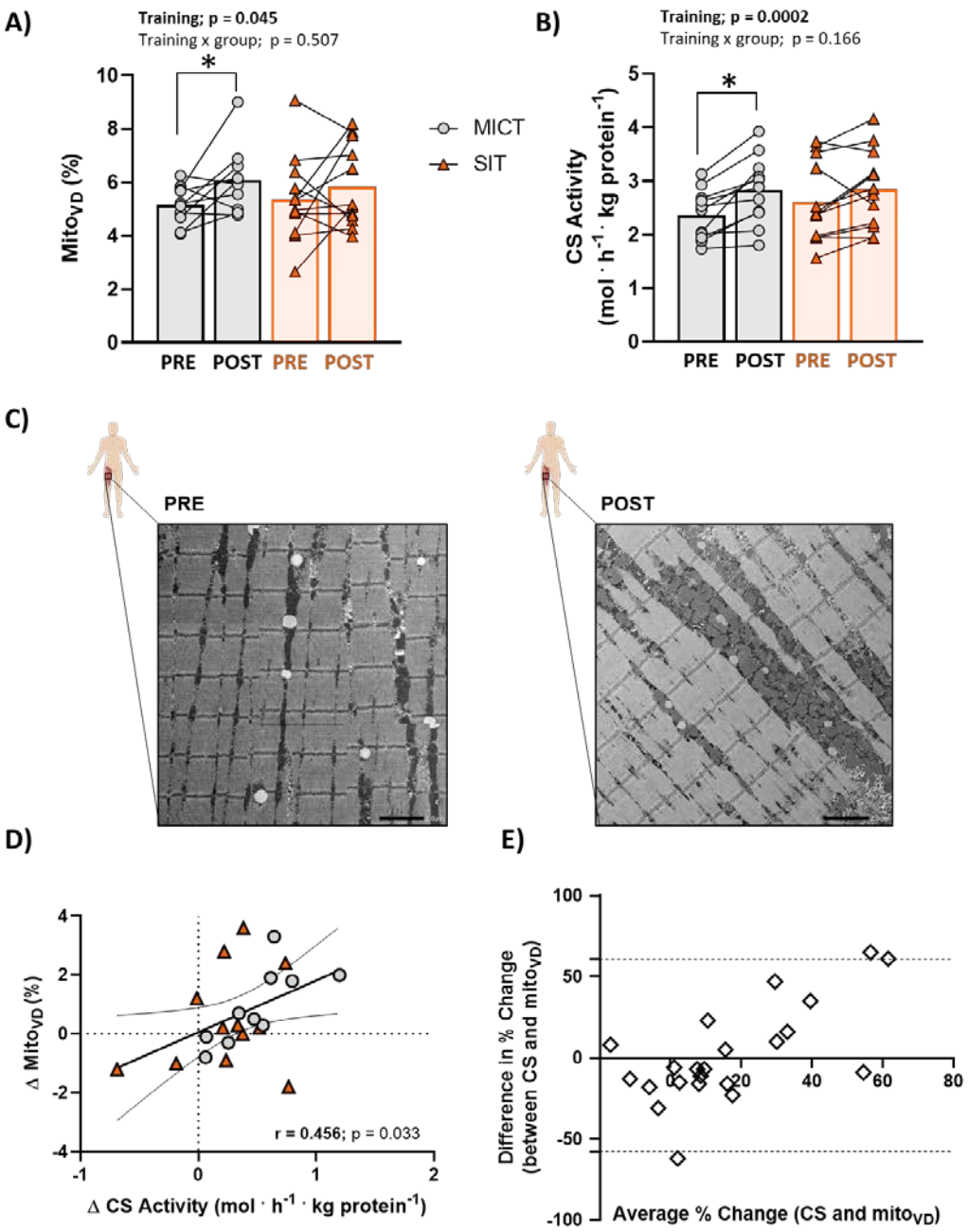

Figure 5.2. A) Changes in mitochondrial volume density (mito$_{VD}$) and B) citrate synthase (CS) activity pre and post 8 weeks of MICT and SIT. C) A representative image of a skeletal muscle sample PRE and POST the training period. Note the difference in the abundance of mitochondria. D) Correlation between absolute changes in CS activity and mito$_{VD}$, and E) Bland-Altman plot of relative changes in both CS activity and mito$_{VD}$. MICT = moderate-intensity continuous training; SIT = sprint-interval training. Orange symbols and bars represent SIT. Grey symbols and bars represent MICT. Circles represent individual participants in MICT group. Triangles represent individual participants in SIT. * = significantly different from PRE ($p < 0.05$). Data are mean + SD.

5.3.2 Oxidative phosphorylation (OXPHOS) protein levels

Given the observed differences between training modalities for $mito_{VD}$ and CS activity, it was important to establish whether the protein levels of OXPHOS subunits were also altered following training. There was a training effect for the subunits of complex I, III and IV ($p < 0.05$). While there was no training x group effects, pre-planned analyses showed increases in complex I (16 ± 19%; 90% CI [6, 25%]; ES = 0.50; p = 0.013) and IV (22 ± 24%; 90% CI [11, 34%]; ES = 0.41; p = 0.028) following MICT, and in complex III (8 ± 11%; 90% CI [3, 13%]; ES = 0.38; p = 0.044) and IV (23 ± 22%; 90% CI [13, 34%]; ES = 0.38; p = 0.011) following SIT. Total OXPHOS, as the sum of the five different complexes, was significantly increased only following MICT (10 ± 15%; 90% CI [3, 18%]; ES = 0.43; p = 0.04), but not following SIT (10 ± 12%; 90% CI [4, 15%]; ES = 0.38; p = 0.06).

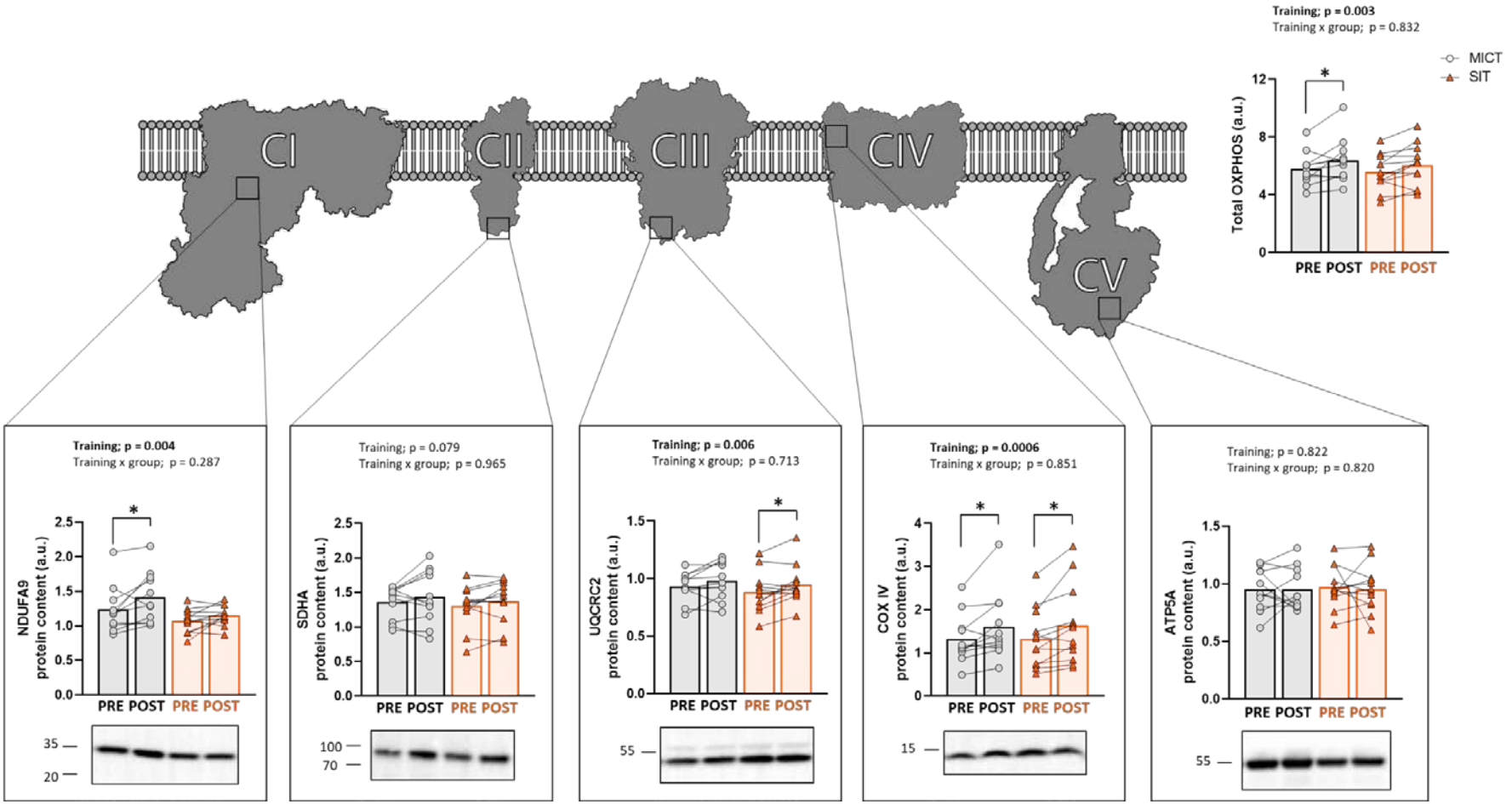

Figure 5.3. Protein levels of different subunits of the electron transport chain complexes pre and post 8 weeks of MICT and SIT. MICT = moderate-intensity continuous training; SIT = sprint-interval training. Orange symbols and bars represent SIT. Grey symbols and bars represent MICT. * = significantly different from PRE. Circles represent individual participants in MICT group. Triangles represent individual participants in SIT group.

5.3.3 Mitochondrial respiratory function

Considering previous literature suggesting a divergent response of markers of mitochondrial content and respiratory function (52), the effects of both training prescriptions on mitochondrial respiratory function were next explored. There was a training effect for electron-transferring flavoprotein (ETF) leak (ETF_L), coupled ETF (ETF_P), and coupled ETF and complex I ($ETF+CI_P$) (all $p < 0.05$) , but no group x training interaction in any variable. Pre-planned analyses showed that ETF_P and $ETF+CI_P$ were significantly changed only following SIT (Figure 5.4.A and Table 5.1). There was no training or group x training effect in maximal mitochondrial respiratory function ($ETF+(CI+II_P)$; Figure 5.4.A), but pre-planned analysis showed that following SIT there was a significant increase in maximal mitochondrial respiratory function ($ETF+(CI+II_P)$) (19 ± 20%; 90% CI [8.5, 29.4%]; ES = 0.56; p = 0.016), while there was no change following MICT (4 ± 26%; 90% CI [-10, 19%]; ES = 0.09; p = 0.854).

While mass-specific mitochondrial respiratory function was increased only following SIT, there was no effect of training on the mitochondrial-specific maximal mitochondrial respiratory function (expressed relative to $mito_{VD}$ and CS activity). There was a group x training effect (p = 0.025) when normalised by CS activity. Post-hoc analyses showed no significant differences ($p > 0.05$).

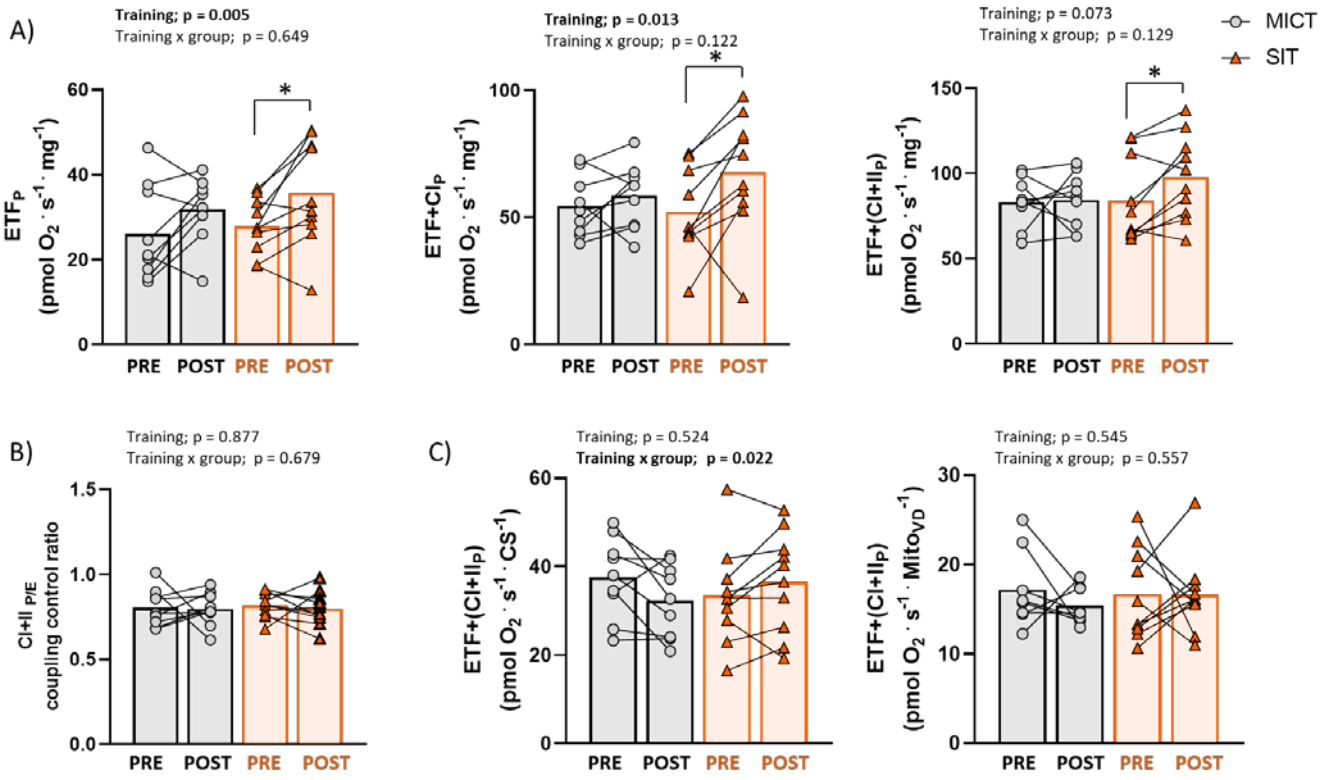

Figure 5.4. A) Mitochondrial respiratory function variables pre and post 8 weeks of MICT and SIT. B) Respiratory coupling efficiency, and C) Mitochondrial-specific changes in maximal respiratory function pre and post MICT and SIT. MICT = moderate-intensity continuous training; SIT = sprint-interval training. Orange symbols and bars represent SIT. Grey symbols and bars represent MICT. Circles represent individual participants in MICT group. Triangles represent individual participants in SIT. * = significantly different from PRE.

Table 5.1. Mitochondrial respiratory function and content changes following 8 weeks of MICT and SIT.

	MICT				SIT				
	PRE	POST	%	n	PRE	POST	%	n	Interaction
Mitochondrial respiratory function									
ETF_L (pmol $O_2 \cdot s^{-1} \cdot mg^{-1}$)	11.0 ± 5.3	16.1 ± 4.6	59 ± 113	9	13.3 ± 2.9	17.3 ± 5.1	34 ± 46	10	0.734
ETF_P (pmol $O_2 \cdot s^{-1} \cdot mg^{-1}$)	26.0 ± 10.6	31.8 ± 7.3	36 ± 49	9	27.9 ± 6.2	**35.6 ± 11.8***	26 ± 31	10	0.650
ETF_P + CI_P(pmol $O_2 \cdot s^{-1} \cdot mg^{-1}$)	54.5 ± 11.3	58.5 ± 12.1	10 ± 23	9	52.1 ± 16.2	**67.8 ± 21.9***	39 ± 58	10	0.122
ETF_P + ($CI+CII_P$) (pmol $O_2 \cdot s^{-1} \cdot mg^{-1}$)	83.2 ± 13.7	84.5 ± 15.2	4 ± 26	9	83.8 ± 23.4	**97.8 ± 23.4***	19 ± 20	10	0.073
Cyt C (pmol $O_2 \cdot s^{-1} \cdot mg^{-1}$)	83.0 ± 12.1	85.1 ± 15.2	5 ± 26	9	86.8 ± 24.6	**102.1 ± 26.4***	20 ± 19	10	0.130
$CI + CII_{ETS}$ (pmol $O_2 \cdot s^{-1} \cdot mg^{-1}$)	106.2 ± 26.8	107.0 ± 19.0	8 ± 37	9	109.8 ± 43.6	120.9 ± 32.8	16 ± 23	10	0.434
$CI + CII_{P/E}$ (a.u.)	0.80 ± 0.1	0.80 ± 0.1	1 ± 19	9	0.80 ± 0.1	0.82 ± 0.1	4 ± 15	10	0.679
ETF_P + ($CI+CII_P$) (pmol $O_2 \cdot s^{-1} \cdot mg^{-1} \cdot CS^{-1}$)	37.6 ± 9.2	32.3 ± 8.2	-12 ± 20	9	33.5 ± 11.0	36.9 ± 11.4	12 ± 21	10	**0.022**
ETF_P + ($CI+CII_P$) (pmol $O_2 \cdot s^{-1} \cdot mg^{-1} \cdot mito_{VD}^{-1}$)	17.2 ± 4.3	15.4 ± 2.1	-5 ± 29	8	17.0 ± 5.1	16.4 ± 4.3	5 ± 38	10	0.557
Mitochondrial content									
CS activity (mol $\cdot h^{-1} \cdot$ kg protein^{-1})	2.4 ± 0.5	**2.8 ± 0.6***	20 ± 16	11	2.6 ± 0.8	2.8 ± 0.7	11 ± 15	12	0.166
$Mito_{VD}$ (%)	5.2 ± 0.7	**6.1 ± 1.3***	19 ± 25	10	5.4 ± 1.6	5.8 ± 1.5	16 ± 41	12	0.507
Total OXPHOS (a.u.)	5.8 ± 1.2	**6.4 ± 1.5***	10 ± 15	11	5.7 ± 1.1	6.2 ± 1.4	10 ± 12	12	0.832

MICT = Moderate-intensity continuous training; SIT = Sprint-interval training

Data are presented as mean ± standard deviation. * = significantly different than PRE ($p < 0.05$); # significant interaction between groups.

% represent the average of relative individual changes.

5.3.4 Mitochondrial morphology

Given the relationship between mitochondrial morphology and function (103, 337), it was next explored whether MICT or SIT led to changes in mitochondrial morphological variables. Multiple morphological characteristics from mitochondria were obtained (Table S5.1). The frequency distribution of mitochondria PRE and POST training remained unchanged following MICT for all the measured variables (all $p > 0.05$). The only measurements that increased following SIT were the average mitochondrial size (+ 26%; ES = 0.20; $p < 0.0001$; Figure 5.4), the Feret's diameter (+17%; ES = 0.24; $p < 0.0001$; Supplemental Table D.1), and the perimeter (+16%; ES = 0.22; $p < 0.0001$; Table S5.1).

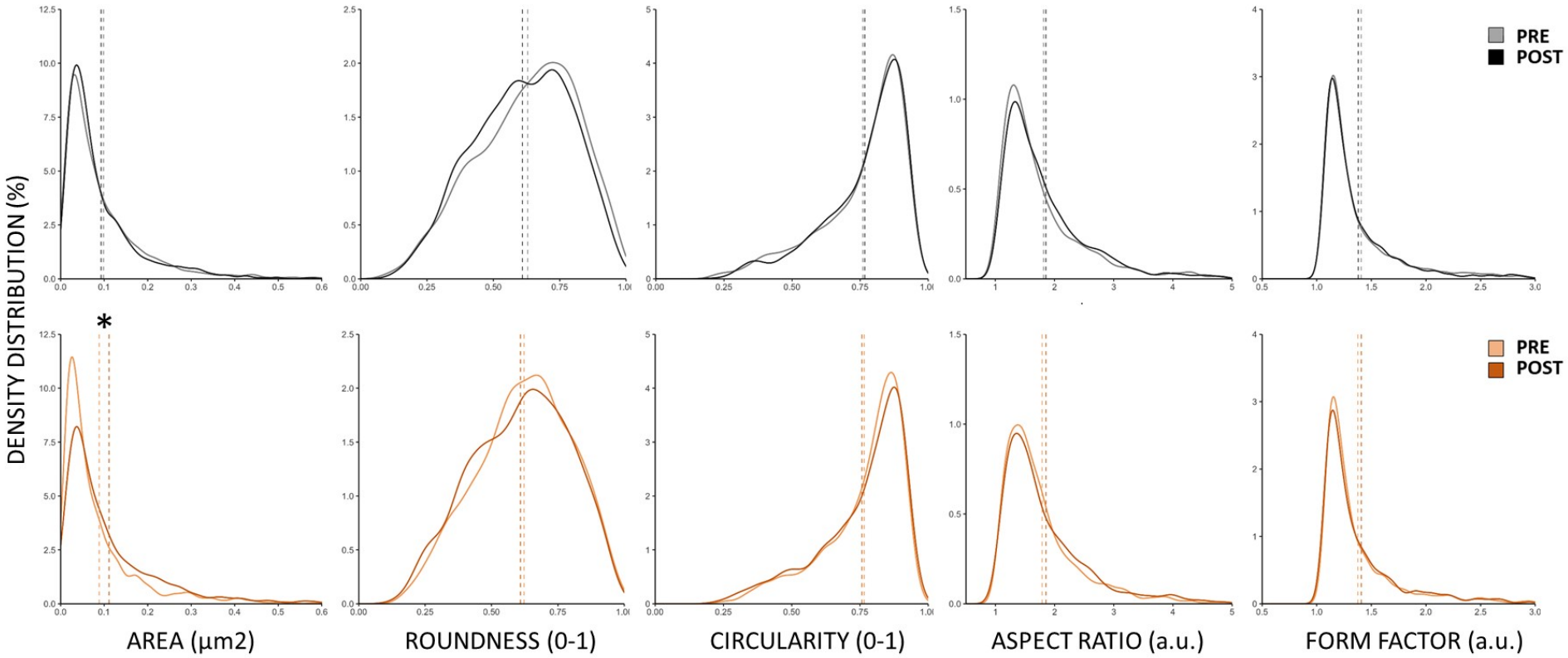

Figure 5.5. Density distribution of mitochondrial morphological characteristics pre and post 8 weeks of MICT and SIT. Light grey represents PRE MICT, black represents POST MICT. Light orange represents PRE SIT, dark orange represents POST SIT. Dashed lines represent the mean at each timepoint. * = significantly different than PRE ($p < 0.05$).

