Effects of different exercise intensity and volume on markers of mitochondrial biogenesis in human skeletal muscle

by

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Abstract

Mitochondria are key components of skeletal muscles as they provide the energy required for almost all cellular activities, and play an important role in ageing and cell pathology. Different forms of exercise training have been associated with mitochondrial adaptations, such as increased mitochondrial content and function, and enhanced mitochondrial biogenesis, as well as improved endurance performance. However, the role of training intensity and training volume, in determining these changes remains elusive. Therefore, the aim of this thesis was to investigate the role of training intensity and volume on changes in mitochondrial content and function (as measured by mitochondrial respiration in permeabilised muscle fibres), in the skeletal muscle of healthy humans, and to study the molecular mechanisms underlying these changes. It was demonstrated that training intensity is a key factor regulating changes in mitochondrial respiration, but not mitochondrial content, and that an apparent dissociation exists between changes in these two parameters. Training consisting of repeated 30-s "all-out" sprints lead to improved mitochondrial (mt)-specific respiration (indicative of improved mitochondrial quality). Conversely, training volume was shown to be a key factor regulating mitochondrial content, with the associated increase in mitochondrial respiration being likely driven by the increase in mitochondrial content (i.e., unchanged mt-specific respiration). A training volume reduction resulted in a rapid decrease in most mitochondrial parameters, underlining the importance of maintaining the training stimulus to preserve training-induced mitochondrial adaptations. The protein content of PGC-1α, p53 and PHF20 was shown to be regulated in a training intensity-dependent manner, and was more strongly associated with changes in mitochondrial respiration rather than content, whereas changes in the protein content of TFAM were primarily associated with changes in mitochondrial content. Moreover, it was demonstrated that exercise intensity induced an increase in nuclear PGC-1α protein content and nuclear p53 phosphorylation, two events that may represent the initial phase of different pathways of the exercise-induced adaptive response. Collectively, this research provides novel information regarding mitochondrial adaptations to different training stimuli, and could have important implications for the design of exercise programs in conditions of compromised mitochondrial function.

Student Declaration

I, Cesare Granata, declare that the PhD thesis entitled “Effects of different exercise intensity and volume on markers of mitochondrial biogenesis 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: 30/04/2015

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List of Publications, Conferences and Awards

Publications

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First Author Conference Abstracts

Granata C, Oliveira RSF, Little JP, Renner K, Bishop DJ. Exercise-induced modulation of PGC-1α and p53 in enriched subcellular fractions of human skeletal muscle. *16th International Biochemistry of Exercise Congress (IBEC).* September 7th – 9th, 2015. São Paulo, Brazil.

Granata C, Oliveira RSF, Little JP, Renner K, Bishop DJ. Training intensity-dependent regulation of mitochondrial respiration in human skeletal muscle: an effect modulated by the transcription factors PGC-1α, p53 and PHF20. *Australian Physiological Society Meeting (AuPS).* November 30th – December 3rd, 2014. Brisbane, Australia.

Granata C, Oliveira RSF, Renner K, Little JP, Bishop DJ. Effects of three different training intensities on mitochondrial respiration and endurance performance in humans. *Canadian Society for Exercise Physiology (CSEP).* October 9th – 16th, 2013. Toronto, Canada.

Granata C, Brentnall EL, Oliveira RSF, Stepto NK, Renner K, Bishop DJ. Mitochondrial adaptations to different training stimuli: the remarkable plasticity of skeletal muscle oxidative capacity. *FEBS workshop & MiP summer school*. July 3rd – 7th, 2012, Cambridge, UK.

Granata C, Oliveira RSF, Stepto NK, Renner K, Bishop DJ. Effects of three weeks of intensified training and two weeks of taper on mitochondrial respiration in humans. *17th Annual Congress of the ECSS*. July 4th – 7th, 2012. Bruges, Belgium.

Co-authored Conference Abstracts

Bishop DJ, **Granata C**, Oliveira RSF, Little JP, Renner K. (2015). Mitochondrial function and content are increased by different types of training in human skeletal muscle. *20th Annual Congress of the ECSS*. June 24th – 27th, 2015. Malmö, Sweden.

Oliveira RSF, McGinley C, **Granata C**, Pilegaard H, Bishop DJ. Effects of high-intensity interval exercise, under either induced metabolic acidosis or alkalosis, on the regulation of genes associated with the acid-base regulation in human skeletal muscle. *Australian Physiological Society Meeting (AuPS).* November 30th – December 3rd, 2014. Brisbane, Australia.

Bishop DJ, **Granata C**, Brentnall EL, Renner K, Oliveira RSF. Mitochondrial adaptations to intense exercise training and detraining. *XXXVII International Union of Physiological Societies (IUPS).* July 21st – 26th, 2013. Birmingham, UK.

Delfour-Peyrethon R, Thomas C, Hanon C, **Granata C**, Bishop DJ. Mitochondrial respiration changing after repeated-cycling-sprints performed under classic acidosis and induced-alkalosis (Poster). *18th Annual Congress of the ECSS*. June 26th – 29th, 2013. Barcelona, Spain.

Bishop DJ, **Granata C**, Eynon N. Can we optimize exercise training to improve mitochondrial respiration? *AussieMit*. December 10th – 12th, 2012. Melbourne, Australia.

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Awards

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^{31}P-MRS	phosphorus magnetic resonance spectroscopy
ACC	acetyl-CoA carboxylase
ADP	adenosine diphosphate
AIF	apoptosis inducing factor
AMP	adenosine monophosphate
AMPK	5' AMP-activated protein kinase
ATF2	activating transcription factor 2
ATP	adenosine triphosphate
BIOPS	biopsy preserving solution;
BM	body mass
Calcineurin	Ca^{2+}/Calmodulin-dependent phosphatase
CaM	Calmodulin
CaMKs	Ca^{2+}/Calmodulin-dependent kinases
CBS	cystathionine β-synthase
cDNA	complementary DNA
CHO	carbohydrate
$CI+II_E$	maximal electron transport system capacity through CI+II
$CI+II_P$	maximal oxidative phosphorylation state through CI+CII
CII_E	maximal electron transport system capacity through CII
CI_L	leak respiration state through Complex I
CI_P	maximal oxidative phosphorylation state through CI
CIV_E	maximal noncoupled respiration through CIV
CIV_{RES}	reserve capacity of CIV
Complex I	NADH-Q reductase or CI
Complex II	succinate-coenzyme Q reductase or CII
Complex III	cytochrome reductase or CIII
Complex IV	cytochrome c oxidase
Complex V	ATP synthase or CV
COX	cytochrome c oxidase
CREB	cAMP response element-binding protein
CS	citrate synthase

CT	continuous training
D_2O	heavy water
DRP1	dynamin-related protein-1
E	maximal ETS capacity
ERK	extracellular-regulated kinase
ERRα	estrogen-related receptor α
ETS	electron transport system
$FADH_2$	flavin adenine dinucleotide
FCCP	carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone;
FCR	respiratory flux control ratio
FOXO3	forkhead box O 3
FSR	fractional protein synthesis rate
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GC-MS	gas chromatography-mass spectrometry
GCN5	general control non-repressed protein 5
GLUT4	glucose transporter type 4
GXT	graded exercise test
HDACs	histone deacetylases
HIIT	high-intensity interval training
IMF	intermyofibrillar mitochondria
IMM	inner membrane
IMS	intermembrane space
INT	intensified training volume
inv-RCR	inverse respiratory control ratio
JNK	c-Jun N-terminal kinase
HSP70	mitochondrial heat-shock protein 70
KO	knockout
L	leak respiration
LC-MS	liquid chromatography-mass spectrometry
LCR	leak control ratio
LKB1	liver kinase B1
MAPR	mitochondrial ATP production rate
MDM2	murine double minute 2
MEF2	myocyte enhancer factor-2

MiR05	respiration medium;
miRNA	micro RNA
mRNA	messenger RNA
mtDNA	mitochondrial DNA
MPS	mitochondrial protein synthesis
mRNA	messenger RNA
MyoD	myogenic determining factor
NADH	nicotinamide adenine dinucleotide
NF-κβ	nuclear factor kappa-light-chain-enhancer of activate B cells
NIRS	near-infrared spectroscopy
NRF	nuclear respiratory factor
NT	normal training volume
OMM	outer membrane
OSPC	oligomycin sensitivity-conferring protein
OXPHOS	oxidative phosphorylation
P	maximal oxidative phosphorylation
PCR	phosphorylation control ratio
PGC-1α	PPARγ coactivator 1-alpha
PHF20	plant homeodomain finger-containing protein 20
PKA	protein kinase A
PKC	Ca^{2+}-dependent kinases C
PMSF	phenylmethanesulfonyl fluoride
PPAR	peroxisome proliferator-activated receptor
PRC	PGC-1 related coactivator rRNA ribosomal RNAs
RT	reduced training volume
ROS	reactive oxygen species
ROX	residual oxygen consumption
SCO2	synthesis of COX 2
SCR	substrate control ratio
SD	standard deviation
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
SIRT1	silent mating type information regulation 2 homolog 1
SIT	sprint interval training

SOD2	superoxide dismutase 2
SS	subsarcolemmal mitochondria
STCT	sub-lactate threshold continuous training
SUIT	substrate-uncoupler-inhibitor titration protocol
TBP	TATA-binding protein
TCA	tricarboxylic acid cycle
TEM	transmission electron microscopy
TFAM	mitochondrial transcription factor A
TIM	translocases of the inner membrane
TMPD	N,N,N',N'-tetramethyl-p-phenylenediamine
TOM	translocase of the outer membrane
tRNA	transfer RNAs
TT	cycling time trial
$\dot{V}O_{2Peak}$	peak oxygen uptake
W_{LT}	power at the lactate threshold
W_{Peak}	peak power output
WT	wild-type
β2M	β-2 microglobulin
β-HAD	β-hydroxy acyl-CoA dehydrogenase

Chapter 1

Review of Literature

The reference for the published version of part of this literature review is as follows:

Bishop DJ, **Granata C**, & Eynon N (2014). Can we optimise the exercise training prescription to maximise improvements in mitochondria function and content? *Biochimica Et Biophysica Acta-General Subjects*, 1840, 1266-1275.

1.1 Skeletal muscle and mitochondria

Skeletal muscle constitutes the largest tissue mass in the human body (40-50% body weight) and is responsible for movement, bioenergetic homeostasis, and energy balance via a wide variety of physiological processes. A key component of skeletal muscles is the mitochondrion, which represents the major source of energy in the human body and provides the energy required for almost all cellular activities. Mitochondria are central to life and death. For example, mitochondria play a key role in cell signalling [115] and apoptosis [3], whereas mitochondrial dysfunction is at the base of aging and cell pathology [60]. Mitochondrial defects have been shown to be central to a series of metabolic disorders such as type 2 diabetes [197], insulin resistance [164], obesity [33, 339], cardiovascular diseases [346], and a wide variety of neurodegenerative diseases such as Huntington's, Alzheimer's, and Parkinson's disease [168].

From a structural standpoint, mitochondria range generally from 0.5 to 1.0 μm in diameter [111], form an integrated reticulum that is continually remodelled by fusion and fission processes [228], and are divided in four main compartments: the outer membrane (OMM), the intermembrane space (IMS), the inner membrane (IMM) and the matrix (Figure 1.1). The OMM consists of a phospholipid bilayer housing protein structures called porins that make it permeable to small molecules (<10,000 Da), including adenosine diphosphate (ADP) and adenosine triphosphate (ATP). Conversely, the IMM is made of a protein-rich, highly-folded complex structure that is only permeable to oxygen, water, and carbon dioxide. The wrinkled shape of the IMM generates several infolds and pockets (cristae) that increase its surface area and maximise its ability to house the four complexes of the electron transport system (ETS) and *ATP synthase* required for the generation of ATP. The compartment inside the IMM is defined as the matrix and hosts mitochondrial (mt) DNA, ribosomes, and the enzymes of the tricarboxylic acid (TCA) cycle. The TCA cycle, which includes the enzyme citrate synthase (CS), is the final common pathway for the oxidation of fuel molecules such as carbohydrates, lipids and amino acids.

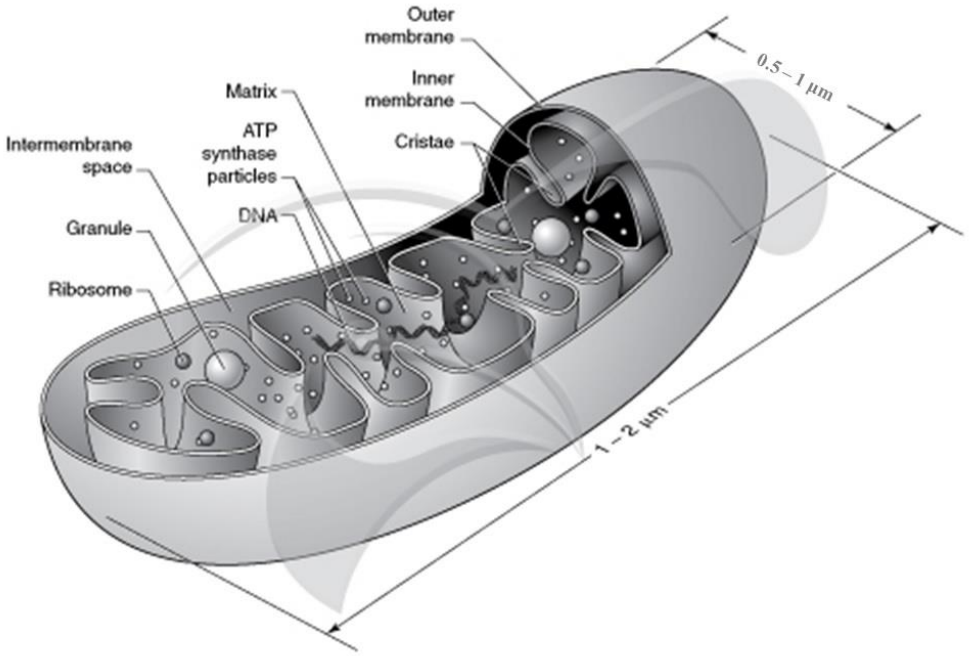

Figure 1.1. Structure of a mitochondrion. *(Image courtesy of www.imgarcade.com)*

During the TCA cycle, a small amount of ATP is generated alongside the reducing equivalents nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$). These high-energy electron carriers can transfer electrons to coenzyme Q in a reaction catalysed by complex I (*NADH-Q reductase* or CI) in the case of NADH, or by complex II (*succinate-coenzyme Q reductase* or CII) in the case of $FADH_2$. From coenzyme Q the electron pair is transferred to cytochrome *c* in a reaction catalysed by the enzyme complex III (*cytochrome reductase* or CIII), and then onto its final acceptor, molecular oxygen (O_2), in a process catalysed by complex IV (*cytochrome c oxidase* (COX) or CIV). During electron transfer, protons are pumped from the matrix to the IMS, with the resulting electrochemical gradient representing the proton-motive force that allows generation of ATP by phosphorylation of ADP in a reaction catalysed by complex V (*ATP synthase* or CV)[1] [269]. The combination of these processes taking place along the IMM is described as oxidative phosphorylation (OXPHOS) (Figure 1.2).

[1] The four complexes of the ETS and ATP synthase, are often grouped together and referred to as the "five complexes of the ETS". For simplicity, this theses will refer to them as the "five complexes of the ETS".

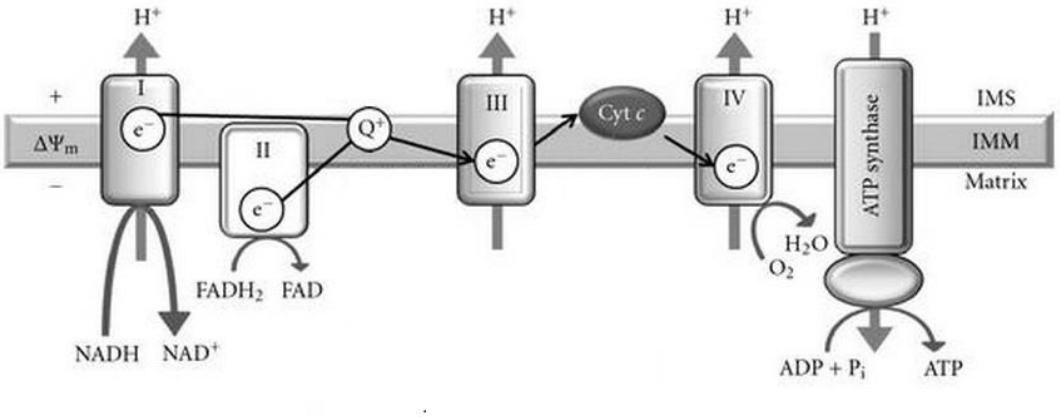

Figure 1.2. The electron transport system (ETS) and oxidative phosphorylation (OXPHOS). cyt *c*: cytochrome *c*; $FADH_2$: flavin adenine dinucleotide; IMM: inner mitochondrial membrane; IMS: intermembrane space; NADH: nicotinamide adenine dinucleotide; Q: coenzyme Q; $\Delta\psi_m$: mitochondrial membrane potential. *(Image courtesy of www.imgarcade.com)*

In skeletal muscles, mitochondria are classified according to their location. Those situated under the sarcolemma are called subsarcolemmal (SS) mitochondria, whilst those interspersed throughout the myofibrils are named intermyofibrillar (IMF) mitochondria (Figure 1.3), with the latter representing approximately 75% of the entire mitochondrial population [59]. The two different mitochondrial sub-fractions differ in biochemical properties and function, with the IMF sub-fraction displaying a 2.5-fold higher maximal respiration rate [59] and an approximately 3-fold higher ATP production rate [59] compared to the SS sub-fraction. Other differences have been demonstrated in reactive oxygen species (ROS) production rates and apoptotic signalling [2], as well as rates of protein import into the mitochondria [303]. This biochemical and functional diversity is reflected in different responses to common signalling events, such that SS mitochondria adapt earlier and to a greater extent than their IMF counterparts in response to a training stimulus [119].

Skeletal muscle is a plastic tissue undergoing continuous remodelling, with the ever changing content and function of skeletal muscle mitochondria constantly adapting to the cellular needs. Several factors such as age, [63], disease [109, 168, 197], and exercise

[360], to name a few, have all been shown to influence mitochondrial quality and quantity. In particular, exercise is a powerful stimulus that has been shown to lead to up to a 50% increase in both mitochondrial content and function [313, 343]. Therefore, the scope of this manuscript is to review the available research examining the importance of, and the mechanisms underlying, exercise-induced mitochondrial adaptations.

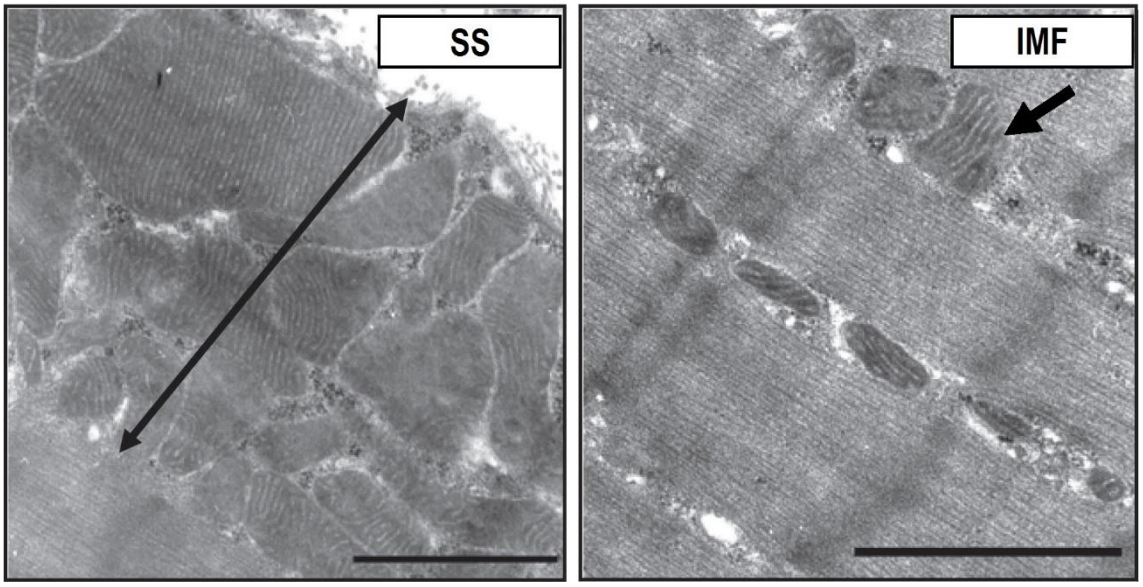

Figure 1.3. Electron micrographs showing (A) subsarcolemmal (SS), and (B) intermyofibrillar (IMF) mitochondria. *(Adapted from [265])*

1.2 Mitochondrial biogenesis

Mitochondrial biogenesis is a process by which an increase in the mitochondrial reticulum takes place within the cell. From its etymology (from the Greek word "*genesis*", meaning: "origin, creation") it would appear to indicate the generation of new mitochondria. However, an important consideration, is that new mitochondria are not made *ex novo* (i.e., from scratch) or *de novo* (i.e., from scratch again), but are rather the result of incorporation of new proteins to pre-existing sub-compartments and protein complexes leading to growth and division by fission [260]. These events take place in conjunction with continuous remodelling by dynamic fusion and fission processes, and the subsequent

alteration of the mitochondrial network morphology that is thought to be determined by the mitochondrial structure-function relationship [228]. However simple the definition may be, scientists refer to "mitochondrial biogenesis" in many different ways, and with different meanings, creating debate within the scientific community as to what this definition really describes [196, 290]. This lack of consensus leads to further controversy about which technique can best assess mitochondrial biogenesis [189, 196, 290].

Following the etymological meaning, the assessment of newly-formed mitochondria as an indicator of mitochondrial biogenesis can be obtained by measuring the rate of mitochondrial protein synthesis (MPS) using stable isotopic tracers [196]. Although MPS is likely the best-suited technique to determine if mitochondria biogenesis has taken place, it seems to lack the ability, if used in isolation, to provide information regarding mitochondria remodelling, net mitochondrial content (the net outcome of mitochondrial synthesis and degradation), and mitochondrial function and quality [189, 290]. It is not surprising that for many the definition "mitochondrial biogenesis" extends beyond its etymological meaning, and is intended to embrace part, or all of the above information [189, 290]. In this respect, many scientists more broadly refer to mitochondrial biogenesis as an increase of mitochondrial content per unit of tissue. For many years mitochondrial content has been measured as the best assessment of mitochondrial biogenesis, either through histological techniques or via biological methods assessing the activity or content of valid biomarkers of mitochondrial content [145, 189].

The outcome of mitochondrial biogenesis is also reflected in a change in mitochondrial function, which can be adaptive or maladaptive. Therefore, assessment of newly-formed mitochondria via MPS, and evidence of variations in mitochondrial content, should be paired with analysis of mitochondrial function to establish if these changes are associated with a gain or loss of function [340]. For example, the importance of measuring mitochondrial function is evident from the findings of a cross-sectional study in healthy individuals showing that differences in skeletal muscle respiratory capacity and aerobic fitness could not be solely explained by only taking into account mitochondrial quantitative differences [128]. The same group reported that skeletal muscle oxidative capacity measured by mitochondrial respiration was the best predictor of time trial performance in highly-trained cyclists [129], further strengthening the argument of

assessing mitochondrial function alongside the more classic estimation of mitochondrial content. In addition, exercise has been shown to regulate mitochondrial turnover by modulation of mitochondrial morphology [266] and autophagy [173, 320], an essential process for the support of skeletal muscle plasticity in response to exercise training [268]. When mitochondrial turnover increases and changes in protein synthesis and degradation are matched, mitochondrial content does not change; however, the replacement of damaged proteins with newly synthesised ones may still result in enhanced mitochondrial function [173]. This underlines an apparent dissociation between mitochondrial function and mitochondrial content, providing a strong argument as to why mitochondrial function should be measured alongside mitochondrial content.

As described in detail later, mitochondrial biogenesis is governed by transcriptional regulation requiring the concerted integration of two genomes (nuclear and mitochondrial), and a series of processes such as signalling, transcription, translation, post-translational events, and protein import, amongst others [116]. Measurements of these processes allow researchers to better understand the events underlying mitochondrial biogenesis. Therefore, it is common to measure gene and protein expression, the activity and location of signalling proteins, transcription factors, regulators or coactivators, and/or the content of mitochondrial proteins as markers of mitochondrial biogenesis. Whilst this certainly expands the knowledge about the mechanisms regulating the transcriptional activity leading to mitochondrial biogenesis, it has been shown that assessing biogenesis with only these measurements may lead to erroneous conclusions [196] and should be supplemented with other measurements.

Following on from the above considerations, it appears evident that a range of analyses should be used to assess if mitochondrial biogenesis has taken place and to examine its consequences for changes in mitochondrial content and function. Therefore, a comprehensive examination of mitochondrial biogenesis should not be limited to the assessment of the rate of mitochondrial protein synthesis, but should also include a thorough examination of changes in both mitochondrial content and function [189, 290]. Furthermore, to expand the knowledge of the mechanisms leading to exercise-induced mitochondrial biogenesis, measurement of changes of key proteins and transcription factors involved in the transcriptional machinery should also be considered.

1.3 Techniques for the assessment of mitochondrial biogenesis

As seen in the above paragraph, there exist several approaches for the assessment of mitochondrial biogenesis. Following is a brief review of the most commonly used techniques for the measurement of mitochondrial biogenesis, along with their main advantages and limitations.

1.3.1 Mitochondrial protein synthesis

Measurement of the MPS rate, which allows the assessment of the fractional protein synthesis rate (FSR) for the determination of newly-synthesized protein, is obtained by a primed and continuous intravenous infusion of a stable, isotopically labelled amino acid such as [^{13}C]leucine or [^{13}C], or [^{2}H]phenylalanine amongst others, or deuterium oxide (D_2O). This is followed by subsequent analysis of the tissue of interest with gas or liquid chromatography-mass spectrometry (GC-MS or LC-MS) [294]. The use of isotopically-labelled amino acids is extensively used to investigate the MPS response that follows a single exercise bout, but the conclusions that can be made when used to asses changes following a training intervention lasting days or week are somewhat limited by the brief duration of the tracer administration [270]. A modification of this method consists in the daily oral administration of D_2O [80], which can be sustained for weeks or months, and allows free-living conditions for the duration of the intervention. The main advantage of measuring MPS is that it represents a true measurement of the generation of newly-formed mitochondria. However, there are several limitations. These include the cost associated with the tracers and the equipment, the inability to detect net changes in mitochondrial content (defined by the rates of protein synthesis and breakdown), and the absence of information about changes in mitochondrial function. Moreover, the timing of the measurement is often a challenging factor given that different proteins follow different temporal patterns, whilst the processes of mitochondrial dynamics (fusion and fission), mitophagy, and protein breakdown, are not detectable by this technique.

1.3.2 Mitochondrial content

The most common methods to determine mitochondrial content are histological and they include transmission electron microscopy (TEM) and florescent microscopy [189]. However, due to the cost and time constraints, a range of more accessible and practical biochemical measurements has been proposed as valid biomarker of mitochondrial content. These include measurements of enzymatic activity, and/or the content of mtDNA, key mitochondrial proteins, and lipids.

1.3.2.1 Histological methods

1.3.2.1.1 Transmission electron microscopy

The gold-standard method to assess mitochondrial content consists of two-dimensional imaging using TEM [145], even though it is well accepted that mitochondria form a three-dimensional network [211]. This rather laborious technique involves four main stages: fixation, dehydration and embedding, sectioning, and staining [189]. Once the images are generated, the morphometric analysis that follows consists of assessing the amount of mitochondria by using "point counting grids" or "dashed lines" [189]. Both these methods require overlaying the grid (or dashed lines) on the image, and determining the volume density of the organelles by counting the number of times that a mitochondria intersects a grid intersection (or the beginning and end of a dashed line). The major drawback of this technique is that it requires a highly-skilled scientist and is very time consuming. For this reason, it is not available in many laboratories.

1.3.2.1.2 Fluorescent microscopy

This technique measures the area occupied by mitochondria and involves the use of dye markers that can enter the mitochondria and, due to their fluorescence, can be analysed under a fluorescent microscope [189]. The major limitation of this technique is that the analysis of the results is somewhat subjective, given that the determination of the colour intensity associated to the different organelles in the cell depends on the judgment of the operator.

1.3.2.2 Biochemical methods

Following, are several validated biomarkers that have been used to assess mitochondrial content [145].

1.3.2.2.1 Protein content of mitochondrial proteins

The protein content of selected subunits of the five complexes of the ETS has been shown to present a "substantial" (CII and CV), or a "moderate" (CIII and CIV) concordance[2] with mitochondrial surface area [145], and is routinely used as a biomarker of mitochondrial content. The method used to determine the protein content of these subunits is the immunoblotting technique (Western Blot), which can be used to determine the content of almost any protein. A small sample of muscle (10-15 mg) is homogenised in a lysis buffer, and the muscle homogenate is then separated by electrophoresis and transferred to a membrane. After blocking, the membrane is probed with a specific antibody recognising the protein of interest and then imaged to obtain a blot that can be quantified (for a more detailed description of this technique the reader is referred to sections 2.2 and 3.2). This technique is very simple and cost effective, and can be used to determine changes in mitochondrial content, for example, following a training intervention. Its major downside is the limited sensitivity and reproducibility, but, regardless of these drawbacks, it is one of the widest used techniques in the modern laboratory. The content of the five complexes of the ETS can also be determined with the use of the Blue Native PAGE technique [275]. The advantage of this technique is that multi-enzyme complexes can be analysed in their native state.

1.3.2.2.2 Mitochondrial enzyme activity

The enzymatic activity of key enzymes involved in the TCA cycle and the oxidative phosphorylation process are often used as biomarkers of mitochondrial content. The

[2] Lin's concordance coefficient (R_C) defines how well the relationship between two variables is represented by a line through the origin at an angle of 45 degrees (slope = 1). The values in the Lin's scale vary between 0 and 1. R_C values between 0.21–0.40 show a "fair" concordance, R_C values between 0.41–0.60 show a "moderate" concordance, R_C values between 0.61–0.80 a "substantial" concordance, and R_C values between 0.81–1.00 show an "almost perfect" concordance. (Lin LI, 1989)

activity of CS, one of the key enzyme of the TCA cycle, has been shown to present a "substantial to almost perfect" concordance with mitochondrial content [145], and it is the most widely used biomarker of mitochondrial content. In addition, the activity of the ETS complexes I to IV has also been shown to present a "substantial" concordance with mitochondrial content [145]. CS activity is determined by homogenising a small amount of sample (3-5 mg) that is then reacted with a series of substrates. This leads to the formation of a coloured chemical, the rate of formation of which is proportional and linear to the rate of CS activity. Changes in absorbance are followed by use of a spectrophotometer (for a more detailed description of this technique the reader is referred to sections 2.2 and 3.2). Determination of the enzymatic activity of the complexes of the ETS follows a similar methodology [344]. The main advantage of these enzymatic assays is that they require a very small amount of sample, and are quick, easy and relatively inexpensive assays.

1.3.2.2.3 Cardiolipin content

Cardiolipin constitutes about 20% of the lipid composition of the IMM and has been shown to be the best biomarker of mitochondrial content, as it presents an "almost perfect" concordance with mitochondrial content [145]. Cardiolipin content is measured with a sensitive and simple method, using an image analyser after separation from other phospholipids by one-dimensional thin-layer chromatography (TLC), and requires only a small amount (3-5 mg) of freeze-dried muscle tissue [256]. The main advantages of this method are the high concordance with mitochondrial content, the small amount of sample required, and that it is a sensitive, reproducible and simple technique.

1.3.2.2.4 Mitochondria DNA content

Mitochondria have their own DNA, and, even though each mitochondrion has between 2 and 10 DNA copies, mtDNA is proportional to the number of mitochondria; therefore mtDNA content has been extensively used as a biomarker of mitochondrial content [189]. mtDNA determination can be achieved by real-time polymerase chain reaction (PCR) or Southern Blots. The PCR method requires a small amount (~10 mg) of muscle tissue for

DNA extraction, and involves the use of a primer selected from the circular mtDNA, and one selected from genomic (nuclear) DNA to correct for cell number; a thermocycler is then used to amplify the segment of DNA and obtain a threshold count that is proportional to the amount of initial DNA. The Southern blot method is very similar to the one described for immunoblotting as it requires electrophoresis of the DNA fragments on an agarose gel followed by transfer to a nitrocellulose (or nylon) membrane. The membrane is then baked (nitrocellulose) or exposed to ultraviolet radiation (nylon) to permanently attach the mtDNA to the membrane, and is then exposed to an hybridization probe with both a mtDNA probe and a genomic DNA probe labelled with radioactive ^{32}P (or a non-radioactive biotinylated probe). After washing, the membrane is then visualized on X-ray film or a phosphoimager for quantitation [189]. Although it has been extensively used up until now, mtDNA content has recently been shown to be unrelated to mitochondrial content; these findings have therefore cast doubts on its validity as a biomarker of mitochondrial content [145].

1.3.2.2.5 Summary

In conclusion, the findings of Larsen *et al.* (2012) demonstrated that the best biomarkers of mitochondrial content are cardiolipin, followed by CS and CI activity. It was also concluded that regardless of its wide-spread use, mtDNA is not a valid biomarker of mitochondrial content.

1.3.3 Mitochondrial function

Mitochondrial function is defined as the ability of mitochondria to maintain cellular energetic homeostasis and is commonly expressed as maximal ATP production rate, oxygen consumption, or oxidative capacity [135]. It can also be normalised to a measure of mitochondrial content [135], as a mean to express mitochondrial quality. There is a lack of consensus on the terminology, with terms such as mitochondrial "function", "respiratory function", "oxidative capacity", "oxidative potential", and several others all used interchangeably. No researcher to date has called for a homogenisation of this terminology across different laboratories. Therefore, for the scope of this literature

review, "mitochondrial function" will be used. In skeletal muscle, mitochondrial function can be measured both invasively and non-invasively, with both methods presenting advantages and disadvantages.

1.3.3.1 Invasive measurements

There are two main approaches to directly assess mitochondrial function from skeletal muscle, by measuring oxygen consumption (mitochondrial respiration) or by measuring the rate of mitochondrial ATP production (MAPR)

1.3.3.1.1 Mitochondrial respiration

The assessment of mitochondrial respiration requires the measurement of the rate of oxygen consumption by mitochondria, using an O_2-sensitive electrode [85]. Following a series of titrations with a range of substrates, uncouplers and inhibitors, this method provides a series of valuable biological information (for a more detailed description of this technique the reader is referred to sections 2.2 and 3.2). In addition to the rate of maximal oxygen consumption, this technique allows determination of the oxygen consumption associated with substrate combinations providing electrons through different complexes, or other qualitative information such as noncoupled and uncoupled respiration, and the degree of respiratory control, amongst others. Therefore, this technique offers a useful tool to study specific complexes of the ETS, and provides valuable information to better understand the potential mechanisms of altered mitochondrial function in states of use and disuse. An obvious limitation however, is that the experiment is often carried out in non-physiological conditions [261]. The measurement of mitochondrial respiration can be performed in cell-free systems (isolated mitochondria or muscle homogenates) or with permeabilised muscle fibres, and requires the extraction of a small amount of skeletal muscle fibres using the percutaneous needle biopsy technique [312].

Cell-free systems

The assessment of mitochondrial respiration was initially carried out in isolated mitochondria. The isolation procedure requires the disruption of the cell structure by the force of a rotating homogeniser and subsequent centrifugation. Following a series of centrifugation steps, a pellet containing the isolated mitochondria is resuspended and placed in the chamber for measurement of oxygen consumption [312]. There are several disadvantages to using isolated mitochondria. One is that a large sample (~100 mg) is required, due to the low mitochondrial density of skeletal muscle. More importantly, during the isolation procedure mitochondria are removed from their cellular environment and their morphology, which is intimately linked to their function [229], is disrupted [230]. As a consequence, the isolation procedure can induce fragmented organelle morphology, increased ROS production, and differentially-altered mitochondrial respiration depending upon the respiratory conditions [230]. Moreover, it is possible that the isolation procedure may disproportionately harvest high-functioning, sturdy mitochondria, compared to more fragile, fragmented, and dysfunctional ones, therefore substantially altering assessment of mitochondrial function. For all these reasons, measurements obtained using isolated mitochondria are starting to be questioned. Mitochondrial respiration can also be measured in crude muscle homogenates. This approach requires a considerably smaller amount of sample (~20 mg of skeletal muscle), however, it does not provide qualitative information about mitochondrial function due to the presence of other cellular compartments. The lack of control over the chemical composition of the crude homogenate is also a cause of potential artefacts [312].

Permeabilised muscle fibres

Mitochondrial respiration can be assessed *in situ* in permeabilised muscle fibres. Briefly, a small amount of muscle fibres (5-10 mg) is mechanically separated with the use of pointed forceps, followed by selective permeabilisation of the cellular membrane with saponin. This method leaves the mitochondria intact and allows for *in situ* measurements of mitochondrial respiration [225] (for a more detailed description of this technique the reader is referred to sections 2.2 and 3.2). The two main advantages of this technique are the small amount of skeletal muscle required (an assay can be performed with as little as

~2 mg of tissue), and that mitochondria structure and function are less affected during preparation, allowing for the preservation of mitochondrial morphology and the functional interaction with other intracellular components [230]. For these reasons mitochondrial respiration in permeabilised muscle fibres is considered by many as the gold-standard measurement of mitochondrial function [85, 230, 261].

1.3.3.1.2 Mitochondrial ATP production rate

MAPR is a technique enabling the measurement of the rate of ATP production in isolated mitochondria using chemiluminescence [342]. This technique allows the determination of ATP production with different substrates or combinations of substrates [342]. Due to the high sensitivity of chemiluminescence, the sample required to run an assay (30-50 mg) is lower than that needed for measurement of oxygen consumption during mitochondrial respiration in isolated mitochondria, but still considerably greater compared to that needed for measurements in permeabilised muscle fibres. The main difference with the mitochondrial respiration technique however, is that MAPR directly measures the amount of ATP production and not oxygen consumption. Therefore, a limitation of MAPR is that the information obtained from this technique is limited to ATP production, as it does not provide the extra qualitative information (noncoupled and uncoupled respiration, respiratory control, etc.) that can be obtained from measuring oxygen consumption [312]. Finally, given that MAPR is measured in isolated mitochondria, it has the same intrinsic disadvantages of the mitochondrial isolation procedure (i.e., mitochondria structure and function disruption due to removal from their cellular environment [230].

1.3.3.2 Non-invasive measurements

Non-invasive measurements are also available to assess mitochondrial function. An obvious advantage of these techniques is the lack of invasive procedure with no discomfort for the patient/participant, and the fact that they take place *in vivo,* within a living organism with an intact biological system [261]. The main disadvantage is that they provide only a limited amount of information regarding the molecular mechanisms of

mitochondrial biochemistry [312]. The two main non-invasive techniques are: phosphorus magnetic resonance spectroscopy (^{31}P-MRS) and near-infrared spectroscopy (NIRS) [312].

1.3.3.2.1 Phosphorus magnetic resonance spectroscopy

^{31}P-MRS is used to measure the concentration of cytosolic phosphate metabolites, and the assessment of kinetic changes in phosphocreatine content during exercise and exercise recovery has been used to determine maximal oxidative power [312]. In spite of several studies validating ^{31}P-MRS as a measurement of mitochondrial function, the limited availability of magnetic resonance scanners, and the very high costs associated with it, have reduced the popularity of this technique [261].

1.3.3.2.2 Near-infrared spectroscopy

NIRS is a widely-used, non-invasive technique that takes advantage of the oxygen-dependent absorption of near-infrared light by the haemoglobin/myoglobin shuttle to monitor tissue oxygenation [134]. NIRS is extensively used to monitor brain function following neural activity [191], and to measure skeletal muscle oxygenation during contractile activity and ischemia [285]. Furthermore, it has been shown that NIRS assessment of in-vivo mitochondrial respiratory capacity correlates well with measurements of maximal ADP-stimulated mitochondrial respiration in permeabilised fibre [261]. Therefore, NIRS provides a cost-effective, non-invasive means of assessing *in vivo* mitochondrial respiratory capacity.

1.3.4 Protein and gene expression of transcription factors, coactivators, and key regulatory proteins

Measurement of gene expression, and/or the protein content of some of the key transcription factors and regulatory proteins modulating transcriptional activity, provides a valuable tool to better understand the mechanisms associated with mitochondrial

biogenesis. The most common technique used to assess changes in gene expression is real-time PCR, whilst immunoblotting is routinely used to determine changes in protein content (for a more detailed description of this technique the reader is referred to sections 2.2, 3.2 and 4.2). Peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) [352], the nuclear respiratory factors 1 (NRF1) and 2 (NRF2) [274], mitochondrial transcription factor A (TFAM) [272], and p53 [266], are amongst some of the most extensively-studied markers of mitochondrial biogenesis [266]. Movement of these transcription factors across subcellular compartments (e.g., nucleus, cytosol, mitochondrion), and a wide array of post-translational events (e.g., phosphorylation, acetylation, deacetylation, and many others), can be assessed by a range of techniques (e.g., immunoblotting, mass spectrometry and others). These translocation and post-translational events have been shown to play a key role in the transcriptional process [75], therefore providing another tool to enhance the knowledge of the intricate processes of mitochondrial biogenesis [189]. For a detailed description of the role and function of these regulatory proteins the reader is referred to the next section.

1.4 Cellular mechanisms of exercise-induced mitochondrial biogenesis

Mitochondria have their own genetic system comprised of a circular DNA (mtDNA) and a basic transcriptional and translational apparatus. In humans, mitochondria possess 1000 to 10,000 copies of a circular genome consisting of 16,569 base-pairs, containing 37 genes encoding for two ribosomal RNAs (rRNA), 22 transfer RNAs (tRNA), and 13 polypeptides that assembles as essential subunits of the complexes of the ETS, and are involved in the oxidative phosphorylation process [76, 121]. As a result, the vast majority of the ~1,000 proteins that are necessary for the complete operation of the oxidative phosphorylation system within the mitochondria are encoded by the nuclear genome [317]. Therefore, mitochondrial biogenesis requires the coordinated expression of both the nuclear and mitochondrial genome [75]. Nuclear proteins are then imported inside the mitochondria compartments via the mitochondria import machinery [317], where they

combine with those encoded by mtDNA. The import machinery consists of specialised import components containing transition pores, such as the translocase of the outer membrane (TOM), allowing proteins to cross the OMM, the translocases of the inner membrane (TIM), allowing protein transport within or across the IMM, the matrix heat shock protein 70 (mtHsp70), allowing access of precursor proteins into the mitochondrial matrix, and the mitochondrial export complex, which is responsible for the insertion of proteins into the inner membrane from the matrix [317].

At the molecular level, exercise-induced mitochondrial biogenesis is the results of, in chronological order: signalling, transcription, translation, and a host of post-translational events culminating with protein incorporation into the mitochondria [116]. The sequence of events leading to mitochondrial biogenesis, and the expansion of the mitochondrial reticulum, begins at the onset of contractile activity with a series of perturbations acting as signals for the activation of signalling proteins such as kinases, deacetylases, phosphatases and others [116]. The most important signalling events generated during an exercise bout are: an increase in cytosolic and mitochondrial calcium [50], greater ADP and adenosine monophosphate (AMP) concentration with a subsequent increase in the AMP:ATP ratio [39], changes in the cellular reduction/oxidation (redox) balance of $[NAD^+]$/[NADH] [75], and changes in the production of ROS [238] (Figure 1.4). These cellular signals lead to the activation of a series of kinases such as Ca^{2+}/Calmodulin-dependent kinases II (CaMKII), 5' AMP-activated protein kinase (AMPK), and p38 mitogen-activated protein kinases (p38 MAPK). These in turn initiate DNA transcription by activating a series of sensor enzymes (transcription factors, coactivators and regulators) by chemical events such as phosphorylation, or by induction of conformational or sub-cellular localization changes amongst others [116]. The transcriptional process is initiated following an increase in the messenger RNA (mRNA) of these sensor enzymes and that of downstream proteins, and is dependent on the type, intensity, frequency and duration of contractile activity, as well as the half-life of the proteins of interest [75]. All of these cellular signals contribute with different pathways to mitochondria biogenesis [75], and are activated with different time courses that range from ~3 up to 24 h and beyond the termination of the exercise stimulus [224, 354]. Following the formation of mRNA, the process of translation leads to the generation of proteins, which can then be imported inside the mitochondria. After a host of post-

translational modifications such as folding or assembly into complexes, proteins become biologically active and can carry out their function [115]. For the 13 proteins encoded by mtDNA the process is similar, and it is under the regulation of a series of transcription factors, with TFAM playing the most prominent role [115].

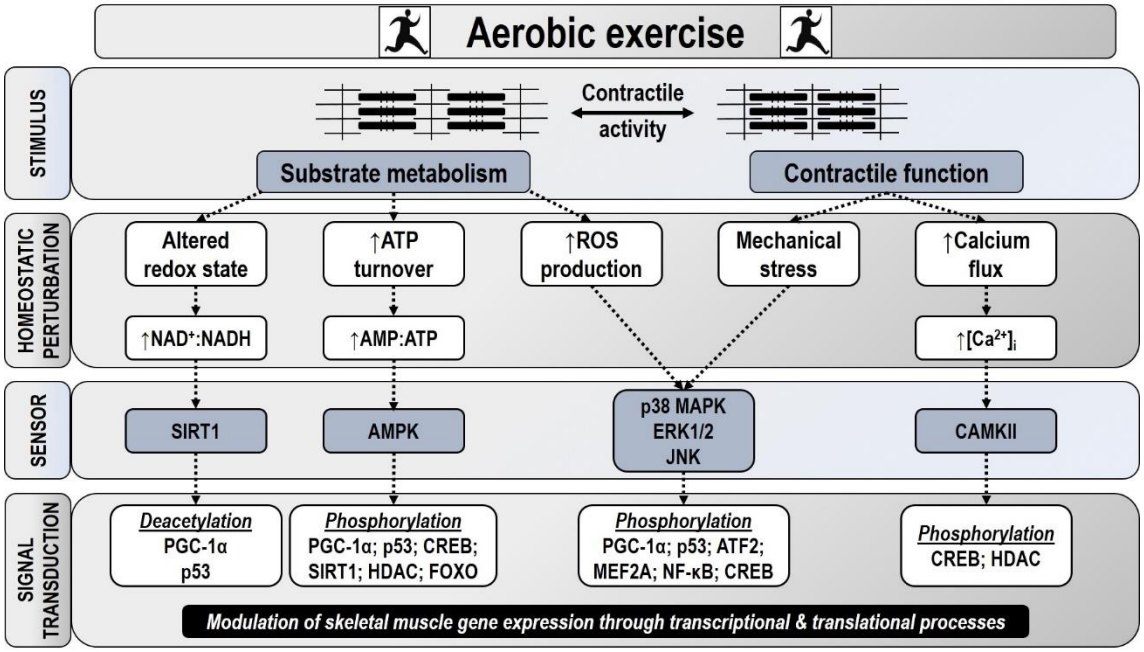

Figure 1.4. Schematic of transcriptional activity leading to exercise-induced mitochondrial biogenesis. NADH: Nicotinamide adenine dinucleotide; ROS: reactive oxygen species; Ca^{2+}: calcium; SIRT1: silent mating type information regulation 2 homolog 1; AMPK: 5' adenosine monophosphate-activated protein kinase; p38 MAPK: p38 mitogen-activated protein kinase; ERK1/2: extracellular-regulated kinase 1 and 2; JNK: *c-jun* N-terminal kinase; CaMKII: Ca^{2+}/calmodulin-dependent protein kinase II; PGC-1α: peroxisome proliferator-activated receptor γ coactivator-1α; CREB: cAMP response element binding protein; HDAC: Histone deacetylases; FOXO: forkhead box O subfamily; ATF2: activating transcription factor 2; MEF2A: myocyte enhancer factor-2A; NF-κB: nuclear factor kappa-light-chain-enhancer of activate B cells. *(Adapted from [75]).*

1.4.1 Cell signalling

1.4.1.1 Calcium and the role of CAMKII

Neural activation of skeletal muscle generates an action potential that results in Ca^{2+} release from the T tubules of the sarcoplasmic reticulum. As well as supporting contractile

activity, Ca^{2+} acts as an important primary messenger activating a cascade of events within different signalling pathways that have been linked with mitochondrial biogenesis [212]. Once Ca^{2+} is released, Calmodulin (CaM), a calcium-binding messenger protein, acts as a signal transducer and activates its downstream targets through conformational, activity, or location changes. Amongst these targets are a family of CaMKs, a Ca^{2+}/Calmodulin-dependent phosphatase (Calcineurin), and the Ca^{2+}-dependent kinases C (PKC) [22]. There is increasing evidence that CaMKs are involved in the regulation of mitochondrial biogenesis, with CAMKII playing a prominent role in transcriptional activity and the regulation of muscle plasticity [51]. Upon elevation of intracellular Ca^{2+}, Ca^{2+} binds to CaM; the Ca^{2+}/CaM complex binds in turn to the CaM-binding domain of CAMKII initiating an auto-phosphorylation process that increases CAMKII activity. The activity of CAMKII remains elevated even after calcium concentration returns to basal levels. This paradigm is described as Ca^{2+}/CaM-independent activity and is responsible for prolonged activation that persists after muscle contraction is terminated [295]. Studies in cultured cells [212] and in vivo [44] have shown that changes in intracellular Ca^{2+} concentration can modulate transcriptional activity. This modulation leads to changes in the content of mitochondrial enzymes, such as CS and cytochrome *c* [79], and transcription factors such as NRF-1, NRF-2, TFAM [212], and cAMP response element-binding protein (CREB) [29]. The transcriptional coactivator PGC-1α is also upregulated in a calcium-dependent manner following exercise. It was shown that an increase in cellular calcium leads to activation of CAMKII which, by phosphorylation of p38 MAPK, increases the activity and expression of PGC-1α [350] and induces mitochondrial biogenesis. A further post-translational mechanism by which CAMK can modulate mitochondrial biogenesis is by favouring the dissociation between myocyte enhancer factor-2 (MEF2) and histone deacetylases (HDACs) [165]. HDACs are a family of deacetylases that repress MEF2 activity by a physical association to it in the nucleus [183]. Following phosphorylation by CAMK, HDACs leave the nucleus so that MEF2 is able to carry out its transcriptional activity [188]. For more comprehensive information on the structure and transcriptional activation mechanism of calcium, the reader is referred to two excellent reviews [51, 295].

1.4.1.2 ATP turnover and the energy sensor AMPK

The change in cellular energy levels during contractile activity is one of the major factors disrupting cellular homeostasis. During metabolic stress, energy demand increases and so does ATP breakdown. When ATP utilisation is greater than ATP production, deamination of adenosine nucleotides, followed by reamination, causes an increase in cellular AMP levels leading to a greater AMP:ATP ratio. The signalling protein AMPK, often referred to as the "master sensor" of cellular energy balance, senses these changes and regulates metabolic processes, energy levels and mitochondrial biogenesis.

AMPK exists as a heterotrimer consisting of α, β, and γ subunits. The AMPK α subunit (of which there are two forms, α1 and α2) contains the catalytic domain whose phosphorylation is necessary for enzymatic activity [107]. Activation of AMPK plays an important role in the regulation of metabolism and mitochondrial biogenesis [245]. When the AMP:ATP ratio is high, AMPK is activated in an AMP-dependent manner by allosteric binding of the AMP molecule to the Cystathionine β-synthase (CBS) domain [279]. However, AMPK can also be activated in an AMP-independent manner, via phosphorylation at the 172 tyrosine residue (Thr^{172}) [107] by the tumour suppressor liver kinase B1 (LKB1) [349], and/or the calcium dependent CAMKKβ [108]. Although the primary role of AMPK is to control energy balance at the cellular level, recent findings have shown that AMPK is also regulated by hormones, growth factors, and cytokines, revealing its role in the maintenance of whole-body energy balance [103]. Finally, it has been shown that ROS can also activate AMPKα1 in an AMP-independent manner in rodent skeletal muscle [315], and that AMPK location and its translocation between different cellular compartments plays a key role in its activity [347]. These mechanisms represent an intricate and complex series of signals that, following contractile activity, contribute in a coordinated and timely manner to the activation of AMPK.

During a bout of exercise AMPK activity mediates increases in fatty acid transport to the mitochondria via phosphorylation of acetyl-CoA carboxylase (ACC) [203]. AMPK activation also supports increases in glucose uptake and fatty acid oxidation in rat muscle [192], as well as the upregulation of genes involved in mitochondrial respiration [345]. A series of metabolic targets, such as CS, glucose transporter type 4 (GLUT4), β-hydroxy acyl-CoA dehydrogenase (β-HAD), COX sub-unit I and IV and superoxide dismutase 2

(SOD2), are upregulated by activation of AMPK following contractile activity [137]. Following exercise, AMPK exerts its control on mitochondrial biogenesis by modulation of gene expression and transcriptional regulation that leads to a more oxidative phenotype. AMPK activates PGC-1α via direct phosphorylation at Thr^{177} and Ser^{538} in vitro and in cells, via a process that was thought to control all of the AMPK-mediated transcriptional activity of PGC-1α [130]. Activation of AMPK was shown to be proportional to exercise intensity [47, 331], and to the level of glycogen depletion [348], therefore suggesting that high-intensity training may be a potent stimulus to increase transcriptional activity and mitochondrial biogenesis. Further studies have also shown the existence of indirect mechanisms of AMPK control on PGC-1α activity. It was reported that AMPK enhances silent mating type information regulation 2 homolog 1 (SIRT1) activity by increasing cellular NAD^+ levels [40]. SIRT1 is a type III deacetylase that following activation modulates the activity of PGC-1α and its downstream targets by deacetylation [40]. Similarly, AMPK can indirectly control PGC-1α expression by HDAC phosphorylation at Ser^{259} and Ser^{498} and the subsequent nuclear export of HDACs [66]. Following chronic upregulation of AMPK activity in rats, NRF-1 DNA-binding activity, muscle mitochondrial density, and cytochrome *c* content have all been reported to be elevated [24]. These findings led the authors to suggest that AMPK plays an important role in training-induced adaptations by promoting mitochondrial biogenesis and respiratory protein expression, via an effect mediated by activation of NRF-1. Regardless of these claims, there is no evidence to date showing direct interaction between AMPK and NRF-1. Hence, it is unclear whether the observed phenotypic adaptations are a consequence of direct AMPK activation of NRF-1 or whether these effects are mediated by the coactivator PGC-1α.

Following contractile activity, AMPK also phosphorylates the tumour suppressor p53 at Ser^{15} [136], a post-translational modification associated with increased p53 activity and stability [71, 289]. Studies have also shown increased AMPK and p53 phosphorylation following a bout of exercise in both mice [265] and humans [18]. In a follow up study in humans, the increased p53 response following a bout of exercise with low carbohydrate availability, resulted in increased phosphorylation of ACC immediately post-exercise, suggesting that AMPK may be the dominant upstream signalling kinase regulating exercise-induced p53 activity [19].

AMPK can also modulate muscle metabolism via transcriptional control of the CREB family of transcription factors. It is known that the CREB family can control muscle metabolism and transcription via regulation of several genes including PGC-1α [7]. CREB transcriptional activity is activated by direct phosphorylation at Ser^{133} by Protein Kinase A (PKA) [86]. However, it has recently been shown that AMPK can also directly phosphorylate CREB at the same residue [306]. The importance of AMPK in the CREB transcriptional machinery is further underlined by the fact that mice deficient in the AMPK-activator LKB1 showed decreased voluntary running and reduced muscle mitochondrial marker enzyme expression including PGC-1α [307]. AMPK control over metabolism can also take place through direct phosphorylation of forkhead box O 3 (FOXO3) [94], a member of the FOXO family of transcription factors that are important regulators of metabolism [95]. Furthermore AMPK activation increases fatty acid oxidation in mouse skeletal muscle by activation of peroxisome proliferator-activated receptors α (PPARα) and PGC-1 [148], a transcriptional activation that is thought to be upregulated by AMPK-dependent PGC-1α activation [39].

As demonstrated here AMPK regulates the transcriptional activity following both a single bout of exercise and exercise training via an intricate network of signalling pathways aimed at the modulation of diverse phenotypic adaptations. For extensive reviews on AMPK, the reader is referred to two excellent reviews [39, 185].

1.4.1.3 Redox state, NAD^+ and the metabolic sensor SIRT1

NAD^+ is a molecule playing a crucial role in energy metabolism. This electron carrier provides the link between ATP synthesis during oxidative phosphorylation and glycolysis as well as the TCA, as previously described. NAD^+ concentration and the NAD^+:NADH ratio are representative of the cellular redox state and reflect the cellular energy status. It has been shown that NAD^+ levels increase in skeletal muscle due to oxidative stress following exercise [40] or calorie restriction [43]. The sirtuin family of enzymes, which includes 7 paralogs (SIRT1–SIRT7), is regulated by the redox state [278], and SIRT1 activity is enhanced in conditions of increased NAD^+ levels [38].

SIRT1, a NAD^+-dependent deacetylase, is mainly located in the nucleus [195], where it carries out its activity on its nuclear targets. However, it can translocate to the cytosol to exert its action on cytosolic targets [305]. SIRT1 exerts its activity by deacetylation [321] and has been described as a "master metabolic sensor" given its ability to sense changes in the redox state. SIRT1 dependence on NAD^+ fluctuations links its activity to the cellular metabolic status, exerting control over energy provision. This takes place mainly via modulation of transcriptional activity and favours a shift towards a more mitochondrially-derived energy production, and an increased antioxidant protection [38].

In the context of this review, the main targets of SIRT1 are the nuclear regulators PGC-1α and p53. Activation of PGC-1α takes place through deacetylation at one of the 13 Lysine residues by SIRT1 [248]. PGC-1α and SIRT1 are both nuclear proteins, but only during situations of energy stress is PGC-1α activated by SIRT1; this suggests the need for a further signal. The low AMP:ATP ratio that follows energy stress, is the signal required to induce phosphorylation of PGC-1α by AMPK, with PGC-1α phosphorylation also being a requirement for subsequent deacetylation and activation by SIRT1 [40]. SIRT1 also regulates p53 by deacetylation at Lys^{382} in humans [321], repressing its transcriptional activity [321], and reducing the rate of p53-dependent apoptosis [169].

The effects of exercise on SIRT1 content are somewhat controversial. It has been reported that 2 weeks of high-intensity interval training (HIIT) increase SIRT1 protein content and mitochondrial capacity, with the authors suggesting the SIRT1/PGC-1α axis as a possible mediator of mitochondrial biogenesis [161]. In contrast, another research group observed that HIIT increases SIRT1 activity and PGC-1α content, despite a decrease in SIRT1 content [96], and that SIRT1 overexpression in muscle reduces mitochondrial biogenesis [97]. In a later study, the same group showed that nuclear SIRT1 activity and not nuclear SIRT1 protein content is correlated with oxidative capacity, and that increased nuclear SIRT1 activity accompanies increases in mitochondrial biogenesis following exercise training [98]. These findings seem to confirm the SIRT1–PGC-1α axis as a possible regulator of mitochondrial biogenesis in skeletal muscle; however, the mechanisms of action still remain elusive. For a more comprehensive review on SIRT1 the reader is referred to an excellent review [38].

1.4.1.4 Oxidative stress, ROS, and the role of the MAPKs

Superoxide, oxygen peroxide and nitric oxide are extremely reactive molecules, known collectively as ROS. Although generated at several locations within muscle fibres, ROS are mainly produced in the mitochondria by complex I and III during oxidative phosphorylation [16]. In order to minimize their effects, skeletal muscles possess anti-oxidant enzymes which act as a defence system enabling removal or inactivation of ROS. When the balance between ROS production and removal shifts towards the former, oxidative stress occurs.

Although it was originally thought that ROS were cytotoxic molecules, it is now well accepted that they play a significant role as signalling events and control the modulation of muscular phenotypic adaptations to exercise and disuse [238]. The duration and magnitude of ROS production seems to influence the transcriptional outcome. A moderate increase in ROS production over a short time period leads to an adaptive response promoting antioxidant defence capacity [247]. In contrast, intense ROS production for longer periods can result in proteolysis, oxidative damage to mtDNA, and cell death [133]. The MAPK family, provides the link between oxidative stress and transcriptional activity by phosphorylating serine/threonine residues of several transcription factors and coactivators [163]. The three main MAPKs subfamilies in skeletal muscle are p38 MAPK, extracellular-regulated kinase 1 and 2 (ERK1/2) and c-Jun N-terminal kinase (JNK) [46].

Of the three MAPKs, p38 plays a prominent role, as it mediates the effects of cytokine stimulation by activating PGC-1α via direct phosphorylation [241]. This in turn increases mitochondrial biogenesis due to PGC-1α-mediated upregulation of NRF-1 and NRF-2, two transcription factors regulating the expression of nuclear-encoded mitochondrial proteins. Activation of the p38γ isoform, but not p38α or p38β, is necessary for exercise-induced mitochondrial biogenesis through the p38-PGC-1α axis [235]. Furthermore, it has been shown that p38 can stimulate PGC-1α-regulated mitochondrial biogenesis by stimulation of activating transcription factor 2 (ATF2) [6], and MEF2 [359], two upstream transcription factors of PGC-1α. ROS also provides MAPK-mediated signalling to activate nuclear factor kappa-light-chain-enhancer of activate B cells (NF-κB), a transcription factor involved in the expression of PGC-1α, NRF1 and other transcription

factors [238]. p38 was also shown to mediate resveratrol-induced activation of p53 [287], and exercise-induced activation of p53 following a single bout of exercise [265], by phosphorylation at Ser^{15}. Moreover, it has been demonstrated that activation of p38 for endurance-trained athletes requires a larger stimulus compared to untrained individuals, showing that the level of exercise-induced p38 activation is training status-dependent [357]. Finally, different authors have also reported that ROS production influences mitochondrial morphology [234, 358], although more research is warranted in this area. Taken together, these findings underline the important role of the ROS and the p38 MAPK pathway in promoting mitochondrial biogenesis.

1.4.2 Coactivators and regulators of mitochondrial biogenesis

1.4.2.1 PGC-1α, the master regulator of mitochondrial biogenesis

The PGC-1 family consists of PGC-1α, PGC-1β and PGC-1-related coactivator (PRC) [273], all of which share similar structures and function. PGC-1α is the most widely studied of the three and is expressed in many tissues, with the highest abundance found in oxidative tissues such as skeletal muscle [273]. PGC-1α has been reported to regulate cellular processes such as metabolic control [140, 156, 273], transcriptional activity [84, 141], mitochondrial turnover [320], and mitochondrial function amongst others [242, 352]. It therefore plays a pivotal role in the adaptive response to exercise and the modulation of mitochondrial biogenesis [213, 242]. As previously described, mitochondrial biogenesis requires the concerted activation of the mitochondrial and nuclear genomes. PGC-1α has emerged as a master coordinator of this interplay through the coactivation of nuclear transcription factors encoding mitochondrial proteins [116, 156]. A fine example of this coordination is via the regulation of the PGC-1α:NRF-1:TFAM axis. In this regard, it has been reported that PGC-1α promotes mitochondrial gene transcription via regulation of NRF-1 and NRF-2 [13, 352], as well as TFAM [151, 352]. Whilst NRF-1 and NRF-2 are nuclear transcription factors mediating the expression of various nuclear-encoded subunits of the ETS, TFAM, a nuclear-encoded transcription factor, exerts its transcriptional role inside the mitochondria where it regulates the

transcription of the 13 essential subunits of the ETS that are mtDNA-encoded [274]. Furthermore, it has been reported that the NRFs, but mainly NRF-1, directly activate TFAM, providing a possible link between nuclear and mitochondrial gene expression [325]. These findings demonstrate that the coordinated regulation of nuclear- and mitochondrial-encoded transcription factors through the PGC-1α:NRF-1:TFAM axis may be under the control of PGC-1α, and that this axis is essential for the control of mitochondrial biogenesis [84, 352]. PGC-1α can also coactivate members of the PPAR family, such as PPARγ [243], and PPARδ [336] - two transcription factors involved in mitochondrial biogenesis, lipid oxidation and fibre type regulation [148, 322, 336]. It has also been reported that PGC-1α exerts control on the expression and activity of the transcription factor estrogen-related receptor α (ERRα) [277], and that inhibition of ERRα compromises the ability of PGC-1α to induce the expression of mitochondrial proteins and to increase mtDNA [276]. The above findings clearly highlight the role of PGC-1α as a master regulator of mitochondrial biogenesis.

