Skeletal muscle mitochondrial and inflammatory signaling in response to a single session of high-intensity interval exercise in hypoxia

by

Jia Li

Victoria University

Institute for Health and Sport (IHeS)

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Abstract

Both high-intensity interval exercise (HIIE) and hypoxia have been associated with mitochondrial biogenesis. However, limited literature has explored the effects of a single session of HIIE in hypoxia on physiological and molecular adaptive responses in human skeletal muscle and blood samples. In the current study, I combined HIIE with simulated hypoxia (3200m, oxygen fraction of 0.14) to investigate those aspects in human participants. Ten healthy male participants (Age, 28 ± 5; BMI, 26.0 ± 3.4) were recruited and completed three HIIE sessions in a random order, including one HIIE session in hypoxia (88.4% of peak oxygen uptake (VO_{2peak}) in hypoxia, HY), one HIIE session in normoxia matched for the relative intensity of hypoxia (88.7% of VO_{2peak} in normoxia, NR), and one HIIE session in normoxia matched for the absolute intensity of hypoxia (74.1% of VO_{2peak} in normoxia, NA). Results from graded exercise tests suggested that hypoxia led to a decrease in VO_{2peak} (by 20.2 ± 9.1%, $p<0.01$), peak power output (PPO, by 9.4 ± 2.1%, $p<0.01$), and lactate threshold (LT, by 13.1 ± 3.0%, $p<0.01$). Skeletal muscle samples were collected before (B), immediately post (P0H), 3 hours post (P3H), and 24 hours post (P24H) exercise. Mitochondrial respiration and citrate synthase activity did not differ significantly among B, P3H and P24H in the any of conditions. HIF-1α and VEGF mRNA increased at P3H in HY and NR. The downstream targets genes, including mitochondrial biogenesis-related genes (PGC-1α, PGC-1α1, PGC-1α4, PPARα), mitochondrial respiration regulators (HSP70, P53), glycolytic enzyme (PDK4, SLC27A4) and fatty-acid related genes (UCP3, CD36) increased at various time points in HY and NR ($p < 0.05$), but not in NA. Gene expression did not differ between HY and NR. PGC-1α protein content increased immediately after the NR session. Besides, RNA-seq was used to identify broad patterns in inflammatory gene expression in skeletal muscle and RT- PCR was then used to verify the expression of some inflammatory markers of human body. To summarise, a single session of HIIE in hypoxia is not enough to change mitochondrial respiratory function or content, but it can induce the expression of mitochondrial biogenesis-related genes, which was comparable to HIIE in normoxia matched for the relative intensity of hypoxia. However, those genes were not induced by the HIIE in normoxia matched for the absolute intensity of hypoxia. A single session of HIIE is not enough to induce changes in protein abundance, except for PGC-1α, p-AMPK and p-MTOR. Results from this study may help a better understanding of the molecular pathway as well as refine exericse protocols of HIIE in hypoxia to achieve a better outcome.

Student Declaration

“I, Jia Li, declare that the PhD thesis entitled “The effect of hypoxia on mitochondrial adaptions to high-intensity interval exercise” 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 abstract and reports of any other journal meeting. Except where otherwise indicated, this thesis is my own work.”

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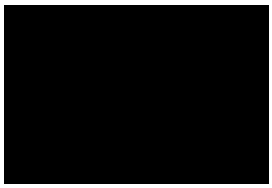
Date: 15 February 2023

Ethics Declaration

All research procedures reported in the thesis were approved by the Victoria University Human Research Ethics Committee (Ethics Approval NO. HRE18-214).

Signature: 

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List of abbreviations

AMPK	5' AMP-activated protein kinase
ANGPTL	angiopoietin-like protein
ANGPT-1	angiopoietin-1
ANGPT-2	angiopoietin-2
ATP	adenosine triphosphate
BDNF	brain-derived neurotrophic factor
BIOPS	biopsy preserving solution
cDNA	complementary DNA
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
CRP	C-reactive protein
CS	citrate synthase
DTNB	59-dithiobis (2-nitrobenzoic acid)
EDL	extensor digitorum longus
EPO	gene encoding erythropoietin
ERRα	estrogen-related receptor α
ETC	electron transport chain
FAD	flavin adenine dinucleotide
FCCP	carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone
FGF	fibroblast growth factor
FiO_2	inspired O_2 fraction
GET	gas exchange threshold
GXT	graded exercise test
HIF	Hypoxia Inducible Factor
HIIE	high-intensity interval exercise
HIIT	high-intensity interval training

HR_{peak}	heart rate peak
HSP70	mitochondrial heat-shock protein 70
HY	hypoxia
IL-6	interleukin
LT	lactate threshold
MiRO5	respiration medium
mRNA	messenger RNA
MPS	mitochondrial protein synthesis
MCT	moderate-intensity continuous training
mtDNA	mitochondrial DNA
NA	normoxia with workload matched to the absolute workload performed in hypoxia
NAD	nicotinamide adenine dinucleotide
NR	normoxia with workload matched for the relative workload Performed in hypoxia
NRF	nuclear respiratory factor
OXPHOS	oxidative phosphorylation
PHDs	prolyl hydroxylases
PGC-1α	proliferator-activated receptor gamma, coactivator 1 alpha
PPAR	peroxisome proliferator-activated receptor
PPO	peak power output
qPCR	quantitative real-time PCR
RCP	respiratory compensation point
ROX	residual oxygen consumption
RPE	perceived exertion
SD	standard deviation
SIE	sprint interval exercise
SIT	sprint interval training
SUIT	substrate–uncoupler–inhibitor titration
TEM	transmission electron microscopy
T2D	type 2 diabetes
TFAM	transcription factor A, mitochondrial
TNF	tumour necrosis factor
TLRs	Toll-like receptors

VEGF	vascular endothelial growth factor
VT	ventilatory threshold
$V\dot{O}_{2max}$	the maximum oxygen consumption
$V\dot{O}_{2Peak}$	peak oxygen uptake

Chapter1
Literature Review

Parts of the literature review is from a published paper as below:

Jia Li, Yanchun Li, Muhammed M. Atakan, Jujiao Kuang, Yang Hu, David J. Bishop, and Xu Yan. "The molecular adaptive responses of skeletal muscle to high-intensity exercise/training and hypoxia." *Antioxidants* 9, no. 8 (2020): 656. https://doi.org/10.3390/antiox9080656

1.1 Overview of Mitochondria

1.1.1 Mitochondrial and cellular respiration

Mitochondria, widely known as the "powerhouses" of cells, play a crucial role in converting nutrients into chemical energy in the form of adenosine triphosphate (ATP) [1]. This process, known as cellular respiration, involves a series of metabolic reactions that convert glucose into ATP [2]. During cellular respiration, glucose undergoes an initial breakdown, known as glycolysis, resulting in the production of pyruvate and ATP. Simultaneously, nicotinamide adenine dinucleotide (NAD+) is reduced to its reduced form, NADH. In the presence of oxygen, pyruvate can be further converted to acetyl-CoA through pyruvate oxidation, further reducing NAD+ to NADH. Acetyl-CoA enters the citric acid cycle, a metabolic pathway that utilizes acetyl-CoA and water to generate NADH and carbon dioxide as waste [3]. In addition to NADH, flavin adenine dinucleotide (FAD) accepts electrons and protons to form FADH2. These electron carriers, NADH and FADH2, are crucial for powering oxidative phosphorylation (OXPHOS), the process responsible for the majority of ATP production [1]. This cellular respiration mostly occurs in the matrix of the mitochondria, while glycolysis occurs in cytoplasm. Mitochondria are responsible for creating more than 90% of the energy needed by the human body to sustain life and support organ function, and also enable cells to produce 15 times more ATP than from glycolysis alone [1]. For tissues that demand high ATP, especially skeletal muscles, limiting the supply of ATP impairs their function. Thus, optimal mitochondrial function appears important for both health and exercise performance.

Mitochondria are small in size [3] and can float freely throughout the cell. The mitochondrial content depends on the type of cells. Muscle cells require a large amount of energy to support daily physiological activities, so they have a high mitochondria content (other cells may not need as many). Furthermore, it is important to note that mitochondria can adapt and modify their content, structure, and function based on the needs of the cell. This adaptability has led researchers to believe that exercise plays a crucial role in improving human health and enhancing athletic performance by inducing favorable changes in mitochondria [2].

1.1.2 The structure and function of mitochondria

Mitochondria consist of distinct components, namely the membrane and the matrix, each playing a vital role in their functionality. The membrane encompasses the organelle, comprising the inner membrane and outer membrane. The outer membrane covers the organelle, with many protein-based pores that are big enough to allow the passage of ions and molecules, such as small proteins. Maximizing productivity, the inner membrane undergoes numerous folds known as "cristae" (Figure 1.1). These intricate folds house the electron transfer system essential for oxidative phosphorylation, referred to as the electron transport chain (ETC) (Figure 1.2). Acting as the primary site for chemical reactions, the enlarged surface area of the inner membrane allows for ample space for these reactions to occur. Meanwhile, the matrix, a fluid-filled compartment within the mitochondria, contains water and proteins, including enzymes. It serves as the location for crucial processes such as pyruvate oxidation and the citric acid cycle. These reactions take place within the matrix, utilizing the enzymes and other components present in this fluid environment.

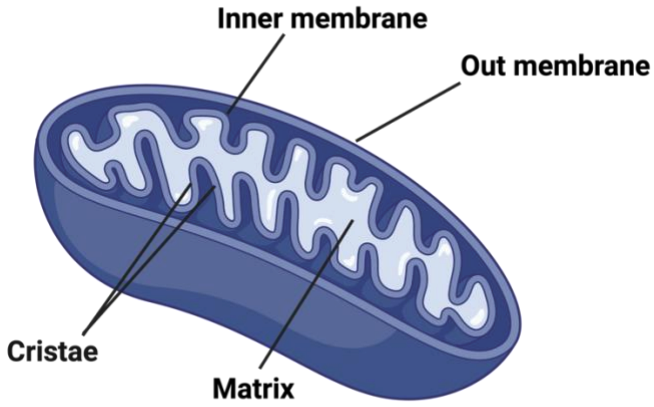

Figure1.1 The structure of mitochondrion. Mitochondria consist of two layers of membranes, matrix. Most of ATP is produced by enzyme-catalysed reactions in the matrix, driven by electron transport processes associated with the inner membrane.

Within the intricate folds of the inner mitochondrial membrane, the ETC operates as a series of five multiprotein enzyme complexes [5]. These complexes, including ubiquinone oxidoreductase (complex I), succinate dehydrogenase (complex II), ubiquinol–cytochrome c oxidoreductase (complex III, or cytochrome bc1 complex), cytochrome c oxidase (complex IV), and ATP synthase (complex V), form the core of the ETC responsible for ATP production

[3]. Complex I is the largest enzyme of the ETC; it oxidizes NADH molecules and uses the two electrons derived from each NADH to reduce ubiquinone and pumps 4 protons from the matrix to the inter membrane space. Complex II is the smallest and simplest complex of the ETC, with a molecular weight of 140 kD, and facilitates the transfer of electrons from succinate to ubiquinone. Ubiquinol is deoxidised by Complex III and transfers electrons to Complex IV. The terminal ETC complex IV combines the transported electrons with inspired oxygen (O_2), which acts as the ultimate electron acceptor. The redox energy released during this process is used to transfer protons from the mitochondrial matrix to the intermembrane space, which generates the proton-motive force across the inner mitochondrial membrane at complex I, III, and IV. Complex V uses this proton-motive force to produce ATP from ADP and inorganic phosphate. This entire process is known as Oxidative Phosphorylation (OXPHOS) [3]. This process establishes the life-sustaining mitochondrial electrochemical activity similar to a charged battery. Generating the mitochondrial electrochemical activity is the ultimate reason why living organisms must eat and breathe [4].

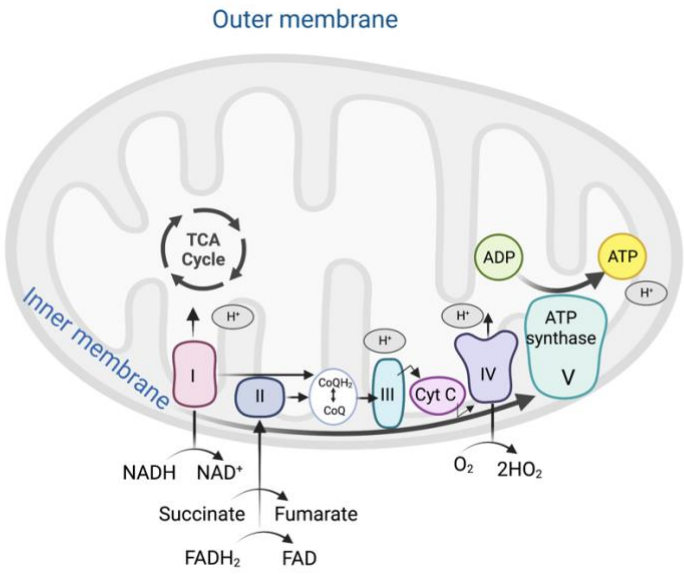

Figure 1.2 The mitochondrial electron transport chain (ETC) and oxidative phosphorylation (OXPHOS). Electrons are transferred through Complex I to IV, resulting in the leakage of electrons and finally the addition of electrons to oxygen (O_2). The proton (H+) gradient generated by the electron transfer provides the energy needed to generate adenosine triphosphate (ATP) by the final Complex V or ATP synthase. oxidized ubiquinone (CoQ); reduced form of ubiquinone (CoQH2).

1.2 Mitochondrial Biogenesis

1.2.1 The conception and regulation process of mitochondrial biogenesis

Mitochondrial biogenesis has been defined as “the making of new components of the mitochondrial reticulum” [5, 6]. Mitochondria have the remarkable ability to increase the content of their components or fuse with other mitochondria, adapting to the specific needs of the cell [7]. During aging, there is a decline in the number and function of muscle mitochondria; however, research has shown that exercise can alleviate this reduction and promote mitochondrial health [7].

The regulation of mitochondrial biogenesis is a complex process that not only requires the synthesis of the mitochondrial building blocks, but also the importation and incorporation of these proteins into the existing mitochondrial reticulum [8, 9]. The mitochondrial reticulum is a net-like structure [10], which is made of proteins and protein complexes that control aerobic energy production. Extending reticulum also requires regulation of the factors controlling the fusion and fission processes [11]. Fusion is a compensation process that mixes the contents of partially damaged mitochondria in response to the cellular stress [12]. On the other hand, fission contributes to creating new mitochondria, while also promoting apoptosis and enabling the removal of damaged mitochondria [12]. Of note, biogenesis by definition is “the making of new”, however, is not making de novo (start from the beginning) but rather recruiting new proteins to the organelle with subsequent division by fission.

Although the mitochondrial remodelling (fusion and fission) was widely accepted to define mitochondrial biogenesis, there are still many deferent ways to interpretate the effect of exercise on mitochondrial biogenesis [5]. It further led the controversial assessment of newly formed mitochondria as an indicator of mitochondrial biogenesis [6]. The rate of mitochondrial protein synthesis (MPS) is suggested to be the reliable indicator of mitochondrial biogenesis [6]. However, there is no evidence to correlate MPS rate to changes of the other indicators such as mitochondrial content, reparatory function, cristae density or supercomplex formation [5].

Taking this into account, a comprehensive evaluation of exercise-induced mitochondrial biogenesis should include mitochondrial content, structure, quality, and respiratory function, and place all the findings of mitochondrial biogenesis within a contextual framework [13, 14]. Besides, measurement of changes in the gene expression and protein content of transcription factors and co-factors can contribute to our understanding of exercise on mitochondrial biogenesis.

1.2.2 Common method to assess mitochondrial biogenesis

There are several methods available to determinate mitochondrial biogenesis, as this present thesis is to assess mitochondrial biogenesis in human exercise study with muscle biopsy, the introduction will primarily focus on the measurement of mitochondrial biogenesis using human skeletal muscle. The following is a brief review on the mechanism, advantages, and disadvantages of the different methods.

1.2.2.1 Defining and measuring "mitochondrial respiratory function"

Mitochondria play a central role in the synthesis of ATP through oxidative phosphorylation. It also contributes to regulating cellular redox homeostasis. Based on these characteristics, mitochondrial function is defined as the ability of mitochondria to maintain cellular energetic homeostasis. This can be expressed through various parameters, such as the maximal rate that an individual consumes oxygen ($\dot{V}O_{2max}$) , maximal ATP production rate (MAPR) and/or oxidative capacity [15]. Assessing mitochondrial function involves measurement of mitochondrial oxygen (O_2) consumption, or respiration.

During the past two decades, there has been an increased interest in determining the intrinsic function of mitochondria. The classic method of assessing mitochondrial respiratory function involves the measurement of oxygen consumption rate using an oxygen-sensitive electrode, originally invented by Leland Clark in 1954 [16, 17]. One approach to assess mitochondrial respiration is through a cell-free system that requires the isolation of mitochondria from skeletal muscle (this technique was originally developed for tissues with a higher mitochondrial density than skeletal muscle, such as the heart) [16]. This technique has been adapted for skeletal muscle by using saponin-permeabilized cardiac fibres where the sarcolemma is removed but the intracellular structures were left intact [18]. This method was later validated for the permeabilization of skeletal muscle fibres using saponin [16]. Currently, two classical methods exist to study mitochondrial respiratory function of skeletal muscles: isolated mitochondria and permeabilized fibres [19, 20]. High-resolution respirometry, such as the Oxygraph-2k from Oroboros (Innsbrunk, Austria), is a commercially available, commonly used system to assess mitochondrial respiratory function in yeast, cells and tissue, including

human skeletal muscle. Both isolated mitochondria and permeabilized muscle fibres can be analysed with this system [21].

Isolation of mitochondria from tissue/cell culture homogenization is a common assessment of mitochondrial O_2 consumption and oxidant emission [20] [22]. This approach allows for the isolation of mitochondria and provids an opportunity to study the effects of cytosolic structures in regulating mitochondrial function [23], in the meanwhile, avoiding the influence of other cellular factors such as cytoskeleton and endoplasmic reticulum. Some studies tend to accept that the best way to assess mitochondrial respiratory function free from other factors is to isolate mitochondria. However, it is important to note that the process of isolating mitochondria involves removing a portion of mitochondria from their cellular environment, which may impact mitochondrial function due to potential loss of samples [19]. The isolation of mitochondria does come with its disadvantages. Firstly, the disruption of mitochondrial structure may impact function. Secondly, the isolated mitochondria may not represent a completely unbiased sample selection [24]. Thirdly, the isolation method typically requires relatively large sample sizes (100–150 mg wet weight of tissue) [23]. Lastly, the isolation of mitochondria results in a loss of interactions with other intracellular components [25][[26].

By contrast, fibre permeabilization can largely preserve the mitochondrial structure and functional interactions with other intracellular components [20]. The permeabilized myofiber approach offers several advantages for studying muscle mitochondria. Firstly, it allows for a retention of the mitochondrial reticular morphology, preserving the structural integrity of mitochondria [27]. Secondly, this approach avoids disrupting the interactions between mitochondria and other subcellular structures, such as the cytoskeleton and endoplasmic reticulum, enabling the examination of mitochondrial function within its native cellular environment [28]. Lastly, this approach only requires 1–2 mg muscle samples, making it a more practical and feasible method for determining mitochondrial respiratory function in human studies.

1.2.2.2 Defining and measuring “mitochondrial content”

Mitochondrial content is considered as an important quantitative indicator of oxidative capacity and is often used to normalize global measurements of muscle bioenergetic capacity. The determination of mitochondrial content can be done by various methods , to date, transmission

electron microscopy (TEM) is still regarded as the golden standard for measuring mitochondrial fractional area (mitochondrial content) [29, 30]. It is capable of visualizing the subcellular structures and organization, such as the size and numbers of the mitochondria pool, which are regulated through the balance between biogenesis and apoptosis. However, the TEM technique is time consuming, high skill and large size of sample required, so it may not be available for many laboratories.