5.3.5 Mitochondrial dynamics protein levels

Given the morphological changes observed following SIT, it was important to identify if any key protein involved in mitochondrial dynamics were concurrently altered following training. There was no training or group x training effect in the mitochondrial inner membrane fusion protein Dynamin-like 120 kDa protein OPA1 (OPA1) and the the mitochondrial outer membrane fusion protein mitofusin 2 (MFN2). Pre-planned analyses showed both OPA1 and MFN2 were unchanged following training ($p > 0.05$). On the other hand, there was training ($p < 0.0001$) and group x training ($p = 0.028$) effect the main protein responsible for mitochondrial fission, dynamin-related protein 1 (DRP1). Post-hoc analysis showed that DRP1 protein level was increased following both MICT (23 ± 15%; 90% CI [15, 31%]; ES = 1.05; $p < 0.0001$) and SIT (10 ± 11%; 90% CI [4, 15%]; ES = 0.50; $p = 0.027$) (Figure 5.6).

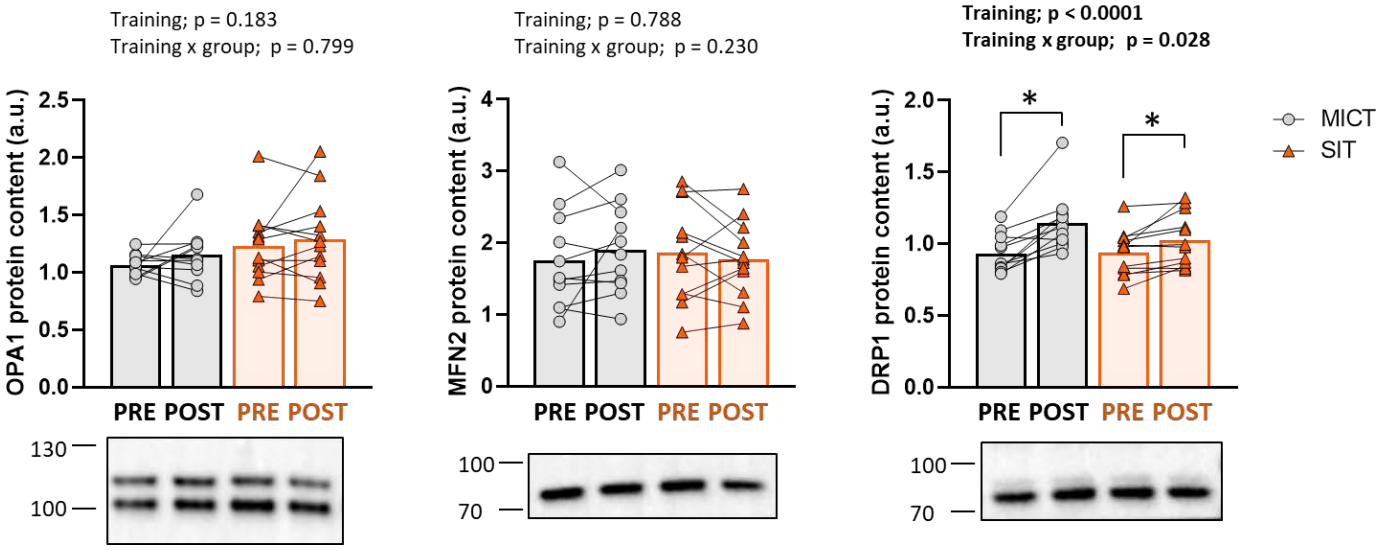

Figure 5.6. Protein levels of components of the fusion and fission branches of mitochondrial dynamics pre and post 8 weeks of MICT and SIT. MICT = moderate-intensity continuous training; SIT = sprint-interval training. * = significantly different from PRE. Orange symbols and bars represent SIT. Grey symbols and bars represent MICT. Circles represent individual participants in the MICT group. Triangles represent individual participants in the SIT group.

5.3.6 Physiological measures

Following the divergent mitochondrial adaptations observed between MICT and SIT, it remained to be established whether there were any differential physiological adaptation. There was no significant effect of training for $\dot{V}O_{2max}$ ($p = 0.055$). There was a significant training effect ($p < 0.0001$) for $\dot{W}_{max}$ following both MICT (11.0 ± 5.9%; 90% CI [8.1, 13.9%]; ES = 0.67; $p < 0.0001$) and SIT (5.9 ± 5.8%; 90% CI [3.1, 8.6]; ES = 0.31; $p = 0.0012$). There was a training x group effect for $\dot{W}_{max}$ ($p = 0.036$), and an unpaired t-test conducted on the relative changes in both groups revealed there was a larger improvement following MICT than SIT (Figure 5.7). There was a training effect for 20-kmTT ($p < 0.0001$) following both MICT (11.9 ± 8.9%; 90% CI [7.5, 16.3%]; ES = 0.52; $p < 0.0001$) and SIT (6.5 ± 5.8%; 90% CI [3.8, 9.3%]; ES = 0.33; $p = 0.003$). Similarly, there was a training effect for 4-kmTT ($p < 0.0001$) following both MICT (11.1 ± 8.5%; 90% CI [6.8, 15.3%]; ES = 0.59; $p = 0.0002$) and SIT (8.4 ± 8.2%; 90% CI [4.4, 12.3%]; ES = 0.46; $p = 0.0006$). Furthermore, a correlation matrix was performed between mitochondrial and physiological characteristics at baseline (Figure 5.8A), as well as relative from PRE to POST (Figure 5.8B) and absolute changes (data not shown) in these measures from PRE to POST. The only mitochondrial parameter found to correlate with both relative and absolute changes in endurance performance markers was the change in NDUFA9 protein levels (a subunit of Complex I) (Figure 5.8.C).

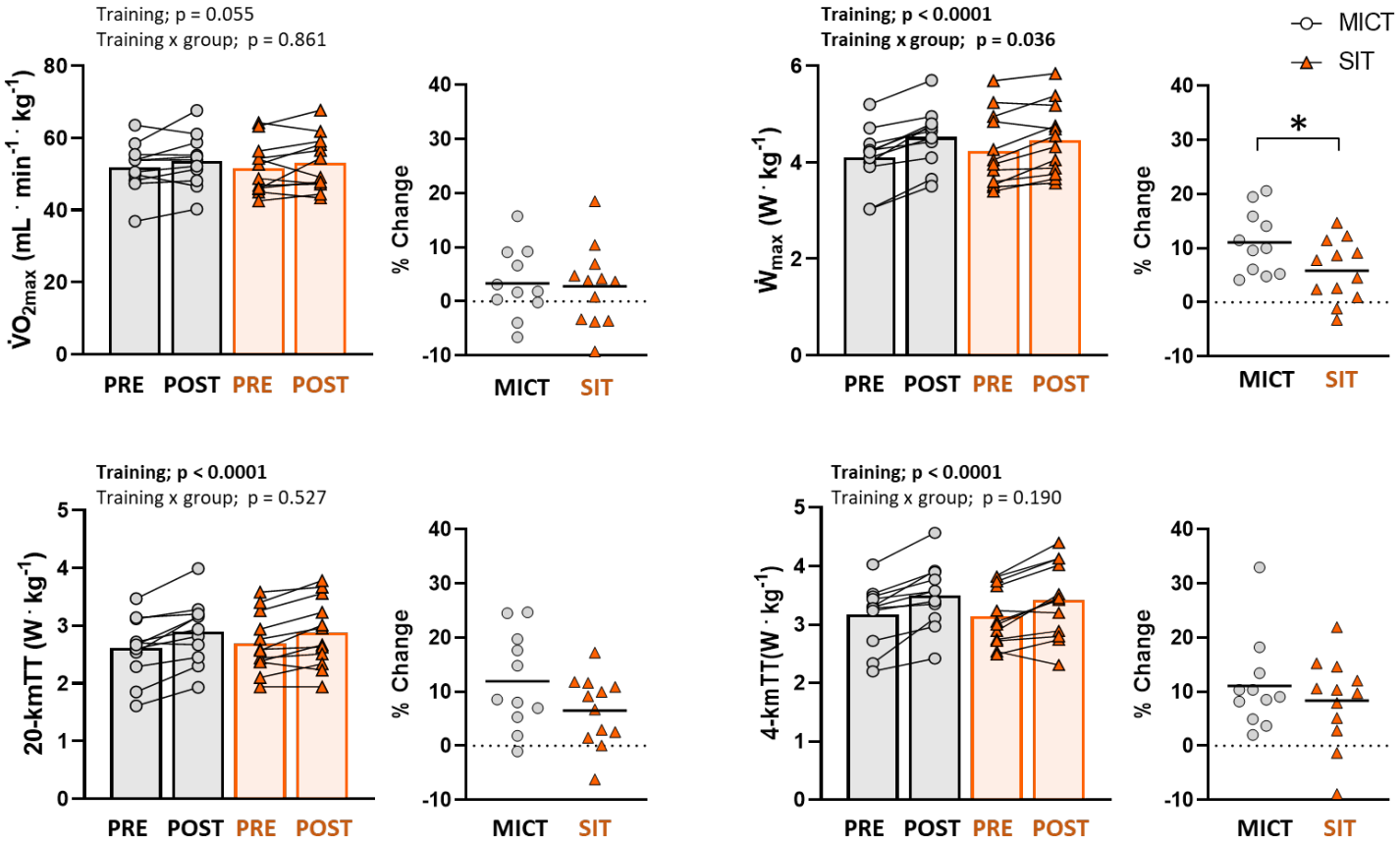

Figure 5.7. Changes in endurance performance markers following 8 weeks of MICT and SIT. MICT = moderate-intensity continuous training; SIT = sprint-interval training. Orange symbols and bars represent SIT. Grey symbols and bars represent MICT. $\dot{V}O_{2max}$ = maximal rate oxygen consumption; $\dot{W}_{max}$ = maximal aerobic power; 20-kmTT = 20-km time-trial; 4-kmTT = 4-km time-trial. * = significantly different between groups ($p < 0.05$).

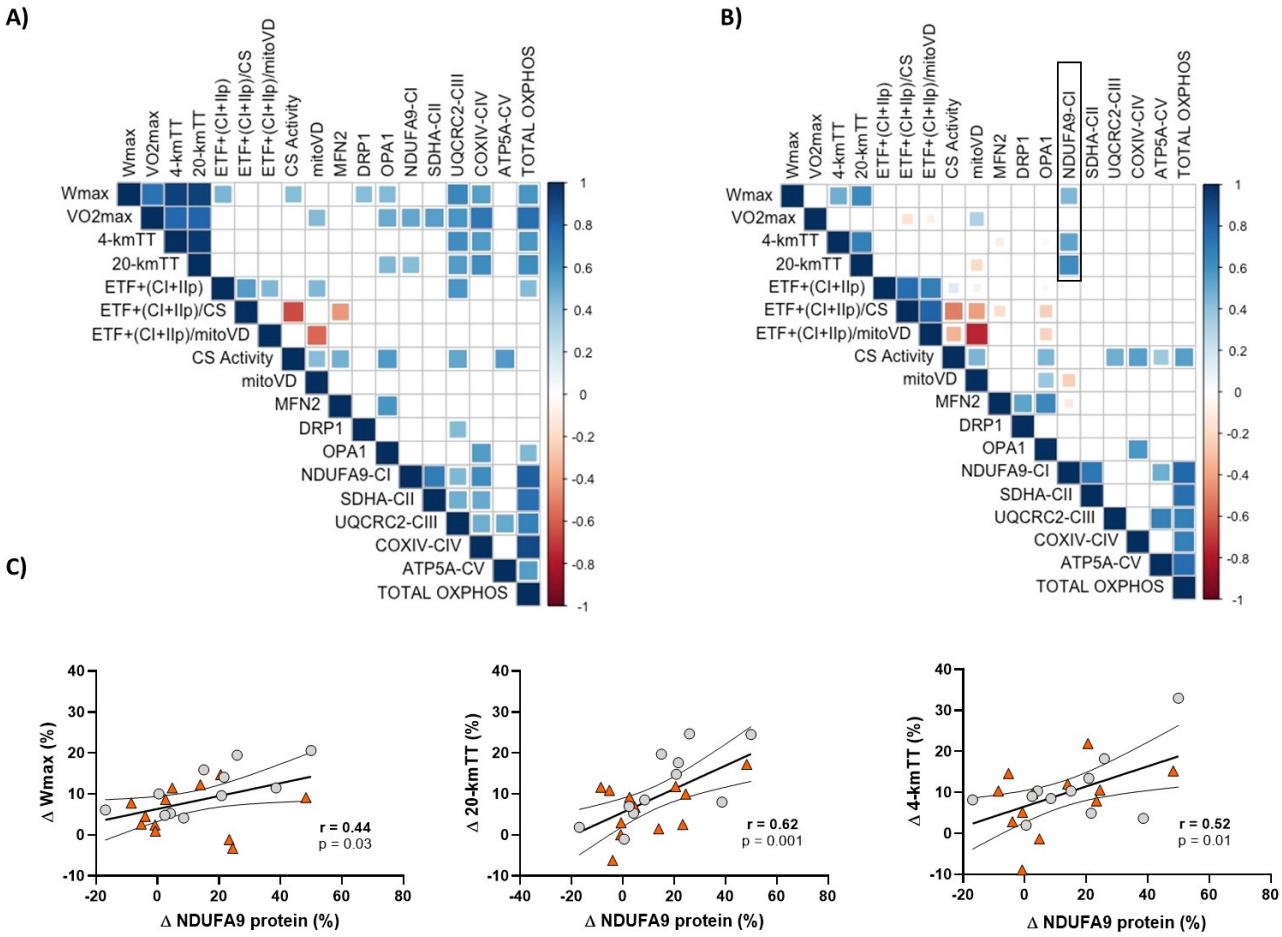

Figure 5.8. A) Correlation matrix of endurance performance markers and mitochondrial characteristics at baseline. B) Correlation matrix of relative changes following training; C) scatter plots of relative changes in NDUFA9 protein levels and endurance performance markers following 8 weeks of MICT and SIT. Empty spaces in A and B reflect non-significant interactions. Darkness and size of the squares reflect the pearson correlation coefficient (-1.0 to 1.0). Endurance performance markers are expressed relative to body mass. CS = citrate synthase; ETF = electron-transferring flavoprotein; CI = complex I; CII = complex II; p = phosphorylation; $\dot{W}_{max}$ = maximal aerobic power; $\dot{V}O_{2max}$ = maximal rate of oxygen consumption; 4-kmTT = 4-km time-trial; 20-kmTT = 20-km time-trial; OXPHOS = oxidative phosphorylation; mitoVD = mitochondrial volume density; MFN2 = mitofusin 2; DRP1 = dynamin-related protein 1; OPA1 = total dynamin-like 120 kDa protein OPA1; CI-V = Complex I to 5.

5.4 Discussion

The findings from the present study suggest that following 8 weeks of two very different training prescriptions (MICT and SIT), a divergent mitochondrial remodelling was observed. There were significant increases in mitochondrial content variables such as $mito_{VD}$, CS activity, and total OXPHOS protein levels only following MICT. On the other hand, SIT led to increases in the maximal mitochondrial respiratory function and mitochondrial size, without significant increases in variables related to mitochondrial content. Changes in $\dot{V}O_{2max}$, 4-kmTT, and 20-kmTT were similar following both MICT and SIT, but $\dot{W}_{max}$ increased to a larger extent following MCIT. Furthermore, the training-induced changes in endurance performance markers were correlated with changes in NUDFA9 protein levels, but not with changes in mitochondrial content or respiratory function.

This study aimed to resolve the ongoing discussion on whether training volume or intensity are more important for training-induced improvements in mitochondrial content (51, 54). To do this, the current study utilised two very different exercise training prescriptions whereby the MICT group performed an ~5-fold higher training volume while the SIT group trained at an ~5-fold higher intensity. It was shown that MICT leads to a significant improvement in $mito_{VD}$, CS activity, and total OXPHOS protein levels. On the other hand, SIT did not result in a significant improvement in either of these markers, despite some variables approaching significance. While no between-group differences were found, these results suggest that MICT led to a greater upregulation of mitochondrial content. Furthermore, it confirms that training intensity is not the primary driver of the training-induced improvements in mitochondrial content (51, 54, 219). These results are in agreement with previous research suggesting that training-induced changes in mitochondrial content were mainly attributable to changes in training volume (42, 51, 218, 219, 338). Moreover, the magnitude of increases observed

following MICT in the present study (~ 20%) suggest that training volume by itself may not be enough to maximise training-induced improvements in mitochondrial content variables. Instead, a combination of a high-volume and a high-intensity exercise training may be needed to achieve greater improvements in mitochondrial content variables, as observed in previous studies (301). Future studies should elucidate the optimal relationship between training volume and intensity to maximise training-induced adaptations in mitochondrial content.

$Mito_{VD}$ and CS activity were correlated at baseline in line with previous findings (62). However, for the first time training-induced changes in both parameters were also found to be significantly correlated (51, 243). Nonetheless, the strength of the correlations between CS activity and $mito_{VD}$ were moderate (R = 0.4 to 0.5), suggesting caution is required when interpreting changes in both of these measures. Instead, the present study proposes the use of a combination of multiple mitochondrial content biomarkers, rather relying on one of them, may be the optimal way of reflecting changes in mitochondrial content.

The present study shows that mitochondrial respiratory function was only improved following SIT, but not following MICT. Interestingly, while mass-specific respiratory function increased following SIT, and supports the idea that a higher exercise intensity is needed for mitochondrial respiratory function improvements (239, 331), mitochondrial-specific (relative to CS activity and $mito_{VD}$) respiratory function did not significantly change in any group. However, a training and group interaction for mitochondrial-specific respiratory function suggested that MICT and SIT may lead to divergent mitochondrial remodelling. Previous studies have shown that SIT increased mitochondrial-specific respiratory function, while it remained unchanged following MICT and HIIT (52). While the current study lasted for 8 weeks with a total of 29 exercise sessions, the previous reported study only performed 12 sessions over a 4-week period (335). It could be suggested that mitochondrial-specific respiratory function can be modified in the

short term, as with sleep restriction (339) or limb immobilisation (340), but over a longer period this remodelling aims at re-establishing a balance between mitochondrial content and function.

The findings of the study showed divergent increases in the abundance of oxidative phosphorylation (OXPHOS) proteins. Following MICT, there was a significant increase in NDUFA9 protein levels (a subunit of complex I), while this was unchanged following SIT. This is in contrast with a previous study showing that high-intensity training led to a greater training-induced increase in NDUFA9 protein level when compared to MICT (238). It has been suggested that complex I is bypassed during high-intensity exercise (213) and this may be especially relevant with training prescriptions such as SIT. Furthermore, in the present study both groups increased the protein abundance of COXIV (subunit of complex IV), while only SIT increased UQCRC2 (subunit of complex III) protein levels.

Mitochondrial morphological changes are a layer of adaptation usually unexplored in human training studies. The current study is the first to comprehensively show morphological changes to two different exercise training interventions. Interestingly, markers of mitochondrial complexity (form factor and aspect ratio) suggest that skeletal muscle mitochondrial complexity was not modified following 8 weeks of MICT or SIT. This suggests that the morphological changes observed in mitochondria following SIE (Chapter 4) are transient in time. Similarly, circularity and roundness, a sign of mitochondrial stress (222), were also unaltered, which would suggest a maintained resting mitochondrial morphology. In contrast to previous research (243), the average mitochondrial size (area) was not increased following MICT, but was increased following SIT. Average mitochondrial size has been related to substrate utilisation at rest and during exercise (341), and has been suggested to increase following 6 weeks of MICT (243). While this may seem contradictory, the previous study led by Meinild-Lundby et al. (243) also included 30-s bouts at 130% of $\dot{W}_{max}$, making it hard to distinguish where the stimulus originated. Furthermore, despite their statement on enlargement

of mitochondria, they found a strong relationship between increased number of mitochondria per profile analysed and increased mitochondrial content (243), suggesting that changes in mitochondrial number, rather than mitochondrial enlargement, was implicated in the training-induced increases in mitochondrial content. Thus, the present findings suggest that changes in the average mitochondrial size were a result of a higher exercise training intensity and not volume. Whether the increased mitochondrial size reflects increased mitochondrial fusion remains to be established.