As described in previous sections, and illustrated in Figure 1.5, the main signals to induce PGC-1α expression are: 1) cellular calcium increases mediated by CAMKII and p38 MAPK phosphorylation [350]; 2) increases in AMP:ATP ratio resulting in phosphorylation by AMPK [130]; 3) increases in cellular NAD^+ resulting in concerted deacetylation by SIRT1 [248], and phosphorylation by AMPK [40]; 4) increases in oxidative stress mediated by p38 MAPK phosphorylation [241], and ATF2 [6] and MEF2 [359] activation, and 5) increases in ROS production mediated by indirect activation of AMPK [123]. It is important to notice that PGC-1α can also control its own expression, as transcription factors coactivated by PGC-1α such as MEF2 and myogenic determining factor (MyoD), can in turn regulate PGC-1α through an autoregulatory loop [8, 102]. Finally, substrate availability following exercise may influence PGC-1α expression, as maintaining low muscular glycogen content during recovery keeps PGC-1α mRNA levels elevated [232].

Studies on PGC-1α overexpression and ablation in cells and transgenic mice, provide further confirmation that PGC-1α plays a prominent role in mitochondrial biogenesis. Briefly, overexpression of PGC-1α stimulates mitochondrial respiration and biogenesis in muscle cells, via upregulation of the gene expression of the NRFs, as well as induction

of TFAM activity [352]. These findings were supported by studies in vivo demonstrating that PGC-1α overexpression in transgenic mice improved endurance performance and peak oxygen uptake, alongside an increase in mitochondrial function and content, as indicated by enhanced mitochondrial gene expression, mitochondrial DNA, and mitochondrial enzyme activity [37]. PGC-1α overexpression has also been associated with an increase in slow-twitch muscle fibres (type I), and a shift towards a more oxidative muscle phenotype [157]. Conversely, studies on PGC-1α ablation showed reduced muscle performance and exercise capacity [101, 151]. This is mediated by increased muscle damage and a shift towards a more glycolytic muscle phenotype [101], reduction in mitochondrial respiration and content, and ATP production [5, 151].

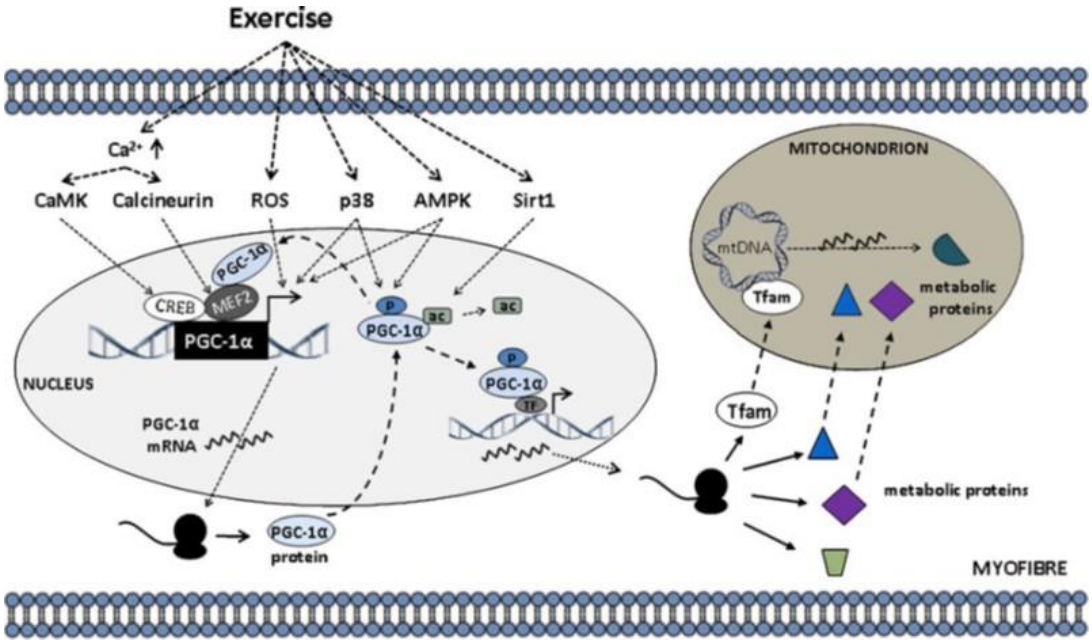

Figure 1.5. Exercise-induced signalling of PGC-1α transcriptional activity. ac: acetylation; AMPK: 5' adenosine monophosphate-activated protein kinase; Ca^{2+}: calcium; CaMK: Ca2+/calmodulin-dependent protein kinase; CREB: cAMP response element binding protein; MEF2: myocyte enhancer factor-2; mtDNA: mitochondrial DNA; p: phosphorylation; p38: p38 mitogen-activated protein kinase; PGC-1α: peroxisome proliferator-activated receptor γ coactivator-1α; ROS: reactive oxygen species; Sirt1: silent mating type information regulation 2 homolog 1; TF: transcription factor; Tfam: mitochondrial transcription factor A. *(From [213])*.

While the above evidence highlights its importance, studies have also reported that PGC-1α is likely not the only mediator of mitochondrial biogenesis. Following exercise training, PGC-1α knockout (KO) mice showed similar increases in mitochondrial proteins

compared to their wild-type (WT) littermates, with the authors suggesting that PGC-1α is not mandatory for exercise-induced muscular adaptations [150, 253]. In support of this notion, it was reported that the decrease in mitochondrial function present in PGC-1α KO mice can be reversed by endurance training [5]. Moreover, studies in murine cells demonstrated the existence of additional pathways regulating mitochondrial transcription that function in parallel with those regulated by PGC-1α [318].

Exercise is one of the most prominent activators of mitochondrial biogenesis. Consequently, PGC-1α activity is strongly modulated by physical activity. The effects of both a single bout of exercise and exercise training on PGC-1α protein and gene expression in human skeletal muscle will be discussed in detail in section 1.5.3.1.

Post-translational modifications of the PGC-1α protein (e.g., acetylation/deacetylation, phosphorylation, sumoylation, methylation) also influence PGC-1α activity and mitochondrial biogenesis by altering protein stability, subcellular localization, and protein activation amongst others. For example, phosphorylation of PGC-1α by p38 MAKP increases PGC-1α stability by increasing protein half-life [241], or increases PGC-1α activity and mitochondrial biogenesis by freeing PGC-1α from a repressor molecule named p160 myb binding protein [142]. As described earlier, SIRT1 activates PGC-1α by deacetylation [248], while general control non-repressed protein 5 (GCN5) negatively regulates PGC-1α transcriptional and biological function through direct acetylation and nuclear localization of PGC-1α [152]. Subcellular location has also been shown to play a key role in the modulation of PGC-1α activity [351]. Research in rodent [351], and human [159, 160] skeletal muscle demonstrated that at rest PGC-1α resides mainly in the cytosol. However, it was shown in rodents that following a single bout of exercise, nuclear PGC-1α increases, in parallel with increased activation of other transcription factors (e.g., NRF-1 and NRF-2) involved in mitochondrial gene transcription [351]. The authors suggested that accumulation of PGC-1α in the nucleus may constitute the initial phase of the exercise-induced adaptive response, whilst the subsequent increase in PGC-1α protein may sustain the increase in mitochondrial biogenesis. Ensuing research in human skeletal muscle also reported an increase in nuclear PGC-1α protein abundance following a bout of continuous [160] or sprint interval [159] training. This was accompanied by increased expression of mitochondrial genes

involved in the adaptive response to exercise. Changes in whole-muscle PGC-1α protein content however, did not take place until 24 h following exercise cessation [159]. Moreover, research in both mouse [11, 293] and human [293] skeletal muscle, demonstrated that PGC-1α also resides in the mitochondria, where it can form a complex in association with TFAM [11]. Subsequent research showed that following a single bout of exercise in mice, both nuclear and mitochondrial PGC-1α abundance are significantly increased [262]. The authors suggested that following an exercise stimulus, PGC-1α may relocate to both the nucleus and the mitochondrion to coactivate nuclear and mtDNA transcription factors respectively. In doing so, PGC-1α regulates the nuclear-mitochondrial DNA cross-talk and promotes mitochondrial biogenesis. These findings suggest that post-translational events leading to PGC-1α sub-cellular translocation, and its subsequent coordination of the nuclear and mitochondrial transcriptional activity, play a key role in exercise-induced mitochondrial biogenesis.

1.4.2.2 p53, "guardian of the genome" and metabolic regulator

p53 is widely known as the "guardian of the genome" for the wide variety of functions that it influences, ranging from cell cycle arrest to senescence, from apoptosis to DNA-damage repair, from angiogenesis to tumour suppression and many others [52, 153, 282]. However, p53 has recently come to the fore as a metabolic regulator and a mediator of mitochondrial biogenesis (Figure 1.6). The mechanisms by which p53 regulates mitochondrial biogenesis are still largely unknown; however, evidence is emerging regarding its transcriptional control of mitochondrial and respiratory genes.

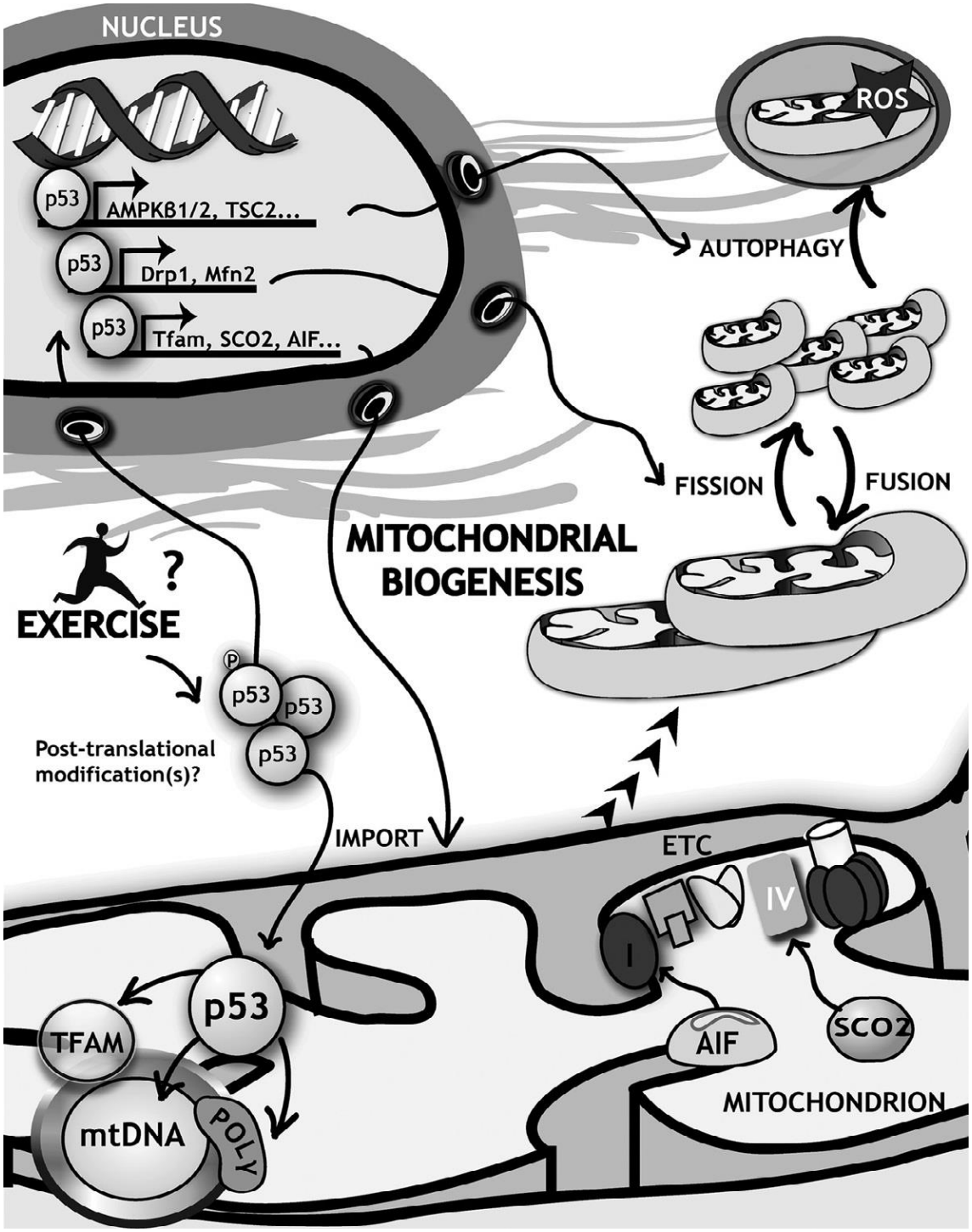

Figure 1.6. Regulation of mitochondrial biogenesis by p53. AIF: apoptosis inducing factor; AMPKβ1/2: 5' adenosine monophosphate-activated protein kinase subunit β 1 & 2; Drp1: dynamin-related protein-1; ETC: electron transport chain; Mfn2: mitofusin 2; mtDNA: mitochondrial DNA; POLγ: polymerase γ; ROS: reactive oxygen species; SCO2: synthesis of cytochrome *c* oxidase 2; Tfam: mitochondrial transcription factor A; TSC2: tuberous sclerosis complex 2. *(From [266])*.

Findings from p53 KO mice studies indicate that absence of p53 leads to reduced endurance performance [217, 265], mitochondrial respiration [181], and mitochondrial content [265], as well as increased fatigability and ROS production [265]. One of the key studies in this area has demonstrated that in mouse liver mitochondria p53 is able to influence the balance between glycolytic and oxidative pathways, via modulation of its downstream transcriptional target synthesis of cytochrome *c* oxidase 2 (SCO2) [181]. SCO2 has been shown to assist the assembly of the mitochondrially-encoded subunit II into the COX complex, the site of oxygen utilization during mitochondrial respiration. This study also showed that ablation of p53 decreased oxygen consumption and aerobic respiration, promoting a switch to glycolytic pathways whilst reducing endurance performance [181]. In a similar manner, p53 can also modulate mitochondrial function via positive transcriptional regulation of the apoptosis inducing factor (AIF) gene [298]. AIF is not only involved in the regulation of mitochondrial structure [284], but can also regulate the ETS by assisting the assembly and stabilisation of complex I and III [284, 319]. It has been demonstrated that lack of AIF in human and mouse cells severely compromised CI activity and promoted a switch to glycolytic pathways [319]. p53 regulation of mitochondrial function also extends to its ability to promote and stabilise the assembly of mature ATP synthase (CV), by interacting within the mitochondrial matrix with oligomycin sensitivity-conferring protein (OSPC) [23], a subunit of CV [254].

p53 mediates mitochondrial biogenesis and exerts control on mitochondrial function in different ways, one of which is via the modulation of mitochondrial morphology. Mitochondrial fusion and fission are dynamic processes necessary for the maintenance of mitochondrial structure and quality. It has been reported that p53 can transcriptionally upregulate the fission protein dynamin-related protein-1 (DRP1), which conveys the p53 apoptotic signal and initiates fission [154]. Furthermore, p53 can also modulate mitochondrial fusion as it was shown that p53 binds directly to the promoter of mitofusin 2, and that mitofusin 2 mRNA and protein content are upregulated in a p53-dependent manner [335]. Mitofusin 2 has also been reported to be sensitive to exercise, as its mRNA is increased following an exercise bout [42]. Mitofusin 2 protein content did not change following seven sessions of HIIT [224]; however, an increase was reported following 12 weeks of continuous training in humans [143], suggesting that greater training workloads

may be required. Mitofusin 2 has also been shown to play an important role in oxidative phosphorylation, given that its loss-of-function is associated with a decreased content of the subunits of complex I, II, III and V, whereas mitofusin 2 overexpression is associated with an increased content of the subunits of complex I, IV and V [231]. From the above evidence, it seems that through transcriptional activation p53 extends its modulation to all of the ETS complexes, therefore playing an important role in the regulation of oxidative capacity. Similar to PGC-1α [320], p53 has also been shown to be an important regulator of autophagy [174], as well as mitochondrial morphology [266]. Autophagy, and fusion and fission processes, have been reported to contribute to the replacement of damaged proteins with de novo synthesised ones, and enhance mitochondrial function independent of changes in mitochondrial content [135, 173]. These findings suggest that modulation of mitochondrial turnover, is yet another mechanism by which p53 can regulate mitochondrial function.

Further insights into the role of p53 as a regulator of mitochondrial function stem from research showing that within the mitochondria, p53 mediates promotion and maintenance of mtDNA integrity, by specifically binding to mtDNA polymerase γ, whilst enhancing its DNA replication function [1]. p53 has also been reported to directly bind to mtDNA [112, 144, 267], or to interact with TFAM [217, 356, 267], therefore exerting control on the mitochondrial transcriptional machinery and mtDNA copy number. p53 can also interact with and influence PGC-1α. It was reported that the PGC-1α promoter region houses a p53 binding site [124], and that p53 KO mice have lower PGC-1α protein content in parallel with a host of mitochondrial deficiencies [181, 217, 265] as previously reported. However, there seems to be some controversy with respect to the effects of p53 on PGC-1α. Whilst a study showed that p53 upregulates PGC-1α expression by binding to its promoter region [10], it has also been demonstrated that p53 binds and represses PGC-1α, although in the setting of telomere dysfunction [263]. From the above findings, it seems evident that more research is needed to elucidate the interplay between the two regulators and to unveil the role of p53 in mitochondrial biogenesis.

p53 is activated by similar signalling events that leads to the activation of PGC-1α, providing further evidence that p53 is likely involved in the control of mitochondrial biogenesis. In this regard, p53 is activated by AMPK [136], as well as ERK2, and p38

MAPK [287]. This activation induces phosphorylation of p53 at Ser^{15}, a post-translational modification that further increases p53 activity by protein stabilisation [289]. Furthermore, it was previously described how SIRT1 deacetylates p53 and inactivates its transcriptional activity [169, 321]. Plant homeodomain finger protein 20 (PHF20), a nuclear transcription factor upstream of p53, has also been shown to upregulate p53 activity at the mRNA level by directly binding to its promoter region [218]. The same research showed a reduction in p53 protein content following PHF20 knockdown, providing further confirmation of PHF20 transcriptional regulation of p53. A second study also demonstrated that PHF20 stabilises and activates p53 protein within the nucleus by associating with p53 and disrupting murine double minute 2 (MDM2)-mediated degradation [65]. Finally, upon Akt phosphorylation of PHF20 at Ser^{291}, PHF20 translocates from the nucleus to the cytoplasm, with consequent abrogation of the PHF20 transactivation of p53 [218]. Despite the above evidence of PHF20 control on p53, no study to date has investigated the effects of a single exercise bout, or of exercise training on the gene and protein expression of PHF20. Future research is therefore required to provide these answers.

Post-translational modifications leading to p53 stabilisation, as well as the cellular translocation of both p53 and its transcriptional partners, seem to play a pivotal role in p53 activity. It is known that p53 is predominantly a nuclear transcription factor [328], but studies have demonstrated its presence inside the mitochondria, where it carries out its activity and promotes mtDNA integrity and replication [1, 112, 356]. Moreover, research in mouse skeletal muscle demonstrated that a single bout of exercise induces translocation of p53 inside the mitochondria where, by interaction with TFAM and mtDNA, it increases mtDNA transcription [267]. These findings are in agreement with those from a previous study showing that p53 determines maximal aerobic capacity and regulates mtDNA content in mouse skeletal muscle via interaction with TFAM [217]. This study has also shown that p53 KO mice exhibit significantly lower adaptations to training compared to wild-type mice, further enhancing the importance of p53 as a possible regulator of training-induced adaptations. Studies have reported that a single bout of exercise induced p53 phosphorylation in conjunction with activation of p38 and AMPK in both mouse [267], and human [18] skeletal muscle, suggesting a possible pathway for p53-induced mitochondrial biogenesis. However, research in mouse skeletal

muscle showed that p53 mRNA does not change within 3 h following a single bout of exercise [267], a fact that may be due to the ability of p53 to auto regulate itself [201]. Although the available research is limited, the effects of both a single bout of exercise and exercise training on p53 protein and gene expression in human skeletal muscle will be discussed in detail in section 1.5.3.2. In conclusion, while p53 has been extensively studied in the area of cancer and apoptosis, there clearly is a gap in the literature on how p53 responds to endurance exercise, and how it modulates mitochondrial biogenesis.

1.4.2.3 TFAM, a mitochondrial transcription factor

TFAM is a nuclear-encoded transcription factor that controls the expression of mtDNA and regulates the transcription of the 13 mtDNA-encoded subunits of the ETS [274]. The role of TFAM has been touched upon in previous sections, therefore it will only be discussed briefly. Studies have demonstrated that TFAM is under the regulation of both PGC-1α [151, 352], and NRF-1 [325], and that the PGC-1α:NRF-1:TFAM axis plays an essential role in the regulation of mitochondrial biogenesis [84, 352]. Studies in rat skeletal muscle indicate that following muscle contraction TFAM mRNA is increased, together with an increase in the rate of mitochondrial protein import, and TFAM protein content [87]. This translates into increased binding to the mtDNA promoter region, and the subsequent upregulation of mtDNA transcription and replication [115]. In addition, research in rodents indicates that following exercise TFAM can form a complex inside the mitochondria with both PGC-1α [262], and p53 [267] and positively drive mtDNA transcriptional activity. Given that some studies in humans failed to show increased TFAM protein or gene expression following exercise [96, 210, 224], it was suggested that TFAM activity rather than content may contribute to mitochondrial biogenesis during exercise [224]. The effect of a single bout of exercise and exercise training on TFAM protein and gene expression in human skeletal muscle will be discussed in detail in section 1.5.3.2.

1.4.3 Summary

A summary of the proposed mechanisms of action of some of the key signalling proteins and transcription factors involved in the regulation of exercise-induced mitochondrial biogenesis, has been presented in the previous sections. These sections have provided evidence that exercise is a powerful stimulus to induce mitochondrial adaptations. Therefore, it is also important to understand how specific training interventions may influence mitochondrial adaptations. An overview of the current knowledge on how exercise intensity and exercise volume (two key determinants of the training stimulus [12]) affect changes in markers of mitochondrial biogenesis, will be presented in the next sections

1.5 Exercise training and mitochondrial adaptations

Exercise training can be defined as a series of exercise bouts over a period of time, and the resulting adaptations are largely the cumulative effect of those obtained following a single exercise session [224]. Repeated disturbances to cellular homeostasis lead to a series of adaptive responses that aim to minimise disruptions when exercise is next performed. It is important to note however, that long-term adaptations are not always the result of the simple addition of those induced by a single exercise bout [55].

Changes in mitochondrial content [128, 239, 257, 360] and mitochondrial function [68, 128, 194, 311, 360] are two important training-induced adaptations, and for this reason will represent the main focus of this literature review. It is well established that maximal mitochondrial oxidative power exceeds oxygen delivery in humans, and is in excess of that required during exercise when half or more of the body muscle mass is engaged during exercise (e.g., cycling or running) [28, 312]. Regardless of this overcapacity, both mitochondrial content [36, 224, 296], and function [68, 127, 343] have been reported to increase following exercise training. Moreover, these adaptations can take place very rapidly, as changes in mitochondrial content have been reported as early as 24 h after a

single bout of exercise [159], whereas changes in mitochondrial function have been shown after only five training sessions [300].

Changes in mitochondrial content and function are not necessarily similar in magnitude [300, 343]. As can be seen in Figure 1.7, an in-depth analysis of the available literature in human skeletal muscle indicates that higher physical activity levels are associated with relatively larger values of mitochondrial function (as measured by mitochondrial respiration) than mitochondrial content (as assessed by CS activity) [26]. This apparent dissociation between mitochondrial function and mitochondrial content has previously been demonstrated in mouse skeletal muscle [252]. It should be noted that two separate studies have reported that the ratio between mitochondrial respiration and mitochondrial content was not related to training status [311, 342]. These two studies however, measured mitochondrial function in isolated mitochondria, a technique that has been reported to disrupt both mitochondrial structure and function [230]. This raises the possibility that mitochondrial damage during the isolation procedure may have altered the outcome. Nonetheless, while further research is required, it appears important that training studies should measure and report changes in both mitochondrial function and mitochondrial content [128].

The origin of this dissociation between training-induced changes in mitochondrial content and function is not readily apparent, and may have many explanations. For example, while one of the primary roles of mitochondrial transcription factors is the regulation of transcriptional activity leading to mitochondrial biogenesis, both PGC-1α [140] and p53 [181] have been shown to act as metabolic regulators, favouring a switch from glycolytic to oxidative energy provision. This indicates that it may be possible to improve mitochondrial function, independent of changes in mitochondrial content. Mitochondrial turnover may also contribute to the dissociation between mitochondrial content and function. Studies in humans have in fact demonstrated that initiation of autophagy [198], and increased fusion [42] and fission [224] processes, constitutes an important physiological response to exercise. Hence, an increase in mitochondrial function may be the result of more efficient mitochondria following autophagic upregulation, or improved mitochondrial morphology, that may not necessarily result in a net increase in mitochondrial content [173]. It has been suggested that if changes in mitochondrial

protein synthesis and degradation are matched, mitochondrial content would not change, nevertheless, replacement of old proteins with newly synthesised one, may still result in an increase in mitochondrial function [173]. Metabolic regulation and modulation of mitochondrial turnover are therefore two potential mechanisms that may contribute to the dissociation between mitochondrial content and respiration. Future research is warranted to confirm this apparent dissociation in human skeletal muscle, and to elucidate the possible mechanisms that could explain it.

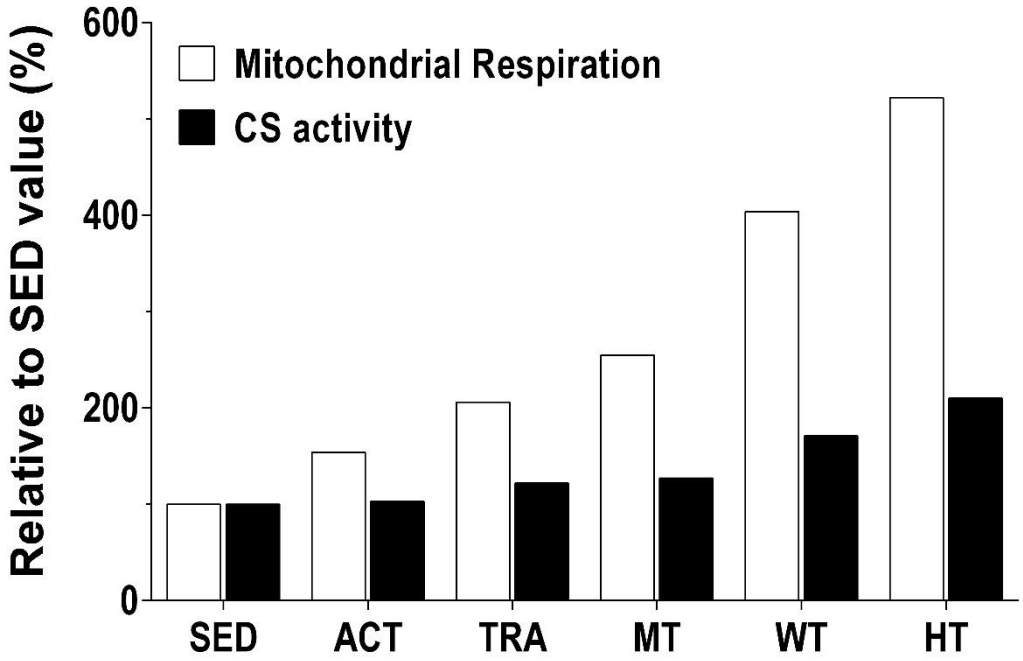

Figure 1.7. Mitochondrial respiration and citrate synthase (CS) activity in humans of differing training status. SED: sedentary, ACT: active, TRA: trained, MT: moderately-trained, WT: well-trained, and HT: highly-trained. *(From [26])*.

While it is well accepted that exercise training represent a powerful stimulus to improve endurance performance, and to increase mitochondrial content and function, it is not yet known which training intervention results in the greatest adaptations. The stimulus provided by exercise training is the combination of training intensity, frequency, duration, volume, and the overall length of the intervention. Manipulation of any of these variables is likely to influence the outcome. Training intensity and training volume have been

previously identified as two of the most important parameters [12]. Considering that training duration is typically inversely related to training intensity, and the paucity of studies examining the role of frequency, duration and the overall length of an intervention, this thesis will focus primarily on the role of training intensity and volume and how they affect exercise- and training-induced changes mitochondrial adaptations.

1.5.1 Exercise- and training-induced changes in mitochondrial content

As described in section 1.3.2, there are several ways to assess changes in mitochondrial content. However, as most studies have utilised the activity of CS as a valid biomarker of mitochondrial content [145], stronger conclusions can be made when pooling these studies together. Therefore, this literature review will focus on studies that measured exercise- and training-induced changes in this biomarker (Table 1.1). Most studies report an increase in CS activity following exercise training in humans (Table 1.1). However, a number of studies have also shown that CS activity does not change significantly following a period of exercise training [55, 88, 90-93, 147, 155, 158, 170, 244]. The reasons for these discrepancies are not readily apparent, and may relate to the training status of the participants, the training protocols used, or the methodological differences in the CS activity assay employed. For example, the timing of muscle biopsy (see below), the method by which the reaction is monitored (spectrophotometer or fluorimeter), the different reagents, the reaction temperature, and the sample preparation employed (wet *vs.* freeze-dried muscle tissue) may all influence the assay results [323].

There are limited and contrasting findings regarding the effects of a single bout of exercise on the activity of CS. While a 12% increase in CS activity was reported immediately after the termination of an ~70-min bout of continuous cycling at 75% $\dot{V}O_{2Peak}$ [309], another study reported unchanged CS activity immediately, and 2 h, after the termination of a HIIT bout consisting of three cycling intervals to fatigue at 130% of $\dot{V}O_{2Peak}$ [314]. CS activity did not change also following 90 min of continuous cycling at 60% of $\dot{V}O_{2Peak}$ in male participants [249]. Similarly, there was no change in CS activity 4 h after the termination of a HIIT bout consisting of ten 4-min cycling intervals at 90% of $\dot{V}O_{2Peak}$ [224]. Moreover, while one study observed increased CS activity 24 h after

the termination of an exercise bout consisting of four "all-out" 30-s cycling sprints (SIT) [159], two different studies employing either HIIT (ten 4-min cycling intervals at 90% of $\dot{V}O_{2Peak}$) [224], or continuous cycling (60 min at 80% of $\dot{V}O_{2Peak}$) [74], reported that CS activity was not changed 24 h after the termination of the bout. These latter studies observed the first significant increase in CS activity only after three training sessions [74, 224]. Although the majority of research seem to agree that changes in CS activity do not take place "within minutes" of the termination of exercise, further research is clearly required to determine the effects of a single bout of exercise on the activity of CS. An interesting corollary to the above findings however, is that the timing of post-exercise (and post-training) muscle sampling needs to be carefully controlled as this may influence the results.

Table 1.1. Changes in citrate synthase (CS) activity from human skeletal muscle biopsies (vastus lateralis) from healthy participants, following a cycle training intervention. Studies with diseased populations (e.g., chronic heart failure, diabetics, and obese patients), studies investigating a single bout of exercise, and studies not providing precise and detailed information about the training prescription, were excluded. Training volume arbitrary units were calculated by multiplying the training intensity (as a percentage of $\dot{V}O_{2Peak}$) by the duration of exercise training (in minutes) and by the total number of training sessions.

Study	**Participants**		**Training**			**ΔCS (%)**
	n - sex	**$\dot{V}O_{2Peak}$**	**TYPE**	**INTENSITY (%$\dot{V}O_{2Peak}$)**	**VOLUME (a.u.)**	
Svedenhag 1983 [302]	8 - M	43	CT	75	96000	62*
Jacobs 1987 [126]	11 - M/F	n/r	SIT	220	9900	12*
Gorostiaga 1991 [88]	6 - M/F	37	CT	50	36000	25*
	6 - M/F	36	HIIT	100	36000	2
Green 1991 [93]	8 - M	53	CT	59	77880	23
Green 1992 [92]	9 - M	55	CT	67	48240	2
Wibom 1992 [343]	9 - M	44	CT	70	60480	40*
Chesley 1996 [48]	8 - M	45	CT	65	46800	20*

Study	Participants		Training			ΔCS (%)
	n - sex	$\dot{V}O_{2Peak}$	TYPE	INTENSITY (%$\dot{V}O_{2Peak}$)	VOLUME (a.u.)	
Spina 1996 [296]	12 - M/F	39	CT	65	66300	32*
Tiidus 1996 [308]	7 - M	48	CT+HIIT	75-100	66000	25*
	6 - F	37	CT+HIIT	75-100	66000	50*
Linossier 1997 [158]	8 - M	52	SIT	280	25200	7
Liljedhal 1998 [155]	16 - M/F	n/r	SIT	250	4500	6
MacDougall 1998 [171]	8 - M	52	SIT	195	16953	36*
Putman 1998 [244]	7 - M	45	CT	60	50400	5
Green 1999 [89]	7 - M	46	CT	68	24480	12
				68	48960	26*
Starritt 1999 [300]	7 - M/F	43	CT+HIIT	75-95	39375	22*
Dubouchaud 2000 [70]	9 - M	45	CT+HIIT	75	243000	75*
McKenzie 2000 [187]	6 - F	38	CT+HIIT	60-100	108216	27*
	6 - M	46	CT+HIIT	60-100	108216	34*
Parra 2000 [220]	5 - M	n/r	SIT	208	10920	38*
	5 - M	n/r	SIT	208	10920	28*
Tonkonogi 2000 [313]	8 - M/F	39	CT+HIIT	75-95	98402	48*
Carter 2001 [41]	8 - M	42	CT	60	126000	40*
	8 - F	32	CT	60	126000	43*
Masuda 2001 [179]	7 - M	45	CT	69	115920	28*
Barnett 2004 [17]	8 - M	48	SIT	218	11772	42*
Howarth 2004 [120]	8 - M	43	CT	80	168000	32*

Study	Participants		Training			ΔCS (%)
	n - sex	$\dot{V}O_{2Peak}$	TYPE	INTENSITY (%$\dot{V}O_{2Peak}$)	VOLUME (a.u.)	
LeBlanc 2004 [147]	8 - M	48	CT	75	180000	40*
				75	22500	15
Burgomaster 2005 [36]	8 - M	45	SIT	212	3392	38*
Messonnier 2005 [193]	8 - M/F	43	CT	75	216000	54*
Burgomaster 2006 [34]	8 - M	49	SIT	212	3392	11*
Ostergard 2006 [216]	19 - M/F	41	CT	70	94500	25*
Bakkman 2007 [14]	8 - M	45	CT	65	31200	21*
Talanian 2007 [304]	8 - F	36	HIIT	90	25200	20*
Burgomaster 2008 [35]	10 - M	41	CT	65	97500	35*
	10 - M	41	SIT	213	9585	28*
Harmer 2008 [104]	7 - M/F	n/r	SIT	204	17736	42*
Perry 2008 [223]	8 - M/F	45	HIIT	90	64800	26*
Green 2009 [91]	9 - M	48	CT	62	37200	14
Gurd 2010 [96]	9 - M/F	45	HIIT	90	64800	28*
Little 2010 [161]	7 - M	46	HIIT	100	6000	16*
Perry 2010 [224]	9 - M	46	HIIT	90	25200	24*
Stannard 2010 [299]	7 - M/F	46	CT	65	81250	18*
	7 - M/F	46	CT	65	81250	19*
Yfanti 2010 [355]	10 - M	50	CT+HIIT	55-100	230760	54*
Gurd 2011 [98]	7 - M/F	n/r	HIIT	90	25200	9*

Study	Participants		Training			ΔCS (%)
	n - sex	$\dot{V}O_{2Peak}$	TYPE	INTENSITY (%$\dot{V}O_{2Peak}$)	VOLUME (a.u.)	
Irving 2011 [125]	8 - M/F	26	CT+HIIT	70-85	33300	20*
Murias 2011 [204]	7 - M	49	CT	70	56700	50*
	7 - M	49	CT	70	113400	67*
Jeppesen 2012 [132]	8 - M	38	CT+HIIT	62.5	121875	50*
Rud 2012 [255]	12 - M/F	57	CT	57	111720	30*
Egan 2013 [74]	8 - M	37	CT	80	67200	32*
Ma 2013 [170]	8 - M	41	SIT	170	7262	8
Cochran 2014 [55]	9 - M/F	47	CT	110	9900	7

$\dot{V}O_{2Peak}$: peak oxygen uptake (mL min^{-1} kg^{-1}); ΔCS: change in citrate synthase (CS) activity M: male; CT: continuous training; F: female; n/r: not reported; SIT: sprint interval training; HIIT: high-intensity interval training. * Significantly different from pre-training values ($P < 0.05$).

Results from Table 1.1 indicate that exercise training is usually associated with an increase in CS activity. Cross-sectional studies provide further support for the above statement by showing greater CS activity values for endurance-trained compared to active individuals, whose values are in turn greater than those of sedentary individuals [128, 246, 257, 360, 249, 199]. Previous research observed that skeletal muscle of previously well-trained individuals requires a greater stimulus to activate the signalling pathways leading to mitochondrial biogenesis [357], consistent with the notion that training adaptations are reduced as training status increases [146, 162, 357]. This would suggest that exercise-induced changes in CS activity may be influenced by a participant's training status. Consistent with this hypothesis, it has been reported that following ten days of continuous cycling at an intensity of 60-90% of maximal heart rate, sedentary participants increased their CS activity by 59% whereas that of trained athletes remained unchanged [199]. Moreover, results from a study in which ten well-trained endurance athletes cycled

outdoor for a total of 3211 km over 21 days (an average of ~170 km/day), showed that CS activity did not increase significantly [292].

Although training-induced changes in CS activity appear to be associated with training status, there does not seem to be a relationship between changes in CS activity and baseline $\dot{V}O_{2Peak}$ values. Using the data set in Table 1.1, no significant correlation was found between $\dot{V}O_{2Peak}$ and ΔCS (r = -0.08). This correlation was then re-calculated with the effects of training volume "partialed out" (i.e., controlled for). This can be achieved by running a partial correlation that allows for the determination of the coefficient of correlation $r_{y1.2}$, where the two independent variables are baseline $\dot{V}O_{2Peak}$ (variable 1) and training volume (variable 2) and the dependent variable is ΔCS (variable Y) [222]. This analysis revealed no correlation between baseline $\dot{V}O_{2Peak}$ and ΔCS, even when the effects of training volume were removed ($r_{y1.2}$ = -0.06).

Training volume and intensity are two of the most important parameters influencing training-induced changes in mitochondrial content [12], as evidenced by the number of available reports (Table 1.1). The following two sections will examine the available literature and try to define the role of training volume and training intensity in promoting changes in mitochondrial content. Finally, there are other factors that may influence both baseline levels and training-induced changes in CS activity, such as age [30, 58, 63, 204, 246], sex [41, 58, 187, 308], or health status [32, 104, 109, 125, 176, 197, 323], but they will not be reviewed in this thesis.

1.5.1.1 Effects of training volume

To date there is limited research that has investigated the effects of different training volumes on changes in CS activity within the one study. However, analysis of the results from 47 studies (including 57 different training groups) with human participants suggests that greater training volumes are associated with a greater increase in CS activity (Figure 1.8). This is in agreement with the observation of a similar relationship when the results of training-induced changes in CS activity in oxidative rat skeletal muscle are pooled [26]. These results indicate that training volume may be a key determinant of training-induced changes in mitochondrial content (as assessed by CS activity). However, caution

is required when pooling the results from different studies, due to small methodological differences between laboratories. Furthermore, data from both humans (Figure 1.8) and rats [26] do not indicate the presence of a plateau, seemingly suggesting the greater the training volume, the greater the increase in CS activity. In contrast with this findings, a study investigating the time course of training-induced changes in CS activity following fourteen sessions of continuous training with humans, showed that CS activity was significantly increased after the third session and did not increase further in the following session, which suggests a possible plateau [74]. Similarly, another study demonstrated no further increase in CS activity in the second half of a 12-week training program in a pool of young participants [204]. Further research directly comparing how different training volumes affects training-induced changes in CS activity in humans is required. In this regard, it would be valuable for future training studies to consider measurements of CS activity in single fibres, and to also consider assessment of changes in mitochondrial content by TEM, the gold-standard technique for this evaluation [189].

1.5.1.2 Effects of training intensity

In contrast to training volume, training intensity does not seem to be a key determinant of training-induced changes in CS activity (Figure 1.9). A similar analysis of findings from studies in rat oxidative skeletal muscle fibres, also indicates that training intensity is not a key factor for changes in CS activity [26]. Conversely, the same review reported that in rat type II glycolytic skeletal muscle fibres training intensity may be a determining factor influencing changes in CS activity. Caution needs to be used when interpreting these results as higher training intensities were also associated with greater training volumes [26]. Another possible explanation for these results is that type II fibres are only recruited at higher training intensities [327].

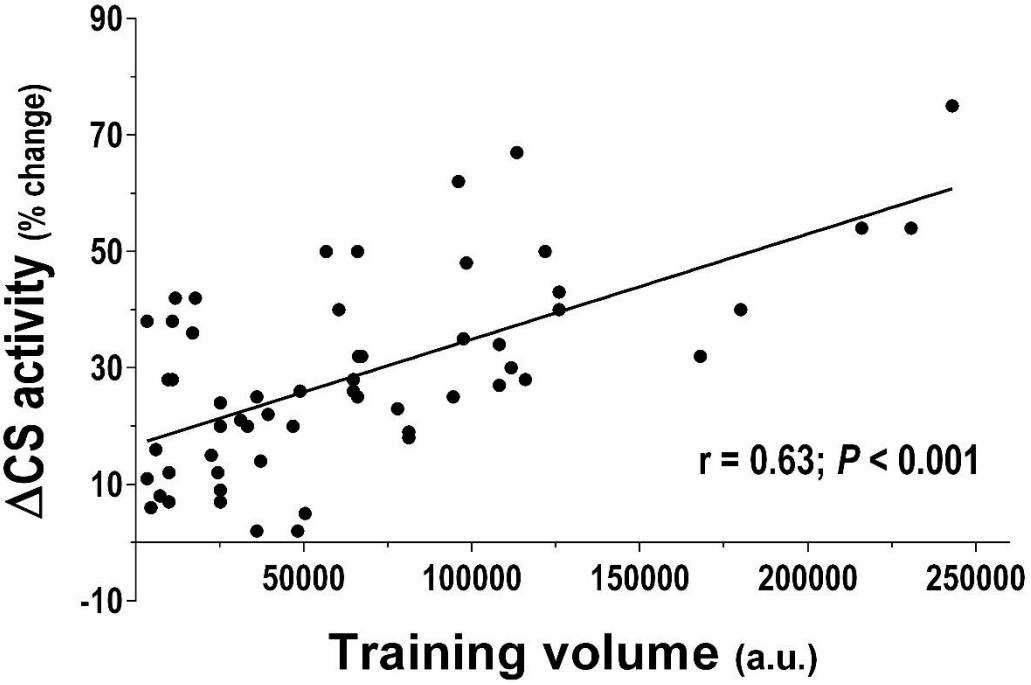

Figure 1.8. The relationship between training volume and training-induced changes in citrate synthase (CS) activity in the vastus lateralis muscle of healthy human participants. All the studies presented in Table 1.3 have been included in this figure. Therefore, studies with diseased populations (e.g., chronic heart failure, diabetics, and obese patients), studies investigating a single bout of exercise, and studies not providing precise and detailed information about the training prescription, were excluded. Training volume arbitrary units were calculated by multiplying the training intensity (as a percentage of $\dot{V}O_{2Peak}$) by the duration of exercise training (in minutes) and by the total number of training sessions. Training intensity for studies employing sprint interval training (SIT) is often not provided; for these studies, a value of training intensity was used based on that attained by participants in the training study presented in Chapter 2, completing the same number of repetitions. These ranged from ~220% to ~205% of $\dot{V}O_{2Peak}$ for four to ten 30-s "all-out' bouts, respectively.

To date, only two studies have investigated the effects of exercise training in human skeletal muscle, by directly comparing different training intensities. While an 8-week training study showed that CS activity was increased following continuous training at 50% $\dot{V}O_{2Peak}$, but not HIIT at 100% $\dot{V}O_{2Peak}$ [88], a second report revealed similar changes in CS activity following six weeks of either continuous training at 65% $\dot{V}O_{2Peak}$, or sprint interval training (SIT) at maximal training intensity (>200% $\dot{V}O_{2Peak}$) [35]. Considering these discrepancies, and that most of the above conclusions were drawn by comparing different studies presenting a series of methodological differences, further research

directly comparing different training intensities is needed to clarify the role of training intensity on training-induced changes in CS activity.

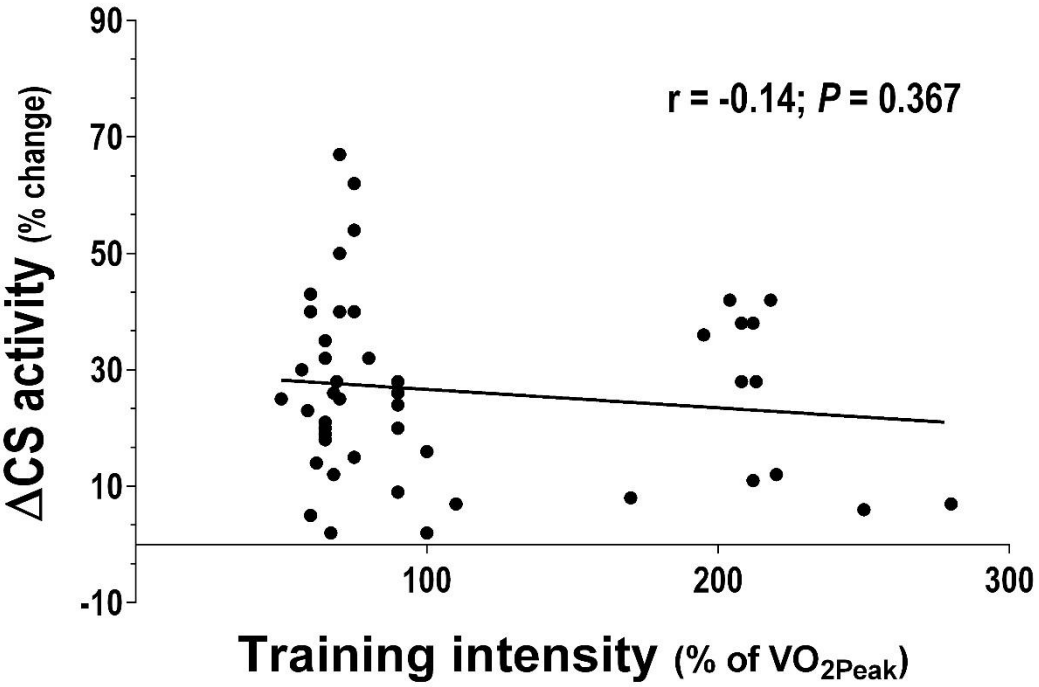

Figure 1.9. The relationship between training intensity and training-induced changes in citrate synthase (CS) activity in the vastus lateralis muscle of healthy human participants. Only studies involving cycling and using a single training intensity were included. Results were obtained from: [14, 17, 34-36, 41, 48, 55, 74, 88, 89, 91-93, 96, 98, 104, 120, 126, 147, 155, 158, 160, 170, 171, 179, 193, 204, 216, 220, 223, 224, 244, 255, 296, 299, 302, 304, 343]. Studies with diseased populations (e.g., chronic heart failure, diabetics, and obese patients), studies investigating a single bout of exercise, and studies not providing precise and detailed information about the training prescription, were excluded. Training intensity was expressed as percent of $\dot{V}O_{2Peak}$. Training intensity for studies employing sprint interval training (SIT) is often not provided; for these studies, a value of training intensity was used based on that attained by participants in the training study presented in Chapter 2, completing the same number of repetitions. These ranged from ~220% to ~205% of $\dot{V}O_{2Peak}$ for four to ten 30-s "all-out' bouts, respectively.

1.5.1.3 Summary

Analysis of the current literature seems to suggest that training volume may be an important determinant of training-induced changes in CS activity, whereas training intensity does not seem to influence these changes. While these conclusions are interesting and offer a good perspective on the effects of these two parameters on changes

in CS activity, caution must be used when comparing results from different studies, due to the many methodological differences. For this reason, future research directly measuring the effects of these two parameters on training-induced changes in CS activity is required.

1.5.2 Exercise- and training-induced changes in mitochondrial respiration

Cross-sectional studies show that greater training status is associated with an increase in mitochondrial function [68, 128, 194, 311, 360], suggesting that exercise training may induce an increase in this parameter. This seems to be confirmed by analysis of the results from the studies presented in Table 1.2, which indicate that exercise training is typically associated with an increase in mitochondrial function. As described in section 1.3.3, there are several way to assess mitochondrial function, and measurement of mitochondrial respiration in permeabilised muscle fibres has been suggested as the gold standard technique for this assessment [85, 230, 261]. However, given the paucity of available research employing this technique, this review will also consider reports that employed different techniques.

Only two studies have investigated the effect of a single bout of exercise on changes in mitochondrial respiration in permeabilised muscle fibres [310, 314]. Both of these studies employed exercise to exhaustion, and revealed that immediately post-exercise mitochondrial respiration through complex I increased by 23% ($P < 0.05$) following HIIT at 130% of $\dot{V}O_{2Peak}$ [314], but remained unchanged following continuous cycling at 75% of $\dot{V}O_{2Peak}$ [310]. From these results, several conclusions can be made. One is that, given the rapid changes in mitochondrial respiration following exercise, the timing of the biopsy in future training studies must be carefully controlled. Moreover, these results indicate that maximal mitochondrial oxidative power is intact or improved after exhaustive exercise. Even more important for the scope of this review, is that these findings seem to suggest that training intensity may be an important determinant of exercise-induced changes in mitochondrial respiration. More research, directly comparing different training intensities and volumes, is required to better understand how these variables affect exercise-induced changes in mitochondrial respiration.

Table 1.2. Training-induced changes in mitochondrial function in humans.

Study	Particip. n - sex ($\dot{V}O_{2Peak}$)		Training protocol	Method	Substrates	MF % change
Wibom 1992 [343]	9 - M (44)	6w (4d/w)	**CT:** 36min @ 70% $\dot{V}O_{2Peak}$	MAPR	PPCKM	70*
					PCM	92*
					PM	50*
					SR	66*
Starritt 1999 [300]	7 - M/F (44)	10d (1 x d)	**CT+HIIT:** [60min @ 75% $\dot{V}O_{2Peak}$] or [6×5min @ 95% $\dot{V}O_{2Peak}$]	MAPR	PPCKM	136*
					PCM	161*
Tonkonogi 2000, [313]	8 - M/F (39)	6w (4d/w)	**CT+HIIT:** [30min @ 70% $\dot{V}O_{2Peak}$] + [5×2min @ 100% $\dot{V}O_{2Peak}$]	IM	PM	40*
Walsh 2001 [332]	8 - M/F (38)	6w (4d/w)	**CT+HIIT:** [30min @ 70% $\dot{V}O_{2Peak}$] + [5×2min @ 100% $\dot{V}O_{2Peak}$]	SF	PM	38*
Daussin 2008 [67]	11 - M/F (29)	8w (3d/w)	**HIIT:** 28min [60s @ 90% W_{Peak} + 4min @ 56% W_{Peak}]	SF	GM	36*
			CT: 28min @ 61% W_{Peak}			13
Jacobs 2013 [127]	16 - M (43)	2w (3d/w)	**HIIT:** 10x [60s @ 100% W_{Peak} + 75s @ 30W]	SF	MOC	22*
					PGM	12*
					PGMS	12*
Vincent 2015 [324]	8 - M (46)	2w (4d/w)	**HIIT:** 12x [1 min @ ~120% W_{Peak} + 90 s rest]	SF	PGM	18
					PGMS	21*

Particip: participants; $\dot{V}O_{2Peak}$: peak oxygen uptake (mL min^{-1} kg^{-1}); MF: mitochondrial function; M: male; w: week; d: day; CT: continuous training; MAPR: maximal ATP production rate; PPCKM: pyruvate + palmitoyl-L-carnitine + α-ketoglutarate + malate; PCM: palmitoyl-L-carnitine + malate; PM: pyruvate + malate; SR: succinate + rotenone; F: female; HIIT: high-intensity interval training; IM: isolated mitochondria; SF: saponin-skinned permeabilised fibres; W_{Peak}: peak power; GM: glutamate + malate; MOC: malate + L-octanoyl-carnitine; PGM: pyruvate + glutamate + malate; PGMS: pyruvate + glutamate + malate + succinate. *Significantly different from pre-training values ($P < 0.05$).

Due to the paucity of available studies, it is difficult to establish the role of training intensity and volume as determinants of training-induced changes in mitochondrial

function. Moreover, the seven studies presented in Table 1.2 assessed mitochondrial function using different techniques, such as MAPR [300, 343] or mitochondrial respiration [67, 127, 313, 332, 324], and a series of different substrates. Of these studies, only four measured mitochondrial respiration in saponin-skinned permeabilised fibres [67, 127, 332, 324]. To complicate matters further, three studies employed a mix of training intensities [300, 313, 332], whereas only one directly compared two training intensities [67]. Nonetheless, some conclusions can be made. Results from Daussin *et al.* (2008) show that HIIT (training intensity = 90% of $\dot{V}O_{2Peak}$) resulted in a significant increase in mitochondrial respiration through complex I (36%; $P < 0.05$), whereas continuous (CT) cycling (training intensity = 61% of $\dot{V}O_{2Peak}$) did not (13%; $P > 0.05$), despite the groups being matched for total work (i.e., volume of training). While only one study, these results are consistent with the hypothesis that higher training intensities are required to enhance mitochondrial respiration. Findings from ensuing research, showing an increase in mitochondrial respiration through complex I and II combined following HIIT at training intensities ≥ 100% of $\dot{V}O_{2Peak}$, support this hypothesis [127, 324].

In their research, Daussin *et al.* (2008) concluded that workload and oxygen uptake fluctuations, rather than training volume or intensity, were the key determinants of these improvements. However, a different study showed that continuous cycling at 70% of $\dot{V}O_{2Peak}$ increased MAPR measured with pyruvate and malate to a similar extent (50%; $P < 0.05$) [343]. This suggests that other factors, such as training intensity, may play a more important role. The hypothesis that training intensity is an important determinant of training-induced changes in mitochondrial respiration is also consistent with findings from studies in rat skeletal muscle showing a trend for greater increases in mitochondrial respiration with higher training intensities [26]. Regardless of the above findings however, more research directly comparing different training intensities within a single study is needed to better define the role of training intensity in the regulation of training-induced improvements in mitochondrial respiration.

For the same reasons explained above, the role of training volume is also difficult to establish. However, one study directly comparing two training groups showed that one group increased mitochondrial respiration by 36% ($P < 0.05$), whereas a second group that was matched for total work did not (13%; $P > 0.05$) [67]. These findings seem to

indicate that training volume may not be a key determinant of training-induced changes in mitochondrial respiration. Comparisons of studies employing MAPR seem to confirm this conclusion. Starritt *et al.* (1999) reported an increase in MAPR, using pyruvate, palmitoyl-L-carnitine, α-ketoglutarate, and malate (PPCKM), that was almost double that reported by Wibom *et al.* (1992) with the same substrates (136% *vs.* 70% respectively; both $P < 0.05$), despite almost half the training volume (~37,000 *vs.* ~60,000 training units, respectively). Changes in MAPR measured with palmitoyl-L-carnitine and malate (PCM) were also almost double in the former study compared to the latter (161% *vs.* 92% respectively; both $P < 0.05$). The major difference in the training protocols of the above studies was that about half of the training in Starritt *et al.* (1999) was carried out using a higher training intensity (95% of $\dot{V}O_{2Peak}$) than that used in Wibom *et al.* (1992) (70% of $\dot{V}O_{2Peak}$). This seems to strengthen the hypothesis that training intensity and not training volume is a key determinant of training-induced changes in mitochondrial function. In conclusion, more research is required to clearly define the role of training volume as a determinant of changes in mitochondrial function, for example by directly comparing the effect of different training volumes on changes in mitochondrial respiration.

1.5.3 Exercise- and training-induced changes in the gene and protein expression of regulators of mitochondrial biogenesis

Understanding the control of gene and protein expression that allows for changes in mitochondrial components, is fundamental for understanding the factor regulating exercise-induced mitochondrial biogenesis. As described in section 1.4.2, PGC-1α, p53 and TFAM are some of the most important regulators of the transcriptional machinery regulating this process. A single bout of exercise, as well as exercise training, have been shown to modulate both the mRNA and protein content of the coactivator PGC-1α, and the transcription factors p53 and TFAM [116, 266]. Therefore, a better understanding of the molecular mechanisms regulating these processes could have important implications for the design of exercise training programs.

1.5.3.1 PGC-1α

PGC-1α is a widely studied coactivator regulating mitochondrial biogenesis [116]. Considering the extensive literature available on PGC-1α, the next two sections will separately examine the effect of either a single bout of exercise or exercise training on its mRNA and protein content. A summary of the results of studies investigating the effects of a single bout of exercise on PGC-1α mRNA and protein content in the vastus lateralis muscle of healthy humans is presented in Table 1.3.

Table 1.3. Changes in PGC-1α mRNA and protein content following a single bout of cycling (unless otherwise specified) in the vastus lateralis muscle of healthy humans.

Study	**Participants n - sex age - $\dot{V}O_{2Peak}$**	**Training bout**	**PGC-1α mRNA**	**PGC-1α p.c.**	**Comments**
Pilegaard 2003 [233]	7 - M 22y - n/r	3 h 2-legged knee extensor @ 100% of 1-leg 2-min max	TR-leg NC @ 0 h ↑10 fold @ 2 h ↑ 4 fold @ 6 h NC @ 24 h	n/r	TR > UT @ 2 h.
			UT-leg NC @ 0 h ↑ 7 fold @ 2 h ↑ 6 fold @ 6 h NC @ 24 h	n/r	
Watt 2004 [337]	7 - M 24y - 54	3 h @ 60% $\dot{V}O_{2Peak}$	↑10 fold @ 3 h	↑48% @ 3 h (P = 0.09)	
McGee 2004, [184]	7 - M 27y - 47	1 h @ 74% $\dot{V}O_{2Peak}$	n/m	NC in WM or Nuc @ 0 h	
Norrbom 2004 [209]	9 - M 22y - n/r	45 min 1-leg knee extensor (10-60 W)	NC @ 0 or ½ h ↑2.4 fold @ 2 h ↑2.7 fold @ 6 h	n/r	Same increase in type I & II fibres @ 2h.
Pilegaard 2005 [232]	9 - M 27y - 58	HC: 75 min @ 75% $\dot{V}O_{2Peak}$	NC @ 2 h ↑5.5 fold @ 5 h NC @ 8 h NC @ 24 h	n/r	LC availability post-exercise extends PGC-1α mRNA upregulation time.
		LC: 75 min @ 75% $\dot{V}O_{2Peak}$	NC @ 2 h ↑5.5 fold @ 5 h ↑4.8 fold @ 8 h NC @ 24 h	n/r	

Study	Participants n - sex age - $\dot{V}O_{2Peak}$	Training bout	PGC-1α mRNA	PGC-1α p.c.	Comments
Russell 2005 [259]	7 - M 23y - 51	2 h @ 50% W_{Peak} (w/ or w/out glucose)	↑4.8 fold @ 0 h ↑12 fold @ 1 h ↑3-6 fold @ 4 h	n/r	No effect of high FFA on PGC-1α activation post-exercise.
Cartoni 2005 [42]	1 - M 36y - 58	10-km cycling time trial	↑NC @ 0 h ↑3.1 fold @ 2 h ↑ NC @ 24 h	n/r	
Vissing 2005 [326]	14 - M 25y - n/r	3 h 1-legged knee extensor @ 50% max work capacity	↑2.5 fold @ 1 h ↑8.5 fold @ 3 h ↑6 fold @ 8 h NC @ 20 h	n/r	
Mahoney 2005, [172]	14 - M 22y - n/r	75 min GXT to exhaustion	↑2.9 fold @ 3 h NC @ 48 h	n/r	
Cluberton 2005 [53]	6 - M 28y - 46	HC: 60 min @ 74% $\dot{V}O_{2Peak}$ LC: 60 min @ 74% $\dot{V}O_{2Peak}$	NC @ 0 h ↑3 fold @ 3 h NC @ 0 h ↑3.8 fold @ 3 h	n/r n/r	CHO ingested pre and post exercise; No effect of CHO on PGC-1α mRNA activation.
Coffey 2006 [57]	ET: 6 - M 29y - 65 ST: 7 - M 31y - 37	60 min @ 70% $\dot{V}O_{2Peak}$	↑8.5 fold @ 3 h ↑10 fold @ 3 h	NC @ 3 h NC @ 3 h	No effect of previous training history on PGC-1α.
Sriwijitkamol 2007 [297]	8 - M/F 45y -21	40 min @ 50% $\dot{V}O_{2Peak}$ 40 min @ 70% $\dot{V}O_{2Peak}$	NC @ 0 h ↑5 fold @ 2.5 h NC @ 0 h ↑14 fold @ 2.5 h	n/r	ND between low *vs.* high-intensity cycling.
Mathai 2008 [180]	7 - M 23y - 48	TTE @ 65% $\dot{V}O_{2Peak}$ (~2h)	↑3 fold @ 0 h ↑6.2 fold @ 2 h NC @ 24 h NC @ 52 h	↑23% @ 0 h ↑23% @ 2 h ↑16% @ 24 h NC @ 52 h	
De Filippis 2008 [69]	6 - M/F 33y - 35	4x [8 min @ ~70% + 2 min @ ~90% HR_{Max} + 2 min rec]	↑1.5 fold @ ½ h ↑8 fold @ 5 h	↑20% @ ½ h ↑50% @ 5 h	

Study	Participants n - sex age - $\dot{V}O_{2Peak}$	Training bout	PGC-1α mRNA	PGC-1α p.c.	Comments
Wang 2009 [334]	9 - M 26y - 41	CT: 90 min @ 67% $\dot{V}O_{2Peak}$	↑10 fold @ 3 h	n/r	No difference in PGC-1α mRNA increase @ 3 h in CT *vs.* HIIT
		HIIT: 90 min as: [12 s @ 120% + 18 s @ 20% $\dot{V}O_{2Peak}$]	↑13 fold @ 3 h	n/r	
Gibala 2009, [83]	6 - M 23y - 47	4x [30 s "all-out" + 4 min rec]	↑2 fold @ 3 h	NC @ 3 h	
Egan 2010 [73]	8 - M 24y - 40	400 kcal @ 40% $\dot{V}O_{2Peak}$ (MFW)	↑3.8 fold @ 3 h	n/r	Intensity-dependent regulation of PGC-1α.
		400 kcal @ 80% $\dot{V}O_{2Peak}$ (MFW)	↑10 fold @ 3 h	n/r	
Nordsborg 2010 [207]	UT: 8 - M 24y - 44	4x [4 min @ 85% $\dot{V}O_{2Peak}$ + 3 min rec]	NC @ 0 h ↑2.5 fold @ 1 h ↑4.3 fold @ 3 h ↑2.9 fold @ 5 h	n/r	Same relative (T_{REL}) & absolute (T_{ABS}) training intensity compared to UT; UT & T_{ABS} MFW; Average 1-5h UT & T_{ABS} > T_{REL}; PGC-1α mRNA response depends on relative ex intensity.
	T_{REL}: 10 - M 28y - 55		↑1.8 fold @ 0 h ↑2.4 fold @ 1 h ↑5.5 fold @ 3 h ↑3 fold @ 5 h	n/r	
	T_{ABS}: 10 - M 28y - 55	4x [4 min @ 70% $\dot{V}O_{2Peak}$ + 3 min rec]	NC @ 0 h NC @ 1 h ↑2.7 fold @ 3 h NC @ 5 h	n/r	
Perry 2010 [224]	9 - M 23y - 46	10x [4 min @ ~90% $\dot{V}O_{2Peak}$ + 2 min rec]	1st session: ↑10 fold @ 4 h 3rd session: ↑9 fold @ 4 h 5th session: ↑8 fold @ 4 h 7th session: ↑5 fold @ 4 h	NC @ 4 h ↑23% @ 24 h	NC @ 24 h following all sessions; PGC-1α mRNA increased/decreased in a "stair-case" type response (reduced activation from 1st to 7th bout);

Study	Participants n - sex age - $\dot{V}O_{2Peak}$	Training bout	PGC-1α mRNA	PGC-1α p.c.	Comments
Cochran 2010 [54]	10 - M 21y - 51	HIIT: 2x [5 x 4 min @ ~95% HR_{Max} +2 min rec] separated by 3h w/ LC	1st session: NC @ 0 h; ↑8 fold @ 3 h 2nd session: NC @ 0 h	1st session: NC @ 0 h; NC @ 3 h 2nd session: NC @ 0 h	3 h post end of 1st session is equiv. to rest of 2nd session; No effect of different CHO supplementation; No change in PGC-1α p.c. throughout.
		HIIT: 2x [5 x 4 min @ ~95% HR_{Max} +2 min rec] separated by 3h w/ HC	1st session: NC @ 0 h; ↑8 fold @ 3 h 2nd session: NC @ 0 h	1st session: NC @ 0 h; NC @ 3 h 2nd session: NC @ 0 h	
Little 2010 [160]	8 - M 29y - 55	CT: 90 min @ 65% $\dot{V}O_{2Peak}$	n/m	WM: NC, Cyt : NC, Nuc: ↑54%, @ 0h	Resting PGC-1α p.c.: Cyt 4x > Nuc.
Little 2011 [159]	8 - M 24y - 45	SIT: 4x [30 s "all-out" + 4 min rec]	NC @ 0 h ↑7.5 fold @ 3 h NC @ 24 h	WM: NC @ 0 & 3 h ↑57% @ 24 h Nuc: NC @ 0 h ↑66% @ 3 h NC @ 24 h	Resting PGC-1α p.c.: Cyt >> Nuc.
Norrbom 2011 [208]	12 - M 24y - 51	45 min 1-leg knee xtensor (max to completion)	NC @ 0 & 2 h	n/r	
Serpiello 2012 [283]	10 - M/F 22y - 54	Pre-training: 3 sets of: 5x [4 s "all-out" + 20 s rec], w/ 4.5 min rec between sets	NC @ 0 & 1 h ↑3.1 fold @ 4 h	n/r	Training: [4w (3d/w), same as single bout; ND in PGC-1α mRNA upregulation @ 0, 1, 4 h between pre & post-training.
		Post-training: 3 sets of: 5x [4 s "all-out" + 20 s rec], w/ 4.5 min rec between sets	NC @ 0 & 1 h ↑2.7 fold @ 4 h	n/r	
Stepto 2012 [301]	9 - M 23y - 44	Pre-training: 60 min @ 72% $\dot{V}O_{2Peak}$	↑11 fold @ 3 h	↑50% @ 0 h ↑40% @ 3 h	Training: [6x CT + 4x HIIT] in 14d; PGC-1α mRNA @ 3h pre > post-training; PGC-1α p.c. @ 0 & 3h pre > post-training.
		Post-training: 60 min @ 72% $\dot{V}O_{2Peak}$	↑4.3 fold @ 3 h	NC @ 0 h NC @ 3 h	

Study	Participants n - sex age - $\dot{V}O_{2Peak}$	Training bout	PGC-1α mRNA	PGC-1α p.c.	Comments
Bartlett 2012 [18]	10 - M 20y – 52	HIIT (MFW): 6x [3 min @ 90% + 3 min @ 50% $\dot{V}O_{2Peak}$]	↑4.3 fold @ 3 h	NC @ 3 h	Exercise was RUN; No difference between HIIT or CT.
		CT (MFW): 50 min @ 70% $\dot{V}O_{2Peak}$	↑4.5 fold @ 3 h	NC @ 3 h	
Bartlett 2013 [19]	8 - M 25y - 55	HC (MFW): 6x [3 min @ 90% + 3 min @ 50% $\dot{V}O_{2Peak}$]	↑3.7 fold @ 3 h	n/r	Exercise was RUN; No effect of HC & LC availability pre-exercise.
		LC: (MFW): 6x [3 min @ 90% + 3 min @ 50% $\dot{V}O_{2Peak}$]	↑2.1 fold @ 3 h	n/r	
Edgett 2013 [72]	8 - M 22y - 53	11x [1 min @ 73% W_{Peak} + 1 min rec]	↑4.3 fold @ 3 h	n/r	3 groups were MFW; 100% > 73% & 133%; PGC-1α increased in intensity-dependent manner but only below W_{Peak}.
		8x [1 min @ 100% W_{Peak} + 1 min rec]	↑7.9 fold @ 3 h	n/r	
		6x [1 min @ 133% W_{Peak} + 1 min rec]	↑4.2 fold @ 3 h	n/r	
Popov 2013 [237]	UT: 6 - M n/r - 42	10x [3 min @ 60% AT +2 min @ 91% AT]	↑9 fold @ 3 h ↑8.5 fold @ 5 h	n/r	No effect of previous training history on PGC-1α mRNA upregulation post-exercise.
	ET: 6 - M n/r - 57		↑8 fold @ 3 h ↑7.5 fold @ 5 h	n/r	
Psilander 2013 [240]	10 - M 28y - 65	LG: 1 h @ 64% $\dot{V}O_{2Peak}$	↑8.1 fold @ 3 h	n/r	PGC-1α mRNA LG > NG @ 3 h.
		NG: 1 h @ 64% $\dot{V}O_{2Peak}$	↑2.5 fold @ 3 h	n/r	
Cochran 2014 [55]	8 - M 22y - 48	SIT (MFW): 4x [30 s "all-out" + 4 min rec]	↑4 fold @ 3 h	n/r	No effect of different training modality on PGC-1α mRNA.
		CT (MFW): ~4 min "all-out"	↑4 fold @ 3 h	n/r	

Study	Participants n - sex age - $\dot{V}O_{2Peak}$	Training bout	PGC-1α mRNA	PGC-1α p.c.	Comments
Popov 2014 [236]	12 - M 21 - 46	HIIT: (MFW): 10x [2 min @ 70% W_{Peak} + 3 min @ 46% W_{Peak}]	NC @ 1 h ↑9 fold @ 3 h ↑8 fold @ 5 h	n/r	PGC-1α mRNA HIIT > CT @ 3 & 5 h; Exercise intensity-dependent regulation of PGC-1α mRNA.
		CT (MFW): 50 min @ 54% W_{Peak}	NC @ 1 h ↑5.6 fold @ 3 h ↑5 fold @ 5 h	n/r	

$\dot{V}O_{2Peak}$: peak oxygen uptake (mL min^{-1} kg^{-1}); PGC-1α: peroxisome proliferator-activated receptor γ coactivator 1α; p.c.: protein content; M: male; TR: trained; NC: no change; @ 0 h: immediately after cessation of exercise; @ 3 (x) h: after 3 (x) h after cessation of exercise; n/r: not reported; UT: untrained; WM: whole-muscle fraction; Nuc: nuclear fraction; HC: high CHO availability; LC: low CHO availability; CHO: carbohydrate; W_{Peak}: peak power; FFA: free fatty acids; GXT: graded exercise test; ET: endurance trained; ST: strength trained; F: female; TTE: time to exhaustion; HR_{Max}: maximal heart rate; rec: recovery; CT: continuous training; HIIT: high-intensity interval training; MFW: matched for total work; Cyt: cytosolic fraction; SIT: sprint interval training; all-out: maximal intensity exercise; ND: no difference; AT: anaerobic threshold; LG: low glycogen availability; NG: normal glycogen availability.