Biochemical measurements of mitochondrial proteins, lipids, tricarboxylic acid cycle enzymes such as citrate synthase (CS) and mitochondrial DNA (mtDNA) copy number have been more often used to measure mitochondrial content (biomarkers). Among them, the most used biomarker is CS activity [30, 31]. Activity of CS , the key enzyme of the Tricarboxylic Acid Cycle (TCA cycle), is strongly associated with mitochondrial content. It is a validated biomarker for mitochondrial density and oxidative adaptation in response to exercise training in skeletal muscle [32]. CS activity can be measured both spectrophotometrically and fluorometrically [32]. Only a small muscle sample (3-5 mg) is required. With a series of reactions between the reagents (acetyl CoA, 5,59-dithiobis (2-nitrobenzoic acid) (DTNB) in Tris buffer, then oxaloacetic acid), this leads to the formation of a coloured chemical. Changes in absorbance is obtained by using the spectrophotometer, which is proportional and linear to the rate of CS activity. In short, the advantage of CS activity is that it only requires very few muscle samples and is easy to measure. Furthermore, it can use frozen tissue while TEM requires fresh muscle samples.

Mitochondria contain their own DNA (mtDNA) that is essential for OXPHOS and ATP production [33]. mtDNA content has been extensively used as a biomarker of mitochondrial content [34]. Mitochondrial DNA copy number (mtDNA-CN), which quantifies the number of mitochondrial genomes per cell [35], is typically assessed using Southern blot hybridization or quantitative real-time PCR (qRT-PCR) [35]. However, Southern blot requires a significant amount of biopsied tissue, involves time-consuming serial steps, and can be challenge to conduct with well-defined quality controls. Consequently, qRT-PCR is increasingly employed as a more convenient and reliable method to determine mtDNA copy number [36].

1.2.3 Defining and measuring "protein and gene expression of transcriptional factor, coactivator and key regulators"

Gene expression is the process by which information from a gene is used for the synthesis of a functional product, protein, or non-coding RNA, and ultimately affects a phenotype. By controlling gene expression, the quantity, quality, and type of proteins can be changed, further producing cell types to allow different cells to carry out different functions. The process of gene expression includes transcription (copy genetic information from genomic DNA into RNA) [37], mRNA splicing (splicing of a newly made precursor RNA into a mature mRNA) [38], translation (produce polypeptide chains based on mRNA) and post translational modification of protein (chemical changes of protein after translation) [39, 40]. Linking the expression of specific genes to a biological process or phenotype helps illustrate gene function, biological pathways, and the genes that regulate development, cell behaviour, cell signalling, and diseases. The specific gene expression patterns can be associated with a biological state and serve as biomarkers for that condition. Currently, the commonly used techniques to assess gene expression include qRT-PCR or RT-PCR, microarrays, and RNA sequenencing (RNA-seq) for the transcriptome analysis. RT-PCR or qRT-PCR is a widely employed technique to measure the expression of target genes, providing valuable insights into changes of gene expression after different interventions (for a more detailed description of this technique the reader is referred to sections 3.2). Northern blotting and *in situ* hybridization are other quantification methods for gene expression, but they are not as rapid or accurate as qRT-PCR.

Western blotting (WB), also named immunoblotting, is a laboratory technique routinely used to investigate presence or absence of a target protein, relative abundance, relative mass, presence of post-translational modifications (PTM), as well as protein-protein interactions in a blood or tissue sample [41] (for a more detailed description of this technique the readers is referred to section 3.2). Furthermore, it can provide a qualitative assessment of changes in protein content in response to different interventions. Western blotting is a technique evolved from Southern blotting, which is utilized to detect specific DNA sequences among DNA fragments, and northern blotting, which is used for RNA quantification and size determination [42].

RNA-Seq is a extensively used technology for profiling transcriptional activity in biological systems [43]. This method is able to demonstrate the transcriptome and many other aspects of

RNA biology, including single-cell gene expression, translation and RNA structure [44]. One of the most common aims of RNA-Seq profiling is to identify genes or molecular pathways that are differentially expressed between two or more biological conditions [43, 45]. The process of RNA-Seq analysis includes sequence alignment, data exploration, differential expression analysis, visualization, and pathway analysis [43]. In comparison to qRT-PCR, this innovative technique has the advantage of being fast and efficient, particularly within the R environment.

In molecular biology, a transcription factor is defined as a protein that controls the rate of converting genetic information from DNA to mRNA, by binding to a specific DNA sequence generally located in the promoter region of genes [39, 46]. Besides transcription factors, there is another level of regulation by a set of proteins that modulate these factors called transcriptional coactivators.

Proliferator-activated receptor gamma, coactivator 1 alpha (PGC-1α) is an extensively studied marker of mitochondrial biogenesis. Hypoxia-Inducible Factor 1 Alpha (HIF- 1α) and Vascular Endothelial Growth Factor (VEGF) are linked to the hypoxic response (please refer to Section 1.4). The gene expression of these transcription factors/cofactors can be investigated by qRT-PCR whilst the post-translational activity can be assessed by Western Blotting. These transcription and post-translational events have been shown to play an important role in the transcriptional process [47], therefore providing new perspective to enhance the knowledge of the intricate processes of mitochondrial biogenesis [34]. Furthermore, RNA-Seq can provide insight into the molecular pathways. Beyond quantifying gene expression, the data generated by RNA-Seq facilitate the discovery of novel transcripts, identification of alternatively spliced genes, and detection of allele-specific expression. To the best of my knowledge, there are s limited studies with RNA-seq being used to detect molecular-level adaptions after exercise and hypoxia interventions.

1.3 High-Intensity Exercise and Physiological Adaptations

1.3.1 High-intensity exercise

High-intensity exercise is used to differentiate from moderate-intensity and low-intensity exercise, however, there is no consensus on its definition [48]. Different terms have been used to describe high exercise intensity, including “high-intensity”, “vigorous”[49], “all out” [50], “near exhaustion” [5], or “supramaximal efforts” [50]. In this thesis, I have adopted the definition of high-intensity from my supervisors’ previous work, in which it was defined as exercise intensity above 75% of the peak power output (PPO) achieved from a graded exercise test (GXT) [48]. Similar to a previous review, high-intensity exercise includes both high-intensity interval exercise (HIIE) and high-intensity interval training (HIIT), as well as sprint interval exercise (SIE) and sprint interval training (SIT) [48]. HIIE and SIE are sometimes referred to a single session of HIIT and SIT, respectively. HIIE has been defined as short bursts of vigorous exercise, with periodic intervals of rest or low-intensity exercise [51]. A meta-analysis conducted in 2016 focused on articles with ‘high-intensity exercise’ mentioned in the title, the analysis revealed a wide range of exercise intensities, ranging from 80-250% of maximal oxygen consumption, or maximal oxygen uptake ($\dot{V}O_{2max}$), and duration of 20-240 sec [52]. Our research group specify HIIE and HIIT as a relatively longer duration of exercise (2–5 min), with “near maximal” effort (~75–95% PPO), separated by resting periods. While SIE and SIT have a duration of less than 30 sec with training intensities exceeding the PPO recorded from a GXT (“supramaximal”).

Compared with continuous exercise, short work duration of interval training is more comfortably adapted for those who lack time and motivation [53]. HIIT has been shown to benefit those with cardiovascular conditions more than moderate-intensity continuous training (MCT), when energy expenditure was matched [54]. Although the exercise intensity is very high, the low intensity during relief intervals helps the body endurre stimulation [55]. Research comparing the effects of HIIT and SIT with MCT have shown that HIIT and SIT have similar improvements as MCT in skeletal muscle metabolic adaptations, cardiorespiratory fitness, vascular function, and body composition, but require less time [5, 56, 57].

1.3.2 Commonly used high-intensity exercise prescriptions

Growing evidence suggests that high-intensity exercise performed with different protocols is a time-efficient exercise strategy to improve cardiorespiratory and metabolic health [58, 59]. Among the SIE protocols , the most frequently used is the Wingate test. This test involes a 30

s ‘all-out’ cycling effort against a supra-maximal workload, which lasts ~20 min in total duration, but only with 2–3 min of intense exercise. However, the Wingate test is extremely demanding and may not be safe to be prescribed to the broad population [7], which necessitated designing new SIT protocols with a wider application tothose who are overweight/obese, older sedentary adults at higher risk for cardiometabolic disorders, and patients with coronary artery disease and type 2 diabetes (T2D). Recent research has focused on the potential for other models of SIT, which may be more feasible but are still time-efficient [60]. For example, 10 x 60 sec at $\dot{V}O_{2max}$, interspersed by 75 sec active recovery, led to an increase in mitochondrial enzymes [60, 61], exercise capacity [60, 61], and reduced hyperglycaemia [60], with less feelings of nausea and discomfort that were usually reported after the Wingate test. Another HIIE protocol commonly used in literature is the 4 x 4 min at 85-90 of $\dot{V}O_{2max}$, separated by 2 min of rest. It was shown to provide sufficient stimulus for exercise capacity, whole-body and skeletal muscle capacity for fatty acid oxidation [62].

1.4 High-Intensity Exercise and mitochondrial adaptions

The results of cross-sectional studies, as well as exercise studies including animals and humans, suggest that there is a tight association between HIIE and increase of mitochondrial function (as determined by mitochondrial respiratory function) [14, 63] [64-66], likely due to high demand of skeletal muscle energy during HIIE. Increasing content and/or respiratory function of skeletal muscle mitochondria can represent positive adaptive response to regular aerobic exercise training [67].

1.4.1 High-intensity exercise and skeletal muscle mitochondrial function

Several excellent studies have covered this topic previously. Here, I summarised the most recent findings in human experiments focusing on the molecular adaptions (including mitochondrial function and content) after HIIE with/without hypoxia. It appears that little is known on hypoxia-induced changes in mitochondrial function, especially when combined with a single session of HIIE. Some studies reported increased mitochondrial respiratory function [63, 64, 68-71] or citrate synthase activity after HIIE/HIIT [64, 68, 69, 72-75], others did not [75, 76]. Furthermore, several studies even suggested that HIIE/HIIT might be associated with negative effects on mitochondria function [77-79]. Compared with exercise in normoxia, most of hypoxic exercise studies revealed unchanged [64, 72, 80-85], or even decreased

mitochondrial respiration [86]. Hypoxia leads to increased cellular O_2 consumption, and a lower tissue partial pressure of oxygen (PO_2), which in turn stabilises and activates the transcription factor HIF-1α [87]. During acute hypoxic exercise, HIF-1α may then transiently suppress mitochondrial function and oxygen consumption [88].

When combining HIIE with hypoxia in an experimental intervention, typically only one intensity is included. This is done by matching the relative intensity while reducing the absolute intensity under hypoxic compared to normoxic conditions [89-92]. For example, Bakkman, L., et al. [81] set up the same relative work intensity corresponding to 65% of Wmax, for hypoxic and normoxic leg, respectively. In this study, maximal Adenosine Diphosphate (ADP) stimulated respiration (state 3) expressed per weight of muscle tended to increase in the N group but did not reach statistical significance, with little changes in the H group (N +31.2%, $p < 0.08$, H +3.2%, ns). Previous research with higher exercise intesnities reported significant changes in mitochondrial function [93-95], which suggests exercise intensity is crucial for the induction of mitochondrial function.

1.4.2 High-intensity exercise and skeletal muscle mitochondrial content

To specificize the alteration of mitochondrial function elicited by a qualitative shift of respiratory control or a general increase of mitochondrial quantity, CS activity has been utilized as a routine biomarker. As illustrated in table 1, most studies performed both mitochondrial respiration and CS. A previous review suggested that training volume is a primary determinant of the exercise- induced increase in mitochondrial content in humans [14], which was supported by measured CS maximal activity before and after exercise programs with different durations. Another review argued that exercise intensity could determine mitochondrial adaptations to short-term training [50]. One of the cited studies compared the effect of HIIT with MICT within the same individual using single leg cycling (six sessions for each leg over 2 weeks, with one leg performing HIIT and the other leg performing MICT) [96]. With the same training volume, HIIT elicited a greater increase in CS activity (a marker of mitochondrial content), when compare to MICT. While OXPHOS capacity (a marker of mitochondrial function) remained unchanged in both interventions. However, other studies reported similar increases [97, 98], or no changes [99] in mitochondrial content after work-matche HIIT and MICT.

Furthermore, exercise intensity can be defined in various ways, such as a percentage to peak power (Wpeak) or lactate threshold (LT). It appears that LT might be a better method to define exercise intensity for individuals with different training levels [99, 100]. Baldwin, J., et al. reported that exercise intensity expressed relative to 70% W_{peak} resulted in different adaptaitons between untrained and trained participants. However, when exercise intensity was defined by LT (at 95% LT), similar increases in the markers of metabolic stress were observed [101]. Based on that, a previous study from my research group suggested determining exercise intensity calculated relative to the LT instead of W_{peak} or the maximum workload (W_{max}) when investigatingexercise-induced mitochondrial biogenesis [100]. Noteworthy, although mitochondrial respiratory function and content are often assumed to increase in parallel with mitochondrial biogenesis, this is not always the case. On the contrary, changes in mitochondrial respiratory function have been reported to occur independently of changes in mitochondrial content [64, 99]. Additionally, changes in mitochondrial content are not always accompanied by an improvement in mitochondrial respiratory function [102]. This suggests the need to assess both aspects in exercise training studies examing mitochondrial adaptions.

Table 1.1 Changes in mitochondrial respiration and citrate synthase (CS) activity from human skeletal muscle biopsies (vastus lateralis) from healthy participants, following exercise training intervention with or without hypoxia. ↑ increased, ↓ decreased, ↔ unchanged, X not tested.

Study	Participants (size)	Training intensity/volume	Mitochondrial Respiration	Citrate Synthesis Activity
Ponsot, E., et al. (2006). [80]	N 7/ HY 8, M	6 wks. FiO_2 14.5%,3000m	↔	↔
Bakkman, L., et al. (2007).[81]	3 F +5 M	4wks, hypobaric hypoxia (526 mmHg), One-legged cycle training in normoxic or hypoxic 65% of maximal power output, W_{max}	↔	↑
Jacobs, R. A., et al. (2012).[103]	8M	28 d, FiO_2 = 15%, 3454m; HA, 100 to 150W, SL, 75 to 125W (25W each session)	HA↑	both ↔
Puype, J., et al. (2013). [89]	N 9/ HY 10, M	6 wks. of SIT, FiO_2 = 14.4%, 3000m, 30-s sprints vs 4.5-min rest intervals; 3d /w	X	↔
Jacobs, R. A., et al. (2013). [64]	24	only hypoxia exposure	↔	X

Study	Participants (size)	Training intensity/volume	Mitochondrial Respiration	Citrate Synthesis Activity
Desplanches, D., et al. (2014). [86]	N 8/ HY 9, M	6 wks. of endurance training, 3–4d /w, 60 min per session),65 % peak aerobic capacity, FiO_2 = 15%, 4000m	↓	X
Tsai, H. H., et al. (2016).[85]	HIIT 20, MICT 20, HE 20	6 wks.HIIT, 3 min intervals at 80% and 40% $\dot{V}O_2$max, MICT, sustained 60% $\dot{V}O_2$max, HE, 100 W under 12% O_2,5 days/wk.	HIIT/MICT ↑ , HE ↔	HIIT/MICT ↔ ,HE ↓
Salvadego, D., et al. (2016). [82]	9M	D1 3000m, D2 3400m, D3 and the after 4000m, D1 3000m, D2 3400m, D3 and the after 4000m	↔	↔
Wu, L. H., et al. (2017). [84]	45M	5d/w, 6wks. HIT, 3 min intervals at 40% and 80%$\dot{V}O_{2max,}$ MCT, sustained 60%$\dot{V}O_{2max}$, CTL, 12% O_2 , HE, 100W under 12% O_2 for 30 min	HIT ↑ , HE/MICT ↔	HIT ↑ , HE/MICT ↔
Meinild Lundby, A. K., et al. (2018). [83]	N 12/ HY 9, M	6 wks of endurance training 3850m	↔	N ↑
Jacobs, R. A., et al. (2013). [72]	16 M	6d in 2 wks. (8-12 X 60S), 100% PPO	↑	↑
Salvadego, D., et al. (2013). [104]	22 M	a single bout of continuous exercise , and 1 bout of knee extensions for an acute RT	↑	↔
Vincent, G., et al. (2015). [68]	8 M	2 wks., 4d in 1wk (12 X 1min), 120% PPO	↑	↑
Granata et al. (2015). [63, 99]	29 M	2d in 4 wks., MICT: 20–36 min 65% W_{max}, SIT: (4–10× 30 s), HIIT:(4–7× 4 min) at ~90% W_{max} and 2 min at 60 W	↑	↔
MacInnis et al. (2017). [50, 96]	10 M	6d in 2 wks., HIIT (4 × 5 min) at 65% $W_{peak,}$ MICT 30min at 50% W_{peak}	X	↑
Christensen, P. M., et al. (2016). [75]	10 M	6d in 2 wks., (8-12 × 60s), 271 ± 52 W	↔	X

Study	Participants (size)	Training intensity/volume	Mitochondrial Respiration	Citrate Synthesis Activity
Wyckelsma, V. L., et al. (2017). [105]	16 older + 8 young	12 wks., 3 d/ wks., (4 × 4 min) at ~90-95% HR_{peak} 4min 50-60% HR_{peak} interval	X	↑
Trewin, A. J., et al. (2018). [76]	8 M	a single bout, CMIE 30min, 50% PPO. HIIE (5 X 4min), 75% PPO; SIE (4X 30 s).	↔	X
Dohlmann, T. L., et al. (2018). [73]	12 F + M	6 wks. (7 × 1 min), 100% $\dot{V}O_{2max}$	↑	↑
Meinild Lundby, A. K., et al. (2018). [83]	21 M	6 wks. endurance training	X	↑
Layec, G., et al. (2018). [78]	8 M	acute bout 5 km cycling time-trial	↓	X
Hedges, C. P., et al. (2019). [106]	10 M	6d in 2 wks. (8-12 X 60s), 100 $\dot{V}O_{2peak}$	↑	X
Chrois, K. M., et al. (2020).	22 (F + M)	18d in 6 wks. (5 X 1min), 2-6 session 124 ± 3% PPO, 7-20 session 135 ± 3%	↑	↑
Vaccari, F., et al. (2020). [107]	32 F + M	34 session (3 mon), HIIT/MICT	Mitochondrial respiration normalized CS ↔	
Newsom, S. A., et al. (2021). [70]	11 F + 4 M	a single bout,1h, 65% peak aerobic capacity	↑	X

1.5 Skeletal Muscle Molecular Responses to High-Intensity Exercise

1.5.1 High-intensity exercise and skeletal muscle mitochondrial biogenesis

Mitochondria can increase the content of their components or fuse with other mitochondria [108], depending on the needs of the cell. Mitochondrial biogenesis has been defined as "the making of new components of the mitochondrial reticulum" [48, 108].

Upon onset of exercise, a number of signalling molecules are induced, including PGC-1α. PGC-1α is considered as a master regulator of mitochondrial biogenesis, and is able to effectively coordinate mitochondrial biogenesis through binding and coactivation of

transcription factors, such as transcription factor A, mitochondrial (TFAM) [109] and nuclear respiratory factor (NRFs) [110].

Previous studies showed that PGC-1α increased in human muscle during prolonged exercise interventions [111] and remained upregulated 24 hours after the exercise session [52]. Comparatively, intermittent exercise (30 of 1-min intervals separated by 1-min of recovery) at 70% $\dot{V}O_{2peak}$ induced a greater activation of PGC-1α signaling pathways when compared to a single bout of continuous exercise of matched work and intensity [111]. Our previous data showed that a single session of HIIE (eight 2-min intervals at around 85% of PPO) induced the mRNA expression of PGC-1α [7] and content of proteins related to mitochondrial biogenesis in human skeletal muscle. A separate study with a different exercise protocol also demonstrated an increase of mRNA and nuclear content of PGC-1α in human skeletal muscle 3 hours after a single session of HIIE (five 4-min intervals at around 80% of PPO), while exercising twice daily for 20 consecutive days blunted this response to HIIE [112]. Another study reported an increase in the nuclear content of PGC-1α protein immediately after a single session of SIE (4 x 30 sec), but not after a session of continuous exercise at 63% of PPO [113]. However, there was also a study reported that a session of SIE (4 x 30 sec Wingate interspersed with 4 min of rest) resulted in similar elevations in PGC-1α mRNA when compared to a session of continuous all-out exercise [114]. An increase in PGC-1 α is able to induce a variety of its target genes, one of them being the citrate synthase (CS) [115].