Given the different training-induced changes in mitochondrial morphology, proteins involved in mitochondrial dynamics were examined. Following both MICT and SIT, the protein levels of mitochondrial fusion proteins OPA1 and MFN2 were unaltered. This is in line with the only study that compared MICT and SIT (335). On the other hand, both groups increased the abundance of DRP1, a protein involved in the last step of mitochondrial fission. An increase of DRP1 protein levels has been previously observed following short-term, high-intensity training (85), but not following 4 weeks of SIT or MICT (52). Recent findings suggest that DRP1 is deemed crucial for a correct muscle function (342) and has been linked to positive training adaptations (343). Future studies should address whether changes in the protein levels of members of mitochondrial dynamics alter the fusion or fission rates in skeletal muscle.

Consistent with the divergent responses observed in mitochondrial remodelling, there was a greater improvement in endurance performance markers such as $\dot{W}_{max}$ following MICT. However, there were similar increases in time trial performance (both 4-kmTT and 20-kmTT) following both MICT and SIT. A novel aspect of the present study was the correlations between changes in endurance performance and mitochondrial variables. Improvements in time-trial performance and $\dot{W}_{max}$ were only correlated with changes in NDUFA9 protein levels. Interestingly, only following MCIT there was a significant increase in NDUFA9 protein levels. This could explain such divergent response in the present study and could also explain the

overall larger magnitude of increase in endurance performance markers observed in the present study following MICT when compared to SIT, in line with previous research (335).

In conclusion, this study demonstrates how the modulation of exercise training prescription, by using a high-volume (MICT) and high-intensity (SIT) training program, can lead to divergent mitochondrial remodelling. It was shown that MICT leads to a significant improvement in markers related to mitochondrial content, while SIT improved mitochondrial respiratory function and average mitochondrial size. It was also shown that MICT elicits an increase in NDUFA9 protein levels, which was correlated with improvements in endurance performance markers - some of which are increased to a greater extent following MICT (e.g., $\dot{W}_{max}$). These divergent adaptations seem independent of changes in proteins related to mitochondrial dynamics. The present study highlights the importance of exercise variables to target specific training-induced mitochondrial adaptations.

5.5. Supplemental information

Table S5.1. Mitochondrial morphological changes following 8 weeks of moderate-intensity continuous training (MICT) and sprint-interval exercise (SIT). Data are presented as mean ± SD. N = number manually traced individual mitochondria.

	MICT PRE (n = 1291)	MICT POST (n = 1538)	Effect Size	SIT PRE (n = 1598)	SIT POST (n = 1297)	Effect Size
Area (μm^2)	0.098 ± 0.111	0.093 ± 0.094	-0.05	0.089 ± 0.107	0.111 ± 0.116*	0.20
Roundness	0.629 ± 0.189	0.609 ± 0.183	-0.11	0.622 ± 0.176	0.609 ± 0.183	-0.08
Circularity	0.760 ± 0.156	0.767 ± 0.149	0.05	0.765 ± 0.144	0.757 ± 0.155	-0.05
Feret's diameter (μm)	0.459 ± 0.311	0.453 ± 0.282	-0.02	0.422 ± 0.286	0.493 ± 0.311*	0.24
Perimeter (μm)	1.221 ± 0.793	1.185 ± 0.715	-0.05	1.124 ± 0.759	1.299 ± 0.815*	0.22
Form factor	1.407 ± 0.473	1.380 ± 0.415	-0.06	1.376 ± 0.390	1.408 ± 0.450	0.08
Aspect ratio	1.815 ± 0.883	1.854 ± 0.804	0.05	1.788 ± 0.705	1.853 ± 0.781	0.09

CHAPTER 6. GENERAL DISCUSSION, CONCLUSIONS, AND FUTURE RESEARCH

This chapter presents an overview of the main findings elucidated in the previous literature reviews and experimental chapters. The importance of these findings will be discussed in the context of scientific advancement and future research recommendations.

6.1 Thesis aims and objectives

The overall aim of this thesis was to investigate the effects of endurance exercise on the regulation of different mitochondrial remodelling processes (e.g., mitochondrial stress, mitochondrial biogenesis, and mitochondrial dynamics). More specifically, I investigated how these remodelling processes differ between two polarised exercise prescriptions that were ~5 fold different in exercise volume and intensity. To explore this, the thesis was separated into three experimental chapters.

1. Is autophagy regulation affected by exercise variables? (Chapter 3)

Human exercise studies had shown that protein levels of LC3-II, a marker of autophagosome content, decreases following a single exercise session (164, 264). These results would suggest a decreased autophagy flux, which seemed counterintuitive and contrary to rodent studies (251). In chapter 3, I show that exercise-induced regulation of LC3B protein levels in skeletal muscle differ between rodents and humans. Furthermore, independent of exercise intensity and fitness level, LC3B-II protein levels decrease following a single exercise session in humans. Despite this, the present data suggests an increased autophagy flux following exercise, based on the adaptation of a method to assess autophagy flux in human skeletal muscle. The findings of this thesis show for the first time that an exercise-induced decrease in LC3B-II protein levels does not indicate a decreased autophagy flux. While exercise-induced changes in autophagosome content did not seem to differ among multiple exercise intensities, this posed the question of whether mitochondrial-specific degradation (mitophagy), and the different processes concurrently altered (mitochondrial dynamics and mitochondrial stress), were differentially regulated following a single exercise session.

2. How are mitochondrial dynamics, and mitochondrial stress regulation, altered following a session of moderate-intensity continuous exercise (MICE) and sprint-interval exercise (SIE)? (Chapter 4)

To explore the influence of the exercise variables (e.g., volume, intensity) on mitochondrial-related processes, two very distinct exercise prescriptions were chosen - high-volume MICE and high-intensity SIE. The transcriptional response to both exercises showed that the unfolded protein response was the most enriched pathway among the genes that responded differently between MICE and SIE. Among the differentially regulated genes, those pertaining to the mitochondrial unfolded protein response (HSPD1, CHOP) were increased in SIE. Furthermore, genes involved in the integrated stress response, the pathway under which the mitochondrial unfolded protein response signals to the nucleus, were increased following SIE. Using micrographs obtained following both MICE and SIE, mitochondrial morphological and structural alterations were observed following SIE but not MICE. These included disrupted cristae density, increased mitochondrial roundness, and a swollen and disrupted phenotype. In line with this, only following SIE was there an increase in genes involved in mitochondrial fission (MIEF2) and mitophagy (p62, PARK2), as well as increased ULK1 phosphorylation at serine 556 - an important phosphorylation site needed for exercise-induced mitophagy (144). Interestingly, the exercise-induced increase in PGC-1α mRNA expression was similar between the two very different exercise prescriptions.

3. Are mitochondria differentially remodelled following MICT or SIT? (Chapter 5)

Since early findings in the field, the exercise prescription is known to dictate some of the observed mitochondrial adaptations following training (241). In this work, by polarising the exercise training stimulus (~5 fold greater volume (MICT) or intensity (SIT)), it was possible to interrogate potential divergent mitochondrial adaptations affected by either the intensity or

volume of training. In accordance with previous research (219), markers of mitochondrial content were only increased following MICT, while mitochondrial respiratory function was only increased following SIT. This divergent response had been previously reported in the literature (52), but remains to be fully understood as it is generally accepted that content and function are intrinsically related (334). This thesis extends the previous literature by incorporating TEM measures, which is important because it provides the gold standard measure and adds information about multiple mitochondrial layers of adaptation (e.g., morphological). These results demonstrated that the only morphological adaptation (an increase in the average mitochondrial size) occurred following SIT. Furthermore, it was demonstrated that training-induced changes in $mito_{VD}$, the 'gold standard' measure of mitochondrial content, and CS activity were significantly correlated. The relationship between mitochondrial characteristics and endurance performance at rest were also explored. While markers of mitochondrial content and respiratory function correlated with peak power, the protein levels of UQCRC2 (Complex III), COXIV (Complex IV), and total OXPHOS protein levels were strongly correlated with multiple markers of endurance performance at baseline. On the other hand, training-induced changes in endurance performance markers were only correlated with changes in NUDFA9 (Complex I), which may underlie an important adaptation for endurance performance. These results highlight the complexity of the mitochondrial adaptations and endurance performance.

6.2 General discussion and key findings

Despite the increase in medical research, exercise remains the most effective lifestyle intervention for the prevention and treatment of multiple diseases. Improving the understanding of the molecular regulation of skeletal muscle following exercise and training not only provides new and valuable knowledge to discover new treatment targets, but also aids at improving the individualised exercise prescription for health and performance.

In Chapter 1 research summarising the role of mitochondrial characteristics in the context of endurance performance was discussed. There was convincing evidence that multiple mitochondrial adaptations represent a necessary and commonly observed adaptation for training-induced improvements in endurance performance. Despite the large body of evidence that suggests mitochondrial characteristics do not limit maximal oxygen consumption (57), there seems to be some support for the idea that mitochondrial characteristics are important for endurance performance (32). This was supported by intervention studies where central adaptations were removed but endurance performance markers remained increased (37, 38). Given the evidence, I have revisited and proposed a new framework for the physiological and biological factors that determine endurance performance that incorporates mitochondrial characteristics as a primary determinant.

In Chapter 2, the effects of exercise on the regulation of the multiple layers of mitochondrial dynamics and mitochondrial stress were explored. Given the limited data in humans, a summary of each pathway was established. Furthermore, and in line with my experimental chapters, a role for the mitochondrial-driven integrated stress response was discussed in the context of skeletal muscle and exercise. Despite limited evidence on the effects of exercise, there is an emerging literature highlighting the importance of mitochondrial stress as this pathway is chronically present in multiple skeletal muscle diseases were mitochondrial dysfunction is a distinctive feature.

In the first experimental chapter (Chapter 3), I discussed the effects of exercise on the regulation of autophagy. Given that a key step in mitochondrial dynamics is mitochondrial-specific autophagy (mitophagy), it was important to answer a standing question in the field of exercise in humans: is autophagy downregulated following exercise? This idea was based on the decreased content of lipidated LC3 (LC3-II) observed in human skeletal muscle but not in rodents (164). I have shown that the exercise-induced decrease in LC3B-II protein levels is a

consistent finding in human skeletal muscle, independent of exercise intensity. Furthermore, I confirm the divergent response observed in LC3B between rodents and humans. However, by using a novel and adapted *ex vivo* autophagy flux assay, I was able to show that autophagy does not decrease following exercise, and follows a similar pattern as previously shown in rodents (273). This chapter suggests that making conclusions regarding autophagy based on basal LC3-II protein levels may lead to an erroneous interpretation. It calls for the use of a more valid approach, with the use of the *ex vivo* autophagy flux assay, as well as to include multiple members of the ATG8 family for a more comprehensive analysis.

In the second experimental chapter (Chapter 4), I discuss the effects of different exercise prescriptions, focusing on high-volume (MICE) or high-intensity (SIE), on mitochondrial-related signalling. Transcriptomic results show that the most enriched pathway divergently activated following exercise is the unfolded protein response. This pathway is enriched following SIE, and included the initial genes linked to the mammalian mitochondrial unfolded protein response (311). This was corroborated by an enrichment analysis of the 'Mitostress' geneset (178), which was significantly enriched following SIE but not MICE. In agreement with this, the micrograph analyses showed that mitochondrial morphology and structure were robustly altered following SIE, while they remained unchanged following MICE. Interestingly, this occurred independently of gene expression levels of the 'master regulator' of mitochondrial biogenesis PGC-1α. Given these results, it was important to elucidate the mitochondrial remodelling that occurs following these divergent exercise prescriptions.

In the last experimental chapter (Chapter 5), I discuss the physiological and mitochondrial adaptations to 8 weeks of exercise training with MICT and SIT. Following MICT there was predominantly an increase in variables associated with mitochondrial content, including $mito_{VD}$, CS activity, and protein levels of subunits of the OXPHOS complexes. On the other hand, SIT resulted in a significant improvement in mitochondrial respiratory function without

concomitant increases in markers of mitochondrial content. Furthermore, SIT resulted in an increased average mitochondrial size. The present results also elucidated correlations between endurance performance markers at rest and following training. A novel finding was that training-induced changes in NDUFA9 protein levels (a subunit of Complex I) was the only mitochondrial variable to correlate with training-induced changes in markers of endurance performance. These results highlight the complexity of mitochondrial adaptations to multiple exercise training prescriptions, and highlights the usefulness of these findings for optimised exercise prescription through the understanding of how training volume and intensity affect mitochondrial adaptations.

6.3 Contribution to literature

The beneficial effects of exercise have been long known. However, the underlying mechanisms by which exercise positively affect health and improves endurance performance remain incomplete. More importantly, how different types of exercise affect the underlying molecular signature in skeletal muscle, and how this may result in different skeletal muscle adaptations, is important to improve exercise prescription.

The work of this thesis has contributed to the body of knowledge showing for the first time that endurance exercise may increase autophagy flux, and that exercise-induced autophagosome changes occur independently of exercise intensity. Furthermore, I have shown that mitochondrial morphological and structural changes, as well as mitochondrial stress, are distinctive feature of SIE and precede an upregulation of a transcriptional response aiming at restoring mitochondrial and cellular homeostasis through the integrated stress response. In line with this, when SIE is repeated over time (SIT) there is an improved mitochondrial respiratory function without changes in mitochondrial content. On the other hand, MICE has been shown to be a more mild cellular stress upregulating a similar number of transcripts, but without

influencing mitochondrial quality control pathways to a large extent. This, when repeated over time (MICT), leads to an increased mitochondrial content without a parallel increase in mitochondrial respiratory function.

Collectively, this work provides multiple important contributions to literature. On one hand, it provides a framework for a mitochondrial basis of endurance performance and muscle adaptations to exercise and training. On the other hand, it shows that human exercise studies are a valuable research model to study stress-induced mitochondrial signalling.

6.4 Research limitations and considerations

In the following bullet points I will highlight what I consider to be some of the most important limitations I have encountered during my PhD research:

- Limitation on experimental techniques in human samples.

Molecular biology is an important field for the advancement of our understanding of disease, and how to prevent disease while extending and improving health. While this relies on the ultimate possibility of utilising our knowledge to treat human diseases, our translational capability does not always succeed. While utilising human skeletal muscle tissue for this PhD thesis has avoided this translational limitation, it has added experimental limitations. One example is during the experiments of chapter 4, as I observed that the ISR was activated following SIE (via the gene expression levels of GADD34) and there was a clear mitochondrial stress. Just as I was performing those experiments two new papers showed that a protein called DELE1 was cleaved following mitochondrial stress and translocated to the nucleus to signal the ISR (168, 169); however, in those papers they showed that none of the commercially available antibodies was valid. This left this chapter with a missing link between mitochondrial stress and the ISR activation, which will require future research using *in vitro* or *in vivo* models until any further improvement in this antibody. I think human research has this problem as it

leaves a lot of room for interpretation, and possibly error, and that by adding multiple research models of work (i.e., cell culture and rodent model) would have helped to narrow down and elucidate the specific molecular events leading to our findings. This was not possible due to time limitation in running a human study with skeletal muscle biopsies and completing the PhD in the 3 to 3.5 year timeframe. However, I aim to implement this approach in my future research.

- Reliance on valid protein antibodies.

In line with the previous limitation, the reliance on commercially available antibodies has made, on occasions, these experimental chapters challenging. While other experimental settings have found a way of overcoming non-valid or non-existent antibodies, human tissue samples can only rely on these antibodies. In my case, multiple non-specific bands due to antibodies or sample preparation have made this work really hard, but more importantly, challenges the notion of our strong reliance on externally validated antibodies. This unknown antibody validation may limit our interpretation of human experimental findings. Future technologies should allow us to move away from commercially available antibodies, and more towards an unbiased approach, which is probably already occurring with the so-called 'omics' approach. Although these technologies may seem expensive at first, research knowledge (as a fundamental principle) will benefit in the long-run from valid and reproducible findings. On the other hand, a strong reliance on unknown antibodies, will complicate the interpretation of our literature and will decrease the rigour of our field.

- Human recruitment, sample size and lifestyles.

We know each human is different to another. In this regards, the same holds true with the human participants recruited for the experiments described in this thesis. While only as much 'standardisation' can be done (e.g., nutrition), human lifestyle is hard to modify and in some

cases even discouraged (to simulate real-life interventions). The present studies did recruit participants with divergent genetic backgrounds (~10 different nationalities), from different areas of the Melbourne metropolitan area, and with very different lifestyles. In efforts to reduce the variability of these studies, nutritional intake was standardised for at least 24 hours before the muscle sample collection; however, any other factors before those 24 h could still affect our findings. Given the diverse nature of our target study population, any significant difference could also be interpreted as a possibly even larger effect if it was performed in a fully controlled setting. Furthermore, it could be argued that a bigger sample size may be needed. However, in my opinion, it is sometimes ethically and morally questionable how important our research questions are to increase even more the number of muscle biopsies and participants needed for the study.

6.5 Future research

Most of the time a research project leads to more questions than answers, and I believe this is the case with this PhD thesis. In an effort to inform future research, I will highlight some potential areas of research that may provide interesting and valuable findings in the future:

1- Autophagy and mitophagy in skeletal muscle

The 'self-eating' process of the cell, called autophagy, is gaining research interest due to its extensive involvement in multiple beneficial adaptations. I believe this thesis has scratched the surface on how autophagy is regulated following exercise. Unfortunately, due to time constraints I have not been able to explore some outstanding questions raised during this thesis in conjunction with my supervisors. While basic research is showing the autophagy machinery is more complex than once anticipated, human research studies are still relying on LC3-II protein levels. Future research should explore the regulation of the different atg8 family members following exercise, and explore whether skeletal muscle relies on one or multiple subfamily members for the exercise-induced increases in autophagy flux. I believe this will be a great starting point for appropriately quantifying autophagy flux levels. Furthermore, due to time I have not been able to explore mitophagy as deeply as I would have loved to. While mitophagy is a challenging process to explore in human tissue, I believe isolated mitochondria and immunofluorescence approaches will provide useful information on how this process is regulated following exercise, and I hope this will be addressed in future research.

2 - Mitochondrial stress and the exercise response

Multiple mitochondrial signalling pathways are relatively well-understood in the context of exercise (e.g., mitochondrial biogenesis). Less attention has been paid to the involvement of other mitochondrial pathways in exercise probably due to the difficulty to measure or quantify (i.e., mitochondrial dynamics). Also, mitochondrial retrograde signalling, by which

mitochondria communicate their status to the nucleus, is an area with abundant research in lower organisms and *in vitro* studies, but remains unexplored in the context of skeletal muscle. I believe this will be a fruitful area of research in the future, especially elucidating the adaptive and maladaptive mechanisms of this intracellular communication.

Another interesting point is the involvement of mitochondrial stress in the very important exercise-induced phosphorylation of AMPK and GLUT4 translocation to the membrane. Interestingly, it was recently shown by Beth Levine's group that the exercise-induced increase in AMPK phosphorylation and GLUT4 translocation, key drivers of the beneficial exercise response, were disrupted in $tlr9^{-/-}$ mice (93). This is important because TLR9 binds to mtDNA following exercise, and seemed a needed interaction to increase the phosphorylation of AMPK and subsequent events (i.e., GLUT4 translocation). This research posed the question as to how the mtDNA arrives to the endosomal compartment and interacts with TLR9. While this is currently unknown, the findings from Chapter 4 (together with those previously published (161, 320)) offer the possibility of mitochondrial disturbances following exercise allowing mtDNA to escape mitochondria and interact with other organelles (e.g., endosome). Furthermore, this could also occur through mitochondrial-derived vesicles that may be targeted to the endosome. Interestingly, this would explain why phosphorylation of AMPK is mainly seen in high-intensity exercises (322), where this mitochondrial stress has been observed, as well as to why in highly-trained athletes this exercise-induced AMPK phosphorylation is blunted (344).

3 - Mitochondrial stress in peripheral tissues

This thesis is skeletal muscle-focused; however, during the preparation of this thesis multiple research studies of excellent quality have brought to my attention the effects of exercise on peripheral tissues. Specifically, a study showed that high-intensity exercise led to an increased

mitochondrial stress in cardiac tissue (319). Although most of the associated benefits of exercise are linked to what occurs in skeletal muscle, I believe we are limiting our perspective to the main tissue involved during exercise. That said, I believe the cardiac muscle is a very important tissue that also contracts to different extents during exercise, and may provide similar or greater beneficial effects to whole-body metabolism. Specially, given the high prevalence of cardiac dysfunction, which paradoxically happens to a lower extent in endurance athletes or highly-trained individuals, it will be an important area of research. Other tissues crucial for metabolism, like liver or adipose tissue, will also provide insightful knowledge to the growing body of evidence of the health benefits of exercise.