1.5.3.1.1 Exercise-induced changes in PGC-1α gene and protein expression

PGC-1α mRNA

In human skeletal muscle, one bout of exercise induces a dramatic increase in PGC-1α mRNA that peaks 2 to 5 h post-exercise (Figure 1.10). While some studies have reported an increase in PGC-1α mRNA immediately post-exercise [180, 207, 259], the majority of research suggests this increase is not significant until ~1 h following exercise cessation, and is maintained until ~8 h post-exercise (Figure 1.10). To date, no study has observed an increase in PGC-1α mRNA content beyond 8 h from exercise cessation (Figure 1.10). An important consideration is that the timing of PGC-1α mRNA upregulation normally presented in the literature (and also in Figure 1.10) is based on the time passed from the termination of exercise. However, especially for longer exercise durations, the time required for the increase in PGC-1α mRNA would likely be reduced, considering the signalling response to exercise has been shown to begin at the onset of exercise [251]. This should be considered when directly comparing exercise bouts of different durations.

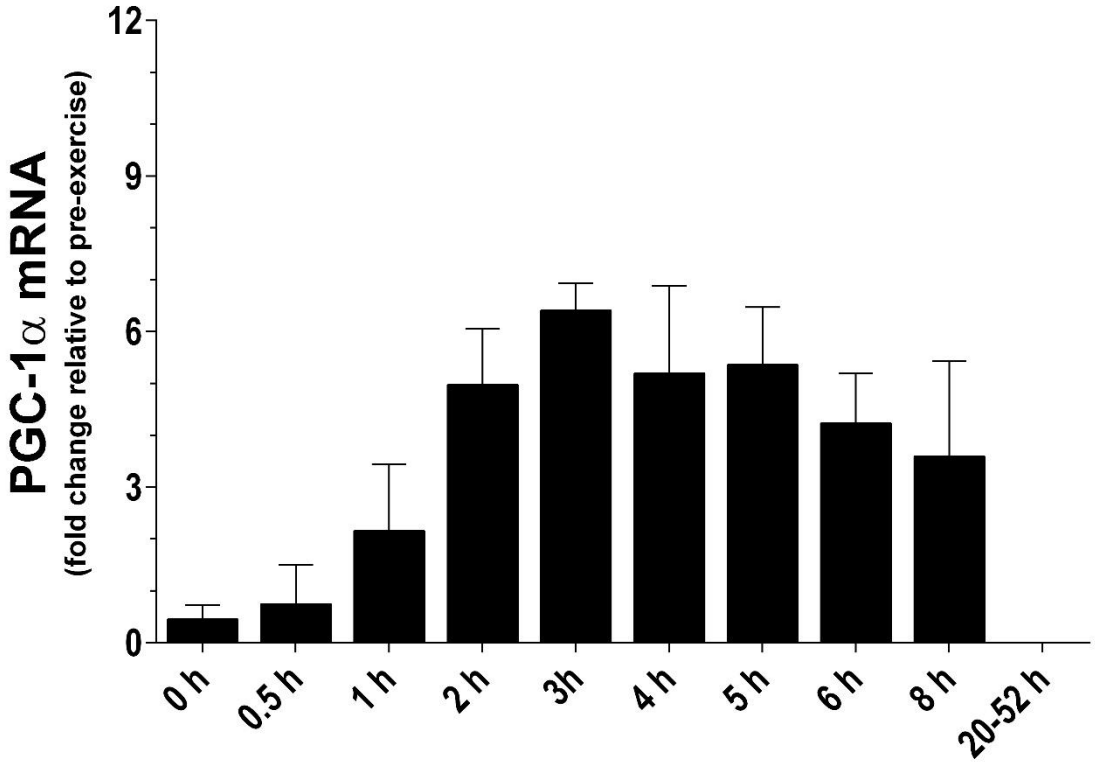

Figure 1.10. Timing of PGC-1α mRNA upregulation from the termination of a single exercise bout in the vastus lateralis muscle of healthy human participants. Results were obtained from: [18, 19, 42, 53-55, 57, 69, 72, 73, 83, 159, 172, 180, 207-209, 224, 232, 233, 236, 237, 240, 259, 283, 297, 301, 326, 334, 337]. All values are mean ± SEM.

Several studies have reported that following an exercise bout, PGC-1α mRNA is temporarily increased and returns to baseline values within 24 h [159, 180, 224, 232, 233]. Further knowledge on the mechanisms regulating exercise-induced increases in PGC-1α mRNA was gained when a training study (seven sessions) observed that the increase in PGC-1α mRNA 4 h post-exercise was progressively reduced with every subsequent exercise bout performed at the same relative exercise intensity [224]. This "saw-tooth" pattern, created by the continuous upregulation (4 h) and return to baseline values (24 h) following each training bout, continued until the seventh bout with smaller increases after each bout (Figure 1.11). This suggests that the increase in mitochondrial proteins that follows a training intervention may be the result of transient bursts in the mRNA of PGC-

1α (and other transcription factors) [224], and that the transcriptional stimulus is reduced as the training intervention progresses.

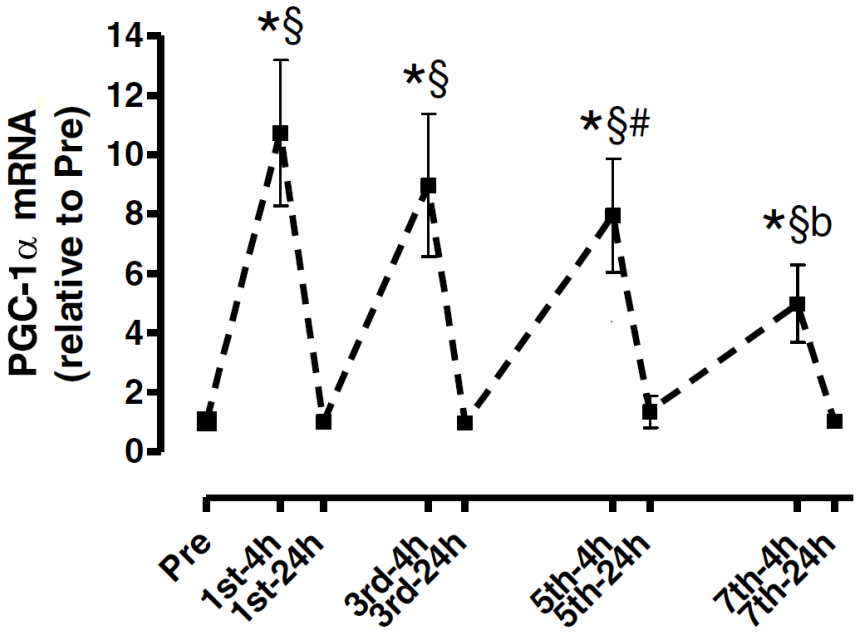

Figure 1.11. . Skeletal muscle PGC-1α mRNA changes following 7 sessions of HIIT (cycling). *Significantly different from Pre, § all 24 h time points, # 1st-4 h, b all 4 h time points. All values are mean ± SEM *(From [224]).*

The effect of training intensity on changes in PGC-1α mRNA is not completely understood. Findings from a study showing a greater increase in PGC-1α mRNA after a cycling bout at 80% of $\dot{V}O_{2Peak}$, compared with an isocaloric cycling bout at 40% of $\dot{V}O_{2Peak}$, indicate that the magnitude of the increase in PGC-1α mRNA is regulated in a training intensity-dependent manner [73]. These findings were confirmed by another study showing a greater increase in PGC-1α mRNA following a bout of HIIT at 70% of W_{Peak}, compared with an isocaloric bout of continuous cycling at 54% of W_{Peak} [236]. Further insight into the training intensity-dependent regulation of PGC-1α mRNA was provided by a study investigating work-matched HIIT bouts at three different training intensities (73%, 100% and 133% of $\dot{V}O_{2Peak}$). This study showed a greater increase in PGC-1α mRNA after a bout of HIIT at 100% of $\dot{V}O_{2Peak}$, compared with that obtained

after a bout of HIIT at either 73% or 133% of $\dot{V}O_{2Peak}$. The authors hypothesised that the exercise-induced increase in PGC-1α mRNA is regulated in a training intensity-dependent manner only at submaximal training intensities, as the apparent intensity effect did not extend to supramaximal intensities [72]. Findings from a study reporting no difference in the PGC-1α mRNA increase following two separate exercise bouts in which participants performed either four 30-s "all-out" bouts, or a maximal continuous work-matched effort lasting ~4 minutes performed at a significantly lower overall training intensity [55], are consistent with this hypothesis. These authors also suggested that the regulation of PGC-1α mRNA does not depend on the training modality (continuous *vs.* intermittent exercise), as previously demonstrated in a study showing a similar increase in PGC-1α mRNA following a single bout of work-matched continuous *vs.* HIIT, performed at the same average training intensity [18].

Further insight into the importance of relative training intensity can be gained from studies comparing changes in PGC-1α mRNA pre- and post-training, and in participants of different training status. A study investigating the effects of a single bout of 2-legged knee extension has shown a significantly greater exercise-induced increase in PGC-1α mRNA in the untrained *vs.* trained leg, when the relative exercise intensity was the same [233]. Similarly, results from a study investigating the effects of a single bout of HIIT, showed a greater exercise-induced increase in the PGC-1α mRNA in untrained compared with endurance-trained participants, when the relative exercise intensity was the same [207]. The authors from the latter study concluded that the relative intensity of brief intermittent exercise is of major importance for the exercise-induced increase in PGC-1α mRNA. Conversely, another study reported no difference in the exercise-induced increase in PGC-1α mRNA of untrained *vs.* endurance-trained participants, following a single bout of HIIT at the same relative exercise intensity [237]. Similarly, no influence of previous training history was also reported by a study comparing endurance trained *vs.* strength trained participants, showing similar exercise-induced increases in PGC-1α mRNA following a single bout of continuous cycling at the same relative intensity [57]. More research is needed to better understand the effect of training status and previous training history on the PGC-1α response to exercise.

PGC-1α protein

A central dogma of molecular biology is that 'DNA makes RNA and RNA makes protein' [64]. It is not surprising therefore, that studies investigating the effects of a single exercise bout on PGC-1α protein content indicate that a longer time is needed until PGC-1α protein is increased; however, different time frames have been reported. There seems to be consensus that a lapse of 24 h from the cessation of exercise is sufficient to increase PGC-1α content, with reported increases ranging from 15 to 60% at this time point [159, 180, 224]. An increase in PGC-1α content has also been reported immediately [180, 301], or half an hour [69] post-exercise. However, the majority of research has reported no change immediately post-exercise [54, 159, 160, 184], or for a period lasting up to 3-5 h post-exercise [18, 54, 57, 83, 159, 224]. The reasons for these differences are difficult to explain and are likely related to the various methodological differences between studies. The somewhat contrasting results presented above suggest that more research is needed to investigate the timing of the response of PGC-1α protein to a single bout of exercise. Nuclear accumulation of PGC-1α protein following an exercise bout has been shown in human skeletal muscle [159, 160], and has been suggested to constitute the initial phase of the exercise-induced adaptive response [351]. However, this topic will not be further discussed at this stage, as it has been presented in detail in section 1.4.2.1.

1.5.3.1.2 Training-induced changes in PGC-1α protein content

Most published research indicates that PGC-1α protein content is increased (~20-100%) following a training intervention (Table 1.4), with only two studies failing to observe a significant change [161, 324]. Studies have also measured the level of resting PGC-1α mRNA pre- and post-training. Results are contrasting, with some studies showing similar increases to those in PGC-1α protein content (~20-70%) [74, 281], whereas others did not observe a change [221, 233, 280]. One study has also assessed PGC-1α protein content changes in single fibres, and reported that changes in PGC-1α content in type IIa fibres are more than 3-fold greater than those in type I and IIx [258]. Finally, studies have assessed changes in PGC-1α protein content in different cellular compartments. While two studies demonstrated a 25-35% increase in nuclear PGC-1α protein content following two weeks of HIIT [98, 161], another study reported no difference compared to pre-

training values after eleven weeks of a mix of HIIT and continuous running in both the nuclear and the cytosolic fraction [221]. While it is difficult to explain these discrepancies, it is possible that the increase in nuclear PGC-1α protein content observed after two weeks of training in the first two studies represents an early training-adaptation that may no longer be present after eleven weeks of training. The type of exercise (cycling *vs.* run) may also have confounded these results. Nonetheless, more research is required to confirm these hypotheses and to better understand training-induced changes in PGC-1α protein content in different subcellular compartments and in different fibre types.

Table 1.4. Changes in PGC-1α protein content measured in whole-muscle homogenates from the vastus lateralis muscle of healthy human participants following cycle exercise training (unless otherwise specified).

Study	**Participants** **n - sex** **age - $\dot{V}O_{2Peak}$**	**Training type**	**Training protocol**	**PGC-1α protein content**	**Comments**
Pilegaard 2003, [233]	7 - M 22y - n/r	~1 h 1-leg knee extensor @ 70% of 1-leg 2-min max	4w (5d/w); increasing resistance		mRNA: NC.
Russell 2003, [258]	7 - M 34y - 54	HIIT: 5-6x [1-3 min @ 70-80% + 1 min @ 50% $\dot{V}O_{2Peak}$] CT: 40 min @ 60% $\dot{V}O_{2Peak}$	6w (3d/w); HIIT-CT-HIIT each week; increasing resistance & reps	type I: ↑50% type IIa: ↑180% type IIx: ↑50%	type IIa > type I & IIx; ↑2.7 fold increase in baseline PGC-1α mRNA.
Short 2003, [291]	65 - M/F 21-87y - 41	20-40 min @ 70-80% HR_{Max}	16w (3-4d/w); increasing resistance, frequency & duration		mRNA: ↑55%;
Burgomaster 2008, [35]	SIT: 5 - M/F 24y - 41	4-6x [30 s 'all-out' + 4.5 min @ 30 W]	6w (3d/w); increasing reps	↑100%	Similar increase in PGC-1α p.c. for both groups (as main effect of time).
	CT: 5 - M/F 23y - 41	40-60 min @ 65% $\dot{V}O_{2Peak}$	6w (5d/w); increasing duration	↑100%	

Study	**Participants n - sex age - $\dot{V}O_{2Peak}$**	**Training type**	**Training protocol**	**PGC-1α protein content**	**Comments**
Morton 2009, [200]	LO+G (MFW): 8 - M 20y - 41	5x [3 min @ 90% + 1.5 min @ 25% & 1.5 min @ 50% $\dot{V}O_{2Peak}$]	6w (2 a day 2d/w); increasing resistance	↑50%	No effect of low glycogen availability or exogenous glucose supplementation (similar for gastrocnemius muscle).
	LO+P (MFW): 7 - M 21y - 41		As above	↑40%	
	NORM (MFW): 8 - M 20y - 41		6w (1 a day 4d/w); increasing resistance	↑50%	
Little 2010, [161]	7 - M 21y - 46	8-12x [60 s @ 100% W_{Peak} + 75 s @ 30 W]	2w (3d/w); increasing reps	WM: NC Nuc: ↑24%	HIIT increases Nuc but not WM PGC-1α p.c.
Perry 2010, [224]	9 - M 23y - 46	10x [4 min @ ~90% $\dot{V}O_{2Peak}$ + 2 min rec]	2w (3.5d/w); increasing resistance	post-session 1: ↑23% post-session 3: ↑35% post-session 5: ↑42% post-session 7: ↑38%	PGC-1α mRNA increased/decreased in a "stair-case" type response (reduced activation from 1st to 7th bout);
Gurd 2010, [96]	9 - M/F 23y - 45	10x [4 min @ ~90% $\dot{V}O_{2Peak}$ +2 min rec]	6w (3d/w); increasing resistance	↑16%	
Hood 2011, [117]	7 - M/F 45y - 30	10x [60 s @ 60% W_{Peak} + 60 s @ 30 W]	2w (3d/w); no progression	↑56%	
Gurd 2011, [98]	7 - M/F n/r - n/r	10x 4 min @ ~90% $\dot{V}O_{2Peak}$ +2 min rec	2w (3.5d/w); increasing resistance	WM: ↑36% Nuc: ↑34%	
Serpiello 2012, [283]	10 - M/F 22y - 54	3 sets of: 5x [4 s 'all-out' + 20 s rec], w/ 4.5 min rec between sets	4w (3d/w); no progression	↑33%	
Stepto 2012, [301]	9 - M 23y - 44	6x [5 min @ ~95% $\dot{V}O_{2Peak}$ + 2 min rec] or: 45, 60 or 90 min @ 75% $\dot{V}O_{2Peak}$	2w (5d/w); (4x HIIT + 6x CT)	↑54%	

Study	**Participants** **n - sex** **age - $\dot{V}O_{2Peak}$**	**Training type**	**Training protocol**	**PGC-1α protein content**	**Comments**
Egan 2013, [74]	8 - M 23y - 37	60 min @ 80% $\dot{V}O_{2Peak}$	2w (7d/w); no progression	post-session 1: ↑52% post-session 3: ↑49% post-session 7: ↑90% post-session 10: ↑80% post-session 14: ↑77%	PGC-1α p.c. increases after 1 bout, and plateaus after 7th bout; PGC-1α mRNA increases only after 3rd session (↑48%) and slowly declines thereafter (still ↑25% after 14th session)
Paulsen 2014, [221]	27 - M 24y – 53	HIIT: 4-6x [4-6 min @ >90% HR_{Max}] CT: 30-60 min @ 70-90% HR_{Max}	11w (3-4d/w); 2x (HIIT+CT) per week; increasing frequency, intervals & duration	WM: ↑50% Cyt: NC Nuc: NC	mRNA: NC; Exercise was RUN.
Konopka 2014, [143]	Young: 7 - M 20y - 41 Old: 6 - M 74y - 21	20-45 min @ 60-80% HR_{Res}	12w (3-4d/w); increasing intensity, duration & frequency	↑62% ↑55%	Aging does not influence the PGC-1α training-induced response.
Scribbans 2014, [280]	9 - M 21y - 48	HIIT: 8x [20 s @ ~170% $\dot{V}O_{2Peak}$ + 10 s rec]	6w (4d/w); no progression		mRNA: NC
	10 - M 21y - 48	CT: 30 min @ 65% $\dot{V}O_{2Peak}$	6w (4d/w); no progression		mRNA: NC
Scribbans 2014, [281]	8 - M 22y - 51	8x [20 s @ ~170% $\dot{V}O_{2Peak}$ + 10 s rec]	4w (3d/w); no progression		mRNA: ↑70%
Vincent 2015, [324]	8 - M 22y - 46	12x [1 min @ ~120% W_{Peak} + 90 s rec]	2w (4d/w); no progression	NC	

$\dot{V}O_{2Peak}$: peak oxygen uptake (mL min^{-1} kg^{-1}); PGC-1α: peroxisome proliferator-activated receptor γ coactivator 1α; M: male; n/r: not reported; w: week; d: day; NC: no change; HIIT: high-intensity interval training; CT: continuous training; F: female; HR_{Max}: maximal heart rate; SIT: sprint interval training; p.c.: protein content; W: Watt; 'all-out': maximal intensity exercise; LO: low glycogen availability; G: glucose supplementation; MFW: matched for total work; P: placebo supplementation; NORM: normal glycogen availability; WM: whole-muscle; Nuc: nuclear; rec: recovery; W_{Peak}: peak power; cyt: cytosolic; HR_{Res}: heart rate reserve.

The magnitude of the increase in whole-muscle PGC-1α protein content following training does not appear dependent on the training volume, as demonstrated by two studies measuring the time course of training-induced changes in PGC-1α protein content [74, 224]. Despite using different training protocols (HIIT at 90% of $\dot{V}O_{2Peak}$ for Perry *et al.* (2010) and continuous cycling at 80% of $\dot{V}O_{2Peak}$ for Egan *et al.* (2013)), also resulting in different training volume amounts (3600 and 4800 training volume units per session, respectively), both studies showed that PGC-1α protein content is significantly increased within one training session, and reached maximum values after 5 to 7 sessions (Figure 1.12). The data in the figure indicate that changes in PGC-1α protein content seems to plateau, or even a decrease, after reaching maximum values following bout 5-7. It would be valuable for future research to assess a longer time course of PGC-1α change. This would allow to explore whether, following the initial increase, the protein content of PGC-1α plateaus, continues to decrease before possibly returning to pre-training levels, or increases again at a later stage.

The role of training intensity as a determinant of changes in PGC-1α protein content has not been addressed. The only study to directly compare two different training intensities reported that continuous training (CT) at 65% of $\dot{V}O_{2Peak}$, and SIT at training intensities greater than 200% of $\dot{V}O_{2Peak}$, increased PGC-1α protein content to a similar extent [35]. However, while training volume does not seem to be a key determinant of training-induced changes in PGC-1α protein content [74, 224], the SIT group in the above study performed less than 10% of the training volume performed by the CT group. This seems to indicate that PGC-1α is sensitive to training intensity, and that SIT at maximal training intensity represents a potent stimulus to increase PGC-1α protein content. In conclusion, little is known about the role of training intensity in the regulation of PGC-1α. Therefore, future research should aim to provide these answers by directly comparing different training intensities, and where possible, trying to match training groups for total work.

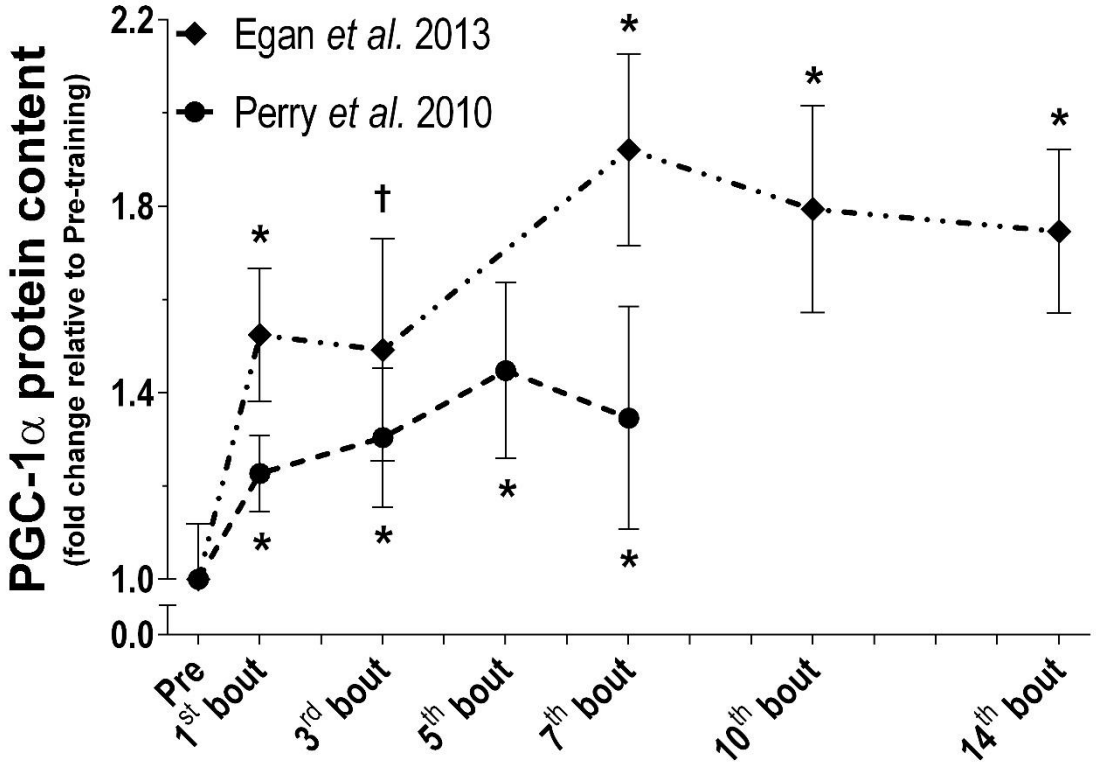

Figure 1.12. Time course of changes in PGC-1α protein content following either fourteen sessions of continuous cycling for 60 min at 80% of $\dot{V}O_{2Peak}$ [74], or seven sessions of high-intensity interval cycling consisting of ten 4-min repetitions at ~90% $\dot{V}O_{2Peak}$ interspersed with two min of recovery [224]. * Significantly different from baseline ($P < 0.05$); † trend for a significant difference from baseline ($0.1 > P > 0.05$). All values are mean ± SD. *(Adapted from [74, 224])*

1.5.3.2 p53

As described in detail in section 1.4.2.2, the tumour suppressor p53 is another important regulator of exercise-induced mitochondrial biogenesis [266]. While its role as a metabolic [329] and transcriptional [266] regulator has been well documented in rodent and cell models, there is a paucity of research on the effects of exercise and exercise training on p53 mRNA and protein expression in human skeletal muscle. A single bout of HIIT, at exercise intensities of 73, 100, and 133% of W_{Peak}, has been reported to increase p53 mRNA, in human skeletal muscle, after 3 hours of recovery [72]. Moreover, both a single bout of HIIT at 90% of $\dot{V}O_{2Peak}$, and continuous running at 70% of $\dot{V}O_{2Peak}$, increased p53 phosphorylation at serine 15 [18] (a post-translational modification

inducing p53 activity [71]), in human skeletal muscle, three hours following the cessation of exercise. The latter study also reported no difference in p53 phosphorylation between low-intensity continuous and HIIT matched for total work and average intensity. It has also been observed that increases in exercise-induced p53 phosphorylation are enhanced by low glycogen availability [19], suggesting a nutritional modulation of p53 activity, and consistent with the notion that cellular stress increases p53 activity [215].

p53 nuclear accumulation is another post-translational modification that has been linked with an increase in p53 activity [178, 215, 286]. However, no study to date has investigated the effects of a single bout of exercise on the nuclear accumulation of p53 in humans. A study in rat skeletal muscle reported a ~50% increase in whole-muscle p53 protein six hours after the termination of a single bout of maximally-activated eccentric contractions, an effect preceded by a ~60% increase in nuclear p53 protein 1 hour post-exercise [45]. Similarly, an increase in nuclear p53 has also been reported in mice immediately after termination of a 60-min running bout at a variable intensity [227]. Conversely, a study in mouse skeletal muscle reported a decrease in nuclear p53 protein content both immediately post-exercise and after 3 h of recovery following a 90-min running bout [267]. Given the importance of the changes in p53 localisation in response to oxidative stress [215], more research is required, specifically in human skeletal muscle, to better understand the effects of exercise on p53 nuclear localisation and the cellular mechanisms that regulate these processes. For an in-depth discussion on the events and mechanisms related to p53 post-translational modifications, the reader is referred to section 4.4.

While the above findings suggest that p53 is involved in the regulation of exercise-induced mitochondrial adaptations, no study to date has measured changes in p53 protein content following a training intervention in humans. Given the critical role that is emerging for p53 [266], more research is needed to determine if exercise training results in a changes in p53 protein content in human skeletal muscle, and its possible consequences. Moreover, determining the role of training intensity and training volume on the regulation of p53 is critical to better understand the mechanisms of exercise and training-induced mitochondrial biogenesis.

1.5.3.3 TFAM

TFAM is also a key player in the modulation of exercise-induced mitochondrial biogenesis is [272]. TFAM mRNA has been shown to be upregulated 4-6 hours post-exercise in human skeletal muscle [224, 233, 237, 236], with no change in TFAM protein content within a 24-hour period [224]. Only one study to date has investigated the effects of different exercise intensities on the modulation of TFAM mRNA [236]. This study compared continuous *vs.* high-intensity intermittent exercise matched for total work at 54 and 70% of peak power output (W_{Peak}) respectively, and reported that TFAM mRNA was upregulated only following exercise at the higher intensities. Further research is needed however, to rule out the possible effects of different training modalities (continuous vs. intermittent training), and to also determine the role of training volume on the regulation of TFAM. On the other hand, there is controversy regarding the effects of a training intervention on TFAM protein content and basal mRNA abundance. Studies have reported no change in TFAM protein content following seven sessions [224], or six weeks [96] of cycling HIIT. Similarly, no change was reported in resting TFAM mRNA levels following 10 days of one-legged, knee-extension exercise [210]. Conversely, an increase in basal TFAM mRNA abundance was reported following 16 weeks of continuous cycling [291], as well as increased TFAM protein content following four weeks of one-legged cycling [21], or six sessions of HIIT cycling [161]. These discrepancies, which may depend on several methodological differences between studies, are difficult to explain. In addition, no study to date has directly investigated the effects of different training intensities or training volumes on the modulation of TFAM following a training intervention. Therefore, to better understand the effect of exercise and exercise training on the modulation of TFAM, further research directly comparing the effects of manipulating these parameters within one study is warranted.

1.5.4 Summary

These sections have provided an extensive review on the current knowledge regarding the effects of training intensity and volume on exercise- and training-induced mitochondrial adaptations. Although when comparing results from different studies it is

difficult and somewhat risky to generalise, the present literature review seem to suggest that while training intensity may be a key determinant of changes in mitochondrial respiration, training volume appears to be an important modulator of mitochondrial content. Conversely, little is known about the effects of exercise intensity and exercise volume on the changes in the mRNA and protein expression of key coactivators and transcription factors. The aim of Chapter 2 to 4 will be to provide a clearer and better understanding of the above hypotheses, by directly comparing different training intensities and volumes within the one study, and investigating their effects on genes and proteins that have been proposed to regulate mitochondrial biogenesis.

1.6 Future research: a holistic approach to training-induced mitochondrial adaptations

Despite the wealth of research investigating the several facets of exercise- and training-induced mitochondrial adaptations, very few studies, if any, have looked at a more holistic approach and measured several of these parameters simultaneously. For example, studies investigating changes in mitochondrial respiration have in general neglected to measure the protein content of key transcription factors reported to influence mitochondrial function [5, 140, 181, 329]. Conversely, mechanistic studies investigating exercise- and training-induced changes in the genes and proteins regulating mitochondrial biogenesis, have not measured mitochondrial function. New evidence is emerging regarding the role of transcription factors such as PGC-1α and p53 in the regulation of metabolism [140, 329], autophagic processes influencing mitochondrial turnover [173, 198, 320], and mitochondrial remodelling events such as fusion and fission processes [42, 266]. Moreover, research in mice and humans indicates that mitochondrial content and function are not necessarily linked [128, 252], and should be differentiated and measured simultaneously [128]. Therefore, future research investigating exercise- and training-induced mitochondrial adaptations should take a more holistic approach and measure markers of mitochondrial content and function in parallel with changes in the protein content of key transcription factors regulating exercise- and training-induced

mitochondrial biogenesis. This would allow a better understanding of the interconnection between mitochondrial content and function and would further define the role of key transcription factors in the regulation of mitochondrial adaptations to training and the remodelling of skeletal muscle phenotype.

Chapter 2

Effects of training intensity on markers of mitochondrial biogenesis

This study is presented as the first in a series of related studies investigating how the manipulation of different training variables affects exercise- and training-induced mitochondrial adaptations.

The specific purpose of this study was to investigate the role of training intensity. The effects of four weeks of moderate-intensity continuous training (STCT), high-intensity interval training (HIIT), and maximal-intensity sprint interval training (SIT), on mitochondrial content, mitochondrial respiration, and the protein content of valid biomarkers of mitochondrial biogenesis, were investigated.

The manuscript for this study is ready for submission:

Granata C, Oliveira RSF, Little JP, Renner K and Bishop DJ (2015). Training intensity modulates changes in mitochondrial respiration, and PGC-1α and p53 protein content, but not markers of mitochondrial content *The FASEB Journal* (under review).

2.1 Introduction

Mitochondria are key components of skeletal muscles, as they provide the energy required for almost all cellular activities and play an important role in ageing and cell pathology. For example, mitochondria have been implicated in many age-related degenerative diseases, cardiomyopathies and atherosclerosis, as well as a large variety of metabolic disorders [168, 333]. Given their pivotal role in energy provision, it is not surprising that mitochondrial adaptations have also been associated with endurance performance [129]. However, while early studies focussed on changes in mitochondrial enzymes, such as citrate synthase (CS) activity, a biomarker of mitochondrial content [145], subsequent studies have suggested that increasing mitochondrial function may contribute more to improvements in both health [333] and endurance performance [129]. It is therefore important to better understand factors that may influence mitochondrial function.

Exercise is one factor that has been shown to provide a powerful stimulus to increase both mitochondrial content [113] and/or function [67, 360], yet little is known about the optimal exercise dose, and whether mitochondrial content and function are altered by the same or different exercise prescriptions. Only a handful of studies have directly investigated the effects of different types of training on mitochondrial function [67, 127, 300, 313, 332, 343]. Moreover, only one study [67] directly compared the effects of two different training intensities on mitochondrial respiration in permeabilised skeletal muscle fibres, a technique presenting several advantages over measurements in isolated mitochondria [264, 312]. This study showed that mitochondrial respiration was increased after high-intensity interval training (HIIT), but did not change following work-matched continuous training at a moderate intensity. This is in contrast with the findings from an earlier study reporting an increase in mitochondrial function, measured via mitochondrial ATP production rate (MAPR), following continuous training at a moderate intensity [343]. Studies have also investigated the effects of all-out sprint interval training (SIT) on markers of mitochondrial content [35, 36, 82], and showed that SIT induces similar increases in CS activity to continuous training at a moderate intensity [35]. However, none of these studies directly measured the effects of SIT on mitochondrial function. Furthermore, it has been hypothesised that changes in mitochondrial content seem to depend on training volume, whilst changes in mitochondrial function seem to be related

to training intensity [26]. To test these hypotheses, and given the sometimes contradictory and limited sample size of available studies, further research directly comparing the effects of different training intensities on mitochondrial content and function is warranted.

The oxidative capacity of mitochondria is determined by the abundance of mitochondrial proteins, and their functional ability to produce ATP [135]. The abundance and functional ability of these proteins is influenced by transcriptional activity. This requires the concerted integration of signalling, transcription and translation events taking place in both the nucleus and the mitochondrion, followed by the import and incorporation of the newly-generated proteins into the mitochondrial reticulum [56]. Monitoring changes in these events not only allows for an indirect assessment of mitochondria biogenesis, but also contributes to a better understanding of the underlying cellular mechanisms. Peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) is a transcriptional coactivator that has been shown to regulate mitochondrial content and respiration in cultured muscle cells [352], and also metabolic control in rat skeletal muscle [140]. Subsequent studies in humans have shown that exercise training leads to an increase in PGC-1α protein content [35, 224, 258], and suggest that modulation of PGC-1α mRNA is sensitive to training intensity [73]. However, no study to date has directly compared the effects of exercise training at different intensities on changes in PGC-1α protein abundance, and both mitochondrial content and respiration.

The tumour suppressor p53, has also been shown to be an important regulator of mitochondrial biogenesis [266]. Studies in cells and mice indicate that ablation of p53 leads to a reduction in mitochondrial respiration and content, and endurance performance [181, 265]. p53 is also up-regulated following a single bout of exercise [18], and, similar to PGC-1α, has been shown to regulate the mitochondrial transcription machinery via modulation of mitochondrial transcription factor A (TFAM) [217, 267]. Furthermore, by regulation of downstream targets, p53 can control mitochondrial function by modulating the balance between glycolytic and oxidative pathways [181, 329], and has been linked with mitochondria dynamics and the maintenance of mitochondrial morphology [266]. Although the signalling mechanisms upstream of p53 are not yet fully known, plant homeodomain finger-containing protein 20 (PHF20) has been shown to up-regulate p53 transcription [218] and to stabilise p53 protein [65], emphasising the role of PHF20 as a

regulator of p53. Despite evidence demonstrating the importance of the p53 pathway in the regulation of mitochondria biogenesis, no study to date has examined the effects of a period of exercise training at different intensities on p53 and its upstream regulators and downstream targets.

The purpose of this research was to compare the effects of four weeks of sub-lactate threshold continuous training (STCT), HIIT and SIT on markers of mitochondrial biogenesis in human skeletal muscle. It was hypothesised that higher training intensities would produce greater improvements in mitochondrial respiration, and that these changes would be associated with increased protein content of transcription factors known to regulate mitochondrial biogenesis.

2.2 Methods

Participants and ethics approval

Participants. Thirty-one healthy men, who were non-smokers, moderately-trained and involved in various individual and team sports, volunteered to take part in this study. After initial screening and testing, they were matched by the power attained at the lactate threshold (W_{LT}) and randomly assigned to the STCT (n=10), HIIT (n=11), or SIT (n=10) group. Twenty-nine participants completed the study, whilst two, one each from the STCT and SIT group, withdrew from the study due to time constraints. Baseline physiological parameters for each group are described in Table 2.1.

Ethical approval. Participants were informed of the study requirements, benefits and risks before giving written informed consent. Approval for the study's procedures, which conformed to the standards set by the latest revision of the Declaration of Helsinki, was granted by the Victoria University Human Research Ethics Committee.

Table 2.1. Baseline characteristics of the participants

Measurement	STCT group (n=9)	HIIT group (n=11)	SIT group (n=9)
Age (y)	21 ± 2	21 ± 1	21 ± 3
Height (cm)	179.3 ± 6.3	180.0 ± 10.7	180.6 ± 7.3
Body Mass (kg)	77.4 ± 10.6	80.2 ± 13.8	84.5 ± 19.4
$\dot{V}O_{2Peak}$ [mL min^{-1} kg^{-1}]	46.7 ± 3.8	45.1 ± 7.2	47.1 ± 7.8

STCT: sub-lactate threshold continuous training; HIIT: high-intensity interval training; SIT: sprint interval training; $\dot{V}O_{2Peak}$: peak oxygen uptake. All values are mean ± SD.

Study design

The experimental protocol began with a familiarisation session, and baseline testing that consisted of, in the following order, a 20-km cycling time trial (20k-TT), a graded exercise test (GXT), and a resting muscle biopsy; all tests were separated by a minimum of 48 h (72 h for the biopsy trial). During the training phase participants trained three times a week for four weeks regardless of group, with the STCT and HIIT groups matched for total work. Due to the nature of sprint training, it was not possible to match the SIT group for total work with the other two groups. Seventy-two hours following the last training session a resting muscle biopsy took place, followed 48 hours later by the 20k-TT test. The testing and training duration was approximately 6 weeks.

Testing procedures

Participants were required to refrain from any strenuous physical activity for the 48 hours preceding each performance test (72 h for the biopsy trial), from alcohol and any exercise for 24 h before testing, and from food and caffeine consumption for the 2 h preceding each test. Each type of test was performed at the same time of the day during the course of the entire study with the aim to avoid variations caused by circadian rhythm changes.

20k-TT. Cycling time trials were used as an indicator of endurance performance and were performed on an electronically-braked cycle ergometer (Velotron, RacerMate, Seattle, WA, USA). Prior to each time trial participants completed a warm-up involving cycling for 4 min at 66% of W_{LT} followed by 2 min at W_{LT} and 2 min of rest. During the time trial, participants were only allowed access to cadence and completed distance, and were provided with similar verbal encouragement during each test. Heart rate monitors (Polar Electro, Kempele, Finland) were used in all exercise trials and training sessions to monitor heart rate.

GXT. Prior to training, a discontinuous graded exercise test was performed on an electronically-braked cycle ergometer (Lode Excalibur, v2.0, Groningen, The Netherlands) to determine the peak oxygen uptake ($\dot{V}O_{2Peak}$), peak power output (W_{Peak}), W_{LT} (using the modified D_{Max} method [25]), and the training intensities for the STCT and HIIT groups. The test consisted of 4-min stages at a constant power output, interspersed with 30 seconds of rest. The test began at a power output of 60, 90 or 120 W depending on participants' fitness levels, and was subsequently increased by 30 W every 4 min. Prior to the test, and during the 30-s rest, capillary blood samples were taken from the fingertip for measurement of blood lactate concentration ($[La^-]$). Participants were instructed to keep a cadence > 60 rpm and were given verbal encouragement throughout the test, during which they were only allowed access to cadence and elapsed time. The test was stopped when a participant reached volitional exhaustion or cadence dropped below 60 rpm. The W_{Peak} was determined as the power of the last completed stage when participants stopped at the end of a stage. If a participant stopped during a stage, W_{Peak} was determined as the power of the last completed stage plus 7.5 W for every completed minute.

Gas Analysis during the GXT. During the GXT, expired air was continuously analysed for O_2 and CO_2 concentrations via a gas analyser (Moxus modular oxygen uptake system, 2010, AEI technologies, Pittsburgh, PA, USA). The gas analysers were calibrated immediately before each test using known gas mixtures (A: 21% O_2, 0% CO_2; B: 16% O_2, 4% CO_2; BOC, Melbourne, Australia). The ventilometer was calibrated using a 3-liter syringe (Hans Rudolph). $\dot{V}O_2$ values were recorded every 15 s and the two highest consecutive 15-s values recorded during the test were averaged and recorded as the participant's $\dot{V}O_{2Peak}$.

Capillary blood sampling. Glass capillary tubes (MultiCap 140 µL, Siemens Healthcare Diagnostics Inc. Deerfield, IL, USA) were used to collect about 50 µL of blood at the various time points during the GXT. Capillary blood [La^-] was determined using a blood-lactate analyser (YSI 2300 STAT Plus Glucose & Lactate Analyser, YSI Inc., Yellow Spring, Ohio, USA). The blood-lactate analyser was regularly calibrated using precision standards and routinely assessed by external quality controls.

Muscle biopsies. All muscle samples were obtained in the morning by an experienced practitioner at a constant depth of around 2-3 cm. Before and after the training period, resting muscle biopsies (approximately 150-300 mg wet weight) were taken from the vastus lateralis muscle using a biopsy needle 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. The same leg was chosen post-training. Once obtained, muscle samples were processed, cleaned of excess blood, fat and connective tissue and split in two portions. One portion (10-20 mg) was immediately immersed in a 5-mL tube containing ~3 mL of biopsy preserving solution kept on ice and was used for in-situ measurements of mitochondrial respiration. The remaining portion was immediately frozen in liquid nitrogen and stored at -80°C for subsequent analyses.

Training intervention

All training sessions were carried out on an electronically-braked cycle ergometer (Velotron, RacerMate, Seattle, WA, USA) and were preceded by an 8-min warm up (as described in the 20k-TT paragraph).

STCT. Training sessions consisted of continuous cycling at a fixed power equivalent to 90, 92.5, 95 and 97.5% of baseline W_{LT} for week one to four respectively (Figure 2.1). The workload was matched to that completed by the HIIT group.

HIIT. Training sessions consisted of 4-min cycling intervals interspersed with a 2-min recovery period at a power of 60 W. The training intensities were defined as [$W_{LT} + x (W_{Peak}-W_{LT})$] (with x equivalent to 0.35, 0.50, 0.65 and 0.75 for week one to four respectively, Figure 2.1). For the above two groups, training intensity was set relative to W_{LT} rather than W_{Peak} as metabolic and cardiac stresses are similar when individuals of

differing fitness levels exercise at a percent of the W_{LT}, but can vary significantly when training at a percent of W_{Peak} [15].

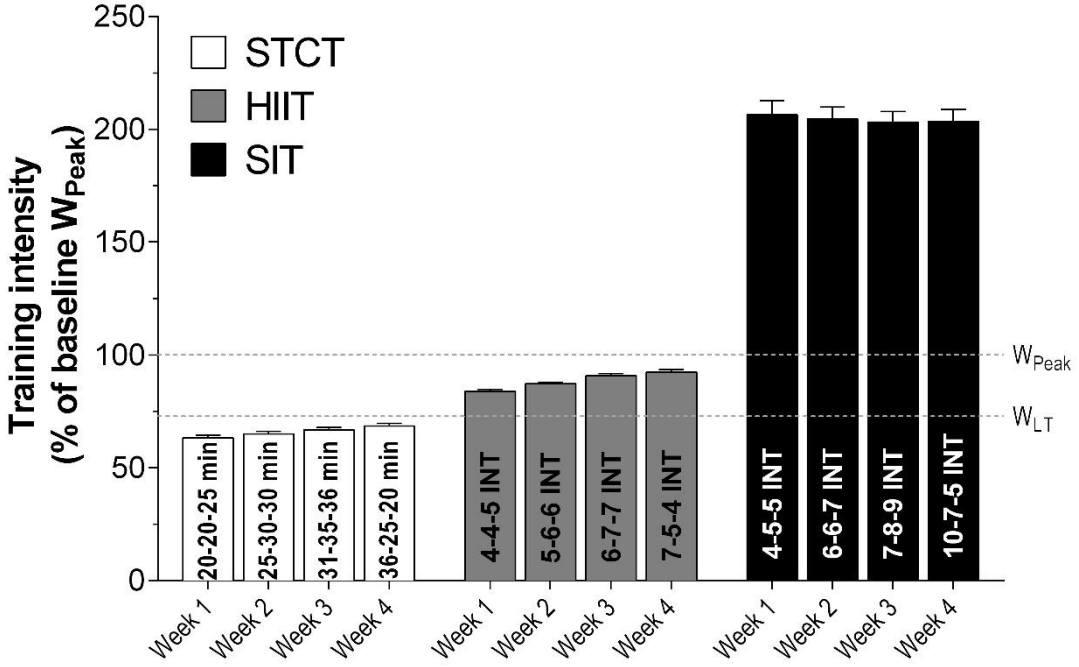

Figure 2.1. Training intensity expressed as percent of the peak power (W_{Peak}) for the sub-lactate threshold continuous training (STCT), high-intensity interval training (HIIT), and sprint interval training (SIT) group. The numbers in each bar represent the duration in minutes (STCT) or the number of intervals (HIIT, SIT) for each of the three weekly training sessions. W_{LT}: power at the lactate threshold attained during the graded exercise test (GXT) calculated using the modified D_{Max} method [25]. All values are mean ± SEM.

SIT. Training sessions consisted of 30-s "all-out" cycling bouts (Wingate test) against a resistance set at 0.075 kg/kg body mass, interspersed with a 4-min recovery period (Figure 2.1). During recovery participants remained on the bikes and were allowed to either rest or cycle against a low resistance (30 W) at low cadence (< 50 rpm). During the last 15 s of the recovery period participants were instructed to begin pedalling and reach a cadence of 80-100 rpm against no resistance (warm up phase) and in the last 2 seconds they were instructed to begin pedalling as fast as possible (speed up phase). 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 keep the cadence as high as possible during the entire duration of the bout.

Physical activity and nutritional control

Participants were instructed to maintain a normal dietary pattern and to keep their routine physical activity at a constant level throughout the entire study. To minimize variability in muscle metabolism attributable to diet, participants were provided with a standardised dinner (55 kJ kg^{-1} body mass (BM), providing 2.1 g carbohydrate kg^{-1} BM, 0.3 g fat kg^{-1} BM, and 0.6 g protein kg^{-1} BM) and breakfast (41 kJ kg^{-1} BM, providing 1.8 g carbohydrate kg^{-1} BM, 0.2 g fat kg^{-1} BM, and 0.3 g protein kg^{-1} BM) prior to the two biopsy trials. These meals were consumed 15 and 3 h prior to the biopsy trials respectively. Participants were also required to fill in a nutrition diary recording the last 3 meals prior to each performance test undertaken during baseline testing and they were asked to replicate the same nutritional intake thereafter prior to the same type of test.

Muscle analyses

Preparation of permeabilised skeletal muscle fibres for mitochondrial respiration. A 10-20 mg fresh muscle sample was placed in ice cold BIOPS, a biopsy preserving solution containing (in mM) 2.77 CaK_2EGTA, 7.23 K_2EGTA, 5.77 Na_2ATP, 6.56 $MgCl_2$, 20 taurine, 50 Mes, 15 Na_2phosphocreatine, 20 imidazole and 0.5 DTT adjusted to pH 7.1 [225]. Samples were transferred to a 6-well plate kept on ice where the fibres were mechanically separated using pointed forceps. Fibres were subsequently permeabilised by gentle agitation for 30 min at 4°C in BIOPS containing 50 µg/mL of saponin. Samples were then washed 3 times for 7 min at 4°C by gentle agitation in MiR05, a respiration medium containing (in mM, unless specified) 0.5 EGTA, 3 $MgCl_2$, 60 K-lactobionate, 20 taurine, 10 KH_2PO_4, 20 Hepes, 110 sucrose and 1 g/l BSA essentially fatty acid-free adjusted to pH 7.1 at 37°C [225]. This method selectively permeabilises the cellular membrane leaving the mitochondria intact and allows for *in-situ* measurements of mitochondrial respiration.

High-Resolution Respirometry. After washing, 3-4 mg wet weight of muscle fibres were assayed in duplicate 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/mL) and oxygen flux (pmol s^{-1} 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/mL and to avoid potential oxygen diffusion limitation.

Mitochondrial respiration protocol. A substrate-uncoupler-inhibitor titration (SUIT) protocol was used and the SUIT sequence, with final chamber concentration in brackets, was as follows: pyruvate (2 mM) and malate (5 mM) in the absence of adenylates were added for measurement of LEAK respiration (L) with electron entry through Complex I (CI) (CI_L). ADP (5 mM) was then added for measurement of maximal oxidative phosphorylation (OXPHOS) capacity (P) with electron input through CI (CI_P), followed by addition of succinate (10 mM) for measurement of P with simultaneous electron supply through CI + Complex II (CII) combined ($CI+II_P$). This respiration state provides convergent electron input to the Q-junction through CI (NADH provided by malate/pyruvate) and CII (flavin adenine dinucleotide reduced ($FADH_2$) provided by succinate) and supports maximal mitochondrial respiration by reconstruction of the tricarboxylic acid cycle function. Cytochrome *c* (10 μM) was then added to test for outer mitochondrial membrane integrity; an exclusion criterion was set such that if a chamber showed an increase in O_2 flux > 6% after addition of cytochrome *c*, it was discarded. This was followed by a series of stepwise carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone titrations (FCCP, 0.75-1.5 μM) for measurement of electron transport system (ETS) capacity (E) with convergent electron input through CI+II ($CI+II_E$). Rotenone (0.5 μM), an inhibitor of CI, was then added to obtain a measurement of E with electron input through CII (CII_E). This was followed by addition of antimycin A (2.5 μM), an inhibitor of Complex III (CIII), to obtain a measurement of residual oxygen consumption capacity (ROX). ROX was subtracted from all other measurements to account for oxidative side reactions. Ascorbate (2 mM) and N,N,N',N'-tetramethyl-p-

phenylenediamine (TMPD, 0.5 mM), artificial electron donors for Complex IV (CIV), followed by sodium azide (> 200 mM), an inhibitor of CIV to account for auto oxidation of ascorbate + TMPD, were then added to measure CIV respiration in the noncoupled state (CIV_E). Respiratory flux control ratios (FCR) were calculated. In brief, the leak control ratio (LCR) is the quotient of CI_L over $CI+II_E$; the phosphorylation control ratio (PCR) is the quotient of $CI+II_P$ over $CI+II_E$; the coupling control ratio is the quotient of CI_L over $CI+II_P$ and is equivalent to the inverse respiratory control ratio (inv-RCR); and the substrate control ratio (SCR) at constant P is the quotient of CI_P over $CI+II_P$. Finally, the reserve capacity of CIV (CIV_{RES}) as the quotient of $CI+II_P$ over CIV_E was determined.

Preparation of whole muscle lysates. Approximately 10-20 mg of frozen muscle was homogenised 4 times for 5 seconds with a hand-held mini-homogenizer (Kontes Pellet Pestle Cordless Motor, Kimble Chase, NJ, USA.) on ice (1:20 w/v) in an ice-cold lysis buffer containing 50 mM Tris, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 1 μg/mL aprotinin, 1 μg/mL leupeptin, 1 mM benzamidine, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 5 mM sodium pyrophosphate, 1 mM DTT, 1 mM phenylmethanesulfonyl fluoride (PMSF), adjusted to pH 7.4. Muscle homogenates were rotated end over end at 4 °C for 60 min, after which they were centrifuged for two cycles of 10 min at 15 000 g at 4 °C separated by a similar re-homogenization step to the initial one. After the second centrifugation the supernatant was taken as the whole muscle lysates for Western Blotting and enzyme activity assay. Protein concentration was determined using a commercial colorimetric assay (Bio-Rad Protein Assay kit II, Bio-Rad Laboratories Pty Ltd, Gladesville, NSW, Australia) and lysates were then diluted with an equal volume of 2x Laemmli buffer containing 10% β-mercaptoethanol.

Western Blotting. For each protein of interest a linearity study was conducted to determine the ideal loading amount (data not shown). Muscle lysates were then loaded in equal amounts (10-25 μg according to target protein) and separated by electrophoresis for 1.5-2.5 h at 100 V using self-cast SDS-PAGE gels (8-12%). Once resolved, the gels were then wet transferred onto PVDF membranes at 100 V for 70-90 min. Transfer efficiency was checked by Coomassie staining the gels together with Ponceau S staining of the membranes. 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). Immunoblotting was carried out using the following antibodies (with the amount loaded and the supplier noted in brackets): apoptosis-inducing factor (AIF) (12 µg; Cell Signaling Tech (CST), 5318), dynamin-related protein 1 (DRP1) (15 µg; CST, 5391), mitofusin 2 (7.5 µg; CST, 9482), p53 (25 µg; CST, 2527), PGC-1α (20 µg; Calbiochem, st-1202), PHF20 (17 µg; CST, 3934), synthesis of cytochrome *c* oxidase (COX) 2 (SCO2) (20 µg; Santa Cruz, sc-49110), TFAM (15 µg; Abcam, ab47517), and Total OXPHOS (7.5 µg; MitoSciences, ab110411). 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 90 min in 1-5% skim milk in TBS-T. Membranes were then washed again for 3 x 5-min in TBS-T followed by a final 5-min wash in TBS before being immersed for 5 min under gentle agitation at room temperature in a chemiluminescent solution (Solution A: 2.5 mM Luminol, 0.36 mM p-Coumaric Acid, 0.1 M Tris (pH 8.5) in 10 mL MilliQ H_2O; Solution B: 6.1 µL H_2O_2, 0.1 M Tris (pH 8.5) in 10 mL MilliQ H_2O; solution A and B prepared separately and mixed just prior to use). Protein bands were visualized 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, Australia). Coomassie Blue staining was performed at the end of the procedure and was used to verify correct loading and equal transfer between lanes [338]. Finally, samples for both time points for each participant were loaded on the same gel together with an identical amount of internal standard (IS). Comparison and statistical analysis between each participant's time points were done using the raw density data normalised to the IS loaded in each gel to reduce gel-to-gel variability. For graphical purposes, each time point was normalised to baseline; for this reason WB graphs throughout this manuscript are presented as fold change compared to baseline, and no error bar is presented for the baseline time point. A representative blot for each protein analysed is presented in Figure 2.2.

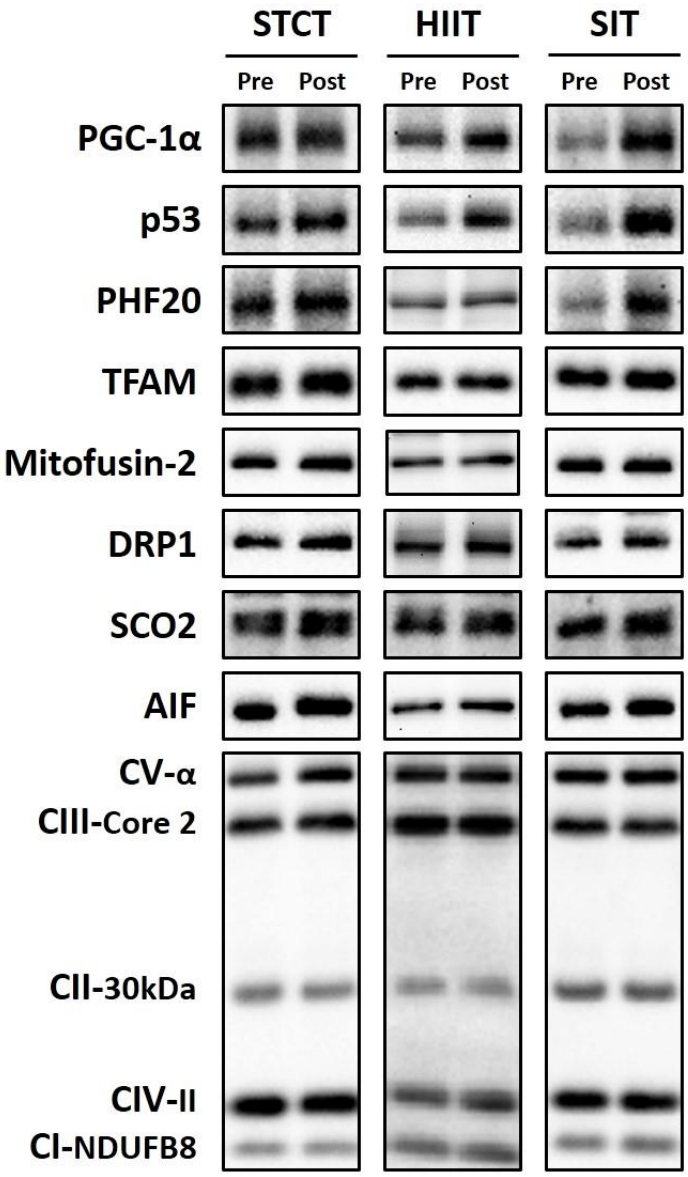

Figure 2.2. Representative immunoblots of transcription factors, downstream targets of p53 and subunits of the five complexes of the electron transport system (ETS), measured in whole muscle lysates prepared from skeletal muscle biopsy samples (vastus lateralis). Biopsies were obtained at rest before (Pre) and after (Post) 4 weeks of training at each of the three training intensities: sub-lactate threshold continuous training (STCT), high-intensity interval training (HIIT) and sprint interval training (SIT).

Citrate Synthase Activity Assay. CS activity was determined in triplicate on a microtiter plate by adding: 5 µL of a 2 mg/mL muscle homogenate, 40 µL of 3mM acetyl CoA in Tris buffer and 25 µL of 1mM 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, Australia). 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 kg of protein^{-1} h^{-1}.

Statistical analysis

All values are reported as means ± SD unless specified otherwise. A one-way ANOVA was used to assess differences between the three groups for age, body mass, height and $\dot{V}O_{2Peak}$, and for total training workload. A one-way RM ANOVA was used to assess differences between the percent changes in the mitochondrial respiration measurements of the SUIT protocol within each training group. To investigate the influence of treatment and time, and the interaction between both of these variables, two-way ANOVA for repeated measure were used. Significant interactions and main effects were further analysed using a Tukey's honestly significant difference post hoc test. Least squares linear regression analysis was used to calculate correlation coefficients between variables using Pearson's product moment (r). SigmaStat software (Jandel Scientific, San Rafael, CA) was used for all statistical analyses. The level of statistical significance was set at $P < 0.05$.

2.3 Results

Training and performance measurements.