During long-term exercise training, AMPK and other signals will facilitate muscle adaptations, and upregulate PGC-1α and the other oxidative enzymes (e.g., hexokinase II, PPAR, TFAM, cytochrome C etc.) [116], to accomplish a metabolic transition to oxidative metabolism. Eight weeks of continuous or intermittent endurance exercise (30 min of continuous exercise or 3 × 10 min intervals with 2 hours between intervals, five times per week) resulted in a similar increase in CS activity of human skeletal muscle, a marker of mitochondrial content [117]. Previous results from our research group have supported that training intensity is important for mitochondrial function adaptations to training, while training duration is essential for mitochondrial content adaptations [118]. While four weeks of HIIT at 80-95% of PPO did not increase skeletal muscle mitochondrial function, four weeks of SIT led to a higher mitochondrial function (as evidenced from the mitochondrial respiration) [119]. A separate 4-week HIIT study also showed similar findings in mitochondrial respiration [120]. The failure

in detecting significant mitochondrial function changes could be due to technical variations [121], or genetic factors[7] [122, 123]. Notably, a modest but significant increase in CS activity was observed after four weeks of HIIT, suggesting a higher mitochondrial content [120].

1.5.2 High-intensity exercise and skeletal muscle angiogenesis

Angiogenesis refers to the growth of new blood vessels from original vessels during natural development, reproduction, or tissue repair [124]. Angiogenesis is linked to exercise, aging, and cancer, while affected by previously mentioned aspects [125]. Angiogenic factors are regulated by vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and hypoxia-inducible factors (HIFs). When these angiogenic factors bind to their receptors on endothelial cells, signals within these cells are initiated to promote the growth and survival of new blood vessels [126]. Two common downstream targets are angiopoietin-1 (ANGPT-1) and angiopoietin-2 (ANGPT-2). ANGPT-1 is known as a pro-angiogenic factor [127], while ANGPT-2 has been reported as an antagonist of ANGPT-1, but also important for angiogenesis [124]. Notably, excessive angiogenesis has been linked with some malignant diseases such as cancer, diabetic retinopathy, and preeclampsia. Interestingly, the VEGF-induced angiogenesis has been linked with an increase in mitochondrial function [128].

Exercise induces a range of adaptations, including an upregulation of angiogenesis, which in turn changes the patterns of the molecules involved [129]. Several studies have been conducted to evaluate the angiogenesis-associated changes after exercise. mRNA level of VEGF was higher in skeletal muscle of rats after eight weeks of moderate-intensity incremental treadmill exercise [130]. Blood VEGF and ANGP-1 were also found to be significantly increased after eight weeks of resistance training [131]. The study also suggested that moderate-intensity resistance training might lead to higher angiogenesis compared to high-intensity resistance training [131]. The interstitial content of VEGF protein increased after a session of moderate-intensity exercise and a session of SIE (24 bouts of 1 min at 117% of $\dot{V}O_{2max}$ separated by 1.5 mins of passive rest), but the increase with moderate-intensity exercise was higher than that of SIE [132]. Endurance exercise has been shown to lead to increased CS activity and mitochondrial signalling in human skeletal muscle, accompanied by a decrease in the protein content of anti-angiogenic regulators and an increase in muscle capillarity [117]. On the other hand, High-intensity exercises have been demonstrated to induce angiogenesis [133]. For

instance, a four-week HIIT, consisting of one-legged extensor at 90% of $\dot{V}O_{2max}$ for one minute with 30-second rests, three times per week, resulted in increased capillaries and proliferating endothelial cells [133]. Similarly, a four-week SIT, involveling in one-legged extensor exercise, at 150% of $\dot{V}O_{2max}$ for 1 min with 3-min rest, also led to increased capillaries and proliferating endothelial cells [133]. Furthermore, an acute exercise session at an intensity at ~70% of the maximal load has shown to induce the mRNA expression of VEGF in human skeletal muscle, indicating a molecular response to hypoxia [134]. Intersetingly, a four-week training attenuated the acute exercise-induced upregulation of VEGF mRNA in human skeletal muscle, suggesting a potential adaptation to chronic hypoxic conditions [134].

1.6 Skeletal Muscle Molecular Response to Hypoxia

1.6.1 Acute hypoxia and physiological adaptations

In terms of the effect of hypoxia on exercise performance, it is well-established that decreased oxygen concentrations, known as "physiological hypoxia" can have a significant impact [134]. It can be caused by some physiological activities such as vasodilation, increasing blood flow, and upregulation of hypoxia response [59]. Both PPO and $\dot{V}O_{2peak}$ obtained from GXTs were lower when inspired O_2 fraction (FiO_2) was decreased to 14% (equivalent to 3,200 m altitude) [135]. Additionally, peak heart rate and $\dot{V}O_{2max}$ achieved in the GXTs are lower with acute hypoxia (FiO_2 of 10.4%, equivalent to 5,500 m altitude) [135]. Siminlar decrease in PPO and $\dot{V}O_{2max}$ have been observed during an incremental exercise test at FiO_2 of 12% (equivalent to 4,450 m altitude) [136], accompanied by a lower LT [136].

1.6.2 Long-Term Hypoxia and Physiological Adaptations

In the 100 m race at the 1968 Olympics in Mexico City (elevation 2250 m), male and female athletes who were living in a high altitude were reported to have a time advantage of about 0.19 s and 0.21 s, respectively, compared to athletes who were living at sea level [137]. Consequently, these observations triggered attention towards research into the long-term effects of hypoxia on exercise performance. Interestingly, it has been shown that high-altitude natives (also known as "highlanders") and sea-level natives have a similar $\dot{V}O_{2max}$ in normoxic conditions (where the FiO_2 is 20.9%), but that highlanders can attain a greater $\dot{V}O_{2max}$ when

the oxygen availability is reduced at altitude exposures of 3600 m (FiO_2 of 13.3%) [138]. High-altitude natives usually have an enhanced performance and/or increased physical work capacity [139]. Highlanders from Tibet (3700–4000 m) seem to possess a better economy of walking, cycling, and treadmill running compared to acclimatised lowlanders (sea level) [140]. These advantages of the high-altitude natives may have occurred as a result of both genetic and developmental basis [139].

Sea-level natives who become acclimatised to high altitudes have been shown to improve their physical work capacity and exercise performance, although not to the extent of highlanders [138]. Altitude training is mostly used with endurance training to increase the exercise capacity at sea level and to improve adaptations during competitions in high-altitude areas [141]. Training at a high altitude has been shown to improve the endurance performance of sea-level natives in hypoxic conditions; however, the improvements in their endurance performance was less conclusive once back at sea-level [142].

1.7 Skeletal Muscle Molecular Response to Hypoxia

Under conditions of insufficient oxygen supply in the human body, oxygen-sensing mechanisms are activated to restore oxygenation and adapt quickly to the hypoxic conditions by initiating survival responses such as increased respiration and blood flow [143]. The major oxygen sensing mechanism is the Hypoxia Inducible Factor (HIF), which is dependent on prolyl hydroxylases (PHDs). HIF-1, first reported in 1997, is a transcription factor of the human gene encoding erythropoietin (EPO) and is the main regulator of genes responsive to hypoxia [144]. For the first time, Semenza et al., predicted the existence of a hypoxia-dependent transcription factor [145] and identified HIF-1 as a transcription factor for the cellular hypoxia response [146]. HIF-1 is composed of two subunits: an oxygen-regulated subunit (HIF-1α) and a constitutively expressed subunit (HIF-1β).

1.7.1 Hypoxia with and without Exercise/Training, and Mitochondrial Biogenesis

The reduction in oxygen availability under hypoxia requires cells to change their metabolism to adapt to the catabolic and anabolic reactions that rely on the availability of ATP, normally supplied by mitochondrial Oxidative Phosphorylation (OXPHOS) [147]. HIF-1α signalling

reduces cell dependence on oxygenated energy products by downregulating OXPHOS [82]. Activated HIF-1α has an important function in metabolic transition under hypoxia [148]. HIF-1α target genes are involved in oxygen transport, glycolysis, glucose transport, and satiety [149]. A study reported that after a 43-day Himalayan Expedition (with 23 days above 5000 m), the slow isoform I of both heavy and light myosin subunits increased and the fast isoform IIa decreased, suggesting chronic hypoxia results in a fast-to-slow muscle fibre transition, which could lead to a faster activation of mitochondrial oxidative metabolism [150].

Both hypoxia and exercise can increase HIF-1α accumulation [144]. A recent study showed that the skeletal muscle HIF-1α protein content was 120% higher after hypoxia exposure and was further induced by exercise [92]. Compared to resting in normoxia, exercise in hypoxia raised HIF-1α protein expression approximately 2.5-fold [92]. When the oxygen supply is insufficient, HIF-1α target genes improve oxygen transport by EPO-mediated erythropoiesis and VEGF-induced angiogenesis mechanisms, and mediate skeletal muscle adaptions to endurance training through optimised glucose transport and glycolytic enzyme activity. Finally, training in hypoxia increases PGC-1α mRNA expression [151], which induces mitochondrial biogenesis. In addition, it has been suggested that the combination of hypoxia and exercise may have a synergistic effect on body composition and metabolism [49].

1.7.2 Hypoxia and Angiogenesis

Angiogenesis is a hallmark adaptation to hypoxia in cells and tissues [152]. Most transcriptional responses to hypoxia are mediated by hypoxia-inducible transcription factors [152], including HIF-1α and HIF-2α. HIFs have been shown to upregulate the pro-angiogenic factor ANGPT-1 and to downregulate ANGPT-2 [153]. A previous study reported that HIF-1α regulated VEGF in response to hypoxia more than HIF-2α [154]. The current literature suggests that hypoxia and HIF-1 expression in adult organisms may promote angiogenesis in the following ways: (1) activating angiogenic genes and their receptors such as VEGF [155], (2) regulating proangiogenic chemokines and receptors [156], and (3) enhancing endothelial cells and regulating genes in the cell cycle and DNA replication [157]. All of these findings offer evidence for the effects of HIF-1 on angiogenesis [152].

VEGF, which promotes the proliferation of endothelial cells, is also a hypoxia-adaptive gene. The translocation of proliferated endothelial cells into the extracellular matrix has an important

effect on angiogenesis. VEGF and EPO respond to acute changes in oxygen demand in human skeletal muscle, suggesting that oxygen-sensitive pathways could be relevant for adaptation to physical activity by increasing capillary growth [94]. In fact, besides regulating mitochondrial biogenesis, PGC-1α protein also increases the VEGF mRNA expression and subsequent angiogenesis [158]. On the other hand, the transcription factors HIF-1α and HIF-2α also regulate the expression of VEGF in response to hypoxia [159]. Most of the evidence on hypoxia and angiogenesis is from studies in cancer and angiogenesis. These studies showed that acute hypoxia may result in a dysregulation of tumour vascular systems. In chronic hypoxia, HIF-1α regulates the proangiogenic activities of VEGF, which control the expression of a multitude of genes, inducing angiogenesis [160]. This can ultimately trigger the adaptive mechanisms of angiogenesis to optimise oxygen delivery [161]. VEGF has been reported in many studies to be the most remarkable factor that stimulates angiogenesis [162]. Of note, hypoxia is known to induce the expression of myoglobin in skeletal muscle [163], an alternate way to increase oxygen availability.

1.7.3 High-Intensity Exercise/Training in Hypoxia and Angiogenesis

It has been shown that the combination of hypoxia and exercise training are capable of improving muscle oxygen delivery and metabolism [164]. Data from an animal study have suggested that exercise-induced angiogenesis can alleviate obesity-induced tissue hypoxia [165]. In human skeletal muscle, 45 min of one-legged knee-extension exercise (~26% of one-legged peak load) increased VEGF mRNA, which was further increased in hypoxia (induced via restricted blood flow by ~15–20%) [166]. Six weeks of high-intensity training twice per week (involving two bouts of 12–20 min of running at ~92% of the maximal running speed, separated by 5 min of recovery) in normobaric hypoxia (FiO_2 of 14.5%, equivalent to 3000 m) increased the mRNA of HIF-1α, PGC-1α, and CS in endurance athlete [166]. Similar findings were also shown after six weeks of moderate-intensity (65.6% of the PPO) and low-intensity (52.4% of the PPO) endurance training, performed five times per week in normobaric hypoxia (FiO_2 of 12.9%, 3500 m); both training programs increased HIF-1α mRNA expression and mitochondrial density [49]. The capillary density also increased after six weeks of moderate-intensity exercise [49].

In horse skeletal muscle, four weeks of high-intensity training (2 min of running at 100% $V\dot{O}_{2max}$, three times per week) in hypoxia (FiO_2 of 15%, ~2620 m) resulted in a greater increase in HIF-1α and VEGF mRNA expression compared to training in normoxia, and was associated with a higher capillary density [167]. In a human study, eight weeks of resistance training (twice per week, five sets of 10 repetitions at 70% of pretraining one-repetition maximum, separated by 90 s of rest between sets) was performed in normoxic and hypoxic conditions (FiO_2 of 14.4%, equivalent to 3000 m) [168], the plasma VEGF protein and capillary-to-fibre-ratio increased only after hypoxic resistance training [168]. A recent study investigated the effect of HIIT on the serum concentrations of pro-angiogenic factors in hypoxia and suggested that HIIT alone resulted in a significant increase in serum VEGF, but exercise in hypoxia did not further influence the VEGF levels [169].

1.8 Inflammatory Response to Exercise/Training and Hypoxia

Inflammation is a complicated physiological process associated with the activation of several signalling pathways, usually in response to stress [170]. Chronic low-grade inflammation is associated with metabolic disorders, such as obesity, insulin resistance, and T2D [171]. This inflammatory state is characterised by increased levels of circulating inflammatory markers, such as tumour necrosis factor (TNF), interleukin-6 (IL-6), and C-reactive protein (CRP) [172]. Of note, the inflammatory response consists of both anti- and pro-inflammatory mediators [170]. TNF- α and interleukin-1 (IL-1) are examples of pro-inflammatory cytokines, while IL-10 and interleukin-1 receptor antagonist (IL-1ra) are anti-inflammatory cytokines [173]. Despite high levels of IL-6 having been associated with obesity and T2D, IL-6 also exhibits anti-inflammatory effects during exercise [173, 174]. Toll-like receptors (TLRs) are highly conserved transmembrane proteins with important functions in detecting exogenous microbial pathogens and endogenous molecules, which are released after tissue damage [175]. TLR signalling has a critical role in mediating systemic inflammation, and its activation leads to the elevated expression and release of pro-inflammatory cytokines [176]. Mitochondria have long been linked with inflammation [177]. The endosymbiotic theory of mitochondrial origin supports the role of mitochondria in the activation of the immune system, and therefore inflammation and the pathogenesis of inflammatory diseases [177]. The immune signalling receptor TLR has been associated with mitochondrial functions, suggesting the role of mitochondria in the initiation and development of inflammation [177]. Mitochondria-

associated membranes are linked with inflammation-mediated diseases [178].The mitochondrial outer membrane permeabilisation has been recently shown to have pro-inflammatory effects via pro-inflammatory NFκB signalling [121]. Through regulating the energetic state of immunological synapses between dendritic cells and lymphocytes, mitochondria can direct the inflammatory response toward immunotolerance or immunogenicity [179]. Mitophagy, the selective degradation of dysfunctional mitochondria by autophagy, can dampen inflammation and prevent unnecessary cell loss [180].

Inflammation seems to be linked with muscle fibre composition. The pro-inflammatory cytokine profile is different between the soleus (oxidative) and extensor digitorum longus (EDL) (glycolytic) muscles of mice [181]. Soleus muscle regeneration is associated with elevated and prolonged inflammation as compared to EDL [181]. In mouse skeletal muscle, an inhibition of the slow-to-fast muscle fibre type transition by pyrroloquinoline quinone was reported to be due to a decrease in the expression of cytokine genes [181]. After treadmill running, mice with muscle-specific PGC-1 α knock-out showed a shift from oxidative type I and IIa toward type IIx and IIb muscle fibres, and this was associated with elevated markers of inflammation [51]. TLR4 signalling is essential for lauric acid-induced glycolytic muscle fiber formation. A downhill running-based overtraining protocol resulted in changes in the inflammatory markers and muscle fibre composition in mice [182]. Chronic inflammation is able to increase the abundance of type II muscle fibres in the diaphragm of mice [183].

Angiogenesis has also been linked with inflammation, which can lead to further angiogenesis [184], and vice versa [130]. Abnormal ischaemia-induced angiogenesis is associated with a transiently increased angiogenesis in the ischaemic skeletal muscle of mice [131]. The angiopoietin-like protein (ANGPTL) protein family is involved in both angiogenesis and inflammation [132]. Obesity-associated inflammation has been reported to promote angiogenesis (and breast cancer) via ANGPTL-4 [133]. Inflammatory reactions are able to regulate angiogenesis through the interplay between HIF1, HIF2, NFκB, and nitric oxide [184].

1.8.1 High-Intensity Exercise and Inflammation

Exercise has long been recognised to have anti-inflammatory effects [172]. There are at least three possible mechanisms proposed, including a lower expression of TLRs on macrophages

and monocytes, a higher production and release of anti-inflammatory cytokines from contracting muscle, and a reduction in the visceral fat mass [173]. Secretory peptides from skeletal muscle have been termed myokines [134], many of which are induced by exercise, such as IL-6, IL-15, and brain-derived neurotrophic factor (BDNF) [135]. Interestingly, some myokines show anti-inflammatory effects after a single session of exercise [173].

1.8.2 Hypoxia and Inflammation

Hypoxia and inflammation are tightly interconnected [143, 185]. On one hand, hypoxia can induce inflammation as evidenced by the increased levels of circulating pro-inflammatory cytokines with mountain sickness [186]. A three-night stay at 3400 m above sea level (FiO_2 of 13.6%) has been shown to increase the levels of circulating pro-inflammatory cytokines, such as C-reactive protein and IL-6 [187]. Mice exposed to short-term, extreme hypoxic conditions (FiO_2 of 8% for 8 h) exhibit mucosal inflammation and elevated circulating pro-inflammatory cytokines [188]. Obesity is usually accompanied by adipose tissue hypoxia, which is associated with chronic low-grade systemic inflammation [189]. On the other hand, tissues with inflammation often become hypoxic [143]. A good example is inflammatory bowel disease, in which the mucosa becomes more hypoxic than in normal conditions [190], which is accompanied by a higher protein content of HIF-1 α [191]. Besides the well-accepted concept that hypoxia causes inflammation, hypoxia might also possess some anti-inflammatory effects [192]. A short stay at 3400 m above sea level (FiO_2 of 13.6%) also resulted in an increase in anti-inflammatory cytokine IL-1Rα [187]. The stabilisation of HIF-1 α, the main mediator of hypoxia signalling, has been demonstrated to control excessive inflammation [193]. Notably, a study found a subtle but non-significant decrease in TNF- α and IL-6 after two hours in simulated hypoxia at 4500 m (FiO_2 of 11.8%) [194].

1.8.3 High-Intensity Exercise/Training in Hypoxia and Inflammation

Only a handful of studies have examined the effects of exercise in hypoxia on inflammation. One study compared the effects of exercise at different intensities in normoxia and hypoxia (2800 m, FiO_2 of 14.65%) on pro-inflammatory cytokines, and found no significant differences in the TNF- α or IL-1 after exercise at 40% or 60% of the $\dot{V}O_{2max}$ [195]. Another study

investigated the level of cytokines after an exercise bout at 70% of the $\dot{V}O_{2max}$ performed until exhaustion and detected no changes in TNF- α immediately after or 2 h after the exercise session, while the IL-6 was higher at both time points [194].

Table1.2 Changes in inflammatory markers and proteins from human skeletal muscle biopsies (vastus lateralis), following exercise training intervention with hypoxia. ↑ increased, ↓ decreased, ↔ unchanged.