4 – Exercise-induced tissue cross-talk

Similarly to the previous question, multiple excellent research groups have shown in the last 20 years how muscle, and other tissues, are able to communicate with each other by secreting peptides, vesicles, and/or proteins. In this regard, mitochondria are known to be transferred between different cell types (345), and even to communicate their 'stress' status to peripheral tissues to prime them for a possible stressful event (346). While exercise has been shown to result in increased levels of circulating mtDNA (347), whether this reflects increased release of mitochondria, or mitochondrial-derived proteins/vesicles, to the circulation remains to be elucidated. In fact, some post-exercise TEM images from chapter 4 had some vesicles between skeletal muscle fibres, which in some cases seemed to contain mitochondria. Furthermore, if this secreted mitochondria originate from the skeletal muscle, and have a positive priming event for other important tissues, this will provide invaluable information for the prevention of multiple mitochondrial-associated diseases and further our understanding of mitochondria.

5 - Beneficial or detrimental effects of mitochondrial stress

Suboptimal mitochondrial characteristics are considered a hallmark of multiple diseases and aging. This would suggest that mitochondrial stress and disturbance (as observed in Chapter 4) will have negative health implications, as it will 'damage' the mitochondrial pool. Multiple studies in lower organisms (i.e., *Caenorhabditis elegans*) have repeatedly shown that a low mitochondrial stress can have long-standing health benefits and lifespan extension (192, 194). More importantly, some pharmacological agents widely used in humans, like metformin, can have a negative effect on mitochondrial enzyme activity, but paradoxically improve health-related parameters (348). I believe my study has shown for the first time, in humans, that this mitochondrial-stress induced by a physiological stimulus (i.e., SIE) may have a positive effect on long-term mitochondrial adaptations (improved mitochondrial respiratory function and increased mitochondrial size; Chapter 5). Furthermore, this may be one of the mechanisms by which SIT has been proposed to be as, or even more, effective than MICT for some health-related adaptations (e.g., insulin sensitivity) (332). Future research should address and comprehensively characterise the beneficial and detrimental effects of mitochondrial stress (some have been highlighted in Chapter 2), which most likely will depend on the severity, persistence, and reversibility of the stimulus. Furthermore, it will help elucidate what pathways are differentially regulated in adaptive and maladaptive mitochondrial stress and could help inform future research and treatments.

6 - Mitochondrial adaptations and endurance performance

It is commonly accepted that increases in both mitochondrial content and respiratory function will positively affect changes in endurance performance. However, the present thesis highlights some complex interrelationships between training-induced adaptations in mitochondrial characteristics and endurance performance. The current thesis raises question as to whether mitochondrial content is more important than respiratory function for improvements in endurance performance. In fact, this aligns with the literature review, where mitochondrial

content has been consistently associated with enhanced endurance performance, while respiratory function seems to be less consistent. Whether these differences are caused by underlying technical issues, or whether these different adaptations have specific roles in endurance performance remain to be fully elucidated. Furthermore, I believe this research will provide a framework for exercise prescription that could differ based on whether the aim of the training is enhancing endurance performance or achieving a 'healthy' mitochondrial profile. While this currently remains speculative, I look forward to seeing this field continue to grow.

6.6 Practical applications

Exercise prescription in an important component of programs aiming at improving endurance performance and cardiorespiratory fitness for health purposes. This thesis provides new information related to the common upregulation of autophagy flux following exercise, and the divergent mitochondrial remodelling responses to high-volume (MICE) and high-intensity exercise (SIE) and training (MICT and SIT).

It can be suggested that MICE stimulates markers of mitochondrial biogenesis resulting in an increased skeletal muscle mitochondrial content following weeks of MICT. This type of exercise does not seem to disrupt or compromise mitochondria, which may also explain why this often does not result in an improved mitochondrial respiratory function. On the other hand, SIE elicits a robust mitochondrial stress with concomitant mitochondrial morphological and cristae alterations. This results in a mitochondrial-stress response together with the upregulation of fission and mitophagy signalling. Despite the increased expression of markers of mitochondrial biogenesis following SIT, the increase in mitochondrial content may be compromised due to the constant homeostatic disturbance and quality control. Alternatively, this results in an increased mitochondrial respiratory function of the mitochondrial pool due to the increased turnover mitochondrial proteins.

The current thesis sets up a framework in which high-volume exercise increases the content of mitochondria while high-intensity exercise challenges those mitochondria and enhances their respiratory function due to the ‘survival of the best’. It could be suggested that the combination of both types of exercise may render the optimal prescription to accomplish both improvements in mitochondrial content and respiratory function, and ultimately endurance performance. This may in fact agree with the commonly observed training prescription in endurance athletes called polarised training.

7. References

1. Coyle EF. Integration of the physiological factors determining endurance performance ability. Exerc Sport Sci Rev. 1995;23:25-63.
2. Coyle EF, Feltner ME, Kautz SA, Hamilton MT, Montain SJ, Baylor AM, et al. Physiological and biomechanical factors associated with elite endurance cycling performance. Medicine and science in sports and exercise. 1991;23(1):93-107.
3. McCormick A, Meijen C, Marcora S. Psychological Determinants of Whole-Body Endurance Performance. Sports medicine (Auckland, NZ). 2015;45(7):997-1015.
4. Hill AV, Lupton H. Muscular Exercise, Lactic Acid, and the Supply and Utilization of Oxygen. QJM: An International Journal of Medicine. 1923;os-16(62):135-71.
5. Blomstrand E, Rådegran G, Saltin B. Maximum rate of oxygen uptake by human skeletal muscle in relation to maximal activities of enzymes in the Krebs cycle. The Journal of physiology. 1997;501 (Pt 2)(Pt 2):455-60.
6. Boushel R, Saltin B. Ex vivo measures of muscle mitochondrial capacity reveal quantitative limits of oxygen delivery by the circulation during exercise. The international journal of biochemistry & cell biology. 2013;45(1):68-75.
7. Gollnick PD, Armstrong RB, C W Saubert t, Piehl K, Saltin B. Enzyme activity and fiber composition in skeletal muscle of untrained and trained men. Journal of Applied Physiology. 1972;33(3):312-9.
8. Rasmussen UF, Rasmussen HN, Krustrup P, Quistorff B, Saltin B, Bangsbo J. Aerobic metabolism of human quadriceps muscle: in vivo data parallel measurements on isolated mitochondria. American journal of physiology Endocrinology and metabolism. 2001;280(2):E301-7.
9. Costill DL, Thomason H, Roberts E. Fractional utilization of the aerobic capacity during distance running. Medicine and science in sports. 1973;5(4):248-52.
10. Foster C, Costill DL, Daniels JT, Fink WJ. Skeletal muscle enzyme activity, fiber composition and VO2 max in relation to distance running performance. European journal of applied physiology and occupational physiology. 1978;39(2):73-80.
11. Ivy JL, Withers RT, Van Handel PJ, Elger DH, Costill DL. Muscle respiratory capacity and fiber type as determinants of the lactate threshold. Journal of applied physiology: respiratory, environmental and exercise physiology. 1980;48(3):523-7.
12. Coyle EF, Coggan AR, Hopper MK, Walters TJ. Determinants of endurance in well-trained cyclists. Journal of applied physiology (Bethesda, Md : 1985). 1988;64(6):2622-30.
13. Coyle EF, Martin WH, 3rd, Bloomfield SA, Lowry OH, Holloszy JO. Effects of detraining on responses to submaximal exercise. Journal of applied physiology (Bethesda, Md : 1985). 1985;59(3):853-9.
14. Hagberg JM, Coyle EF. Physiological determinants of endurance performance as studied in competitive racewalkers. Medicine and science in sports and exercise. 1983;15(4):287-9.
15. Joyner MJ, Coyle EF. Endurance exercise performance: the physiology of champions. The Journal of physiology. 2008;586(1):35-44.
16. Hill AV, Long CNH, Lupton H. Muscular exercise, lactic acid and the supply and utilisation of oxygen.— Parts VII–VIII. Proceedings of the Royal Society of London Series B, Containing Papers of a Biological Character. 1924;97(682):155-76.
17. Pedersen BK, Saltin B. Exercise as medicine - evidence for prescribing exercise as therapy in 26 different chronic diseases. Scandinavian journal of medicine & science in sports. 2015;25 Suppl 3:1-72.
18. Lundby C, Montero D, Joyner M. Biology of VO2 max: looking under the physiology lamp. Acta physiologica (Oxford, England). 2017;220(2):218-28.

19. Lundby C, Montero D, Joyner M. Biology of VO(2) max: looking under the physiology lamp. Acta physiologica (Oxford, England). 2017;220(2):218-28.
20. Skattebo Ø, Calbet JAL, Rud B, Capelli C, Hallén J. Contribution of oxygen extraction fraction to maximal oxygen uptake in healthy young men. Acta physiologica (Oxford, England). 2020:e13486.
21. Kumagai S, Tanaka K, Matsuura Y, Matsuzaka A, Hirakoba K, Asano K. Relationships of the anaerobic threshold with the 5 km, 10 km, and 10 mile races. European journal of applied physiology and occupational physiology. 1982;49(1):13-23.
22. Morgan DW, Baldini FD, Martin PE, Kohrt WM. Ten kilometer performance and predicted velocity at V02max among well-trained male runners. Medicine & Science in Sports & Exercise. 1989;21(1).
23. Jamnick NA, Pettitt RW, Granata C, Pyne DB, Bishop DJ. An Examination and Critique of Current Methods to Determine Exercise Intensity. Sports medicine (Auckland, NZ). 2020.
24. Jamnick NA, Botella J, Pyne DB, Bishop DJ. Manipulating graded exercise test variables affects the validity of the lactate threshold and [Formula: see text]. PloS one. 2018;13(7):e0199794.
25. Barnes KR, Kilding AE. Running economy: measurement, norms, and determining factors. Sports medicine - open. 2015;1(1):8.
26. Barnes KR, McGuigan MR, Kilding AE. Lower-body determinants of running economy in male and female distance runners. Journal of strength and conditioning research. 2014;28(5):1289-97.
27. Kunimasa Y, Sano K, Oda T, Nicol C, Komi PV, Locatelli E, et al. Specific muscle-tendon architecture in elite Kenyan distance runners. Scandinavian journal of medicine & science in sports. 2014;24(4):e269-74.
28. Moseley L, Achten J, Martin JC, Jeukendrup AE. No differences in cycling efficiency between world-class and recreational cyclists. International journal of sports medicine. 2004;25(5):374-9.
29. Conley DL, Krahenbuhl GS. Running economy and distance running performance of highly trained athletes. Medicine and science in sports and exercise. 1980;12(5):357-60.
30. Weibel ER, Taylor CR, Hoppeler H. The concept of symmorphosis: a testable hypothesis of structure-function relationship. Proceedings of the National Academy of Sciences of the United States of America. 1991;88(22):10357-61.
31. Wagner PD. Determinants of maximal oxygen transport and utilization. Annual review of physiology. 1996;58:21-50.
32. Bassett DR, Jr., Howley ET. Limiting factors for maximum oxygen uptake and determinants of endurance performance. Medicine and science in sports and exercise. 2000;32(1):70-84.
33. Amann M. Pulmonary system limitations to endurance exercise performance in humans. Experimental physiology. 2012;97(3):311-8.
34. Dempsey JA, Wagner PD. Exercise-induced arterial hypoxemia. Journal of applied physiology (Bethesda, Md : 1985). 1999;87(6):1997-2006.
35. Hellsten Y, Nyberg M. Cardiovascular Adaptations to Exercise Training. Comprehensive Physiology. 2015;6(1):1-32.
36. Convertino VA. Heart rate and sweat rate responses associated with exercise-induced hypervolemia. Medicine and science in sports and exercise. 1983;15(1):77-82.
37. Montero D, Cathomen A, Jacobs RA, Fluck D, de Leur J, Keiser S, et al. Haematological rather than skeletal muscle adaptations contribute to the increase in peak oxygen uptake induced by moderate endurance training. The Journal of physiology. 2015;593(20):4677-88.
38. Bonne TC, Doucende G, Fluck D, Jacobs RA, Nordsborg NB, Robach P, et al. Phlebotomy eliminates the maximal cardiac output response to six weeks of exercise training. American journal of physiology Regulatory, integrative and comparative physiology. 2014;306(10):R752-60.
39. Convertino VA, Mack GW, Nadel ER. Elevated central venous pressure: a consequence of exercise training-induced hypervolemia? The American journal of physiology. 1991;260(2 Pt 2):R273-7.
40. Lundby C, Robach P. Performance Enhancement: What Are the Physiological Limits? Physiology (Bethesda, Md). 2015;30(4):282-92.

41. Bloor CM. Angiogenesis during exercise and training. Angiogenesis. 2005;8(3):263-71.
42. Bishop DJ, Granata C, Eynon N. Can we optimise the exercise training prescription to maximise improvements in mitochondria function and content? Biochimica et biophysica acta. 2014;1840(4):1266-75.
43. Bishop DJ, Botella J, Genders AJ, Lee MJ, Saner NJ, Kuang J, et al. High-Intensity Exercise and Mitochondrial Biogenesis: Current Controversies and Future Research Directions. Physiology (Bethesda, Md). 2019;34(1):56-70.
44. Mitchell EA, Martin NRW, Bailey SJ, Ferguson RA. Critical power is positively related to skeletal muscle capillarity and type I muscle fibers in endurance-trained individuals. Journal of applied physiology (Bethesda, Md : 1985). 2018;125(3):737-45.
45. Egginton S. Invited review: activity-induced angiogenesis. Pflugers Archiv : European journal of physiology. 2009;457(5):963-77.
46. Skorjanc D, Jaschinski F, Heine G, Pette D. Sequential increases in capillarization and mitochondrial enzymes in low-frequency-stimulated rabbit muscle. The American journal of physiology. 1998;274(3):C810-8.
47. Takakura H, Furuichi Y, Yamada T, Jue T, Ojino M, Hashimoto T, et al. Endurance training facilitates myoglobin desaturation during muscle contraction in rat skeletal muscle. Sci Rep. 2015;5:9403.
48. Hickson RC. Skeletal muscle cytochrome c and myoglobin, endurance, and frequency of training. Journal of applied physiology: respiratory, environmental and exercise physiology. 1981;51(3):746-9.
49. Yamada T, Furuichi Y, Takakura H, Hashimoto T, Hanai Y, Jue T, et al. Interaction between myoglobin and mitochondria in rat skeletal muscle. Journal of applied physiology (Bethesda, Md : 1985). 2013;114(4):490-7.
50. Granata C, Oliveira RSF, Little JP, Bishop DJ. Forty high-intensity interval training sessions blunt exercise-induced changes in the nuclear protein content of PGC-1α and p53 in human skeletal muscle. American journal of physiology Endocrinology and metabolism. 2020;318(2):E224-e36.
51. Bishop DJ, Botella J, Granata C. CrossTalk opposing view: Exercise training volume is more important than training intensity to promote increases in mitochondrial content. The Journal of physiology. 2019;597(16):4115-8.
52. Granata C, Oliveira RS, Little JP, Renner K, Bishop DJ. Training intensity modulates changes in PGC-1alpha and p53 protein content and mitochondrial respiration, but not markers of mitochondrial content in human skeletal muscle. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 2016;30(2):959-70.
53. Hood DA, Uguccioni G, Vainshtein A, D'Souza D. Mechanisms of exercise-induced mitochondrial biogenesis in skeletal muscle: implications for health and disease. Comprehensive Physiology. 2011;1(3):1119-34.
54. MacInnis MJ, Skelly LE, Gibala MJ. CrossTalk proposal: Exercise training intensity is more important than volume to promote increases in human skeletal muscle mitochondrial content. The Journal of physiology. 2019;597(16):4111-3.
55. Jacobs RA, Rasmussen P, Siebenmann C, Diaz V, Gassmann M, Pesta D, et al. Determinants of time trial performance and maximal incremental exercise in highly trained endurance athletes. Journal of applied physiology (Bethesda, Md : 1985). 2011;111(5):1422-30.
56. Hoppeler H, Luthi P, Claassen H, Weibel ER, Howald H. The ultrastructure of the normal human skeletal muscle. A morphometric analysis on untrained men, women and well-trained orienteers. Pflugers Archiv : European journal of physiology. 1973;344(3):217-32.
57. Boushel R, Gnaiger E, Calbet JA, Gonzalez-Alonso J, Wright-Paradis C, Sondergaard H, et al. Muscle mitochondrial capacity exceeds maximal oxygen delivery in humans. Mitochondrion. 2011;11(2):303-7.

58. Boushel R, Gnaiger E, Larsen FJ, Helge JW, González-Alonso J, Ara I, et al. Maintained peak leg and pulmonary VO2 despite substantial reduction in muscle mitochondrial capacity. Scandinavian journal of medicine & science in sports. 2015;25 Suppl 4:135-43.
59. Gifford JR, Garten RS, Nelson AD, Trinity JD, Layec G, Witman MA, et al. Symmorphosis and skeletal muscle V̇O2 max : in vivo and in vitro measures reveal differing constraints in the exercise-trained and untrained human. The Journal of physiology. 2016;594(6):1741-51.
60. Sloniger MA, Cureton KJ, Prior BM, Evans EM. Lower extremity muscle activation during horizontal and uphill running. Journal of Applied Physiology. 1997;83(6):2073-9.
61. Chapman AR, Vicenzino B, Blanch P, Hodges PW. Patterns of leg muscle recruitment vary between novice and highly trained cyclists. Journal of electromyography and kinesiology : official journal of the International Society of Electrophysiological Kinesiology. 2008;18(3):359-71.
62. Larsen S, Nielsen J, Hansen CN, Nielsen LB, Wibrand F, Stride N, et al. Biomarkers of mitochondrial content in skeletal muscle of healthy young human subjects. The Journal of physiology. 2012;590(14):3349-60.
63. Jacobs RA, Lundby C. Mitochondria express enhanced quality as well as quantity in association with aerobic fitness across recreationally active individuals up to elite athletes. Journal of applied physiology (Bethesda, Md : 1985). 2013;114(3):344-50.
64. Zoll J, Sanchez H, N'Guessan B, Ribera F, Lampert E, Bigard X, et al. Physical activity changes the regulation of mitochondrial respiration in human skeletal muscle. The Journal of physiology. 2002;543(1):191-200.
65. Mettauer B, Zoll J, Sanchez H, Lampert E, Ribera F, Veksler V, et al. Oxidative capacity of skeletal muscle in heart failure patients versus sedentary or active control subjects. Journal of the American College of Cardiology. 2001;38(4):947-54.
66. Broskey NT, Boss A, Fares EJ, Greggio C, Gremion G, Schlüter L, et al. Exercise efficiency relates with mitochondrial content and function in older adults. Physiological reports. 2015;3(6).
67. Mogensen M, Bagger M, Pedersen PK, Fernström M, Sahlin K. Cycling efficiency in humans is related to low UCP3 content and to type I fibres but not to mitochondrial efficiency. The Journal of physiology. 2006;571(Pt 3):669-81.
68. van der Zwaard S, de Ruiter CJ, Noordhof DA, Sterrenburg R, Bloemers FW, de Koning JJ, et al. Maximal oxygen uptake is proportional to muscle fiber oxidative capacity, from chronic heart failure patients to professional cyclists. Journal of applied physiology (Bethesda, Md : 1985). 2016;121(3):636-45.
69. Mallory LA, Scheuermann BW, Hoelting BD, Weiss ML, McAllister RM, Barstow TJ. Influence of peak VO2 and muscle fiber type on the efficiency of moderate exercise. Medicine and science in sports and exercise. 2002;34(8):1279-87.
70. Coyle EF, W. H. Martin r, Sinacore DR, Joyner MJ, Hagberg JM, Holloszy JO. Time course of loss of adaptations after stopping prolonged intense endurance training. Journal of Applied Physiology. 1984;57(6):1857-64.
71. Skattebo Ø, Bjerring AW, Auensen M, Sarvari SI, Cumming KT, Capelli C, et al. Blood volume expansion does not explain the increase in peak oxygen uptake induced by 10 weeks of endurance training. European Journal of Applied Physiology. 2020;120(5):985-99.
72. Davies KJ, Maguire JJ, Brooks GA, Dallman PR, Packer L. Muscle mitochondrial bioenergetics, oxygen supply, and work capacity during dietary iron deficiency and repletion. The American journal of physiology. 1982;242(6):E418-27.
73. Wüst RCI, Houtkooper RH, Auwerx J. Confounding factors from inducible systems for spatiotemporal gene expression regulation. Journal of Cell Biology. 2020;219(7).
74. Bartlett MF, Fitzgerald LF, Nagarajan R, Hiroi Y, Kent JA. Oxidative ATP synthesis in human quadriceps declines during 4 minutes of maximal contractions. The Journal of physiology. 2020;598(10):1847-63.
75. Gray MW, Burger G, Lang BF. Mitochondrial Evolution. Science (New York, NY). 1999;283(5407):1476-81.