Participants were matched for age, body mass, height, $\dot{V}O_{2Peak}$ (Table 2.1), and W_{LT} (Table 2.2) (all $P > 0.05$). Participants in the SIT group completed a significantly lower amount of total work (~35%) compared to the HIIT and STCT groups (1.3 ± 0.2, 3.6 ± 0.6 and 3.8 ± 0.7 MJ for the SIT, HIIT and STCT groups respectively, $P < 0.001$). There were no changes in body mass following training (data not shown). W_{LT} increased after

training following all 3 training interventions (all $P < 0.001$), with no difference between conditions ($P = 0.754$, Table 2.2). W_{Peak} increased significantly following SIT ($P = 0.011$) and HIIT ($P < 0.001$), and there was a similar trend following STCT although it failed to reach significance ($P = 0.063$) (Table 2.2). 20k-TT time improved significantly following STCT and HIIT (both $P < 0.001$), but did not change following SIT ($P = 0.177$) (Table 2.2).

Table 2.2. Participants' endurance performance and physiological measurements before and after 4 weeks of training

Measurement		STCT (n=9)	HIIT (n=11)	SIT (n=9)
W_{LT} [W]	pre	194.9 ± 46.1	198.1 ± 27.4	204.4 ± 39.7
	post†	208.9 ± 50.0	214.7 ± 29.7	222.3 ± 45.4
W_{Peak} [W]	pre	275.6 ± 54.6	264.1 ± 37.4	280.8 ± 48.2
	post†	284.4 ± 62.5	293.2 ± 34.3*	293.3 ± 51.5*
20k-TT time [s]	pre	2216.7 ± 183.8	2247.7 ± 147.5	2162.3 ± 143.1
	post†	2130.9 ± 176.0*	2138.1 ± 90.7*	2131.9 ± 165.1
CS activity [mol h^{-1} kg $prot^{-1}$]	pre	9.3 ± 1.6	8.0 ± 2.2	9.4 ± 1.8
	post	9.8 ± 0.8	8.6 ± 1.8	9.6 ± 1.9

STCT: sub-lactate threshold continuous training; HIIT: high-intensity interval training; SIT: sprint interval training; W_{LT}: power at the lactate threshold; W_{Peak}: peak power output; 20k-TT: 20-km time trial; CS: citrate synthase. † Significant main effect of time ($P < 0.05$). *Significantly different ($P < 0.05$) *vs.* pre. All values are mean ± SD

Mass-specific mitochondrial respiration (all values in the text are expressed as pmol O_2 s^{-1} mg^{-1})

Mass-specific mitochondrial respiration changes are shown in Figure 2.3A. SIT was the only group increasing maximal coupled ($CI+II_P$) and noncoupled ($CI+II_E$) respiration (interaction, $P = 0.008$ and 0.028 respectively). $CI+II_P$ increased significantly following SIT (85.6 ± 12.1 *vs.* 106.9 ± 19.0, $P < 0.001$) and did not change following HIIT (68.1 ± 11.6 *vs.* 65.6 ± 7.7, $P = 0.625$) or STCT (88.6 ± 19.3 *vs.* 89.5 ± 15.5, $P = 0.873$). Similarly, $CI+II_E$ increased significantly following SIT (107.4 ± 12.0 *vs.* 139.6 ± 36.7, $P = 0.001$)

and did not change following HIIT (87.4 ± 16.2 *vs.* 87.9 ± 15.9, $P = 0.966$) or STCT (111.4 ± 24.1 *vs.* 110.7 ± 21.1, $P = 0.935$). CII_E was also significantly increased following SIT (47.0 ± 6.3 *vs.* 60.9 ± 17.5, $P = 0.002$) and did not change following HIIT (41.7 ± 7.7 *vs.* 41.1 ± 4.5, $P = 0.860$) or STCT (44.2 ± 11.9 *vs.* 46.9 ± 13.7, $P = 0.524$; interaction, $P = 0.027$). Although it failed to reach statistical significance, the same trend was observed for CI_P (SIT: 59.1 ± 8.0 *vs.* 70.8 ± 13.8; HIIT: 45.2 ± 8.4 *vs.* 45.1 ± 6.2; STCT: 63.2 ± 15.3 *vs.* 60.1 ± 10.6; interaction, $P = 0.055$) and CIV_E (SIT: 132.3 ± 22.3 *vs.* 160.9 ± 49.2; HIIT: 124.0 ± 17.9 *vs.* 125.1 ± 14.0; STCT: 132.6 ± 30.9 *vs.* 128.3 ± 36.6; interaction, $P = 0.061$). CI_L did not change (SIT: 8.1 ± 1.5 *vs.* 10.0 ± 1.8; HIIT: 4.9 ± 2.2 *vs.* 5.5 ± 1.6; STCT: 9.0 ± 2.8 *vs.* 8.3 ± 3.1; interaction, $P = 0.271$). The data presented in this study showed no effect of the addition of cytochrome *c* as a control for outer mitochondrial membrane integrity. There were no significant differences between the percentage changes of the six measurements of the SUIT protocol within each training intervention, or in the FCRs measured (all $P > 0.05$, Table 2.3).

Mitochondrial-specific respiration (all values in the text below were obtained by normalising mass-specific mitochondrial respiration by CS activity and are expressed as: pmol O_2 s^{-1} CS^{-1})

CS activity did not change significantly following any of the training interventions (8.2 ± 20.6%, 12.4 ± 33.9%, and 2.8 ± 11.7% for STCT, HIIT and SIT respectively, all $P > 0.05$; Table 2.2). Mitochondrial (mt)-specific respiration changes are shown in Figure 2.3B. SIT was once again the only training intervention to significantly increase maximal coupled ($CI+II_P$) and noncoupled ($CI+II_E$) respiration (interaction, $P = 0.019$ and $P = 0.045$ respectively). Specifically, $CI+II_P$/CS increased significantly following SIT (9.4 ± 2.1 *vs.* 11.3 ± 1.4, $P = 0.014$) and did not change following HIIT (8.8 ± 1.5 *vs.* 7.9 ± 1.8, $P = 0.218$) or STCT (9.8 ± 2.8 *vs.* 9.1 ± 1.5, $P = 0.375$). Similarly $CI+II_E$/CS increased significantly following SIT (11.7 ± 2.1 *vs.* 14.5 ± 2.0, $P = 0.013$) and did not change following HIIT (10.7 ± 1.7 *vs.* 11.0 ± 2.5, $P = 0.840$) or STCT (12.4 ± 3.8 *vs.* 11.3 ± 1.8, $P = 0.302$). CII_E/CS was also increased significantly following SIT (5.3 ± 0.9 *vs.* 6.6 ± 0.9, $P = 0.004$) and did not change following HIIT (5.3 ± 0.8 *vs.* 4.9 ± 0.9, $P = 0.272$) or STCT (4.9 ± 1.7 *vs.* 4.9 ± 1.3, $P = 0.946$; interaction, $P = 0.013$). Despite not reaching

statistical significance, the same trend was observed for CI_P/CS (SIT: 6.5 ± 1.5 *vs.* 7.5 ± 1.3; HIIT: 5.8 ± 1.0 *vs.* 5.4 ± 1.3; STCT: 7.0 ± 2.2 *vs.* 6.1 ± 1.0; interaction, $P = 0.107$) and CIV_E/CS (SIT: 14.4 ± 2.8 *vs.* 16.7 ± 3.4; HIIT: 16.1 ± 3.1 *vs.* 15.2 ± 3.9; STCT: 14.6 ± 4.3 *vs.* 13.0 ± 3.2; interaction, $P = 0.072$). CI_L/CS did not change (SIT: 0.9 ± 0.3 *vs.* 1.1 ± 0.3; HIIT: 0.6 ± 0.3 *vs.* 0.6 ± 0.2; STCT: 1.0 ± 0.4 *vs.* 0.9 ± 0.3; interaction, $P = 0.313$). There were no significant differences between the magnitudes of change of the six respiratory parameters measured with the SUIT protocol within each training intervention.

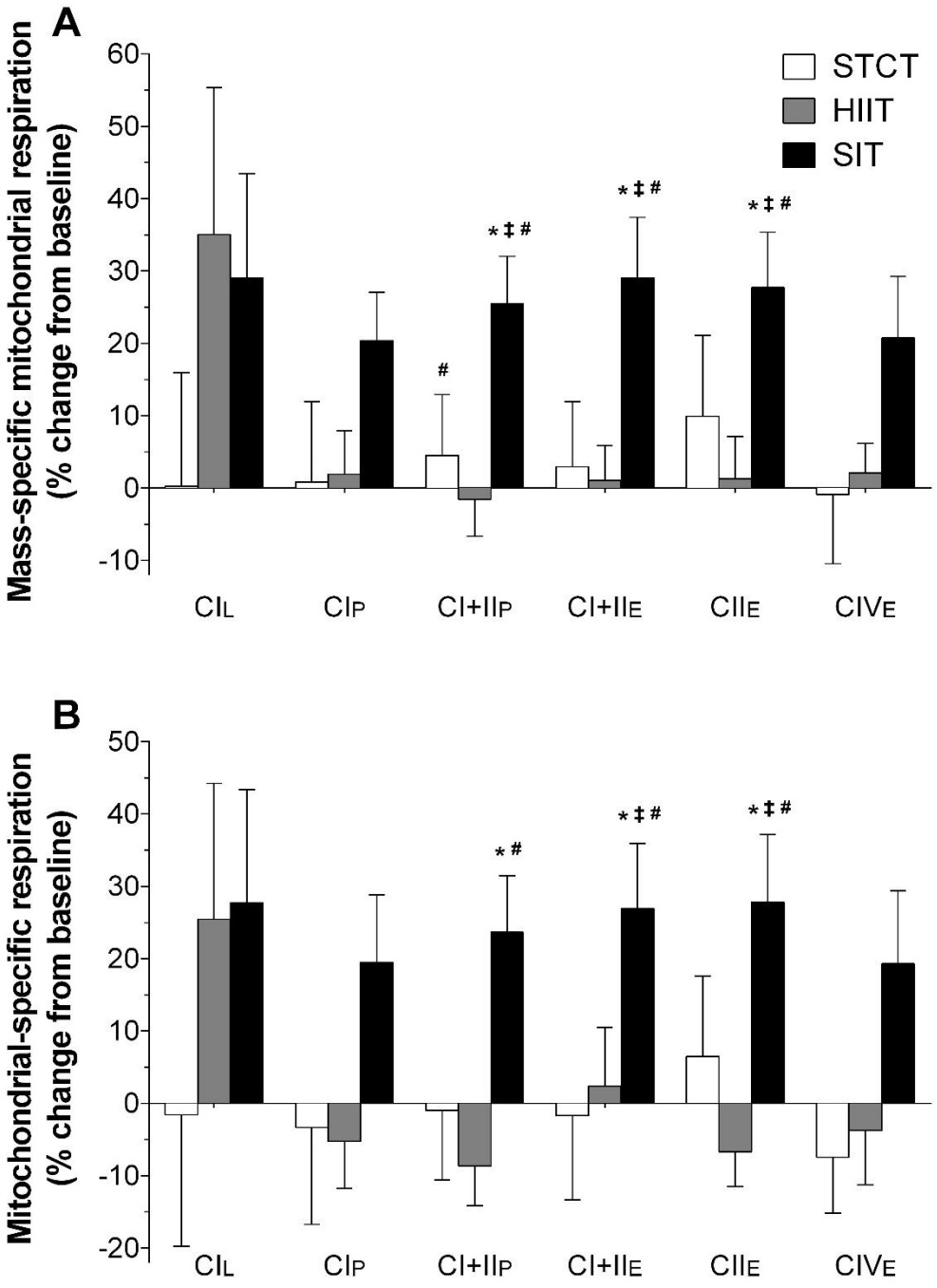

Figure 2.3. Mitochondrial respiration changes compared to baseline. A: percent change of mass-specific mitochondrial respiration (pmol O_2 s^{-1} mg^{-1}), and B: percent change of mitochondrial-specific respiration (obtained by normalising mass-specific mitochondrial respiration values by citrate synthase activity expressed per kg of protein). Respiration was measured in permeabilised fibres prepared from muscle biopsy samples (vastus lateralis) obtained at rest before and after 4 weeks of training at each of the three training intensities: sub-lactate threshold continuous training (STCT), high-intensity interval training (HIIT) and sprint interval training (SIT). Respiration values are: CI_L: Leak respiration state (L) in the absence of adenylates and limitation of flux by electron input through Complex I (CI); CI_P: maximal oxidative phosphorylation state (P) with

saturating levels of ADP and limitation of flux by electron input through CI; $CI+II_P$: P with saturating levels of ADP and limitation of flux by convergent electron input through CI + Complex II (CII); $CI+II_E$: maximal electron transport system (ETS) capacity (E) with saturating levels of ADP and limitation of flux by convergent electron input through CI+II; CII_E: E with saturating levels of ADP and limitation of flux by electron input through CII; CIV_E: maximal Complex IV noncoupled respiration with saturating levels of ADP and limitation of flux by electron input through CIV. * Significantly different ($P < 0.05$) *vs.* pre, # *vs.* post-HIIT, ‡ *vs.* post-STCT. All values are mean ± SEM.

Table 2.3. Respiratory flux control ratios

FCR		STCT (n=9)	HIIT (n=11)	SIT (n=9)
LCR (L/E)	pre	0.08 ± 0.03	0.04 ± 0.02	0.08 ± 0.02
	post	0.08 ± 0.03	0.07 ± 0.04	0.07 ± 0.02
PCR (P/E)	pre	0.80 ± 0.07	0.81 ± 0.07	0.80 ± 0.08
	post	0.81 ± 0.05	0.74 ± 0.03	0.78 ± 0.08
Inv-RCR (L/P)	pre	0.10 ± 0.04	0.07 ± 0.03	0.10 ± 0.02
	post	0.10 ± 0.04	0.09 ± 0.03	0.09 ± 0.02
SCR (Constant P)	pre	0.71 ± 0.10	0.67 ± 0.05	0.69 ± 0.05
	post	0.67 ± 0.02	0.69 ± 0.05	0.66 ± 0.04
CIV_{RES}	pre	0.67 ± 0.08	0.55 ± 0.05	0.65 ± 0.07
	post	0.73 ± 0.14	0.53 ± 0.09	0.69 ± 0.09

FCR: respiratory flux control ratio; STCT: sub-lactate threshold continuous training; HIIT: high-intensity interval training; SIT: sprint interval training; L: leak respiration; P: oxidative phosphorylation capacity; E: electron transport system capacity; LCR: leak control ratio ($CI_L/CI+II_E$); PCR: phosphorylation control ratio ($CI+II_P/CI+II_E$); inv-RCR: inverse of respiratory control ratio ($CI_L/CI+II_P$); SCR: substrate control ratio at constant P ($CI_P/CI+II_P$); CIV_{RES}: CIV reserve capacity ($CI+II_P/CIV_E$). CI: electron input through complex I; CI+II: convergent electron input through complex I and II; CIV: electron input through complex IV. FCR were calculated from mass-specific respiration measurements in permeabilised muscle fibres (vastus lateralis), obtained from muscle biopsies taken before and after 4 weeks of training at each of the three training intensities. All values are mean ± SD.

Protein content of transcription factors

PGC-1α protein content increased by 61 ± 58% following SIT ($P = 0.011$), but did not change significantly following either STCT ($P = 0.143$) or HIIT ($P = 0.137$) (Figure

2.4A). The protein content of p53 increased by 93 ± 77% following SIT (P = 0.004), but did not change significantly following STCT (P = 0.554) or HIIT (P = 0.204) (Figure 2.4B). PHF20 protein content increased by 84 ± 76% following SIT (P = 0.003), but did not change significantly following STCT (P = 0.175) or HIIT (P = 0.856) (Figure 2.4C). The protein content of TFAM did not change after training (main effect of time, P = 0.364, Figure 2.4D).

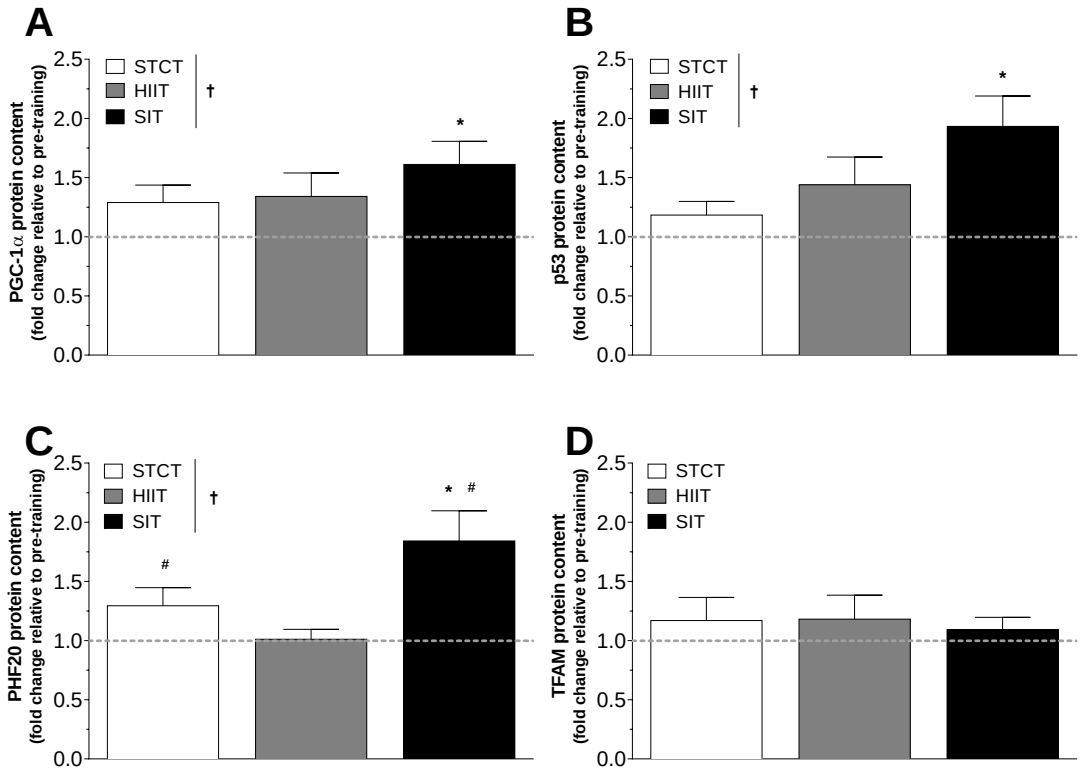

Figure 2.4. Fold change compared to baseline of the protein content of peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α), (A), p53 (B), plant homeodomain finger-containing protein 20 (PHF20) (C), and mitochondrial transcription factor A (TFAM) D) in whole muscle lysates prepared from skeletal muscle biopsy samples (vastus lateralis). Biopsies were obtained at rest at baseline (dotted line) and after 4 weeks of training at each of the three training intensities: sub-lactate threshold continuous training (STCT), high-intensity interval training (HIIT) and sprint interval training (SIT). † Significant main effect of time (P < 0.05). *Significantly different (P < 0.05) *vs.* pre, # *vs.* post-HIIT. All values are mean ± SEM.

Protein content of subunits from the five ETS complexes

The protein content of subunits from the five ETS complexes did not change significantly following training (all $P > 0.05$, Figure 2.5).

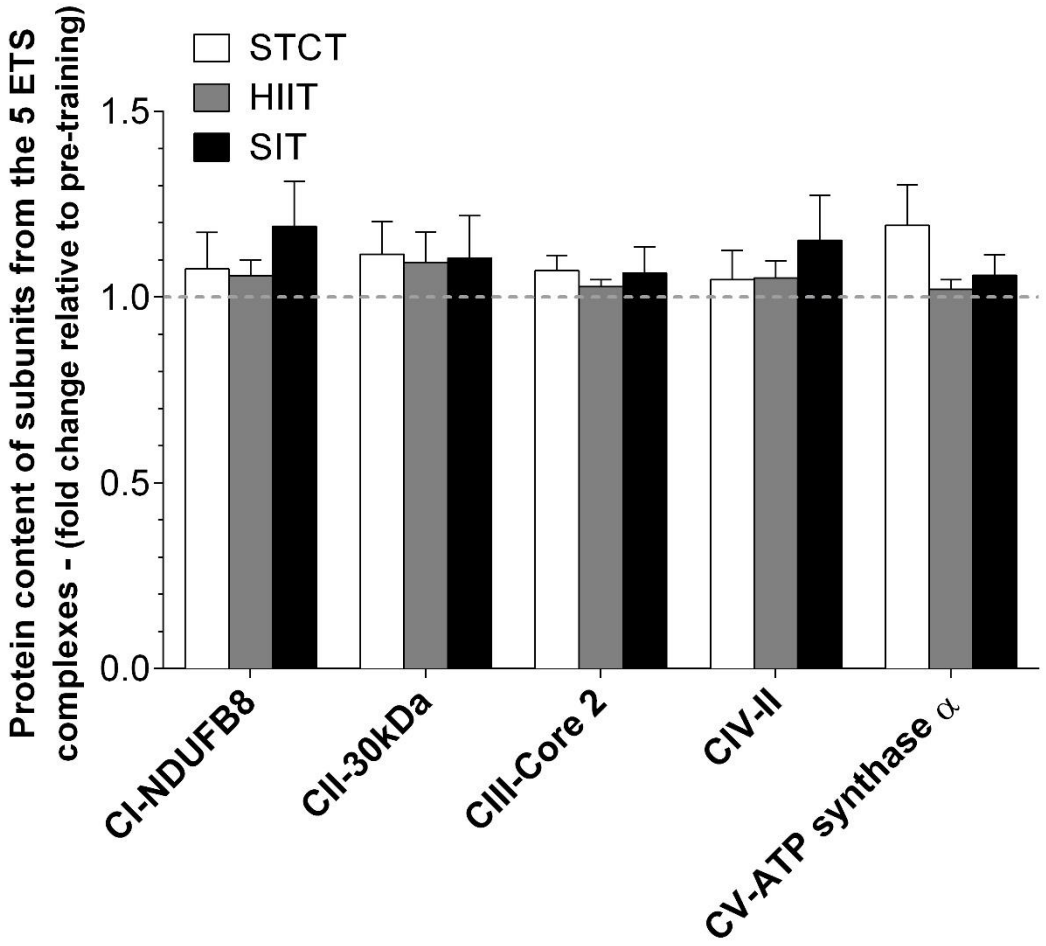

Figure 2.5. Fold change compared to baseline of the protein content of subunits from the five complexes of the electron transport system (ETS), complex I to V (CI to CV), in whole muscle lysates prepared from skeletal muscle biopsy samples (vastus lateralis). Biopsies were obtained at rest at baseline (dotted line) and after 4 weeks of training at each of the three training intensities: sub-lactate threshold continuous training (STCT), high-intensity interval training (HIIT) and sprint interval training (SIT). All values are mean ± SEM.

Protein content of mitofusin 2, DRP1, SCO2 and AIF

The protein content of mitofusin 2 increased after training with no significant difference between groups (main effect of time, $P = 0.047$, Figure 2.6A). The protein content of DRP1 showed a trend to increase following training with no difference between groups (main effect of time, $P = 0.063$, Figure 2.6B). The protein content of SCO2 and AIF did not change with training (both $P > 0.05$, Figure 2.6C and 6D respectively).

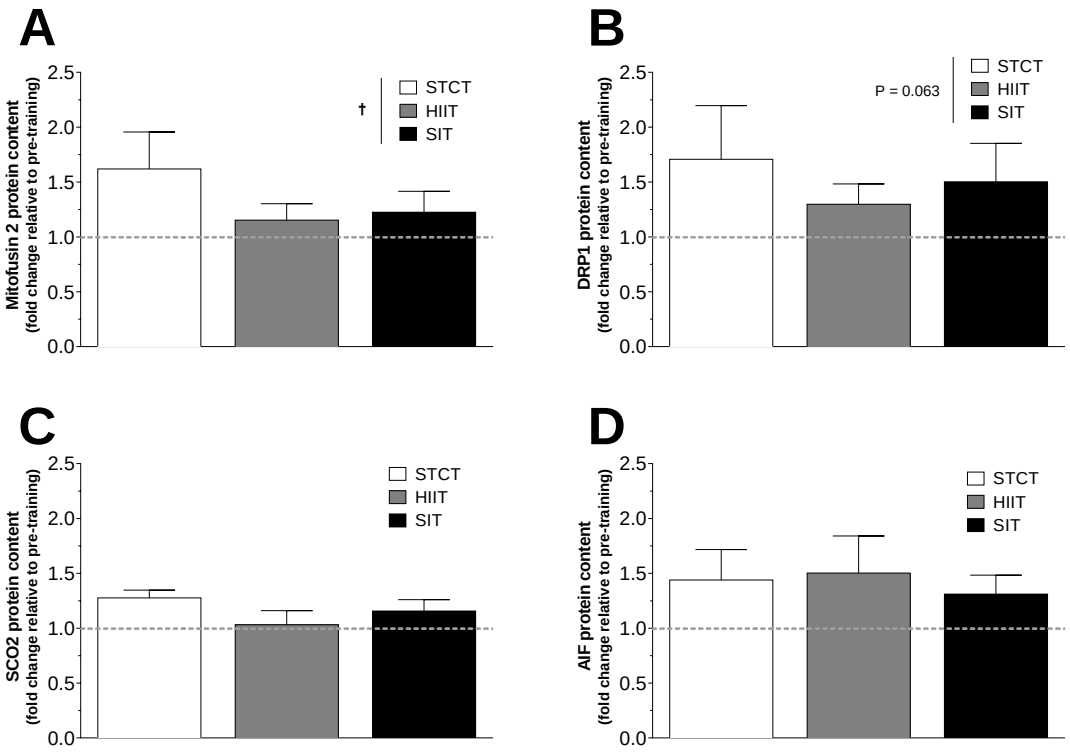

Figure 2.6. Fold change compared to baseline of the protein content of: Mitofusin 2 (A), dynamin related protein 1 (DRP1) (B), synthesis of cytochrome *c* oxidase (COX) 2 (SCO2) (C) and apoptosis inducing factor (AIF) (D) in whole muscle lysates prepared from skeletal muscle biopsy samples (vastus lateralis). Biopsies were obtained at rest at baseline (dotted line) and after 4 weeks of training at each of the three training intensities: sub-lactate threshold continuous training (STCT), high-intensity interval training (HIIT) and sprint interval training (SIT). † Significant main effect of time ($P < 0.05$); a P value next to a legend indicates a trend for a main effect of time. All values are mean ± SEM.

Correlation between mass-specific mitochondrial respiration and performance measurements

There was a significant linear correlation between $CI+II_P$ and 20k-TT performance before training (r = -0.65, $P < 0.001$, Figure 2.7A). No correlation was found between changes in $CI+II_P$ and 20k-TT performance with training (r = 0.21, $P = 0.279$), and the initial correlation was no longer significant after training (r = -0.26, $P = 0.188$, Figure 2.7B). Similarly $CI+CII_P$ was significantly correlated with W_{LT} and W_{Peak} before training (r = 0.60 and r = 0.67 respectively, both $P < 0.001$), but there was no correlation after training (r = 0.27 and r = 0.16 respectively, both $P > 0.05$).

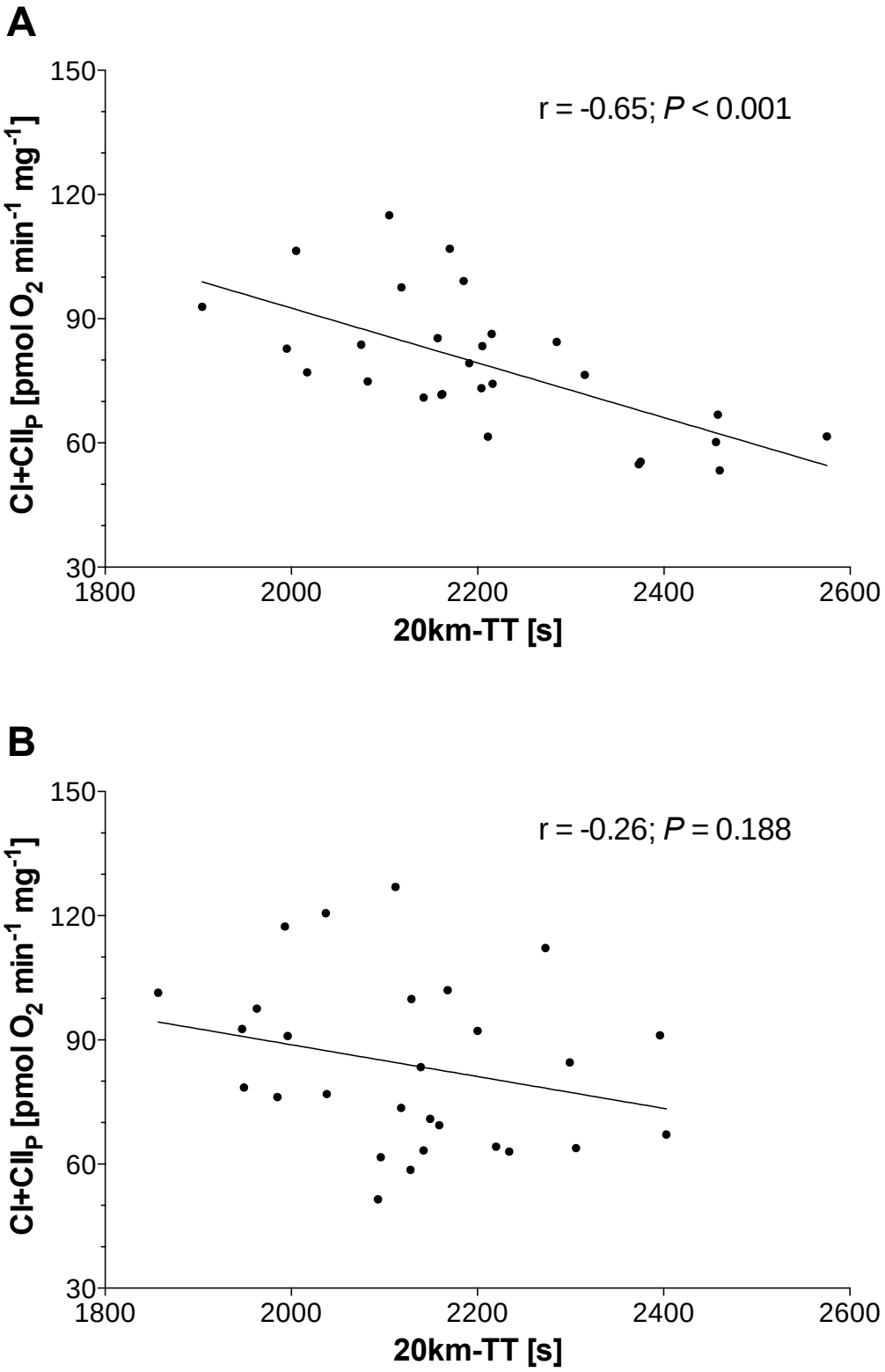

Figure 2.7. Correlation between 20-km cycling time trial (20k-TT) time and maximal ADP-stimulated mass-specific mitochondrial respiration ($CI+II_P$), (A) before and (B) after training. Data include participants from all three groups

2.4 Discussion

The present study shows that four weeks of SIT increased mitochondrial respiration, whilst the same training duration at lower exercise intensities (STCT, HIIT) resulted in no change despite much higher training volumes. None of the interventions increased CS activity. In addition, this is the first study to show that four weeks of SIT increased the protein content of p53; this, along with changes in PGC-1α protein content may help to explain the intensity-dependent changes in mitochondrial respiration. Furthermore, these findings indicate that the protein content of PHF20, a transcription factor upstream of p53, was increased following SIT, providing further mechanistic insights into the cellular events that may mediate the adaptive response to exercise training. Finally, despite being the only training intensity to improve mitochondrial respiration, SIT was the only type of training that did not improve endurance performance.

Mass-specific mitochondrial respiration

A novel aspect of this research was to directly compare whether gains in mitochondrial respiration were related to exercise intensity. The results provided indicate that $CI+II_P$ increased by 25% following four weeks of SIT, whereas neither HIIT nor STCT were associated with an increase in mass-specific respiration. These findings highlight the role of training intensity as a key determinant of changes in mitochondrial respiration. Although caution is required due to various methodological differences and populations examined between studies, it is relevant to compare the present results with previous literature using only one type of training intensity, and that assessed mitochondrial respiration in permeabilised fibres. The training intensities reported below were re-calculated based on unpublished data from this laboratory suggesting that the continuous incremental tests used in those studies yields a value for W_{Peak} that is ~15% higher compared to that obtained with GXT protocol used in the present research. In the studies of Daussin *et al.* (2008) and Jacobs *et al.* (2013) interval training at intensities of ~105-115% of W_{Peak} led to increases in mass-specific respiration of 11-40%, which is in line with the 25% increase reported after SIT. The lower training intensities associated with the HIIT and STCT groups in the present study (89 and 66% of W_{Peak} respectively), did

not improve mass-specific mitochondrial respiration, which agrees with results for the CT group (71% of W_{Peak}) from Daussin *et al.* (2008).

Daussin *et al.* (2008) concluded that workload and oxygen uptake fluctuations, rather than training volume or intensity, were the key determinants of exercise-induced improvements in oxidative capacity. However, the present study provides evidence that, despite workload and oxygen uptake fluctuations being a hallmark of both SIT and HIIT protocols, only SIT, which elicits a much higher training intensity, increased mass-specific mitochondrial respiration in the present study. This further strengthens the notion that training intensity is likely the key factor behind exercise-induced improvements in mitochondrial respiration. The present findings agree with those of Daussin *et al.* (2008) and confirm that training volume is not a key factor in determining changes in mitochondrial respiration. Neither STCT nor HIIT improved mass-specific mitochondrial respiration, despite a training volume almost three-fold larger than that of the SIT group.

While further research is required, possible explanations for the present results relate to differences in fibre recruitment, the activation of oxidative phosphorylation, and increases in common stressors associated with training intensity. For example, studies monitoring glycogen depletion revealed that the number (and type) of fibres activated during an exercise bout increases in response to increased exercise intensity [327]. In addition, studies measuring oxidative enzymes indicate that type II fibres, which are only maximally recruited at greater training intensities, present similar metabolic adaptability to high oxidative demand as type I fibres [49, 131]. It has been shown that SIT elicits full activation and greater reliance on oxidative phosphorylation within each bout and with each subsequent bout [219]. Therefore, it is possible that the higher training intensity associated with the SIT protocol in the current study, led to improved mitochondrial respiration by maximally stressing the oxidative phosphorylation system and by recruiting a wider fibre pool when compared to HIIT and STCT. However, the lack of change in mitochondrial respiration at the lower intensities may depend on the nature of a muscle biopsy. Due the mosaic pattern of human muscle fibres, it is entirely possible that during the biopsy a smaller portion of the muscle fibres obtained may have been recruited at the lower training intensities. This would give the impression that the lower exercise intensity had no effect on mitochondrial parameters, when in fact, if one

measured the recruited fibres only, the outcome would likely show a positive adaptation. This would dilute the magnitude of the effect observed, and it is the result of the limitation that a mixed biopsy may not reveal the maximal adaptation that has taken place in regularly recruited fibres. Finally, an intensity-dependent increase in common stressors associated with exercise (e.g., the AMP:ATP ratio, calcium release, the redox state, and reactive oxygen species (ROS) production) may affect upstream signalling and modulate the response to different training intensities. Future research investigating changes in single fibres, and upstream signalling is warranted.

Mitochondrial qualitative and quantitative changes

To separate qualitative and quantitative changes, mt-specific respiration was determined by normalising mass-specific respiration values to CS activity, a biomarker of mitochondrial content [145]. Results from the present study showed that CS activity was not altered by any of the three training interventions. Although many studies have reported increased CS activity following training at various intensities, the literature also provides evidence of unaltered or even decreased CS activity following training [323]. The reasons for these discrepancies are not readily apparent, but may relate to methodological differences in the CS activity assay and the sample preparation employed [323], or could be related to the fitness level of the participants and differences in study design.

As can be seen in Figure 2.3B, changes in mt-specific respiration mirror those for mass-specific respiration. This indicates that SIT resulted in an increased mitochondrial respiration relative to mitochondrial content. Whilst cross-sectional research has reported that differences in the maximal aerobic capacity of individuals of various fitness levels are accompanied by modifications in mt-specific respiration [128], the present study is the first to show that these modifications can be achieved in as little as 4 weeks of SIT in active individuals. In addition, the lack of change in the protein content of subunits from the five ETS complexes (similar to CS activity), provides further confirmation that changes in mitochondrial function can take place before, or independent of, changes in mitochondrial content. This is perhaps not surprising, as the protein content of subunits from the ETS complexes has been shown to poorly correlate to mitochondrial respiration

and is not considered a valid biomarker of OXPHOS capacity [145]. The present research demonstrate that mitochondrial respiration changes following SIT were independent of changes in mitochondrial content, and highlight the dissociation between mitochondrial respiration and content, as previously demonstrated in mice [252]. While further research is required, it is possible that the lack of change in mitochondrial content may be the result of matched changes in protein synthesis and degradation, whereas replacement of damaged proteins with newly synthesised ones may be responsible for the increase in mitochondrial function [173].

To further analyse the origin of these qualitative changes, FCRs were calculated. These ratios provide an internal normalization expressing respiratory control independent of a marker of mitochondrial content, and minimize experimental errors given they are determined from measurements collected within the same experiment [225]. The present findings indicate that FCRs did not change post-training. Specifically, there was no change in the index of uncoupling (LCR), in the limitation of OXPHOS capacity by the phosphorylation system (PCR), and in the degree of coupling between oxidation and phosphorylation (inv-RCR). This is similar to the findings of Jacobs *et al.* (2013). On the contrary, Daussin *et al.* (2008) showed improved coupling between oxidation and phosphorylation (as measured by RCR) following IT; however, this may be due to the use of sedentary individuals, for whom training adaptations are easier to obtain [146, 162]. The current results also show that improvements in mass-specific respiration following SIT were not driven by changes in substrate control, as evidenced by the lack of change in the $CI_P/CI+II_P$ (SCR), the $CII_E/CI+II_E$ ($P > 0.05$, data not shown), and the CIV_{RES} ratio. Moreover, no significant difference between mass-specific respiration changes at the different substrate control states within each training group was found. Therefore, the present findings suggest that increased oxidative capacity following SIT was mediated by an improvement in mitochondrial respiration relative to mitochondrial content, without changes in the coupling and substrate control of the respiratory system. Although evidence of improved mitochondrial respiration following only 4 weeks of SIT was provided, it was not possible to identify the adaptations responsible for these changes. While further research investigating the possible underlying mechanisms is required, this could be the result of metabolite changes within the organelle, alterations in protein import of selective ETS subunits, and/or assembly of the ETS complexes.

Cellular mechanism of mitochondrial adaptations

To provide a mechanistic insight into the cellular events modulating the adaptive response to exercise training, the protein content of key regulators of mitochondrial biogenesis was measured. PGC-1α protein content was significantly increased only following SIT, in agreement with previous research reporting increased PGC-1α protein content in whole muscle fractions of male participants following 3 weeks of SIT [270]. Although separate studies have shown that PGC-1α protein content can be increased following SIT, HIIT or continuous training [35, 224, 270], the present study is the first to directly compare the effects of different training intensities and to report a training-intensity dependent regulation of PGC-1α protein. It has previously been suggested that PGC-1α mRNA is regulated in an intensity-dependent manner following a single bout of exercise [73], but that this intensity-dependent regulation is limited to exercise intensities below peak power [72]. However, findings from the present study indicate that the intensity-dependent regulation of PGC-1α protein content extends to intensities above peak power. The importance of training intensity is strengthened further when considering that despite the much greater training volumes of the STCT and HIIT protocols, these interventions were not associated with an increase in PGC-1α content.

A novel finding of the present study was that increased PGC-1α content was associated with an increase in mitochondrial function, but not mitochondrial content. PGC-1α has been reported to mediate the downregulation of glycogenolytic and glycolytic enzymes following exercise in rats [140]. In addition, PGC-1α has been shown to coordinate the upregulation of autophagy and mitophagy following exercise in mice [320], with studies in humans demonstrating that autophagy constitutes an important physiological response to exercise [198]. Metabolic regulation and modulation of mitochondrial turnover are therefore two potential mechanisms by which increased PGC-1α content may enhance mitochondrial function independent of changes in mitochondrial content.

The tumour suppressor p53 has also been reported to be an important regulator of mitochondrial biogenesis [266]. In the present study, only SIT increased p53 protein content, indicating a possible intensity-dependent regulation similar to that of PGC-1α. These findings are in agreement with previous research showing that a single bout of

high-intensity interval exercise activates p53 protein via phosphorylation in humans [18], and that a single bout of maximally-activated eccentric contractions increase p53 protein content in rats [45]. The increase in p53 protein content, coupled with greater mitochondrial respiration following SIT, is consistent with the role of p53 as a metabolic regulator [329], and its ability to modulate the balance of energy provision favouring a switch from glycolytic to oxidative pathways [181]. The latter study revealed that in mouse liver mitochondria this modulation is mediated downstream of p53 by SCO2, a protein involved in the assembly of the mtDNA-encoded COX II subunit into the COX complex. However, subsequent research failed to provide evidence of p53 control on SCO2 in both mouse [217] and human [19] skeletal muscle. In line with these findings, results from the present study indicate that the protein content of SCO2 does not change following exercise training and adds to literature suggesting that p53 regulates mitochondrial function in human skeletal muscle via modulation of proteins other than SCO2 [217]. Another point of p53 control on mitochondrial function is through transcriptional regulation of AIF [298], a protein assisting in the assembly and stabilization of complex I [319]. The present results indicate that AIF protein content did not change following training, consistent with previous research showing AIF protein content to be unaffected following 14 weeks of continuous endurance training in humans with mtDNA defects [4]. It is possible that the subcellular localization of AIF, whose principal role is the induction of apoptosis, may be a more important factor in the assessment of training-induced adaptations.

p53 has also been linked with mitochondrial dynamics [266], a series of processes regulating mitochondrial morphology by maintaining a tight balance between fusion and fission rates, which is crucial for a healthy mitochondrial pool [341]. Studies in cells have shown that p53 can up-regulate transcription of both the fusion protein mitofusin 2 [335], and the fission protein DRP1 [154]. In a study conducted with humans, seven sessions of HIIT were reported to increase DRP1 but not mitofusin 2 content [224]. In the present study, mitofusin 2 content was increased after training, with DRP1 showing a similar trend (P = 0.063), but there were no differences between groups for either protein. An additional novel finding was that although SIT was associated with a greater increase in p53 content, this was not the case for mitofusin 2 or DRP1.

TFAM is a nuclear-encoded gene that controls mtDNA transcription and maintenance [272]. p53 can interact with TFAM directly or indirectly, and through modulation of its expression can regulate the mitochondrial transcription machinery [217, 267]. The present results indicate that TFAM protein content was unchanged following training; this is consistent with previous findings [224], although increased TFAM protein content has also been reported [21, 161]. The present findings seem consistent with the hypotheses that activation of existing TFAM may promote mitochondrial adaptations [224], and that it is the interaction of p53 with TFAM and mtDNA inside the mitochondria that positively drives mtDNA transcription [267], rather than the protein content of TFAM itself.

PHF20 is a novel transcription factor that binds directly to the p53 promoter and transcriptionally up-regulates [218] and stabilises [65] p53. The present study indicates that, similar to p53 and mass-specific respiration, PHF20 protein content was increased only following SIT. Together, these findings seem to indicate that four weeks of SIT may induce improved mitochondrial function via a mechanism involving the co-ordinated up-regulation of the PHF20-p53 pathway. Future research investigating these hypotheses and confirming the association of the PHF20-p53 pathway to training-induced changes in mitochondrial respiration is warranted.

In conclusion, changes in the protein content of PGC-1α, p53, and PHF20 mirrored those in mitochondrial respiration, and seem to be mediated by training intensity. Nonetheless, despite an increase in PGC-1α, p53 and PHF20 protein content, no significant change in markers of mitochondrial content was observed. Therefore, these results indicate that changes in the content of these transcription factors may be more strongly associated with changes in mitochondrial respiration rather than content. Moreover, the metabolic adaptations (e.g., glycogen sparing, reduced lactic acid accumulation, increased fatty acid oxidation) that follow exercise training in humans [92, 226], have also been shown to precede increases in mitochondrial content. These findings therefore suggest that these transcription factors may regulate mitochondrial function and content following different pathways or kinetics.

Mitochondrial respiration and endurance performance

Factors such as $\dot{V}O_{2Peak}$, W_{LT}, and exercise efficiency, have all been demonstrated to be good predictors of endurance performance [20]. Central to these factors, mitochondrial respiration has been reported to be the strongest determinant of time-trial performance in highly-trained athletes [129]. Consistent with these findings, the present results reveal a significant linear correlation between pre-training $CI+II_P$ and 20k-TT performance across the twenty-nine participants completing this study. However, despite being the only protocol increasing mitochondrial respiration, SIT did not improve 20k-TT performance following training. This discrepancy between changes in mitochondrial respiration and endurance performance is further supported by the lack of correlation between changes in $CI+II_P$ and 20k-TT time, and the subsequent loss of correlation between the same two parameters post-training. This indicates that mitochondrial respiration was not the key mediator of training-induced changes in endurance performance for the active population in this study, which is in line with the concept that muscle oxidative capacity far exceeds maximal oxygen delivery in humans [28].

Conclusions

The present study demonstrates that training intensity is a key factor determining exercise-induced changes in mitochondrial respiration. Evidence was provided that as little as four weeks of SIT were sufficient to induce improvements in mitochondrial quality, such as an increased mt-specific respiration, a finding that could have implications in the treatment of diseases associated with compromised mitochondrial function. Furthermore, a dissociation between changes in mitochondrial content and respiration was shown, suggesting that these changes may be mediated by different molecular mechanisms. From a cellular mechanism standpoint, these results highlight the potential role of PHF20 in the regulation of mitochondrial respiration in humans via a mechanism involving the co-ordinated upregulation of the PHF20/p53 pathway. The present research indicates that PHF20, p53, and PGC-1α are transcription factors that may be involved in the exercise-induced regulation of mitochondrial respiration, as they are all upregulated after SIT, but not STCT or HIIT. Finally, the present findings confirm that mitochondrial respiration is a good determinant of baseline endurance performance.

However, a discordance between changes in these two parameters was observed, suggesting that mitochondria respiration is not the key mediator of training-induced changes in endurance performance in active individuals.

Chapter 3

Effects of training volume on markers of mitochondrial biogenesis

The main findings from Chapter 2 were that training intensity is a key factor regulating training-induced changes in mass-specific mitochondrial respiration, but not mitochondrial content, and that four weeks of SIT were sufficient to induce improvements in mt-specific respiration (an indicator of mitochondrial quality). In parallel, it was observed that changes in the protein content of key transcription factors, such as PGC-1α, p53, and PHF20, were more strongly associated with changes in mass-specific mitochondrial respiration than mitochondrial content. These findings highlight the apparent dissociation between changes in mitochondrial content and mitochondrial respiration, and indicate that these changes may not be mediated by the same molecular mechanisms.

The purpose of this second study was to investigate the role of training volume. The effects of four weeks of normal training volume (NT), followed by three weeks of a very large training volume (INT) and two weeks of reduced training volume (RT), on mitochondrial content, mitochondrial respiration, and the protein content of valid biomarkers of mitochondrial biogenesis, were investigated.

The manuscript for this study is ready for submission:

Granata C, Oliveira RSF, Little JP, Renner K and Bishop DJ (2015). Training-induced mitochondrial adaptations to intensive training are rapidly reversed following a reduction in training volume in human skeletal muscle. *FASEB Journal* (ready for submission).

3.1 Introduction

Skeletal muscle is a remarkably plastic tissue, rapidly responding to different physiological stimuli. As a consequence of this plasticity, the repeated physiological alterations associated with exercise training lead to a host of adaptations, such as greater mitochondrial function and content [26]. However, studies in mouse [252] and findings in human skeletal muscle described in Chapter 2, have also observed an apparent dissociation between training-induced changes in mitochondrial content and function, highlighting the importance of measuring and differentiating these two parameters [128]. Mitochondrial adaptations have repeatedly been associated with improvements in both health [31, 190, 333] and endurance performance [128], whereas mitochondrial dysfunction has been linked with a wide range of metabolic and neurodegenerative diseases [168, 333]. Collectively, these findings highlight the importance of better understanding how mitochondria adapt to the physiological stress of exercise training.

Recently, there has been increased emphasis on the use of high-intensity exercise training to promote mitochondrial adaptations [35, 81]. However, excessive high-intensity training, without adequate recovery, can lead to maladaptation and overtraining syndrome (OTS) [149]. The symptoms of OTS have many similarities with those of chronic fatigue syndrome, which has been associated with reduced muscle oxidative capacity [182] and mitochondrial dysfunction [205]. Studies in overtrained rats have also shown reduced citrate synthase (CS) activity, a valid biomarker of mitochondrial content [145], in the red gastrocnemius [78], and decreased mitochondrial respiration in the myocardium [138]. However, despite the above findings in rats, no study has investigated the effects of the physiological stress associated with a large volume of high-intensity training without adequate recovery on both mitochondrial respiration and content in humans.

If excessive training does result in mitochondrial maladaptation, it is important to know if this is just a transient effect. Studies indicate that, if training intensity is maintained or increased, a reduction in training volume following intense training is accompanied by a series of positive physiological adaptations [202]. For example, the CS activity of highly-trained athletes increased by ~18% after one week of reduced-volume, increased-intensity training [288]. Conversely, following one week of complete detraining CS activity was

reduced by ~13% [288], and mitochondrial respiration measured in muscle homogenates of elite swimmers decreased by 50% [62]. While these findings highlight the rapid and specific adaptability of skeletal muscle to different training stimuli, no study has directly compared the effects of a period of reduced training volume on both mitochondrial respiration in permeabilised muscle fibres and markers of mitochondrial content in parallel.

The underlying molecular mechanisms by which changes in the level of physical activity mediate mitochondrial adaptations are only beginning to be understood. A key player in this process, peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α), is a transcriptional coactivator regulating both mitochondrial content and respiration in muscle cells [352]. PGC-1α mediates mitochondrial biogenesis by coactivation of, amongst others, nuclear respiratory factor 1 (NRF1), which in turns activates mitochondrial transcription factor A (TFAM), providing a link between nuclear and mitochondrial gene expression [352, 325]. In humans, PGC-1α is sensitive to exercise training, as indicated by the increase in PGC-1α protein content following seven sessions of exercise training [224]. Similar to PGC-1α, the tumour suppressor p53 is another key transcription factor that modulates mitochondrial biogenesis [266], and can regulate the mitochondrial transcriptional machinery via interaction with TFAM [217, 267]. Greater p53 protein content following a 4-week training intervention, as described in Chapter 2 demonstrates that p53 is also sensitive to exercise training in humans. p53 can be upregulated [218] and stabilised [65] by plant homeodomain finger-containing protein 20 (PHF20), a transcription factor that is also sensitive to exercise training, as observed in Chapter 2. However, despite the above evidence, no study has investigated how altering the training-induced physiological stress, via manipulation of training volume, affects the protein content of these transcription factors, and their possible association with changes in mitochondrial respiration and content.

Therefore, the aim of this research was to investigate the effects of intensified (INT), followed by reduced (RT) training volume, on mitochondrial adaptations. It was hypothesised that markers of mitochondrial content, mitochondrial respiration, and the content of key proteins involved in the regulation of mitochondrial biogenesis, would be reduced following INT, alongside a decrease in endurance performance, and for these

changes to be reversed following RT. It was also expected that changes in the protein content of these transcription factors would be associated with changes in both mitochondrial respiration and content.

3.2 Methods

Participants and ethics approval

Ten healthy men (20 ± 2 y; 179.7 ± 11.3 cm; 80.6 ± 14.5 kg), who were non-smokers and involved in various individual and team sports, volunteered to take part in this study. Their baseline peak oxygen uptake ($\dot{V}O_{2Peak}$) was 45.1 ± 7.6 mL min^{-1} kg^{-1} (Table 3.1). Participants were informed of the study requirements, benefits and risks involved before giving written informed consent. Approval for the study's procedures, which conformed to the standards set by the latest revision of the Declaration of Helsinki, was granted by the Victoria University Human Research Ethics Committee.

Table 3.1. Participants' physiological and endurance performance measurements before and after each training phase

Measurement	Pre-NT	Post-NT	Post-INT	Post-RT
$\dot{V}O_{2Peak}$ [mL min^{-1} kg^{-1}]	45.1 ± 7.6	46.9 ± 7.6	52.2 ± 7.7$^{*\#}$	48.9 ± 8.0*†
W_{LT} [W]	199.9 ± 28.1	216.5 ± 30.5*	233.5 ± 34.9$^{*\#}$	228.0 ± 33.3*
W_{Peak} [W]	265.8 ± 39.0	294.0 ± 36.1*	313.7 ± 40.2$^{*\#}$	302.6 ± 41.7*
20k-TT time [s]	2235.2 ± 149.2	2136.0 ± 95.4*	2033.2 ± 84.1$^{*\#}$	2047.2 ± 98.8$^{*\#}$

NT: normal training; INT: intensive training; RT: reduced training; $\dot{V}O_{2Peak}$: peak oxygen uptake; W_{LT}: power at the lactate threshold; W_{Peak}: peak power output; 20k-TT: 20-km time trial. * Significantly different ($P < 0.05$) *vs.* pre-NT, $^{\#}$ *vs.* post-NT, † *vs.* post-INT. n=10. All values are mean ± SD.

Study design

The experimental protocol began with familiarization and baseline testing, followed by three consecutive training phases, the normal training (NT), INT and RT phase, lasting 4, 3 and 2 weeks respectively. Each training phase was preceded (and followed) by a testing period to monitor changes in physiological and performance parameters. During each testing phase participants performed a 20-km cycling time trial (20k-TT), a graded exercise test (GXT), and had a resting muscle biopsy. A series of 10-km cycling time trial (10k-TT) were performed during the INT phase to monitor the participants' state of fatigue. The study duration was approximately 13 weeks.

Testing procedures

Participants were required to refrain from any strenuous physical activity for the 48 h preceding each performance test (72 h for the biopsy trial), from alcohol and any exercise for 24 h before testing, and from food and caffeine consumption for the 2 h preceding each test. Each type of test was performed at the same time of the day during the course of the entire study with the aim to avoid variations caused by circadian rhythm changes.

20k-TT and 10k-TT. Cycling time trials were used as an indicator of endurance performance (20k-TT) or to monitor the state of fatigue during INT (10k-TT), and were performed on an electronically-braked cycle ergometer (Velotron, RacerMate, Seattle, WA, USA). Prior to each time trial participants completed a warm-up involving cycling for 4 min at 66% of the power attained at the lactate threshold (W_{LT}) followed by 2 min at W_{LT} and then 2 min of rest. During the time trial participants were only allowed access to cadence and completed distance, and were provided with similar verbal encouragement during each test. Heart rate monitors (Polar Electro, Kempele, Finland) were used in all exercise trials and training sessions to monitor heart rate.

GXT. A discontinuous graded exercise test was performed on an electronically-braked cycle ergometer (Lode Excalibur, v2.0, Groningen, The Netherlands) to determine $\dot{V}O_{2Peak}$, peak power output (W_{Peak}), W_{LT} (using the modified D_{Max} method [25]), and the training intensities for the three training phases. The test consisted of 4-min stages at a constant power output, interspersed with 30 s of rest. The test began at a power output of

60, 90 or 120 W, depending on a participant's fitness level, and was subsequently increased by 30 W every 4 min. Prior to the test, and during the 30-s rest, capillary blood samples were taken from the fingertip for measurement of blood lactate concentration ([La^-]). Participants were instructed to keep a cadence > 60 rpm and were given verbal encouragement throughout the test, during which they were only allowed access to cadence and elapsed time. The test was stopped when a participant reached volitional exhaustion or cadence dropped below 60 rpm. The W_{Peak} was determined as the power of the last completed stage when participants stopped at the end of a stage. If a participant stopped during a stage, W_{Peak} was determined as the power of the last completed stage plus 7.5 W for every completed minute.

Gas Analysis during the GXT: During the GXT, expired air was continuously analysed for O_2 and CO_2 concentrations via a gas analyser (Moxus modular oxygen uptake system, 2010, AEI technologies, Pittsburgh, PA, USA). The gas analysers were calibrated immediately before each test using known gas mixtures (A: 21% O_2, 0% CO_2; B: 16% O_2, 4% CO_2; BOC, Melbourne, AUS). The ventilometer was calibrated using a 3-liter syringe (Hans Rudolph). $\dot{V}O_2$ values were recorded every 15 s and the two highest consecutive 15-s values recorded during the test were averaged and recorded as the participant's $\dot{V}O_{2Peak}$.

Capillary blood sampling. Glass capillary tubes (MultiCap 140 µL, Siemens Healthcare Diagnostics Inc. Deerfield, IL, USA) were used to collect about 50 µL of blood at the various time points during the GXT. Capillary blood [La^-] was determined using a blood-lactate analyser (YSI 2300 STAT Plus Glucose & Lactate Analyser, YSI Inc., Yellow Spring, Ohio, USA). The blood-lactate analyser was regularly calibrated using precision standards and routinely assessed by external quality controls.

Muscle biopsies

All muscle samples were obtained in the morning, at a constant depth of around 2-3 cm, by an experienced practitioner. Resting muscle biopsies (approximately 150-300 mg wet weight) were taken from the vastus lateralis muscle using a biopsy needle with suction at the following four time points: pre-NT, post-NT, post-INT and post-RT. 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. The opposite leg was chosen for each biopsy. Once obtained, muscle samples were processed, cleaned of excess blood, fat and connective tissue and split in two portions. One portion (10-20 mg) was immediately immersed in a 5-mL tube containing ~3 mL of biopsy preserving solution (BIOPS) kept on ice, and then used for in-situ measurements of mitochondrial respiration. The remaining portion was immediately frozen in liquid N_2 and stored at -80°C for subsequent analyses.

Training intervention

All training sessions were performed on an electronically-braked cycle ergometer (Velotron, RacerMate, Seattle, WA, USA), and were preceded by an 8-min warm up (see 20k-TT). Participants performed high-intensity interval training (HIIT), with a work-to-rest ratio of 2:1, at a training intensity set between W_{LT} and W_{Peak} (i.e., within the same training zone) throughout the entire study. This allowed for training volume to be the only parameter manipulated between the three training phases. Training intensity was set relative to W_{LT}, rather than W_{Peak}, as metabolic and cardiac stresses are similar when individuals of differing fitness levels exercise at a percent of the W_{LT}, but can vary significantly when training at a percent of W_{Peak} [15].

NT phase. Participants performed HIIT three times a week for four weeks. Training sessions consisted of four to seven 4-min cycling intervals interspersed with a 2-min recovery period at a power of 60 W. The training intensities were defined as [W_{LT} + x (W_{Peak}-W_{LT})] (with x equivalent to 0.35, 0.50, 0.65 and 0.75 for week one to four respectively). This initial phase was conducted with the aim of conditioning the participants to the rigours of the INT phase.

INT phase. Participants performed HIIT twice a day (morning and afternoon) for 20 consecutive days. Training sessions consisted of either five to twelve repetitions of the 4-min cycling set with working intensities ranging from [W_{LT} + 0.3 (W_{Peak}-W_{LT})] to [W_{LT} + 0.8 (W_{Peak}-W_{LT})], or eight to twenty-two 2-min cycling intervals interspersed with a 1-min recovery period at a power of 60 W, with working intensities ranging from [W_{LT} +

0.5 (W_{Peak}-W_{LT})] to [W_{LT} + 0.8 (W_{Peak}-W_{LT})]. Single session duration throughout this phase increased from 30-35 min to 70-80 min. A 10k-TT was performed prior to, and at regular weekly intervals during this phase (for a total of four 10k-TT tests), to monitor the participants' state of fatigue. An INT-termination criterion, not disclosed to participants, was set so that if a participant's performance on two consecutive 10k-TT decreased by more than 15% compared to the initial test the participant would be declared overreached and, following completion of the post-INT testing, would begin the RT phase. No participant met this INT-termination criterion, and all completed the 20 consecutive days of intensive training.

RT phase. The RT phase consisted of a total of 5 sessions spread over 14 days. Participants took part in HIIT sessions consisting of either four repetitions of the 4-min cycling intervals at a working intensity set to [W_{LT} + 0.7 (W_{Peak}-W_{LT})], or five to one repetitions of the 2-min cycling interval set, with working intensities ranging from [W_{LT} + 0.8 (W_{Peak}-W_{LT})] to [W_{LT} + 0.9 (W_{Peak}-W_{LT})].

Physical activity and nutritional control

Participants were instructed to maintain a normal dietary pattern and to keep their routine physical activity at a constant level throughout the entire study. For this reason, participants were required to fill in a food and activity diary that was regularly monitored. To minimize variability in muscle metabolism attributable to diet, participants were provided with a standardised dinner (55 kJ kg-1 body mass (BM), providing 2.1 g carbohydrate (CHO) kg-1 BM, 0.3 g fat kg-1 BM, and 0.6 g protein kg-1 BM) and breakfast (41 kJ kg-1 BM, providing 1.8 g carbohydrate kg-1 BM, 0.2 g fat kg-1 BM, and 0.3 g protein kg-1 BM) prior to the four biopsy trials. These meals were consumed 15 and 3 h prior to the biopsy trial respectively. Participants were also required to fill in a nutrition diary recording the last 3 meals prior to each performance test undertaken during baseline testing and they were asked to replicate the same nutritional intake thereafter prior to the same type of test. During the INT phase, participants were asked to fill in a food diary, which was collected and examined daily so that CHO intake was individually monitored to prevent overtraining due to low body carbohydrate stores [100]. Following previous research [100], all participants were administered 1-2 g kg^{-1} body mass of Poly-

Joule (Nutricia Australia Pty Ltd, Macquarie Park, NSW, AUS), a CHO powder dissolved in water, during each training session, and a further 1-2 g kg^{-1} body mass in the first hour after each training session for the entire duration of the INT phase. Hence, in an effort to ensure optimum levels of muscle glycogen were maintained throughout this phase, a total of approximately 4-6 g kg^{-1} body mass of CHO was provided each day with the aim to reach a total daily CHO intake of 10-12 g kg^{-1} body mass.

Muscle analyses

Preparation of whole-muscle lysates. Approximately 10-20 mg of frozen muscle was homogenised 4 times for 5 seconds with a hand-held mini-homogenizer (Kontes Pellet Pestle Cordless Motor, Kimble Chase, NJ, USA.) on ice, in an ice-cold lysis buffer (1:20 w/v) containing 50 mM Tris, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 1 μg/mL aprotinin, 1 μg/mL leupeptin, 1 mM benzamidine, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 5 mM sodium pyrophosphate, 1 mM DTT, 1 mM phenylmethanesulfonyl fluoride (PMSF), adjusted to pH 7.4. Muscle homogenates were rotated end over end at 4 °C for 60 min, after which they were centrifuged for two cycles of 10 min at 15 000 g at 4 °C separated by a similar re-homogenization step to the initial one. After the second centrifugation the supernatant was taken as the whole-muscle lysates for Western Blotting and enzyme activity assay. 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.

Western Blotting. For each protein of interest a linearity study was conducted to determine the ideal loading amount (data not shown). Muscle lysates were then loaded in equal amounts (10-25 μg according to target protein) and separated by electrophoresis for 1.5-2.5 h at 100 V using self-cast SDS-PAGE gels (8-12%). Once resolved, the gels were then wet transferred onto PVDF membranes at 100 V for 70-90 min. Transfer efficiency was checked by Coomassie staining the gels together with Ponceau S staining of the membranes. 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). Immunoblotting was carried out using the following antibodies: apoptosis-inducing factor (AIF) (Cell Signaling Tech (CST), 5318), dynamin-related protein 1 (DRP1) (CST, 5391), mitofusin 2 (CST, 9482), NRF1 (Abcam, ab34682), p53 (CST, 2527), PGC-1α (Calbiochem, st-1202), PHF20 (CST, 3934), synthesis of cytochrome *c* oxidase (COX) 2 (SCO2) (Santa Cruz, sc-49110), TFAM (Abcam, ab47517), and Total OXPHOS (MitoSciences, ab110411). 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 90 min in 1-5% skim milk in TBS-T. Membranes were then washed again for 3 x 5-min in TBS-T followed by a final 5-min wash in TBS before being immersed for 5 min under gentle agitation at room temperature in a chemiluminescent solution (Solution A: 2.5 mM Luminol, 0.36 mM p-Coumaric Acid, 0.1 M Tris (pH 8.5) in 10 mL MilliQ H_2O; Solution B: 6.1 µL H_2O_2, 0.1 M Tris (pH 8.5) in 10 mL MilliQ H_2O; solution A and B prepared separately and mixed just prior to use). Protein bands were visualized 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). Coomassie Blue staining was performed at the end of the procedure and was used to verify correct loading and equal transfer between lanes [338]. Finally, samples for all four time points for each participant were loaded on the same gel together with an identical amount of internal standard (IS). Comparison and statistical analysis between each participant's time points were done using the raw density data normalised to the IS loaded in each gel to reduce gel-to-gel variability. For graphical purposes, each time point was normalised to baseline; for this reason WB graphs throughout this manuscript are presented as fold change compared to baseline, and no error bar is presented for the baseline time point. A representative blot for each protein analysed is presented in Figure 3.1.