Study	Participants size	Exercise intensity/volume	Markers / proteins
Blegen, M., et al. (2008). [195]	9 M	Hy: FiO_2: 14.65% Nor: FiO_2 = 20.94%, 40% or 60% of the $\dot{V}O_{2max}$	TNF- α, IL-1 ↔
Caris, A. V., et al. (2014).[194]	9 M	Altitude: 4500m, at 70% $\dot{V}O_{2peak}$ until exhaustion	IL-6 ↑, TNF- α ↔, IL-4↓
Richardson, A. J., et al. (2016). [196]	Nor 14, Hy 14, Con, 14 (F+M)	2 Wks. 30 s maximal work, 4 min recovery; 4 –7 repetitions, Hy: FiO_2: 15% Nor: FiO_2 = 21 %	TNF-α ↑ (Hy, Nor and Con), IL-6↑ (Hy, Nor)
Sumi, D., et al. (2018). [197]	9 trained endurance athletes	H / N: 10 X 3 at 95% $\dot{V}O_{2max,}$ + 30min continuous at 85% $\dot{V}O_{2max,}$ Hy: FiO_2 = 14.5% Nor: FiO_2 = 20.9 %	Myoglobin (Nor) > (Hy), IL-6 ↑ in both
Morrison, J., et al. (2018). [198]	11 amateur athletes	4 sets of 4x4-s SIT	IL-10 ↓, IL-6 ↑ 0h post, then ↓ 3h post, IL-8 ↓ 0h post, then further ↓ 3h post
Kasai, N., et al. (2019). [199]	10 M	3 sets of 5 s × 6 s maximal SIT, 30s rest, Hy: FiO_2 = 14.5% Nor: FiO_2 = 20.9 %	IL-1, myoglobin, VEGF ↔
Britto, F. A., et al. (2020). [200]	20 M	8 sets of 8 repetitions at 80% 1 maximum, FiO_2: 15%	Myoglobin ↑, IL-6 ↑, TNF-α ↔
Kammerer, T., et al. (2020). [201]	5 F + 6 M	Hy: 3883 m, Nor: 520 m, 90 min endurance	IL-1β ↑ and IL-3 ↑, IL-6 ↑, IL-10 ↑ in Hy
Woo, J., et al. (2020). [202]	18 M	60 min at 75–80% HR_{max} , FiO_2: 14.5%	LDH ↓ in Hy, IL-6 ↓ in Nor and Hy
Zebrowska, A., et al. (2019). [169]	12 M	6 X 5min 120% LT, 3/wk., 3wk. for total, Hy: FiO_2: 15.2%	IL-1β↑, TGF-β, IL-6, TNF-α ↔ , HIF-1α ↑, VEGF ↑ (Hy)
Baygutalp, F., et al. (2021). [203]	23 athletes, M	2h/day, 5d/wk., 1850m, 50% -75% -100% $\dot{V}O_{2\ max}$ until exhaustion	HIF-1α ↑, CRP ↑, TNF-α ↑, IL-10 ↓
Maciejczyk, M., et al. (2022). [204]	15 athletes	GE until exhaustion and 30 km TT FiO_2: 16.5%	TNF-α, HEX, GLU ↑, MPO ↔

1.9 Glucose Response to Exercise/Training and Hypoxia

In addition to mitochondrial biogenesis and inflammation, the glucose response is a crucial factor that needs to be considered when examining the effects of HIIE in hypoxic environment.

1.9.1 High intensity Exercise and Glucose Response

As exercise intensity increases, there is a progressive increase in the activation of muscle fibers, accompanied by a further elevation in the utilisation of both plasma glucose and muscle glycogen [205][212]. In a study conducted by van Loon, L. J., et al. (2001) with eight male cyclists, it was observed that increasing exercise intensity resulted in elevated rates of muscle glycogen and plasma glucose oxidation[212]. These findings highlight the interplay between exercise intensity, muscle fiber activation, and glucose utilization, supporting the notion that skeletal muscle plays a critical role in energy metabolism during exercise, further emphasising the importance of exercise intensity when studying glucose utilisation. HIIE has been shown to cause notable depletion of muscle glycogen, as observed in a study by Gyntelberg, F., et al. [206]. This depletion is expected to stimulate greater uptake of glucose by skeletal muscles. Little et al. found that HIIE can reduce post-meal glucose and decrease the duration of hyperglycemia during the 24 hours after exersice sessions, in obese individuals with type 2 diabetes mellitus [207].

1.9.2 High intensity interval Exercise in hypoxia and Glucose Response

Exercising in hypoxic environment decreases the availability of oxygen, resulting in a proportional shift in the flux of metabolic pathways compared to normoxia [205]. Furthermore, the exercise intensity is substantially higher when performed in hypoxia than in normoxia, further emphasising the metabolic adaptations associated with this condition. In response to decreased efficiency of ATP generation and incomplete oxidation of glucose, exercising in hypoxia leads to an increased dependency on glucose and glycogen utilisation [208]. Additionally, hypoxia may activate signaling molecules responsible for glucose transport activity, similar to high-intensity exercise. A study by Mackenzie et al. demonstrated that a 60-minute cycling bout at an absolute intensity of 90% LT, conducted in normobaric hypoxia (FiO_2 =14.6%), resulted in a greater improvement in insulin sensitivity among individuals with type 2 diabetes mellitus, compared to exercise performed in normoxia [209]. By combining the benefits of hypoxia and exercise, individuals with type 2 diabetes may experience a synergistic

effect on glucose regulation, offering a safe and effective approach. While it is generally accepted that exercise in hypoxia enhances intracellular processes related to glucose uptake and the utilisation of glycogen and glucose-derived metabolites [210, 211], it is important to note that findings from some studies suggest contrasting results. For instance, De Groote et al. reported that under hypoxic conditions, plasma triglyceride levels remained stable, whereas triglyceride increased when exercise was performed at the same relative intensity in a normoxic environment [212]. These contrasting observations highlight the complexity of the metabolic responses to exercise in different oxygen level environments and warrant further investigation.

1.10 Summary

High-intensity exercise, especially interval exercises, has gained popularity recently. Even though no consensus on the definition of "high-intensity" has been reached, exercise intensities higher than 75% of the $\dot{V}O_{2max}$ or PPO are commonly used [5, 92]. High-intensity exercise leads to similar, if not greater, improvements in skeletal muscle metabolic adaptations, cardiorespiratory fitness, vascular function, and body composition, but is more time efficient, when compared with moderate-intensity exercises. Within skeletal muscle, high-intensity exercise is associated with similar or great adaptations in mitochondrial biogenesis and angiogenesis in a muscle fibre type-specific manner. The main molecular mediators involved in these adaptations include PGC-1 α, HIF1- α, and VEGF, which are also linked to the hypoxic response [158]. A speculative model has been proposed for the role of PGC-1 α, HIF1- α, and VEGF in the adaptive responses of skeletal muscle to both high-intensity exercise and hypoxia (Figure 1).

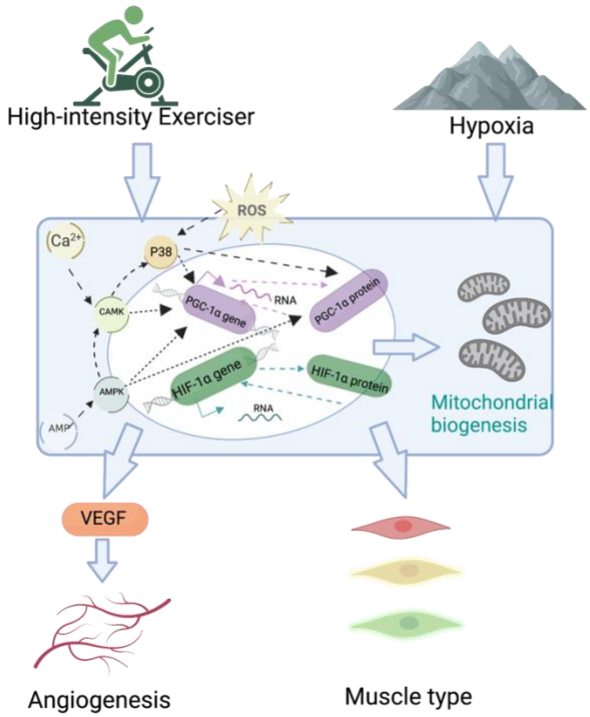

Figure 1.3 A speculative model for the molecular adaptive responses of skeletal muscle to both high-intensity exercise/training and hypoxia. Both exercise/training and hypoxia induce a range of adaptations, including an upregulation in angiogenesis and mitochondrial biogenesis and a shift in the skeletal muscle fibre type. Peroxisome proliferator activated receptor gamma coactivator 1 alpha (PGC-1α), hypoxia-inducible factor 1-alpha (HIF-1 α), and vascular endothelial growth factor (VEGF) play important roles in the regulation of the adaptive response to both high-intensity exercise/training and hypoxia within skeletal muscle.

Exercise is also known to exhibit anti-inflammatory effects [213]. A single exercise session leads to an immediate increase in IL-6 (an anti-inflammatory myokine), without an increase in the pro-inflammatory cytokines TNF- α and IL-1 [213]. Training has been associated with the reduced activation of TLR signalling and reductions in the abdominal and visceral fat, which can both lead to lower inflammation. Both a single exercise session of exercise and training mediate reductions in inflammatory signalling in skeletal muscle, which may be related to the expression of anti-inflammatory myokines, as well as reductions in TLR signalling. Hypoxia is associated with an upregulation of inflammatory signalling, such as a higher pro-inflammatory cytokine [143]. On the other hand, HIF-1α is critical to control excessive inflammation. A speculative model has been proposed for the inflammatory responses to high-

intensity exercise and hypoxia (Figure 2) [185]. Few studies have explored the effects of exercise on inflammatory signalling in hypoxia, but this limited research reported no differences in pro-inflammatory cytokines and increases in anti-inflammatory cytokines. Future studies are required to explore the effects of high-intensity exercise in hypoxia on inflammatory signalling, especially in a tissue-specific manner, which will lead to a more comprehensive scientific basis for maximising the benefits of high-intensity exercise.

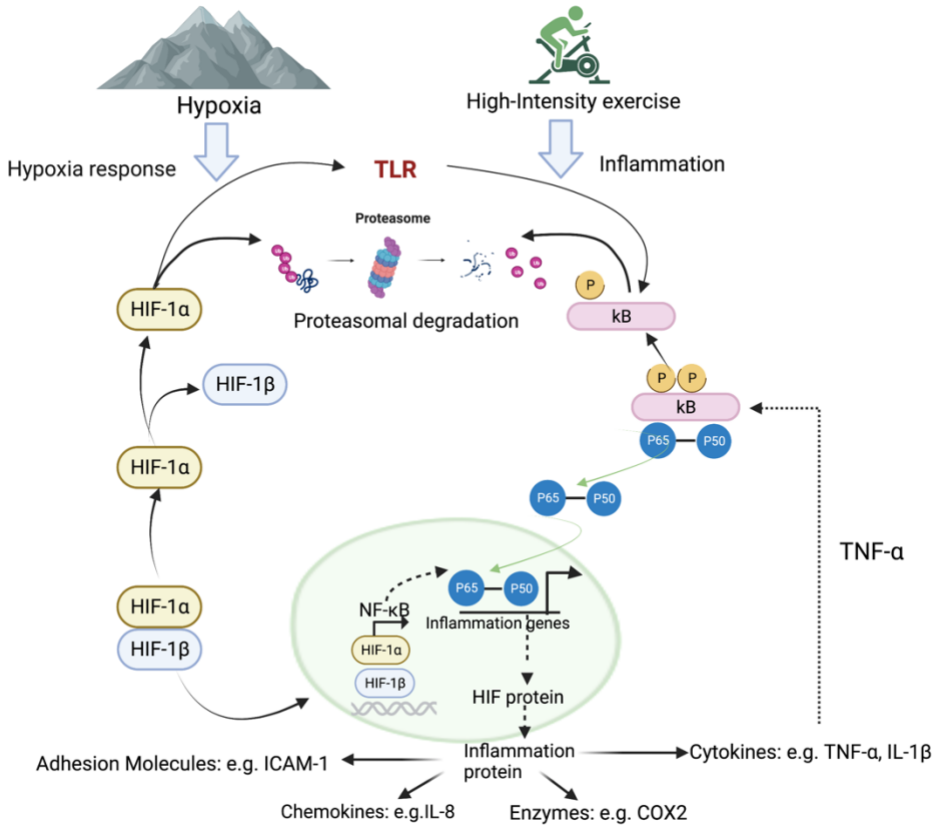

Figure 1.4 A speculative model for the inflammatory responses to high-intensity exercise/training and hypoxia. Exercise/Training exhibits anti-inflammatory effects via the induction of anti-inflammatory cytokines and downregulating toll-like receptor (TLR) signalling. Hypoxia is pro-inflammatory and mediates the upregulation of TLR signalling. HIF-1α is important in regulating the inflammatory response to high-intensity exercise/training and hypoxia.

Chapter 2

Effects of a single session of high-intensity interval exercise in hypoxia based on peak power output and blood lactate threshold

This study is used meticulously designed exercise intensity in both normoxia and hypoxia to investigate whether HIIE combined hypoxia further induce more physiological and biochemical adaptations, and which factor (exercise/ hypoxia) plays more important role in the impact of mitochondrial adaptions.

The aim of this chapter was to compare high-intensity interval exercise (HIIE) sessions prescribed based on two values: peak power output (PPO), which represents the maximal value, and lactate threshold (LT), which represents the submaximal value. In conclusion, the PPO and lactate threshold LT are appropriate parameters for prescribing exercise intensities for high-intensity interval exercise in hypoxia.

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* These authors contributed equally to this work and are co-first authors.

[#] These authors contributed equally to this work and are co-corresponding authors.

2.1 Introduction

Prescribing exercise intensity remains a challenging task. A review from 2020 has summarised current practices in determining exercise intensities [214]. Classic approaches of prescribing exercise intensity are based on percentage of maximal anchors, such as $\dot{V}O_{2max}$ or $\dot{V}O_{2peak}$, maximum (HR_{max}) or peak heart rate (HR_{peak}), and W_{max}/ PPO [214]. However, using fixed percentages of the above-mentioned maximal anchors could result in large variability in the physiological responses [214]. Alternatively, submaximal anchors can be used for the purpose of deriving exercise intensity, such as the first and second lactate threshold (LT1 and LT2), the maximal lactate steady state (MLSS), the ventilatory threshold (VT), the gas exchange threshold (GET), the respiratory compensation point (RCP), critical speed (CS), and critical power (CP) [215-218]. However, current evidence suggests that prescribing exercise intensity relative to the submaximal anchors remains to be validated in different settings and environments [214].

Hypoxia is defined as reduced oxygen supply to tissues leading to decreased oxygen availability [219] which in turn results in decreased $\dot{V}O_{2peak}$ and PPO derived from incremental tests [220-222]. Training in hypoxic conditions is widely used by athletes who aim to increase physical performance at sea level and to improve exercise tolerance during competitions held at terrestrial altitude [141]. With minimal costs and small disturbance to daily routine, interval hypoxic training is often used by athletes to increase performance [223]. High-intensity interval exercise (HIIE) is comprised of bursts of vigorous activity lasting several minutes, and performed at a workload eliciting either ≥75% of $\dot{V}O_{2max}$ [224], ≥75% maximal power output [5], ≥ 90% minimal running speed required to elicit $\dot{V}O_{2max}$ [192, 225], or a rating of perceived exertion (RPE) of "hard" to "very hard" (RPE ≥6 or ≥15 on the 0-10 and 6-20 Borg scales, respectively) [192] interspersed with brief rest periods. Performing HIIE under hypoxic conditions has gained popularity lately, due to the possibility to achieve larger performance gains and associated metabolic and cardiorespiratory benefits compared to similar training near sea level [224].

In general, exercise intensity in hypoxia is prescribed based on PPO [226-230] maximal aerobic speed [231], HR_{max} [232] [233, 234], $\dot{V}O_{2max}$ [235, 236], RPE [237] or LT [229, 238] Different methods of prescribing exercise intensity across studies may limit the possibility to directly compare performance and physiological outcomes between training studies [214]. A lower-

than-expected exercise intensity can also lead to a detraining effect in highly trained individuals. In contrast, prescribing exercise intensity inclusive of maximal, submaximal, and resting values is likely to result in a more homogeneous physiological response [214]. For example, recent studies by our group have suggested that prescription of HIIE in normoxia based on both a maximal value (PPO) and a submaximal value (LT2) resulted in comparable physiological adaptations, when compared with studies based on only a maximal value or a submaximal value [99, 117, 239, 240]. Exercising at similar workloads (same absolute exercise intensity such as pedalling at 100 W), hypoxia will increase physiological and perceptual responses. However, since hypoxia decreases $\dot{V}O_{2max}$, pedalling at a given physiological intensity (for example, 75% $\dot{V}O_{2max}$) represents a reduced mechanical output (power sustained). To our knowledge, however, no study has explored the prescription of exercise intensities for HIIE in hypoxia, based on the combination of a maximal value (i.e., PPO) and a submaximal value (i.e., LT2), derived from a graded exercise test (GXT). Therefore, the aim of this study was to explore and evaluate the prescription of HIIE intensity based on PPO and LT on physiological parameters in simulated hypoxia and compare them with two HIIE sessions (matched for absolute and relative intensity in hypoxia) performed in normoxia.

2.2 Method

Participants

A total of ten males (aged 18-37) were recruited from the Victoria University student population and residents of nearby communities. Inclusion criteria were 1) males aged 18-45 years old; 2) a body mass index of 20-30 kg/m2; 3) no hypertension (resting systolic blood pressure >140 mmHg and/or diastolic blood pressure > 90 mmHg); 4) free of medications before and during the study, without unstable angina or diagnosed reversible cardiac ischemia, diagnosed uncontrolled cardiac arrhythmia with recurrent episodes or symptoms on exertion, heart failure, symptomatic aortic stenosis; 5) non-smokers; 6) not having been to an altitude greater than 1000 m for more than 24 h in the last three months. A risk factor assessment questionnaire (assessing the medical history, symptoms during or after exercise, family medical history, and exercise participation) was obtained from each participant before enrolling in the study. Informed consent was obtained from the participants before participation. The study was approved by Victoria University Human Research Ethics Committee (Ethics Approval NO. HRE18-214). Calculation of the required sample was based on the assessment

of a main effect for difference in gene expression, at a significance level $\alpha = 0.05$ and power $1 - \mathcal{B} = 80\%$. Using peroxisome proliferator-activated receptor gamma coactivator 1- α mRNA levels reported as fold changes after an acute exercise in normoxia and hypoxia by Slivka et al. we performed simulations in R, assuming a random exponential distribution of the fold-change data for the normoxia and hypoxia groups, sample size was estimated as 12 for the current study. Due to the COVID pandemic, we had to terminate the human exercise trial in early 2020, with a final sample size of ten participants.

Table2.1 Participant characteristics (n =10)

Parameter	**Mean ± SD**
Age (ys)	26 ± 4
Body mass (kg)	71.3 ± 2
Height (cm)	175 ± 1
BMI ($kg \cdot m^{-2}$)	23.3 ± 2
$\dot{V}O_{2peak}$ ($L \cdot min^{-1}$)	3.05 ± 0.21
$\dot{V}O_{2peak}$ ($mL \cdot kg^{-1} \cdot min^{-1}$)	42.8 ± 7.3
Peak power output (W)	259 ± 26
Peak HR ($beat \cdot min^{-1}$)	185 ± 7

BMI, Body mass index. $\dot{V}O_{2max}$, Peak oxygen uptake. HR, Heart rate. W, watt.

Study design

The experimental protocol included a familiarization procedure, two GXTs in normoxia (FiO_2 = 20.9%) and two GXTs in normobaric hypoxia (FiO_2 =14%, corresponding to a simulated altitude of ~3200 m, similar to our previous study [241], and three HIIE sessions performed in a random order at the same time of the day (± 2 h). The three HIIE sessions included one in hypoxia (HY), one in normoxia with workload matched to the relative workload performed in hypoxia (NR), and one in normoxia with workload matched to the absolute workload performed in hypoxia (NA). The study duration, inclusive of the familiarization procedure, was about 6 weeks. During the study, participants were asked to maintain their normal daily diet and physical activities. Participants were required to refrain from any strenuous physical activity for 48 h before the familiarization and GXTs and from alcohol and any exercise for 24 h before HIIE sessions. Additionally, participants were advised to restrict eating 2 h before the

GXTs and HIIE sessions and completed a physical activity and dietary intake questionnaire before each test (Fig. 2.1).