76. Trewin AJ, Berry BJ, Wojtovich AP. Exercise and Mitochondrial Dynamics: Keeping in Shape with ROS and AMPK. Antioxidants (Basel, Switzerland). 2018;7(1).
77. Chan DC. Fusion and fission: interlinked processes critical for mitochondrial health. Annual review of genetics. 2012;46:265-87.
78. Chan DC. Mitochondrial Dynamics and Its Involvement in Disease. Annu Rev Pathol. 2020;15:235-59.
79. Lee JE, Westrate LM, Wu H, Page C, Voeltz GK. Multiple dynamin family members collaborate to drive mitochondrial division. Nature. 2016;540(7631):139-43.
80. Saitoh T, Igura M, Obita T, Ose T, Kojima R, Maenaka K, et al. Tom20 recognizes mitochondrial presequences through dynamic equilibrium among multiple bound states. EMBO J. 2007;26(22):4777-87.
81. Pfanner N, Meijer M. Mitochondrial biogenesis: The Tom and Tim machine. Current Biology. 1997;7(2):R100-R3.
82. Weidberg H, Amon A. MitoCPR—A surveillance pathway that protects mitochondria in response to protein import stress. Science (New York, NY). 2018;360(6385):eaan4146.
83. Wrobel L, Topf U, Bragoszewski P, Wiese S, Sztolsztener ME, Oeljeklaus S, et al. Mistargeted mitochondrial proteins activate a proteostatic response in the cytosol. Nature. 2015;524(7566):485-8.
84. Topf U, Uszczynska-Ratajczak B, Chacinska A. Mitochondrial stress-dependent regulation of cellular protein synthesis. Journal of cell science. 2019;132(8):jcs226258.
85. Perry CG, Lally J, Holloway GP, Heigenhauser GJ, Bonen A, Spriet LL. Repeated transient mRNA bursts precede increases in transcriptional and mitochondrial proteins during training in human skeletal muscle. The Journal of physiology. 2010;588(Pt 23):4795-810.
86. Miller BF, Hamilton KL. A perspective on the determination of mitochondrial biogenesis. American journal of physiology Endocrinology and metabolism. 2012;302(5):E496-9.
87. Schwanhäusser B, Gossen M, Dittmar G, Selbach M. Global analysis of cellular protein translation by pulsed SILAC. PROTEOMICS. 2009;9(1):205-9.
88. Weibel ER, Hoppeler H. Exercise-induced maximal metabolic rate scales with muscle aerobic capacity. The Journal of experimental biology. 2005;208(Pt 9):1635-44.
89. Granata C, Oliveira RSF, Little JP, Renner K, Bishop DJ. Sprint-interval but not continuous exercise increases PGC-1α protein content and p53 phosphorylation in nuclear fractions of human skeletal muscle. Scientific reports. 2017;7:44227-.
90. Fiorenza M, Gunnarsson TP, Hostrup M, Iaia FM, Schena F, Pilegaard H, et al. Metabolic stress-dependent regulation of the mitochondrial biogenic molecular response to high-intensity exercise in human skeletal muscle. The Journal of physiology. 2018;596(14):2823-40.
91. Herzig S, Shaw RJ. AMPK: guardian of metabolism and mitochondrial homeostasis. Nature reviews Molecular cell biology. 2018;19(2):121-35.
92. McConell GK. It's well and truly time to stop stating that AMPK regulates glucose uptake and fat oxidation during exercise. American journal of physiology Endocrinology and metabolism. 2020;318(4):E564-e7.
93. Liu Y, Nguyen PT, Wang X, Zhao Y, Meacham CE, Zou Z, et al. TLR9 and beclin 1 crosstalk regulates muscle AMPK activation in exercise. Nature. 2020;578(7796):605-9.
94. Barrès R, Yan J, Egan B, Treebak Jonas T, Rasmussen M, Fritz T, et al. Acute Exercise Remodels Promoter Methylation in Human Skeletal Muscle. Cell metabolism. 2012;15(3):405-11.
95. Egan B, Carson BP, Garcia-Roves PM, Chibalin AV, Sarsfield FM, Barron N, et al. Exercise intensity-dependent regulation of peroxisome proliferator-activated receptor γ coactivator-1α mRNA abundance is associated with differential activation of upstream signalling kinases in human skeletal muscle. The Journal of physiology. 2010;588(10):1779-90.
96. Eisner V, Lenaers G, Hajnóczky G. Mitochondrial fusion is frequent in skeletal muscle and supports excitation-contraction coupling. The Journal of cell biology. 2014;205(2):179-95.

97. Guillery O, Malka F, Landes T, Guillou E, Blackstone C, Lombes A, et al. Metalloprotease-mediated OPA1 processing is modulated by the mitochondrial membrane potential. Biology of the cell. 2008;100(5):315-25.
98. Chan DC. Mitochondrial fusion and fission in mammals. Annual review of cell and developmental biology. 2006;22:79-99.
99. Gordaliza-Alaguero I, Canto C, Zorzano A. Metabolic implications of organelle-mitochondria communication. EMBO reports. 2019;20(9):e47928.
100. Giacomello M, Pyakurel A, Glytsou C, Scorrano L. The cell biology of mitochondrial membrane dynamics. Nature reviews Molecular cell biology. 2020.
101. Franco A, Kitsis RN, Fleischer JA, Gavathiotis E, Kornfeld OS, Gong G, et al. Correcting mitochondrial fusion by manipulating mitofusin conformations. Nature. 2016;540(7631):74-9.
102. Liesa M, Palacín M, Zorzano A. Mitochondrial dynamics in mammalian health and disease. Physiol Rev. 2009;89(3):799-845.
103. Liesa M, Shirihai OS. Mitochondrial dynamics in the regulation of nutrient utilization and energy expenditure. Cell metabolism. 2013;17(4):491-506.
104. Mattie S, Riemer J, Wideman JG, McBride HM. A new mitofusin topology places the redox-regulated C terminus in the mitochondrial intermembrane space. The Journal of cell biology. 2018;217(2):507-15.
105. Frezza C, Cipolat S, Martins de Brito O, Micaroni M, Beznoussenko GV, Rudka T, et al. OPA1 controls apoptotic cristae remodeling independently from mitochondrial fusion. Cell. 2006;126(1):177-89.
106. MacVicar T, Langer T. OPA1 processing in cell death and disease – the long and short of it. Journal of cell science. 2016;129(12):2297-306.
107. Ban T, Ishihara T, Kohno H, Saita S, Ichimura A, Maenaka K, et al. Molecular basis of selective mitochondrial fusion by heterotypic action between OPA1 and cardiolipin. Nature cell biology. 2017;19(7):856-63.
108. Anand R, Wai T, Baker MJ, Kladt N, Schauss AC, Rugarli E, et al. The i-AAA protease YME1L and OMA1 cleave OPA1 to balance mitochondrial fusion and fission. The Journal of cell biology. 2014;204(6):919-29.
109. Picard M, McManus MJ, Csordás G, Várnai P, Dorn GW, 2nd, Williams D, et al. Trans-mitochondrial coordination of cristae at regulated membrane junctions. Nature communications. 2015;6:6259.
110. Vincent AE, White K, Davey T, Philips J, Ogden RT, Lawless C, et al. Quantitative 3D Mapping of the Human Skeletal Muscle Mitochondrial Network. Cell reports. 2019;27(1):321.
111. Pham AH, McCaffery JM, Chan DC. Mouse lines with photo-activatable mitochondria to study mitochondrial dynamics. Genesis. 2012;50(11):833-43.
112. Picard M, Gentil BJ, McManus MJ, White K, St Louis K, Gartside SE, et al. Acute exercise remodels mitochondrial membrane interactions in mouse skeletal muscle. Journal of applied physiology (Bethesda, Md : 1985). 2013;115(10):1562-71.
113. Eisner V, Lenaers G, Hajnóczky G. Mitochondrial fusion is frequent in skeletal muscle and supports excitation-contraction coupling. The Journal of cell biology. 2014;205(2):179-95.
114. Nielsen J, Gejl KD, Hey-Mogensen M, Holmberg HC, Suetta C, Krustrup P, et al. Plasticity in mitochondrial cristae density allows metabolic capacity modulation in human skeletal muscle. The Journal of physiology. 2017;595(9):2839-47.
115. Mishra P, Varuzhanyan G, Pham AH, Chan DC. Mitochondrial Dynamics is a Distinguishing Feature of Skeletal Muscle Fiber Types and Regulates Organellar Compartmentalization. Cell metabolism. 2015;22(6):1033-44.
116. Arribat Y, Broskey NT, Greggio C, Boutant M, Conde Alonso S, Kulkarni SS, et al. Distinct patterns of skeletal muscle mitochondria fusion, fission and mitophagy upon duration of exercise training. Acta physiologica (Oxford, England). 2019;225(2):e13179-e.

117. Kruse R, Pedersen AJT, Kristensen JM, Petersson SJ, Wojtaszewski JFP, Højlund K. Intact initiation of autophagy and mitochondrial fission by acute exercise in skeletal muscle of patients with Type 2 diabetes. Clinical science (London, England : 1979). 2017;131(1):37-47.
118. Tarpey MD, Davy KP, McMillan RP, Bowser SM, Halliday TM, Boutagy NE, et al. Skeletal muscle autophagy and mitophagy in endurance-trained runners before and after a high-fat meal. Mol Metab. 2017;6(12):1597-609.
119. Balan E, Schwalm C, Naslain D, Nielens H, Francaux M, Deldicque L. Regular Endurance Exercise Promotes Fission, Mitophagy, and Oxidative Phosphorylation in Human Skeletal Muscle Independently of Age. Frontiers in physiology. 2019;10:1088-.
120. Schwalm C, Deldicque L, Francaux M. Lack of Activation of Mitophagy during Endurance Exercise in Human. Medicine and science in sports and exercise. 2017;49(8):1552-61.
121. Jamart C, Francaux M, Millet GY, Deldicque L, Frère D, Féasson L. Modulation of autophagy and ubiquitin-proteasome pathways during ultra-endurance running. Journal of applied physiology (Bethesda, Md : 1985). 2012;112(9):1529-37.
122. Cartoni R, Leger B, Hock MB, Praz M, Crettenand A, Pich S, et al. Mitofusins 1/2 and ERRalpha expression are increased in human skeletal muscle after physical exercise. The Journal of physiology. 2005;567(Pt 1):349-58.
123. Huertas JR, Ruiz-Ojeda FJ, Plaza-Díaz J, Nordsborg NB, Martín-Albo J, Rueda-Robles A, et al. Human muscular mitochondrial fusion in athletes during exercise. The FASEB Journal. 2019;33(11):12087-98.
124. Banfi G, Dolci A, Verna R, Corsi MM. Exercise raises serum heat-shock protein 70 (Hsp70) levels. Clinical Chemistry and Laboratory Medicine (CCLM). 2004;42(12):1445.
125. Arribat Y, Broskey NT, Greggio C, Boutant M, Conde Alonso S, Kulkarni SS, et al. Distinct patterns of skeletal muscle mitochondria fusion, fission and mitophagy upon duration of exercise training. Acta physiologica (Oxford, England). 2019;225(2):e13179.
126. Hernández-Alvarez MI, Thabit H, Burns N, Shah S, Brema I, Hatunic M, et al. Subjects with early-onset type 2 diabetes show defective activation of the skeletal muscle PGC-1{alpha}/Mitofusin-2 regulatory pathway in response to physical activity. Diabetes Care. 2010;33(3):645-51.
127. Meinild Lundby AK, Jacobs RA, Gehrig S, de Leur J, Hauser M, Bonne TC, et al. Exercise training increases skeletal muscle mitochondrial volume density by enlargement of existing mitochondria and not de novo biogenesis. Acta physiologica (Oxford, England). 2018;222(1):10.1111/apha.12905.
128. Fealy CE, Mulya A, Lai N, Kirwan JP. Exercise training decreases activation of the mitochondrial fission protein dynamin-related protein-1 in insulin-resistant human skeletal muscle. Journal of applied physiology (Bethesda, Md : 1985). 2014;117(3):239-45.
129. Axelrod CL, Fealy CE, Mulya A, Kirwan JP. Exercise training remodels human skeletal muscle mitochondrial fission and fusion machinery towards a pro-elongation phenotype. Acta physiologica (Oxford, England). 2019;225(4):e13216-e.
130. Chen H, Vermulst M, Wang YE, Chomyn A, Prolla TA, McCaffery JM, et al. Mitochondrial fusion is required for mtDNA stability in skeletal muscle and tolerance of mtDNA mutations. Cell. 2010;141(2):280-9.
131. Ross JM, Coppotelli G, Branca RM, Kim KM, Lehtiö J, Sinclair DA, et al. Voluntary exercise normalizes the proteomic landscape in muscle and brain and improves the phenotype of progeroid mice. Aging Cell.0(0):e13029.
132. Losón OC, Song Z, Chen H, Chan DC. Fis1, Mff, MiD49, and MiD51 mediate Drp1 recruitment in mitochondrial fission. Molecular biology of the cell. 2013;24(5):659-67.
133. Osellame LD, Singh AP, Stroud DA, Palmer CS, Stojanovski D, Ramachandran R, et al. Cooperative and independent roles of the Drp1 adaptors Mff, MiD49 and MiD51 in mitochondrial fission. Journal of cell science. 2016;129(11):2170-81.
134. Zhang Z, Sliter DA, Bleck CKE, Ding S. Fis1 deficiencies differentially affect mitochondrial quality in skeletal muscle. Mitochondrion. 2019;49:217-26.

135. Pei S, Minhajuddin M, Adane B, Khan N, Stevens BM, Mack SC, et al. AMPK/FIS1-Mediated Mitophagy Is Required for Self-Renewal of Human AML Stem Cells. Cell Stem Cell. 2018;23(1):86-100.e6.
136. Taguchi N, Ishihara N, Jofuku A, Oka T, Mihara K. Mitotic phosphorylation of dynamin-related GTPase Drp1 participates in mitochondrial fission. The Journal of biological chemistry. 2007;282(15):11521-9.
137. Cereghetti GM, Stangherlin A, Martins de Brito O, Chang CR, Blackstone C, Bernardi P, et al. Dephosphorylation by calcineurin regulates translocation of Drp1 to mitochondria. Proceedings of the National Academy of Sciences of the United States of America. 2008;105(41):15803-8.
138. Toyama EQ, Herzig S, Courchet J, Lewis TL, Jr., Losón OC, Hellberg K, et al. Metabolism. AMP-activated protein kinase mediates mitochondrial fission in response to energy stress. Science (New York, NY). 2016;351(6270):275-81.
139. Palmer CS, Elgass KD, Parton RG, Osellame LD, Stojanovski D, Ryan MT. Adaptor proteins MiD49 and MiD51 can act independently of Mff and Fis1 in Drp1 recruitment and are specific for mitochondrial fission. The Journal of biological chemistry. 2013;288(38):27584-93.
140. Cho B, Cho HM, Jo Y, Kim HD, Song M, Moon C, et al. Constriction of the mitochondrial inner compartment is a priming event for mitochondrial division. Nature communications. 2017;8:15754.
141. Spier A, Sachse M, Tham NT, Matondo M, Cossart P, Stavru F. Bacterial FtsZ induces mitochondrial fission in human cells. bioRxiv. 2020:2020.01.24.917146.
142. Lavorato M, Loro E, Debattisti V, Khurana TS, Franzini-Armstrong C. Elongated mitochondrial constrictions and fission in muscle fatigue. Journal of cell science. 2018;131(23):jcs221028.
143. Moore TM, Zhou Z, Cohn W, Norheim F, Lin AJ, Kalajian N, et al. The impact of exercise on mitochondrial dynamics and the role of Drp1 in exercise performance and training adaptations in skeletal muscle. Mol Metab. 2019;21:51-67.
144. Laker RC, Drake JC, Wilson RJ, Lira VA, Lewellen BM, Ryall KA, et al. Ampk phosphorylation of Ulk1 is required for targeting of mitochondria to lysosomes in exercise-induced mitophagy. Nature communications. 2017;8(1):548.
145. Pickles S, Vigie P, Youle RJ. Mitophagy and Quality Control Mechanisms in Mitochondrial Maintenance. Current biology : CB. 2018;28(4):R170-r85.
146. Villa E, Marchetti S, Ricci JE. No Parkin Zone: Mitophagy without Parkin. Trends in cell biology. 2018;28(11):882-95.
147. Vincow ES, Thomas RE, Merrihew GE, Shulman NJ, Bammler TK, MacDonald JW, et al. Autophagy accounts for approximately one-third of mitochondrial protein turnover and is protein selective. Autophagy. 2019:1-14.
148. Lazarou M, Sliter DA, Kane LA, Sarraf SA, Wang C, Burman JL, et al. The ubiquitin kinase PINK1 recruits autophagy receptors to induce mitophagy. Nature. 2015;524(7565):309-14.
149. Nguyen TN, Padman BS, Lazarou M. Deciphering the Molecular Signals of PINK1/Parkin Mitophagy. Trends in cell biology. 2016;26(10):733-44.
150. Carter HN, Kim Y, Erlich AT, Zarrin-Khat D, Hood DA. Autophagy and mitophagy flux in young and aged skeletal muscle following chronic contractile activity. The Journal of physiology. 2018;596(16):3567-84.
151. Scorrano L, De Matteis MA, Emr S, Giordano F, Hajnóczky G, Kornmann B, et al. Coming together to define membrane contact sites. Nature communications. 2019;10(1):1287.
152. McWilliams TG, Prescott AR, Montava-Garriga L, Ball G, Singh F, Barini E, et al. Basal Mitophagy Occurs Independently of PINK1 in Mouse Tissues of High Metabolic Demand. Cell metabolism. 2018;27(2):439-49.e5.
153. Chen CCW, Erlich AT, Hood DA. Role of Parkin and endurance training on mitochondrial turnover in skeletal muscle. Skelet Muscle. 2018;8(1):10-.
154. Gouspillou G, Godin R, Piquereau J, Picard M, Mofarrahi M, Mathew J, et al. Protective role of Parkin in skeletal muscle contractile and mitochondrial function. The Journal of physiology. 2018;596(13):2565-79.

155. Leduc-Gaudet JP, Reynaud O, Hussain SN, Gouspillou G. Parkin overexpression protects from ageing-related loss of muscle mass and strength. The Journal of physiology. 2019;597(7):1975-91.
156. Bock FJ, Tait SWG. Mitochondria as multifaceted regulators of cell death. Nature reviews Molecular cell biology. 2020;21(2):85-100.
157. Sliter DA, Martinez J, Hao L, Chen X, Sun N, Fischer TD, et al. Parkin and PINK1 mitigate STING-induced inflammation. Nature. 2018;561(7722):258-62.
158. Seabright AP, Fine NHF, Barlow JP, Lord SO, Musa I, Gray A, et al. AMPK activation induces mitophagy and promotes mitochondrial fission while activating TBK1 in a PINK1-Parkin independent manner. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 2020.
159. Drake JC, Laker RC, Wilson RJ, Zhang M, Yan Z. Exercise-induced mitophagy in skeletal muscle occurs in the absence of stabilization of Pink1 on mitochondria. Cell Cycle. 2019;18(1):1-6.
160. Chen CCW, Erlich AT, Crilly MJ, Hood DA. Parkin is required for exercise-induced mitophagy in muscle: impact of aging. American journal of physiology Endocrinology and metabolism. 2018;315(3):E404-E15.
161. P. D. Gollnick CDI, D. W. King. Advances in Experimental Medicine and Biology

1971. 69-85 p.
162. Brandt N, Gunnarsson TP, Bangsbo J, Pilegaard H. Exercise and exercise training-induced increase in autophagy markers in human skeletal muscle. Physiological reports. 2018;6(7):e13651-e.
163. Moller AB, Vendelbo MH, Christensen B, Clasen BF, Bak AM, Jorgensen JO, et al. Physical exercise increases autophagic signaling through ULK1 in human skeletal muscle. Journal of applied physiology (Bethesda, Md : 1985). 2015;118(8):971-9.
164. Fritzen AM, Madsen AB, Kleinert M, Treebak JT, Lundsgaard AM, Jensen TE, et al. Regulation of autophagy in human skeletal muscle: effects of exercise, exercise training and insulin stimulation. The Journal of physiology. 2016;594(3):745-61.
165. Needham EJ, Humphrey SJ, Cooke KC, Fazakerley DJ, Duan X, Parker BL, et al. Phosphoproteomics of Acute Cell Stressors Targeting Exercise Signaling Networks Reveal Drug Interactions Regulating Protein Secretion. Cell reports. 2019;29(6):1524-38.e6.
166. Hoffman NJ, Parker BL, Chaudhuri R, Fisher-Wellman KH, Kleinert M, Humphrey SJ, et al. Global Phosphoproteomic Analysis of Human Skeletal Muscle Reveals a Network of Exercise-Regulated Kinases and AMPK Substrates. Cell metabolism. 2015;22(5):922-35.
167. Nelson ME, Parker BL, Burchfield JG, Hoffman NJ, Needham EJ, Cooke KC, et al. Phosphoproteomics reveals conserved exercise-stimulated signaling and AMPK regulation of store-operated calcium entry. EMBO J. 2020;39(8):e104246.
168. Fessler E, Eckl EM, Schmitt S, Mancilla IA, Meyer-Bender MF, Hanf M, et al. A pathway coordinated by DELE1 relays mitochondrial stress to the cytosol. Nature. 2020;579(7799):433-7.
169. Guo X, Aviles G, Liu Y, Tian R, Unger BA, Lin YT, et al. Mitochondrial stress is relayed to the cytosol by an OMA1-DELE1-HRI pathway. Nature. 2020;579(7799):427-32.
170. Pakos-Zebrucka K, Koryga I, Mnich K, Ljujic M, Samali A, Gorman AM. The integrated stress response. EMBO reports. 2016;17(10):1374-95.
171. Melber A, Haynes CM. UPR(mt) regulation and output: a stress response mediated by mitochondrial-nuclear communication. Cell research. 2018;28(3):281-95.
172. Baker BM, Nargund AM, Sun T, Haynes CM. Protective coupling of mitochondrial function and protein synthesis via the eIF2α kinase GCN-2. PLoS Genet. 2012;8(6):e1002760.
173. Gal-Ben-Ari S, Barrera I, Ehrlich M, Rosenblum K. PKR: A Kinase to Remember. Frontiers in Molecular Neuroscience. 2019;11(480).
174. Miyake M, Nomura A, Ogura A, Takehana K, Kitahara Y, Takahara K, et al. Skeletal muscle-specific eukaryotic translation initiation factor 2α phosphorylation controls amino acid metabolism and fibroblast growth factor 21-mediated non-cell-autonomous energy metabolism. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 2016;30(2):798-812.