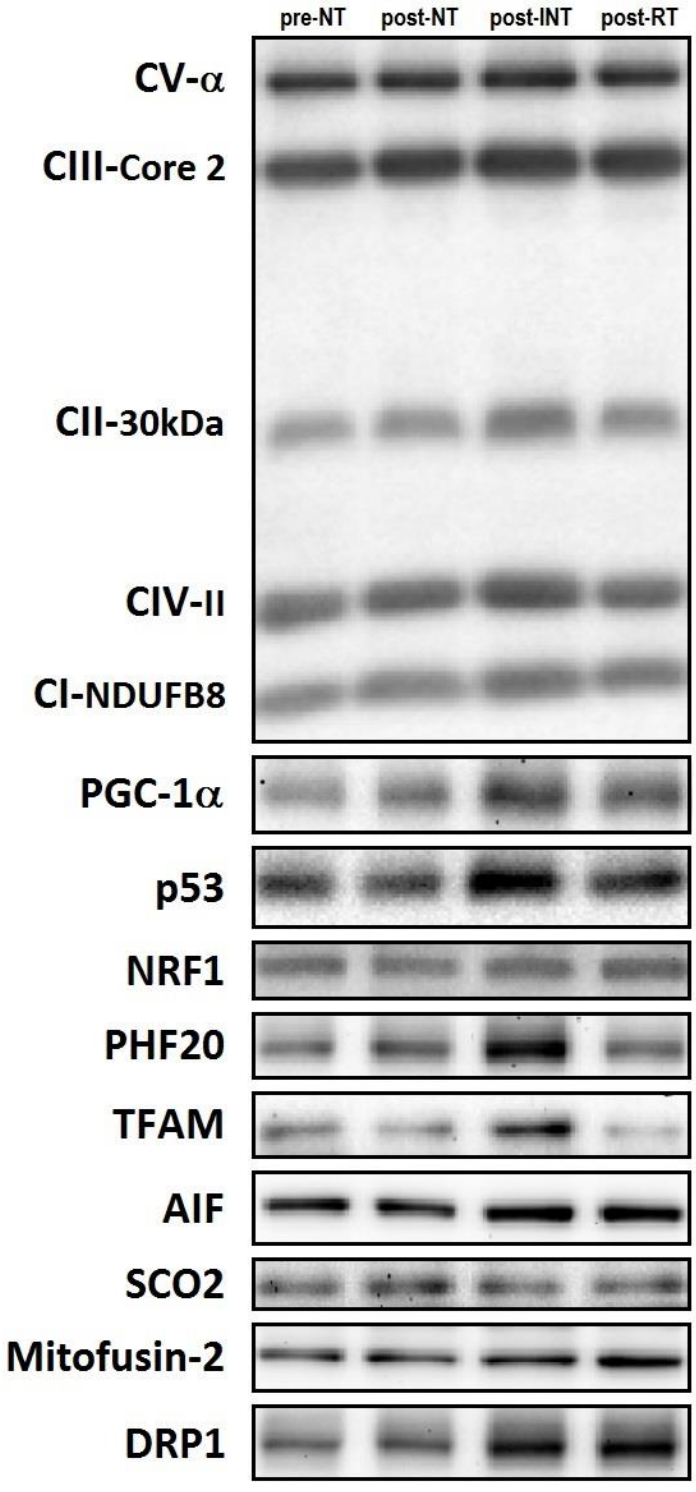

Figure 3.1. Representative immunoblots of subunits from the five complexes of the electron transport system (ETS), transcription factors, and downstream targets of p53 measured in whole-muscle lysates prepared from skeletal muscle biopsy samples (vastus lateralis). Biopsies were obtained at rest at baseline (pre-NT), and following the normal (post-NT), intensive (post-INT), and reduced (post-RT) training phase.

Citrate Synthase Activity Assay. CS activity was determined in triplicate on a microtiter plate by adding: 5 µL of a 2 mg/mL muscle homogenate, 40 µL of 3mM acetyl CoA in Tris buffer and 25 µL of 1mM 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 kg of protein^{-1} h^{-1}.

Preparation of permeabilised skeletal muscle fibres for mitochondrial respiration. A 10-20 mg fresh muscle sample was placed in ice cold BIOPS, a biopsy preserving solution containing (in mM) 2.77 CaK_2EGTA, 7.23 K_2EGTA, 5.77 Na_2ATP, 6.56 $MgCl_2$, 20 taurine, 50 Mes, 15 Na_2phosphocreatine, 20 imidazole and 0.5 DTT adjusted to pH 7.1 [225]. Samples were transferred to a 6-well plate kept on ice where the fibres were mechanically separated using pointed forceps. Fibres were subsequently permeabilised by gentle agitation for 30 min at 4°C in BIOPS containing 50 µg/mL of saponin. Samples were then washed 3 times for 7 min at 4°C by gentle agitation in MiR05, a respiration medium containing (in mM, unless specified) 0.5 EGTA, 3 $MgCl_2$, 60 K-lactobionate, 20 taurine, 10 KH_2PO_4, 20 Hepes, 110 sucrose and 1 g/l BSA essentially fatty acid-free adjusted to pH 7.1 at 37°C [225]. This method selectively permeabilises the cellular membrane leaving the mitochondria intact and allows for in-situ measurements of mitochondrial respiration.

High-Resolution Respirometry. After washing, 3-4 mg wet weight of muscle fibres were assayed in duplicate 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/mL) and oxygen flux (pmol s^{-1} 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/mL, so as to avoid potential oxygen diffusion limitation.

Mitochondrial respiration protocol. A substrate-uncoupler-inhibitor titration (SUIT) protocol was used and the SUIT sequence, with final chamber concentration in brackets, was as follows: pyruvate (2 mM) and malate (5 mM) in the absence of adenylates were added for measurement of LEAK respiration (L) with electron entry through Complex I (CI) (CI_L). ADP (5 mM) was then added for measurement of maximal oxidative phosphorylation (OXPHOS) capacity (P) with electron input through CI (CI_P), followed by addition of succinate (10 mM) for measurement of P with simultaneous electron supply through CI + Complex II (CII) combined ($CI+II_P$). This respiration state provides convergent electron input to the Q-junction through CI (NADH provided by malate/pyruvate) and CII (flavin adenine dinucleotide reduced ($FADH_2$) provided by succinate) and supports maximal mitochondrial respiration by reconstruction of the tricarboxylic acid cycle function. Cytochrome *c* (10 μM) was then added to test for outer mitochondrial membrane integrity; an exclusion criterion was set such that if a chamber showed an increase in O_2 flux > 6% after addition of cytochrome *c*, it was discarded. This was followed by a series of stepwise carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone titrations (FCCP, 0.75-1.5 μM) for measurement of electron transport system (ETS) capacity (E) with convergent electron input through CI+II ($CI+II_E$). Rotenone (0.5 μM), an inhibitor of CI, was then added to obtain a measurement of E with electron input through CII (CII_E). This was followed by addition of antimycin A (2.5 μM), an inhibitor of Complex III (CIII), to obtain a measurement of residual oxygen consumption capacity (ROX). ROX was subtracted from all other measurements to account for oxidative side reactions. Ascorbate (2 mM) and N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD, 0.5 mM), artificial electron donors for Complex IV (CIV), followed by sodium azide (> 200 mM), an inhibitor of CIV to account for auto oxidation of ascorbate + TMPD, were then added to measure CIV respiration in the noncoupled state (CIV_E). Respiratory flux control ratios (FCR) were calculated. In brief, the leak control ratio (LCR) is the quotient of CI_L over $CI+II_E$; the phosphorylation control ratio (PCR) is the quotient of $CI+II_P$ over $CI+II_E$; the coupling control ratio is the quotient of CI_L over $CI+II_P$ and is equivalent to the inverse respiratory control ratio (inv-RCR); and

the substrate control ratio (SCR) at constant P is the quotient of CI_P over $CI+II_P$. Finally the reserve capacity of CIV (CIV_{RES}) was determined as the quotient of $CI+II_P$ over CIV_E.

Statistical analysis

All values are reported as means ± SD, unless specified otherwise. One-way ANOVA with repeated measures for time were used to test for main effects. Significant main effects were further analysed using a Tukey's honestly significant difference post hoc test. SigmaStat software (Jandel Scientific, San Rafael, CA) was used for all statistical analyses. The level of statistical significance was set at $P < 0.05$.

3.3 Results

Training and performance measurements

Participants completed a significantly higher training volume during the INT phase (14.7 ± 3.7 MJ), when compared to both the NT and RT phase (3.6 ± 0.6 and 0.6 ± 0.1 MJ respectively, both $P < 0.001$, Figure 3.2). The training volume of the RT phase was also significantly lower when compared to that of the NT phase ($P = 0.015$). There were no changes in body mass throughout the study (data not shown). Performance results ($\dot{V}O_{2Peak}$, W_{LT}, W_{Peak}, 20k-TT) are shown in Table 3.1. When compared to baseline, all performance parameters improved following INT (9 to 19%, all $P < 0.001$), and remained elevated at the end of the study (8 to 15%, all $P < 0.05$).

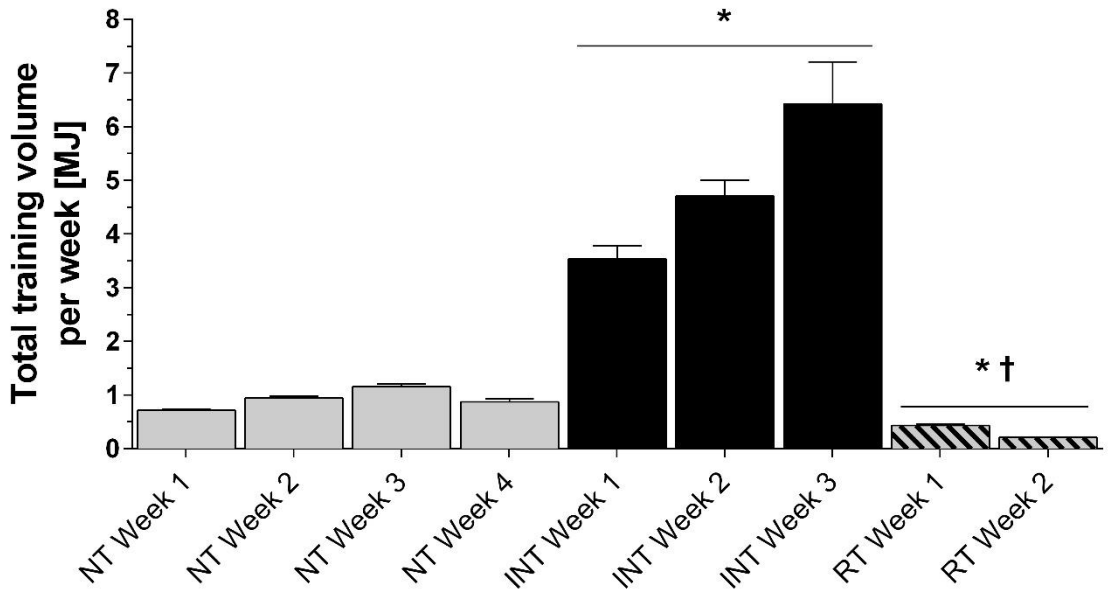

Figure 3.2. Total training volume per week for the entire study. MJ: megajoule; NT: normal training; INT: intensive training; RT: reduced training. * Significantly different ($P < 0.05$) *vs.* pre-NT, † *vs.* post-INT. n=10. All values are mean ± SEM.

Muscle analyses

Following NT, there were no significant changes in any of the measurements from the muscle analyses (Figure 3.3 to Figure 3.7).

Citrate Synthase activity. Results are shown in Figure 3.3. Following INT, CS activity increased by 49.9 ± 49.0% ($P = 0.002$) when compared to baseline (and 38.1 ± 32.4% when compared to post-NT, both $P = 0.007$), and did not change significantly following RT (-5.3 ± 28.2%, $P = 0.645$), where it was still elevated when compared to baseline (36.2 ± 47.0%, $P = 0.040$).

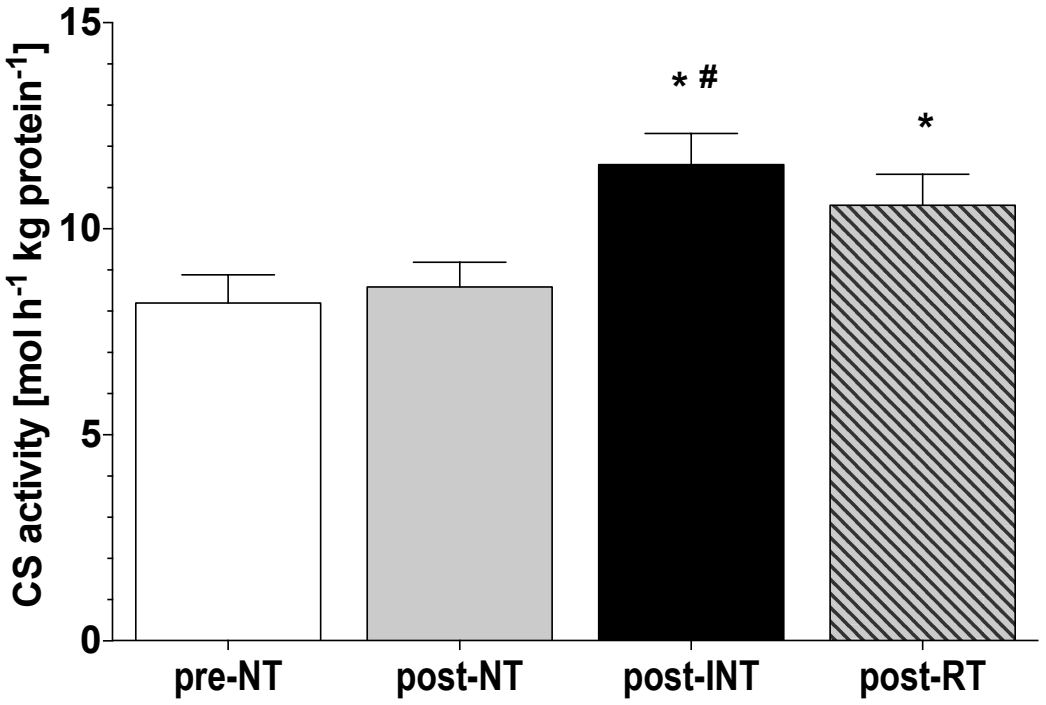

Figure 3.3. Citrate synthase activity expressed per kg of protein. CS activity was measured in whole-muscle lysates prepared from muscle biopsy samples (vastus lateralis) obtained at rest at baseline (pre-NT), and following the normal (post-NT), intensive (post-INT), and reduced (post-RT) training phase. * Significantly different ($P < 0.05$) *vs.* pre-NT, $^{\#}$ *vs.* post-NT. n=10. All values are mean ± SEM.

Protein content of subunits of the five ETS complexes. Results are shown in Figure 3.4. Following INT, the protein content of all subunits increased when compared to baseline (9 to 41%, all $P < 0.05$). With the exception of the CIII subunit (6%, $P = 0.138$), the protein content of all subunits also increased as an effect of the INT phase alone (9 to 31%, all $P < 0.05$). During the RT phase, the protein content of the subunits of CI, CII and CIV was reduced (10 to 20%, all $P < 0.05$), while that of CIII and CV did not change significantly (-3 to -5%, both $P > 0.05$). By study end however, the protein content of all subunits was not significantly different when compared to baseline (4 to 13%, all $P > 0.05$).

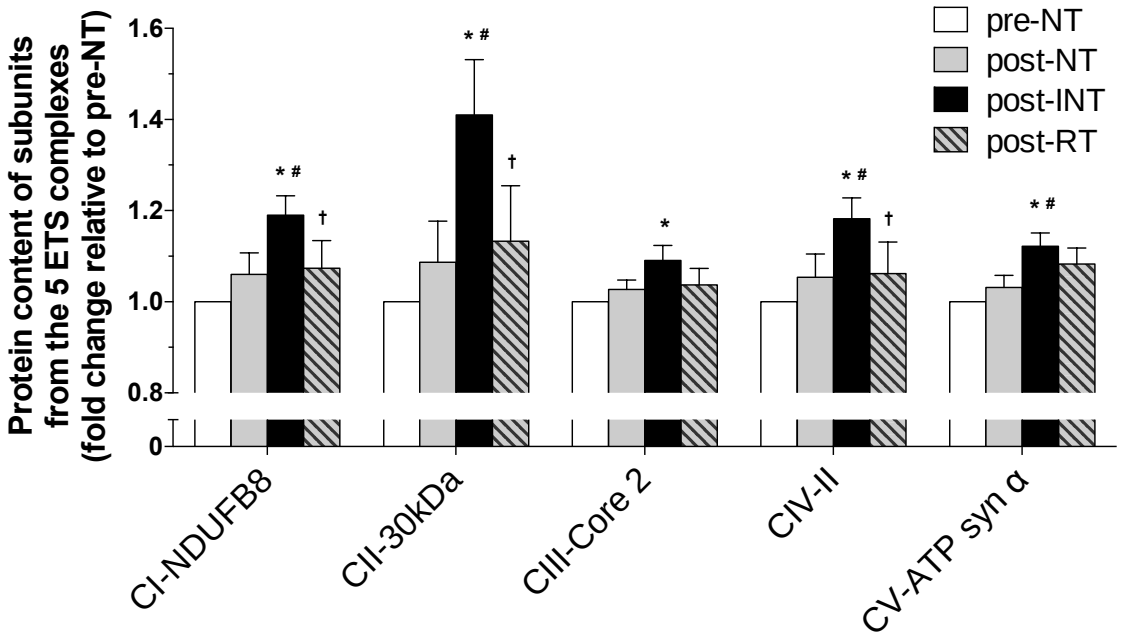

Figure 3.4. Fold change compared to baseline of the protein content of subunits from the five complexes of the electron transport system (ETS), complex I to V (CI to CV) in whole-muscle lysates prepared from skeletal muscle biopsy (vastus lateralis). Biopsies were obtained at rest at baseline (pre-NT), and following the normal (post-NT), intensive (post-INT), and reduced (post-RT) training phase. *Significantly different ($P < 0.05$) *vs.* pre-NT, $^{\#}$ *vs.* post-NT, † *vs.* post-INT. n=10. All values are mean ± SEM.

Mass-specific mitochondrial respiration (expressed as pmol O_2 s^{-1} mg^{-1}). Results are shown in Figure 3.5A. There were no significant changes in CI_L throughout the study (all $P > 0.05$). Following INT, CI_P increased by 43.7 ± 26.9% when compared to baseline (and 47.0 ± 18.3% when compared to post-NT, both $P < 0.001$), and decreased by 14.1 ± 12.7% following RT ($P = 0.019$); however, the post-RT value was still elevated by 23.7 ± 31.6% when compared to baseline ($P = 0.034$). Following INT, $CI+II_P$ increased by 41.0 ± 23.5% when compared to baseline (and 48.5 ± 20.0% when compared to post-NT, both $P < 0.001$), and decreased by 17.0 ± 12.7% following RT ($P = 0.003$), where it was not significantly different from baseline ($P = 0.131$). Following INT, $CI+II_E$ increased by 41.3 ± 22.9% when compared to baseline (and 39.6 ± 16.4% when compared to post-NT, both $P = 0.002$), and decreased by 25.0 ± 11.1% following RT ($P = 0.003$), where it was not significantly different from baseline ($P = 0.980$). Following INT, CII_E increased by 34.5 ± 25.1% ($P = 0.002$) when compared to baseline (and 36.8 ± 23.4% when compared

to post-NT, $P < 0.001$), and decreased by 20.9 ± 15.1% following RT ($P = 0.005$), where it was not significantly different when compared to baseline ($P = 0.970$). Following INT, CIV_E increased by 21.5 ± 24.4% when compared to baseline ($P = 0.049$), whereas a 20.5 ± 26.4% increase from post-NT failed to reach statistical significance ($P = 0.058$). CIV_E did not change significantly following RT (-12.8 ± 18.0%, $P = 0.100$), where it was not significantly different when compared to baseline ($P = 0.986$). The data presented in this study showed no effect of the addition of cytochrome *c* as a control for outer mitochondrial membrane integrity. FCRs changes are shown in Table 3.2. Following INT, CIV_{RES} increased by 19.3 ± 24.4% ($P = 0.028$), when compared to baseline (and 26.6 ± 20.7% when compared to post-NT, $P = 0.003$), and did not change significantly following RT (-3.1 ± 13.4%, $P = 0.848$), where it was not significantly different from baseline (both $P = 0.155$). There were no significant changes in all the other FCRs.

Mitochondrial (mt)-specific respiration (mass-specific mitochondrial respiration normalised by CS activity, expressed as: pmol O_2 s^{-1} CS^{-1}). When normalised to CS activity, mitochondrial respiration did not change throughout the study for any of the six measurements of the SUIT protocol as shown in Figure 3.5B (all $P > 0.05$).

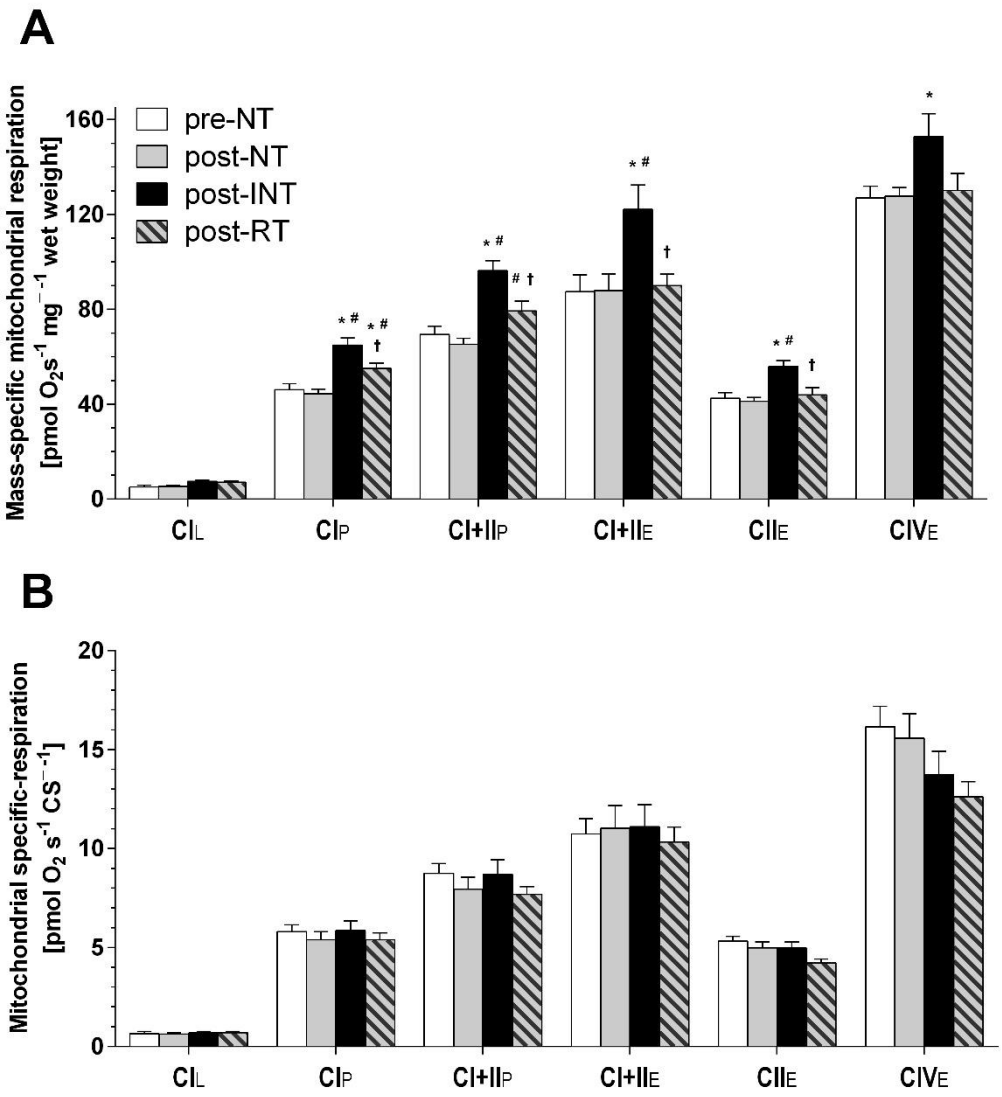

Figure 3.5. Mitochondrial respiration measurements at different coupling control states. A: mass-specific mitochondrial respiration, and B: mitochondrial-specific respiration (obtained by normalising mass-specific mitochondrial respiration by CS activity expressed per kg of protein). Respiration was measured in permeabilised fibres prepared from muscle biopsy samples (vastus lateralis) obtained at rest at baseline (pre-NT), and following the normal (post-NT), intensive (post-INT), and reduced (post-RT) training phase. Respiration values are: CI_L: Leak respiration state (L) in the absence of adenylates and limitation of flux by electron input through Complex I (CI); CI_P: maximal oxidative phosphorylation state (P) with saturating levels of ADP and limitation of flux by electron input through CI; $CI+II_P$: P with saturating levels of ADP and limitation of flux by convergent electron input through CI + Complex II (CII); $CI+II_E$: maximal electron transport system (ETS) capacity (E) with saturating levels of ADP and limitation of flux by convergent electron input through CI+II; CII_E: E with saturating levels of ADP and limitation of flux by electron input through CII; CIV_E: maximal Complex IV noncoupled respiration with saturating levels of ADP and limitation of flux by electron input through CIV. *Significantly different ($P < 0.05$) *vs.* pre-NT, $^{\#}$ *vs.* post-NT, † *vs.* post-INT. n=10. All values are mean ± SEM.

Table 3.2. Respiratory flux control ratios measurements before and after each training phase

FCR	Pre-NT	Post-NT	Post-INT	Post-RT
LCR (L/E)	0.04 ± 0.02	0.07 ± 0.04	0.06 ± 0.02	0.08 ± 0.01
PCR (P/E)	0.81 ± 0.07	0.74 ± 0.03	0.77 ± 0.04	0.81 ± 0.03
Inv-RCR (L/P)	0.07 ± 0.04	0.09 ± 0.04	0.08 ± 0.02	0.09 ± 0.02
SCR (Constant P)	0.66 ± 0.05	0.68 ± 0.04	0.67 ± 0.03	0.70 ± 0.05
CIV_{RES}	0.55 ± 0.05	0.51 ± 0.08	0.64 ± 0.09$^{*\#}$	0.62 ± 0.08$^{\#}$

FCR: respiratory flux control ratio; NT: normal training; INT: intensive training; RT: reduced training. L: leak respiration; P: oxidative phosphorylation capacity; E: electron transport system capacity; LCR: leak control ratio ($CI_L/CI+II_E$); PCR: phosphorylation control ratio ($CI+II_P/CI+II_E$); inv-RCR: inverse of respiratory control ratio ($CI_L/CI+II_P$); SCR: substrate control ratio at constant P ($CI_P/CI+II_P$); CIV_{RES}: CIV reserve capacity ($CI+II_P/CIV_E$). CI: electron input through complex I; CI+II: convergent electron input through complex I and II; CIV: electron input through complex IV.FCR were calculated from mass-specific mitochondrial respiration measurements in permeabilised muscle fibres (vastus lateralis), obtained from muscle biopsies taken at baseline and after each training phase. *Significantly different ($P < 0.05$) *vs.* pre-NT, $^{\#}$ *vs.* post-NT. n=10. All values are mean ± SD.

Protein content of transcription factors. Following INT: the protein content of PGC-1α increased by 73 ± 87% when compared to baseline ($P = 0.026$, Figure 3.6A); PHF20 protein content was increased by 96 ± 146% ($P = 0.022$) when compared to baseline (and 93 ± 109% when compared to post-NT, $P = 0.011$) (Figure 3.6B); the protein content of NRF1 was increased by 63 ± 58% when compared to baseline ($P < 0.001$, Figure 3.6C); the protein content of p53 increased by 168 ± 234% when compared to baseline ($P = 0.002$, Figure 3.6D); TFAM protein content was increased by 76 ± 69% when compared to baseline ($P = 0.047$, Figure 3.6E). During RT, there was a decrease in the protein content of NRF1 (19 ± 25%, $P = 0.045$, Figure 3.6C), and TFAM (49 ± 30%, $P = 0.005$, Figure 3.6E). Following RT, the protein content of all the transcription factors was not significantly different when compared to baseline (all $P > 0.05$, Figure 3.6A to E).

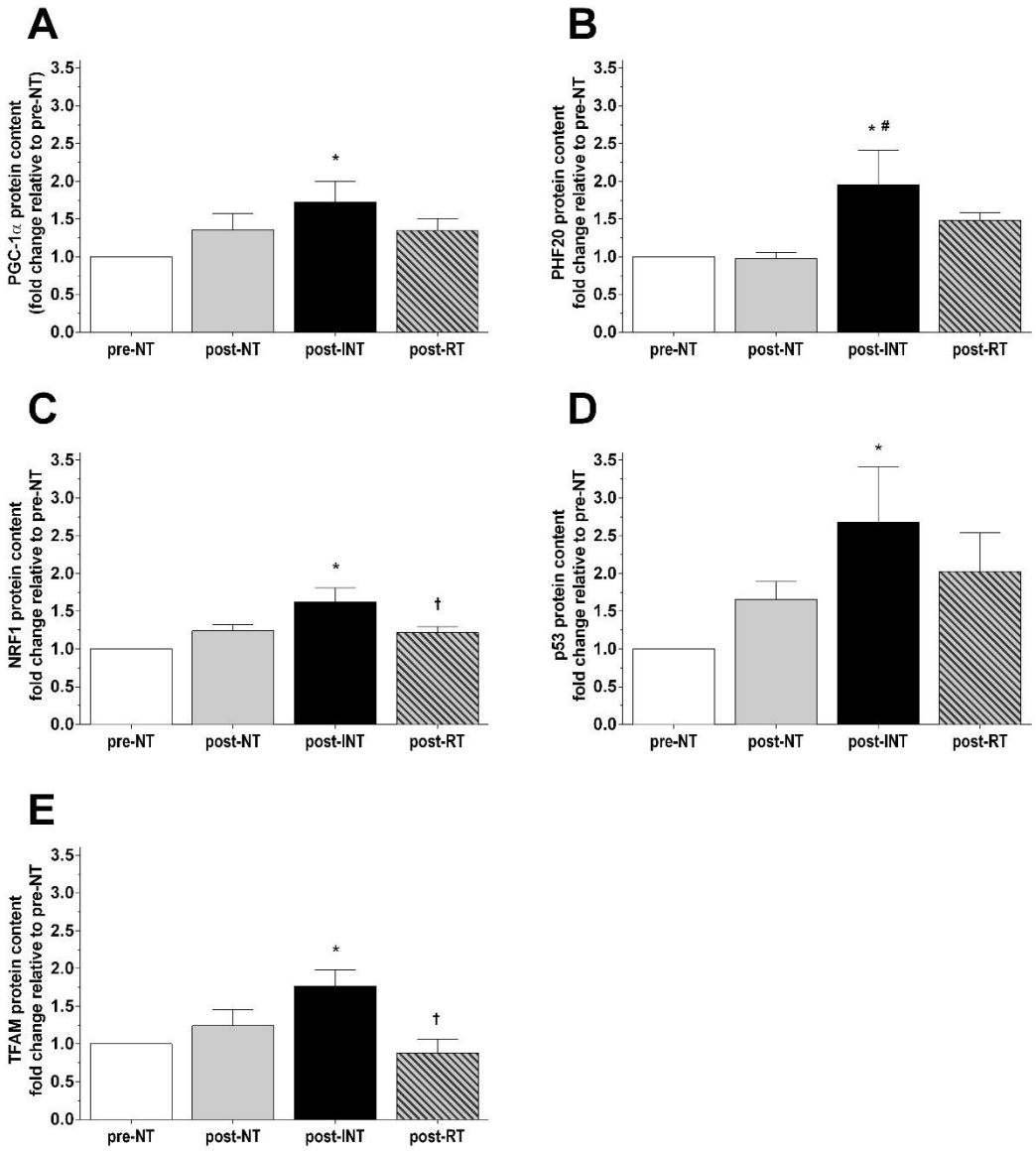

Figure 3.6. Fold change compared to baseline of the protein content of (A) PGC-1α, (B) PHF20, (C) NRF1, (D) p53, and (E) TFAM in whole-muscle lysates prepared from skeletal muscle biopsy samples (vastus lateralis). Biopsies were obtained at rest at baseline (pre-NT), and following the normal (post-NT), intensive (post-INT), and reduced (post-RT) training phase. *Significantly different ($P < 0.05$) *vs.* pre-NT, $^{\#}$ *vs.* post-NT, † *vs.* post-INT. n=10. All values are mean ± SEM.

Protein content of AIF, SCO2, mitofusin 2, and DRP1. The protein content of AIF, SCO2, mitofusin 2, and DRP1 did not change significantly throughout the study (all $P > 0.05$, Figure 3.7A to D).

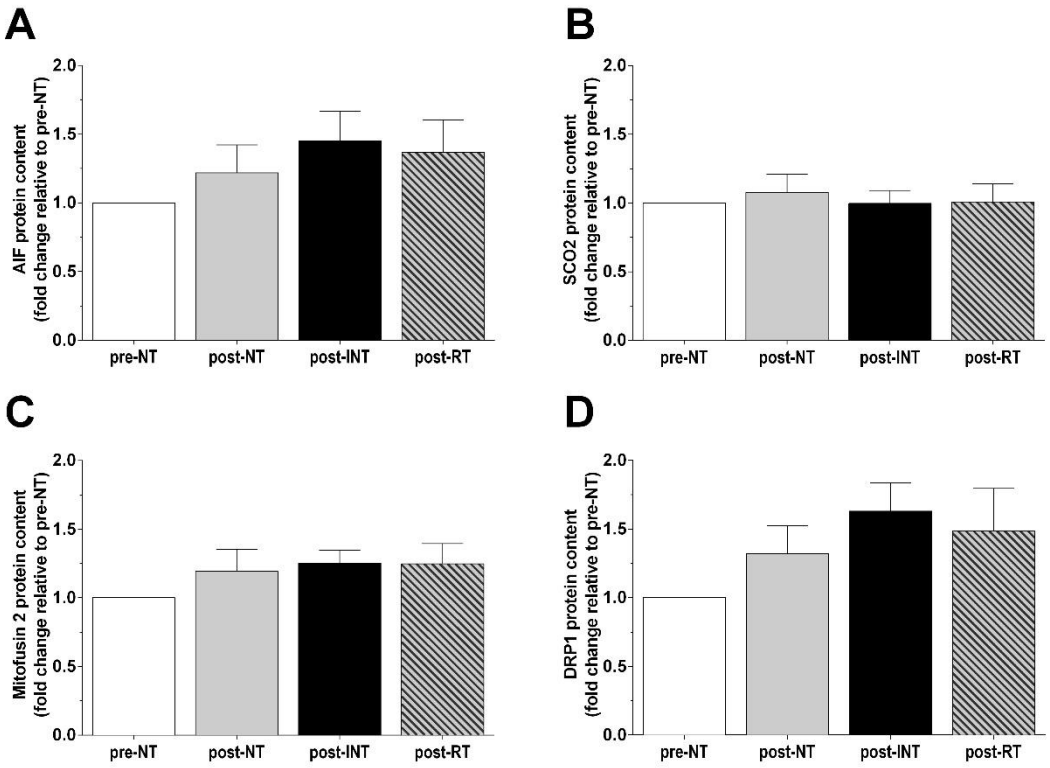

Figure 3.7. Fold change compared to baseline of the protein content of (A) AIF, (B) SCO2, (C) mitofusin 2 and (D) DRP1 in whole-muscle lysates prepared from skeletal muscle biopsy samples (vastus lateralis). Biopsies were obtained at rest at baseline (pre-NT), and following the normal (post-NT), intensive (post-INT), and reduced (post-RT) training phase; n=10. All values are mean ± SEM.

3.4 Discussion

In contrast to what hypothesised, three weeks of very high-volume, high-intensity training resulted in improved endurance performance, mitochondrial content and mitochondrial respiration. These improvements were accompanied by an increase in the protein content of key transcription factors regulating mitochondrial biogenesis. Two weeks of reduced training however, were sufficient to revert the majority of the gains obtained during INT, with most of the parameters measured in muscle returning to values that were not significantly different from baseline. These results provide further evidence that training volume is an important determinant of changes in mitochondrial content, and that human skeletal muscle is a remarkably plastic tissue that rapidly adapts to an increase or decrease in training volume.

NT phase

The NT phase resulted in no significant changes in any of the measured muscle parameters, and resulted in a small improvement in W_{LT}, W_{Peak}, and 20k-TT time. Results from the NT phase will not be discussed further in this chapter as these represent a subset of the HIIT group whose results have already been presented as part of Chapter 2.

Performance

Indicators of endurance performance ($\dot{V}O_{2Peak}$, W_{Peak}, W_{LT}, and 20k-TT time) improved during INT (~5 to 12%), where all participants registered peak values for the study. These findings are in contrast with what was hypothesised, and previous research showing a reduction in markers of endurance performance after two weeks of intensified training [99]. These discrepancies may relate to CHO supplementation during the INT phase reducing or delaying the symptoms of overreaching [100]. Furthermore, in the study from Halson *et al.* (2002), the increase in training volume was accompanied by an overall increase in training intensity. However, training intensity was not changed in the present study, allowing to isolate the manipulation of training volume, which had previously been hypothesised as a key contributor to OTS in athletes [118]. Therefore, this research indicates that when CHO levels are kept high, moderately-trained individuals are able to positively adapt to a period of increased training volume (two HIIT sessions per day, each lasting up to 80 min, for 20 consecutive days) at the same training intensity. As a consequence of the positive adaptations that followed INT, the RT phase, which was associated with a heavily reduced training volume, resulted in a very modest and mostly insignificant loss of performance (~1 to 6%). This is consistent with previous research showing that unless training intensity is increased, a subsequent reduction in training volume does not improve endurance performance [288].

Markers of mitochondrial content

The increase in CS activity following the INT phase in the present study is consistent with previous research reporting that exercise training is usually associated with an increase in CS activity [323]. However, the magnitude of the change in CS activity varies

considerably following different training interventions [323], and has been suggested to depend on the training volume employed [26]. The large increase in CS activity in the present study (50%) is consistent with that from previous research employing very large volumes of training [41, 70, 132, 204], and agrees well with the values predicted from the relationship between training volume and training-induced changes in CS activity reported in Bishop *et al.* (2014). This provides further support that training volume is an important determinant of increases in mitochondrial content (as assessed by changes in CS activity).

Following RT, CS activity showed a modest, non-significant decrease (5%). Previous research, investigating various one-week protocols of reduced training volume, has reported that CS activity increased only if training intensity increased during RT [288]. When training intensity remained constant, CS activity showed a modest non-significant decrease [288], consistent with this research. These findings are also in agreement with the modest (~5-10%) non-significant reduction in CS activity reported following a 3-week reduction in training volume in both recreational [167, 316] and competitive distance runners [166]. Studies investigating the effects of complete detraining on mitochondrial enzymes have observed a reduction in CS activity ranging from 10% in 1 week [288] to 45% in as little as 3 weeks [199]. However, at the end of the present study CS activity was still elevated when compared to baseline (36%), suggesting that, despite a dramatic 23-fold reduction, maintenance of a minimal amount of training volume may help to preserve CS activity and to avoid the loss that is associated with complete detraining.

The protein content of the subunits of the complexes of the ETS is also considered a valid biomarker of mitochondrial content, although the strength of this relationship varies for each of the five complexes and is weaker than that for CS activity [145]. Following INT, changes in the protein content of the subunits of the ETS complexes showed a similar pattern to CS activity, and increased by 9-41%. No research to date has investigated changes in the content of mitochondrial proteins following a period of intensified (or reduced) training volume. In contrast to the results for CS activity however, the RT phase induced a 3-20% reduction in the protein content of the subunits of the ETS complexes. Moreover, by study end, the content of all five subunits was not significantly different

from baseline. This suggests that the training volume during the RT phase did not provide a strong enough physiological stimulus to prevent the decrease in mitochondrial proteins that is associated with detraining. It may also indicate that the protein content of the ETS complexes is more sensitive to a reduction in training volume, when compared to CS activity; this observation may relate to the different half-lives of mitochondrial complexes and CS [27, 351]. This research highlights the rapid plasticity and adaptability of human skeletal muscle to different training stimuli, and further supports the hypothesis that training volume is an important determinant of changes in mitochondrial content [26].

Mitochondrial respiration

To assess adaptations in mitochondrial function, mitochondrial respiration in permeabilised muscle fibres was measured. Following INT, all measurements of the SUIT protocol following the addition of ADP showed a greater mass-specific mitochondrial respiration (pmol O_2 s^{-1} mg^{-1}) when compared to both baseline and post-NT values (Figure 3.5A). To determine the origin of these changes, mt-specific respiration was determined by normalising mass-specific mitochondrial respiration by CS activity (pmol O_2 s^{-1} CS^{-1}). As shown in Figure 3.5, mt-specific respiration did not change throughout the study. This suggests that changes in mass-specific mitochondrial respiration in the present study were mainly the result of changes in mitochondrial content, consistent with previous research employing HIIT [127, 324]. However, when training intensity is maximal ("all-out" sprint interval training), mass-specific mitochondrial respiration has been shown to improve despite no change in mitochondrial content, but as an effect of improved mitochondrial quality (i.e., greater mt-specific respiration, as described in Chapter 2). Taken together, these findings seem to indicate that mitochondrial respiration can be improved by either increasing training intensity, which leads to greater mt-specific respiration (as described in Chapter 2), and/or by increasing training volume, which leads to greater mitochondrial content with no apparent changes in mt-specific respiration. Finally, findings from Chapter 2 have shown that training at an intensity between W_{LT} and W_{Peak} was not sufficient to increase mitochondrial respiration. However, the results of the present study indicate that, when

training volume is markedly increased, training at intensity between W_{LT} and W_{Peak} is sufficient to enhance mitochondrial respiration (via increased mitochondrial content).

No study to date has investigated the influence of intensified training on changes in mitochondrial respiration. However, these results demonstrate a similar increase in CI_P to that of the interval training group in Daussin *et al.* (2008) (~40 *vs.* 47% respectively), even though the training volume was ~30% of that employed during INT in the present study. Similarly, increases of 11% and 21% in $CI+II_P$, although smaller when compared to the increase associated with the INT phase in this study (49%), were obtained with training volumes equivalent to only ~5% [127] and ~10% [324] respectively, of that employed during INT. Thus, although minor differences in training intensity, methodology, and participant's fitness may have influenced the outcome, these results seem to suggest that mitochondrial respiration is sensitive, but not proportional, to the training volume, as previously hypothesised [26].

The much reduced training volume of the RT phase was associated with an ~15-25% reduction in mass-specific mitochondrial respiration across the SUIT protocol measurements that followed the addition of ADP. No study to date has measured mitochondrial respiration in permeabilised fibres following a reduction in training volume. Nevertheless, it has been reported that mitochondrial respiration measured in deltoid muscle homogenates of elite swimmers is reduced by 50% following one week of detraining [62]. The greater decrease in mitochondrial respiration reported by Costill *et al.* (1985) compared to that reported in this research may depend on factors such as muscle type, fitness levels and training history, the use of complete detraining rather than reduced training volume, and the use of muscle homogenates instead of permeabilised fibres. The ~15-25% decrease in mass-specific mitochondrial respiration that followed RT in this study is comparable with the 12-28% decrease in mitochondrial ATP production rate (MAPR) reported following 3 weeks of detraining in active humans [343]. A remarkable finding of this research was that, at the end of the 13-week study, only CI_P was significantly higher compared to baseline, whereas all the other respiration states were not significantly different from baseline despite the large increases that followed the INT phase. These findings emphasise the reversibility of increases in mitochondrial respiration following a period of reduced training, further highlighting the plasticity of

human skeletal muscle and the importance of maintaining training volume in order to preserve the adaptations gained during exercise training.

Analysis of the FCRs showed that there was no change in the index of uncoupling (LCR), the limitation of OXPHOS capacity by the phosphorylation system (PCR), the degree of coupling between oxidation and phosphorylation (inv-RCR), and substrate control (SCR) throughout the study. CIV_{RES} however, was significantly elevated after INT when compared to baseline. It is known that CIV oxidative capacity measured in permeabilised fibres in the presence of ascorbate and TMPD is in excess of maximal coupled respiration [225]; therefore, it is possible that the smaller increase in CIV_E compared to $CI+II_P$ (22 vs. 41% respectively) after INT, may be due to this overcapacity and the ability of CIV to already sustain a higher overall mitochondrial respiration. Further research is needed to confirm these hypotheses.

Cellular mechanisms of mitochondrial adaptations to changes in training volumes

To provide a better understanding of the cellular mechanisms involved in the regulation of mitochondrial adaptations to different training volumes, the protein content of PGC-1α, p53, NRF1, PHF20 and TFAM, five transcription factors involved in the regulation of mitochondrial biogenesis [266, 65, 271], was also measured. The protein content of all five transcription factors responded to the large volume of HIIT, and was significantly greater (~65-170%) following INT. The similar and concomitant increase in PGC-1α, NRF1 and TFAM protein content (73, 63, and 76% respectively), is consistent with the changes in the same transcription factors (1.8-, 1.9-, and 1.4-fold respectively) following four days of contractile activity in muscle cells [122], and is also in agreement with the notion that NRF1 may provide a link between nuclear and mitochondrial gene expression [325, 352]. It has been suggested that existing concentrations of NRF1 are sufficient to meet the needs of the cell [233]. However, the increase in NRF1 protein content following INT seems to indicate that when training volume is greatly increased, the existing concentrations of this transcription factor may not be sufficient to support cellular needs.

The increase in PGC-1α, p53 and PHF20 protein content following INT mirrored that of mass-specific mitochondrial respiration, and is consistent with findings in from Chapter

2 showing an association between these parameters following training. These results are also consistent with the role of PGC-1α [352] and p53 [181] as modulators of mitochondrial respiration. Although no significant changes in these transcription factors following moderate volume HIIT (3x/week for 4 weeks) have been reported in Chapter 2, the current findings indicate that when training volume is markedly increased, the training intensity associated with HIIT is sufficient to increase the protein content of these transcription factors in healthy males. In contrast, results from Chapter 2 indicate that training-induced changes in the protein content of TFAM are not associated with changes in mass-specific mitochondrial respiration. Following INT however, an increase in TFAM protein content in parallel with increased mitochondrial content and mass-specific mitochondrial respiration was also reported. Taken together, these findings seem to suggest that changes in the protein content of TFAM may be associated primarily with changes in mitochondrial content, and may only associate with changes in mitochondrial respiration when these are driven by a change in mitochondrial content, as was the case following INT. This would also be consistent with the role of TFAM as a key factor regulating the mitochondrial transcriptional machinery [272]. Further research is needed to investigate these hypotheses.

Following RT, only the content of TFAM and NRF1 was significantly decreased; however, the protein content of all five transcription factors decreased to a level that was not significantly different from baseline. This is the first report to demonstrate that these transcription factors are sensitive to reductions in training volume. One of the most striking findings of this research is that a reduction in training volume lasting as little as two weeks was sufficient to reverse the majority of the gains obtained following eleven weeks of testing and training at various intensities. The rapid changes in the content of these transcription factors indicate that, even at the cellular level, human skeletal muscle quickly responds to a change in training stimulus rapidly adapting to the new metabolic and energy requirements, and highlights once again its remarkable plasticity.

A secondary aim of this research was to determine if different training volumes would alter the protein content of selected downstream targets of p53. Through transcriptional regulation of the COX assembly protein SCO2 [181], the apoptotic protein AIF [298, 319], the fusion protein mitofusin 2 [335], and the fission protein DRP1 [154] amongst

others, p53 has been linked with processes associated with the regulation of both mitochondrial function and mitochondrial remodelling [266]. Data from this research reveal that in humans the protein content of these downstream targets of p53 did not change following INT, nor after RT. This lack of change is consistent with results from the previous chapter showing that these targets did not respond to four weeks of HIIT in human skeletal muscle. These results further support the notion that in skeletal muscle p53 seems to regulate mitochondrial function via modulation of proteins other than SCO2, as previously shown in mice [217] and in humans (Chapter 2). The present study did not measure the mitochondrial (or nuclear) fraction of these proteins; nonetheless, it may be possible that their location is linked with their activity. Therefore, future research exploring how a single bout of exercise or exercise training alters the location of these downstream targets of p53 is warranted.

Conclusions

The main finding of this research is that training volume is a key determinant of exercise-induced mitochondrial adaptations. The present study demonstrated that a training volume as large as 40 sessions of HIIT (lasting up to 80 min each) in 20 days increased both markers of mitochondrial content and mass-specific mitochondrial respiration, without changes in mt-specific respiration. Improvements in whole-body exercise performance also mirrored these changes. A remarkable finding was that following a 23-fold reduction in training volume (RT), and the maintenance of endurance performance, all parameters measured in the muscle were not significantly different from pre-study values (with the exception of CI_P and CS activity). This suggests that maximal mass-specific mitochondrial respiration may be reversed more rapidly than mitochondrial content, providing further evidence that these two parameters are not necessarily associated. Overall, these findings emphasise the rapid reversibility of mitochondrial adaptations following a period of reduced training, and the importance of maintaining the training stimulus to preserve the gains obtained during exercise training. The present study shows that even at the cellular level human skeletal muscle is a malleable tissue rapidly adapting to a change in training stimulus and to the metabolic and energy requirements of the cell. This research provides further evidence that changes in the

protein content of PGC-1α, p53 and PHF20 may be associated with changes in mass-specific mitochondrial respiration, whereas it suggests that changes in the protein content of TFAM may be primarily associated with changes in mitochondrial content. In conclusion, this research highlights the remarkable plasticity of human skeletal muscle, and provides valuable tools to better design exercise protocols for the prevention and cure of diseases linked to impaired mitochondrial function.

Chapter 4

Effect of exercise intensity on PGC-1α and p53 in subcellular fractions of human skeletal muscle

Findings from previous chapters have highlighted the role of training intensity and training volume in the modulation of training-induced mitochondrial adaptations. However, the cellular mechanisms regulating these processes are not yet fully understood.

The purpose of this third study was to investigate how training intensity regulates the initial response to exercise. Previous research indicates that the initial phase of the exercise-induced adaptive response may take place by nuclear accumulation of PGC-1α [351]. Moreover, while p53 has also been shown to regulate the exercise-induced adaptive response, little is known on the molecular mechanisms that regulate its activity. Therefore, the effects of a single bout of moderate-intensity continuous exercise (STCT) and maximal-intensity sprint interval training (SIT) on changes in the protein content of PGC-1α and p53 in enriched nuclear and cytosolic fractions, were investigated.

The manuscript for this study is in the final stages of preparation:

Granata C, Oliveira RSF, Little JP, Renner K and Bishop DJ (2015). Modulation of the exercise-induced adaptive response mediated by PGC-1α and p53, in enriched subcellular fractions of human skeletal muscle. *Journal of Physiology* (final stages of preparation).

4.1 Introduction

Exercise is a powerful stimulus to induce mitochondrial biogenesis [224]. The result of mitochondrial biogenesis is an increase in mitochondrial content [296] and/or mitochondrial respiration [127], and an improved capacity for substrate oxidation [114] and ATP production [343]. As a consequence, aerobic exercise training has been associated with improved endurance performance [128], and has been used as a valuable tool to help prevent and treat a host of chronic diseases linked to mitochondrial dysfunction [106, 190, 206, 246]. Therefore, a better understanding of the molecular mechanisms regulating exercise-induced mitochondrial adaptations provides important information that may help to improve both endurance performance and health.

A key regulator of exercise-induced mitochondrial biogenesis is peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α), a transcriptional coactivator regulating the expression of nuclear genes encoding mitochondrial proteins [242]. While initial research focused on the increase in whole-muscle PGC-1α protein content in the hours following the end of exercise [180, 224], more recent research has investigated changes in the nuclear content of PGC-1α. Research in rat skeletal muscle has demonstrated that the increase in whole-muscle PGC-1α protein content following 3 hours of recovery is preceded by an increase in nuclear PGC-1α protein content in muscle samples taken immediately after the termination of exercise [351]. The authors suggested that the increase in nuclear PGC-1α protein content may constitute the initial phase of the exercise-induced adaptive response [351]. There is controversy however, on the timing and magnitude of the increase in nuclear PGC-1α content in human skeletal muscle following different exercise intensities. While a single bout of "all-out" sprint interval exercise (SIT) has been shown to induce an increase in nuclear PGC-1α content after 3 hours of recovery [159], continuous exercise at a lower intensity has been reported to not change [184] or to increase [160] nuclear PGC-1α content immediately post-exercise. Moreover, the two latter studies did not investigate further changes in the subsequent hours of recovery, potentially missing any delayed responses. Participants' fitness levels in the three studies were also different. Therefore, to better understand the influence of different exercise intensities on the timing and magnitude of the change in nuclear PGC-

1α protein content, it is important to directly compare participants of similar fitness levels within the same study.

p53 is another key transcription factor involved in the regulation of exercise-induced mitochondrial adaptations, as demonstrated by a reduction in mitochondrial respiration and content, as well as endurance performance, in p53 knockout mice [217, 265]. p53 has been implicated in the regulation of autophagy [174], mitochondrial remodelling [266] and mitochondrial function [181]. Induction of the p53 response to physiological stressors, such as exercise, occurs largely through alterations in the p53 protein [215]. For example, in human skeletal muscle, a single bout of endurance exercise increases p53 phosphorylation at serine 15 [18], a post-translational modification enhancing p53 activity and stability [71, 289]. Subcellular localisation is also a key factor regulating the activity of p53 [215, 266, 286]. An increase in p53 protein content in the nucleus, where p53 can exert its transcriptional and biochemical activity [178, 286], has been reported in cell culture following exposure to stress [177], and also in mouse skeletal muscle immediately after a 60-min bout of running [227] or 1 hour after a 20-min bout of intermittent eccentric muscle contractions [45]. Although the consensus is that cellular stress induces p53 protein stabilisation and nuclear accumulation [178, 215], a decrease in nuclear p53 protein content has been reported in mice following an exhaustive 90-min running bout [267]. To date however, no study has investigated possible exercise-induced changes in nuclear p53 content (or nuclear p53 phosphorylation) in human skeletal muscle, and whether these changes may be regulated in an exercise intensity-dependent manner. Similar to PGC-1α, measuring changes in nuclear p53 may provide greater physiological insight into its role in the modulation of exercise-induced mitochondrial adaptations.

This study investigated, for the first time in human skeletal muscle, whether a single bout of exercise can influence the protein content and phosphorylation of p53 in the nucleus. It was hypothesised that, similar to PGC-1α, exercise would represent a physiological stress that leads to an increase in the nuclear content (and phosphorylation) of p53. It was also hypothesised that the greater physiological stress associated with an increase in exercise intensity would further increase these changes.

4.2 Methods

Participants and ethics approval

Participants. Twenty healthy men, who were non-smokers, moderately-trained, and involved in various individual and team sports, volunteered to participate in this study. After initial screening and testing, participants were ranked by the power attained at the lactate threshold (W_{LT}) and assigned in reversed counterbalanced (ABBA) order to the sub-lactate threshold continuous training (STCT) or SIT group (both n=10), in a between-subjects study design. Nineteen participants completed the study, with one participant from the SIT group withdrawing due to other commitments. Baseline physiological parameters for each group are described in Table 4.1.

Table 4.1. Baseline characteristics of the participants.

Variable	Study (n=19)	STCT (n=10)	SIT (n=9)
Age (y)	21 ± 2	21 ± 2	21 ± 3
Body Mass (kg)	82.0 ± 15.9	79.8 ± 12.7	84.5 ± 19.4
Height (cm)	180.1 ± 6.5	179.6 ± 6.1	180.6 ± 7.3
$\dot{V}O_{2Peak}$ [mL min^{-1} kg^{-1}]	47.0 ± 5.8	47.0 ± 3.7	47.1 ± 7.8
W_{LT}	199.4 ± 40.8	194.8 ± 43.4	204.4 ± 39.7
W_{Peak}	278.6 ± 48.6	276.5 ± 51.5	280.8 ± 48.2

STCT: sub-lactate threshold continuous training; SIT: sprint interval training; $\dot{V}O_{2Peak}$: peak oxygen uptake; W_{LT}: power at the lactate threshold; W_{Peak}: peak power output. All values are mean ± SD.

Ethical approval. Participants were informed of the study requirements, benefits and risks before providing written informed consent. Approval for the study's procedures, which conformed to the standards set by the latest revision of the Declaration of Helsinki, was granted by the Victoria University Human Research Ethics Committee.

Study design

The experimental protocol consisted of, in the following order, a graded exercise test (GXT), and the biopsy trial. Each of these two tests was preceded by a familiarisation trial. All tests, including familiarisation tests, were separated by a minimum of 48 h (72 h prior to the biopsy trial). Due to the nature of sprint training, it was not possible to match the two groups for total work. The study duration was approximately two weeks.

Experimental protocol and testing procedures

Participants were required to refrain from any strenuous physical activity for the 48 h preceding each performance test (72 h for the biopsy trial), from alcohol and any exercise for 24 h before testing, and from food and caffeine consumption for the 2 h preceding each test (3 h for the biopsy trial).

GXT. A discontinuous graded exercise test was performed on an electronically-braked cycle ergometer (Lode Excalibur, v2.0, Groningen, The Netherlands) to determine the peak oxygen uptake ($\dot{V}O_{2Peak}$), peak power output (W_{Peak}), and W_{LT} (using the modified D_{Max} method [25]) for all the participants. The test consisted of 4-min stages at a constant power output, interspersed with 30 s of rest. The test began at a power output of 60, 90 or 120 W depending on participants' fitness levels, and was subsequently increased by 30 W every 4 min. Prior to the test, and during the 30-s rest, capillary blood samples were taken from the fingertip for the measurement of blood lactate concentration ($[La^-]$). Participants were instructed to keep a cadence > 60 rpm and were given verbal encouragement throughout the test, during which they were only allowed access to cadence and elapsed time. The test was stopped when a participant reached volitional exhaustion or cadence dropped below 60 rpm. The W_{Peak} was determined as the power of the last completed stage when participants stopped at the end of a stage. If a participant stopped during a stage, W_{Peak} was determined as the power of the last completed stage plus 7.5 W for every completed minute.

Gas Analysis during the GXT. During the GXT, expired air was continuously analysed for O_2 and CO_2 concentrations via a gas analyser (Moxus modular oxygen uptake system, 2010, AEI technologies, Pittsburgh, PA, USA). The gas analysers were calibrated

immediately before each test using known gas mixtures (A: 21% O_2, 0% CO_2; B: 16% O_2, 4% CO_2; BOC, Melbourne, Australia). The ventilometer was calibrated using a 3-liter syringe (Hans Rudolph). $\dot{V}O_2$ values were recorded every 15 s and the two highest consecutive 15-s values recorded during the test were averaged and recorded as the participant's $\dot{V}O_{2Peak}$.

Capillary blood sampling. Glass capillary tubes (MultiCap 140 µL, Siemens Healthcare Diagnostics Inc. Deerfield, IL, USA) were used to collect about 50 µL of blood at various time points during the GXT. Capillary blood [La^-] was determined using a blood-lactate analyser (YSI 2300 STAT Plus Glucose & Lactate Analyser, YSI Inc., Yellow Spring, Ohio, USA). The blood-lactate analyser was regularly calibrated using precision standards and routinely assessed by external quality controls.

Biopsy trial. Trials were always performed in the morning to avoid variations caused by circadian rhythm changes. To minimize variability in muscle gene and protein expression attributable to diet, participants were provided with a standardised dinner (55 kJ kg^{-1} body mass (BM), providing 2.1 g carbohydrate kg^{-1} BM, 0.3 g fat kg^{-1} BM, and 0.6 g protein kg^{-1} BM) and breakfast (41 kJ kg^{-1} BM, providing 1.8 g carbohydrate kg^{-1} BM, 0.2 g fat kg^{-1} BM, and 0.3 g protein kg^{-1} BM) prior to the biopsy trial. Participants consumed these meals 15 and 3 h prior to the biopsy trials respectively, and reported to the laboratory thirty minutes before the first biopsy was scheduled. Participants rested in the supine position, and after injection of a local anaesthetic into the skin and fascia (1% xylocaine, Astra Zeneca) three small incisions, one for each biopsy to be taken during the biopsy trial, were made about 2-3 cm apart. Fifteen minutes prior to the beginning of the exercise protocol, a resting muscle biopsy (Pre) was obtained. All muscle biopsies (approximately 150-300 mg wet weight) were taken by an experienced practitioner at a constant depth of around 2-3 cm, and were obtained from the vastus lateralis muscle using a biopsy needle with suction. Once obtained, muscle samples were immediately processed, cleaned of excess blood, fat, and connective tissue, were rapidly frozen in liquid nitrogen, and stored at -80°C for subsequent analyses. At this stage participants were helped to an electronically-braked cycle ergometer (Velotron, RacerMate, Seattle, WA, USA) where, after completion of a warm-up involving cycling for 6 min at 66% of W_{LT} followed by 2 min of rest, they began either the STCT or SIT protocol. Immediately upon completion

of the exercise bout (< 2 s), a second muscle biopsy (+0 h) was obtained, with the participant still sitting on the ergometer and while leaning his back towards a spotter. Participants were then helped to a laboratory bed where they rested in the supine position for 3 hours, at which stage a third muscle biopsy (+3 h) was obtained. Capillary blood samples to measure [La^-] were collected at rest, and immediately after the completion of exercise. During this period participants had no access to food, and were permitted to consume only water *ab libitum*.

STCT. Exercise consisted of 24 min of continuous cycling at a fixed power equivalent to 90% of baseline W_{LT}. Exercise was set relative to W_{LT} rather than W_{Peak} as metabolic and cardiac stresses are similar when individuals of differing fitness levels exercise at a percent of the W_{LT}, but can vary significantly when training at a percent of W_{Peak} [15]. Participants were verbally encouraged for the duration of the exercise bout.

SIT. Exercise consisted of four repetitions of a 30-s "all-out" cycling bout against a resistance set at 0.075 kg/kg body mass, interspersed with a 4-min recovery period. During recovery participants remained on the bike and were allowed to either rest or cycle against a low resistance (30 W) at a low cadence (< 50 rpm). During the last 15 s of the recovery period participants were instructed to begin pedalling and reach a cadence of 80-100 rpm against no resistance (warm up phase), and in the last 2 seconds they were instructed to begin pedalling as fast as possible (speed up phase). At this time, the load was applied via the software (Velotron Wingate Software, Velotron, RacerMate, Seattle, WA, USA) loaded on a computer and interfaced with the cycle ergometer. Participants were verbally encouraged to keep the cadence as high as possible during the entire duration of the bout.

Muscle analyses

Preparation of nuclear and cytosolic fractions. Nuclear and cytosolic fractions were prepared from 40-60 mg of wet muscle using a commercially-available nuclear extraction kit (NE-PER #78833; Pierce, Rockford, IL). Muscle samples were minced on ice with sharp scissors in PBS containing a protease/phosphatase inhibitor cocktail (Cell Signaling Technology (CST), 5872), and then centrifuged at 500 g for 5 min at 4°C. After discarding

the supernatant, the muscle was homogenised 5 times for 5 seconds with a hand-held mini-homogenizer (Kontes Pellet Pestle Cordless Motor, Kimble Chase, NJ, USA.) on ice in CER-I buffer containing the same inhibitor cocktail. Homogenates were vortexed vigorously and let sit on ice for 5-10 minutes. CER II buffer was then added. Homogenates were vortexed vigorously and let sit on ice for 1 minute; this procedure was repeated twice. Homogenates were centrifuged for 5 min at 16000 g at 4°C, and the supernatant was taken as the crude cytosolic fraction and stored at -80°C. Pellets containing nuclei were subsequently washed five times in PBS containing the same inhibitor cocktail, to remove cytosolic contaminating proteins. Pellets were re-homogenized with ~30 strokes using a Teflon pestle by hand in NER buffer supplemented with the same inhibitor cocktail. Homogenates were vortexed vigorously and let sit on ice for 10 minutes; this procedure was repeated four times. Following this 40-min incubation step, samples were centrifuged at 16000 g at 4°C, and the supernatant was taken as the soluble nuclear fraction and stored at -80°C. Sufficient biopsy material was available to prepare and analyse nuclear and cytosolic fractions from nine participants in each group. Determination of subcellular enrichment is described in the Results section.