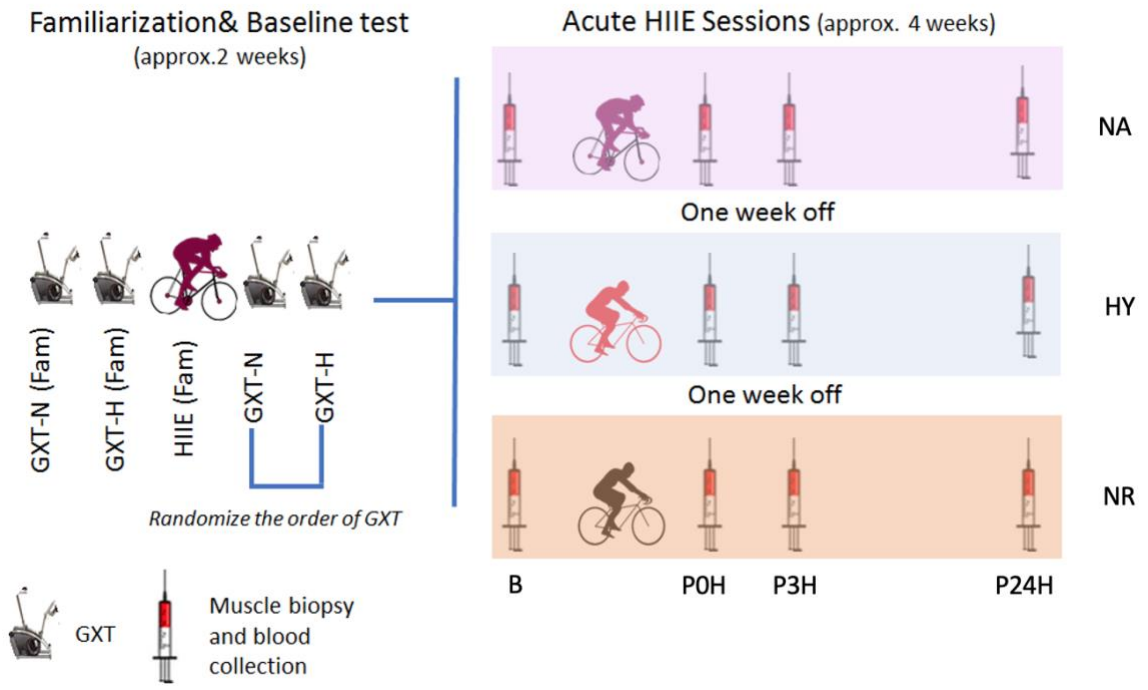


Figure 2.1 Outline of Procedures for HIIE study in Hypoxia: Familiarization & baseline tests will be performed before the acute High-Intensity Interval exercise sessions. Each participant has three sessions including four biopsies and a single bout of HIIE. Abbreviation: GXT, Graded exercise test; GXT-N, GXT at Normoxia; GXT-H, GXT at Hypoxia; Baseline (B); immediately post-exercise (P0H); 3hrs-post exercise (P3H); 24 hrs post-exercise (P24H); NA, workload matched to the absolute workload performed in hypoxia; HY, hypoxia; NR, normoxia with workload matched to the relative workload performed in hypoxia.

Testing procedures

Familiarization and graded exercise tests.

At least 1 week before baseline measurements, all participants performed two familiarization tests, including a GXT in normoxia and a HIIE session to become accustomed with all the testing procedures on two occasions, with at least two days between each session. Following the familiarization sessions, participants performed two GXTs in normoxia or two GXTs in hypoxia, in a randomised and counterbalanced order. A fifth GXT (normoxia and/or hypoxia) was conducted if PPO reached for the two GXTs in the same condition (normoxia or hypoxia) differed by more than 10%. All tests were performed, at least 48 h apart. The PPO, LT, and $\dot{V}O_{2peak}$ data from the two closest GXTs were averaged and then used to determine the exercise intensity of HIIE. The GXTs were conducted on an electronically braked cycle ergometer (Excalibur Sport, Lode B.V., The Netherland), as previously reported, with some modifications

(starting at 25% of each participant's PPO, with an increment of an eleventh of their PPO every 4 min, aiming to achieve 10 completed 4-min stages for each participant) [117, 214]. A 30-s break was given after each completed stage during which capillary blood samples were taken to analyse blood lactate (YSI 2300 Stat; Yellow Springs Instruments, Yellow Springs, USA). The blood lactate data were utilized to calculate the lactate threshold (LT, expressed in W) with the modified D-max formula. The test was terminated when the participants reported 20 on the RPE scale and/or when the participants voluntarily stopped cycling. Exhaled gas during the GXTs was collected and analysed using a MOXUS Metabolic Cart (AEI Technologies, Bastrop, TX, United States) or a Cosmed Quark CPET (COSMED, Rome, Italy) based on equipment availability. In our laboratory, the differences in $\dot{V}O_{2peak}$ values between the MOXUS and Cosmed analysers were shown to be nonsignificant, as evidenced by coefficient of variations (6.4% vs 3.9%) and technical error of measurements (7.4% vs 7.1%). Since the $\dot{V}O_2$ data were collected every 15 s (Cosmed, Rome, Italy) or 30s (Moxus, AEI Technologies Inc., Naperville, IL, USA), the 1-min mean values for $\dot{V}O_2$, $\dot{V}CO_2$, and ventilation (VE) were obtained. The MOXUS Metabolic Cart was the preferred system to measure the gas composition, due to its capacity to input FiO_2 prior to the test. However, the system was occupied by other researchers and unavailable some of the days, when the Cosmed Quark CPET was used to measure the gas composition. Heart rate was monitored by a Polar heart rate monitor throughout the whole test [117]. For the GXTs in hypoxia, participants were acclimatised to the environmental chamber for 10 -15 min before performing the test.

HIIE Sessions.

Each HIIE session consisted of 6x4-min exercise bouts, separated by 2 min of rest. For the HY session, the exercise workload was based on the PPO achieved in hypoxia (PPO_H) and the LT achieved in hypoxia (LT_H) and was calculated as 50% PPO_H + 50% LT_H. For the NR session, the exercise workload was based on the PPO achieved in normoxia (PPO_N) and the LT achieved in normoxia (LT_N) and was calculated as 50% PPO_N + 50% LT_N. For NA, the exercise workload was the same as HY, equalling 50% PPO_H + 50% LT_H. For the HIIE in hypoxia, participants stayed in the simulated hypoxia chamber for 75-85 min, which consisted of 30-40 min of acclimatisation to the environment, baseline blood sampling (after approximately 30 min into the chamber), a 5-min warm-up, a 34-min exercise session, and a 5-min post-exercise blood sampling. All participants then rested for 3 h in normoxia and also reported to the lab the next morning. Blood samples were collected before (B), immediately post (P0), 3 h post

(P3) and 24 h post (P24) each HIIE session. Participants consumed a controlled diet for 48 h before and 24 h following the HIIE session to avoid any confounding effects of different dietary habits and arrived at the lab fasted in the morning. The energy requirement of each participant was estimated using the Mifflin St-Jeor equation, by including each participant's body mass, height, and age [239]. The controlled diet included approximately 53-56% of energy from carbohydrates, 22-24% from fats, and 18-21% from protein.

Plasma blood lactate

Venous blood samples were collected from the antecubital vein and allocated into 3 mL BD Vacutainer® heparin blood collection tubes (BD, Franklin Lakes, United States), inverted 6-10 times, and centrifuged at 3500 rpm at 4℃ for 10-min. The supernatant plasma was collected and carefully aliquoted into 1.5 mL Eppendorf tubes. Plasma lactate was measured with YSI 2300 Stat (YSI Incorporated, Yellow Springs, OH, USA). The plasma lactate level immediately post-exercise (P0) was normalised in fold-change and percentage to the baseline (B) and the corresponding peak blood lactate reached during the GXTs (Peak), respectively.

Statistical analyses

All data in text, figures, and tables are presented as mean ± standard deviation (SD). Student's t-tests and two-way analyses of variance (ANOVA) were used to assess differences between samples. Statistical analyses were conducted using the statistical software package GraphPad Prism (V8.0, GraphPad Software, Inc., San Diego, CA, USA), except for the calculation of effect size, which was performed using the R software. Statistical significance was accepted as $p < 0.05$.

2.3 Results

The characteristics of the participants are presented in Table 2.1. All participants showed a lower absolute and relative $\dot{V}O_{2peak}$, PPO, and LT during the GXT in hypoxia compared to the GXT performed in normoxia, with the biggest differences in $\dot{V}O_{2peak}$. Absolute $\dot{V}O_{2peak}$ decreased by 20.2 ± 8.8% ($p < 0.001$; effect size = 0.87; Fig. 2.2A), while $\dot{V}O_{2peak}$ relative to body mass decreased by 20.2 ± 9.1% in hypoxia when compared with normoxia ($p < 0.001$; effect size = 0.84; Fig. 2.2B). End-exercise RER was 12.5 ± 10.1% higher in hypoxia than in

normoxia ($p < 0.001$; effect size = 0.75) (Fig. 2.2C). End-exercise HR_{peak} was lower in hypoxia compared to normoxia (181 ± 6 vs. 187 ± 6 beats.min^{-1}; $p < 0.001$; effect size = 0.77; Fig. 2.2D). PPO decreased by 9.4 ± 2.1% in hypoxia ($p < 0.001$; effect size = 0.33; Fig. 2.2E) and power at LT was 13.1 ± 3.0% lower in hypoxia than normoxia ($p < 0.001$; effect size = 0.40; Fig. 2.2F). During the GXTs, there was no difference between the peak blood lactate level between normoxia (8.7 ± 1.9 mmol/L) and hypoxia (8.8 ± 2.0 mmol/L).

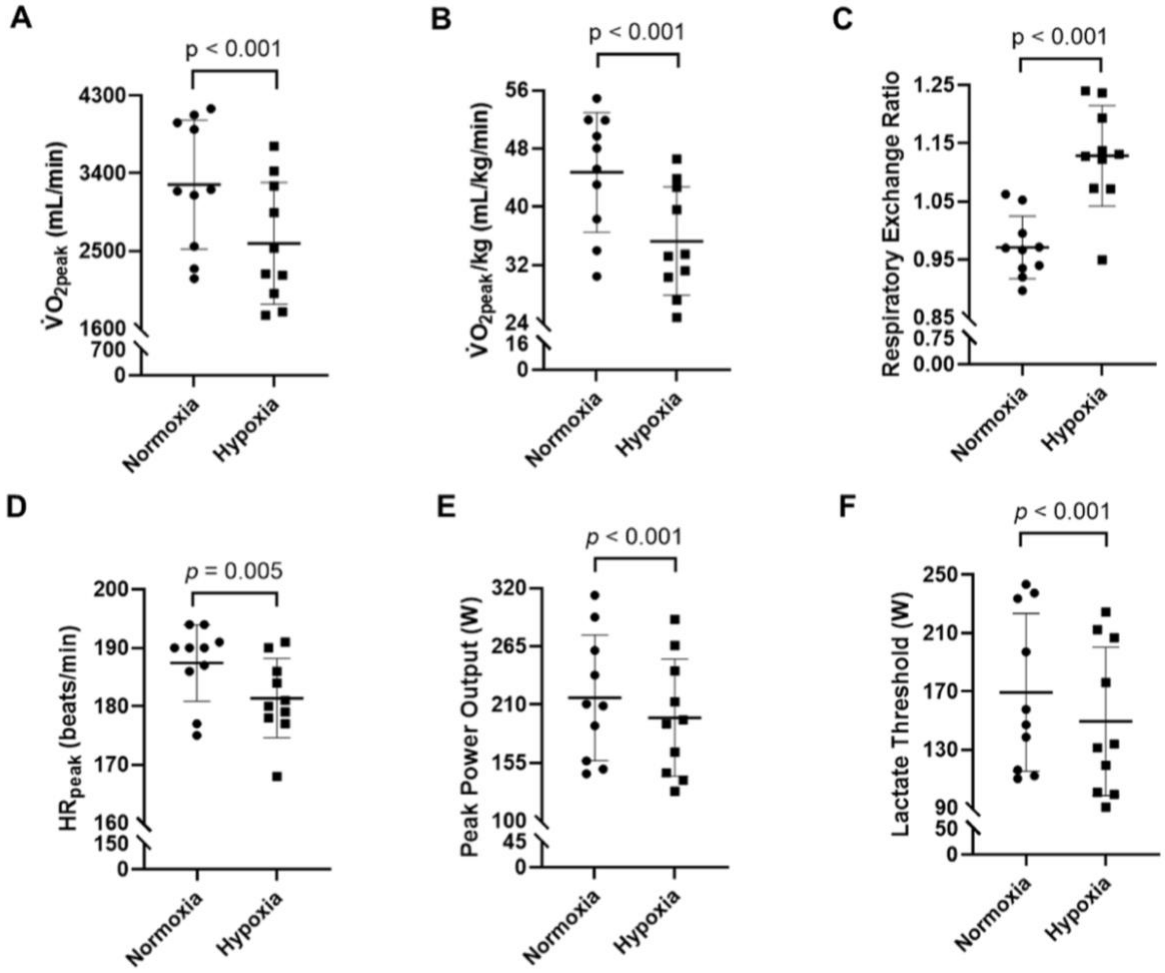


Figure2.2 The effects of hypoxia on physiological data during the graded exercise tests. The graphs represented individual participant's data in normoxia and hypoxia. Under hypoxia, there was an overall decrease in $\dot{V}O_2$peak (A), $\dot{V}O_{2peak}$/kg (B), heart rate peak (D), peak power output (E) and, lactate threshold (F), with an increase in respiratory exchange ratio (C). $\dot{V}O_{2peak}$, Peak oxygen uptake; W, Watt.

The workload was 170.1 ± 50.9 W for NA and HY (Fig. 2.3A), equivalent to 78.2 ± 2.4% and 85.8 ± 1.9% of PPO in normoxia and hypoxia, respectively (Fig. 2.3B). The workload for NR was significantly higher (189.3 ± 53.7 W, $p < 0.001$, Fig. 2.3A), equivalent to 87.4 ± 1.7% of the PPO in normoxia (different from both the NA and HY, Fig. 2.3B). The percentage of HR_{peak} was higher after the HY (92.4 ± 6.6%, $p = 0.026$) and NR sessions (94.4 ± 5.0%, $p = 0.002$), when compared with NA session ((85.0 ± 7.5%; Fig. 2.3C). Compared with NA (15.3 ± 2.6),

RPE was higher in HY (17.2 ± 2.7; p = 0.017) and NR (17.9 ± 3.2, p = 0.005), with no difference between HY and NR (p = 0.15) (Fig. 2.3D). The percentage of $\dot{V}O_{2peak}$ was higher after NR when compared with NA (p = 0.005), with no difference between NA and HY (p = 0.29) (Fig. 2.3E). No significant difference was observed in RER among the three conditions (Fig. 2.3F).

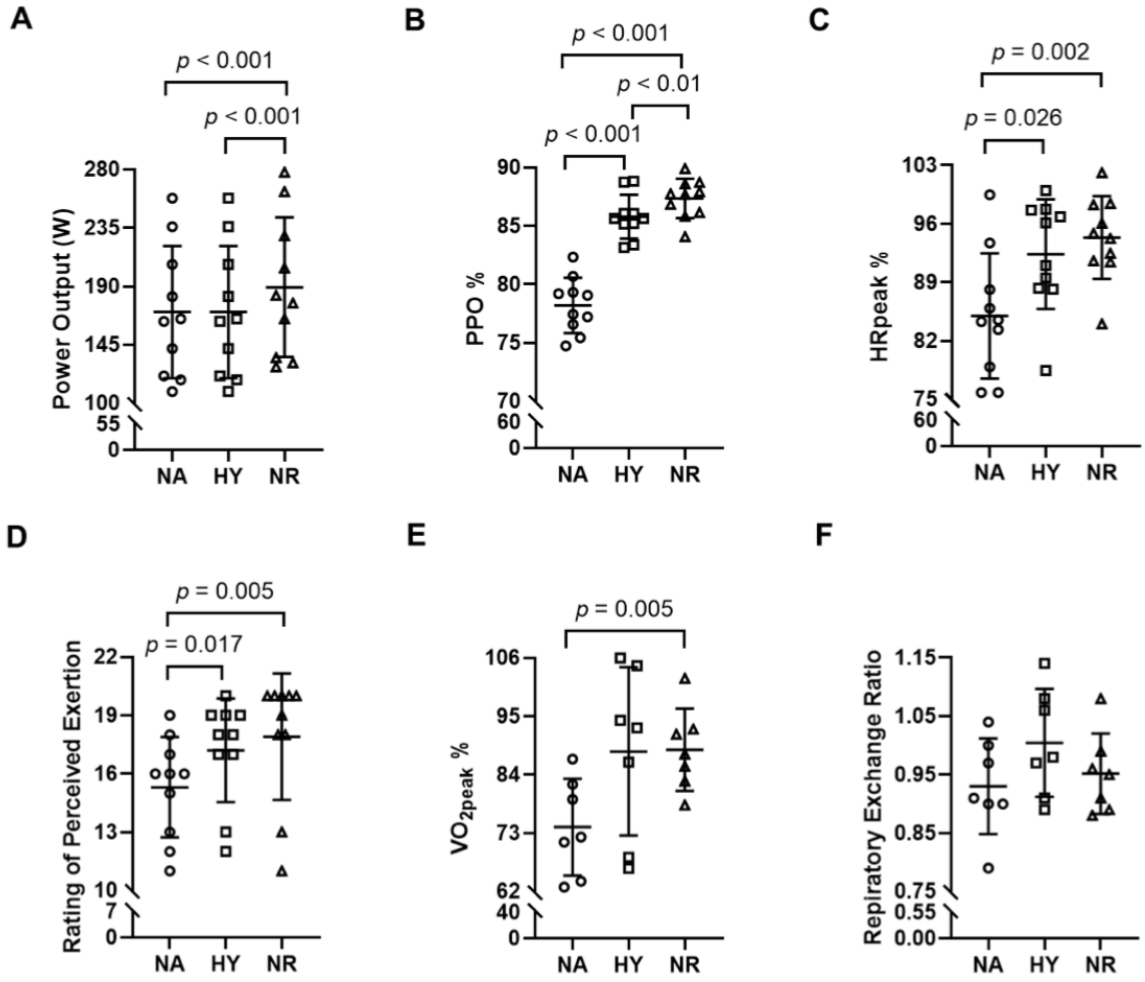


Figure2. 3 HIIE in hypoxia compared with HIIE in normoxia with absolute and relative matched intensities. The graphs represented individual participant's data. NA, HIIE at normoxia matched for absolute intensity to that of hypoxia; HY, HIIE in hypoxia; NR, HIIE at normoxia matched for relative intensity to that of hypoxia. The power output of HIIE sessions (A), the percentage of power out to peak power out (PPO) (B), the percentage of heart rate to heart rate peak HRpeak (C), the rating of perceived exertion (D), the percentage of V̇ O2 to V̇ O2peak of the three HIIE sessions (E), the respiratory exchange ratio reached for the three HIIE sessions (F).

There was no difference for the baseline plasma lactate level among the three HIIE sessions (Fig. 2.4A). Immediately after the HIIE sessions, plasma lactate level increased significantly, by 5.8 ± 1.8, 10.1 ± 3.2, and 8.5 ± 3.1-fold for NA, HY, and NR, respectively (Fig. 2.4B and E) (p < 0.01). The plasma lactate concentration was higher in the HY (p < 0.001) and NR sessions (p =0.005) than that of NA immediately after HIIE (Fig. 2.4B). The plasma lactate

level returned to near baseline 3 h after the exercise interventions (Fig. 2.4C). When plasma lactate level at P0 was normalised to baseline values, a significant difference in plasma lactate occurred between NA and HY sessions (p = 0.01) (Fig. 2.4E), but not between NA and NR sessions (p = 0.11) (Fig. 2.4E). Once the plasma lactate from the HIIE sessions was normalised to the corresponding peak blood lactate reached during the GXTs, significant difference between NA and HY sessions (p = 0.003) (Fig. 2.4F) and between NA and NR sessions (p < 0.001) (Fig. 2.4F) were observed.