175. Baker BM, Nargund AM, Sun T, Haynes CM. Protective coupling of mitochondrial function and protein synthesis via the eIF2α kinase GCN-2. PLoS Genet. 2012;8(6):e1002760-e.
176. Martinus RD, Garth GP, Webster TL, Cartwright P, Naylor DJ, Høj PB, et al. Selective induction of mitochondrial chaperones in response to loss of the mitochondrial genome. Eur J Biochem. 1996;240(1):98-103.
177. Zhao Q, Wang J, Levichkin IV, Stasinopoulos S, Ryan MT, Hoogenraad NJ. A mitochondrial specific stress response in mammalian cells. EMBO J. 2002;21(17):4411-9.
178. Quiros PM, Prado MA, Zamboni N, D'Amico D, Williams RW, Finley D, et al. Multi-omics analysis identifies ATF4 as a key regulator of the mitochondrial stress response in mammals. The Journal of cell biology. 2017;216(7):2027-45.
179. Michel S, Canonne M, Arnould T, Renard P. Inhibition of mitochondrial genome expression triggers the activation of CHOP-10 by a cell signaling dependent on the integrated stress response but not the mitochondrial unfolded protein response. Mitochondrion. 2015;21:58-68.
180. Khan NA, Nikkanen J, Yatsuga S, Jackson C, Wang L, Pradhan S, et al. mTORC1 Regulates Mitochondrial Integrated Stress Response and Mitochondrial Myopathy Progression. Cell metabolism. 2017;26(2):419-28.e5.
181. Forsstrom S, Jackson CB, Carroll CJ, Kuronen M, Pirinen E, Pradhan S, et al. Fibroblast Growth Factor 21 Drives Dynamics of Local and Systemic Stress Responses in Mitochondrial Myopathy with mtDNA Deletions. Cell metabolism. 2019.
182. Tyynismaa H, Carroll CJ, Raimundo N, Ahola-Erkkilä S, Wenz T, Ruhanen H, et al. Mitochondrial myopathy induces a starvation-like response. Hum Mol Genet. 2010;19(20):3948-58.
183. Lehtonen JM, Forsström S, Bottani E, Viscomi C, Baris OR, Isoniemi H, et al. FGF21 is a biomarker for mitochondrial translation and mtDNA maintenance disorders. Neurology. 2016;87(22):2290-9.
184. Montero R, Yubero D, Villarroya J, Henares D, Jou C, Rodríguez MA, et al. GDF-15 Is Elevated in Children with Mitochondrial Diseases and Is Induced by Mitochondrial Dysfunction. PloS one. 2016;11(2):e0148709.
185. Favaro G, Romanello V, Varanita T, Andrea Desbats M, Morbidoni V, Tezze C, et al. DRP1-mediated mitochondrial shape controls calcium homeostasis and muscle mass. Nature communications. 2019;10(1):2576.
186. Kim KH, Jeong YT, Oh H, Kim SH, Cho JM, Kim YN, et al. Autophagy deficiency leads to protection from obesity and insulin resistance by inducing Fgf21 as a mitokine. Nature medicine. 2013;19(1):83-92.
187. Fu T, Xu Z, Liu L, Guo Q, Wu H, Liang X, et al. Mitophagy Directs Muscle-Adipose Crosstalk to Alleviate Dietary Obesity. Cell reports. 2018;23(5):1357-72.
188. Rodríguez-Nuevo A, Díaz-Ramos A, Noguera E, Díaz-Sáez F, Duran X, Muñoz JP, et al. Mitochondrial DNA and TLR9 drive muscle inflammation upon Opa1 deficiency. EMBO J. 2018;37(10).
189. Ebert SM, Monteys AM, Fox DK, Bongers KS, Shields BE, Malmberg SE, et al. The transcription factor ATF4 promotes skeletal myofiber atrophy during fasting. Molecular endocrinology (Baltimore, Md). 2010;24(4):790-9.
190. Wang X, Middleton FA, Tawil R, Chen XJ. Cytosolic Adaptation to Mitochondrial Precursor Overaccumulation Stress Induces Progressive Muscle Wasting. bioRxiv. 2020:733097.
191. Ebert SM, Dyle MC, Kunkel SD, Bullard SA, Bongers KS, Fox DK, et al. Stress-induced skeletal muscle Gadd45a expression reprograms myonuclei and causes muscle atrophy. The Journal of biological chemistry. 2012;287(33):27290-301.
192. Houtkooper RH, Mouchiroud L, Ryu D, Moullan N, Katsyuba E, Knott G, et al. Mitonuclear protein imbalance as a conserved longevity mechanism. Nature. 2013;497(7450):451-7.
193. Borch Jensen M, Qi Y, Riley R, Rabkina L, Jasper H. PGAM5 promotes lasting FoxO activation after developmental mitochondrial stress and extends lifespan in Drosophila. eLife. 2017;6:e26952.
194. Mottis A, Herzig S, Auwerx J. Mitocellular communication: Shaping health and disease. Science (New York, NY). 2019;366(6467):827-32.

195. Wu J, Ruas JL, Estall JL, Rasbach KA, Choi JH, Ye L, et al. The unfolded protein response mediates adaptation to exercise in skeletal muscle through a PGC-1alpha/ATF6alpha complex. Cell metabolism. 2011;13(2):160-9.
196. Fernández-Verdejo R, Vanwynsberghe AM, Essaghir A, Demoulin JB, Hai T, Deldicque L, et al. Activating transcription factor 3 attenuates chemokine and cytokine expression in mouse skeletal muscle after exercise and facilitates molecular adaptation to endurance training. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 2017;31(2):840-51.
197. Cordeiro AV, Peruca GF, Braga RR, Brícola RS, Lenhare L, Silva VRR, et al. High-intensity exercise training induces mitonuclear imbalance and activates the mitochondrial unfolded protein response in the skeletal muscle of aged mice. GeroScience. 2020.
198. KIM HJ, JAMART C, DELDICQUE L, AN G-L, LEE YH, KIM CK, et al. Endoplasmic Reticulum Stress Markers and Ubiquitin-Proteasome Pathway Activity in Response to a 200-km Run. Medicine & Science in Sports & Exercise. 2011;43(1):18-25.
199. Ogborn DI, McKay BR, Crane JD, Parise G, Tarnopolsky MA. The unfolded protein response is triggered following a single, unaccustomed resistance-exercise bout. American journal of physiology Regulatory, integrative and comparative physiology. 2014;307(6):R664-9.
200. Hart CR, Ryan ZC, Pfaffenbach KT, Dasari S, Parvizi M, Lalia AZ, et al. Attenuated activation of the unfolded protein response following exercise in skeletal muscle of older adults. Aging. 2019;11(18):7587-604.
201. Kleinert M, Clemmensen C, Sjøberg KA, Carl CS, Jeppesen JF, Wojtaszewski JFP, et al. Exercise increases circulating GDF15 in humans. Mol Metab. 2018;9:187-91.
202. Laurens C, Parmar A, Murphy E, Carper D, Lair B, Maes P, et al. Growth and differentiation factor 15 is secreted by skeletal muscle during exercise and promotes lipolysis in humans. JCI insight. 2020;5(6).
203. Kim KH, Kim SH, Min YK, Yang HM, Lee JB, Lee MS. Acute exercise induces FGF21 expression in mice and in healthy humans. PloS one. 2013;8(5):e63517.
204. Boushel R, Gnaiger E, Schjerling P, Skovbro M, Kraunsoe R, Dela F. Patients with type 2 diabetes have normal mitochondrial function in skeletal muscle. Diabetologia. 2007;50(4):790-6.
205. Broskey NT, Daraspe J, Humbel BM, Amati F. Skeletal muscle mitochondrial and lipid droplet content assessed with standardized grid sizes for stereology. Journal of applied physiology (Bethesda, Md : 1985). 2013;115(5):765-70.
206. Nielsen J, Suetta C, Hvid LG, Schroder HD, Aagaard P, Ortenblad N. Subcellular localization-dependent decrements in skeletal muscle glycogen and mitochondria content following short-term disuse in young and old men. American journal of physiology Endocrinology and metabolism. 2010;299(6):E1053-60.
207. Sjöström M, Angquist KA, Bylund AC, Fridén J, Gustavsson L, Scherstén T. Morphometric analyses of human muscle fiber types. Muscle Nerve. 1982;5(7):538-53.
208. Schiaffino S, Reggiani C. Fiber types in mammalian skeletal muscles. Physiol Rev. 2011;91(4):1447-531.
209. Wiesner RJ, Rüegg JC, Morano I. Counting target molecules by exponential polymerase chain reaction: copy number of mitochondrial DNA in rat tissues. Biochem Biophys Res Commun. 1992;183(2):553-9.
210. Horvath SE, Daum G. Lipids of mitochondria. Progress in Lipid Research. 2013;52(4):590-614.
211. Funai K, Summers SA, Rutter J. Reign in the membrane: How common lipids govern mitochondrial function. Current opinion in cell biology. 2020;63:162-73.
212. Gonzalez-Franquesa A, Stocks B, Chubanava S, Hattel HB, Moreno-Justicia R, Treebak JT, et al. Mass-spectrometry based proteomics reveals mitochondrial supercomplexome plasticity. bioRxiv. 2019:860080.
213. Nilsson A, Björnson E, Flockhart M, Larsen FJ, Nielsen J. Complex I is bypassed during high intensity exercise. Nature communications. 2019;10(1):5072.

214. Howald H, Hoppeler H, Claassen H, Mathieu O, Straub R. Influences of endurance training on the ultrastructural composition of the different muscle fiber types in humans. Pflugers Archiv : European journal of physiology. 1985;403(4):369-76.
215. Montero D, Cathomen A, Jacobs RA, Flück D, de Leur J, Keiser S, et al. Haematological rather than skeletal muscle adaptations contribute to the increase in peak oxygen uptake induced by moderate endurance training. The Journal of physiology. 2015;593(20):4677-88.
216. Meinild Lundby AK, Jacobs RA, Gehrig S, de Leur J, Hauser M, Bonne TC, et al. Exercise training increases skeletal muscle mitochondrial volume density by enlargement of existing mitochondria and not de novo biogenesis. Acta physiologica (Oxford, England). 2018;222(1).
217. TURNER DL, HOPPELER H, CLAASSEN H, VOCK P, KAYSER B, SCHENA F, et al. Effects of endurance training on oxidative capacity and structural composition of human arm and leg muscles. Acta Physiologica Scandinavica. 1997;161(4):459-64.
218. Shepherd SO, Cocks M, Meikle PJ, Mellett NA, Ranasinghe AM, Barker TA, et al. Lipid droplet remodelling and reduced muscle ceramides following sprint interval and moderate-intensity continuous exercise training in obese males. International journal of obesity (2005). 2017;41(12):1745-54.
219. Granata C, Jamnick NA, Bishop DJ. Training-Induced Changes in Mitochondrial Content and Respiratory Function in Human Skeletal Muscle. Sports medicine (Auckland, NZ). 2018;48(8):1809-28.
220. Vigelsø A, Andersen NB, Dela F. The relationship between skeletal muscle mitochondrial citrate synthase activity and whole body oxygen uptake adaptations in response to exercise training. International journal of physiology, pathophysiology and pharmacology. 2014;6(2):84-101.
221. Sprenger HG, Langer T. The Good and the Bad of Mitochondrial Breakups. Trends in cell biology. 2019;29(11):888-900.
222. Picard M, Shirihai OS, Gentil BJ, Burelle Y. Mitochondrial morphology transitions and functions: implications for retrograde signaling? American journal of physiology Regulatory, integrative and comparative physiology. 2013;304(6):R393-406.
223. Diaz-Vegas A, Sanchez-Aguilera P, Krycer JR, Morales PE, Monsalves-Alvarez M, Cifuentes M, et al. Is mitochondrial dysfunction a common root of noncommunicable chronic diseases? Endocrine reviews. 2020.
224. Wang C, Taki M, Sato Y, Tamura Y, Yaginuma H, Okada Y, et al. A photostable fluorescent marker for the superresolution live imaging of the dynamic structure of the mitochondrial cristae. Proceedings of the National Academy of Sciences. 2019;116(32):15817-22.
225. Kondadi AK, Anand R, Hänsch S, Urbach J, Zobel T, Wolf DM, et al. Cristae undergo continuous cycles of membrane remodelling in a MICOS-dependent manner. EMBO reports. 2020;21(3):e49776.
226. Groennebaek T, Billeskov TB, Schytz CT, Jespersen NR, Bøtker HE, Olsen RKJ, et al. Mitochondrial Structure and Function in the Metabolic Myopathy Accompanying Patients with Critical Limb Ischemia. Cells. 2020;9(3).
227. Acin-Perez R, Benador IY, Petcherski A, Veliova M, Benavides GA, Lagarrigue S, et al. A novel approach to measure mitochondrial respiration in frozen biological samples. EMBO J. 2020;39(13):e104073.
228. Cardinale DA, Gejl KD, Ortenblad N, Ekblom B, Blomstrand E, Larsen FJ. Reliability of maximal mitochondrial oxidative phosphorylation in permeabilized fibers from the vastus lateralis employing high-resolution respirometry. Physiological reports. 2018;6(4).
229. Ryan TE, Brophy P, Lin CT, Hickner RC, Neufer PD. Assessment of in vivo skeletal muscle mitochondrial respiratory capacity in humans by near-infrared spectroscopy: a comparison with in situ measurements. The Journal of physiology. 2014;592(15):3231-41.
230. Jacques M, Kuang J, Bishop DJ, Yan X, Alvarez-Romero J, Munson F, et al. Mitochondrial respiration variability and simulations in human skeletal muscle: The Gene SMART study. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 2020;34(2):2978-86.

231. Cardinale DA, Gejl KD, Ørtenblad N, Ekblom B, Blomstrand E, Larsen FJ. Reliability of maximal mitochondrial oxidative phosphorylation in permeabilized fibers from the vastus lateralis employing high-resolution respirometry. Physiological reports. 2018;6(4).
232. Andreux PA, van Diemen MPJ, Heezen MR, Auwerx J, Rinsch C, Groeneveld GJ, et al. Mitochondrial function is impaired in the skeletal muscle of pre-frail elderly. Sci Rep. 2018;8(1):8548.
233. Andreux PA, van Diemen MPJ, Heezen MR, Auwerx J, Rinsch C, Jan Groeneveld G, et al. Mitochondrial function is impaired in the skeletal muscle of pre-frail elderly. Scientific Reports. 2018;8(1):8548.
234. Picard M, Prather AA, Puterman E, Cuillerier A, Coccia M, Aschbacher K, et al. A Mitochondrial Health Index Sensitive to Mood and Caregiving Stress. Biological psychiatry. 2018;84(1):9-17.
235. Zorova LD, Popkov VA, Plotnikov EY, Silachev DN, Pevzner IB, Jankauskas SS, et al. Mitochondrial membrane potential. Analytical biochemistry. 2018;552:50-9.
236. Scaduto RC, Jr., Grotyohann LW. Measurement of mitochondrial membrane potential using fluorescent rhodamine derivatives. Biophysical journal. 1999;76(1 Pt 1):469-77.
237. Hey-Mogensen M, Gram M, Jensen MB, Lund MT, Hansen CN, Scheibye-Knudsen M, et al. A novel method for determining human ex vivo submaximal skeletal muscle mitochondrial function. The Journal of physiology. 2015;593(17):3991-4010.
238. MacInnis MJ, Zacharewicz E, Martin BJ, Haikalis ME, Skelly LE, Tarnopolsky MA, et al. Superior mitochondrial adaptations in human skeletal muscle after interval compared to continuous single-leg cycling matched for total work. The Journal of physiology. 2017;595(9):2955-68.
239. Daussin FN, Zoll J, Dufour SP, Ponsot E, Lonsdorfer-Wolf E, Doutreleau S, et al. Effect of interval versus continuous training on cardiorespiratory and mitochondrial functions: relationship to aerobic performance improvements in sedentary subjects. American journal of physiology Regulatory, integrative and comparative physiology. 2008;295(1):R264-72.
240. Hearn GR, Wainio WW. Succinic dehydrogenase activity of the heart and skeletal muscle of exercised rats. The American journal of physiology. 1956;185(2):348-50.
241. Holloszy JO. Biochemical adaptations in muscle. Effects of exercise on mitochondrial oxygen uptake and respiratory enzyme activity in skeletal muscle. The Journal of biological chemistry. 1967;242(9):2278-82.
242. Burgomaster KA, Howarth KR, Phillips SM, Rakobowchuk M, Macdonald MJ, McGee SL, et al. Similar metabolic adaptations during exercise after low volume sprint interval and traditional endurance training in humans. The Journal of physiology. 2008;586(1):151-60.
243. Meinild Lundby AK, Jacobs RA, Gehrig S, de Leur J, Hauser M, Bonne TC, et al. Exercise training increases skeletal muscle mitochondrial volume density by enlargement of existing mitochondria and not de novo biogenesis. Acta physiologica (Oxford, England). 2018;222(1).
244. Tarnopolsky MA, Rennie CD, Robertshaw HA, Fedak-Tarnopolsky SN, Devries MC, Hamadeh MJ. Influence of endurance exercise training and sex on intramyocellular lipid and mitochondrial ultrastructure, substrate use, and mitochondrial enzyme activity. American journal of physiology Regulatory, integrative and comparative physiology. 2007;292(3):R1271-8.
245. Jacobs RA, Flück D, Bonne TC, Bürgi S, Christensen PM, Toigo M, et al. Improvements in exercise performance with high-intensity interval training coincide with an increase in skeletal muscle mitochondrial content and function. Journal of applied physiology (Bethesda, Md : 1985). 2013;115(6):785-93.
246. Greggio C, Jha P, Kulkarni SS, Lagarrigue S, Broskey NT, Boutant M, et al. Enhanced Respiratory Chain Supercomplex Formation in Response to Exercise in Human Skeletal Muscle. Cell metabolism. 2017;25(2):301-11.
247. Gillen JB, Martin BJ, MacInnis MJ, Skelly LE, Tarnopolsky MA, Gibala MJ. Twelve weeks of sprint interval training improves indices of cardiometabolic health similar to traditional endurance training despite a five-fold lower exercise volume and time commitment. PloS one. 2016;11(4):e0154075.
248. Choi AM, Ryter SW, Levine B. Autophagy in human health and disease. The New England journal of medicine. 2013;368(7):651-62.

249. Lo Verso F, Carnio S, Vainshtein A, Sandri M. Autophagy is not required to sustain exercise and PRKAA1/AMPK activity but is important to prevent mitochondrial damage during physical activity. Autophagy. 2014;10(11):1883-94.
250. Call JA, Wilson RJ, Laker RC, Zhang M, Kundu M, Yan Z. Ulk1-mediated autophagy plays an essential role in mitochondrial remodeling and functional regeneration of skeletal muscle. American journal of physiology Cell physiology. 2017;312(6):C724-c32.
251. He C, Bassik MC, Moresi V, Sun K, Wei Y, Zou Z, et al. Exercise-induced BCL2-regulated autophagy is required for muscle glucose homeostasis. Nature. 2012;481(7382):511-5.
252. Lira VA, Okutsu M, Zhang M, Greene NP, Laker RC, Breen DS, et al. Autophagy is required for exercise training-induced skeletal muscle adaptation and improvement of physical performance. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 2013;27(10):4184-93.
253. Melia TJ, Lystad AH, Simonsen A. Autophagosome biogenesis: From membrane growth to closure. The Journal of cell biology. 2020;219(6).
254. Slobodkin MR, Elazar Z. The Atg8 family: multifunctional ubiquitin-like key regulators of autophagy. Essays in biochemistry. 2013;55:51-64.
255. Tanida I, Ueno T, Kominami E. LC3 conjugation system in mammalian autophagy. The international journal of biochemistry & cell biology. 2004;36(12):2503-18.
256. Mizushima N, Yoshimori T. How to Interpret LC3 Immunoblotting. Autophagy. 2007;3(6):542-5.
257. Sánchez-Martín P, Komatsu M. p62/SQSTM1 – steering the cell through health and disease. Journal of cell science. 2018;131(21):jcs222836.
258. Birgisdottir AB, Lamark T, Johansen T. The LIR motif - crucial for selective autophagy. Journal of cell science. 2013;126(Pt 15):3237-47.
259. Shang L, Chen S, Du F, Li S, Zhao L, Wang X. Nutrient starvation elicits an acute autophagic response mediated by Ulk1 dephosphorylation and its subsequent dissociation from AMPK. Proceedings of the National Academy of Sciences of the United States of America. 2011;108(12):4788-93.
260. Jaspers RT, Zillikens MC, Friesema EC, delli Paoli G, Bloch W, Uitterlinden AG, et al. Exercise, fasting, and mimetics: toward beneficial combinations? FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 2017;31(1):14-28.
261. Vainshtein A, Tryon LD, Pauly M, Hood DA. Role of PGC-1alpha during acute exercise-induced autophagy and mitophagy in skeletal muscle. American journal of physiology Cell physiology. 2015;308(9):C710-9.
262. Zhang D, Lee JH, Kwak SE, Shin HE, Zhang Y, Moon HY, et al. Effect of a Single Bout of Exercise on Autophagy Regulation in Skeletal Muscle of High-Fat High-Sucrose Diet-Fed Mice. Journal of Obesity & Metabolic Syndrome. 2019;28(3):175-85.
263. Brandt N, Dethlefsen MM, Bangsbo J, Pilegaard H. PGC-1α and exercise intensity dependent adaptations in mouse skeletal muscle. PloS one. 2017;12(10):e0185993.
264. Kruse R, Pedersen AJ, Kristensen JM, Petersson SJ, Wojtaszewski JF, Hojlund K. Intact initiation of autophagy and mitochondrial fission by acute exercise in skeletal muscle of patients with Type 2 diabetes. Clinical science (London, England : 1979). 2017;131(1):37-47.
265. Schwalm C, Jamart C, Benoit N, Naslain D, Premont C, Prevet J, et al. Activation of autophagy in human skeletal muscle is dependent on exercise intensity and AMPK activation. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 2015;29(8):3515-26.
266. Brandt N, Gunnarsson TP, Bangsbo J, Pilegaard H. Exercise and exercise training-induced increase in autophagy markers in human skeletal muscle. Physiological reports. 2018;6(7):e13651.
267. Yoshii SR, Mizushima N. Monitoring and Measuring Autophagy. Int J Mol Sci. 2017;18(9):1865.
268. Martinez-Lopez N, Tarabra E, Toledo M, Garcia-Macia M, Sahu S, Coletto L, et al. System-wide Benefits of Intermeal Fasting by Autophagy. Cell metabolism. 2017;26(6):856-71.e5.