Western Blotting. Protein concentration was determined using a commercial colorimetric assay (Bio-Rad Protein Assay kit II, Bio-Rad, Hercules, CA), and lysates were then diluted with a 3x Laemmli buffer, containing 1 mM DTT and 10% β-mercaptoethanol. For each protein of interest, a linearity study was conducted to determine the ideal loading amount (data not shown). Muscle lysates were then loaded in equal amounts (10-45 μg according to target protein and the fraction analysed) and separated by electrophoresis for 1.5-2.5 h at 100 V using self-cast SDS-PAGE gels (8-12%). Once resolved, the gels were wet transferred onto PVDF membranes at 100 V for 70-90 min. Transfer efficiency was checked by Coomassie staining the gels together with Ponceau S staining of the membranes. 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). Immunoblotting was carried out using the following antibodies (supplier and catalogue number noted in brackets): histone H3 (CST, 9715), lactate dehydrogenase A (LDHA) (CST, 2012), p53 (CST, 2527), PGC-1α (Calbiochem, st-1202), p-Acetyl-CoA Carboxylase (ACC)^{Ser79} (CST, 3661), p-

$p53^{Ser15}$ (CST, 9284), and plant homeodomain finger-containing protein 20 (PHF20) (CST, 3934). 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 90 min in 1-5% skim milk in TBS-T. Membranes were then washed again for 3 x 5-min in TBS-T followed by a final 5-min wash in TBS before being immersed for 5 min under gentle agitation at room temperature in a chemiluminescent solution [Solution A: 2.5 mM Luminol, 0.36 mM p-Coumaric Acid, 0.1 M Tris (pH 8.5) in 10 mL MilliQ H_2O; Solution B: 6.1 µL H_2O_2, 0.1 M Tris (pH 8.5) in 10 mL MilliQ H_2O; solution A and B prepared separately and mixed just prior to use]. Protein bands were visualized using a Bio-Rad Versa-Doc imaging system and band densities were determined using Bio-Rad Quantity One image analysis software (Bio-Rad, Hercules, CA). Coomassie Blue staining was performed at the end of the procedure and was used to verify correct loading and equal transfer between lanes [338]. Finally, samples for the three time points for each participant were loaded on the same gel together with an identical amount of internal standard (IS). Comparison and statistical analysis between each participant's time points were done using the raw density data normalised to the IS loaded in each gel to reduce gel-to-gel variability. For graphical purposes, each time point was normalised to baseline; for this reason WB graphs throughout this manuscript are presented as fold change compared to baseline, and no error bar is presented for the baseline time point. A representative blot for each protein analysed is presented in Figure 4.1.

Total RNA isolation. Total RNA was isolated from approximately 15-25 mg of muscle tissue using the RNeasy® Mini kit (Qiagen, Mississauga, ON, Canada) according to the manufacturer's instructions. Briefly, muscle samples were homogenised using the TissueLyser II (Qiagen, Mississauga, ON, Canada), and total RNA was isolated from the aqueous phase following precipitation with 600 µL of 70% ethanol using RNeasy® Mini kit. On-column DNA digestion was performed. RNA concentration was determined by spectrophotometry (Nanodrop, ND1000, Thermo Fisher Scientific, Wilmington, DE) by measuring the absorbance at 260 nm (A260) and 280 nm (A280), with A260/A280 ratios above 1.8 indicating high-quality RNA. Sufficient biopsy material was available to isolate and analyse total RNA from nine participants in each group.

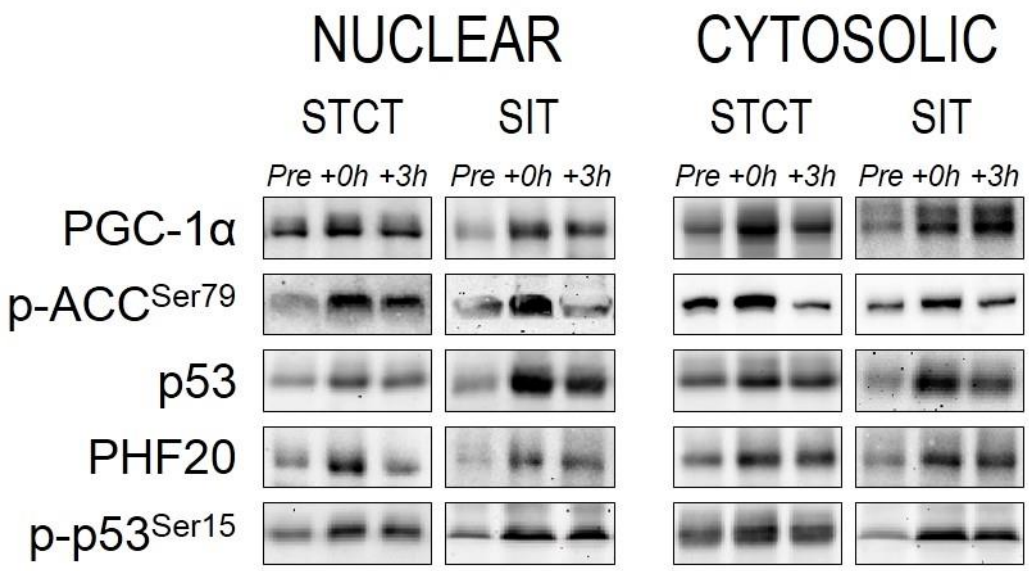

Figure 4.1. Representative immunoblots corresponding to total and phosphorylated protein expression measured in the nuclear and cytosolic fractions, before (Pre), and immediately (+0 h) and 3 h (+3 h) following the STCT and SIT exercise trials. PGC-1α: peroxisome proliferator-activated receptor γ coactivator-1α; p-ACC^{Ser79}: Acetyl-Coenzyme A carboxylase phosphorylated at serine 79; PHF20: plant homeodomain finger-containing protein 20; p-p53^{Ser15}: p53 phosphorylated at serine 15.

Real-time RT-PCR. First-strand cDNA synthesis from 500 ng of total RNA was performed with random hexamer primers using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA), according to manufacturer's directions. All samples and reverse transcriptase (RT) negative controls were run together to prevent technical variation. Forward and reverse primers for the target and housekeeping genes (Table 4.2) 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 generated by the PCR instrument. The mRNA expression of apoptosis inducing factor (AIF), dynamin-related protein-1 (DRP1), mitofusin 2 (MFN2), p53, and PGC-1α were quantified by quantitative real-time RT-PCR (Mastercycler® RealPlex2, Eppendorf, Hamburg, Germany), using a 10 µL PCR reaction volume and SYBR Green chemistry (iTaqTM Universal SYBR® Green Supermix, Bio-Rad, Hercules, CA). All samples were run in duplicate simultaneously with template free controls, using an automated pipetting system (epMotion 5070,

Eppendorf, Hamburg, Germany). The following PCR cycling patterns were used: initial denaturation at 95°C for 3 min, 40 cycles of 95°C for 15 s and 60°C for 60 s. To account for the efficiency of RT and initial RNA concentration, the mRNA expression of the housekeeping genes cyclophilin, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and TATA-binding protein (TBP), which did not change across time, were quantified, and target genes were normalised to these housekeeping genes. Relative changes in mRNA abundance were calculated using the normalized relative quantities (NRQs) method as described previously [110].

Table 4.2. Primers used for real-time RT-PCR analyses of mRNA expression.

Gene	Forward primer (5'→3')	Reverse primer (5'→3')
AIF	GATTGCAACAGGAGGTACTCCAAGA	GATTTGACTTCCCGTGAAATCTTCTC
DRP1	CACCCGGAGACCTCTCATTC	CCCCATTCTTCTGCTTCCAC
MFN2	CCCCCTTGTCTTTATGCTGATGTT	TTTTGGGAGAGGTGTTGCTTATTTC
p53	GTTCCGAGAGCTGAATGAGG	TTATGGCGGGAGGTAGACTG
PGC-1α	GGCAGAAGGCAATTGAAGAG	TCAAAACGGTCCCTCAGTTC
cyclophilin	GTCAACCCCACCGTGTTCTTC	TTTCTGCTGTCTTTGGGACCTTG
GAPDH	AATCCCATCACCATCTTCCA	TGGACTCCACGACGTACTCA
TBP	CAGTGACCCAGCAGCATCACT	AGGCCAAGCCCTGAGCGTAA

AIF: apoptosis-inducing factor; DRP1: dynamin-related protein 1; MFN2: mitofusin 2; PGC-1α: peroxisome proliferator-activated receptor γ coactivator-1α; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; TBP: TATA-binding protein.

Statistical analysis

All values are reported as means ± SD unless specified otherwise. One-way ANOVA were used to assess differences between the groups for age, height, body mass, W_{LT}, W_{Peak}, $\dot{V}O_{2Peak}$, and for total work during the biopsy trial. To investigate the influence of exercise type and time, and the interaction between both of these variables, two-way ANOVA with repeated measures for time were used. Significant interactions and main

effects were further analysed using a Tukey's honestly significant difference post-hoc test. Sigma Stat software (Jandel Scientific, San Rafael, CA) was used for all statistical analyses. The level of statistical significance was set a priori at $P < 0.05$.

4.3 Results

Energy expenditure and performance parameters during the biopsy trial

Energy expenditure during the biopsy trial was 3.6-fold higher ($P < 0.001$) for the STCT group compared with SIT (Table 4.3). In contrast, 1-s maximum and mean exercise intensity during the biopsy trial, expressed in both absolute values and relative to W_{Peak}, were higher for the SIT group compared with STCT (all $P < 0.001$, Table 4.3). The exercise-induced increase in blood lactate concentration ([La^-], both $P < 0.001$, Table 4.3) was also greater in SIT compared with STCT ($P < 0.001$, Table 4.3).

Table 4.3. Energy expenditure, performance parameters, and blood lactate concentration, during the STCT and SIT exercise trials.

Measurement		**STCT (n=10)**	**SIT (n=9)**
Energy expenditure	[kJ]	252.6 ± 59.7‡	69.5 ± 9.1
1-s max exercise intensity	[W] (%W_{Peak})	175.4 ± 39.1‡ 63.1 ± 3.1‡	823.4 ± 153.5 294.3 ± 33.7
Mean exercise intensity	[W] (%W_{Peak})	175.4 ± 39.1‡ 63.1 ± 3.1‡	578.8 ± 75.6 207.7 ± 14.0
Blood [La^-] [mmol/L]	Pre +0 h	0.9 ± 0.2 3.9 ± 0.3*‡	0.9 ± 0.2 11.6 ± 1.4*

STCT: sub-lactate threshold continuous training; SIT: sprint interval training; W_{Peak}: peak power output; blood [La^-]: blood lactate concentration. * Significantly different ($P < 0.05$) *vs.* Pre, ‡ *vs.* SIT at the same time point. All values are mean ± SD.

Muscle analyses

Cellular fractions enrichment, and antibody specificity. The enrichment and purity of both nuclear and cytosolic fractions was confirmed by blotting the separated fractions against the nuclear protein histone H3, and the cytosolic protein LDHA. Histone H3 was only detected in nuclear fractions (Figure 4.2A), whereas LDHA was only detected in cytosolic fraction (Figure 4.2B), confirming the cellular fractionation protocol was successful. The specificity of the p53 antibody was confirmed by blotting samples beside a commercially-available, untagged, full-length p53 recombinant human protein (Aviva Systems Biology, AEP00002). The results in Figure 4.2C illustrate the specificity of the chosen antibody. The same full-length p53 recombinant human protein, which was expressed in E. coli and was not phosphorylated, was also used as a negative control against the chosen p-p53^{Ser15} antibody. Results from Figure 4.2C show the p-p53^{Ser15} antibody did not recognise this protein, suggesting it is phospho-specific.

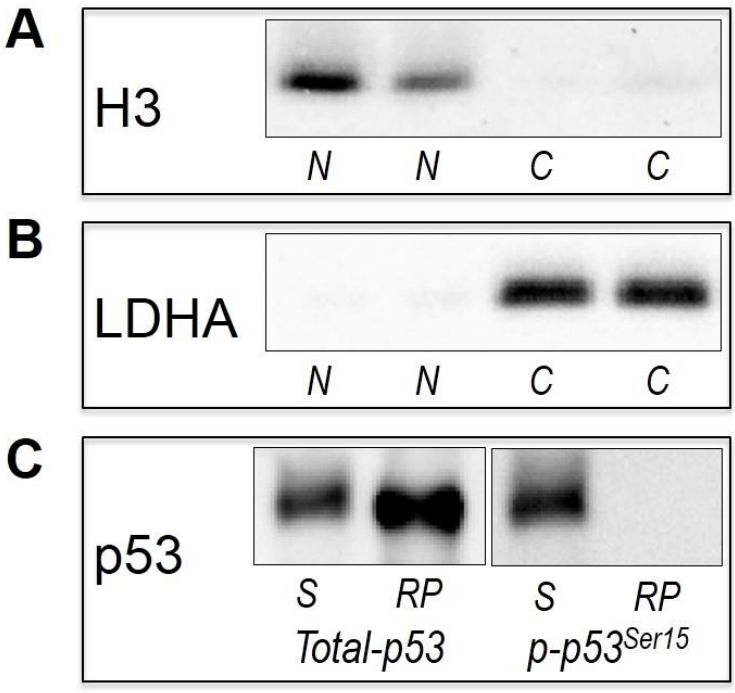

Figure 4.2. Subcellular fraction enrichment, and p53 antibody specificity. Histone H3 (A) and lactate dehydrogenase A (LDHA) (B) were used as indicators of cytosolic and nuclear enrichment, respectively. Confirmation of the specificity of the p53 antibody (C). Samples were run beside an untagged full length p53 recombinant human protein and then blotted against both Total-p53 (positive control) and p-p53^{Ser15} (negative control) antibodies. N: nuclear fractions; C: cytosolic fractions; S: sample; RP: p53 recombinant protein.

PGC-1α protein content. Nuclear PGC-1α protein content increased 2.3-fold immediately following SIT ($P < 0.001$), but not after STCT (1.4-fold, $P = 0.219$), resulting in a difference between groups at this time point ($P = 0.043$). Nuclear PGC-1α protein content was still elevated, when compared to baseline, after 3 h of recovery following SIT only (1.7-fold, $P = 0.037$) (Figure 4.3A). There was an interaction ($P = 0.047$) between group and time for cytosolic PGC-1α protein content, and after 3 h of recovery cytosolic PGC-1α protein content was 1.9-fold higher ($P = 0.037$) than baseline in the SIT group only (Figure 4.3B).

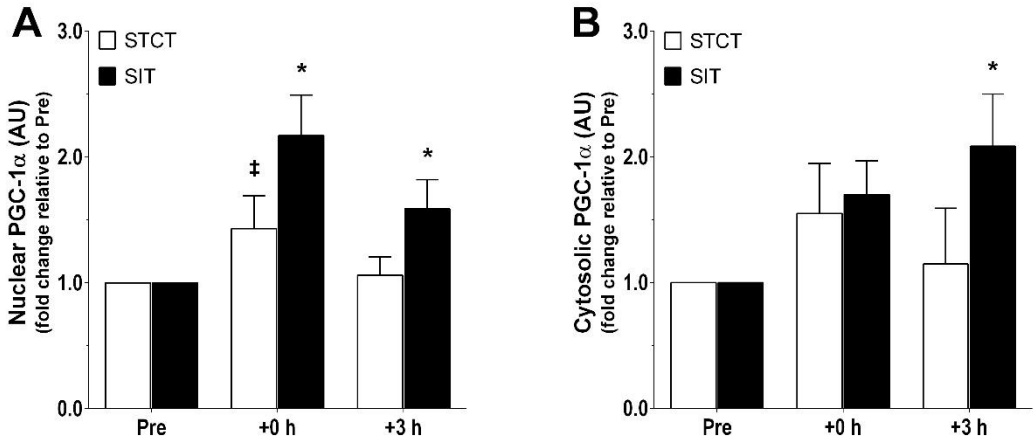

Figure 4.3. Nuclear (A) and cytosolic (B) PGC-1α protein content before (Pre), and immediately (+0 h) and 3 h (+3 h) following the STCT and SIT exercise trials. * Significantly different ($P < 0.05$) *vs.* Pre, ‡ *vs.* SIT at the same time point. n=9 for each group. All values are mean ± SEM.

PGC-1α mRNA. Muscle PGC-1α mRNA content increased 6-fold ($P < 0.001$) after 3 h of recovery, with no difference between groups ($P = 0.701$, Figure 4.4A).

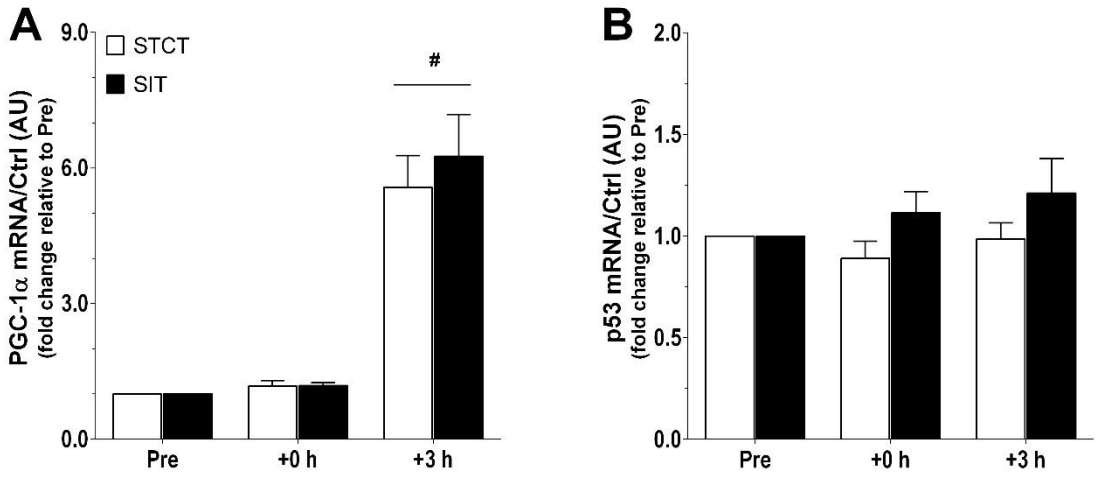

Figure 4.4. PGC-1α (A), and p53 (B) mRNA content before (Pre), and immediately (+0 h) and 3 h (+3 h) following the STCT and SIT exercise trials, expressed relative to cyclophilin, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and TATA-binding protein (TBP) housekeeping genes. # Significant main effect of time ($P < 0.05$) *vs.* Pre. n=9 for each group. All values are mean ± SEM.

P-ACC^{Ser79} protein content. P-ACC^{Ser79} content was increased in both the nuclear (2-fold, $P = 0.015$, Figure 4.5A) and cytosolic (1.7-fold $P = 0.017$, Figure 4.5B) fraction immediately post-exercise only, with no difference between groups (both $P > 0.05$).

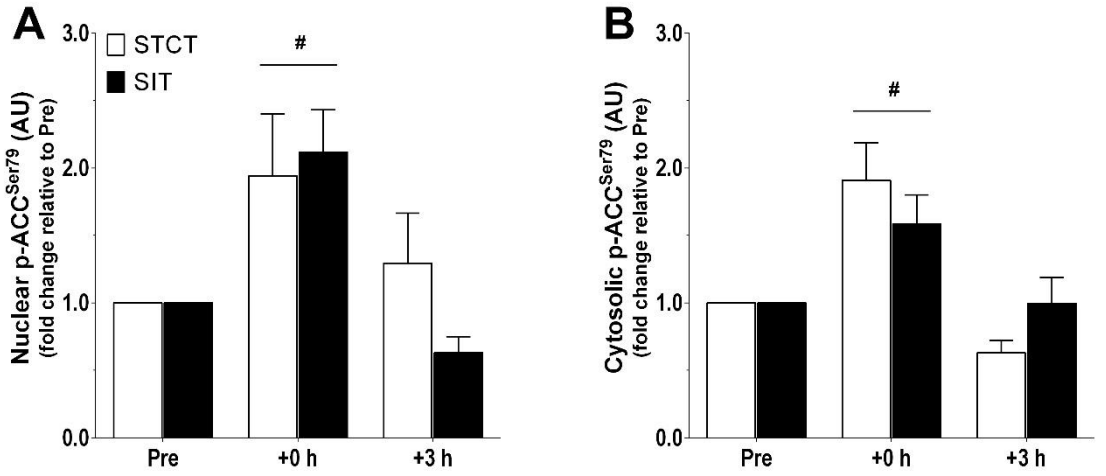

Figure 4.5. Nuclear (A) and cytosolic (B) p-ACC^{Ser79} protein content before (Pre), and immediately (+0 h) and 3 h (+3 h) following the STCT and SIT exercise trials. # Significant main effect of time ($P < 0.05$) *vs.* Pre. n=9 for each group. All values are mean ± SEM.

p53 protein content. The content of p53 was increased in both the nuclear (2-fold, P = 0.001, Figure 4.6A) and cytosolic (1.8-fold, P = 0.014, Figure 4.6B) fraction immediately post-exercise only, with no difference between groups (both $P > 0.05$).

PHF20 protein content. The content of PHF20 was increased in both the nuclear (1.7-fold, P = 0.004, Figure 4.6C) and cytosolic (1.7-fold $P < 0.001$, Figure 4.6D) fraction immediately post-exercise only, with no difference between groups (both $P > 0.05$).

P-p53^{Ser15} protein content. Nuclear p-p53^{Ser15} content increased immediately post-exercise (3.1-fold, $P < 0.001$), and after 3 h of recovery (2.1-fold, P = 0.007), following SIT but not STCT (both $P > 0.05$); it was significantly greater in SIT compared with STCT at both time points ($P < 0.001$ and P = 0.044, at +0 and +3 h, respectively) (Figure 4.6E). Cytosolic p-p53^{Ser15} content increased immediately post-exercise (2.3-fold, $P <$ 0.001), and after 3 h of recovery (2-fold, P = 0.020), with no difference between groups (P = 0.304) (Figure 4.6F).

mRNA of p53, AIF, DRP1 and MFN2. There was no change in p53 mRNA content throughout (P = 0.380, Figure 4.4B). The mRNA content of AIF was increased by 1.4-fold (P = 0.040) after 3 h of recovery, with no difference between groups (P = 0.460) (Table 4.4). The mRNA content of DRP1 and MFN2 was increased by 1.2- (P = 0.038) and 1.4-fold (P = 0.014) respectively, at the end of exercise, with no difference between groups (both $P > 0.05$) (Table 4.4).

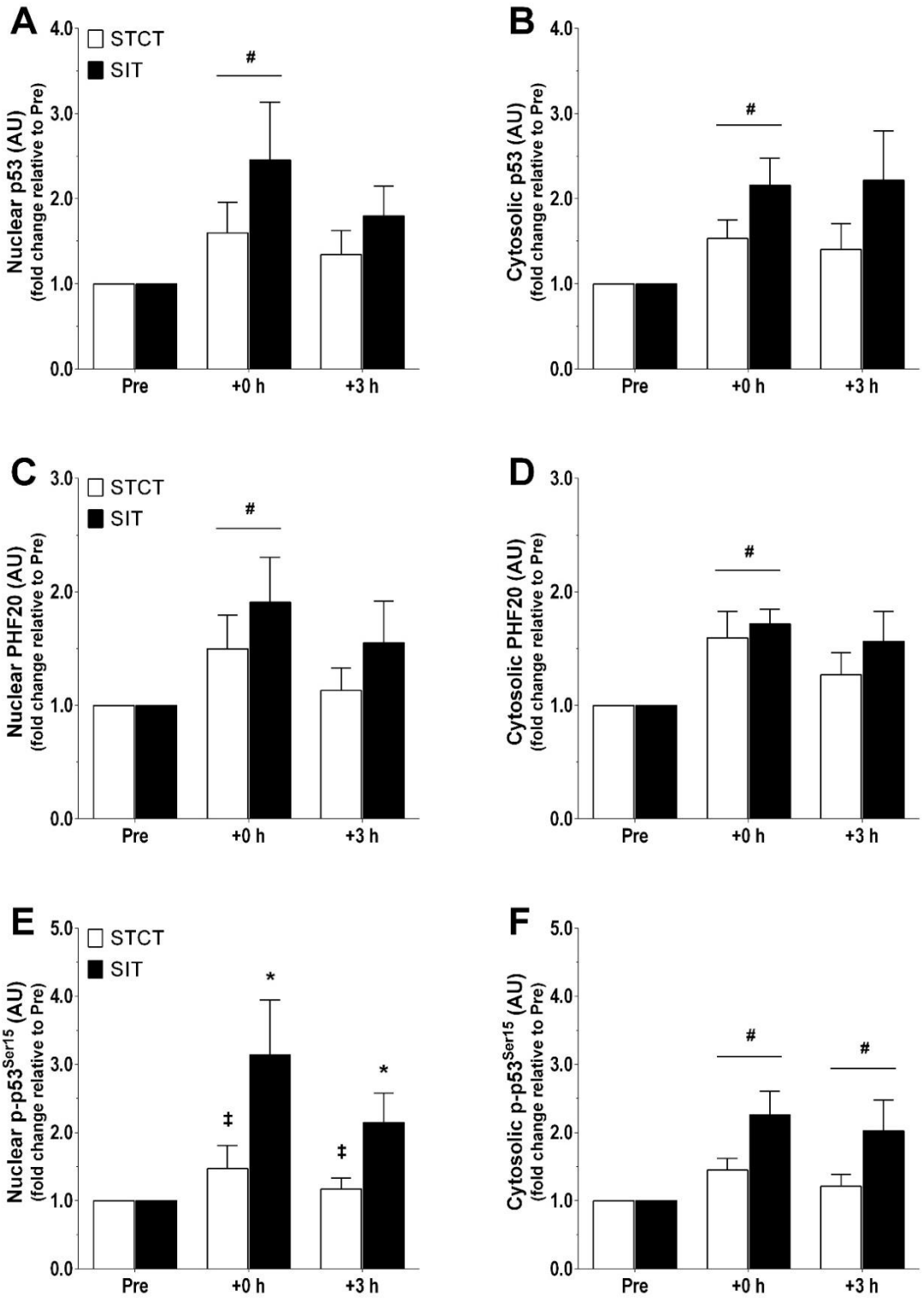

Figure 4.6. Protein content of nuclear (A) and cytosolic (B) p53, of nuclear (C) and cytosolic (D) PHF20, and of nuclear (E) and cytosolic (F) p-p53^{Ser15} before (Pre), and immediately (+0 h) and 3 h (+3 h) following the STCT and SIT exercise trials. $^{\#}$Significant main effect of time ($P < 0.05$) *vs.* Pre. *Significantly different ($P < 0.05$) *vs.* Pre, ‡ *vs.* SIT at the same time point. n=9 for each group. All values are mean ± SEM.

Table 4.4. Fold change compared to Pre, following the STCT and SIT exercise trials, for the mRNA content of AIF, DRP1, and MFN2 expressed relative to cyclophilin, GAPDH and TBP housekeeping genes.

Gene	Time	STCT (n=9)	SIT (n=9)
AIF	+0 h	1.24 ± 0.36	1.47 ± 0.98
	+3 h[#]	1.19 ± 0.28	1.56 ± 0.98
DRP1	+0 h[#]	1.16 ± 0.34	1.22 ± 0.31
	+3 h	1.03 ± 0.22	1.20 ± 0.43
MFN2	+0 h[#]	1.36 ± 0.50	1.37 ± 0.57
	+3 h	1.27 ± 0.41	1.18 ± 0.72

STCT: sub-lactate threshold continuous training; SIT: sprint interval training; AIF: apoptosis-inducing factor; DRP1: dynamin-related protein 1; MFN2: mitofusin 2; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; TBP: TATA-binding protein. [#] Significant main effect of time ($P < 0.05$) *vs.* Pre. All values are mean ± SD.

4.4 Discussion

The aim of this study was to investigate whether, like PGC-1α, a single bout of exercise would increase the protein content (and phosphorylation) of p53 in the nucleus. A second aim was to determine whether the greater physiological stress associated with an increase in exercise intensity would influence these changes. This study reports, for the first time, an exercise-induced increase in the nuclear abundance of p53 in human skeletal muscle, which was also accompanied by an increase in the nuclear abundance of PHF20, a protein that stabilises and activates p53 [65]. Although there was not a clear effect of intensity on the exercise-induced increase in nuclear p53 content, only SIT, and not STCT, was associated with a post-exercise increase in the phosphorylation of p53 at serine 15 (a post-translational modification that regulates p53 activity and stability [71, 289]), in the nuclear fraction. Similarly, only SIT, and not STCT, was associated with a post-exercise increase in the nuclear content of PGC-1α.

PGC-1α

This study demonstrates an increase in nuclear PGC-1α protein content immediately after the termination of SIT, but not STCT, with this increase still apparent 3 hours after the cessation of SIT. These findings are in agreement with previous research showing that a similar bout of SIT increased nuclear PGC-1α protein content to a similar magnitude after 3 h of recovery [159]. In the present study however, this increase was also present immediately post-exercise. The rapid increase in nuclear PGC-1α protein content following SIT in this study provides further support that nuclear PGC-1α accumulation may represent the initial phase of the adaptive response to exercise [351].

In contrast to SIT, STCT (24 min at ~65% of $\dot{V}O_{2Peak}$) did not appear to provide a sufficient physiological stimulus to induce a significant increase in nuclear PGC-1α accumulation. Findings from previous studies are conflicting; whereas cycling for 60 min at 74% of $\dot{V}O_{2Peak}$ was shown not to alter the nuclear content of PGC-1α [184], cycling for 90 min at 65% of $\dot{V}O_{2Peak}$ resulted in a 54% increase immediately post-exercise [160]. While the reasons for these discrepancies are not readily apparent, they may relate to the many methodological differences (e.g., bout duration, the participants' fitness levels, the subcellular fractionation protocol employed) between studies. Moreover, due to the large amount of skeletal muscle required for nuclear fractionation, the results from Little *et al.* (2010) were based on a small number of participants (n=4); therefore, the intrinsic variations between participants' responses may have confounded the outcome. While further research is required, results to date seem to indicate that there is not a large increase in nuclear PGC-1α protein content immediately after moderate-intensity (65-75% of $\dot{V}O_{2Peak}$) exercise.

This study provides evidence that the exercise-induced increase in nuclear PGC-1α protein content might be influenced by exercise intensity. Results from Table 4.3 show that while exercise duration and energy expenditure were 1.7- and 3.6-fold greater during STCT, mean and maximal exercise intensity were 3.3- and 4.7-fold greater during SIT. This seems to suggest that exercise intensity, rather than exercise duration or energy expenditure, may be important factors affecting nuclear PGC-1α accumulation. Moreover, the 3-fold greater blood [La^-] values recorded following SIT may also indicate that physiological stress is another important factor associated with the increase in nuclear

PGC-1α protein. Results from the cytosolic pool, are also consistent with an exercise-intensity effect in the regulation of PGC-1α protein. Similar to the nuclear fraction, only SIT induced an increase in cytosolic PGC-1α protein content; however, this increase took place only after 3 h of recovery. The delayed increase in cytosolic PGC-1α compared with nuclear PGC-1α, seems to provide further evidence that the initial phase of the exercise-induced adaptive response may indeed take place in the nucleus [351].

The combined results from the nuclear and cytosolic pool do not seem to directly support translocation of PGC-1α between the two cellular compartments, as previously hypothesised [351]. However, no study to date has provided convincing evidence of PGC-1α translocation from the cytosol to the nucleus using cellular fractionation and immunoblotting. Studies have either not measured PGC-1α in the cytosol and only reported an increase in nuclear PGC-1α content [351], or have observed no change [160], or an increase [9], in cytosolic PGC-1α alongside an increase in the nuclear fraction. The greater PGC-1α content in the cytosol, coupled with the lack of sensitivity of the immunoblotting technique to detect small changes from the larger cytosolic pool, have been suggested as a possible reason for failing to show evidence of PGC-1α translocation [160]. Another possible explanation for the concomitant increase in nuclear and cytosolic PGC-1α content in this study may relate to an increase in PGC-1α stability. It has been demonstrated that PGC-1α protein has a short half-life [9, 214], and that direct PGC-1α phosphorylation by p38 MAPK increases its half-life by stabilising the PGC-1α protein [241]. Unfortunately, phospho-specific PGC-1α antibodies are not commercially available so further testing of this hypothesis is currently limited by technical challenges. Any factor promoting PGC-1α stability, such as p38 phosphorylation, would likely result in greater PGC-1α protein content independent of translocation. It was hypothesised that SIT would be associated with greater p38 phosphorylation compared to STCT; unfortunately, due to time constraints, it was not possible to test this hypothesis prior to submission of this thesis. Although p38 phosphorylation does not seem to be regulated in an intensity-dependent manner in whole-muscle lysates [55, 73], it may be possible that exercise intensity differentially regulates nuclear and cytosolic phosphorylation of p38. Finally, a model has been suggested whereby phosphorylation of p38 following exercise results in the activation of PGC-1α and its subsequent translocation to the nucleus where it initiates the adaptive response [351]. It is therefore important that future research should

also measure the association between PGC-1α and the import proteins that potentially regulate PGC-1α nuclear/cytosolic shuttling.

The PGC-1α protein has been shown to activate its own promoter through a feed-forward loop [102]; as a result, increased stability of nuclear PGC-1α would further enhance PGC-1α transcriptional activity before degradation. However, although nuclear PGC-1α protein content was only increased following SIT, no effect of exercise intensity on the exercise-induced increase in PGC-1α mRNA after 3 h of recovery was observed. The timing and magnitude of this increase are in accordance with findings from previous research employing similar exercise intensities [57, 159]. The similar increase in PGC-1α mRNA following STCT and SIT is also in agreement with findings from previous research [72]. By comparing high-intensity interval training (HIIT) at 73, 100 and 133% of W_{Peak}, these authors demonstrated that the exercise intensity-dependent regulation of PGC-1α gene expression, which had been previously reported [73], is limited to submaximal (i.e., < W_{Peak}) exercise intensities. The similar increase in PGC-1α mRNA following STCT (exercise intensity < W_{Peak}) and SIT (exercise intensity > W_{Peak}) in this study, is consistent with the findings from Edgett *et al.* (2013).

The similar increase in PGC-1α mRNA between groups also seems to suggest that factors other than increased nuclear PGC-1α may play a role in the upregulation of PGC-1α gene expression. A possible factor is the activation of AMPK following exercise, a key signalling event in the modulation of exercise-induced mitochondrial biogenesis [185]. This modulation leads to an increase in PGC-1α mRNA, either by direct AMPK phosphorylation of the PGC-1α protein [130], or via AMPK regulation of multiple control points in the PGC-1α feed-forward loop [185]. Therefore, the phosphorylation of ACC, a downstream target and commonly used marker of AMPK activation [130] was measured. While previous studies reported increased cytosolic p-ACC^{Ser79} following both continuous [160], and sprint interval [159] exercise, or increased nuclear AMPK phosphorylation following continuous exercise [186], the present research directly compared the effects of different exercise intensities on p-ACC^{Ser79} in subcellular fractions. This study shows that STCT and SIT similarly increased nuclear (and cytosolic) p-ACC^{Ser79} post-exercise, followed 3 h later by an increase in PGC-1α mRNA that was also similar between groups. The similar increase in p-ACC^{Ser79} in this study is in

agreement with the hypothesis that the blunted response of PGC-1α mRNA at supramaximal intensities may indicate that its signalling-mediated activation may also be blunted [72].

p53

The tumour suppressor p53 is another key transcription factor influencing the metabolic and transcriptional control of mitochondrial biogenesis [266], which is modulated by exercise [18, 265]. The present study demonstrates that nuclear (and cytosolic) p53 protein content was increased immediately post-exercise, with no significant difference between the two exercise intensities. Although Saleem & Hood, (2013) observed a decrease in nuclear p53 protein content after a 90-min exhaustive exercise bout in mice, the results from this study agree with previous findings in mice reporting increased nuclear p53 protein content immediately after a 60-min bout of running [227], and 1 hour after a 20-min bout of intermittent eccentric muscle contractions [45]. These findings are also in agreement with the generally-accepted notion that cellular stress is associated with accumulation of p53 protein in the nucleus [178, 215], a process that has been attributed to post-translational events such as nuclear/cytosolic shuttling, and/or increased p53 protein stability [215].

While the C terminus of p53 contains a cluster of several nuclear localisation signals (NLS) that mediate the migration of p53 into the cell nucleus [286], the concomitant increase in nuclear and cytosolic p53 in this study does not provide direct evidence of translocation. However, p53 cellular translocation is a tightly-regulated process that requires a complex series of protein-protein interactions [178], and the help of import and export receptors [330]. This suggests that p53 trafficking between these two compartments cannot simply be assessed by measuring changes in p53 protein content in the two separated pools. A possible explanation for this concomitant increase may relate to an increase in p53 stability [215]. In this regard, p53 has been shown to have an extremely short half-life [250], and cellular stress has been reported to increase its half-life and stability [139, 175]. Consistent with these findings, p53 accumulation in both cellular pools may have been the result of an increased p53 stability in response to the increased physiological stress imparted to the cell by a single bout of exercise.

PHF20 is a multidomain protein responsible for increasing p53 stability through direct interaction in a methylation-dependent manner [65]. This study reports an increase in both nuclear and cytosolic PHF20 protein content immediately post-exercise, with no difference between exercise intensities. In unstressed cells, nuclear p53 is bound to murine double minute-2 (MDM2), a p53 negative regulator that ubiquitinates p53 and induces p53 cytosolic degradation by the proteasome [105, 177, 330]. Upon cellular stress PHF20 interacts with p53 and enhances p53 stability by diminishing the MDM2-mediated p53 degradation [65]. Although the direct interaction between PHF20 and p53 has not been measured in this study, the concomitant and similar increase in these two proteins in both compartments may indicate an increased PHF20-p53 interaction, and a subsequent increase in p53 stability [65]. Future research is required to better understand the molecular events regulating p53 stability, degradation, and the subsequent nuclear/cytosolic shuttling following exercise.

An additional factor affecting p53 stability is phosphorylation at serine 15 (p-p53^{Ser15}) [289]. The present study demonstrates, that nuclear p-p53^{Ser15} was increased immediately post-exercise only following SIT - an increase that persisted until 3 h of recovery. Cytosolic p-p53^{Ser15} was also increased at both time points, but the difference between the groups did not reach significance. The increase in p-p53^{Ser15} only following SIT, which was associated with greater physiological stress than STCT, supports the notion that cellular stress leads to an increase in p-p53^{Ser15} [289]. The only two studies investigating p-p53^{Ser15} following endurance exercise with humans observed whole-muscle p-p53^{Ser15} to increase only after 3 h of recovery, regardless of training modality [18] or carbohydrate availability [19]. The earlier increase in nuclear p-p53^{Ser15} in this research (+0 h), compared with that in whole-muscle p-p53^{Ser15} in the two studies above (+3 h) [18, 19], indicates that, similar to PGC-1α, an increase in nuclear p-p53^{Ser15} may represent the initial phase of the p53-mediated exercise-induced adaptive response. The signalling kinases AMPK [136] and p38 MAPK [287] have also been shown to regulate p-p53^{Ser15}. The lack of an exercise intensity effect on the increase in p-ACC^{Ser79}, a marker of AMPK activation [130], could indicate that other signalling proteins upstream of p53 (e.g., p38 MAPK) may have contributed to the different upregulation of p53 phosphorylation between STCT and SIT. Further research to test this hypothesis is warranted. Overall, these findings seem to further support that an increase in p53 stability

may have been responsible for the increase in p53 protein content observed in the present study.

PHF20 can also act as a transcription factor, and has been shown to activate and regulate p53 gene expression [218]. Nonetheless, despite an increase in PHF20 protein content following both type of exercise, there was no significant change in p53 mRNA content within 3 h from termination of exercise. However, studies in cells have observed upregulation of p53 by PHF20 to take place only after 6 or 12 h [218]; this indicates that any increase in p53 mRNA following STCT or SIT in the present study, may have not taken place until after the third hour of recovery. Future research investigating exercise-induced changes in p53 mRNA 6-24 h post-exercise is warranted, especially as previous reports are scarce and controversial. While research in mice has observed a reduction in p53 mRNA immediately post-exercise [267], a study with humans reported a small increase (~1.3-fold) following 3 h of recovery, with no difference between the three exercise intensities investigated [72]. When considering these findings, two observations must be made. First, changes in the rate of p53 gene transcription have been suggested to play a minor role in the induction of the p53 response to cellular stress [215]; second, regulation of p53 activity is mainly related to the stress-induced post-translational stabilisation of the p53 protein [177, 215]. The lack of change in p53 mRNA, and the increase in p53 and p-p53^{Ser15} in this study, are consistent with these observations. This proposed mechanism of action is especially attractive, as a rapid increase in p53 protein content without the need for de novo transcription, would be particularly advantageous in cells undergoing oxidative stress [215].

p53 is an inducible transcription factor regulating a host of mitochondrial genes [266]. Studies have revealed the presence of a p53 binding site in the promoter region of the PGC-1α gene [124], as well as DRP1 [154] and MFN2 [335] - two key proteins involved in the regulation of mitochondrial remodelling [266]. A p53 response element has also been shown in the AIF gene [298], an apoptotic protein assisting in the assembly and stabilization of complex I [319]. In agreement with an increase in the nuclear content of p53, there was an increase in the mRNA of these 4 genes in response to exercise, with no difference between the two exercise protocols. It has been suggested that the activity of p53 as an inducible transcription factor depends on its rapid nuclear stabilization after

stress [177]; findings from this study seem to agree with this notion, and indicate that the physiological stress associated with exercise is also associated with the induction of p53 transcriptional activity.

Conclusion and significance

This research observed an increase in nuclear PGC-1α protein content following SIT but not STCT, suggesting that exercise intensity could play a key role in the induction of PGC-1α nuclear accumulation, an event thought to mediate the initial adaptive response to exercise. Moreover, and for the first time, this study demonstrates that a single exercise bout induces nuclear accumulation of p53, a transcription factor also involved in the activation of exercise-induced mitochondrial adaptations. It is suggested that this increase may relate to greater p53 stability, as demonstrated by an increase in PHF20 protein content, a p53 regulatory protein that induces p53 stability. In addition, phosphorylation of p53 at serine 15, a post-translational event also associated with increased p53 stability, increased only following SIT. Collectively, this suggests that the greater physiological stress associated with SIT may play a key role in the regulation of the p53 response, consistent with the notion that greater cellular stress induces p53 activity. Considering the important role of PGC-1α and p53 in the regulation of exercise-induced mitochondrial biogenesis, these findings could have important implications for the design of therapeutic exercise programs to prevent and treat a variety of metabolic and age-related diseases.

Chapter 5

Conclusions and future directions

The overall aim of this thesis was to investigate the effects of both training intensity and training volume on markers of mitochondrial biogenesis in human muscle, and to provide a better understanding of the molecular mechanisms mediating exercise-induced mitochondrial adaptations. The aim of the first study (described in Chapter 2) was to investigate the role of training intensity, by comparing a 4-week training protocol at either moderate (STCT), high (HIIT), or maximal (SIT) training intensity. The purpose of the second study (described in Chapter 3) was to examine the role of training volume by comparing three subsequent periods of normal (NT), intensified (INT), and reduced (RT) training volume. Finally, the third study (described in Chapter 4) compared the effects of two different exercise intensities (moderate and maximal) on the molecular mechanisms mediating the exercise-induced mitochondrial response in enriched cytosolic and nuclear fractions.

5.1 Summary of key findings

Chapter 2:

- Training intensity is a key factor regulating training-induced changes in mass-specific mitochondrial respiration, as demonstrated by an increase in this parameter only following four weeks of SIT but not HIIT or STCT.
- Four weeks of SIT are sufficient to induce improvements in mt-specific respiration (an indicator of mitochondrial quality).

- Training intensity is not a key determinant of changes in mitochondrial content, as suggested by the lack of change in CS activity following all three training protocols.

- Changes in the protein content of key transcription factors, such as PGC-1α, p53, and PHF20, seem to be more strongly associated with changes in mass-specific mitochondrial respiration than mitochondrial content.

- There appears to be an apparent dissociation between changes in mitochondrial content and mitochondrial respiration, suggesting that these changes may not be mediated by the same molecular mechanisms.

- Mitochondrial respiration is a good determinant of baseline endurance performance, but is not strongly associated with training-induced changes in endurance performance.

Chapter 3:

- Training volume is an important determinant of exercise-induced changes in mitochondrial content

- Increased mass-specific mitochondrial respiration following INT was the result of an increase in mitochondrial content, as indicated by the unchanged mt-specific respiration.

- Following a reduction in training volume, mass-specific mitochondrial respiration is reversed more rapidly than mitochondrial content, providing further evidence that these two parameters are not necessarily associated.

- Training-induced mitochondrial adaptations are rapidly reversed following a period of reduced training, suggesting that maintenance of the training stimulus is necessary to preserve the gains obtained during exercise training.

- Changes in the protein content of TFAM may be primarily associated with changes in mitochondrial content, whereas, consistent with results from Chapter

2, changes in the protein content of PGC-1α, p53 and PHF20 seem to be associated with changes in mass-specific mitochondrial respiration.

- Human skeletal muscle is a remarkably plastic tissue, rapidly adapting to changes in training stimulus and to the metabolic and energy requirements of the cell.

Chapter 4:

- Exercise intensity is an important determinant of the induction of the initial adaptive response to exercise mediated by PGC-1α, as demonstrated by the rapid increase in the nuclear content of PGC-1α following SIT, but not STCT.
- p53 protein content is increased in the nucleus immediately following exercise, suggesting that nuclear accumulation of p53 may mediate the initial phase of the p53-regulated response to exercise in human skeletal muscle.
- Nuclear PHF20 protein content also increased immediately post-exercise, indicating that nuclear accumulation of p53 following exercise may relate to increased p53 stability, an effect that could be mediated by an increase in the PHF20-p53 interaction [65].
- Exercise intensity is also an important determinant of the exercise-induced phosphorylation of p53 at serine 15 in the nucleus, a post-translational event also associated with increased p53 stability and activity [71].
- The greater physiological stress associated with SIT may play an important role in the regulation of both the PGC-1α and p53 response to exercise.

5.2 The role of exercise intensity: findings and future directions

This research has provided extensive evidence that exercise intensity is an important factor regulating exercise- and training-induced changes in markers of mitochondrial

biogenesis. Specifically, study 1 (described in Chapter 2) showed that 4 weeks of SIT, but not STCT or HIIT, induced an increase in mitochondrial respiration. It has been shown that mitochondrial oxidative capacity is in excess of maximal oxygen delivery in humans [28]; however, this study shows that maximal-intensity training was still able to increase mitochondrial respiration, despite this apparent overcapacity. Future research aiming to explain this phenomenon is warranted. While only maximal training intensity was associated with an increase in mitochondrial respiration, previous studies have shown that lower training intensities can induce an increase in mitochondrial respiration [67, 127, 324]. It must also be noted that the training intensity associated with HIIT in this research, which did not induce an increase in mitochondrial respiration, was 89% of W_{Peak}, whereas previous studies observing an increase in mitochondrial respiration adopted a training intensity of ~105-120% of W_{Peak}. Therefore, it is important that future research attempts to determine if an intensity threshold is required to promote changes in mitochondrial respiration, and if individuals' fitness levels may influence these changes.

A key finding of this research was that SIT improved mt-specific respiration, an indicator of mitochondrial quality. While previous cross-sectional research observed differences in mitochondrial quality (as well as quantity) across a range of aerobic fitness levels [128], this research is the first to show that improvements in mitochondrial quality can be achieved in as little as four weeks. Considering SIT was associated with a training intensity of ~200% of W_{Peak}, and that training intensities of ~105-120% of W_{Peak} [67, 127, 324] did not results in increased mt-specific respiration, future research should aim to determine if only maximal training intensity results in an improved mitochondrial quality, or if lower training intensities are still able to provide the same benefits.

The study described in Chapter 2 also showed an apparent dissociation between mitochondrial content and mitochondrial respiration, consistent with previous finding in mouse skeletal muscle [252]. While leading authors in the field have clearly suggested that "it is important and necessary to differentiate mitochondrial content from function in future studies" [128], this research provides the first evidence of such apparent dissociations in human skeletal muscle. It is important for future research to better understand the mechanisms behind this dissociation. This could be achieved by assessing the exercise-induced regulation of mitochondrial quality [353], in parallel with direct

measurement of mitochondrial content by TEM. Given evidence is starting to emerge about the effects of exercise on the modulation of mitochondrial turnover processes such as autophagy [173, 198, 268], and mitochondrial morphology [42, 224, 266], future research should focus on these topics, and also try to define the role of training intensity (and training volume) on these important mechanisms. The exercise-induced replacement of damaged proteins with de novo synthesised ones, would also induce an increase in mitochondrial respiration without a change in mitochondrial content [173]. Therefore, measurements of mitochondrial protein synthesis [61] and degradation should also be integrated in future training studies investigating if training intensity (and training volume) modulate this dissociation in human skeletal muscle.

From a cellular standpoint, this research suggests that the transcription factors PGC-1α, p53, and PHF20 may be more strongly associated with changes in mitochondrial respiration than mitochondrial content. While studies investigating the transcriptional activity of PGC-1α and p53 are extensive, research on their role as regulators of metabolism [140, 181, 329] and mitochondrial turnover [42, 77, 266, 320] is starting to emerge. A better understanding of their metabolic and mitochondrial turnover regulatory function would not only help explain their associations with improved mitochondrial respiration, but could also explain the dissociation between mitochondrial function and content. It is known that exercise intensity dictates the number and type of fibres recruited during exercise [327]; therefore a better understanding of the mechanisms of activation of PGC-1α and p53 transcriptional and regulatory activity in single muscle fibres in response to different exercise intensities is required.

Results from the study described in Chapter 4 seem to confirm that the initial phase of the exercise-induced adaptive response may be modulated by increased nuclear content of PGC-1α [351]. Moreover, this is the first research to show that this modulation is affected by exercise intensity. In addition, and for the first time in human skeletal muscle, this study demonstrates that nuclear p53 protein content is also rapidly increased following exercise (although the intensity effect did not reach significance). This suggests that nuclear activation of p53, similar to PGC-1α, may represent the initial phase of the adaptive response to exercise. Future research is needed to test this hypothesis. This study also provides preliminary evidence that the increase in nuclear p53 may have been the

result of increased p53 stability, as suggested by the increase in nuclear PHF20 [65]. Similarly, increased phosphorylation of p53 at serine 15 is also indicative of enhanced p53 stability [289] as well as an increase in p53 activity [71]. This is the first report to show that in human skeletal muscle the physiological stress associated with exercise may induce p53 stabilisation, and promote the activation of the p53 response, in agreement with the notion that cellular stress is an activator of p53 [215]. Contrary to the increase in p53 and PHF20 however, the nuclear increase in p-p53^{Ser15} showed a significant effect of exercise intensity. Considering these events are closely linked, future research should aim to reveal the cellular mechanisms leading to this differentiations. Finally, not only increased stability, but also nuclear translocation, may represent a possible mechanism for nuclear p53 accumulation. While this has been demonstrated in cells [177], future research is needed to better understand the effects of exercise on the MDM2-mediated inhibitory effect on p53 [105] and how this would influence p53 stability and nuclear/cytosolic shuttling.

5.3 The role of training volume: findings and future directions

Study 2 (described in Chapter 3) clearly highlights that human skeletal muscle is a remarkably plastic tissue, rapidly adapting to changes in training stimulus and to the metabolic and energy requirements of the cell. A key finding of this study was that exercise volume is an important factor regulating training-induced changes in markers of mitochondrial content. Specifically, it was shown that 3 weeks of a very large training volume induced an increase in CS activity and the protein content of subunits of the ETS, two valid biomarkers of mitochondrial content [145]. Future research employing TEM, which provides a direct measurement of mitochondrial content, would further strengthen this hypothesis. Moreover, and contrary to the initial hypotheses and previous literature [99], the large training volume in study 2 did not reduce endurance performance nor markers of mitochondrial biogenesis. The likely explanation for these findings may relate to CHO supplementation having delayed the symptoms of overreaching [100]. While this constitutes a finding in itself (i.e., the symptoms of overreaching may be prevented, or at

least delayed, if high CHO levels during a period of intensified training are maintained), future research is needed to confirm these hypotheses.

This research shows that a large training volume was also associated with an increase in mass-specific mitochondrial respiration. However, in contrast to a greater training intensity, which induced an increase in mt-specific respiration, an increase in training volume did not alter mt-specific respiration. These findings indicate that training volume induces improvements in mitochondrial respiration by an effect mediated by increased mitochondrial content (unchanged mitochondrial quality). When combined, findings from study 1 and 2 suggest that mitochondrial respiration can be improved either as an effect of higher training intensity (resulting in increased mt-specific respiration), or as an effect of larger training volumes (resulting in unchanged mt-specific respiration). These findings also indicate that when training intensity is similar (HIIT in study 1 and INT in study 2), a marked increase in training volume represents a sufficient stimulus to improve mitochondrial respiration. Further research confirming these hypotheses is required.

Another important finding from study 2 is that training-induced mitochondrial adaptations are rapidly reversed following a period of reduced training, as all the parameters measured in the muscle (except for CS activity) were not different from baseline after the 2-week reduced-volume phase. This clearly highlights that maintenance of the training stimulus is necessary to preserve the gains obtained during exercise training. Future research investigating the kinetics and mechanisms of this reversibility is required. The different kinetics of reversibility between mitochondrial content and mitochondrial respiration presented in study 2, further confirmed the presence of an apparent dissociations between these two parameters. The reader is referred to section 5.2 for a discussion on this topic

This study also indicates that the protein content of key transcription factors associated with mitochondrial biogenesis rapidly responded to a large increase (and a reduction) in training volume. Specifically, study 2 shows that changes in the protein content of TFAM may be primarily associated with changes in mitochondrial content, and confirms results from study 1 about a possible association between changes in the protein content of PGC-1α, p53, and PHF20 and mass-specific mitochondrial respiration. Moreover, the similar changes in the protein content of PGC-1α, NRF1 and TFAM suggest that training volume

may increase mitochondrial content through the activation of the PGC-1α-NRF1-TFAM axis [352, 122]. Considering that the relationships between transcription factors and markers of mitochondrial biogenesis suggested above can, for obvious reasons, only be associative in human skeletal muscle, future research in cell or animal models is needed to provide a clear mechanistic insight confirming these hypotheses.

5.4 Significance and practical applications

This research has helped define the role of training intensity and volume in the adaptive response to endurance exercise, and has provided important mechanistic insights into the cellular processes involved. The above findings provide an important contribution to the body of available literature, and will have a significant impact in the design of therapeutic exercise protocols and athletic training programs.

In a society plagued by diseases linked with mitochondrial dysfunction [164, 168, 197, 339], the increased knowledge provided by this thesis could represent a powerful tool for the prevention and treatment of an ever-growing number of lifestyle and metabolic diseases. Prevention and management of these diseases would undoubtedly help relieve the health and economic burden of the 21st century society.

Moreover, the greater financial incentives and growing pressure on elite athletes to perform at a high level, have brought an increased focus on developing new training strategies to optimise training-induced adaptations. Given the pivotal role of training intensity and training volume in the design of athletic training programs [12], the findings of this research provide information that will influence and improve the current best practice.

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Appendices

GENERIC APPLICATION FORMS

VICTORIA UNIVERSITY | A NEW SCHOOL OF THOUGHT

PRE – STUDY QUESTIONNAIRE

Personal Information

NAME:_____

ADDRESS: _____

_____

PHONE: _____(H) _____(W)

_____(MOB)

AGE:_____ DATE OF BIRTH:_____ SEX: M F

EMAIL ADDRESS: _____

Training Background

-Main Sport (s):_____

-Years Training or Competing: _____

-Other Sporting Background (running, gym, team events):_____

_____

-A typical week of training for you is: (please refer to a recent and representative week)

Day	Morning	Afternoon
Monday		
Tuesday		
Wednesday		
Thursday		
Friday		
Saturday		
Sunday		

1

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PHYSIOLOGICAL TESTING

Pre-Test Instructions

To ensure controlled pre-test preparation and to minimise those factors which can affect your performance during physiological testing, please follow the guidelines set out below.

1. No training inducing severe fatigue in the 36 hours prior to testing.
2. No physical activity on the day of the test prior to the appointment.
3. No food, cigarettes or caffeine intake 2 hours prior to testing.
4. No alcohol on the day of the test.
5. Restrict fluid intake to water (~200mL/hour) for 2 hours prior to testing.
6. Empty your bowel and bladder immediately prior to testing.
7. Wear light, comfortable clothing and your normal jogging/cycling shoes.
8. Do not take any dietary supplements (e.g., iron tablets) on the day of the test.

- Bring with you any medication you may require for a running exercise test to exhaustion, e.g. Ventolin inhaler.
- Shower facilities are available, so you may wish to bring with you the appropriate gear (soap, towel etc.) and a change of clothing.

Please inform the person in charge of testing if you are currently taking any form of medication or have any injury or illness which may affect test performance.

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[*please note: Where the participant/s are aged under 18, separate parental consent is required; where the participant/s are unable to answer for themselves due to mental illness or disability, parental or guardian consent may be required.]

CARDIOVASCULAR AND OTHER RISK FACTORS QUESTIONNAIRE

In order to be eligible to participate in the experiment investigating:

"Effects of different training interventions on endurance performance and muscle adaptations"

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** **Gender:** **M** **F**

Give a brief description of your average activity pattern in the past 2 months:

_____

_____

_____

Circle the appropriate response to the following questions.

1.	Are you overweight?	Yes	No	Don't know
2.	Do you smoke?	Yes	No	Social
3.	Are you an asthmatic?	Yes	No	Don't Know
4.	Are you a diabetic?	Yes	No	Don't Know
5.	Does your family have a history of diabetes?	Yes	No	Don't Know
6.	Do you have a thyroid disorder?	Yes	No	Don't Know
7.	Does your family have a history of thyroid disorders?	Yes	No	Don't Know
8.	Do you have a pituitary disorder?	Yes	No	Don't Know
9.	Does your family have a history of pituitary disorders?	Yes	No	Don't Know
10.	Do you have a heart rhythm disturbance?	Yes	No	Don't Know
11.	Do you have a high blood cholesterol level?	Yes	No	Don't Know

1

12. Do you have elevated blood pressure? Yes No Don't Know

13. Are you being treated with diuretics? Yes No

14. Are you on any other medications? Yes No

List all medications? (including oral contraceptives) _____

_____

15. Do you think you have any medical complaint or any other reason which you know of which you think may prevent you from participating in strenuous exercise? Yes No

If Yes, please elaborate _____

16. Have you had any musculoskeletal problems that have required medical treatment (e.g. broken bones, joint reconstruction etc)? Yes No

If Yes, please provide details (including dates) _____

17. Are you currently pregnant or expect to become pregnant during the time in which this experiment is conducted?

Yes No

18. Does your family have a history of premature cardiovascular problems (e.g. heart attack, stroke)? Yes No Don't Know

I, _____, believe that the answers to these questions are true and correct.

Signed: _____ Date: _____

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MUSCLE BIOPSY, ARTERIAL & VENOUS CATHETERISATION QUESTIONNAIRE:

Effects of different training interventions on endurance performance and muscle adaptations

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 _____

1

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 Raynauds 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: _____

FORMS FOR INDIVIDUAL RESEARCH PROJECTS

In the following pages the "Information to Participants Involved in Research" and the "Consent form to Participants Involved in Research" forms are presented for the 2 research projects completed during the course of this PhD (ethics clearance number: HRETH 11/94, and HRETH 12/189).

Findings described in Chapter 2 use data obtained from project HRETH 11/94 (the phase described in research project A as NT was presented as the HIIT group for the purpose of this Chapter), and project HRETH 12/189 (STCT and SIT groups).

Findings described in Chapter 3 use data obtained entirely from project HRETH 11/94 (NT, INT and RT phases). Results for the NT phase have been presented in this chapter, but have not been discussed, as they have been discussed as part of Chapter 2 (as the HIIT group).

Findings described in Chapter 4 use data obtained entirely from project HRETH 12/189 (STCT and SIT groups).

Project HRETH 11/94 was conducted prior to project HRETH 12/189.

Project HRETH 11/94

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INFORMATION TO PARTICIPANTS INVOLVED IN RESEARCH

You are invited to participate in a research project entitled:

Effects of different training interventions on endurance performance and muscle adaptations

This project is being conducted by a student researcher Cesare Granata as part of a PhD at Victoria University under the supervision of Prof. David Bishop [School of Sport and Exercise Science and ISEAL].

Project explanation

The project aims to investigate the effects of different amounts of interval training on endurance performance and changes in your mitochondria (note: Mitochondria are our muscles' power station, producing the energy required to enable your muscles to contract). The reason we are conducting this study is to be able to understand the physiological mechanisms involved in generating the changes in endurance performance and the mitochondria. This information will be used to design better training programs to improve endurance performance. This information also has important implications for health as the mitochondria have been shown to be involved in a wide range of neurodegenerative diseases (such as Alzheimer's, Parkinson's and Gehrig's) and lifestyle diseases (such as diabetes and obesity).

What will I be asked to do?

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

- 11 testing session spread across approximately 14 weeks (see below for details) lasting approximately 1 h (60 min) each. In 2 of these 11 sessions you are required to spend about 4 h for the testing session to be completed.
- About 55 to 60 training sessions divided in 3 different phases across approximately 14 weeks (see below for details). Each training session will last between 20 and 50 min

We will first ask you to fill in several short questionnaires about your family medical history and your exercise habits to determine your ability to participate in this study which is divided in three phases:

In phase one you will be asked to take part in four testing sessions separated by at least 48 h before the first training phase will commence. These testing sessions will involve a familiarization of a 20-km cycling Time Trial (where you are required to cover 20 km in the shortest possible time; this should last between 35 and 50 min). The second testing session consists of a VO_{2Peak} test, (where you will cycle at increasing velocity until you are not able to sustain the effort; this test usually lasts 25-35 min). You will then be asked to repeat the 20 km cycling Time Trial, which will be used as the baseline performance test. In your last visit we will ask your permission for a medical doctor to take a muscle biopsy (for details see below) and then you will take part in a 10km cycling Time Trial familiarization test (which should last 15 to 25 min). At least 48 to 72 hours after this test, the Interval Training period will begin and you will be required to train 3 times a week for 4 weeks. Each session will consist of a series of 4-min periods of high intensity cycling followed by 2 min of low-intensity cycling. This will be followed by three testing sessions (similar to the pre-interval training sessions) in order to monitor changes in your fitness levels and muscle adaptations since the beginning of the training.
In the second training phase you will be asked to continue the interval training sessions in an intensified but controlled manner for a period lasting approximately 3 weeks. This will involve the same type of training as before but you will be required to train twice a day for the whole period. At the end of this phase you will take part in two more testing sessions (again similar to the previous ones) in order to monitor changes in your muscle and fitness levels.

1

In the third and last phase, you will take part in a period of reduced training lasting two weeks where you will be asked to train three times a week. This period is then followed by 2 final tests (the 20km cycle Time trial and a muscle biopsy) to monitor performance and physiological changes.

All testing and training sessions will be performed on a stationary cycle connected to a computer showing to you all the details of your ride. These are located in the exercise physiology laboratory at Victoria University and all sessions will run under the supervision of an exercise physiologist. At four different time points, you will be asked for your permission to let a medical doctor take a small sample of your muscle (the equivalent of 3-4 rice grains) using a needle biopsy for a total of 8 biopsies. You will be asked to provide one muscle biopsy before the start of phase one, three biopsies (on the same days) before and after phase two, and a final muscle biopsy at the end of the third phase. The muscle biopsies (approximately ~80-120 mg wet mass) will be taken from the vastus lateralis muscle (the external side of your thigh) using a biopsy needle with suction. You will lie in the supine position on a bed; after injection of a local anaesthetic into the skin and fascia a small incisions will be made (when multiple biopsies are required they will be taken 1 cm apart). An experienced medical practitioner will take all biopsies. After the muscle biopsy has been performed you will continue to lie on the bed or sit in a chair with compression and ice on the small incision to reduce the possibility of bruising. Capillary blood samples will also be taken during testing sessions. This involves collection of a few drops of blood by pricking of the skin with a small lancet in either your fingertip or earlobe. A qualified scientist will perform all blood sampling.

There will be a reimbursement to all participants in order to cover for costs incurred for travel, parking or other expenses. The amount will be a total of $350 upon completion of the entire study. Should you withdraw for any reason prior to the completion of the study, a sum of $115 will be paid if you have completed the study up to the second testing phase (i.e.: post-IT testing phase; see Figure 1a); a sum of $230 will be paid if you withdraw after having completed the third testing phase (i.e.: pre-TP testing phase, see Figure 1b)

What will I gain from participating?

From participating in this study you can expect to gain good benefits for your aerobic fitness as you are going to be followed by sport scientists and will train in a state-of-the-art purpose-built training facility. You will also increase your understanding of fitness and fitness tests. You will also gain the experience of having participated in an exercise science experiment designed to increase knowledge about muscles and genes.

How will the information I give be used?

Your samples will be stored under alphanumeric codes (i.e. without your name or personal details) and only the researchers will be able to connect the samples to you. All of the muscle sample collected will be used to measure mitochondrial respiration and analyse some proteins, genes and energy sources involved with the function of your thigh muscle. In the event of any tissue remaining it will be disposed of in a de-identified container (i.e. no coding present) via incineration using Victoria University's waste disposal contractor. The blood samples, once again stored with an alphanumeric code, will be used for determination of lactate and glucose levels as well as haemoglobin and other blood parameters like haematocrit, pH ion levels etc. Other parameters we will collect like, blood pressure, Heart rate, or questionnaire data will once again stored with an alphanumeric code and only the scientists 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.