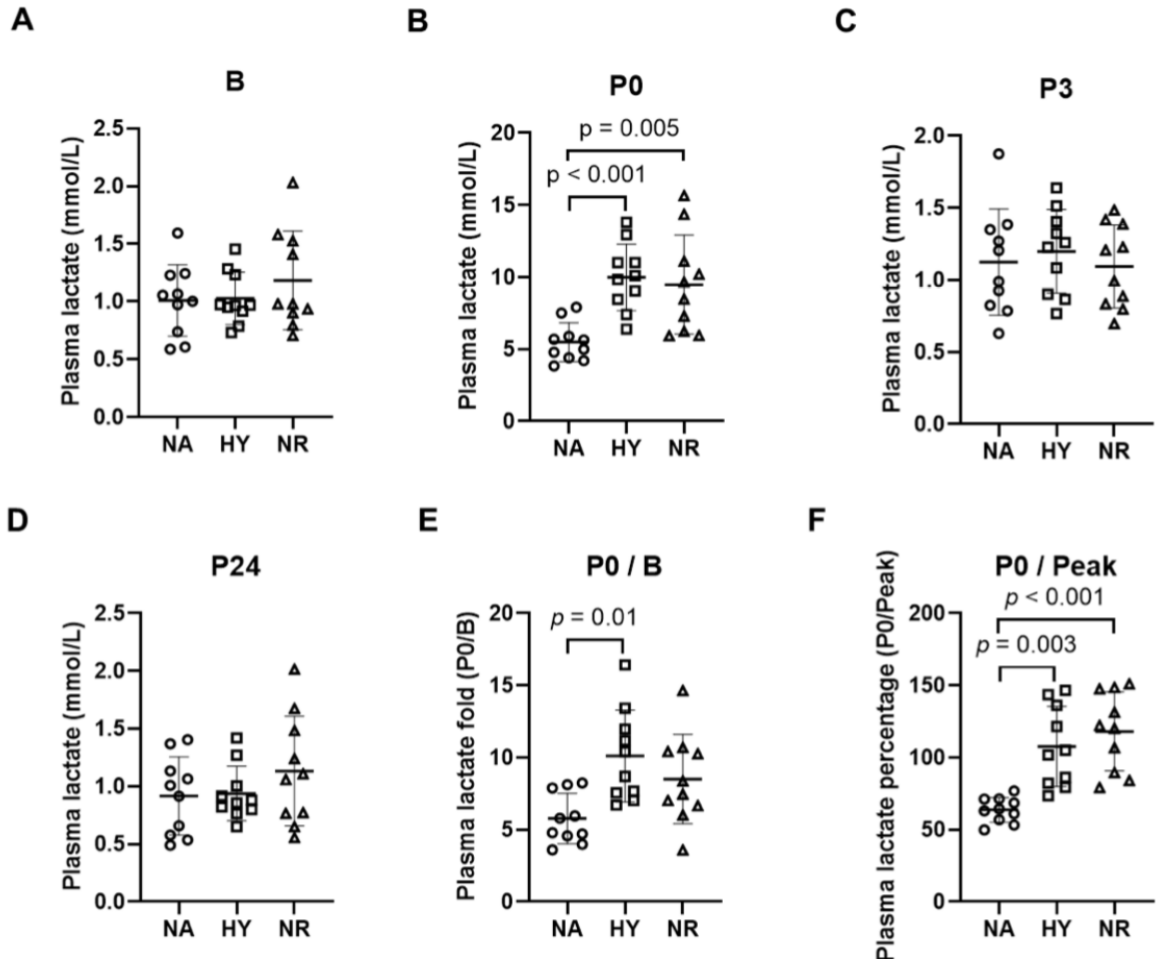


Figure2.4 Plasma lactate level before and after the three HIIE sessions. The graphs represented individual participant's data. NA, HIIE at normoxia matched for absolute intensity to that of hypoxia; HY, HIIE in hypoxia; NR, HIIE at normoxia matched for relative intensity to that of hypoxia. The baseline (B) plasma lactate level (A), plasma lactate level immediately post (P0, B), 3 hours post (P3, C), 24 hours post (P24, D) each HIIE session. The fold of plasma lactate level immediately post each HIIE session when compared with that of baseline (P0/B, E). The percentage of plasma lactate level immediately post each HIIE session to the peak blood lactate reached during the GXTs (P0/Peak, F).

2.4 Discussion

In the current study, we prescribed three HIIE sessions based on PPO and LT derived from GXTs, one HIIE in hypoxia (FiO_2 = 14%, HY) and two in normoxia (FiO_2 = 20.9%, one matched for the absolute intensity in hypoxia, NA; and one matched for the relative intensity in hypoxia, NR). Our study showed that when the work- load during the HIIE session was matched to the absolute value in hypoxia, a lower percentage of HRpeak and $\dot{V}O_{2peak}$, and a lower RPE were observed. On the contrary, when the workload was matched to the relative workload in hypoxia, the percentage of HRpeak and $\dot{V}O_{2peak}$, as well as RPE and RER, were not different. Whilst plasma lactate level increased immediately after all the three HIIE sessions, it was higher after the HY and NR sessions, when compared with that of NA. All the findings suggest that the strategy of matching intensity between normoxia and hypoxia was effective. Interestingly, the percentage of PPO was slightly but significantly higher in the NR than in the HY session. The data suggested if the exercise intensity were prescribed solely based on PPO in normoxia and hypoxia, it would lead to differences in RPE, HR_{peak}%, and $\dot{V}O_{2peak}$ %.

We also observed a decrease in $\dot{V}O_{2peak}$, PPO, LT, and HR_{peak}, with an increase in RER, from the GXTs in hypoxia, when compared with GXTs in normoxia. The observed decrease of $\dot{V}O_{2peak}$ was in agreement with the literature [221] The 8.8% decrease of PPO from GXTs was comparable to a previous study, with a 5.5% decrease when exposure to simulated hypoxia (FiO_2 1⁄4 14.2% or ~3000 m) and a 11.1% decrease when exposure to higher simulated hypoxia (FiO_2 = 12.5% or ~4000 m).39 However, the observed decrease in PPO was less than what was reported by Ozcelik et al. (by 20.2%) [221] It is worth noting that the FiO_2 in the Ozcelik study was 0.120, much less than that in the current study. It is expected that the lower the FiO_2, the lower PPO achieved by the participants. Another explanation for the discrepancy is the differences in the GXT protocols, the current study adopted an increment every 4 min, while the study by OZcelik used an increment every minute. However, the observed decrease of power associated with LT (by 11.8%) was not different from the study by Ozcelik (by 12.5%).8 An early study reported a larger decrease in LT (expressed as the $\dot{V}O_2$ at the LT, by 16.1%) than our study, but the study included only highly trained athletes.40 The lower HRpeak and higher RER were both similar to previously reported [242].

The peak blood lactate values reached after the GXTs in normoxia, and hypoxia were not different, which was supported by the previous research reporting peak/maximal blood was similar from incremental tests in normoxia and acute hypoxia [243]. Yet the blood lactate level during the GXTs was higher at a given power output, in agreement with a previous study [244].

Since the intensities of all the three HIIE sessions were above the power of corresponding lactate threshold, it is not surprising to observe an increase in plasma lactate concentration immediately after the exercise. The increase in blood lactate concentration was not different immediately after HY and NR sessions, but higher after than that after the NA session, suggesting the exercise intensities were well matched in the HY and NR sessions. This increase in plasma lactate was transient, as it returned to baseline 3 hours after the HIIE sessions.

2.5 Conclusions

The prescription of HIIE intensity based on a maximal value and a submaximal value in hypoxia warrants further study. Our data suggest that when matched for relative intensity based on PPO and LT, there was no difference in the percentage of HR_{peak} and $\dot{V}O_{2peak}$, or RPE and RER. All of these parameters were higher than those after HIIE in normoxia matched for absolute intensity. We conclude that the combining of PPO and LT can be used to prescribe exercise intensities of HIIE in hypoxia, and HIIE matched for absolute and relative intensity in normoxia will lead to distinct adaptations when compared with HIIE in hypoxia.

Chapter 3
Mitochondrial adaptations to a single session of high-intensity interval exercise in hypoxia and normoxia matched for different intensities

This chapter aims to determine whether a single session of HIIE in hypoxia induces synergetic adaptations on mitochondrial respiration and content, as well as the mRNA and protein content of genes related to mitochondrial biogenesis. It explores the mitochondrial biogenesis through a series of comprehensive assessment of exercise-induced changes in mitochondrial content, and respiratory function, including mitochondrial respiration, CS activity, Western blots and genes expression analysis, in order to elucidate the upstream and downstream pathway involved.

This chapter is under preparation for submission:
Jia Li, Jujiao Kuang, Yanchun Li, Zhenhuan Wang, Navabeh Zarekookandeh, Kangli Cui, Andrew Garnham, David J Bishop, Li Peng, Olivier Girard , Xu Yan (2023), "Mitochondrial Adaptions to a single session of high-intensity interval exercise in hyoxia and normoxia matched for different intensities". Journal of Science in Sports and Exercise.

3.1 Introduction

High-intensity exercise (HIIE) provides a powerful stimulus to increase mitochondria content [30], respiratory function [63, 245, 246], protein content and enzyme activity [247, 248]. Furthermore, these changes are transient, distinct, and highly dependent on the biopsy timing [5, 249]. A previous study suggested that the greatest changes in mRNA expression were observed between 3 and 72 h post-exercise [249]. Genes specifically related to mitochondrial adaptations to training, such as PGC-1α, PGC-1α1, PGC-1α4 and PPAR α have highest expression at 3h after exercise. Genes associated with metabolism response to training, such as PDK4 and COX4 show the greatest expression at 9 h post-exercise, while CD36, UCP3 and CS showed great changes at 24h after exercise [249].These genes encode mitochondrial proteins and contribute to mitochondrial protein synthesis [5]. The most substantial change in mitochondrial protein synthesis occurs between immediately after and 72 h after exercise [5]. To ensure that the timing of muscle biopsy capture the changes of mRNA expression and protein in response to HIIE, I chose to focus on 14 genes (two isoforms) and 11 proteins that are related to mitochondrial and metabolic adaptations to HIIE, based on the reported time points of the peak expression reported in a previous study.

Hypoxia has long been associated to exercise performance, as evidenced by studies on maximal oxygen uptake ($\dot{V}O_{2max}$) and peak power output (PPO) in hypoxia studies [81, 235, 250-252]. It is widely accepted that hypoxia induce mitochondrial adaptions via activating hypoxia-inducible factor 1-alpha (HIF-1α), which reduce cell dependence on oxygenated energy products by downregulating OXPHOS [224]. In the presence of hypoxia, cellular metabolism undergoes alterations to adapt to the catabolic and anabolic reactions that rely on ATP from mitochondrial Oxidative Phosphorylation (OXPHOS) [147]. PGC-1α serves as a crucial regulator of mitochondrial biogenesis in response to exercise, while HIF-1α is the main regulator of gene expression in hypoxia [144]. The activation of HIF-1α leads to a reduced reliance on oxygenated energy products by downregulating OXPHOS [253]. Under hypoxic conditions, HIF-1α plays a significant role in metabolic transition including oxygen transport [144], glycolysis and glucose transport [254]. VEGF is a HIF-1-dependent gene that induces angiogenesis to enhance oxygen transport when oxygen supply is limited. Moreover, training in hypoxia increases PGC-1α mRNA expression [151], which in turn promotes mitochondrial biogenesis [255]. Finally, it has been suggested that both hypoxia and exercise are able to increase HIF-1α protein accumulation [144]. However, the advantage of adding hypoxia as a

supplement to exercise for performance in normoxia is not consistently supported [256]. This inconsistency may be attributed to the limited number of studies that have compared the effect of HIIE in hypoxia and in normoxia, considering both relative matched intensity and absolute matched intensity.

Previous studies on HIIE in hypoxia have often utilized a single exercise intensity, either a relative matched intensity with reduced absolute intensity compared to normoxia conditions [89, 90, 92, 237] or the same absolute intensity under both hypoxic and normoxic conditions [92, 169, 257]. However, employing a relative intensity protocol in hypoxia, the additional effects on muscle oxidative capacity and mitochondrial adaptions were not consistently observed, whereas differences were found when hypoxic and normoxic training was performed at the same absolute intensity [250]. One of the few studies that examined the effect of acute hypoxia on muscle activation during whole-body dynamic exercise used both absolute and relative exercise intensities was conducted by Torres-Peralta, R., et al. (2014) [258]. The authors suggested that FiO_2 and the relative intensity of exercise played more important roles in muscle activation when compared with absolute intensity [258], Their findings revealed that a linear increases in muscle activation with exercise intensity. Another study by Nordsborg, N. B., et al. (2010). compared the mRNA expression response of intermittent exercise with the matched absolute exercise intensity (70% $\dot{V}O_{2peak}$) and relative intensity (85% $\dot{V}O_{2peak}$). The results indicated that relative exercise intensity was decisive for changes in mRNA expression of PGC-1 α after acute intermittent exercise, thereby inducing skeletal muscle mitochondrial biogenesis [259]. It is worth noting that this study aimed to investigate the differences in the exercise-induced increase of several mRNAs between trained and untrained subjects, instead of hypoxia and normoxia conditions.

The current study is potentially the first one to use both absolute matched intensity in normoxia matched to HY, and the relative matched intensity matched to HY to measure the mitochondrial respiration, CS activity, mitochondrial related genes, and genes coding protein expression. The purpose of this study was to determine whether a single session of HIIE in hypoxia induces extra effects on mitochondrial adaptions. I hypothsised posited that a single session of HIIE in hypoxia would increase PGC-1α 0-3 h post-HIIE, consequently activating mitochondrial biogenesis. This activation would be evidenced by increased mRNA expression, protein content, and changes in a range of downstream genes [92, 151]. If future studies confirm the enhancement of mitochondrial adaption through acute HIIE hypoxia, this type of exercise

performed in conditions of reduced oxygen availability could prove beneficial not only for athletes seeking performance improvements but also for patients with compromised oxygen capability.

3.2 Method

Participants and Ethics

The study design and participant information have been previously reported (Li, Y., et al. 2022). Ten healthy men (age: 28 ± 5 yrs, BMI: 26 ± 3.4 kg/m^2, height: 175 ± 7cm, body mass: 73.5 ± 9.7kg, $\dot{V}O_{2peak}$: 44.7 ± 7.8 ml·kg^{-1}·min^{-1}) were recruited for this study. Written informed consent was obtained from all participants. The study was approved by Victoria University Human Research Ethics Committee (Ethics Approval NO. HRE18-214).

Study design

Please refer to Chapter 2.

Nutritional and physical activity control

During the study, participants were asked to maintain their normal daily diet and physical activities. Additionally, participants were advised to restrict eating two hours before the tests and completed a diet and exercise questionnaire before each test. After each exercise test, a high carbohydrate drink (with 29.7 g of carbohydrates) was provided to help prevent a drop in blood sugar in the participants. A 72h-control diet was provided two days before the trial day. Participants were asked to refrain from strenuous exercise for 24 h before all tests.

Muscle Biopsies

Participants refrained from vigorous physical activities for 48 hours visited the laboratory fasted between 7 am and 8 am. After local anaesthesia of the skin and fascia on the lateral thigh (single leg) by 1-2% Lidocaine, muscle biopsies were taken from the *vastus lateralis* muscle by the same medical doctor via Bergstrom needles with suction (Bergstrom Muscle Biopsy Cannula with 5-millimetre diameter and 101-millimetre length, no. 119-29187-50) [99, 260]. A total of four muscle biopsies were collected, before (B), immediately (P0H), 3 hours (P3H) and 24 hours post (P24H) exercise. Biopsies were taken randomly in opposite legs (i.e., left–

right–left or vice versa). For HY, the P0H biopsy was collected in the hypoxia chamber. Participants were allowed to do light activities (sit or walk) and water, but not food intake for the next 3 hours, a third biopsy was obtained 3 h after. Participants were then provided with a standardized lunch and allowed to leave the laboratory. Participants visited the lab and fasted for the P24H biopsy the next morning. Muscle samples were dried and excess blood, fat, and connective tissue were removed immediately. Around 5-10 mg of fresh muscle piece were immersed in BiOPS solution ice-cold for mitochondrial respiration analysis. A small pieces of muscle was embedded in TissueTek immersed in freezing cold 2-methylbutane. The remaining muscle tissue portion was immediately frozen in liquid nitrogen and stored at -80℃ until required for analysis.

Mitochondria respiration

Muscle fibres were separated with sharpen forceps in ice-cold biopsy preservation solution (BiOPS, with 2.77 mM CaK_2EGTA, 7.23 mM K_2EGTA, 5.77 mM Na_2ATP, 6.56 mM $MgCl_2 \cdot 6H_2O$, 20 mM Taurine, 15 mM Na_2Phosphocreatine, 20 mM Imidazole, 0.5 mM Dithiothreitol, and 50 mM K^+-MES at pH 7.1) immediately after muscle biopsy and incubated in BIOPS with 50ug/ml saponin for 30 min on a shaker. Muscle samples were then washed in MiR05 (0.5 mM EGTA, 3 mM $MgCl_2 \cdot 6H_2O$, 60 mM K-lactobionate, 20 mM Taurine, 10 mM KH_2PO_4, 20 mM Hepes, 110 mM sucrose, and 1 g/L bovine serum albumin at pH 7.1) for seven minutes, three times, on ice. Mitochondrial respiration was measured in triplicate in 2 ml MiR05 at 37℃ by using high-resolution Oxygraph-2K (Oroboros, Innsbruck, Austria). Oxygen concentration and flux were recorded with DatLab software (Oroboros) [261, 262].

A substrate–uncoupler–inhibitor titration (SUIT) protocol was used, and the SUIT sequence was as follows: octanoyl-carnitine (0.2 mM) and malate (2 mM) in the absence of ADP were added to measure the leak respiration (L) via electron transferring flavoprotein (ETF), (ETF)L, $MgCl_2$ (3 mM) and ADP (5 mM) were then be added to measure oxidative phosphorylation capacity (P), (ETF)P. This was followed by the addition of pyruvate (5 mM) for measurement of respiration through Complex I (CI), (ETF+CI)P, then by the addition of succinate (10 mM) for measurement of P through CI (CI) and CI+II combined (ETF+CI+II)P. Cytochrome c (10 mM) was then added to check the integrity of the mitochondrial outer membrane, followed by titration of stepwise carbonyl cyanide 4-(trifluoromethoxy) phenyl-hydrazone (FCCP) titrations to plateau (0.75–1.5 mM), for measurement of electron transport system (ETS)

capacity through (E) through (ETF+CI+II)E followed. Antimycin A (2.5 mM), an inhibitor of CIII, was added to measure residual oxygen consumption (ROX), which is representative of nonmitochondrial oxygen consumption [76, 261].

Preparation of whole-muscle lysates

Frozen muscle (10 - 20mg) was homogenised twice for 2 minutes with a Tissuelyser (Qiagen), in ice-cold buffer (1:10, w/v; 50mM Tris-HCl, pH 7.0; 270 mM sucrose; 5 mM Ethylene Glycol-Bis(β-aminoethyl Ether)-N,N,N',N'-tetraacetic Acid (EGTA); 1 mM EDTA; 1 mM sodium orthovanadate; 50 mM glycerophosphate; 5 mM sodium pyrophosphate; 50mM sodium fluoride; 1mM dithiothreitol; 0.1% Triton X-100) with protease/phosphatase inhibitor cocktail (cell signalling 5871s). The lysate was then rotated at 4 ℃ for 1 h. The protein concentration was measured using a Bradford protein assay (Bio-Rad Laboratories, Hercules, United States). Muscle lysate was stored at −80 ℃ before further analyses. Muscle homogenate was used for both CS activity assay and western blot.

CS activity assay

CS activity was determined in triplicates on a 96-well microtiter plate by adding 10 μg muscle homogenate, 40 μl of 3 mM acetyl CoA, and 25 ul of 1 mM 5,59-dithiobis (2-nitrobenzoic acid) (DTNB) in Tris buffer to 165 ul 100 mM Tris buffer (pH 8.3) kept at 30℃. After the addition of 15 μl of 10 mM oxaloacetic acid, the plate was immediately placed in an xMark-Microplate spectrophotometer (Bio-Rad) at 30℃, and after 30 s of linear agitation, absorbance at 412 nm was recorded every 15 s for 3 min [99]. All samples were measured at the same time with the same reagents and CS activity is reported as moles per hour per kilogram of protein.