269. Billat VL, Sirvent P, Py G, Koralsztein JP, Mercier J. The concept of maximal lactate steady state: a bridge between biochemistry, physiology and sport science. Sports medicine (Auckland, NZ). 2003;33(6):407-26.
270. Granata C, Oliveira RSF, Little JP, Renner K, Bishop DJ. Mitochondrial adaptations to high-volume exercise training are rapidly reversed after a reduction in training volume in human skeletal muscle. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 2016;30(10):3413-23.
271. Granata C, Oliveira RSF, Little JP, Renner K, Bishop DJ. Sprint-interval but not continuous exercise increases PGC-1α protein content and p53 phosphorylation in nuclear fractions of human skeletal muscle. Scientific Reports. 2017;7(1):44227.
272. Yamada E, Singh R. Mapping autophagy on to your metabolic radar. Diabetes. 2012;61(2):272-80.
273. Vainshtein A, Tryon LD, Pauly M, Hood DA. Role of PGC-1α during acute exercise-induced autophagy and mitophagy in skeletal muscle. American journal of physiology Cell physiology. 2015;308(9):C710-9.
274. Jamart C, Naslain D, Gilson H, Francaux M. Higher activation of autophagy in skeletal muscle of mice during endurance exercise in the fasted state. American journal of physiology Endocrinology and metabolism. 2013;305(8):E964-74.
275. Pagano AF, Py G, Bernardi H, Candau RB, Sanchez AM. Autophagy and protein turnover signaling in slow-twitch muscle during exercise. Medicine and science in sports and exercise. 2014;46(7):1314-25.
276. Fritzen AM, Frøsig C, Jeppesen J, Jensen TE, Lundsgaard AM, Serup AK, et al. Role of AMPK in regulation of LC3 lipidation as a marker of autophagy in skeletal muscle. Cellular signalling. 2016;28(6):663-74.
277. Nguyen TN, Padman BS, Usher J, Oorschot V, Ramm G, Lazarou M. Atg8 family LC3/GABARAP proteins are crucial for autophagosome–lysosome fusion but not autophagosome formation during PINK1/Parkin mitophagy and starvation. Journal of Cell Biology. 2016;215(6):857-74.
278. Gonzalez-Freire M, Semba RD, Ubaida-Mohien C, Fabbri E, Scalzo P, Højlund K, et al. The Human Skeletal Muscle Proteome Project: a reappraisal of the current literature. Journal of Cachexia, Sarcopenia and Muscle. 2017;8(1):5-18.
279. Jamart C, Francaux M, Millet GY, Deldicque L, Frère D, Féasson L. Modulation of autophagy and ubiquitin-proteasome pathways during ultra-endurance running. Journal of applied physiology (Bethesda, Md : 1985). 2012;112(9):1529-37.
280. Behrends C, Fulda S. Receptor proteins in selective autophagy. Int J Cell Biol. 2012;2012:673290-.
281. Lee IM, Shiroma EJ, Lobelo F, Puska P, Blair SN, Katzmarzyk PT. Effect of physical inactivity on major non-communicable diseases worldwide: an analysis of burden of disease and life expectancy. Lancet (London, England). 2012;380(9838):219-29.
282. Pillon NJ, Gabriel BM, Dollet L, Smith JAB, Sardón Puig L, Botella J, et al. Transcriptomic profiling of skeletal muscle adaptations to exercise and inactivity. Nature communications. 2020;11(1):470.
283. Eisner V, Picard M, Hajnóczky G. Mitochondrial dynamics in adaptive and maladaptive cellular stress responses. Nature cell biology. 2018;20(7):755-65.
284. Nunnari J, Suomalainen A. Mitochondria: in sickness and in health. Cell. 2012;148(6):1145-59.
285. Zierath Juleen R, Wallberg-Henriksson H. Looking Ahead Perspective: Where Will the Future of Exercise Biology Take Us? Cell metabolism. 2015;22(1):25-30.
286. Boyman L, Karbowski M, Lederer WJ. Regulation of Mitochondrial ATP Production: Ca(2+) Signaling and Quality Control. Trends in molecular medicine. 2020;26(1):21-39.
287. Egan B, Zierath JR. Exercise metabolism and the molecular regulation of skeletal muscle adaptation. Cell metabolism. 2013;17(2):162-84.

288. Goodyear LJ, Kahn BB. Exercise, glucose transport, and insulin sensitivity. Annual review of medicine. 1998;49:235-61.
289. Egan B, O'Connor PL, Zierath JR, O'Gorman DJ. Time course analysis reveals gene-specific transcript and protein kinetics of adaptation to short-term aerobic exercise training in human skeletal muscle. PloS one. 2013;8(9):e74098.
290. Hood DA, Memme JM, Oliveira AN, Triolo M. Maintenance of Skeletal Muscle Mitochondria in Health, Exercise, and Aging. Annual review of physiology. 2019;81:19-41.
291. Glancy B, Hartnell LM, Malide D, Yu ZX, Combs CA, Connelly PS, et al. Mitochondrial reticulum for cellular energy distribution in muscle. Nature. 2015;523(7562):617-20.
292. Glancy B, Hartnell LM, Combs CA, Femnou A, Sun J, Murphy E, et al. Power Grid Protection of the Muscle Mitochondrial Reticulum. Cell reports. 2017;19(3):487-96.
293. Picard M, Gentil BJ, McManus MJ, White K, St Louis K, Gartside SE, et al. Acute exercise remodels mitochondrial membrane interactions in mouse skeletal muscle. Journal of applied physiology (Bethesda, Md : 1985). 2013;115(10):1562-71.
294. Lavorato M, Loro E, Debattisti V, Khurana TS, Franzini-Armstrong C. Elongated mitochondrial constrictions and fission in muscle fatigue. Journal of cell science. 2018;131(23).
295. Jamart C, Naslain D, Gilson H, Francaux M. Higher activation of autophagy in skeletal muscle of mice during endurance exercise in the fasted state. American Journal of Physiology-Endocrinology and Metabolism. 2013;305(8):E964-E74.
296. Dohm GL, Tapscott EB, Barakat HA, Kasperek GJ. Measurement of in vivo protein synthesis in rats during an exercise bout. Biochemical Medicine. 1982;27(3):367-73.
297. Melber A, Haynes CM. UPR(mt) regulation and output: a stress response mediated by mitochondrial-nuclear communication. Cell research. 2018;28(3):281-95.
298. Khan NA, Nikkanen J, Yatsuga S, Jackson C, Wang L, Pradhan S, et al. mTORC1 Regulates Mitochondrial Integrated Stress Response and Mitochondrial Myopathy Progression. Cell metabolism. 2017;26(2):419-28.e5.
299. Forsstrom S, Jackson CB, Carroll CJ, Kuronen M, Pirinen E, Pradhan S, et al. Fibroblast Growth Factor 21 Drives Dynamics of Local and Systemic Stress Responses in Mitochondrial Myopathy with mtDNA Deletions. Cell metabolism. 2019;30(6):1040-54.e7.
300. Lehtonen JM, Forsstrom S, Bottani E, Viscomi C, Baris OR, Isoniemi H, et al. FGF21 is a biomarker for mitochondrial translation and mtDNA maintenance disorders. Neurology. 2016;87(22):2290-9.
301. Granata C, Oliveira RS, Little JP, Renner K, Bishop DJ. Mitochondrial adaptations to high-volume exercise training are rapidly reversed after a reduction in training volume in human skeletal muscle. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 2016;30(10):3413-23.
302. Place N, Ivarsson N, Venckunas T, Neyroud D, Brazaitis M, Cheng AJ, et al. Ryanodine receptor fragmentation and sarcoplasmic reticulum Ca2+ leak after one session of high-intensity interval exercise. Proceedings of the National Academy of Sciences of the United States of America. 2015;112(50):15492-7.
303. BISHOP D, JENKINS DG, McENIERY M, CAREY MF. Relationship between plasma lactate parameters and muscle characteristics in female cyclists. Medicine & Science in Sports & Exercise. 2000;32(6):1088-93.
304. LucÍA A, Hoyos J, PÉRez M, Chicharro JL. Heart rate and performance parameters in elite cyclists: a longitudinal study. Medicine & Science in Sports & Exercise. 2000;32(10).
305. Kuang J, Yan X, Genders AJ, Granata C, Bishop DJ. An overview of technical considerations when using quantitative real-time PCR analysis of gene expression in human exercise research. PloS one. 2018;13(5):e0196438.
306. Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, et al. STAR: ultrafast universal RNA-seq aligner. Bioinformatics. 2013;29(1):15-21.

307. Li B, Dewey CN. RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. BMC Bioinformatics. 2011;12(1):323.
308. Law CW, Alhamdoosh M, Su S, Dong X, Tian L, Smyth GK, et al. RNA-seq analysis is easy as 1-2-3 with limma, Glimma and edgeR. F1000Res. 2016;5:ISCB Comm J-1408.
309. Zhou Y, Zhou B, Pache L, Chang M, Khodabakhshi AH, Tanaseichuk O, et al. Metascape provides a biologist-oriented resource for the analysis of systems-level datasets. Nature communications. 2019;10(1):1523.
310. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, et al. Gene set enrichment analysis: A knowledge-based approach for interpreting genome-wide expression profiles. Proceedings of the National Academy of Sciences. 2005;102(43):15545-50.
311. Zhao Q, Wang J, Levichkin IV, Stasinopoulos S, Ryan MT, Hoogenraad NJ. A mitochondrial specific stress response in mammalian cells. EMBO J. 2002;21(17):4411-9.
312. Anderson NS, Haynes CM. Folding the Mitochondrial UPR into the Integrated Stress Response. Trends in cell biology.
313. Munday MR. Regulation of mammalian acetyl-CoA carboxylase. Biochemical Society transactions. 2002;30(Pt 6):1059-64.
314. Fenyvesi R, Rácz G, Wuytack F, Zádor E. The calcineurin activity and MCIP1.4 mRNA levels are increased by innervation in regenerating soleus muscle. Biochem Biophys Res Commun. 2004;320(2):599-605.
315. Parker L, Trewin A, Levinger I, Shaw CS, Stepto NK. The effect of exercise-intensity on skeletal muscle stress kinase and insulin protein signaling. PloS one. 2017;12(2):e0171613-e.
316. Trewin AJ, Parker L, Shaw CS, Hiam DS, Garnham A, Levinger I, et al. Acute HIIE elicits similar changes in human skeletal muscle mitochondrial H2O2 release, respiration, and cell signaling as endurance exercise even with less work. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology. 2018;315(5):R1003-R16.
317. Nargund AM, Pellegrino MW, Fiorese CJ, Baker BM, Haynes CM. Mitochondrial import efficiency of ATFS-1 regulates mitochondrial UPR activation. Science (New York, NY). 2012;337(6094):587-90.
318. Boos F, Krämer L, Groh C, Jung F, Haberkant P, Stein F, et al. Mitochondrial protein-induced stress triggers a global adaptive transcriptional programme. Nature cell biology. 2019;21(4):442-51.
319. King DW, Gollnick PD. Ultrastructure of rat heart and liver after exhaustive exercise. The American journal of physiology. 1970;218(4):1150-5.
320. Nimmo MA, Snow DH. Time course of ultrastructural changes in skeletal muscle after two types of exercise. Journal of applied physiology: respiratory, environmental and exercise physiology. 1982;52(4):910-3.
321. Vincent AE, Ng YS, White K, Davey T, Mannella C, Falkous G, et al. The Spectrum of Mitochondrial Ultrastructural Defects in Mitochondrial Myopathy. Sci Rep. 2016;6:30610.
322. Kristensen DE, Albers PH, Prats C, Baba O, Birk JB, Wojtaszewski JF. Human muscle fibre type-specific regulation of AMPK and downstream targets by exercise. The Journal of physiology. 2015;593(8):2053-69.
323. Mulero MC, Aubareda A, Schlüter A, Pérez-Riba M. RCAN3, a novel calcineurin inhibitor that down-regulates NFAT-dependent cytokine gene expression. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research. 2007;1773(3):330-41.
324. Gabriel BM, Zierath JR. The Limits of Exercise Physiology: From Performance to Health. Cell metabolism. 2017;25(5):1000-11.
325. Pettersen IKN, Tusubira D, Ashrafi H, Dyrstad SE, Hansen L, Liu X-Z, et al. Upregulated PDK4 expression is a sensitive marker of increased fatty acid oxidation. Mitochondrion. 2019;49:97-110.
326. Fan W, Waizenegger W, Lin CS, Sorrentino V, He MX, Wall CE, et al. PPARδ Promotes Running Endurance by Preserving Glucose. Cell metabolism. 2017;25(5):1186-93.e4.

327. Cheang WS, Wong WT, Zhao L, Xu J, Wang L, Lau CW, et al. PPARδ Is Required for Exercise to Attenuate Endoplasmic Reticulum Stress and Endothelial Dysfunction in Diabetic Mice. Diabetes. 2017;66(2):519-28.
328. Hargreaves M, Spriet LL. Skeletal muscle energy metabolism during exercise. Nature Metabolism. 2020.
329. Leermakers PA, Kneppers AEM, Schols AMWJ, Kelders MCJM, de Theije CC, Verdijk LB, et al. Skeletal muscle unloading results in increased mitophagy and decreased mitochondrial biogenesis regulation. Muscle & Nerve. 2019;60(6):769-78.
330. Gram M, Vigelsø A, Yokota T, Hansen CN, Helge JW, Hey-Mogensen M, et al. Two weeks of one-leg immobilization decreases skeletal muscle respiratory capacity equally in young and elderly men. Experimental gerontology. 2014;58:269-78.
331. MacInnis MJ, Zacharewicz E, Martin BJ, Haikalis ME, Skelly LE, Tarnopolsky MA, et al. Superior mitochondrial adaptations in human skeletal muscle after interval compared to continuous single-leg cycling matched for total work. The Journal of physiology. 2017;595(9):2955-68.
332. Gibala MJ, Hawley JA. Sprinting Toward Fitness. Cell metabolism. 2017;25(5):988-90.
333. Jacobs RA, Lundby AK, Fenk S, Gehrig S, Siebenmann C, Flück D, et al. Twenty-eight days of exposure to 3454 m increases mitochondrial volume density in human skeletal muscle. The Journal of physiology. 2016;594(5):1151-66.
334. Friedman JR, Nunnari J. Mitochondrial form and function. Nature. 2014;505(7483):335-43.
335. Granata C, Oliveira RSF, Little JP, Renner K, Bishop DJ. Sprint-interval but not continuous exercise increases PGC-1α protein content and p53 phosphorylation in nuclear fractions of human skeletal muscle. Scientific Reports. 2017;7:44227.
336. Granata C, Jamnick NA, Bishop DJ. Principles of Exercise Prescription, and How They Influence Exercise-Induced Changes of Transcription Factors and Other Regulators of Mitochondrial Biogenesis. Sports medicine (Auckland, NZ). 2018;48(7):1541-59.
337. Picard M, Shirihai OS, Gentil BJ, Burelle Y. Mitochondrial morphology transitions and functions: implications for retrograde signaling? American journal of physiology Regulatory, integrative and comparative physiology. 2013;304(6):R393-R406.
338. Granata C, Oliveira RSF, Little JP, Renner K, Bishop DJ. Mitochondrial adaptations to high-volume exercise training are rapidly reversed after a reduction in training volume in human skeletal muscle. The FASEB Journal. 2016;30(10):3413-23.
339. Saner NJ, Lee MJ-C, Kuang J, Pitchford NW, Roach GD, Garnham A, et al. Exercise mitigates sleep-loss-induced changes in glucose tolerance, mitochondrial function, sarcoplasmic protein synthesis, and circadian rhythms. bioRxiv. 2020:2020.06.21.163733.
340. Miotto PM, Mcglory C, Bahniwal R, Kamal M, Phillips SM, Holloway GP. Supplementation with dietary ω-3 mitigates immobilization-induced reductions in skeletal muscle mitochondrial respiration in young women. The FASEB Journal. 2019;33(7):8232-40.
341. Castro-Sepulveda M, Jannas-Vela S, Fernández-Verdejo R, Ávalos-Allele D, Tapia G, Villagrán C, et al. Relative lipid oxidation associates directly with mitochondrial fusion phenotype and mitochondria-sarcoplasmic reticulum interactions in human skeletal muscle. American journal of physiology Endocrinology and metabolism. 2020;318(6):E848-e55.
342. Dulac M, Leduc-Gaudet JP, Reynaud O, Ayoub MB, Guérin A, Finkelchtein M, et al. Drp1 knockdown induces severe muscle atrophy and remodelling, mitochondrial dysfunction, autophagy impairment and denervation. The Journal of physiology. 2020.
343. Moore TM, Zhou Z, Cohn W, Norheim F, Lin AJ, Kalajian N, et al. The impact of exercise on mitochondrial dynamics and the role of Drp1 in exercise performance and training adaptations in skeletal muscle. Mol Metab. 2019;21:51-67.
344. McConell GK, Wadley GD, Le Plastrier K, Linden KC. Skeletal muscle AMPK is not activated during 2 h of moderate intensity exercise at ∼65% V̇O2peak in endurance trained men. The Journal of physiology. 2020.

345. Alarcon-Martinez L, Villafranca-Baughman D, Quintero H, Kacerovsky JB, Dotigny F, Murai KK, et al. Interpericyte tunnelling nanotubes regulate neurovascular coupling. Nature. 2020;585(7823):91-5.
346. Shao L-W, Niu R, Liu Y. Neuropeptide signals cell non-autonomous mitochondrial unfolded protein response. Cell research. 2016;26(11):1182-96.
347. Stawski R, Walczak K, Kosielski P, Meissner P, Budlewski T, Padula G, et al. Repeated bouts of exhaustive exercise increase circulating cell free nuclear and mitochondrial DNA without development of tolerance in healthy men. PloS one. 2017;12(5):e0178216.
348. LaMoia TE, Shulman GI. Cellular and Molecular Mechanisms of Metformin Action. Endocrine reviews. 2020.

APPENDIX – RESEARCH STUDY DOCUMENTATION

1 – Participant information sheet

2 – Participant consent form

3 – Risk factor assessment questionnaire

4 – Muscle biopsy and venous catheterisation questionnaire

VICTORIA UNIVERSITY
MELBOURNE AUSTRALIA

INFORMATION TO PARTICIPANTS INVOLVED IN RESEARCH

You are invited to participate in a research project entitled:

The effects of exercise on markers of mitochondrial dynamics and mitochondrial remodelling in men.

This project is being conducted by Mr. Javier Botella Ruiz at Victoria University under the supervision of Prof. David Bishop [College of Sport and Exercise Science and ISEAL].

Project Explanation

The project aims to investigate the effects of different types of endurance exercise typically performed by endurance athletes on changes in mitochondria (note: Mitochondria are our muscles' power houses, producing the energy required to enable your muscles to contract). This will allow us to understand what type of endurance exercise increases the mitochondrial reticulum (network of interconnected mitochondria) and what type of exercise stimulates a higher quality control (recycling of mitochondria). The information obtained from this research has implications for both performance and health, as it will allow better prescribe exercise and to best promote mitochondrial adaptations.

What will I be asked to do?

You will be asked to take part in the following testing and training sessions

- 4 familiarisation sessions and 6 testing session spread across approximately 3 weeks at the beginning and 1 week at the end of the training period (see below for details) lasting approximately 60 min each.
- 30 training sessions. Each training session will last between 30 and 150 min, based on the group you are allocated to.