What are the potential risks of participating in this project?

The maximal incremental exercise test (GXT), the 10- and 20-km cycling Time Trial test (TT) and Interval Training all involve risks of vasovagal episodes, muscle soreness and stiffness, and sudden death due to myocardial infarction. The risk of such events is in reality very low. Due to the intense nature of the training program, there is a small risk of illness (such as colds and flu) and injury (such as muscle strains). Risk of injuries should be avoided by using the correct warm up procedures. This type of training protocol can be accompanied in rare cases by the possibility of mood changes such as irritability and depression, anxiety or stress associated with multiple muscle biopsies, blood tests, extensive time commitment to the study and intensive exercise and training protocols. These changes are however short term and can be reversed with a maximum of two to three weeks of recovery. Risks associated with muscle biopsy include discomfort, pain, bruising, bleeding, soreness, localised altered sensation

of skin (reduced/absent/tingle/hypersensitive) and infection. Risks associated with blood sample include slight discomfort, with the possibility of bruising and infection

How will this project be conducted?

Participants will initially be screened for cardiovascular risk factors and any health issues of relevance to the study. There will be three main phases in the study: Phase 1 (Interval Training), Phase 2 (Intensified Training) and phase 3 (Taper) which are summarised in Figure 1a and 1b.

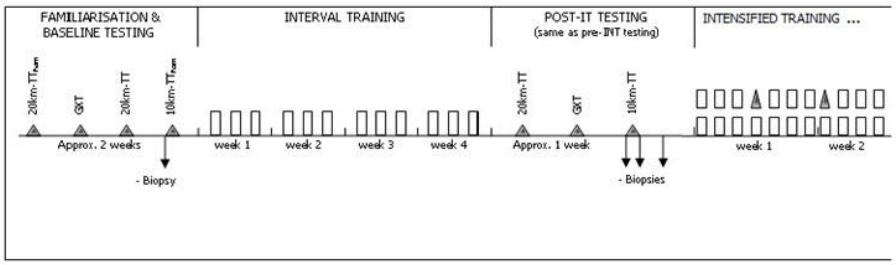

Figure 1a: study first half

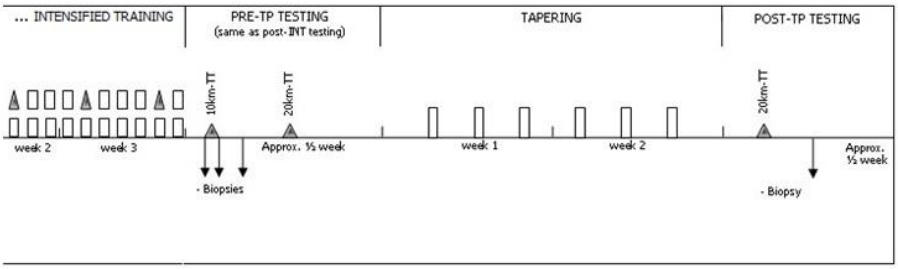

Figure 1b: study second half

Phase 1: Interval Training (IT)

Prior to commencement of the training you will visit the lab 4 times. All visits will be separated by a minimum of 48 to 72 hours. During the first visit you will be asked to perform a 20-km cycle time trial familiarisation test. During the second you will undertake a maximal incremental exercise test to exhaustion for baseline determination of peak oxygen uptake (VO_{2Peak}) and the lactate threshold on a stationary cycle. A capillary blood sample will be taken at rest, during exercise and in recovery. During your third visit you will perform the 20-km cycle time trial which will be used as a baseline performance measurement. During the last visit a muscle biopsy will be taken from the thigh muscle at rest and you will then be asked to perform a 10-km cycle Time Trial familiarisation test. You will be required to refrain from exercise, alcohol and caffeine consumption for 24 h before all tests and biopsies. 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 following tests and biopsies. The initial familiarisation and baseline testing period will be completed in a time of approximately two weeks.

The interval training phase will commence 48 to 72 hours after the last testing session. During this phase you will be required to train 3 times per week during 4 weeks. All training sessions will be completed on a stationary cycle and will be preceded by a 5-min warm up at 50 W. Due to the intense nature of interval training, the 4-week training program will follow a periodised plan to allow progression, to prevent overtraining and subject attrition, and also to simulate athletic training programs. Following the initial warm up, each session will consist of three to seven 4-min intervals performed at different intensities ranging from 120% to 140% of your individually determined pre-training lactate threshold, interspersed with a 2-min recovery period at very low power output (<30 W) giving a work-to-rest ratio of 2:1. In order to maintain progression, the workload will be altered by via manipulation

of both the number of intervals and the intensity in each session. At the end of this first training phase you will visit the lab three times for the post-interval training testing phase. During your first visit, you will perform a 20-km cycle time trial in order to evaluate your performance improvements. During the second visit a maximal incremental exercise test will take place to measure changes in your VO_{2Peak} and lactate threshold and in order to determine the training intensity for the intensified training phase which follows. During your third visit, you will complete a 10-km cycle Time Trial test. This test should last approximately 15 to 25 minutes. In order to measure the response of your muscle to the intensified training phase a series of three muscle biopsies will be taken before and after the 10-km cycle time trial. The first biopsy will be taken at rest prior to the start of the test, a second one at the end of the test and the last one 3 hours after the cessation of the test. During this 3-hour period, you will be allowed to rest in the supine position but will not be allowed food or drinks except for water.

Phase 2: Intensified Training (INT)

During this phase you will continue the interval training sessions but the frequency and intensity will be increased. You will be required to train 12 to 14 times a week (there will be training sessions at w/e) at the same intensity as before. Each interval session will consist of 6 (morning session) or 5 (afternoon session) 4-min intervals interspersed with 2-min of low-intensity cycling (<30 W) as for the previous phase. Furthermore, during the intensified training phase you will be asked to complete a 10-km cycle time trial test on every fourth morning. Training will continue for 2 to 3 weeks (depending on your adaptation to the training stimulus). You will also be required to fill in a questionnaire each day prior to training to monitor your state of fatigue. In order to prevent depletion of muscle glycogen, which could negatively influence performance, you will be encouraged to ingest 1-2 g/kg of carbohydrate two hours prior to the time to fatigue and training sessions, 1-2g/kg in the first hour after each time to fatigue and training session, and a total of 10-12g/kg/day in an effort to ensure optimum levels of muscle glycogen are maintained throughout the study. This will be provided by the University and you will be supplied with Polycose, a liquid carbohydrate supplement, before and after each of the testing sessions. You will also be given a handout that provides suggestions for foods that are high in carbohydrate and low in fat.

The day after the end of the intensified training phase you will be required to perform the last 10-km cycle time trial. During this test, three muscles biopsies will be taken in order to measure changes in your muscle in response to the intensified training phase. As before, the first muscle biopsy will be taken at rest prior to the start of the test, the second one at the end of the test and the last one 3 hours after the cessation of the test. At least 48 hours after the last 10-km cycle time trial test you will be required to perform a 20-km cycle time trial to observe changes in endurance performance due to the period of intensified training.

Phase 3: Taper (TP)

The taper phase will last approximately 2 weeks. Sessions will run every second or third day, will be progressively reduced in duration, and will be kept at the same intensity as the previous phases. At least 48 hours after the last training bout in this phase you will be asked to perform the final 20-km cycle time trial in order to measure performance changes following the taper phase. A further 48 to 72 hours later, you will be asked to undertake the last muscle biopsy which will be used to measure changes in your muscle.

Who is conducting the study?

The study is conducted by the School 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

Dr. Nigel K Stepto, Telephone number: 9919 5416, Mobile: 0409 338 696, email: nigel.stepto@vu.edu.au

Mr. Cesare Granata, Mobile: 0466 615 224, email: triatciccio@yahoo.co.uk

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.

VICTORIA UNIVERSITY | A NEW SCHOOL OF THOUGHT

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 "Effects of different training interventions on endurance performance and muscle adaptations"

INVESTIGATORS:
Prof. David Bishop
Dr. Nigel Stepto
Mr. Cesare Granata

(a) AIMS OF THE STUDY: The major aims of this project are to investigate the effects of three different training workloads on endurance performance and changes in the muscle in healthy young adults.

(b) PARTICIPANT INVOLVEMENT AND OVERVIEW OF TESTING: Participants will be requested to attend the Exercise Physiology Laboratory at Victoria University, Footscray Park Campus (School of Sport and Exercise Science and ISEAL, building P). The study is divided in three phases. During phase one, participants are required to visit the labs on four separate occasions for exercise testing trials, followed by a series of 4 weeks of training and 3 more visits for testing. For the second phase participants will take part in a further 3 weeks of training at an increased workload followed by 2 more testing trials. The third phase of the study consists of a reduced workload period of training lasting 2 weeks followed by the last two testing trials. Whilst each tests and training session may be tiring, participants will recover very quickly and their aerobic fitness will greatly benefit from this training.

(c) EXERCISE TESTING PROCEDURES: Participants will be asked to undertake high-intensity tests and training. This training involves cycling exercises on a stationary cycle over several visits. The tests participants will be asked to perform are the following:

- 20-km cycle time trial: involves cycling 20km on a stationary bike as fast as possible.
- 10-km cycle time trial: involves cycling 10km on a stationary bike as fast as possible.
- Graded test to exhaustion (GXT): involves cycling at regularly increasing speed until no longer sustainable.
- Mood and physical state scale test (MAPSS): a questionnaire used to monitor both physical and mental state of fatigue during the intensified training phase.

The training participants will be asked to perform involves cycling on a stationary bike for a series of intervals lasting 4-min each at high intensity, followed by a 2-min period at very low intensity. According to the different phases of the training the number of repetitions participants are required to complete will vary between 2 and 7.

(d) TRAINING PROGRAMME: Participants will be asked to train:
- 3 times a week for 4 weeks during phase one,
- twice a day for 3 weeks for phase two,
- 3 times a week for 2 weeks on phase 3,
in order to observe improvements in their endurance performance and to observe important skeletal muscle adaptation to training. The training participants will be required to undertake involves a series of interval training repetitions of 4 minutes at 120-140% of lactate threshold (previously measured in a testing session) separated by 2 minutes of recovery cycling at very low intensity. The number of repetitions varies during each phase and participants will be informed prior to the study of the exact number of repetitions required.

1

(e) MUSCLE BIOPSIES - The muscle biopsy procedure is used to obtain small samples of muscle tissue for analysis of enzymes and energy sources. On four separate occasions, muscle biopsies will be taken from the thigh muscle of participants, at rest, or following testing. A muscle biopsy at rest will be taken before the first training phase; three muscle biopsies will be taken at rest, at the end and 3 hours after a testing session both before and after the second phase of training. A final muscle biopsy will be taken at the end of the study, giving an overall total of eight biopsies. 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 about 1-2 seconds. Muscle biopsies are routinely carried out in our laboratory, with no serious adverse effects.

(f) CAPILLARY BLOOD SAMPLING – Capillary blood samples will be taken during testing sessions. This involves collection of a few drops of blood by pricking of the skin with a small lancet in either your fingertip or earlobe. A qualified scientist will perform all blood sampling. Capillary blood sampling is not painful, is rarely uncomfortable and with remote possibility of infection.

CERTIFICATION BY SUBJECT

I, _____

of _____

certify that I am at least 18 years old* and that I am voluntarily giving my consent to participate in the study:
EFFECTS OF DIFFERENT TRAINING INTERVENTIONS ON ENDURANCE PERFORMANCE AND MUSCLE ADAPTATIONS
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 Cesare Granata and Professor David Bishop

and that I freely consent to participation involving the below mentioned procedures:

- Screening.
- VO_{2Peak} test (GXT)
- 20 km Cycling Time Trial (TT)
- 10 km Cycling Time Trial (TT)
- Interval Training (IT)
- Muscle Biopsies
- Capillary 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 researcher
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.

Project HRETH 12/189

VICTORIA UNIVERSITY | A NEW SCHOOL OF THOUGHT

INFORMATION TO PARTICIPANTS INVOLVED IN RESEARCH

You are invited to participate in a research project entitled:

Effects of Sprint Interval Training (SIT) versus sub-threshold continuous training (STCT) and tapering (TP) on endurance performance and muscle adaptations in active people

This project is being conducted by a student researcher Cesare Granata as part of a PhD at Victoria University under the supervision of Prof. David Bishop [School of Sport and Exercise Science and ISEAL].

Project explanation

The project aims to understand the effects of two different types of training intervention, Sub-lactate Threshold Continuous Training (STCT) and supra-maximal Sprint Interval Training (SIT) lasting 4 weeks, and a taper lasting 2 weeks, on mitochondrial respiration and markers of mitochondrial biogenesis (note: "mitochondria" are the muscles' power station, producing the energy required to enable the muscles to contract, "respiration" is an estimate of an individual aerobic capacity, "biogenesis" signifies the ability of mitochondria to increase in number and size). In addition, at the beginning of the study there will be a single exercise session, with muscle biopsies before and after exercise, to investigate the effects of the different types of training on factors responsible for increasing mitochondria size and number.
We will also investigate whether variations in your genes ("polymorphisms") are associated with the changes that you experience following training; to do this we require only a small blood sample.
The reason we are conducting this study is to be able to understand the physiological mechanisms involved in generating changes in endurance performance and the mitochondria. This information will be used to design better training programs to improve endurance performance.

What will I be asked to do?

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

- 6 visits for pre-testing (about 1 h each with the last one lasting about 4 h - Total of approximately 10 h)
- 12 visits for the main training phase
  - ✓ about 25-40 min each - Total of approximately 7 hours for the SIT group; or
  - ✓ about 25-60 min each - Total of approximately 9 hours for the STCT group
- 3 visits for post-training testing (about 1 h each - Total of approximately 3 h)
- 6 visits for the taper phase
  - ✓ about 15-30 min each - Total of approximately 2.5 hours for the SIT group; or
  - ✓ about 10-30 min each - Total of approximately 2.5 hours for the STCT group
- 3 visits for post-taper testing (about 1 h each - Total of approximately 3 h)

A total of approximately 25 hours (SIT group) or 28 hours (STCT group) will be required to complete all sessions within the study.

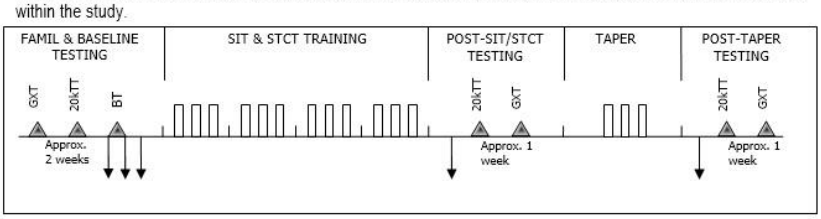

Figure 1: experimental design (each arrow indicates a muscle biopsy)

We will first ask you to fill in several short questionnaires about your family medical history and your exercise habits to determine your ability to participate in this study (shown in Figure 1) which is divided in 2 main phases:

Phase one:

- This will begin with a series of tests, separated by at least 48 each, before the training phase will commence. These testing sessions will involve a familiarization of a 20-km cycling Time Trial (20kTT) lasting between 35 and 45 min. The second testing session will involve a familiarization with a graded exercise test (GXT); this test usually lasts 25-35 min. You will then be asked to repeat the 20kTT and the GXT which will be used as the baseline performance tests. In your fifth visit you will perform a familiarization of the "acute trial test" which will involve either 40 min of continuous cycling at sub-lactate threshold intensity if you are assigned to the STCT group, or 4x30 s all-out bouts at maximal intensity with 4 min of recovery between each, if you are assigned to the SIT group. You will be assigned to one of the two groups after your GXT in order to have the two groups matched for lactate threshold values. On your last pre-training visit you will repeat the same acute trial test, and we will ask your permission for a medical doctor to take three blood samples (two at rest and one immediately at the end of exercise) and three muscle biopsies, one at rest, one immediately after the end of the training bout and one after a period of 3 h of rest (for details see below).
- During the training period you will be required to train 3 times a week for 4 weeks. Each session will consist of either 20 to 50 min (periodized) of continuous cycling at an intensity equivalent to 95% of your lactate threshold for the STCT group or of a series of 4 to 8 30-s bouts (again periodized) at maximal intensity interspersed with 4 min of recovery at very low intensity (60W) for the SIT group.
- This will be followed by three testing sessions, the 20kTT, the GXT and a resting biopsy trial (only one muscle biopsy at rest will be required this time) in order to monitor changes in your fitness levels and muscle adaptations since the beginning of the training.

Phase two:

- During this second phase lasting two weeks, you will be asked to train again three times a week. Each session will consist of either 10 to 30 min of continuous cycling for the STCT group, or of a series of 2 to 5 30-sec bouts for the SIT group set at in the same way and the same intensities of the previous phase.
- This period will be followed by 3 final tests (the 20kTT, the GXT and the resting muscle biopsy trial, again with just one resting biopsy) to monitor performance and physiological changes.

All testing and training sessions will be performed on a stationary cycle connected to a computer showing to you all the details of your ride. These are located in the exercise physiology laboratory at Victoria University and all sessions will run under the supervision of an exercise physiologist. At three different time points, you will be asked for your permission to let a medical doctor take a small sample of your muscle using a needle biopsy for a total of 5 biopsies. . On the same day the medical doctor will take two venous blood samples before exercise and one more at the end of exercise. Capillary blood samples will also be taken during testing sessions (please see below for a better description of these procedures).

There will be a reimbursement to all participants in order to cover for costs incurred for travel, parking or other expenses. The amount will be a total of $200 upon completion of the entire study. Should you withdraw for any reason prior to the completion of the study a sum will be given to you according to which stage you have completed as shown here:

- 0% or $0 if you withdraw before the post-SIT/STCT testing phase is completed (see Figure 1)
- 50% or $100 upon completion of the post-SIT/STCT testing phase (see Figure 1)
- 100% or $200 upon completion of the entire study

Finally for scientific and health reason we will have to exclude participants who:

- Are females
- Have current or previous musculoskeletal problems of either leg
- Are aware that they have an infectious blood disease
- Have a disorder that will affect blood clotting or are taking medication affecting blood clotting
- Have an allergy to the anaesthetic to be employed

- Have a condition or disease which affects cardiovascular function

Explanation of procedures involved in this study

1. Pre-experiment subject screening

Only male participants 18-35 years old will be allowed to undergo the pre-experiment screening. We will first assess your health with a questionnaire and by measuring your blood pressure, heart rate and ECG rhythm at rest. Participants suffering from any pulmonary disorders (i.e. asthma, bronchitis, etc.), high blood pressure, diabetes or arrhythmias, or with a risk of an adverse event from conditions such as bleeding disorders, skin and anaesthesia allergies and musculoskeletal injuries will be excluded from participating in this study. If you pass the pre-screening we will then request that you complete the two phases involved in this study.

2. 20kTT: 20-km cycling time-trial

You will be asked to complete a warm up consisting of 6 min of cycling. Following a 2-min rest, you will be asked to complete the 20-km time-trial in the quickest possible time. You may receive verbal encouragement during the test.

3. GXT: graded exercise test (with VO_{2Max})

These tests involve continuous exercise on a cycle-ergometer with the exercise intensity (effort) being progressively increased until you reach exhaustion. During the graded exercise test the intensity will be increased every 4 minutes and capillary blood samples will be taken at the end of each stage throughout the test. This test usually lasts 25-35 min. You will receive verbal encouragement during the tests. We will closely monitor you, your heart rate and blood pressure during exercise to ensure your safety. The tests will be completed when you become tired (or wish to stop before you become tired), or unless we stop the test earlier for safety due to you displaying an abnormal response to exercise, inappropriate heart rate or sweating responses, chest pain, or severe shortness of breath.
During the VO_{2max} test which will be ran 15 min after completion of the GXT the intensity will be maintained at a fixed percentage of your maximal power attained during the GXT and you will be breathing via a mouth piece to allow us to collect expired gases and determine your VO_{2Max}

4. - Effects of a single session of exercise

All exercise sessions will be completed on a stationary cycle and will be preceded by a 6-min warm up. Following the initial warm up, the single session of exercise test will consist of:

- 4x30 s at maximal intensity with 4 min of recovery at low intensity (60 W) for the SIT group
- 40' of continuous cycling at 95% of lactate threshold for the STCT group.

In order to measure the response of your muscle associated with each training type, a series of three muscle biopsies and 3 blood samples will be taken prior to, immediately after and 3 hours after completion of the trial. During this 3-hour period following the exercise session, you will be allowed to rest lying on your back or seated, but will not be allowed food or drinks except for water.

5. Muscle biopsy

The muscle biopsy procedure is used to obtain small samples of your muscle tissue which can be used for analysis of proteins, genes and muscle function. The muscle biopsy will be performed by an experienced medical doctor under sterile conditions, and will consist of an injection of a local anaesthetic in the skin overlying the thigh muscle, and then a small incision (approx. 0.6 cm long) will be made in the skin. The biopsy needle will be then inserted into your vastus lateralis muscle (the external side of your thigh) and a small piece of tissue will be removed. During this part of the procedure you will feel pressure and this will be quite uncomfortable and you may also experience some pain, but it will last for only about 1-2 seconds. When the small piece of muscle is removed you may also experience a mild muscle cramp, but this only persists for a few seconds. The size of muscle removed by the biopsy needle is similar to 3-4 grains of rice. This poses no long-term effects for your muscle and will not be noticeable to others apart from a small scar on the skin for a few months. Following the biopsy the incision will be closed using a steri-strip and covered by a transparent waterproof dressing. Then a pressure bandage will be applied which should be maintained for 24-48 hours. Steri-strip

closure should be maintained for a few days. You should avoid heavy knocks for 24 hours after the biopsies. It is common for participants to experience some soreness in the muscle over the next 2-3 days, however this passes and does not restrict movement. The soreness is due to slight bleeding within the muscle and is best treated by "ice, compression and elevation". An ice pack will be applied over the biopsy site after the biopsy procedure to minimise any bleeding and therefore soreness. In some rare cases mild haematomas have been reported, but these symptoms disappear within a week. On very rare occasions, some people have reported altered sensation (numbness or tingling) in the skin near the site of the biopsy. This is due to a very small nerve being cut, but this sensation disappears over a period of a few weeks-to-months. Although the possibility of infection, significant bruising and altered sensation is quite small, if by chance it does occur, please inform us immediately and we will immediately consult the doctor who performed the biopsy to review the reported problems and recommend appropriate action.

6. Capillary and venous blood sampling

During the GXT and the 20kTT we will ask for your permission to take capillary blood samples. This consists of a small prick on your fingertip (or earlobe) and few drops of blood will be taken. Capillary blood sampling is only slightly uncomfortable, with very little possibility of bruising. Furthermore, three venous blood samples will be during the acute trial for mitochondrial biogenesis and genetic analysis (approximately 10 ml of blood, 30 ml in total). This procedure is slightly uncomfortable, with the possibility of bruising and infection (for example puss, tenderness and/or redness), although the possibility of infection is very unlikely.

Genetic Testing

We will extract genomic DNA from your blood samples using a standard protocol. Genotyping, which is a description of your genetic type will be performed.

Important things to understand and what are the potential risks of participating in this project?

Before you volunteer to this study, make sure you read carefully the items below:

- You are free to withdraw from this study at any time without any consequences or need for explanation.
- All exercise activity carries a risk of injury and risks of suffering a heart attack or stroke. It is important that you tell us if you have any medical condition.
- The maximal incremental exercise test (GXT), the 20-km cycling Time Trial test (20kTT) and SIT and STCT training all involve risks of vasovagal episodes, muscle soreness and stiffness, and sudden death due to myocardial infarction.
- Due to the intense nature of the training program, there is a small risk of illness (such as colds and flu) and injury (such as muscle strains). Risk of injuries should be avoided by using the correct warm up procedures. This type of training protocol can be accompanied in rare cases by the possibility of mood changes such as irritability and depression, anxiety or stress associated with multiple muscle biopsies, blood tests and extensive time commitment to the study. These changes are however short term and can be reversed with a maximum of two to three weeks of recovery.
- For any medical emergencies a call to 000 will be made, and the researchers will commence appropriate resuscitation methods while waiting for an emergency team to arrive.
- During the exercise sessions, you may experience some muscle or other soft tissue soreness / injury or faint.
- Muscle biopsy risks include possibility of infection and localized altered sensation of skin and/or muscle. However significant adverse effects are rare and if by chance it does occur, please inform us immediately and we will immediately consult the doctor who performed the biopsy to review the reported problems and recommend appropriate action.
- Risks associated with blood sample include slight discomfort, with the possibility of bruising and infection

What will I gain from participating?

We cannot promise or guarantee that you will have a direct benefit from participation in the study. However from participating in this study it is possible you will gain benefits for your aerobic fitness as you are going to be followed by sport scientists and will train in a state-of-the-art purpose-built training facility. You will also increase

your understanding of fitness and fitness tests. You will also gain the experience of having participated in an exercise science experiment designed to increase knowledge about muscles and genes.

How will the information I give be used?

Your samples will be stored under alphanumeric codes (i.e. without your name or personal details) and only the researchers at VU will be able to connect the samples to you. All of the muscle sample collected will be used to measure mitochondrial respiration and analyse some proteins, genes and energy sources involved with the function of your thigh muscle. The analyses of your blood and muscles samples will be performed at Victoria University (ISEAL) and University of British Columbia, Canada (Faculty of Health and Social Development) and the Murdoch Children's Research Institute (Melbourne, Australia). A part of the blood samples, once again stored with an alphanumeric code, will be used for determination of gene polymorphisms. We will then extract Genomic DNA from your blood which will subsequently be analysed for variations in your genes. The results will be analysed by two experienced and independent investigators who will be blind to your personal details (e.g., name, sex, age etc).In the event of any blood or muscle tissue remaining it will be disposed of in a de-identified container (i.e. no coding present) via incineration using Victoria University, University of British Columbia, or the Murdoch Children's Research Institute waste disposal contractor. Lactate and glucose levels, as well as haemoglobin and other blood parameters like haematocrit, pH ion levels etc will also be analysed in these venous blood samples. Other parameters we will collect like, blood pressure, Heart rate, or questionnaire data will once again stored with an alphanumeric code and only the scientists 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 unless required by law.

Who is conducting the study?

The study's main investigator is Professor David Bishop who has more than 15 years of experience in running academic research projects. The day to day running of the study is going to be carried out by Mr. Cesare Granata, a 3rd year PhD student at Victoria University. Furthermore Dr. Nigel Stepto and Dr. Nir Eynon will also participate in the decision making and study design of this research study. The study will be conducted by the School of Sport and Exercise Science, Victoria University, Footscray Park Campus.

Chief Investigator:
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Student investigator:
Mr. Cesare Granata, Telephone number: 99199185, Mobile: 0466 615 224, email: triatciccio@yahoo.co.uk

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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Section .01 ***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:
Effects of Sprint Interval Training (SIT) versus sub-threshold continuous training (STCT) and tapering (TP) on endurance performance and muscle adaptations in active people

INVESTIGATORS:
Prof. David Bishop
Dr. Nigel Stepto
Mr. Cesare Granata
Dr. Nir Eynon

(a) AIMS OF THE STUDY: The major aims of this project are to investigate the effects of two different training intensities on endurance performance and changes in the muscle in healthy young adults both at the acute (1 session) and chronic level (4 weeks). Furthermore we aim to investigate the effect of a reduced training workload using the same training intensities on the same muscular adaptations. Another aim of this project is to verify whether changes observed following acute and chronic sessions of high-intensity interval training are associated with small gene variations ("polymorphisms").

(b) PARTICIPANT INVOLVEMENT AND OVERVIEW OF TESTING: Participants will be requested to attend the Exercise Physiology Laboratory at Victoria University, Footscray Park Campus (School of Sport and Exercise Science and ISEAL, building P). The study is divided in two phases. During phase one, participants are required to visit the labs on six separate occasions for exercise testing trials, followed by 4 weeks of training and 3 more visits for testing. In the second phase participants will continue to train for 2 weeks at a markedly reduced workload (taper) followed by 3 testing trials. Whilst each tests and training session may be tiring, participants will recover very quickly and their aerobic fitness will greatly benefit from this training.

(c) EXERCISE TESTING PROCEDURES: Participants will be asked to undertake high-intensity tests and training. This training involves cycling exercises on a stationary cycle over several visits. The tests participants will be asked to perform are the following:

- 20-km cycle time trial (20kTT): involves cycling 20km on a stationary bike as fast as possible.
- Graded test to exhaustion (GXT): involves cycling at regularly increasing speed until no longer sustainable for the determination of the lactate threshold and $VO2_{Max}$.

(a) TRAINING PROGRAMME:
After completion of the testing phases participants will be assigned to one of two groups and matched for Lactate Threshold. The training participants will be asked to perform involves cycling on a stationary bike and will be one of the following:

- SIT group: a series of intervals lasting 30 seconds each at maximal intensity, followed by a 4-min period at very low intensity (60 W). The number of repetitions will vary between 2 and 8
- STCT group: a period of continuous cycling at an intensity equalling 95% of lactate threshold. The duration of these sessions will be between 10 and 50 minutes

Participants of both groups will be asked to train 3 times a week for 4 weeks during phase one and for 2 weeks during phase.
The number of repetitions and the duration of the continuous cycling will vary during each phase and participants will be informed prior to the study of the exact number of repetitions or minutes of continuous cycling required.

(d) MUSCLE BIOPSIES - The muscle biopsy procedure is used to obtain small samples of muscle tissue for analysis of genes, proteins and energy sources. On three separate occasions, muscle biopsies will be taken from the thigh muscle of participants, at rest, or following testing. Three muscle biopsies will be taken at rest, at the end and 3

hours after completion of the exercise bout during the last of the pre-training testing sessions. Finally a muscle biopsy at rest will be taken at the end of the first training phase (4 weeks) and at the very end of the study (2 more weeks) giving an overall total of five biopsies. 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 about 1-2 seconds. Muscle biopsies are routinely carried out in our laboratory, with no serious adverse effects.

(e) VENOUS AND CAPILLARY BLOOD SAMPLING - Blood samples will be taken during rest and exercise from the finger during the performance tests. Three venous samples will be taken twice at rest and once at the end of exercise during the acute trial.

CERTIFICATION BY SUBJECT

I,
of

certify that I am at least 18 years old* and that I am voluntarily giving my consent to participate in the study:
"Effects of Sprint Interval Training (SIT) versus sub-threshold continuous training (STCT) and tapering (TP) on endurance performance and muscle adaptations in active people"
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 Cesare Granata

and that I freely consent to participation involving the below mentioned procedures:

- VO_{2Peak} test (GXT)
- 20 km Cycling Time Trial (20kTT)
- Sprint Interval Training (SIT) and continuous sub-lactate threshold training (STCT)
- Muscle Biopsies
- Venous and capillary 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 researcher
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.

please note: Where the participant/s are aged under 18, separate parental consent is required; where the participant/s are unable to answer for themselves due to mental illness or disability, parental or guardian consent may be required.]

CHAPTER 2 – RAW DATA

Physiological parameters

Age [y] - Mass [kg] - Height [cm] - Total Work [MJ]

HIIT					STCT					SIT				
Pcpt	**Age**	**Mass**	**Height**	**Tot Work**	**Pcpt**	**Age**	**Mass**	**Height**	**Tot Work**	**Pcpt**	**Age**	**Mass**	**Height**	**Tot Work**
HIT 1	20.2	94.7	191.6	4.1	STCT 1	23.3	77.7	180.5	3.3	SIT 1	19.7	70.7	174.4	1.3
HIT 2	24.0	69.4	169.0	3.2	STCT 2	18.8	86.5	181.6	4.9	SIT 2	21.7	93.1	191.9	1.5
HIT 3	20.9	72.5	174.0	4.2	STCT 3	21.6	75.5	177.6	3.7	SIT 3	24.9	84.9	183.5	1.7
HIT 4	19.6	112.0	180.3	3.4	STCT 4	19.8	76.2	178.0	4.1	SIT 4	21.6	67.9	176.9	1.0
HIT 5	20.6	80.4	183.0	3.1	STCT 5	24.4	79.6	182.2	4.3	SIT 5	22.1	125.5	180.4	1.6
HIT 6	19.3	66.1	170.7	3.7	STCT 6	20.5	84.7	180.5	3.7	SIT 6	19.6	87.0	184.7	1.4
HIT 7	20.6	86.5	193.0	4.0	STCT 7	18.8	92.0	191.0	4.6	SIT 7	18.5	97.6	188.3	1.4
HIT 8	19.9	82.8	198.6	4.0	STCT 8	20.8	67.7	174.4	2.5	SIT 8	18.4	66.6	176.8	1.1
HIT 9	21.8	76.6	180.5	3.8	STCT 9	20.6	56.3	167.5	3.2	SIT 9	25.7	67.1	168.3	1.2
HIT 10	18.7	65.5	165.8	3.6										
HIT 11	19.9	75.6	173.4	2.1										
Mean	**20.5**	**80.2**	**180.0**	**3.6**	**Mean**	**21.0**	**77.4**	**179.3**	**3.8**	**Mean**	**21.3**	**84.5**	**180.6**	**1.3**
SD	***1.4***	***13.8***	***10.7***	***0.6***	***SD***	***1.9***	***10.6***	***6.3***	***0.7***	***SD***	***2.6***	***19.4***	***7.3***	***0.2***
SE	***0.4***	***4.1***	***3.2***	***0.2***	***SE***	***0.6***	***3.5***	***2.1***	***0.2***	***SE***	***0.9***	***6.5***	***2.4***	***0.1***

W_{LT} [W]

HIIT				STCT				SIT			
Pcpt	**Pre**	**Post**	**% Δ**	**Pcpt**	**Pre**	**Post**	**% Δ**	**Pcpt**	**Pre**	**Post**	**% Δ**
HIT 1	216.0	238.9	10.60	STCT 1	172.0	179.5	4.39	SIT 1	193.2	200.4	3.71
HIT 2	163.2	187.5	14.89	STCT 2	259.8	277.8	6.91	SIT 2	233.6	262.0	12.13
HIT 3	246.3	266.4	8.16	STCT 3	176.6	190.2	7.70	SIT 3	271.7	297.3	9.42
HIT 4	184.8	203.4	10.06	STCT 4	219.6	237.0	7.89	SIT 4	139.2	164.1	17.87
HIT 5	179.7	196.0	9.07	STCT 5	225.6	255.8	13.37	SIT 5	226.0	260.0	15.03
HIT 6	209.4	207.8	-0.76	STCT 6	194.9	206.2	5.78	SIT 6	213.5	201.1	-5.82
HIT 7	209.8	217.7	3.77	STCT 7	242.7	251.6	3.67	SIT 7	186.2	212.9	14.33
HIT 8	211.6	247.5	16.97	STCT 8	128.5	137.1	6.75	SIT 8	161.1	165.6	2.77
HIT 9	208.0	226.1	8.70	STCT 9	134.6	145.3	7.93	SIT 9	215.0	237.2	10.33
HIT 10	202.4	210.0	3.75								
HIT 11	147.5	159.9	8.41								
Mean	**198.1**	**214.7**	**8.5**	**Mean**	**194.9**	**208.9**	**7.2**	**Mean**	**204.4**	**222.3**	**8.9**
SD	***27.4***	***29.6***	***5.0***	***SD***	***46.1***	***50.0***	***2.8***	***SD***	***39.7***	***45.4***	***7.4***
SE	***8.3***	***8.9***	***1.5***	***SE***	***15.4***	***16.7***	***0.9***	***SE***	***13.2***	***15.1***	***2.5***

W_{peak} [W]

HIIT				STCT				SIT			
Pcpt	**Pre**	**Post**	**%Δ**	**Pcpt**	**Pre**	**Post**	**%Δ**	**Pcpt**	**Pre**	**Post**	**%Δ**
HIT 1	300.0	320.0	6.67	STCT 1	262.5	255.0	-2.86	SIT 1	262.5	270.0	2.86
HIT 2	230.0	252.5	9.78	STCT 2	345.0	367.5	6.52	SIT 2	315.0	345.0	9.52
HIT 3	340.0	361.9	6.43	STCT 3	255.0	262.5	2.94	SIT 3	367.5	382.5	4.08
HIT 4	237.5	275.0	15.79	STCT 4	307.5	315.0	2.44	SIT 4	217.5	240.0	10.34
HIT 5	247.5	285.0	15.15	STCT 5	307.5	337.5	9.76	SIT 5	315.0	337.5	7.14
HIT 6	262.5	285.0	8.57	STCT 6	270.0	285.0	5.56	SIT 6	285.0	255.0	-10.53
HIT 7	275.0	305.0	10.91	STCT 7	337.5	345.0	2.22	SIT 7	262.5	285.0	8.57
HIT 8	287.5	317.5	10.43	STCT 8	185.0	197.5	6.76	SIT 8	217.5	232.5	6.90
HIT 9	262.5	307.5	17.14	STCT 9	210.0	195.0	-7.14	SIT 9	285.0	292.5	2.63
HIT 10	262.5	277.5	5.71								
HIT 11	200.0	238.0	19.00								
Mean	**264.1**	**293.2**	**11.4**	**Mean**	**275.6**	**284.4**	**2.9**	**Mean**	**280.8**	**293.3**	**4.6**
SD	***37.4***	***34.3***	***4.6***	***SD***	***54.6***	***62.5***	***5.2***	***SD***	***48.2***	***51.5***	***6.3***
SE	***11.3***	***10.4***	***1.4***	***SE***	***18.2***	***20.8***	***1.7***	***SE***	***16.1***	***17.2***	***2.1***

20 km TT [s]

HIIT				STCT				SIT			
Pcpt	**Pre**	**Post**	**%Δ**	**Pcpt**	**Pre**	**Post**	**%Δ**	**Pcpt**	**Pre**	**Post**	**%Δ**
HIT 1	2191.0	2096.0	-4.34	STCT 1	2185.0	2129.0	-2.56	SIT 1	2285.0	2168.0	-5.12
HIT 2	2460.0	2234.0	-9.19	STCT 2	2005.0	1949.0	-2.79	SIT 2	2017.0	1947.0	-3.47
HIT 3	1995.0	1985.0	-0.50	STCT 3	2162.0	2200.0	1.76	SIT 3	1904.0	1857.0	-2.47
HIT 4	2375.0	2220.0	-6.53	STCT 4	2075.0	1993.0	-3.95	SIT 4	2319.0	2355.0	1.55
HIT 5	2373.0	2159.0	-9.02	STCT 5	2170.0	1996.0	-8.02	SIT 5	2082.0	2037.0	-2.16
HIT 6	2161.0	2142.0	-0.88	STCT 6	2215.0	2149.0	-2.98	SIT 6	2216.0	2273.0	2.57
HIT 7	2204.0	2128.0	-3.45	STCT 7	2105.0	1963.0	-6.75	SIT 7	2205.0	2139.0	-2.99
HIT 8	2142.0	2038.0	-4.86	STCT 8	2575.0	2403.0	-6.68	SIT 8	2315.0	2299.0	-0.69
HIT 9	2211.0	2093.0	-5.34	STCT 9	2458.0	2396.0	-2.52	SIT 9	2118.0	2112.0	-0.28
HIT 10	2157.0	2118.0	-1.81								
HIT 11	2456.0	2306.0	-6.11								
Mean	**2247.7**	**2138.1**	**-4.7**	**Mean**	**2216.7**	**2130.9**	**-3.8**	**Mean**	**2162.3**	**2131.9**	**-1.5**
SD	***147.5***	***90.7***	***2.9***	***SD***	***183.8***	***176.0***	***3.0***	***SD***	***143.1***	***165.1***	***2.5***
SE	***44.5***	***27.4***	***0.9***	***SE***	***61.3***	***58.7***	***1.0***	***SE***	***47.7***	***55.0***	***0.8***

VO_{2Peak} [mL min^{-1} kg^{-1}]

HIIT				STCT				SIT			
Pcpt	**Pre**	**Post**	**%Δ**	**Pcpt**	**Pre**	**Post**	**%Δ**	**Pcpt**	**Pre**	**Post**	**%Δ**
HIT 1	38.2	39.6	3.63	STCT 1	51.2	51.5	0.65	SIT 1	51.5	54.7	6.25
HIT 2	47.1	46.9	-0.40	STCT 2	48.7	54.9	12.88	SIT 2	44.6	48.4	8.46
HIT 3	55.3	59.7	7.93	STCT 3	46.3	50.7	9.47	SIT 3	59.4	62.4	5.03
HIT 4	33.3	35.8	7.53	STCT 4	51.5	54.5	5.96	SIT 4	44.9	48.8	8.65
HIT 5	44.9	46.8	4.39	STCT 5	47.9	55.2	15.13	SIT 5	37.5	37.0	-1.22
HIT 6	52.6	51.0	-3.09	STCT 6	47.8	45.6	-4.67	SIT 6	39.1	40.8	4.42
HIT 7	39.2	42.9	9.58	STCT 7	43.9	51.5	17.40	SIT 7	41.2	42.5	3.27
HIT 8	45.8	44.2	-3.55	STCT 8	41.8	41.2	-1.49	SIT 8	48.5	51.7	6.50
HIT 9	47.9	53.8	12.31	STCT 9	41.1	46.9	14.06	SIT 9	57.4	59.8	4.14
HIT 10	53.5	54.3	1.55								
HIT 11	38.1	41.3	8.40								
Mean	**45.1**	**46.9**	**4.4**	**Mean**	**46.7**	**50.2**	**7.7**	**Mean**	**47.1**	**49.6**	**5.1**
SD	***7.2***	***7.2***	***5.3***	***SD***	***3.8***	***4.8***	***8.0***	***SD***	***7.8***	***8.6***	***3.0***
SE	***2.2***	***2.2***	***1.6***	***SE***	***1.3***	***1.6***	***2.7***	***SE***	***2.6***	***2.9***	***1.0***

Mitochondrial respiration (expressed as pmol O_2 s^{-1} mg^{-1})

$CI_{L(PM)}$

HIIT				STCT				SIT			
Pcpt	**Pre**	**Post**	**% Δ**	**Pcpt**	**Pre**	**Post**	**% Δ**	**Pcpt**	**Pre**	**Post**	**% Δ**
HIT 1	8.5	6.0	-29.28	STCT 1	13.6	7.4	-45.50	SIT 1	8.6	9.1	5.95
HIT 2	5.8	7.1	21.52	STCT 2	5.3	7.4	39.78	SIT 2	8.9	10.9	22.74
HIT 3	5.5	3.5	-36.60	STCT 3	6.4	12.1	89.95	SIT 3	8.6	7.4	-13.56
HIT 4	7.6	4.5	-41.14	STCT 4	6.2	4.9	-21.29	SIT 4	8.5	12.1	42.50
HIT 5	3.4	7.2	114.63	STCT 5	9.2	6.0	-34.59	SIT 5	6.0	9.1	50.28
HIT 6	4.5	4.9	9.98	STCT 6	8.2	4.3	-47.70	SIT 6	5.8	12.7	120.41
HIT 7	2.3	4.8	107.34	STCT 7	12.5	8.8	-29.13	SIT 7	9.2	8.6	-7.01
HIT 8	2.0	3.4	73.41	STCT 8	8.9	11.6	30.82	SIT 8	10.2	8.9	-13.10
HIT 9	3.5	8.7	146.25	STCT 9	10.5	12.5	19.69	SIT 9	7.1	10.9	53.13
HIT 10	7.6	5.4	-29.36								
HIT 11	3.5	5.2	48.46								
Mean	**4.9**	**5.5**	**35.0**	**Mean**	**9.0**	**8.3**	**0.2**	**Mean**	**8.1**	**10.0**	**29.0**
SD	***2.2***	***1.6***	***67.5***	***SD***	***2.8***	***3.1***	***47.2***	***SD***	***1.5***	***1.8***	***43.3***
SE	***0.7***	***0.5***	***20.4***	***SE***	***0.9***	***1.0***	***15.7***	***SE***	***0.5***	***0.6***	***14.4***

$CI_{P(PM)}$

HIIT				STCT				SIT			
Pcpt	**Pre**	**Post**	**% Δ**	**Pcpt**	**Pre**	**Post**	**% Δ**	**Pcpt**	**Pre**	**Post**	**% Δ**
HIT 1	46.7	41.3	-11.46	STCT 1	81.2	67.5	-16.87	SIT 1	55.5	68.8	24.00
HIT 2	36.5	44.5	22.05	STCT 2	63.5	51.3	-19.20	SIT 2	52.1	64.0	22.73
HIT 3	50.9	50.1	-1.62	STCT 3	60.1	62.7	4.49	SIT 3	62.5	56.3	-9.82
HIT 4	36.7	40.1	9.04	STCT 4	52.0	79.3	52.60	SIT 4	73.2	92.6	26.42
HIT 5	37.0	52.3	41.26	STCT 5	75.1	60.8	-19.01	SIT 5	59.6	77.6	30.16
HIT 6	44.4	37.8	-14.88	STCT 6	72.9	47.9	-34.29	SIT 6	51.0	78.5	53.96
HIT 7	49.3	40.5	-17.92	STCT 7	79.7	62.6	-21.47	SIT 7	60.8	54.3	-10.65
HIT 8	44.6	52.2	17.03	STCT 8	44.1	45.0	1.97	SIT 8	49.5	58.6	18.29
HIT 9	43.7	36.6	-16.07	STCT 9	40.0	63.6	59.04	SIT 9	67.7	86.9	28.28
HIT 10	65.7	53.4	-18.72								
HIT 11	41.9	46.9	12.14								
Mean	**45.2**	**45.1**	**1.9**	**Mean**	**63.2**	**60.1**	**0.8**	**Mean**	**59.1**	**70.8**	**20.4**
SD	***8.4***	***6.2***	***19.9***	***SD***	***15.3***	***10.6***	***33.4***	***SD***	***8.0***	***13.8***	***20.1***
SE	***2.5***	***1.9***	***6.0***	***SE***	***5.1***	***3.5***	***11.1***	***SE***	***2.7***	***4.6***	***6.7***

$CI+CII_{P(PMS)}$

HIIT				STCT				SIT			
Pcpt	**Pre**	**Post**	**% Δ**	**Pcpt**	**Pre**	**Post**	**% Δ**	**Pcpt**	**Pre**	**Post**	**% Δ**
HIT 1	79.3	61.7	-22.23	STCT 1	99.1	99.9	0.75	SIT 1	84.4	102.0	20.86
HIT 2	53.4	63.0	18.04	STCT 2	106.4	78.5	-26.24	SIT 2	77.1	92.6	20.21
HIT 3	82.7	76.2	-7.94	STCT 3	71.8	92.2	28.40	SIT 3	92.9	101.4	9.21
HIT 4	55.5	64.2	15.72	STCT 4	83.7	117.4	40.20	SIT 4	109.6	137.9	25.82
HIT 5	54.8	69.4	26.52	STCT 5	106.9	90.9	-14.94	SIT 5	74.9	120.6	61.14
HIT 6	71.7	63.3	-11.67	STCT 6	86.3	70.9	-17.85	SIT 6	74.3	112.2	51.08
HIT 7	73.2	58.6	-20.01	STCT 7	115.0	97.5	-15.19	SIT 7	83.4	83.4	0.07
HIT 8	70.9	76.9	8.39	STCT 8	61.6	67.1	8.99	SIT 8	76.5	84.6	10.60
HIT 9	61.5	51.5	-16.33	STCT 9	66.8	91.1	36.31	SIT 9	97.6	127.0	30.11
HIT 10	85.3	73.5	-13.79								
HIT 11	60.2	63.8	6.04								
Mean	**68.0**	**65.6**	**-1.6**	**Mean**	**88.6**	**89.5**	**4.5**	**Mean**	**85.6**	**106.9**	**25.5**
SD	***11.6***	***7.7***	***17.0***	***SD***	***19.3***	***15.5***	***25.3***	***SD***	***12.1***	***19.0***	***19.8***
SE	***3.5***	***2.3***	***5.1***	***SE***	***6.4***	***5.2***	***8.4***	***SE***	***4.0***	***6.3***	***6.6***

$CI+CII_{E(PMS)}$

HIIT				STCT				SIT			
Pcpt	**Pre**	**Post**	**%Δ**	**Pcpt**	**Pre**	**Post**	**%Δ**	**Pcpt**	**Pre**	**Post**	**%Δ**
HIT 1	59.2	89.3	50.89	STCT 1	116.4	138.0	18.49	SIT 1	100.4	129.8	29.23
HIT 2	50.9	75.0	47.41	STCT 2	132.3	92.4	-30.14	SIT 2	96.5	109.1	13.04
HIT 3	81.6	98.4	20.57	STCT 3	99.4	123.9	24.72	SIT 3	120.6	131.4	8.90
HIT 4	50.4	85.9	70.49	STCT 4	121.2	137.6	13.55	SIT 4	117.6	186.3	58.39
HIT 5	50.1	85.7	71.12	STCT 5	126.5	110.9	-12.33	SIT 5	101.7	168.7	65.85
HIT 6	67.2	86.6	28.95	STCT 6	105.2	83.4	-20.72	SIT 6	113.5	124.4	9.57
HIT 7	91.0	80.3	-11.78	STCT 7	147.3	115.1	-21.87	SIT 7	97.7	104.1	6.48
HIT 8	86.4	101.5	17.45	STCT 8	80.5	82.1	2.05	SIT 8	92.5	101.9	10.11
HIT 9	66.4	68.2	2.67	STCT 9	73.7	112.6	52.82	SIT 9	125.5	200.6	59.88
HIT 10	111.2	106.7	-3.99								
HIT 11	81.9	82.7	1.00								
Mean	**87.4**	**87.9**	**1.1**	**Mean**	**111.4**	**110.7**	**3.0**	**Mean**	**107.4**	**139.6**	**29.1**
SD	***16.2***	***15.9***	***10.7***	***SD***	***24.1***	***21.1***	***27.0***	***SD***	***12.0***	***36.7***	***25.2***
SE	***7.2***	***7.1***	***4.8***	***SE***	***8.0***	***7.0***	***9.0***	***SE***	***4.0***	***12.2***	***8.4***

$CII_{E(PMS+Rot)}$

HIIT				STCT				SIT			
Pcpt	**Pre**	**Post**	**%Δ**	**Pcpt**	**Pre**	**Post**	**%Δ**	**Pcpt**	**Pre**	**Post**	**%Δ**
HIT 1	49.1	38.7	-21.28	STCT 1	36.8	62.7	70.40	SIT 1	44.2	54.2	22.57
HIT 2	35.2	38.8	10.18	STCT 2	58.9	38.3	-34.97	SIT 2	39.6	48.5	22.41
HIT 3	49.6	41.1	-17.16	STCT 3	21.5	52.1	142.39	SIT 3	45.7	59.6	30.45
HIT 4	31.6	39.8	26.10	STCT 4	50.1	70.8	41.36	SIT 4	54.4	86.2	58.42
HIT 5	34.4	39.9	16.07	STCT 5	49.8	47.3	-5.07	SIT 5			
HIT 6	51.4	48.8	-5.18	STCT 6	28.0	42.5	51.88	SIT 6	52.6	59.1	12.42
HIT 7	40.1	37.6	-6.25	STCT 7	56.3	58.1	3.15	SIT 7	43.4	42.3	-2.57
HIT 8	46.9	44.8	-4.47	STCT 8	31.3	30.6	-2.22	SIT 8	40.8	48.0	17.56
HIT 9	35.3	48.9	38.48	STCT 9	35.2	40.6	15.40	SIT 9	55.6	89.1	60.19
HIT 10	49.8	39.3	-21.03								
HIT 11	35.5	34.8	-1.91								
Mean	**41.72**	**41.13**	**1.23**	**Mean**	**44.22**	**46.89**	**9.93**	**Mean**	**47.05**	**60.87**	**27.68**
SD	***7.65***	***4.50***	***19.46***	***SD***	***11.87***	***13.70***	***34.67***	***SD***	***6.27***	***17.53***	***21.76***
SE	***2.31***	***1.36***	***5.87***	***SE***	***4.20***	***4.84***	***12.26***	***SE***	***2.22***	***6.20***	***7.69***

$CIV_{Res(Tm+As)}$

HIIT				STCT				SIT			
Pcpt	**Pre**	**Post**	**%Δ**	**Pcpt**	**Pre**	**Post**	**%Δ**	**Pcpt**	**Pre**	**Post**	**%Δ**
HIT 1	124.5	141.0	13.25	STCT 1	173.5	195.3	12.58	SIT 1	131.6	137.8	4.71
HIT 2	101.9	115.5	13.32	STCT 2	165.9	116.6	-29.68	SIT 2	101.5	144.8	42.59
HIT 3	140.2	116.7	-16.79	STCT 3	98.0	144.2	47.22	SIT 3	126.6	128.0	1.16
HIT 4	122.5	146.8	19.85	STCT 4	118.8	169.7	42.86	SIT 4	179.5	231.5	28.94
HIT 5	94.3	98.7	4.65	STCT 5	135.8	117.7	-13.28	SIT 5	126.3	188.1	48.92
HIT 6	144.4	119.6	-17.15	STCT 6	154.1	114.5	-25.70	SIT 6	137.2	142.7	4.03
HIT 7	120.0	139.7	16.44	STCT 7	153.7	126.7	-17.56	SIT 7	116.6	111.9	-4.04
HIT 8	134.8	128.2	-4.90	STCT 8	98.3	79.8	-18.77	SIT 8	121.1	117.9	-2.60
HIT 9	116.5	119.2	2.28	STCT 9	95.7	90.2	-5.77	SIT 9	150.6	245.5	63.01
HIT 10	152.7	131.7	-13.77								
HIT 11	111.9	118.6	5.99								
Mean	**124.0**	**125.1**	**2.1**	**Mean**	**132.6**	**128.3**	**-0.9**	**Mean**	**132.3**	**160.9**	**20.7**
SD	***17.9***	***14.0***	***13.5***	***SD***	***30.9***	***36.6***	***28.8***	***SD***	***22.3***	***49.2***	***25.5***
SE	***5.4***	***4.2***	***4.1***	***SE***	***10.3***	***12.2***	***9.6***	***SE***	***7.4***	***16.4***	***8.5***

Enzyme activity

CS activity (mol/h/kg protein)

HIIT				STCT				SIT			
Pcpt	**Pre**	**Post**	**% Δ**	**Pcpt**	**Pre**	**Post**	**% Δ**	**Pcpt**	**Pre**	**Post**	**% Δ**
HIT 1	11.7	9.2	-21.22	STCT 1	8.1	10.4	27.89	SIT 1	10.0	8.5	-14.71
HIT 2	6.2	11.8	91.33	STCT 2	11.9	9.2	-22.20	SIT 2	8.4	7.5	-11.29
HIT 3	7.3	7.0	-3.84	STCT 3	10.2	11.3	10.64	SIT 3	9.7	10.9	12.81
HIT 4	5.4	7.6	38.63	STCT 4	7.5	9.7	28.52	SIT 4	10.1	10.5	4.72
HIT 5	6.2	8.7	40.54	STCT 5	10.3	9.8	-4.94	SIT 5	11.2	12.9	14.65
HIT 6	10.1	8.9	-11.74	STCT 6	9.7	9.7	-0.70	SIT 6	9.1	8.9	-2.04
HIT 7	6.7	6.1	-8.31	STCT 7	7.6	10.2	35.55	SIT 7	5.9	7.1	20.18
HIT 8	8.5	8.0	-6.88	STCT 8	7.7	8.9	15.68	SIT 8	8.1	8.4	3.01
HIT 9	7.2	9.3	29.80	STCT 9	10.8	9.0	-16.58	SIT 9	11.9	11.7	-2.24
HIT 10	11.4	11.3	-0.63								
HIT 11	7.4	6.6	-11.40								
Mean	**8.0**	**8.6**	**12.4**	**Mean**	**9.3**	**9.8**	**8.2**	**Mean**	**9.4**	**9.6**	**2.8**
SD	***2.2***	***1.8***	***33.9***	***SD***	***1.6***	***0.8***	***20.6***	***SD***	***1.8***	***2.0***	***11.7***
SE	***0.6***	***0.5***	***10.2***	***SE***	***0.5***	***0.3***	***6.9***	***SE***	***0.6***	***0.7***	***3.9***

Protein content (expressed as arbitrary units)

PGC-1α

HIIT - raw data				STCT - raw data				SIT - raw data			
Pcpt	**Pre**	**Post**	**% Δ**	**Pcpt**	**Pre**	**Post**	**% Δ**	**Pcpt**	**Pre**	**Post**	**% Δ**
HIT 1	1.73	1.39	-20.0	STCT 1	1.52	1.25	-17.7	SIT 1	1.15	1.35	17.5
HIT 2	1.84	2.09	13.8	STCT 2	0.73	1.25	71.6	SIT 2	0.63	1.71	172.4
HIT 3	1.77	1.52	-14.2	STCT 3	1.74	0.91	-48.1	SIT 3	1.09	1.66	51.2
HIT 4	0.94	2.51	167.0	STCT 4	1.12	1.60	42.3	SIT 4	1.13	1.60	41.7
HIT 5	0.97	1.76	81.4	STCT 5	0.74	1.43	92.4	SIT 5	1.06	1.37	28.9
HIT 6	0.86	1.70	97.9	STCT 6	0.90	1.20	33.0	SIT 6	0.62	1.28	107.0
HIT 7	1.25	1.14	-8.8	STCT 7	2.25	2.50	11.2	SIT 7	1.86	1.61	-13.7
HIT 8	0.80	1.53	90.9	STCT 8	2.13	3.37	58.1	SIT 8	1.08	1.40	30.3
HIT 9	1.36	0.78	-42.6	STCT 9	1.52	1.80	18.9	SIT 9	1.29	2.77	115.6
HIT 10	0.75	0.90	19.5								
HIT 11	0.73	0.67	-8.1								
Mean	**1.2**	**1.5**	**34.3**	**Mean**	**1.4**	**1.7**	**29.1**	**Mean**	**1.1**	**1.6**	**61.2**
SD	***0.4***	***0.6***	***65.3***	***SD***	***0.6***	***0.8***	***43.9***	***SD***	***0.4***	***0.5***	***58.5***
SE	***0.1***	***0.2***	***19.7***	***SE***	***0.2***	***0.3***	***14.6***	***SE***	***0.1***	***0.2***	***19.5***

p53

HIT - raw data				STCT - raw data				SIT - raw data			
Pcpt	**Pre**	**Post**	**% Δ**	**Pcpt**	**Pre**	**Post**	**% Δ**	**Pcpt**	**Pre**	**Post**	**% Δ**
HIT 1	0.82	1.08	31.0	STCT 1	1.54	1.50	-3.1	SIT 1	0.74	1.89	154.3
HIT 2	1.58	1.58	0.0	STCT 2	0.72	1.09	50.5	SIT 2	0.79	1.86	133.8
HIT 3	1.62	1.58	-2.1	STCT 3	1.53	0.76	-50.3	SIT 3	1.39	1.97	42.1
HIT 4	1.02	1.64	60.5	STCT 4	1.01	1.39	37.9	SIT 4	0.70	1.21	72.3
HIT 5	0.81	1.96	143.0	STCT 5	0.92	1.38	50.8	SIT 5	0.79	1.03	30.6
HIT 6	0.37	1.08	189.8	STCT 6	0.92	1.20	30.3	SIT 6	0.52	1.24	138.1
HIT 7	0.49	0.93	87.7	STCT 7	1.76	1.41	-20.0	SIT 7	1.58	0.80	-49.2
HIT 8	0.93	0.98	6.0	STCT 8	1.28	1.72	33.9	SIT 8	0.45	0.96	111.3
HIT 9	1.14	0.38	-67.0	STCT 9	0.52	0.71	35.1	SIT 9	0.46	1.41	206.2
HIT 10	0.48	0.87	79.6								
HIT 11	1.19	0.64	-46.1								
Mean	**1.0**	**1.2**	**43.8**	**Mean**	**1.1**	**1.2**	**18.3**	**Mean**	**0.8**	**1.4**	**93.3**
SD	***0.4***	***0.5***	***77.9***	***SD***	***0.4***	***0.3***	***34.9***	***SD***	***0.4***	***0.4***	***77.2***
SE	***0.1***	***0.1***	***23.5***	***SE***	***0.1***	***0.1***	***11.6***	***SE***	***0.1***	***0.1***	***25.7***

PHF20

HIT - raw data				STCT - raw data				SIT - raw data			
Pcpt	**Pre**	**Post**	**% Δ**	**Pcpt**	**Pre**	**Post**	**% Δ**	**Pcpt**	**Pre**	**Post**	**% Δ**
HIT 1	1.20	1.31	8.9	STCT 1	2.32	3.09	33.2	SIT 1	1.57	2.26	43.7
HIT 2	1.51	1.29	-14.2	STCT 2	1.35	1.81	33.4	SIT 2	0.95	3.01	217.7
HIT 3	1.74	1.42	-18.5	STCT 3	2.94	1.26	-57.1	SIT 3	3.02	3.41	13.0
HIT 4	0.96	0.92	-4.0	STCT 4	1.29	2.14	65.3	SIT 4	1.00	2.09	109.4
HIT 5	0.92	0.84	-8.6	STCT 5	1.01	1.63	61.6	SIT 5	0.98	1.36	38.0
HIT 6	0.55	0.44	-20.8	STCT 6	1.49	1.48	-0.4	SIT 6	0.74	1.77	138.0
HIT 7	0.94	1.24	32.1	STCT 7	2.53	2.23	-11.8	SIT 7	2.17	1.50	-30.8
HIT 8	1.52	1.96	29.1	STCT 8	2.05	3.05	48.6	SIT 8	0.79	1.49	89.2
HIT 9	1.91	0.90	-52.7	STCT 9	1.29	2.48	92.3	SIT 9	0.91	2.19	140.1
HIT 10	0.69	0.87	25.9								
HIT 11	0.94	1.27	34.7								
Mean	**1.2**	**1.1**	**1.1**	**Mean**	**1.8**	**2.1**	**29.5**	**Mean**	**1.3**	**2.1**	**84.3**
SD	***0.4***	***0.4***	***27.7***	***SD***	***0.7***	***0.7***	***45.8***	***SD***	***0.8***	***0.7***	***76.3***
SE	***0.1***	***0.1***	***8.3***	***SE***	***0.2***	***0.2***	***15.3***	***SE***	***0.3***	***0.2***	***25.4***

TFAM

HIT - raw data				STCT - raw data				SIT - raw data			
Pcpt	**Pre**	**Post**	**% Δ**	**Pcpt**	**Pre**	**Post**	**% Δ**	**Pcpt**	**Pre**	**Post**	**% Δ**
HIT 1	0.96	1.50	55.6	STCT 1	2.04	1.56	-23.5	SIT 1	2.25	1.41	-37.1
HIT 2	1.43	0.90	-37.1	STCT 2	1.54	1.79	15.9	SIT 2	1.56	1.57	0.6
HIT 3	1.74	1.56	-10.2	STCT 3	1.96	2.29	16.5	SIT 3	2.32	3.68	58.5
HIT 4	1.39	1.08	-22.2	STCT 4	1.32	1.65	25.0	SIT 4	1.42	1.93	35.5
HIT 5	0.97	2.07	113.3	STCT 5	1.31	1.18	-9.4	SIT 5	1.46	1.65	12.4
HIT 6	1.09	1.06	-3.0	STCT 6	1.30	1.42	9.2	SIT 6	1.28	1.03	-19.4
HIT 7	1.02	0.76	-25.4	STCT 7	1.11	1.00	-9.9	SIT 7	1.36	1.07	-20.7
HIT 8	1.20	2.91	143.2	STCT 8	0.71	1.86	163.7	SIT 8	0.95	1.10	15.6
HIT 9	0.86	0.46	-46.2	STCT 9	2.08	1.33	-36.1	SIT 9	1.36	1.87	37.3
HIT 10	0.41	0.72	74.9								
HIT 11	1.69	0.93	-44.9								
Mean	**1.2**	**1.3**	**18.0**	**Mean**	**1.5**	**1.6**	**16.8**	**Mean**	**1.6**	**1.7**	**9.2**
SD	***0.4***	***0.7***	***67.3***	***SD***	***0.5***	***0.4***	***58.7***	***SD***	***0.5***	***0.8***	***31.4***
SE	***0.1***	***0.2***	***20.3***	***SE***	***0.2***	***0.1***	***19.6***	***SE***	***0.2***	***0.3***	***10.5***