Western blot

Protein lysate (30 or 50 μg) was loaded in 26-well Criterion ™ 4-20% TGX Stain-free ™ precast gels (Bio-Rad, Australia). Four internal standards (a mixture of every sample homogenate in equal volume) were loaded with varying dilutions in each gel for a calibration curve for quantification of the protein abundance. Protein was transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were blocked with 5% non-fat milk for 1 h and washed with 1 × TBST four times. Membranes were then incubated overnight with primary antibodies (1:1000 dilution in TBST with 5% BSA and 0.02 sodium azide). The key primary antibodies include PGC-1α (ab 3145040), HIF-1α (CST 3716S), VEGF (ab 214424), P38 (CST

9212), P-P38 (CST 9211), AMPK (CST 5832), P-AMPK (CST 2535), CAMKII (CST 3862), P-CAMKII (CST 12716), MTOR (CST 2972), P-MTOR (CST 5536S), ERR (ab 70228). Following four times wash with 1 ×TBST, the membranes were incubated in species-specific secondary antibodies with 5% non-fat milk for 60 min, then chemiluminescent detection with ECL (Bio-Rad). The secondary antibody was from Cell Signalling Technologies, Rabbit (CST 7074S), and Mouse (CST 7076S). Images were taken with a ChemiDoc Imaging System fitted (Bio-Rad). Densitometry was performed with the Image Lab 5.0 software (Bio-Rad). Images are typically displayed with at least five bandwidths above and below the band of interest.

RNA extraction

Total RNA was extracted from frozen samples (10-15 mg) with Qiagen AllPrep DNA/RNA Kits, according to the manufacturer's instructions (Qiagen, Germany). Briefly, muscle samples were homogenised using the TissueLyser II (Qiagen, Mississauga, ON, Canada) for 2 min at 20 Hz, and total RNA was isolated from the aqueous phase following precipitation with 600 µL of 70% ethanol. Prior to storage, separate aliquots were taken for RNA assessments. The RNA concentration and purity were measured using a Nanodrop spectrophotometer (ND1000, Thermo Fisher Scientific, Wilmington, DE). Absorbance at 260 nm (*A*260) gave a specific measurement of nucleic acid concentration, and the absorbance at 280 nm (*A*280) and 230 nm (*A*230) measured protein and background absorption, respectively, as an indication of possible contaminants [37]. RNA integrity was checked using the Agilent RNA ScreenTape (Agilent Technologies, USA). 5 µl RNA sample buffer and 1 µl RNA were mixed for 1 min and spun down another 1 min. Before loading samples into the TapeStation instrument, samples were heated at 72℃ for 3 min and left on ice for 2 min. Samples with RNA Integrity Number (RIN) no less than 7 were accepted for qPCR preparation.

Reverse Transcription

cDNA was synthesised from 1µg of RNA using the iScript™ Reverse transcription supermix for qPCR kit (Bio-Rad Laboratories) [37]. The cDNA synthesis reaction components include 4 µl iScript RT supermix, 1 µg RNA template and Nuclear-free water to make a total of 20 µl. The complete reaction in a thermal cycler includes priming (5 min at 25℃), reverse transcription (20 min at 46 ℃), and RT inactivation (1 min at 95℃) (C1000 Touch Thermal Cycler). Use the same amount (1µg) RT negative control containing no cDNA, but the same amount of genomic DNA contamination as the cDNA sample for the primer specificity test.

The cDNA was then diluted 5 to 10 times with nuclease-free water and stored at -20℃ for subsequent analysis.

Quantitative RT-PCR

Relative mRNA expression was measured by qPCR (QuantStudio 7 Flex, Applied Biosystems, Foster City, CA) using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad). Primers were designed using Primer-BLAST (Ye et al., 2012) to include all splice variants, and were purchased from Sigma-Aldrich. All reactions were performed in duplicate on 384-well MicroAmp optical plates (Applied Biosystems) using an epMotion M5073 automated pipetting system (Eppendorf AG, Hamburg, Germany). The total reaction volume of 5 µl contained cDNA template (concentration depending on the gene targets), 2.5 µl of 2 × mastermix, and 0.3 µM or 0.9 µM primers (concentration depending on the gene targets). All assays were run for 10 min at 95°C, followed by 40 cycles of 15 s at 95℃ and 60 s at 60°C using a (C1000 Touch Thermal Cycler). The list of primers is shown in table 2. The expression of each target gene was normalised to the geometric mean of expression of the two most stably expressed reference genes (from B2M, ACTB, TBP, Cyclophilin, GAPDH) and using the $2^{-\Delta\Delta Ct}$ method (where Ct is the cycle threshold) [263].

Table 3.1 Primer sequences and amplicon details. F, forward primer; R, reverse Primer

Gene Target	Primer Sequence	Efficiency	Accession no.
PGC-1 α	F: GGCAGAAGGCAATTGAAGAG	103.6	NM_01362
	R: GGCAGAAGGCAATTGAAGAG		
PGC1 α 1	F: TCACACCAAACCCACAGAGA	102.9	Adapted from
	R: CTGGAAGATATGGCACAT		(Ruas et.al 2012)
PGC1 α 4	F: TCTCGCTGACACGCAGGGT	91.6	Adapted from
	R: GCACCACTGCAGCTCCCC		(Ruas et.al 2012)
PPAR α	F: GGCAGAAGAGCCGTCTCTACTTA	92.7	NM_001330751.1
	R: TTTGCATGGTTCTGGGTACTGA		
HIF-1 α	F: TATGAGCCAGAAGAACTTTTAGGC	90.1	NM_001530.1
	R: CACCTCTTTTGGCAAGCATCCTG		
VEGF	F: ACAACAAATGTGAATGCAGACC	144	NM_001025366.3
	R: CGTTTTTGCCCCTTTCCCTT		
HSP70	F: ACCTTCGACGTGTCCATCCTGA	99.2	NM_005345.5
	R: TCCTCCACGAAGTGGTTCACCA		

Gene Target	Primer Sequence	Efficiency	Accession no.
P53	F: GTTCCGAGAGCTGAATGAGG	101.8	NM_00546.5
	R: TTATGGCGGGAGGTAGACTG		
UCP3	F: CCACAGCCTTCTACAAGGGATTTA	89.5	NM_003356.4
	R: ACGAACATCACCACGTTCCA		
CS	F: TCAGGAAGTGCTTGTCTGGC	113.5	NM_003201.2
	R: ATAGCCTGGAACAACCCGTC		
PDK4	F: GCAGCTACTGGACTTTGGTT	99.7	NM_002612.3
	R: GCGAGTCTCACAGGCAATTC		
COX4	F: GAGCAATTTCCACCTCTGC	103.6	NM_001861.6
	R: CAGGAGGCCTTCTCCTTCTC		
SLC27A4	F: GCCCTCATCAACACCAACCT	104.8	NM_ 00509.4
	R: CAGGAGGCCTTCTCCTTCTC		
CD36	F: ACAGATGCAGCCTCATTTCCA	119	NM_001371075.1
	R: TACAGCATAGATTGACCTGCAA		
Housekeeping gene			
B2M	F: TGCTGTCTCCATGTTTGATGTATCT	98	NM_004048.2
	R: TCTCTGCTCCCCACCTCTAAGT		
ACTB	F: GAGCACAGAGCCTCGCCTTT	107	NM_001101.3
	R: TCATCATCCATGGTGAGCTGGC		
TBP	F: CAGTGACCCAGCAGCATCACT	99	NM_003194.4
	R: AGGCCAAGCCCTGAGCGTAA		
Cyclophilin	F: GTCAACCCCACCGTGTTCTTC	100	NM_021130.4
	R: TTTCTGCTGTCTTTGGGACCTTG		
GAPDH	F: AATCCCATCACCATCTTCCA	106	NM_001289746.1
	R: TGGACTCCACGACGTACTCA		

Data and statistical analyses

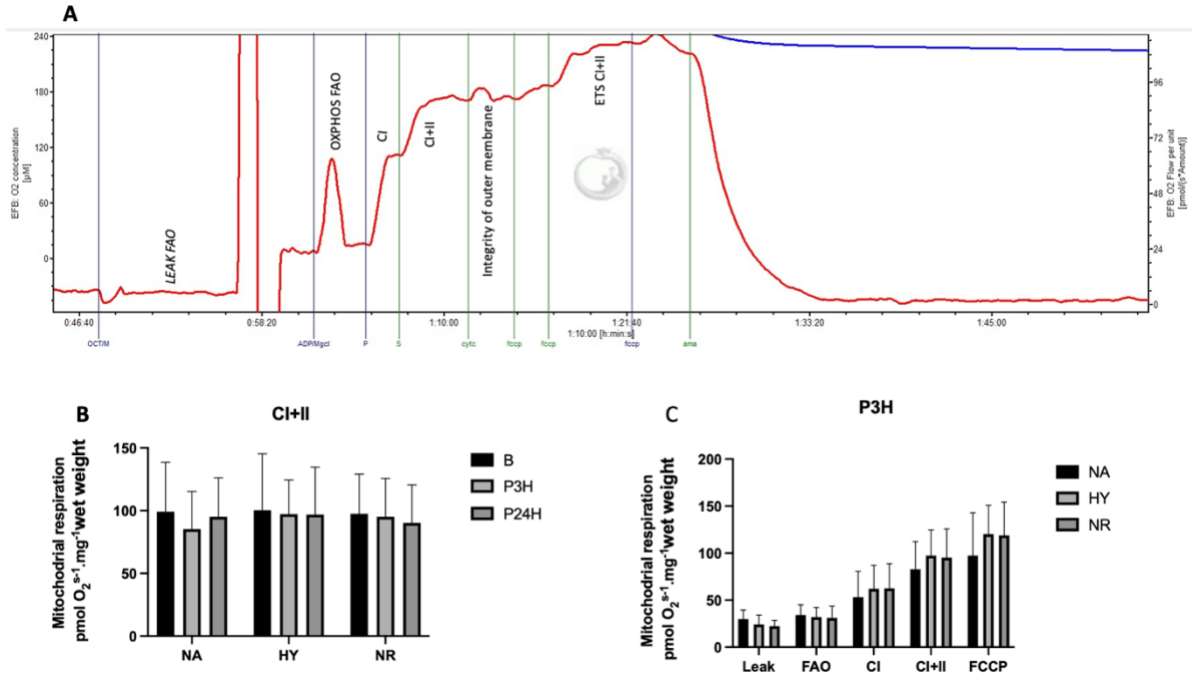
All data in text, figures, and tables were presented as mean ± standard deviation (SD). Because of large inter-individual differences in absolute mitochondrial genes values, post-intervention values are expressed as percent changes in means (Baseline vs. P0H, P3H and P24H respectively), while the mean of the Before values was assigned to the arbitrary value of 1.00 ± 0.00. For gene analysis, a Mann-Whitney U test was used to compare the difference in time points between baseline and post-exercise values. Differentially expressed gene targets post-

exercise were first determined by a posteriori information fusion scheme that combines the fold change and the statistical significance (p value); significance was defined as a Xiao value < 0.05 [264, 265]. A one-way analysis of variance (ANOVA) with the Dunnett test was used to assess the differentially expressed gene targets and proteins post-exercise, and significance was defined as adjusted p value <0.05. Two-way repeated measures ANOVA (time × condition) was used to compare the mitochondrial responses before, immediately after, 3 h and 24 h with p values ≤ 0.05 indicating statistical significance. Statistical analyses were conducted using the statistical software package GraphPad Prism (version 8.1.2, Inc., San Diego, CA, USA).

3.3 Results

Skeletal muscle mitochondrial respiration

The maximum respiration rate (CI + II) did not differ between the three conditions before exercise (B) (p = 0.96) (Fig.3,1B). In all conditions, there were no significant changes in respiration rate either at P3H (p = 0.37) or P24H (p = 0.87) (Fig.3.1B). The fold changes of the respiration rate related to baseline was not different between conditions (Fig, 3.1C). As shown in Fig. 3.1C , D, there was no interaction in mitochondrial respiration between biopsy time and exercise conditions (p = 0.67).



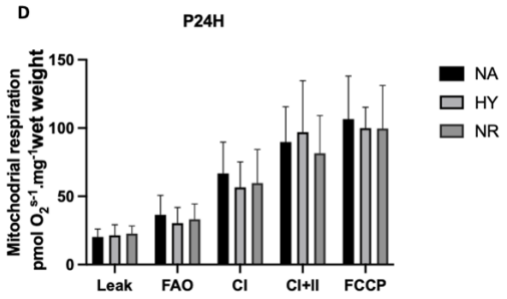


Figure 3.1 Mitochondrial respiration from permeabilized human muscle fibres. A: representative mitochondrial trace depicts one sample in a single chamber. The blue line is chamber O_2 concentration, the red line is the mitochondrial O_2 consumption rate throughout the substrate inhibitor uncoupled titration protocol. Respiratory states were induced as follows: (LEAK FAO) supported by fatty acid oxidise substrates (M) and addition of ADP and Magnesium chloride ($MgCl_2$) stimulated OXPHOS, and complex I substrates pyruvate (P), and succinate (S) for complex II and cytochrome *c* (Cyt.) tested the integrity of the mitochondrial outer membrane and uncoupled (ETS) states with complexes I + II substrate FCCP input. Muscle samples has been taken on the baseline, 3h post-training (P3H); post, 24h post-training (P24H). NA, workload matched to the absolute workload performed in hypoxia; HY, hypoxia; NR, normoxia with workload matched to the relative workload performed in hypoxia. B: Maximum respiration rate (CI+II). C: Fold changes of the respiration rate (represented by CI+II P3H. D: Maximum respiration rate (CI+II). C: Fold changes of the respiration rate (represented by CI+II P24H**.** Data are presented as means ± SD.

Mitochondrial content and skeletal muscle oxidative capacity

CS activity remained unchanged at all time points in all conditions (p = 0.63, Fig.3.2A). No interaction was observed between time and conditions. No difference was observed when the maximal respiration rate was normalized to CS activity in the three conditions (p = 0.57, Fig. 3.2B).

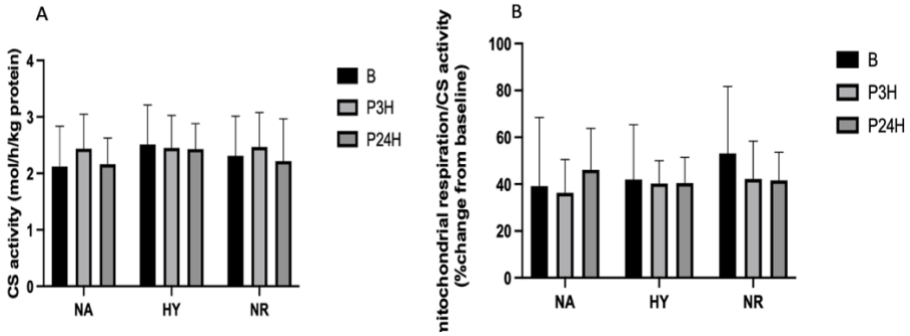


Figure3.2 Citrate Synthesis Activity A: Changes in citrate synthase activity (CS) in three conditions (NA, HY, NR) at the baseline (B) post-exercise immediately (P0H), 3h post-exercise(P3H) and 24h post- exercise (P24H). B: Arbitrary unit of mitochondrial-specific respiration (obtained by normalizing mass-specific mitochondrial respiration values by CS activity expressed per kilogram of protein). Data are presented as means ± SD.

Gene Expression

Gene expression data are depicted in Fig. 3.3. The main target genes include HIF-1α, VEGF and PGC-1 α all increased at P3H for both HY and NR ($p < 0.05$), but only the fold change of HIF-1α mRNA at P24H in HY is significantly higher than that in NR ($p < 0.05$). HY and NR demonstrated larger changes in gene expression compared to NA, while HY and NR induced similar changes in gene expression. The larger changes are seen at P3H, but some exceptional genes such as Pyruvate Dehydrogenase Kinase 4 (PDK4) maintained a high level of expression from P3H to P24H in HY and NR ($P < 0.01$). PDK4 and PPAR α showed an increase in three conditions and no difference in fold changes showed at any time points between NA, HY and NR ($p > 0.05$).

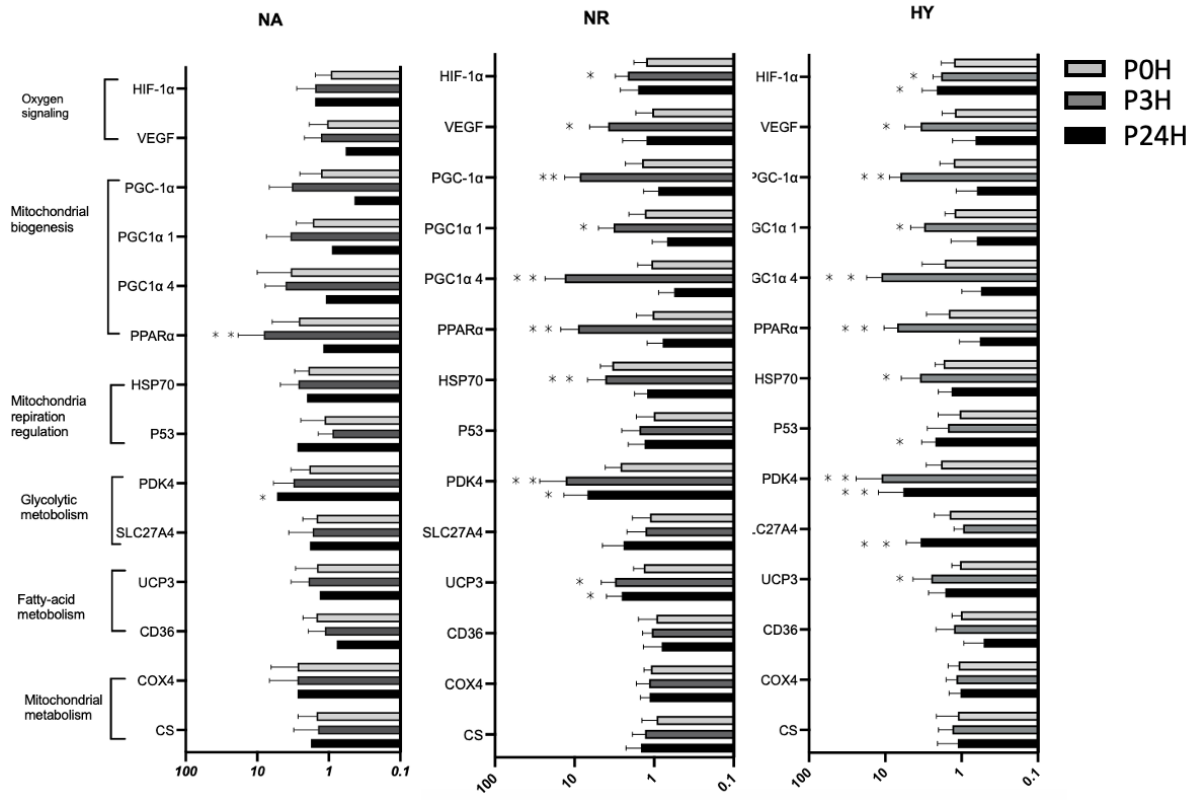


Figure3.**3** Fold changes of mRNA content at immediately post (P0H), 3h post exercise (P3H) and 24h post exercise (P24H) at each of conditions: normoxia absolute intensity (NA), normoxia relative intensity (NR) and hypoxia (HY). Individual data points (triangle, black circle, square and diamond). Mean (horizontal bars) ± standard deviation (SD) is plotted for each graph. * P<0.05, ** P<0.01.

Mitochondrial biogenesis-related protein content

The phosphorylation of Mammalian Target of Rapamycin (*p*-mTOR) decreased significantly at P3H in reference to B in NR ($p = 0.04$) (Fig.3.5B). The protein level of *p*-mTOR at P3H for HY is significantly greater than in that of NA ($P=0.05$) (Fig.3.5B). Compared to respective baseline values, The phosphorylation of Adenosine Monophosphate-Activated Protein Kinase

(*p*-AMPK) decreased at P24H in NA (p = 0.007) and increased at P0H in HY (p = 0.04) (Fig.3.5H). No interaction was observed between biopsy time and conditions (p > 0.05) (Fig.3.5).