Prior to the initial visit, you will be given the opportunity to provide written and oral consent. We will then ask you to fill in a short questionnaire about your medical history and your exercise habits to determine your ability to participate in this project. At each subsequent visit, you will provide oral consent for participating in the exercise testing sessions, muscle biopsies, and blood sampling procedures. You will also be reminded of your right to withdraw from the project at any time.

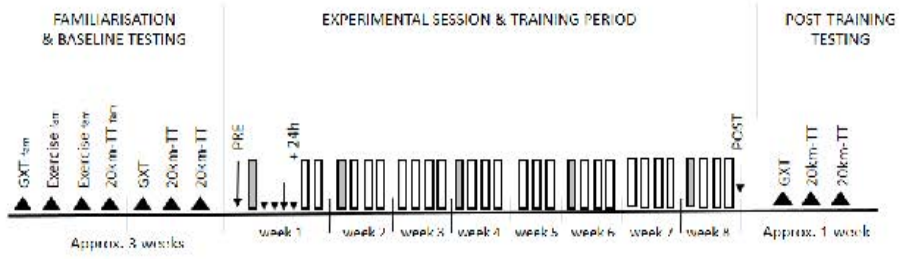

Figure 1. Schematic overview of the study. GXT = graded exercise test; 20km-TT = 20 km time-trial; fam = familiarisation session; arrows point at timing of blood samples and muscle biopsies; grey exercise sessions refer to sessions where physiological monitoring (i.e., VO2, lactate, HR) is also obtained.

All testing sessions will be performed on a stationary bicycle connected to a computer displaying all the details of your ride. These are located in the exercise physiology laboratory at Victoria University Footscray Park campus, and all sessions will be run under the supervision of qualified personnel.

How will this project be conducted?

As a participant, you will initially be screened via questionnaires for your current physical activity, cardiovascular risk factors, and any health issues of relevance to the study. This will be checked by the investigators to make sure you are eligible to take part in this study. You may be required to meet Dr. Garnham for further tests such as blood pressure and ECG tests. If you are eligible, you can take part in the study. Also, you will be asked to complete a dietary report, which consists of the detailed description of the last three meals consumed before the initial maximal incremental exercise test. You will be asked to then replicate the same diet before performing the tests described below.

Prior to commencement of training you will visit the lab **7 times**. During these visits you will be required to refrain from exercise, as well as alcohol and caffeine consumption, for 24 h before all tests. Visits will be separated by a minimum of 48 to 72 hours. During the first four visits, you will be asked to perform a familiarisation of the graded exercise test (GXT) and 20-km time trial (20k-TT), as well as 2 familiarisation sessions of the exercise training that you will perform throughout the 8-week period. The GXT will begin at a low intensity and every 4 minutes the intensity will increase until you cannot continue (i.e., exhaustion) or your cadence declines more than 10 revolutions per minute for 10 seconds. Five minutes after each graded exercise test you will perform a brief, exhaustive verification bout to determine VO2Peak (typically lasting between 2-5 minutes). The 20km-TT will be performed on a stationary bike. You will be required to cover the 20 km distance as fast as possible. Following these four sessions, you will perform a second GXT, which will be used for the determination of your peak oxygen uptake (VO2Peak), peak power (WPeak), and power at the lactate threshold (WLT); these parameters will be used to set your training intensity for the training phase. During the sixth and seventh visit you will perform the 20k-TT cycle time trial, which will be used as a baseline performance measurement.

After the completion of this baseline testing, you will begin the training intervention. The first training session will also be an experimental session with muscle biopsies taken before and after exercise. You will be provided with a full dinner and breakfast to be consumed 15 and 3 hours prior to the scheduled appointment. This diet will be repeated before each of the 3 morning biopsy trials (PRE [before beginning training], +24 h [24 h after the first training session], POST [after 8 weeks of training]). Three additional muscle biopsies will be taken during the three-hour timeframe following the first exercise training session. During this period you will be allowed to rest in the supine position but will not be allowed to consume food or drinks, except for water. All muscle biopsies (6 in total) will be taken from the external side of your thigh ("*vastus lateralis*") using a biopsy needle with suction. You will lie in the supine position on a bed; the skin from where the muscle will be extracted from will be shaven and cleaned. A local anaesthetic will be injected into the skin; after the anaesthetic has taken effect a small incision will be made through the skin and fascia. On the experimental session day, four incisions will be made for four different biopsies. Following the incisions, the muscle biopsy needle will penetrate through the incision through the fascia into the thigh muscle. The doctor will make about 5-10 'snips' to cut about 150 mg. of muscle, while another experienced technician uses a 50 mL syringe to draw the muscle into the biopsy needle. The muscle biopsy needle will remain in your leg for ~10 seconds. While the needle is in your leg you will feel minimal to no pain; however, you will feel pressure inside your leg (similar to a knuckle being firmly pushed into your leg). Once the needle is removed a bandage will be placed on the incision site.

You will be required to train 3 to 4 times per week during 8 weeks (see Figure 1). All training sessions will be completed on a stationary bicycle. The 8-week training program will follow a periodised plan to allow progression. Each training session will consist of either continuous exercise at 90% of W_{LTI} during 60 to 150 min, or a 30-60 min sessions composed of 30-second all-out sprints (4 to 10 sprints of same length per training session). In order to maintain progression, the workload will be altered via manipulation of the duration of the exercise or the number of sprints respectively. During each training session, heart rate (HR) and rate of perceived exertion (RPE) will be obtained in order to monitor your progression. Furthermore, every two weeks, during one training session progression will be monitored by means of oxygen consumption, blood lactate levels and muscle oxygenation (measured with near-infrared spectroscopy). At the end of this training phase you will visit the lab three times for the post-training

testing phase. During your first visit, you will perform a GXT to measure changes in your VO2Peak, WPeak and WLT. During your second and third visit, the 20k-TT will be performed to evaluate your endurance performance. During the training phase, you will be able to perform your normal additional training if it is of low volume (< 3 h per week).

Venous blood samples will be taken during the GXT testing sessions and when muscle biopsies will be obtained. Venous blood sampling involves inserting a small tube (cannula) in your forearm vein, which remains in the vein during the testing session. The cannula needle pierces the vein and then is removed and the small tube remains in the vein during testing. The tube is rarely noticed by participants during testing, is not usually painful, and there is minimal/no discomfort during blood withdrawals. A qualified scientist will perform all blood sampling.

What will I gain from participating?

We anticipate that participation will benefit you by providing information about your physical fitness and that information can be used to inform your own training prescription. Due to the length of the study, we expect you to improve your fitness level and your health status. Furthermore, you will gain knowledge on the different types of training usually performed by well-trained endurance athletes.

How will the information I give be used?

Your metabolic data, as well as muscle and blood samples, will be stored under alphanumeric codes (i.e. without your name or personal details) and only the researchers involved in this study will be able to connect the muscle and blood samples to you. The blood samples will be used for determination of circulating myokines levels (proteins produced in the muscle and subsequently released to the blood), and muscle samples will be used to determine the remodelling of mitochondria (through electron microscopy) following training (see picture below). Other parameters we will collect such as oxygen consumption, heart rate, or questionnaire data, will also be stored with an alphanumeric code and only the scientists involved in this study will have access to these parameters.

The data that will be collected during the study will be used/published in peer-reviewed journals and conference presentations and at no stage will your personal details be revealed or disclosed without your written consent.

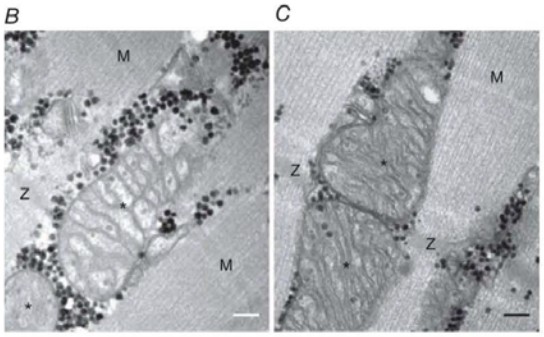

Figure 2. Example of a skeletal muscle electron microscopy image obtained from (B) an obese participant, and (C) a well-trained participant (Nielsen et al., 2016)

What are the potential risks of participating in this project?

Muscle biopsy:
For a few seconds during the procedure you will experience a feeling of pressure, which subsides when the muscle sample is removed. There can be a level of pain involved with the procedure. Additionally, once the local anaesthetic has worn off, you may experience some pain associated with the procedure. It is also possible that you might experience a slight muscle ache whilst walking one or two days after each biopsy. However, this will not be sufficient to cause undue distress or influence your normal lifestyle, and will have no measurable effect on subsequent performance of the quadriceps muscle group. There is a small chance of temporary altered sensation around the muscle biopsy site. This is a risk with any skin incision, when very small nerve endings may be interfered with. Those

nerve endings only supply sensation to the skin, normally regenerate, and have no effect on muscle function. As with all surgical procedures there is a small possibility of infection. There is a very small but real possibility of more serious complications with this technique. It has been previously reported that participants have suffered haematoma of the muscle after biopsy.

Venous blood collection:
Cannulation of the antecubital vein may result in potential complications to both the patient and the practitioner. Complications may include infection, phlebitis and thrombophlebitis, emboli, pain, haematoma or haemorrhage, extravasation, arterial cannulation and needlestick injuries. Careful adherence to guidelines and procedures can minimise these risks.

Graded Exercise Tests (GXT), 20 km time-trial (20km-TT) and training sessions:
You will experience some fatigue (feeling of sore in legs and body) when performing the exercise tests and training sessions. Furthermore, there is a small risk that you could become injured and this includes, muscle, ligament or tendon damage. Lastly, there are also risks of breathing irregularities, dizziness, angina and sudden death. However, the risk of such events happening are extremely low.

Risk Minimisation
Venous Blood Collection:
To minimise risks involved with venous blood collection it is imperative that the investigators follow the procedures and training of the Western Health Sunshine Hospital, the Institute of Sport, Exercise and Active Living Exercise Physiology Lab, and the instructions of Dr. Andrew Garnham.

Muscle biopsy:
Injection of a local anaesthetic in the skin and subcutaneous tissue overlying the muscle is used to minimise pain. The risk of localised altered skin sensation is minimised by (i) the choice of biopsy site – the site that is used above the *vastus lateralis* muscle avoids major branches of subcutaneous sensory nerve branches and (ii) using the smallest possible incision in the skin. The risk of infection is managed by performing the procedure under local sterile conditions. Overall risks are minimised by the biopsy procedure being performed by a qualified and experienced medical practitioner.

Graded Exercise Test, 20 km Time Trial and training sessions:
To minimise risks involved with the GXT, 20km-TT and training sessions, we will exclude participants with pre-existing injuries. All testing will be attended by staff with current CPR and First Aid certification. If we note any unexpected and unusual distress, we will stop the training session. To ensure your safety during exercise testing, all procedures will also be terminated immediately before completion if any of the following criteria are present: you wish to stop, chest pain, severe shortness of breath, or any other pain related to, or caused by exercise; you wish to continue but there are abnormal changes in heart rate (below or above age estimated % of maximum heart rate associated with the given exercise intensity used, monitored by heart rate monitors and watch) or other signs of metabolic, cardiorespiratory or thermoregulatory distress (e.g. facial pallor); your sweating responses are inappropriate to the environmental conditions in the laboratory; you display apprehension or mental confusion.

Right to Withdraw and Right to Withdraw Information

You have the right to withdraw from the study at any time without consequence.

Who is conducting the study?

The study is conducted by the College of Sport and Exercise Science, Victoria University, Footscray Park Campus.

Main investigators:

Prof. David Bishop, Telephone number: 9919 9471, Mobile: 0435 962 364, email: david.bishop@vu.edu.au

Mr. Javier Botella Ruiz, Telephone number: 0490 534 229, email: javier.botellaruiz@live.vu.edu.au

Any queries about your participation in this project may be directed to the Principal Researcher listed above.
If you have any queries or complaints about the way you have been treated, you may contact the Research Ethics and Biosafety Manager, Victoria University Human Research Ethics Committee, Victoria University, PO Box 14428, Melbourne, VIC, 8001 or phone (03) 9919 4148.

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CONSENT FORM FOR PARTICIPANTS INVOLVED IN RESEARCH

INFORMATION TO PARTICIPANTS:
We would like to invite you to be a part of a study investigating the
"The effects of exercise on markers of mitochondrial dynamics and mitochondrial remodelling in men"

INVESTIGATORS: Prof. David Bishop and Mr. Javier Botella Ruiz

AIMS OF THE STUDY: To investigate how mitochondria are remodelled following different types of endurance exercise. Furthermore, changes in mitochondria will be linked with endurance performance variables to gain knowledge on the relation between endurance performance and mitochondria.

PARTICIPANT INVOLVEMENT AND OVERVIEW OF TESTING: You will be requested to attend the Exercise Physiology Laboratory at Victoria University, Footscray Park Campus (College of Sport and Exercise Science and ISEAL, building P). The project involves a minimum of twelve weeks. Visits 1 to 4 will consist of familiarisation of the exercise sessions and the physiological testing. Visits 5 to 7 will consist of a graded-exercise test (GXT) and two 20 km time trials (20km-TT). Visit 8 will be the first training session, which will also be an experimental session, where muscle biopsies will be taken before exercise (PRE), at the end of the exercise (+ 0 h), twice during the following 3 h, and once more the day after (+ 24 h). Following this experimental session, you will be requested to continue the weekly training 3 to 4 times per week for 8 weeks, and another muscle sample will be taken at rest at the end of the study. Following the 8 weeks of training, physiological testing will be repeated (one GXT and two 20km-TT).

- **FAMILIARISATION EXERCISE BOUTS** (VISITS 1 to 4)
- **GRADED EXERCISE TEST** (VISIT 5 & AFTER THE TRAINING INTERVENTION)
- **20 KM TIME TRIAL** (VISITS 6 & 7; & AFTER THE TRAINING INTERVENTION)
- **EXPERIMENTAL SESSION** (VISIT 8)
- **TRAINING INTERVENTION** (8 WEEKS)
- **RESTING MUSCLE SAMPLE**

MUSCLE BIOPSIES: The muscle biopsy procedure is used to obtain small samples of muscle tissue for analysis of mitochondrial proteins, enzymes, and metabolism. Three muscle samples will be taken at rest (PRE [before beginning training], +24 h [24 h after the first training session], POST [after 8 weeks of training]) while another three muscle samples will be taken in the 3 h timeframe following exercise. A total of 6 biopsies will be taken over 9 weeks). During the procedure you will feel pressure and this can be quite uncomfortable and you may also experience some pain, but this will last for only a few seconds. Muscle biopsies are routinely carried out in our laboratory by a qualified medical doctor, with no serious adverse effects.

VENOUS BLOOD SAMPLING: Venous blood samples will be taken right before every muscle biopsy sample. Venous blood sampling involves placing a cannula into your antecubital vein, which remains in the vein during the testing session. The cannula needle pierces the vein and then is removed and the flexible plastic cannula remains in the vein during testing. The cannula is rarely noticed by participants during testing, is not usually painful, and there is minimal/no discomfort during blood withdrawals. A qualified scientist will perform all blood sampling.

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CERTIFICATION BY PARTICIPANT

I, _____

of_____

certify that I am at least 18 years old* and that I am voluntarily giving my consent to participate in the study: **"The effects of exercise on markers of mitochondrial dynamics and mitochondrial remodelling."** being conducted at Victoria University by: Professor David Bishop

I certify that the objectives of the study, together with any risks and safeguards associated with the procedures listed hereunder to be carried out in the research, have been fully explained to me by:
Mr Javier Botella Ruiz or **Professor David Bishop**
and that I freely consent to participation involving the below mentioned procedures:

- Health History Screening
- Graded Exercise Tests (GXT)
- 20 km Cycling Time Trials (20km-TT)
- Training sessions
- Muscle Biopsies
- Venous blood sampling

I certify that I have had the opportunity to have any questions answered and that I understand that I can withdraw from this study at any time and that this withdrawal will not jeopardise me in any way.

I have been informed that the information I provide will be kept confidential.

Signed:

Date:

Any queries about your participation in this project may be directed to the researchers
Mr Javier Botella Ruiz
Telephone number: 0490 534 229
Email: javier.botellaruiz@live.vu.edu.au
Professor David Bishop
Telephone number: 03 9919 9471
Mobile number: 0435 962 364
Email: david.bishop@vu.edu.au

If you have any queries or complaints about the way you have been treated, you may contact the Research Ethics and Biosafety Manager, Victoria University Human Research Ethics Committee, Victoria University, PO Box 14428, Melbourne, VIC, 8001 or phone (03) 9919 4148.

RISK FACTOR ASSESSMENT QUESTIONNAIRE

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In order to be eligible to participate in the experiment investigating: **"The effects of exercise on markers of mitochondrial dynamics and mitochondrial remodelling"** you are required to complete the following questionnaire which is designed to assess the risk of you having a cardiovascular event occurring during an exhaustive exercise bout.

Name: _____ **Date:** _____

Age: _____ **years** **Weight:** _____ **kg** **Height:** _____ **cm** **Sex (Circle One): Male Female**

e-mail:_____ **Telephone (mobile):** _____

MEDICAL HISTORY:

In the past have you ever had (tick No or Yes. Also tick Current if you still have the illness or injury).

Medical Condition	NO	YES	CURRENT	Medical Condition	NO	YES	CURRENT
Heart Attack	❑	❑	n/a	Congenital Heart Disease	❑	❑	n/a
Chest Pain (angina)	❑	❑	❑	Disease of Arteries/Veins	❑	❑	❑
Heart Murmur	❑	❑	❑	Asthma	❑	❑	❑
Heart Rhythm Disturbance	❑	❑	❑	Lung Disease (eg. emphysema)	❑	❑	❑
Heart Valve Disease	❑	❑	❑	Epilepsy	❑	❑	❑
Heart Failure	❑	❑	❑	Stroke	❑	❑	n/a
*Back or neck injury	❑	❑	❑	*Shoulder, elbow or wrist injury	❑	❑	❑
*Hip injury	❑	❑	❑	*Knee or ankle injury	❑	❑	❑
Bleeding Disorder	❑	❑	❑	Diabetes	❑	❑	❑
Smoker	❑	❑	❑				

***Give details of injuries to your back, neck, shoulders, elbows, wrists, hips, knees, or ankles in your medical history**

_____

_____

_____

List any prescribed medications being taken

_____

List any surgical procedures that you have had (write the year in brackets):

_____

_____

ALLERGIES: Do you have any allergies **NO** ❑ **YES** ❑ If yes, _____

SYMPTOMS DURING OR AFTER EXERCISE

As a result of exercise, have you ever experienced any of the following:

Symptom during exercise	**NO**	**YES**	***Symptom during exercise***	**NO**	**YES**
Pain or discomfort in the chest, back, arm, or jaw	❑	❑	Palpitations (heart rhythm disturbance)	❑	❑
Severe shortness of breath or problems with breathing during mild exertion	❑	❑	Pain in the legs during mild exertion	❑	❑
Dizziness, nausea or fainting	❑	❑	Severe heat exhaustion	❑	❑

Current Exercise:

List the sports, exercise or physically active hobbies (eg. gardening or playing with the kids) that you are **currently** engaged in:

Sport/Activity	**Time of the day eg. 6 p.m.**	**Approximate duration eg. 30 minutes**
Monday:		
Tuesday:		
Wednesday		
Thursday		
Friday		
Saturday		
Sunday:		

Client Declaration
I declare that the above information is to my knowledge true and correct, and that I have not omitted any information that is requested on this form.

SIGNED: _____

DATE: _____

OFFICE USE ONLY
CLEARANCE TO UNDERGO AN EXERCISE TEST
This person has been cleared to undergo a fitness test:
❑ Without medical supervision
❑ With medical supervision
❑ A fitness test is not advisable at this time

Signed: Dr/Mr/Mrs/Ms _____

(Circle appropriate title: Physician/exercise physiologist)

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MUSCLE BIOPSY & VENOUS CATHETERISATION QUESTIONNAIRE

For the study entitled: **"The effects of exercise on markers of mitochondrial dynamics and mitochondrial remodelling".**

NAME: _____

ADDRESS: _____

_____

DATE: _____ **AGE:** _____ **years**

1. Have you or your family suffered from any tendency to bleed excessively? (e.g. Haemophilia) or bruise very easily? Yes No Don't Know

   If yes, please elaborate

   _____

2. Are you allergic to local anaesthetic? Yes No Don't Know

   If yes, please elaborate

   _____

3. Do you have any skin allergies? Yes No Don't Know

   If yes, please elaborate

   _____

4. Have you any other allergies? Yes No Don't Know

   If yes, please elaborate

   _____

5. Are you currently on any medication? Yes No

If yes, what is the medication?

_____

6. Do you have any other medical problems? Yes No

If yes, please elaborate

_____

7. Have you ever fainted when you had an injection or blood sample taken?

Yes No Don't know

If yes, please elaborate

_____

8. Have you previously had heparin infused or injected?

Yes No Don't know

If yes, please elaborate

_____

9. Do you or other members of your family have Raynaud's disease, or suffer from very poor circulation in the fingers, leading to painful fingers that turn white/blue?

Yes No Don't know

If yes, please elaborate

_____

To the best of my knowledge, the above questionnaire has been completely accurately and truthfully.

Signature: _____ Date: _____