Complex I

HIT - raw data				STCT - raw data				SIT - raw data			
Pcpt	**Pre**	**Post**	**% Δ**	**Pcpt**	**Pre**	**Post**	**% Δ**	**Pcpt**	**Pre**	**Post**	**% Δ**
HIT 1	1.05	1.07	1.6	STCT 1	1.37	1.36	-1.0	SIT 1	1.33	1.19	-10.4
HIT 2	0.93	1.22	31.3	STCT 2	1.07	1.03	-3.7	SIT 2	0.92	0.99	7.4
HIT 3	1.25	1.05	-16.0	STCT 3	1.18	1.25	5.7	SIT 3	1.66	1.58	-4.7
HIT 4	1.02	1.21	19.4	STCT 4	1.09	1.37	26.2	SIT 4	1.07	1.03	-3.9
HIT 5	1.36	1.24	-8.7	STCT 5	1.04	0.92	-12.2	SIT 5	1.11	1.31	17.5
HIT 6	1.15	1.26	9.6	STCT 6	1.23	1.14	-7.3	SIT 6	0.77	0.75	-2.9
HIT 7	1.29	1.15	-11.1	STCT 7	0.71	1.01	41.8	SIT 7	0.56	1.05	88.6
HIT 8	1.10	1.15	5.2	STCT 8	0.36	0.57	57.1	SIT 8	0.30	0.53	74.9
HIT 9	1.20	1.42	17.7	STCT 9	1.16	0.71	-38.7	SIT 9	1.06	1.11	4.4
HIT 10	1.05	1.17	11.0								
HIT 11	0.87	0.89	3.2								
Mean	**1.1**	**1.2**	**5.8**	**Mean**	**1.0**	**1.0**	**7.6**	**Mean**	**1.0**	**1.1**	**19.0**
SD	***0.2***	***0.1***	***14.2***	***SD***	***0.3***	***0.3***	***29.4***	***SD***	***0.4***	***0.3***	***36.7***
SE	***0.0***	***0.0***	***4.3***	***SE***	***0.1***	***0.1***	***9.8***	***SE***	***0.1***	***0.1***	***12.2***

Complex II

HIT - raw data				STCT - raw data				SIT - raw data			
Pcpt	**Pre**	**Post**	**% Δ**	**Pcpt**	**Pre**	**Post**	**% Δ**	**Pcpt**	**Pre**	**Post**	**% Δ**
HIT 1	1.26	1.42	12.7	STCT 1	1.02	1.37	34.4	SIT 1	1.27	1.21	-4.7
HIT 2	1.07	1.56	46.4	STCT 2	1.03	0.85	-17.3	SIT 2	0.99	0.88	-10.7
HIT 3	1.34	1.20	-9.8	STCT 3	1.22	1.18	-3.5	SIT 3	1.25	1.23	-1.9
HIT 4	1.11	1.29	16.0	STCT 4	1.37	2.03	48.2	SIT 4	1.34	1.22	-9.5
HIT 5	1.42	1.23	-13.2	STCT 5	1.59	1.34	-16.1	SIT 5	1.82	1.80	-0.6
HIT 6	1.08	1.19	10.3	STCT 6	1.48	1.37	-7.3	SIT 6	1.20	0.91	-23.9
HIT 7	1.87	1.06	-43.0	STCT 7	0.68	1.01	48.3	SIT 7	0.72	1.30	80.4
HIT 8	0.94	1.42	51.0	STCT 8	0.46	0.54	17.9	SIT 8	0.57	0.88	53.5
HIT 9	1.34	1.68	25.3	STCT 9	1.00	1.00	-0.1	SIT 9	0.66	0.75	14.5
HIT 10	0.72	0.67	-7.9								
HIT 11	0.79	0.92	16.2								
Mean	**1.2**	**1.2**	**9.5**	**Mean**	**1.1**	**1.2**	**11.6**	**Mean**	**1.1**	**1.1**	**10.8**
SD	***0.3***	***0.3***	***27.2***	***SD***	***0.4***	***0.4***	***26.4***	***SD***	***0.4***	***0.3***	***34.1***
SE	***0.1***	***0.1***	***8.2***	***SE***	***0.1***	***0.1***	***8.8***	***SE***	***0.1***	***0.1***	***11.4***

Complex III

HIT - raw data				STCT - raw data				SIT - raw data			
Pcpt	**Pre**	**Post**	**% Δ**	**Pcpt**	**Pre**	**Post**	**% Δ**	**Pcpt**	**Pre**	**Post**	**% Δ**
HIT 1	1.20	1.14	-4.9	STCT 1	1.38	1.46	6.1	SIT 1	1.34	1.44	7.2
HIT 2	1.09	1.22	12.0	STCT 2	1.32	1.39	5.9	SIT 2	1.39	1.18	-15.3
HIT 3	1.20	1.28	6.2	STCT 3	1.40	1.69	20.3	SIT 3	1.44	1.70	18.0
HIT 4	1.13	1.27	12.4	STCT 4	2.02	2.25	11.4	SIT 4	1.38	2.09	51.2
HIT 5	1.22	1.24	1.5	STCT 5	1.91	1.71	-10.4	SIT 5	2.12	2.02	-5.1
HIT 6	1.30	1.29	-0.6	STCT 6	1.94	2.12	9.1	SIT 6	1.86	1.56	-16.0
HIT 7	1.06	1.04	-1.8	STCT 7	1.69	2.00	18.6	SIT 7	1.01	1.12	11.4
HIT 8	1.04	1.10	6.2	STCT 8	1.58	1.86	17.8	SIT 8	1.71	1.94	13.9
HIT 9	1.05	1.09	3.7	STCT 9	1.83	1.55	-15.1	SIT 9	2.02	1.91	-5.5
HIT 10	1.01	0.94	-6.5								
HIT 11	1.02	1.05	3.8								
Mean	**1.1**	**1.2**	**2.9**	**Mean**	**1.7**	**1.8**	**7.1**	**Mean**	**1.6**	**1.7**	**6.6**
SD	***0.1***	***0.1***	***6.2***	***SD***	***0.3***	***0.3***	***12.5***	***SD***	***0.4***	***0.4***	***20.8***
SE	***0.0***	***0.0***	***1.9***	***SE***	***0.1***	***0.1***	***4.2***	***SE***	***0.1***	***0.1***	***6.9***

Complex IV

HIT - raw data				STCT - raw data				SIT - raw data			
Pcpt	**Pre**	**Post**	**% Δ**	**Pcpt**	**Pre**	**Post**	**% Δ**	**Pcpt**	**Pre**	**Post**	**% Δ**
HIT 1	0.98	1.08	10.2	STCT 1	1.12	1.26	13.2	SIT 1	1.25	1.09	-13.0
HIT 2	0.91	1.27	39.9	STCT 2	0.89	0.86	-3.4	SIT 2	0.74	0.79	6.1
HIT 3	1.41	1.32	-6.4	STCT 3	1.22	1.24	1.8	SIT 3	1.28	1.25	-2.0
HIT 4	1.06	1.15	8.5	STCT 4	1.01	1.33	31.4	SIT 4	1.00	1.10	10.3
HIT 5	1.35	1.12	-17.0	STCT 5	1.15	0.99	-13.8	SIT 5	1.18	1.18	-0.4
HIT 6	1.07	1.22	14.7	STCT 6	1.20	1.26	5.6	SIT 6	1.00	0.81	-19.1
HIT 7	1.15	1.01	-12.2	STCT 7	0.78	1.08	39.1	SIT 7	0.71	1.06	49.2
HIT 8	1.05	1.01	-3.4	STCT 8	0.04	0.04	9.5	SIT 8	0.40	0.80	99.3
HIT 9	1.07	1.16	8.4	STCT 9	1.87	1.08	-42.3	SIT 9	1.14	1.22	6.9
HIT 10	0.80	0.88	9.8								
HIT 11	0.81	0.83	2.8								
Mean	**1.1**	**1.1**	**5.0**	**Mean**	**1.0**	**1.0**	**4.6**	**Mean**	**1.0**	**1.0**	**15.2**
SD	***0.2***	***0.2***	***15.4***	***SD***	***0.5***	***0.4***	***24.0***	***SD***	***0.3***	***0.2***	***36.9***
SE	***0.1***	***0.0***	***4.6***	***SE***	***0.2***	***0.1***	***8.0***	***SE***	***0.1***	***0.1***	***12.3***

Complex V

HIT - raw data				STCT - raw data				SIT - raw data			
Pcpt	**Pre**	**Post**	**% Δ**	**Pcpt**	**Pre**	**Post**	**% Δ**	**Pcpt**	**Pre**	**Post**	**% Δ**
HIT 1	1.18	1.26	7.2	STCT 1	1.18	1.66	40.1	SIT 1	1.18	1.31	10.5
HIT 2	1.12	1.26	12.5	STCT 2	1.29	1.40	8.8	SIT 2	1.22	1.15	-5.9
HIT 3	1.30	1.25	-4.2	STCT 3	1.14	1.63	42.9	SIT 3	1.51	1.53	1.2
HIT 4	1.25	1.26	1.0	STCT 4	1.45	1.60	10.0	SIT 4	1.06	1.21	13.8
HIT 5	1.21	1.27	5.4	STCT 5	1.36	1.31	-3.9	SIT 5	1.51	1.61	6.4
HIT 6	1.21	1.22	1.3	STCT 6	1.85	1.59	-14.4	SIT 6	1.20	0.98	-18.0
HIT 7	1.07	0.98	-8.5	STCT 7	1.23	2.04	65.5	SIT 7	0.92	1.04	12.9
HIT 8	0.95	1.13	19.4	STCT 8	0.92	1.43	54.8	SIT 8	1.12	1.57	39.9
HIT 9	0.97	1.01	4.2	STCT 9	2.10	1.47	-30.0	SIT 9	1.66	1.53	-7.5
HIT 10	0.89	0.83	-7.1								
HIT 11	0.86	0.79	-8.0								
Mean	**1.1**	**1.1**	**2.1**	**Mean**	**1.4**	**1.6**	**19.3**	**Mean**	**1.3**	**1.3**	**5.9**
SD	***0.2***	***0.2***	***8.9***	***SD***	***0.4***	***0.2***	***32.9***	***SD***	***0.2***	***0.2***	***16.6***
SE	***0.0***	***0.1***	***2.7***	***SE***	***0.1***	***0.1***	***11.0***	***SE***	***0.1***	***0.1***	***5.5***

MFN2

HIT - raw data				STCT - raw data				SIT - raw data			
Pcpt	**Pre**	**Post**	**% Δ**	**Pcpt**	**Pre**	**Post**	**% Δ**	**Pcpt**	**Pre**	**Post**	**% Δ**
DR	2.86	1.75	-38.8	4MM	1.67	2.17	29.8	1EA	1.36	1.47	7.6
EA	1.49	3.09	107.8	5JR	1.27	1.34	5.5	2DR	1.55	1.70	9.5
AG	1.45	1.62	11.8	7MR	1.23	1.95	58.4	8SM	1.48	1.77	19.9
MV	0.96	1.16	21.2	10JK	1.91	2.23	16.7	9MA	2.26	2.37	4.9
HB	1.23	1.66	35.0	12SA	1.81	2.75	52.1	11FS	2.23	2.64	18.0
EC	1.41	1.05	-25.4	13DD	1.97	2.04	3.6	17JS	1.96	2.07	5.7
MP	2.22	1.36	-38.8	14MT	0.63	2.44	285.2	18MF	0.78	0.74	-4.6
MM	2.01	3.12	55.4	16HM	0.59	1.53	158.5	19SB	0.61	1.66	170.1
PV	1.44	1.24	-14.1	21SO	2.04	0.98	-52.1	23TS	1.05	0.74	-29.1
BO	0.79	1.41	79.0								
PA	0.96	0.72	-24.7								
Mean	**1.5**	**1.7**	**15.3**	**Mean**	**1.5**	**1.9**	**61.9**	**Mean**	**1.5**	**1.7**	**22.4**
SD	***0.6***	***0.8***	***49.6***	***SD***	***0.6***	***0.6***	***101.2***	***SD***	***0.6***	***0.6***	***57.2***
SE	***0.2***	***0.2***	***15.0***	***SE***	***0.2***	***0.2***	***33.7***	***SE***	***0.2***	***0.2***	***19.1***

DRP1

HIT - raw data				STCT - raw data				SIT - raw data			
Pcpt	**Pre**	**Post**	**% Δ**	**Pcpt**	**Pre**	**Post**	**% Δ**	**Pcpt**	**Pre**	**Post**	**% Δ**
DR	0.59	0.62	5.7	4MM	1.90	1.14	-40.0	1EA	2.52	1.12	-55.4
EA	0.92	0.71	-22.7	5JR	1.14	2.33	104.3	2DR	1.39	1.95	40.2
AG	0.78	0.70	-10.1	7MR	1.49	2.16	45.3	8SM	1.70	3.97	133.2
MV	0.62	1.14	84.3	10JK	3.37	9.04	168.5	9MA	2.90	10.46	260.8
HB	0.58	1.12	92.7	12SA	4.11	2.97	-27.6	11FS	3.82	4.21	10.1
EC	0.33	0.90	173.1	13DD	6.36	7.23	13.7	17JS	6.04	3.88	-35.8
MP	0.52	0.48	-8.4	14MT	5.14	4.36	-15.1	18MF	2.59	1.19	-54.1
MM	0.84	0.99	17.9	16HM	0.84	4.31	414.7	19SB	1.91	2.42	27.0
PV	0.77	0.63	-18.0	21SO	3.53	2.60	-26.4	23TS	1.39	3.13	125.7
BO	0.51	0.54	4.2								
PA	0.57	0.63	9.5								
Mean	**0.6**	**0.8**	**29.8**	**Mean**	**3.1**	**4.0**	**70.8**	**Mean**	**2.7**	**3.6**	**50.2**
SD	***0.2***	***0.2***	***61.1***	***SD***	***1.9***	***2.6***	***146.5***	***SD***	***1.5***	***2.8***	***105.2***
SE	***0.1***	***0.1***	***18.4***	***SE***	***0.6***	***0.9***	***48.8***	***SE***	***0.5***	***0.9***	***35.1***

SCO2

HIT - raw data				STCT - raw data				SIT - raw data			
Pcpt	**Pre**	**Post**	**% Δ**	**Pcpt**	**Pre**	**Post**	**% Δ**	**Pcpt**	**Pre**	**Post**	**% Δ**
DR	2.29	3.87	68.9	4MM	2.08	3.10	48.9	1EA	2.43	2.24	-7.7
EA	2.64	3.31	25.3	5JR	1.70	2.53	49.0	2DR	2.57	2.08	-19.2
AG	1.70	1.21	-28.8	7MR	1.50	1.69	12.5	8SM	1.59	2.19	37.6
MV	2.03	2.53	24.7	10JK	1.85	2.26	22.1	9MA	1.07	1.84	71.8
HB	3.17	3.38	6.7	12SA	1.95	2.29	17.6	11FS	1.94	2.30	18.9
EC	3.13	1.73	-44.6	13DD	1.74	1.52	-12.5	17JS	1.68	1.83	9.0
MP	1.96	1.19	-39.4	14MT	2.80	4.16	48.3	18MF	1.85	2.58	39.4
MM	1.48	1.29	-12.6	16HM	1.98	2.80	41.4	19SB	3.00	2.26	-24.6
PV	1.20	1.25	4.0	21SO	1.55	1.91	23.3	23TS	2.48	2.88	16.3
BO	1.40	2.45	75.1								
PA	2.68	1.46	-45.5								
Mean	**2.2**	**2.2**	**3.1**	**Mean**	**1.9**	**2.5**	**27.8**	**Mean**	**2.1**	**2.2**	**15.7**
SD	***0.7***	***1.0***	***42.7***	***SD***	***0.4***	***0.8***	***21.0***	***SD***	***0.6***	***0.3***	***30.9***
SE	***0.2***	***0.3***	***12.9***	***SE***	***0.1***	***0.3***	***7.0***	***SE***	***0.2***	***0.1***	***10.3***

AIF

HIT - raw data				STCT - raw data				SIT - raw data			
Pcpt	**Pre**	**Post**	**% Δ**	**Pcpt**	**Pre**	**Post**	**% Δ**	**Pcpt**	**Pre**	**Post**	**% Δ**
DR	1.94	1.88	-3.3	4MM	2.42	3.59	48.5	1EA	1.98	2.70	36.5
EA	1.49	2.76	85.5	5JR	1.87	2.24	19.6	2DR	1.44	2.67	85.2
AG	1.49	1.68	12.7	7MR	4.06	2.29	-43.6	8SM	3.28	2.32	-29.2
MV	0.99	1.34	35.5	10JK	5.24	4.80	-8.4	9MA	2.25	4.23	88.3
HB	0.82	1.22	48.0	12SA	1.91	3.19	67.2	11FS	6.09	4.15	-31.9
EC	0.60	0.45	-25.1	13DD	1.97	6.47	228.1	17JS	1.66	3.41	106.2
MP	2.32	2.08	-10.5	14MT	2.29	2.95	28.6	18MF	2.05	2.27	10.9
MM	1.31	3.40	160.5	16HM	1.57	3.09	97.1	19SB	1.92	2.40	25.0
PV	3.16	2.24	-29.1	21SO	2.47	1.44	-41.7	23TS	2.74	2.44	-10.9
BO	2.15	0.92	-57.3								
PA	0.34	1.46	334.3								
Mean	**1.5**	**1.8**	**50.1**	**Mean**	**2.6**	**3.3**	**43.9**	**Mean**	**2.6**	**3.0**	**31.1**
SD	***0.8***	***0.8***	***112.2***	***SD***	***1.2***	***1.5***	***83.6***	***SD***	***1.4***	***0.8***	***52.1***
SE	***0.3***	***0.3***	***33.8***	***SE***	***0.4***	***0.5***	***27.9***	***SE***	***0.5***	***0.3***	***17.4***

CHAPTER 3 – RAW DATA

Physiological parameters

Age [y] - Mass [kg] - Height [cm]

Pcpt	Age	Mass	Height
Ptcp 1	20.2	94.7	191.6
Ptcp 2	24.0	69.5	169.0
Ptcp 3	20.9	72.5	174.0
Ptcp 4	19.6	112.0	180.3
Ptcp 5	19.3	66.1	170.7
Ptcp 6	20.6	86.5	193.0
Ptcp 7	19.9	84.7	198.6
Ptcp 8	21.8	76.6	180.5
Ptcp 9	18.7	65.9	165.8
Ptcp 10	19.9	77.0	173.4
Mean	**20.5**	**80.6**	**179.7**
SD	***1.5***	***14.5***	***11.3***
SE	***0.5***	***4.6***	***3.6***

Total Work [MJ]

Pcpt	NT	INT	RT
Ptcp 1	4.1	20.5	0.7
Ptcp 2	3.2	16.8	0.6
Ptcp 3	4.2	12.1	0.8
Ptcp 4	3.4	12.2	0.6
Ptcp 5	3.7	15.2	0.6
Ptcp 6	4.0	18.3	0.7
Ptcp 7	4.0	17.6	0.7
Ptcp 8	3.8	8.2	0.6
Ptcp 9	3.6	14.1	0.6
Ptcp 10	2.1	11.8	0.5
Mean	**3.6**	**14.7**	**0.6**
SD	***0.6***	***3.7***	***0.1***
SE	***0.2***	***1.2***	***0.0***

W_{LT} [w]

Pcpt	pre-NT	post-NT	post-INT	post-RT
Ptcp 1	216.0	238.9	272.4	271.6
Ptcp 2	163.2	187.5	187.0	200.2
Ptcp 3	246.3	266.4	283.4	270.7
Ptcp 4	184.8	203.4	225.9	202.4
Ptcp 5	209.4	207.8	214.6	203.0
Ptcp 6	209.8	217.7	259.1	264.3
Ptcp 7	211.6	247.5	264.1	250.0
Ptcp 8	208.0	226.1	222.3	202.3
Ptcp 9	202.4	210.0	222.7	229.1
Ptcp 10	147.5	159.9	183.2	186.0
Mean	**199.9**	**216.5**	**233.5**	**228.0**
SD	***28.1***	***30.5***	***34.9***	***33.3***
SE	***8.9***	***9.6***	***11.0***	***10.5***

W_{peak} [w]

Pcpt	pre-NT	post-NT	post-INT	post-RT
Ptcp 1	300.0	320.0	370.0	330.0
Ptcp 2	230.0	252.5	275.0	283.0
Ptcp 3	340.0	361.9	374.2	390.0
Ptcp 4	237.5	275.0	297.5	260.0
Ptcp 5	262.5	285.0	285.0	277.5
Ptcp 6	275.0	305.0	335.0	335.0
Ptcp 7	287.5	317.5	347.5	325.0
Ptcp 8	262.5	307.5	300.0	285.0
Ptcp 9	262.5	277.5	292.5	285.0
Ptcp 10	200.0	238.0	260.0	255.0
Mean	**265.8**	**294.0**	**313.7**	**302.6**
SD	***39.0***	***36.1***	***40.2***	***41.7***
SE	***12.3***	***11.4***	***12.7***	***13.2***

20k-TT [s]					VO_{2Peak} [mL min^{-1} kg^{-1}]				
Pcpt	**pre-NT**	**post-NT**	**post-INT**	**post-RT**	**Pcpt**	**pre-NT**	**post-NT**	**post-INT**	**post-RT**
Ptcp 1	2191.0	2096.0	1932.0	1987.0	Ptcp 1	38.2	39.6	48.0	43.5
Ptcp 2	2460.0	2234.0	2092.0	2094.0	Ptcp 2	47.1	46.9	54.2	51.5
Ptcp 3	1995.0	1985.0	1963.0	1925.0	Ptcp 3	55.3	59.7	62.9	63.1
Ptcp 4	2375.0	2220.0	2063.0	2052.0	Ptcp 4	33.3	35.8	38.7	37.2
Ptcp 5	2161.0	2142.0	2064.0	2140.0	Ptcp 5	52.6	51.0	53.9	47.4
Ptcp 6	2204.0	2128.0	1963.0	1978.0	Ptcp 6	39.2	42.9	45.4	40.7
Ptcp 7	2142.0	2038.0	1932.0	1927.0	Ptcp 7	45.8	44.2	55.8	47.1
Ptcp 8	2211.0	2093.0	2079.0	2041.0	Ptcp 8	47.9	53.8	58.9	56.3
Ptcp 9	2157.0	2118.0	2051.0	2087.0	Ptcp 9	53.5	54.3	59.6	56.8
Ptcp 10	2456.0	2306.0	2193.0	2241.0	Ptcp 10	38.1	41.3	44.5	45.2
Mean	**2235.2**	**2136.0**	**2033.2**	**2047.2**	**Mean**	**45.1**	**46.9**	**52.2**	**48.9**
SD	***149.2***	***95.4***	***84.1***	***98.8***	***SD***	***7.6***	***7.6***	***7.7***	***8.0***
SE	***47.2***	***30.2***	***26.6***	***31.2***	***SE***	***2.4***	***2.4***	***2.4***	***2.5***

Enzyme activity

CS activity (mol/h/kg protein)

Pcpt	pre-NT	post-NT	post-INT	post-RT
Ptcp 1	11.7	9.2	8.5	13.5
Ptcp 2	6.2	11.8	14.7	14.5
Ptcp 3	7.3	7.0	10.9	12.3
Ptcp 4	5.4	7.6	11.1	10.3
Ptcp 5	10.1	8.9	13.7	10.8
Ptcp 6	6.7	6.1	13.0	7.1
Ptcp 7	8.5	8.0	9.6	9.5
Ptcp 8	7.2	9.3	10.4	9.9
Ptcp 9	11.4	11.3	15.0	10.1
Ptcp 10	7.4	6.6	8.7	7.7
Mean	**8.2**	**8.6**	**11.6**	**10.6**
SD	***2.2***	***1.9***	***2.4***	***2.4***
SE	***0.7***	***0.6***	***0.8***	***0.7***

Mitochondrial respiration (expressed as pmol O_2 s^{-1} mg^{-1})

$CI_{L(PM)}$

Pcpt	pre-NT	post-NT	post-INT	post-RT
Ptcp 1	8.5	6.0	7.0	4.2
Ptcp 2	5.8	7.1	7.5	8.6
Ptcp 3	5.5	3.5	7.8	7.8
Ptcp 4	7.6	4.5	8.0	5.0
Ptcp 5	4.5	4.9	7.6	8.2
Ptcp 6	2.3	4.8	5.8	6.7
Ptcp 7	2.0	3.4	11.7	5.9
Ptcp 8	3.5	8.7	5.8	8.1
Ptcp 9	7.6	5.4	5.2	9.2
Ptcp 10	3.5	5.2	8.3	7.1
Mean	**5.1**	**5.3**	**7.5**	**7.1**
SD	***2.3***	***1.6***	***1.8***	***1.6***
SE	***0.7***	***0.5***	***0.6***	***0.5***

$CI_{P(PM)}$

Pcpt	pre-NT	post-NT	post-INT	post-RT
Ptcp 1	46.7	41.3	75.2	53.0
Ptcp 2	36.5	44.5	72.1	71.0
Ptcp 3	50.9	50.1	76.7	59.5
Ptcp 4	36.7	40.1	54.6	46.6
Ptcp 5	44.4	37.8	57.4	59.3
Ptcp 6	49.3	40.5	49.8	47.4
Ptcp 7	44.6	52.2	67.0	51.8
Ptcp 8	43.7	36.6	59.2	54.5
Ptcp 9	65.7	53.4	73.6	48.1
Ptcp 10	41.9	46.9	63.0	59.3
Mean	**46.0**	**44.3**	**64.9**	**55.0**
SD	***8.3***	***6.0***	***9.5***	***7.5***
SE	***2.6***	***1.9***	***3.0***	***2.4***

$CI+CII_{P(PMS)}$

Pcpt	pre-NT	post-NT	post-INT	post-RT
Ptcp 1	79.3	61.7	115.7	80.3
Ptcp 2	53.4	63.0	105.0	109.8
Ptcp 3	82.7	76.2	110.5	83.4
Ptcp 4	55.5	64.2	82.7	70.4
Ptcp 5	71.7	63.3	84.9	86.2
Ptcp 6	73.2	58.6	79.5	63.0
Ptcp 7	70.9	76.9	99.7	70.5
Ptcp 8	61.5	51.5	87.9	71.7
Ptcp 9	85.3	73.5	109.8	77.6
Ptcp 10	60.2	63.8	87.6	80.6
Mean	**69.4**	**65.3**	**96.3**	**79.3**
SD	***11.3***	***8.0***	***13.3***	***12.8***
SE	***3.6***	***2.5***	***4.2***	***4.1***

$CI+CII_{E(PMS)}$

Pcpt	pre-NT	post-NT	post-INT	post-RT
Ptcp 1	59.2	89.3	154.0	118.0
Ptcp 2	50.9	75.0	145.8	137.8
Ptcp 3	81.6	98.4	167.0	110.3
Ptcp 4	50.4	85.9	111.3	85.3
Ptcp 5	67.2	86.6	109.5	107.3
Ptcp 6	91.0	80.3	96.9	75.9
Ptcp 7	86.4	101.5	132.9	84.2
Ptcp 8	66.4	68.2	111.6	89.5
Ptcp 9	111.2	106.7	156.5	100.4
Ptcp 10	81.9	82.7	112.6	100.4
Mean	**87.4**	**87.9**	**122.1**	**90.1**
SD	***16.2***	***15.9***	***23.1***	***10.6***
SE	***7.2***	***7.1***	***10.3***	***4.7***

$CII_{E(PMS+Rot)}$

Pcpt	pre-NT	post-NT	post-INT	post-RT
Ptcp 1	49.1	38.7	54.7	46.5
Ptcp 2	35.2	38.8	63.5	66.9
Ptcp 3	49.6	41.1	69.1	48.5
Ptcp 4	31.6	39.8	51.2	41.3
Ptcp 5	51.4	48.8	48.5	42.7
Ptcp 6	40.1	37.6	54.6	35.0
Ptcp 7	46.9	44.8	56.4	32.6
Ptcp 8	35.3	48.9	53.1	38.5
Ptcp 9	49.8	39.3	63.6	43.9
Ptcp 10	35.5	34.8	43.7	42.9
Mean	**42.5**	**41.3**	**55.8**	**43.9**
SD	***7.6***	***4.7***	***7.7***	***9.4***
SE	***2.4***	***1.5***	***2.4***	***3.0***

$CIV_{E\ (Tm+As)}$

Pcpt	pre-NT	post-NT	post-INT	post-RT
Ptcp 1	124.5	141.0	179.4	140.3
Ptcp 2	101.9	115.5	155.9	173.2
Ptcp 3	140.2	116.7	186.3	139.8
Ptcp 4	122.5	146.6	125.9	135.5
Ptcp 5	144.4	119.6	108.1	109.0
Ptcp 6	120.0	139.7	141.1	92.4
Ptcp 7	134.8	128.2	127.7	108.6
Ptcp 8	116.5	119.2	160.3	129.5
Ptcp 9	152.7	131.7	207.0	128.2
Ptcp 10	111.9	118.6	136.4	144.9
Mean	**126.9**	**127.7**	**152.8**	**130.1**
SD	***15.8***	***11.5***	***30.9***	***22.7***
SE	***5.0***	***3.6***	***9.8***	***7.2***

Protein content (expressed as arbitrary units)

Complex I

Pcpt	pre-NT	post-NT	post-INT	post-RT
Ptcp 1	1.05	1.07	1.19	1.14
Ptcp 2	0.93	1.22	1.27	1.41
Ptcp 3	1.25	1.05	1.35	1.19
Ptcp 4	1.02	1.21	1.44	1.25
Ptcp 5	1.36	1.24	1.45	1.27
Ptcp 6	1.15	1.26	1.37	1.21
Ptcp 7	1.29	1.15	1.28	1.07
Ptcp 8	1.10	1.15	1.31	1.21
Ptcp 9	1.20	1.42	1.48	1.18
Ptcp 10	1.05	1.17	1.31	1.11
Mean	**1.14**	**1.19**	**1.34**	**1.21**
SD	***0.14***	***0.10***	***0.09***	***0.09***
SE	***0.04***	***0.03***	***0.03***	***0.03***

Complex II

Pcpt	pre-NT	post-NT	post-INT	post-RT
Ptcp 1	1.26	1.42	1.51	1.38
Ptcp 2	1.07	1.56	1.56	1.51
Ptcp 3	1.34	1.20	1.64	1.68
Ptcp 4	1.11	1.29	1.90	1.32
Ptcp 5	1.42	1.23	1.54	1.12
Ptcp 6	1.08	1.19	1.35	1.08
Ptcp 7	1.87	1.06	1.53	0.85
Ptcp 8	0.94	1.42	1.96	1.80
Ptcp 9	1.34	1.68	2.55	1.68
Ptcp 10	0.72	0.67	0.99	0.69
Mean	**1.21**	**1.27**	**1.65**	**1.31**
SD	***0.31***	***0.28***	***0.42***	***0.37***
SE	***0.10***	***0.09***	***0.13***	***0.12***

Complex III

Pcpt	pre-NT	post-NT	post-INT	post-RT
Ptcp 1	1.20	1.14	1.26	1.08
Ptcp 2	1.09	1.22	1.29	1.35
Ptcp 3	1.20	1.28	1.25	1.26
Ptcp 4	1.13	1.27	1.44	1.30
Ptcp 5	1.22	1.24	1.48	1.23
Ptcp 6	1.30	1.29	1.39	1.49
Ptcp 7	1.06	1.04	1.04	1.07
Ptcp 8	1.04	1.10	1.07	1.06
Ptcp 9	1.05	1.09	1.16	0.98
Ptcp 10	1.01	0.94	0.97	0.92
Mean	**1.13**	**1.16**	**1.24**	**1.17**
SD	***0.10***	***0.12***	***0.17***	***0.18***
SE	***0.03***	***0.04***	***0.05***	***0.06***

Complex IV

Pcpt	pre-NT	post-NT	post-INT	post-RT
Ptcp 1	0.98	1.08	1.15	1.16
Ptcp 2	0.91	1.27	1.33	1.41
Ptcp 3	1.41	1.32	1.56	1.35
Ptcp 4	1.06	1.15	1.39	1.06
Ptcp 5	1.35	1.12	1.34	1.07
Ptcp 6	1.07	1.22	1.27	1.10
Ptcp 7	1.15	1.01	1.19	0.94
Ptcp 8	1.05	1.01	1.16	1.18
Ptcp 9	1.07	1.16	1.20	1.26
Ptcp 10	0.80	0.88	1.08	0.80
Mean	**1.09**	**1.12**	**1.27**	**1.13**
SD	***0.18***	***0.13***	***0.14***	***0.18***
SE	***0.06***	***0.04***	***0.04***	***0.06***

Complex V

Pcpt	pre-NT	post-NT	post-INT	post-RT
Ptcp 1	1.18	1.26	1.43	1.26
Ptcp 2	1.12	1.26	1.36	1.39
Ptcp 3	1.30	1.25	1.38	1.65
Ptcp 4	1.25	1.26	1.48	1.27
Ptcp 5	1.21	1.27	1.51	1.24
Ptcp 6	1.21	1.22	1.39	1.36
Ptcp 7	1.07	0.98	1.06	1.02
Ptcp 8	0.95	1.13	0.97	1.03
Ptcp 9	0.97	1.01	1.06	1.06
Ptcp 10	0.89	0.83	0.93	0.83
Mean	**1.11**	**1.15**	**1.26**	**1.21**
SD	***0.14***	***0.16***	***0.23***	***0.23***
SE	***0.04***	***0.05***	***0.07***	***0.07***

PGC-1α

Pcpt	pre-NT	post-NT	post-INT	post-RT
Ptcp 1	0.96	0.77	1.03	1.52
Ptcp 2	1.02	1.16	1.31	1.81
Ptcp 3	0.98	0.84	1.51	1.29
Ptcp 4	0.52	1.35	1.11	0.82
Ptcp 5	0.49	0.97	1.82	1.07
Ptcp 6	0.52	0.98	1.05	0.34
Ptcp 7	0.69	0.63	0.75	0.45
Ptcp 8	0.44	0.85	0.72	0.43
Ptcp 9	0.66	0.24	0.41	0.70
Ptcp 10	0.42	0.50	0.91	0.72
Mean	**0.67**	**0.83**	**1.06**	**0.92**
SD	***0.24***	***0.32***	***0.41***	***0.49***
SE	***0.07***	***0.10***	***0.13***	***0.16***

p53

Pcpt	pre-NT	post-NT	post-INT	post-RT
Ptcp 1	1.56	1.87	2.89	2.58
Ptcp 2	1.86	2.72	2.65	2.72
Ptcp 3	1.71	2.58	2.71	2.74
Ptcp 4	0.58	1.43	1.39	0.66
Ptcp 5	0.71	1.52	1.50	0.46
Ptcp 6	0.31	0.92	0.81	1.39
Ptcp 7	0.22	0.44	0.58	0.27
Ptcp 8	0.55	0.51	1.66	0.65
Ptcp 9	0.68	0.27	0.18	0.95
Ptcp 10	0.30	0.48	2.65	1.65
Mean	**0.85**	**1.27**	**1.70**	**1.41**
SD	***0.62***	***0.90***	***0.98***	***0.97***
SE	***0.20***	***0.29***	***0.31***	***0.31***

PHF20

Pcpt	pre-NT	post-NT	post-INT	post-RT
Ptcp 1	0.57	0.62	0.78	0.89
Ptcp 2	0.72	0.62	0.80	0.89
Ptcp 3	0.83	0.67	0.97	0.90
Ptcp 4	0.46	0.44	0.80	0.86
Ptcp 5	0.44	0.40	0.76	0.71
Ptcp 6	0.26	0.21	0.62	0.50
Ptcp 7	0.45	0.59	1.00	0.53
Ptcp 8	0.72	0.93	1.09	0.92
Ptcp 9	0.91	0.43	0.46	1.14
Ptcp 10	0.33	0.41	1.90	0.60
Mean	**0.57**	**0.53**	**0.92**	**0.79**
SD	***0.22***	***0.20***	***0.39***	***0.20***
SE	***0.07***	***0.06***	***0.12***	***0.06***

NRF1

Pcpt	pre-NT	post-NT	post-INT	post-RT
Ptcp 1	1.71	1.62	1.91	1.99
Ptcp 2	1.68	2.43	2.27	2.60
Ptcp 3	2.32	2.13	2.26	2.12
Ptcp 4	1.45	1.81	1.90	1.40
Ptcp 5	0.69	1.06	1.63	0.79
Ptcp 6	0.56	0.53	1.58	0.69
Ptcp 7	0.77	1.10	1.15	1.22
Ptcp 8	1.13	1.36	1.44	1.22
Ptcp 9	1.31	2.18	2.36	1.36
Ptcp 10	1.36	1.44	2.39	2.04
Mean	**1.30**	**1.57**	**1.89**	**1.54**
SD	***0.54***	***0.59***	***0.43***	***0.62***
SE	***0.17***	***0.19***	***0.14***	***0.20***

TFAM

Pcpt	pre-NT	post-NT	post-INT	post-RT
Ptcp 1	0.96	1.50	1.65	1.14
Ptcp 2	1.43	0.90	2.59	1.00
Ptcp 3	1.74	1.56	0.80	0.22
Ptcp 4	1.39	1.08	1.85	2.03
Ptcp 5	0.97	2.07	2.64	1.27
Ptcp 6	1.09	1.06	3.08	0.54
Ptcp 7	1.02	0.76	1.58	0.56
Ptcp 8	1.20	2.91	1.58	0.76
Ptcp 9	0.86	0.46	1.56	0.41
Ptcp 10	0.41	0.72	0.86	0.78
Mean	**1.11**	**1.30**	**1.82**	**0.87**
SD	***0.36***	***0.74***	***0.75***	***0.52***
SE	***0.11***	***0.23***	***0.24***	***0.17***

AIF

Pcpt	pre-NT	post-NT	post-INT	post-RT
Ptcp 1	1.94	1.88	2.63	2.46
Ptcp 2	1.49	2.76	2.00	1.34
Ptcp 3	1.49	1.68	1.84	2.27
Ptcp 4	0.99	1.34	1.17	1.85
Ptcp 5	0.82	1.22	0.80	1.46
Ptcp 6	0.60	0.45	1.85	0.38
Ptcp 7	2.32	2.08	1.96	3.07
Ptcp 8	1.31	3.40	2.76	3.91
Ptcp 9	3.16	2.24	4.74	1.34
Ptcp 10	2.15	0.92	1.91	2.06
Mean	**1.63**	**1.80**	**2.17**	**2.02**
SD	***0.78***	***0.88***	***1.07***	***0.99***
SE	***0.25***	***0.28***	***0.34***	***0.31***

SCO2

Pcpt	pre-NT	post-NT	post-INT	post-RT
Ptcp 1	2.29	3.87	2.71	3.27
Ptcp 2	2.64	3.31	2.51	2.83
Ptcp 3	1.70	1.21	1.75	1.31
Ptcp 4	2.03	2.53	3.02	3.65
Ptcp 5	3.17	3.38	3.88	2.59
Ptcp 6	3.13	1.73	1.80	1.25
Ptcp 7	1.96	1.19	0.99	1.19
Ptcp 8	1.48	1.29	1.35	1.39
Ptcp 9	1.20	1.25	1.10	1.08
Ptcp 10	1.40	2.45	1.64	1.89
Mean	**2.10**	**2.22**	**2.08**	**2.05**
SD	***0.70***	***1.03***	***0.93***	***0.96***
SE	***0.22***	***0.33***	***0.29***	***0.30***

MFN-2

Pcpt	pre-NT	post-NT	post-INT	post-RT
Ptcp 1	2.86	1.75	2.99	2.24
Ptcp 2	1.49	3.09	2.26	3.24
Ptcp 3	1.45	1.62	2.53	2.22
Ptcp 4	0.96	1.16	1.41	1.63
Ptcp 5	1.23	1.66	1.19	1.42
Ptcp 6	1.41	1.05	2.15	1.11
Ptcp 7	2.22	1.36	2.00	2.07
Ptcp 8	2.01	3.12	2.35	2.46
Ptcp 9	1.44	1.24	1.82	1.12
Ptcp 10	0.79	1.41	0.70	1.11
Mean	**1.59**	**1.75**	**1.94**	**1.86**
SD	***0.62***	***0.75***	***0.68***	***0.71***
SE	***0.20***	***0.24***	***0.22***	***0.22***

DRP1

Pcpt	pre-NT	post-NT	post-INT	post-RT
Ptcp 1	0.59	0.62	0.97	1.04
Ptcp 2	0.92	0.71	0.82	1.03
Ptcp 3	0.78	0.70	0.88	0.84
Ptcp 4	0.62	1.14	1.27	0.58
Ptcp 5	0.58	1.12	1.08	0.41
Ptcp 6	0.33	0.90	0.64	1.37
Ptcp 7	0.52	0.48	0.94	0.46
Ptcp 8	0.84	0.99	1.13	1.09
Ptcp 9	0.77	0.63	0.54	1.11
Ptcp 10	0.51	0.54	1.52	0.77
Mean	**0.65**	**0.78**	**0.98**	**0.87**
SD	***0.18***	***0.24***	***0.29***	***0.31***
SE	***0.06***	***0.08***	***0.09***	***0.10***

CHAPTER 4 – RAW DATA

Physiological parameters and measurements of work

Pcpt	Age [y]	Mass [kg]	Height [cm]	Pcpt	Age [y]	Mass [kg]	Height [cm]
STCT 1	23.3	77.7	180.5	SIT 1	19.7	70.7	174.4
STCT 2	18.8	86.5	181.6	SIT 2	21.7	93.1	191.9
STCT 3	21.6	75.5	177.6	SIT 3	24.9	84.9	183.5
STCT 4	19.8	76.2	178.0	SIT 4	21.6	67.9	176.9
STCT 5	24.4	79.6	182.2	SIT 5	22.1	125.5	180.4
STCT 6	20.5	84.7	180.5	SIT 6	19.6	87.0	184.7
STCT 7	18.8	92.0	191.0	SIT 7	18.5	97.6	188.3
STCT 8	20.8	67.7	174.4	SIT 8	18.4	66.6	176.8
STCT 9	20.6	56.3	167.5	SIT 9	25.7	67.1	168.3
STCT 10	19.9	101.9	182.6				
Mean	***20.9***	***79.8***	***179.6***	***Mean***	***21.3***	***84.5***	***180.6***
SD	***1.8***	***12.7***	***6.1***	***SD***	***2.6***	***19.4***	***7.3***
SE	***0.6***	***4.0***	***1.9***	***SE***	***0.9***	***6.5***	***2.4***

Pcpt	W_{LT} [w]	W_{Peak} [w]	VO_{2Peak} [ml min^{-1} kg^{-1}]	En Expen [kJ]	Pcpt	W_{LT} [w]	W_{Peak} [w]	VO_{2Peak} [ml min^{-1} kg^{-1}]	En Expen [kJ]
STCT 1	172.0	262.5	51.2	222.9	SIT 1	193.2	262.5	51.5	64.5
STCT 2	259.8	345.0	48.7	336.7	SIT 2	233.6	315.0	44.6	79.8
STCT 3	176.6	255.0	46.3	228.9	SIT 3	271.7	367.5	59.4	80.7
STCT 4	219.6	307.5	51.5	284.7	SIT 4	139.2	217.5	44.9	57.4
STCT 5	225.6	307.5	47.9	292.4	SIT 5	226.0	315.0	37.5	80.4
STCT 6	194.9	270.0	47.8	252.6	SIT 6	213.5	285.0	39.1	70.9
STCT 7	242.7	337.5	43.9	314.6	SIT 7	186.2	262.5	41.2	67.6
STCT 8	128.5	185.0	41.8	166.5	SIT 8	161.1	217.5	48.5	59.2
STCT 9	134.6	210.0	41.1	174.4	SIT 9	215.0	285.0	57.4	64.6
STCT 10	194.0	285.0	49.7	251.4					
Mean	***194.8***	***276.5***	***47.0***	***252.5***	***Mean***	***204.4***	***280.8***	***47.1***	***69.5***
SD	***43.4***	***51.5***	***3.7***	***56.3***	***SD***	***39.7***	***48.2***	***7.8***	***9.1***
SE	***13.7***	***16.3***	***1.2***	***17.8***	***SE***	***13.2***	***16.1***	***2.6***	***3.0***

Exercise intensity

Pcpt	Mean ex intensity [% WPeak]	Max ex intensity [% WPeak]	Mean ex intensity [W]	Max ex intensity [W]	Pcpt	Mean ex intensity [% WPeak]	Max ex intensity [% WPeak]	Mean ex intensity [W]	Max ex intensity [W]
STCT 1	59.0	59.0	154.8	154.8	SIT 1	204.9	266.5	537.8	699.5
STCT 2	67.8	67.8	233.8	233.8	SIT 2	211.2	312.5	665.3	984.4
STCT 3	62.3	62.3	158.9	158.9	SIT 3	183.0	261.3	672.7	960.4
STCT 4	64.3	64.3	197.7	197.7	SIT 4	220.0	293.2	478.4	637.6
STCT 5	66.0	66.0	203.1	203.1	SIT 5	212.7	338.8	670.0	1067.4
STCT 6	65.0	65.0	175.4	175.4	SIT 6	207.2	245.7	590.5	700.2
STCT 7	64.7	64.7	218.5	218.5	SIT 7	214.6	333.0	563.2	874.2
STCT 8	62.5	62.5	115.6	115.6	SIT 8	226.8	321.6	493.2	699.5
STCT 9	57.7	57.7	121.1	121.1	SIT 9	188.9	276.5	538.4	788.0
STCT 10	61.3	61.3	174.6	174.6					
Mean	***63.1***	***63.1***	***175.4***	***175.4***	***Mean***	***207.7***	***294.3***	***578.8***	***823.4***
SD	***3.1***	***3.1***	***39.1***	***39.1***	***SD***	***14.0***	***33.7***	***75.6***	***153.5***
SE	***1.0***	***1.0***	***12.4***	***12.4***	***SE***	***4.7***	***11.2***	***25.2***	***51.2***

Blood Lactate concentration

Pcpt	$[La^-]_b$ pre [mmol/L]	$[La^-]_b$ +0h [mmol/L]	Pcpt	$[La^-]_b$ pre [mmol/L]	$[La^-]_b$ +0h [mmol/L]
STCT 1	1.0	4.1	SIT 1	0.8	12.7
STCT 2	0.8	3.2	SIT 2	0.8	12.5
STCT 3	1.2	3.8	SIT 3	1.3	11.7
STCT 4	1.0	4.4	SIT 4	1.0	9.0
STCT 5	0.7	4.1	SIT 5	0.9	10.8
STCT 6	0.9	3.9	SIT 6	0.7	10.1
STCT 7	0.8	4.1	SIT 7	0.9	11.7
STCT 8	1.2	3.9	SIT 8	1.1	12.4
STCT 9	0.9	3.7	SIT 9	0.9	13.8
STCT 10	0.7	3.8			
Mean	***0.9***	***3.9***	***Mean***	***0.9***	***11.6***
SD	***0.2***	***0.3***	***SD***	***0.2***	***1.4***
SE	***0.1***	***0.1***	***SE***	***0.1***	***0.5***

Protein content (expressed as arbitrary units)

PGC-1α Nuc

STCT				SIT			
Pcpt	Pre	+0 h	+3 h	Pcpt	Pre	+0 h	+3 h
STCT 1	0.26	0.35	0.49	SIT 1	0.29	0.74	0.25
STCT 2	0.27	0.87	0.36	SIT 2	0.13	0.33	0.19
STCT 3	0.53	0.58	0.38	SIT 3	0.77	1.87	1.22
STCT 4	0.64	1.05	0.78	SIT 4	0.35	0.45	0.78
STCT 5	0.28	0.31	0.22	SIT 5	0.38	0.60	0.62
STCT 6	0.11	0.14	0.13	SIT 6	0.29	0.70	0.59
STCT 8	0.20	0.14	0.09	SIT 7	0.45	0.74	0.34
STCT 9	0.39	0.25	0.22	SIT 8	0.22	0.98	0.66
STCT 10	0.43	0.63	0.44	SIT 9	0.32	0.55	0.41
Mean	***0.35***	***0.48***	***0.35***	***Mean***	***0.36***	***0.78***	***0.56***
SD	***0.17***	***0.32***	***0.21***	***SD***	***0.18***	***0.45***	***0.32***
SE	***0.06***	***0.11***	***0.07***	***SE***	***0.06***	***0.15***	***0.11***

PGC-1α Cyt

STCT				SIT			
Pcpt	Pre	+0 h	+3 h	Pcpt	Pre	+0 h	+3 h
STCT 1	3.84	1.41	1.48	SIT 1	1.69	2.20	3.47
STCT 2	1.88	3.70	1.85	SIT 2	0.98	1.71	1.34
STCT 3	1.62	3.38	0.49	SIT 3	1.60	2.98	2.43
STCT 4	2.10	3.11	2.03	SIT 4	0.89	1.17	2.11
STCT 5	1.09	1.70	1.34	SIT 5	0.54	1.08	0.91
STCT 6	1.55	1.25	1.46	SIT 6	6.55	10.71	11.87
STCT 8	0.99	0.52	0.31	SIT 7	1.83	1.62	0.87
STCT 9	1.95	1.65	1.23	SIT 8	0.55	2.01	2.76
STCT 10	0.58	2.51	2.67	SIT 9	0.88	1.02	1.13
Mean	***1.73***	***2.14***	***1.43***	***Mean***	***1.72***	***2.72***	***2.99***
SD	***0.93***	***1.09***	***0.73***	***SD***	***1.87***	***3.06***	***3.45***
SE	***0.31***	***0.36***	***0.24***	***SE***	***0.62***	***1.02***	***1.15***

p-ACC Nuc

STCT				SIT			
Pcpt	Pre	+0 h	+3 h	Pcpt	Pre	+0 h	+3 h
STCT 1	1.06	2.95	1.45	SIT 1	0.62	2.08	0.63
STCT 2	0.54	0.05	0.85	SIT 2	0.96	2.54	1.04
STCT 3	0.23	0.67	0.24	SIT 3	0.23	0.50	0.12
STCT 4	0.31	0.34	0.12	SIT 4	0.19	0.29	0.16
STCT 5	1.05	1.68	0.16	SIT 5	1.27	3.92	1.11
STCT 6	0.90	1.53	1.16	SIT 6	0.25	0.61	0.01
STCT 8	0.24	1.16	0.97	SIT 7	0.67	0.55	0.37
STCT 9	0.80	1.22	0.62	SIT 8	1.55	1.06	0.70
STCT 10	2.79	2.93	2.95	SIT 9	3.05	7.06	0.83
Mean	***0.88***	***1.39***	***0.95***	***Mean***	***0.98***	***2.07***	***0.55***
SD	***0.79***	***1.03***	***0.88***	***SD***	***0.91***	***2.23***	***0.41***
SE	***0.26***	***0.34***	***0.29***	***SE***	***0.30***	***0.74***	***0.14***

p-ACC Cyt

STCT				SIT			
Pcpt	Pre	+0 h	+3 h	Pcpt	Pre	+0 h	+3 h
STCT 1	32.61	78.08	31.39	SIT 1	26.27	40.04	18.90
STCT 2	12.99	5.88	9.42	SIT 2	20.75	30.68	15.06
STCT 3	2.49	6.79	2.22	SIT 3	4.69	4.49	1.60
STCT 4	1.58	2.88	0.95	SIT 4	2.08	2.85	2.30
STCT 5	8.08	15.01	1.08	SIT 5	3.44	10.09	7.39
STCT 6	6.87	14.18	6.21	SIT 6	4.24	9.70	2.69
STCT 8	506.54	1644.0	202.62	SIT 7	436.19	683.11	702.84
STCT 9	31.79	40.27	14.69	SIT 8	17.37	18.84	20.13
STCT 10	6.24	8.39	3.82	SIT 9	13.82	14.65	7.04
Mean	***67.69***	***201.72***	***30.27***	***Mean***	***58.76***	***90.49***	***86.44***
SD	***164.98***	***541.39***	***65.35***	***SD***	***141.80***	***222.56***	***231.26***
SE	***54.99***	***180.46***	***21.78***	***SE***	***47.27***	***74.19***	***77.09***

p53 Nuc

STCT				SIT			
Pcpt	Pre	+0 h	+3 h	Pcpt	Pre	+0 h	+3 h
STCT 1	0.49	1.31	1.64	SIT 1	0.16	1.22	0.66
STCT 2	0.27	1.00	0.35	SIT 2	0.38	0.63	0.17
STCT 3	0.45	0.38	0.59	SIT 3	0.41	0.96	0.92
STCT 4	0.27	0.60	0.45	SIT 4	0.27	0.48	0.43
STCT 5	0.28	0.37	0.42	SIT 5	0.38	0.51	0.46
STCT 6	0.28	0.26	0.26	SIT 6	0.42	0.49	0.78
STCT 8	0.41	0.18	0.25	SIT 7	0.61	0.95	0.76
STCT 9	0.77	0.53	0.44	SIT 8	0.61	1.58	1.18
STCT 10	0.34	0.54	0.31	SIT 9	0.33	0.64	0.49
Mean	***0.40***	***0.57***	***0.52***	***Mean***	***0.40***	***0.83***	***0.65***
SD	***0.16***	***0.36***	***0.43***	***SD***	***0.15***	***0.38***	***0.30***
SE	***0.05***	***0.12***	***0.14***	***SE***	***0.05***	***0.13***	***0.10***

p53 Cyt

STCT				SIT			
Pcpt	Pre	+0 h	+3 h	Pcpt	Pre	+0 h	+3 h
STCT 1	0.55	0.89	0.84	SIT 1	0.31	0.64	0.37
STCT 2	0.72	1.93	0.51	SIT 2	0.31	0.75	0.56
STCT 3	0.85	0.95	0.74	SIT 3	1.14	2.27	1.72
STCT 4	0.88	1.14	1.10	SIT 4	0.61	0.99	1.01
STCT 5	0.53	1.21	1.86	SIT 5	0.80	1.25	0.98
STCT 6	0.89	1.00	0.85	SIT 6	0.64	2.36	3.86
STCT 8	1.25	0.80	0.62	SIT 7	2.52	2.70	1.86
STCT 9	0.31	0.33	0.48	SIT 8	0.31	1.15	1.33
STCT 10	0.67	1.31	1.18	SIT 9	0.52	0.72	0.83
Mean	***0.74***	***1.06***	***0.91***	***Mean***	***0.80***	***1.43***	***1.39***
SD	***0.27***	***0.43***	***0.43***	***SD***	***0.70***	***0.80***	***1.05***
SE	***0.09***	***0.14***	***0.14***	***SE***	***0.23***	***0.27***	***0.35***

PHF20 Nuc

STCT				SIT			
Pcpt	**Pre**	**+0 h**	**+3 h**	**Pcpt**	**Pre**	**+0 h**	**+3 h**
STCT 1	0.42	0.81	1.04	SIT 1	0.45	0.79	0.50
STCT 2	0.25	0.84	0.32	SIT 2	0.06	0.27	0.24
STCT 3	0.25	0.26	0.23	SIT 3	0.26	0.57	0.46
STCT 4	0.25	0.57	0.32	SIT 4	0.21	0.49	0.30
STCT 5	0.19	0.24	0.13	SIT 5	0.25	0.18	0.23
STCT 6	0.24	0.24	0.22	SIT 6	0.42	0.51	0.44
STCT 8	0.65	0.41	0.45	SIT 7	0.73	0.88	0.58
STCT 9	0.49	0.27	0.23	SIT 8	0.22	0.28	0.22
STCT 10	0.90	1.38	1.34	SIT 9	0.70	1.16	1.07
Mean	***0.41***	***0.56***	***0.48***	***Mean***	***0.37***	***0.57***	***0.45***
SD	***0.24***	***0.39***	***0.42***	***SD***	***0.23***	***0.32***	***0.27***
SE	***0.08***	***0.13***	***0.14***	***SE***	***0.08***	***0.11***	***0.09***

PHF20 Cyt

STCT				SIT			
Pcpt	**Pre**	**+0 h**	**+3 h**	**Pcpt**	**Pre**	**+0 h**	**+3 h**
STCT 1	1.48	2.34	2.47	SIT 1	1.16	2.38	1.37
STCT 2	0.51	1.36	0.46	SIT 2	0.40	0.63	0.43
STCT 3	2.00	2.73	1.91	SIT 3	3.17	5.79	4.36
STCT 4	0.58	0.84	0.68	SIT 4	0.48	0.98	1.12
STCT 5	0.30	0.87	0.77	SIT 5	0.75	1.43	0.88
STCT 6	0.38	0.46	0.60	SIT 6	0.43	0.98	1.44
STCT 8	0.54	0.59	0.41	SIT 7	0.90	1.12	0.80
STCT 9	1.56	1.51	1.55	SIT 8	1.05	1.40	1.12
STCT 10	0.73	0.78	0.60	SIT 9	0.36	0.44	0.60
Mean	***0.90***	***1.28***	***1.05***	***Mean***	***0.97***	***1.68***	***1.35***
SD	***0.61***	***0.79***	***0.74***	***SD***	***0.88***	***1.64***	***1.18***
SE	***0.20***	***0.26***	***0.25***	***SE***	***0.29***	***0.55***	***0.39***

p-p53 Nuc

STCT				SIT			
Pcpt	**Pre**	**+0 h**	**+3 h**	**Pcpt**	**Pre**	**+0 h**	**+3 h**
STCT 1	0.65	0.96	1.03	SIT 1	0.65	1.54	0.78
STCT 2	0.18	0.69	0.23	SIT 2	0.20	0.34	0.26
STCT 3	0.25	0.22	0.25	SIT 3	0.34	0.83	0.65
STCT 4	0.71	0.72	0.85	SIT 4	0.16	1.18	0.74
STCT 5	0.37	0.48	0.80	SIT 5	0.63	0.95	0.92
STCT 6	0.12	0.26	0.12	SIT 6	0.70	1.40	1.75
STCT 8	0.68	0.40	0.51	SIT 7	0.52	1.24	0.89
STCT 9	0.70	0.48	0.33	SIT 8	0.25	1.84	0.96
STCT 10	0.57	0.68	0.61	SIT 9	0.85	1.01	0.63
Mean	***0.47***	***0.54***	***0.52***	***Mean***	***0.48***	***1.15***	***0.84***
SD	***0.24***	***0.24***	***0.32***	***SD***	***0.25***	***0.43***	***0.40***
SE	***0.08***	***0.08***	***0.11***	***SE***	***0.08***	***0.14***	***0.13***

p-p53 Cyt

STCT				SIT			
Pcpt	**Pre**	**+0 h**	**+3 h**	**Pcpt**	**Pre**	**+0 h**	**+3 h**
STCT 1	0.75	1.66	1.48	SIT 1	0.29	0.84	0.45
STCT 2	0.92	1.96	0.77	SIT 2	0.49	0.65	0.64
STCT 3	0.45	0.58	0.49	SIT 3	0.40	1.03	0.85
STCT 4	1.00	1.80	1.50	SIT 4	0.59	0.89	1.19
STCT 5	0.90	1.26	1.68	SIT 5	0.64	0.99	0.75
STCT 6	1.39	1.43	1.95	SIT 6	0.46	2.09	2.51
STCT 8	1.66	1.01	0.75	SIT 7	6.54	8.44	7.15
STCT 9	1.05	1.32	0.68	SIT 8	0.61	1.64	1.35
STCT 10	1.63	2.15	1.82	SIT 9	0.29	0.56	0.37
Mean	***1.08***	***1.46***	***1.24***	***Mean***	***1.15***	***1.90***	***1.69***
SD	***0.40***	***0.49***	***0.56***	***SD***	***2.03***	***2.50***	***2.14***
SE	***0.13***	***0.16***	***0.19***	***SE***	***0.68***	***0.83***	***0.71***

mRNA content (expressed as arbitrary units)

Pcpt	PGC-1a	p53	AIF	DRP1	MFN2
STCT 1 pre	1.00	1.00	1.00	1.00	1.00
STCT 1 +0h	0.90	1.05	0.86	0.96	1.21
STCT 1 +3h	3.93	1.12	0.95	0.93	1.01
STCT 2 pre	1.00	1.00	1.00	1.00	1.00
STCT 2 +0h	0.66	0.60	0.67	0.55	0.48
STCT 2 +3h	4.05	0.90	1.03	0.79	1.01
STCT 3 pre	1.00	1.00	1.00	1.00	1.00
STCT 3 +0h	1.05	0.88	1.18	1.67	1.53
STCT 3 +3h	6.93	1.02	0.87	1.12	0.92
STCT 4 pre	1.00	1.00	1.00	1.00	1.00
STCT 4 +0h	1.96	1.34	1.40	1.56	1.35
STCT 4 +3h	8.40	1.24	1.19	1.51	1.32
STCT 5 pre	1.00	1.00	1.00	1.00	1.00
STCT 5 +0h	1.19	1.00	1.45	1.24	1.85
STCT 5 +3h	3.55	1.17	0.95	1.00	1.47
STCT 6 pre	1.00	1.00	1.00	1.00	1.00
STCT 6 +0h	1.47	1.08	1.52	1.37	2.01
STCT 6 +3h	4.25	1.19	1.38	1.10	1.60
STCT 7 pre	1.00	1.00	1.00	1.00	1.00
STCT 7 +0h	1.27	0.85	1.08	1.01	1.42
STCT 7 +3h	7.10	0.94	1.43	1.09	1.62
STCT 8 pre	1.00	1.00	1.00	1.00	1.00
STCT 8 +0h	1.16	0.57	1.86	1.01	1.64
STCT 8 +3h	8.49	0.81	1.74	0.94	1.85
STCT 10 pre	1.00	1.00	1.00	1.00	1.00
STCT 10 +0h	0.92	0.65	1.14	1.10	0.73
STCT 10 +3h	3.43	0.48	1.20	0.75	0.59
Mean STCT pre	***1.00***	***1.00***	***1.00***	***1.00***	***1.00***
Mean STCT + 0h	***1.17***	***0.89***	***1.24***	***1.16***	***1.36***
Mean STCT + 3h	***5.57***	***0.99***	***1.19***	***1.03***	***1.27***
SD STCT pre	***0.00***	***0.00***	***0.00***	***0.00***	***0.00***
SD STCT + 0h	***0.38***	***0.26***	***0.36***	***0.34***	***0.50***
SD STCT + 3h	***2.12***	***0.24***	***0.28***	***0.22***	***0.41***
SEM STCT pre	***0.00***	***0.00***	***0.00***	***0.00***	***0.00***
SEM STCT + 0h	***0.13***	***0.09***	***0.12***	***0.11***	***0.17***
SEM STCT + 3h	***0.71***	***0.08***	***0.09***	***0.07***	***0.14***

Pcpt	PGC-1a	p53	AIF	DRP1	MFN2
SIT 1 pre	1.00	1.00	1.00	1.00	1.00
SIT 1 +0h	1.23	1.06	0.99	1.45	1.22
SIT 1 +3h	9.17	1.44	1.32	1.39	1.27
SIT 2 pre	1.00	1.00	1.00	1.00	1.00
SIT 2 +0h	0.98	0.78	1.19	1.11	1.10
SIT 2 +3h	5.79	1.02	1.20	1.21	1.15
SIT 3 pre	1.00	1.00	1.00	1.00	1.00
SIT 3 +0h	1.00	1.03	1.24	1.27	1.62
SIT 3 +3h	7.45	1.57	1.63	1.33	1.55
SIT 4 pre	1.00	1.00	1.00	1.00	1.00
SIT 4 +0h	1.18	1.42	1.23	1.03	2.07
SIT 4 +3h	2.76	0.88	1.52	1.09	0.89
SIT 5 pre	1.00	1.00	1.00	1.00	1.00
SIT 5 +0h	1.50	1.05	1.32	1.16	1.29
SIT 5 +3h	5.29	1.22	1.08	0.97	0.86
SIT 6 pre	1.00	1.00	1.00	1.00	1.00
SIT 6 +0h	1.14	0.69	1.12	1.07	0.74
SIT 6 +3h	4.78	0.61	1.45	1.01	0.49
SIT 7 pre	1.00	1.00	1.00	1.00	1.00
SIT 7 +0h	1.29	1.52	4.04	1.43	2.32
SIT 7 +3h	11.21	2.22	4.03	2.06	2.80
SIT 8 pre	1.00	1.00	1.00	1.00	1.00
SIT 8 +0h	0.85	0.93	0.72	0.68	0.55
SIT 8 +3h	2.95	0.63	0.52	0.45	0.35
SIT 9 pre	1.00	1.00	1.00	1.00	1.00
SIT 9 +0h	1.48	1.56	1.35	1.79	1.41
SIT 9 +3h	6.95	1.32	1.23	1.29	1.23
Mean SIT pre	***1.00***	***1.00***	***1.00***	***1.00***	***1.00***
Mean SIT + 0h	***1.18***	***1.12***	***1.47***	***1.22***	***1.37***
Mean SIT + 3h	***6.26***	***1.21***	***1.56***	***1.20***	***1.18***
SD SIT pre	***0.00***	***0.00***	***0.00***	***0.00***	***0.00***
SD SIT + 0h	***0.22***	***0.31***	***0.98***	***0.31***	***0.57***
SD SIT + 3h	***2.77***	***0.51***	***0.98***	***0.43***	***0.72***
SE SIT pre	***0.00***	***0.00***	***0.00***	***0.00***	***0.00***
SE SIT + 0h	***0.07***	***0.10***	***0.33***	***0.10***	***0.19***
SE SIT + 3h	***0.92***	***0.17***	***0.33***	***0.14***	***0.24***