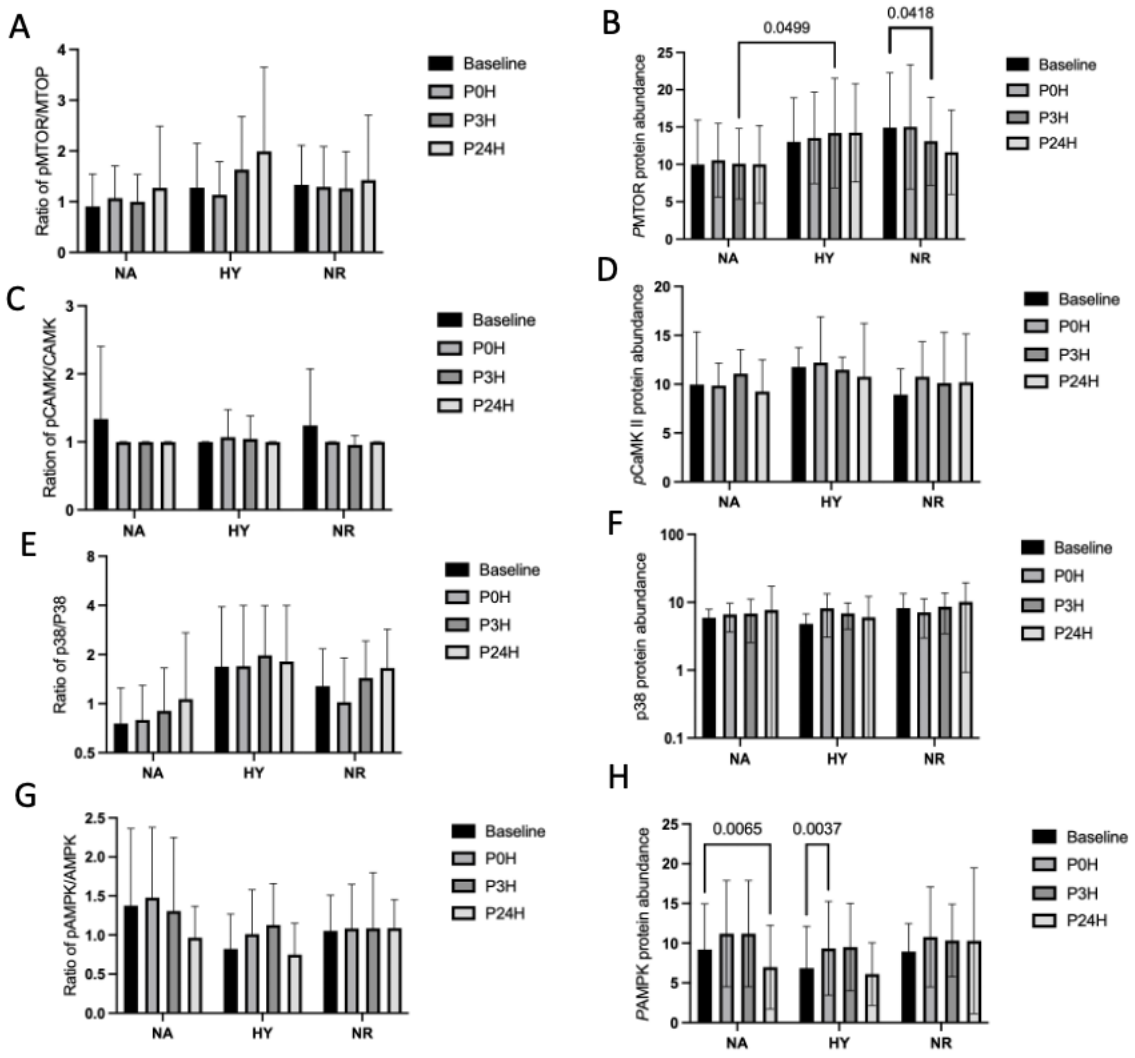


Figure 3.2 Muscle protein abundance responses to exercise. A, C, E, G: Ratio of phosphorylated protein/ total protein. B, D, F, H representative blots were quantified for *p*MTOR, *p*CAMK, and *p*P38, *p*AMPK, at the baseline (B), immediately post (P0H), 3h post-exercise (P3H) and 24h-post exercise (P24H) at each of conditions: normoxia absolute intensity (NA), hypoxia (HY) and normoxia relative intensity (NR). Blot densitometry was normalized to stain-free total protein and quantified relative to standard curves generated on each membrane (not shown). Mean (horizontal bars) ± standard deviation (SD) is plotted for each graph.

The protein abundance of PGC-1 α increased at P0H when compared with baseline for NR (p < 0.01) (Fig. 3.6A). None of the other time points showed significant difference (Fig. 3.6A). No differences in fold changes were observed in PGC-1 α between three conditions in any time

point. Both VEGF and Estrogen-Related Receptor (ERR) protein remained unchanged (Fig.3.6B, C). There was no interaction between biopsy time and exercise conditions for VEGF or ERR.

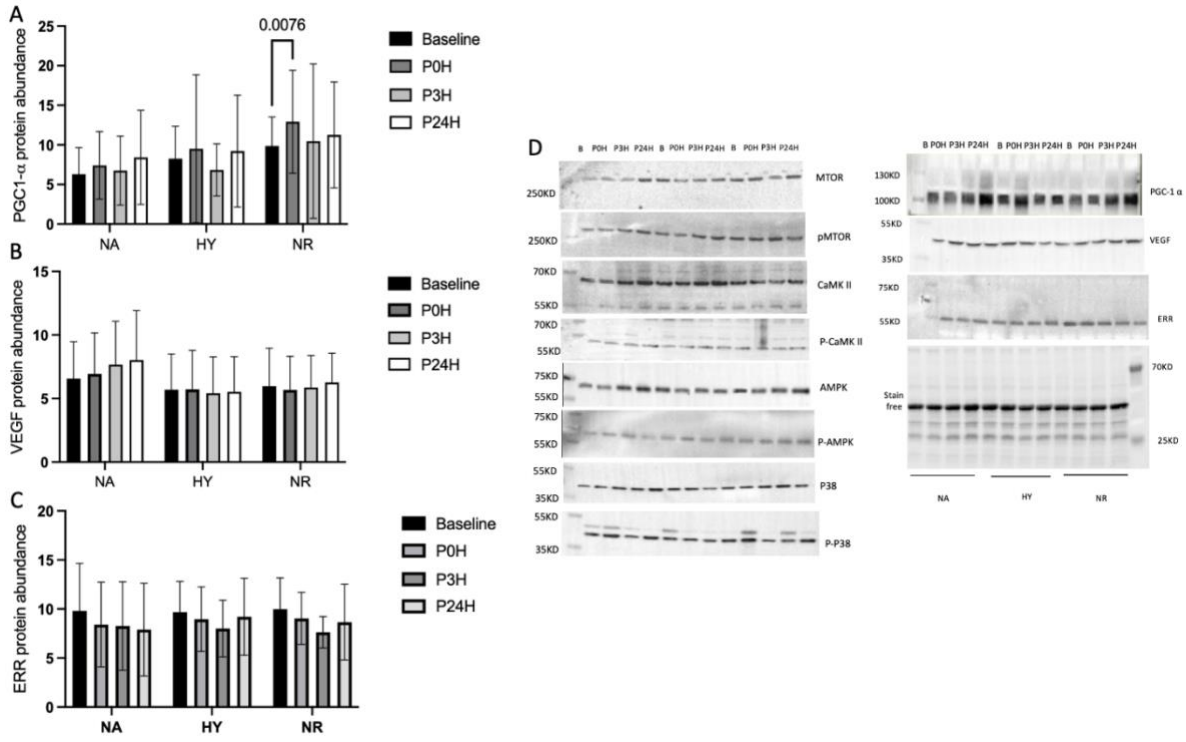


Figure 3.3 Muscle protein abundance responses to exercise. A-C: blots were quantified for PGC-1α, VEGF and ERR at the baseline (B), immediately post (P0H), 3h post-exercise (P3H) and 24h post-exercise (P24H) at each of conditions: normoxia absolute intensity (NA), hypoxia (HY) and normoxia relative intensity (NR). Blot densitometry was normalized to stain-free total protein and quantified relative to standard curves generated on each membrane (not shown). Mean (horizontal bars) ± standard deviation (SD) is plotted for each graph. D: Representative protein blots of protein expression measured at baseline (B) post exercise immediately (P0H), 3h post exercise (P3H) and 24h post exercise (P24H) in three conditions (NA, HY, NR). MTOR, Mammalian target of rapamycin; CaMKII, Ca^{2+} /calmodulin-dependent protein kinase isoform II; p38, p38 MAPK; AMPK, AMP-activated protein kinase; PGC-1 α, peroxisome proliferator-activated receptor- α coactivator-1 α; VEGF, Vascular endothelial growth factor; Estrogen-related receptor alpha, ERR.

3.4 *Discussion*

To investigate the effect of HIIE in hypoxia on mitochondrial adaption, especially mitochondrial biogenesis, the current study included ten young healthy men who completed three HIIE sessions in normoxia and hypoxia. This design allowed for a direct comparison of

skeletal muscle mitochondrial response to a single HIIE session in HY with HIIE in normoxia with relatively matched intensity (NR), as well as that of HY with HIIE in normoxia with absolutely matched intensity (NA). The major finding in the current study was that changes in PGC-1 α mRNA expression in response to HIIE are more dependent on the relative exercise intensity rather than absolute exercise intensity or hypoxia. In addition, PGC-1 α protein was only different immediately after HIIE in NR.

Mass-specific mitochondrial respiration

Both mitochondrial respiration and content remained unchanged in all conditions. Several studies have investigated exercise-induced changes in mitochondrial respiration in permeabilised human muscle fibres using the same technique as this current study [68-70, 72, 73, 75, 76, 99, 106]. However, very few of these studies checked mitochondrial respiration after a single exercise session. Trewin, A. J., et al. reported no significant changes in mitochondrial respiration after one session of HIIE in normoxia (5 × 4 min, 75% PPO), low-volume sprint interval exercise (4 × 30 s), or continuous moderate-intensity (30 m, 50% PPO) [76]. In the current study, the exercise intensity was 78.2 ± 2.4% of PPO in NA and 87.4 ± 1.7% of the PPO in NR [266], and similar results were observed as reported byTrewin, A.J. et al., with no significant differences in mitochondrial respiration. On the other hand, Layec, G., et al. suggested that acute high-intensity aerobic exercise might transiently impair mitochondrial respiratory capacity in skeletal muscle [78], assuming that it would be restored within several hours. Contrastingly, an increase in mitochondrial respiration was reported following a single session of moderate-intensity exercise (1 h cycling at 65% peak aerobic capacity), but the exercise session was longer [70] and involved initially sedentary females and males (n=11F/4M). The differences in gender composition and different baseline training status may contribute to these discrepancies. Notably, these results were observed under the assumption that hypoxia does not seem to affect mitochondrial function [267]. The current study may be the first one to report available data on mitochondrial respiration after a single HIIE session in hypoxia. Furthermore, most studies only assessed mitochondrial respiration before and after exercise, while the current study included additional time-points, including baseline, P3H and P24H which gave more information on mitochondrial bioenergetics, such as rates of ATP synthesis via oxidative phosphorylation, in response to HIIE.

Mitochondrial qualitative and quantitative changes

I then measured CS activity as the marker of mitochondrial content [30]. In this study, none of the exercise interventions (NA, NR, HY) altered CS activity at any time point. A single HIIE session can activate the pathways responsible for mitochondrial biogenesis, which is expected to occur in a dose-response manner, requiring a succession of exercise sessions to increase mitochondrial content [268, 269]. To better characterize mitochondrial respiratory function [261], I normalized mass-specific respiration with CS activity. Specific changes in OXPHOS flux did not show significant diffrences in three conditions at any time point. I am aware of eight studies that measured both mitochondrial respiration and CS activity after training in hypoxia. Among these, three reported increased CS activity [81, 83, 84], while five observed no changes [80, 82, 85, 89, 103]. It is worth noting that the three studies reporting increased CS activity involved hypoxia training over 4 to 6 weeks, rather than a single session of exercise in hypoxia. Consistent with the previous studies, the current study reflected that a single session of HIIE, regardless of hypoxia and/or intensity, is not enough to induce changes in mitochondrial content or function.

Genes and protein expression related to mitochondrial biogenesis and metabolism

Despite no changes in mitochondrial respiration and content, several crucial events related to mitochondrial biogenesis and metabolism, including protein phosphorylation, gene expression and protein expression of oxygen-sensitive targets, were observed immediately or three hours post-exercise in both HY and NR, with minimal changes in NA. When combined, hypoxia and exercise training have been shown to have a greater capacity for improving muscle oxygen delivery and metabolism than exercise alone [164]. Hypoxia can induce mitochondrial biogenesis and increase cellular O_2 consumption, leading to a lower tissue partial pressure of oxygen (PO_2), which in turn stabilises and activates HIF-1α [87]. This transcription factor primarily activates the transcription of genes coding for glycolytic enzymes and angiogenic factors [270], but has limited effects on mitochondrial proteins [51] related to mitochondrial function. Since VEGF is the downstream target of HIF-1α [144, 271, 272], increased mRNAs levels in HIF-1α and VEGF were found after HIIE in both HY and NR, but not in NA. However, there were no significant differences for HIF-1α and VEGF mRNA content between HY and NR. Our observation is consistent with previous finding that HIF-1 α mRNA expression can be induced by hypoxia and further increased by exercise [92, 166], as well as VEGF mRNA expression [166, 167]. In human skeletal muscle, 45 minutes of one-legged knee-extension exercise (~26% of one-legged peak load) for 45 min increased VEGF mRNA levels, which

were further increased in hypoxia (induced via restricted blood flow by ~15–20%) [166]. When the oxygen supply is insufficient, HIF-1 α target genes enhance oxygen transport by EPO-mediated erythropoiesis and VEGF-induced angiogenesis mechanisms and mediate skeletal muscle adaptations to exercise through optimised glucose transport and glycolytic enzyme activity [166]. This may in turn explain the higher PDK4 and Solute Carrier Family 27 Member 4 (SLC27A4) mRNA expression level in HY than NR in this study.

PGC-1α is a transcription coactivator [273] that controls the genes related to energy metabolism [274], as well as mitochondrial biogenesis [275-277] [246, 278]. Previous data in human skeletal muscle demonstrated that a single session of HIIE (8 × 2-min intervals at ~85% of PPO) increased the PGC-1 α mRNA expression 3 h after a single session of HIIE (5 × 4-min intervals at ~80% of PPO) [118]. In the current study, PGC-1α gene expression increased at P3H for HY and NR, but not for NA. Exercise intensity appears to play a crucial role in inducing the expression of PGC-1 α. It is worth noting that a single session of HIIE, with or without hypoxia, does not seem to have an effect on mitochondrial metabolism related genes such as Cytochrome c Oxidase Subunit 4 (Cox4) and Citrate Synthase (CS). It is likely that HIF-1 α in hypoxia primarily induces glycolytic enzymes and angiogenic factors, while not signalling mitochondrial metabolism.

Tumor Protein 53 (p53) can upregulate mitochondrial biogenesis by improving the oxidative capacity of skeletal muscle [279, 280], and modulating aerobic respiration as a direct transcriptional target [281]. A previous study reported that a single session of acute resistance exercise (10 sets of six repetitions, each repetition lasting 3 s) is sufficient to initiate transcriptional signalling for mitochondrial biogenesis with the assistance of p53 [282]. However, it is important to note that the mentioned exercise protocol is not cardiovascular exercise but rather resistance exercise. Additionally, p53 can also regulate the cellular responses to hypoxic stress. In another study involving hypoxia treatment (1% O_2), primary myoblasts were placed in gas-tight modular incubator chambers that were flushed with a custom gas mixture containing 5% air, 5% CO_2, and 90% N_2, 30 p.s.i./min, for 2.5 min each day, two days for total. This study found that hypoxia upregulated 641 genes involved in the cell cycle and metabolism (HIF1-α and glycolysis). Of the specific upregulated genes, p53 was enhanced by hypoxia [283]. In the current study, we observed changes in p53 gene expression in HY, but not in NR or NA.

As a member of the mitochondrial transporter superfamily, UCP3 stands for Uncoupling Protein 3 (UCP3) and plays a role in altering metabolic function under conditions of energy depletion caused by exercise [284]. During acute exercise, there is a need for tight metabolic coupling, which leads to an increase in UCP3 mRNA expression to meet the short-term regulation of energy expenditure [285]. On the other hand, endurance training decreases UCP3 mRNA to enhance metabolic efficiency in skeletal muscles, thereby reducing energy dissipation in these tissues [286]. It has been reported that an acute bout of exercise elevates blood free fatty acid levels and caused an increase in UCP3 gene expression [287]. In an animal study, rats were requested to perform an acute bout of treadmill running (at 24 m/min for ≤ 200 min without rest) or swimming (same as treadmill protocol) in hypoxia (90% N_2-10% O_2 (Medical-Technical Gas) for ≤ 200 min) by rats. Increases in skeletal muscle UCP-3 mRNA expression were observed in all conditions, with exercise and exercise/hypoxia-induced mRNA expression showing both rapid and substantial changes [284]. In our study, I found the same increase in both exercise and hypoxia conditions; however, compared with HY, UCP3 mRNA maintained high expression at 24HP in NR.

In the current study, PGC-1α protein increased immediately post-HIIE in NR compared with baseline. Previous human exercise training studies have reported higher PGC-1α mRNA content was found 0 - 3h after acute HIIE [116, 288-291], while increased PGC-1α protein content has been mainly observed during prolonged exercise sessions lasting 3-12 weeks [114, 118, 292]. This result is consistent with our previous data that a single session of HIIE (8 × 2-minute (LT + 40% × [W_{peak} – LT]) with a 1-minute interval at 60 W) increased the PGC-1α protein content related to mitochondrial biogenesis [116]. Several acute exercise studies have reported increases in both PGC-1α mRNA and protein content in skeletal muscle [113, 293, 294] highlighting their involvement in multiple signalling pathways controlling mitochondrial biogenesis. First, I examined exercise-induced mitochondrial response (Ca^{2+}-dependent signalling) [295]. Calmodulin-dependent protein kinase isoform II (CaMKII) acts as an upstream kinase in exercise-induced regulation of PGC-1α by regulating p38 Mitogen-Activated Protein Kinase (p38 MAPK) and AMPK activation [295]. Recent evidence suggests that p38 MAPK is downstream of CaMKII in a signalling pathway where increased cytosolic calcium leads to an increase in PGC-1 α, and inhibition of p38 MAPK prevents the calcium-induced increase in mitochondrial biogenesis [296]. However, in the current study, I did not find changes in either CaMKII or p38 protein. Additionally, I investigated hypoxia- induced

mitochondrial response through AMPK/mTOR signalling. AMPK serves as an important sensor of decreased energy charge in cells, inhibiting ATP synthesis or accelerating ATP consumption [297]. In the current study, I observed that the gene changes enriched in the AMPK/mTOR signalling pathway, including PGC-1 α and PPAR α, suggesting the involvement of AMPK/mTOR signalling in gene responses. Chen et al. have suggested that the hypoxia-induced increase in PPARα and PGC-1α is AMPK dependent [298]. In our study, phosphorylated AMPK increased immediately post HIIE in HY. The upregulated phosphorylated AMPK may have induced the increase in PGC-1α gene expression, subsequently leading to increased PGC-1α protein levels. Interestingly, PGC-1α proteins upregulation was observed only in NR and not in HY, which aligns with a previous observation suggesting that the induction of PGC-1α by hypoxia appears be independent of HIF-1 activity [273].

In most pathways involving mitochondrial biogenesis-related genes, mitochondrial respiration regulators, glycolytic enzyme and fatty-acid-related genes, there is no evidence to suggest that HY has additional effects on mitochondrial adaptions compared to NR. However, notable differences have been observed between NA and the other two conditions. This leads us to speculate that the divergent results observed in HY could be attributed to the intensity of the exercise rather than hypoxia alone. It is commonly perceived that exercising at the same absolute intensity is more challenging in hypoxia than in normoxia [299]. Keeping this in mind, Mackenzie et al argued that the changes observed in hypoxia studies might be due to an increase in the relative intensity of exercise, as the same absolute intensity was chosen for both the normoxic and hypoxic groups [257]. A study by Brocherie, F., et al. used absolute intensity (four sets of 5 × 5-s maximal sprints interspersed with 25 s recover) in both hypoxia (live in FiO_2 14.5–14.2%, train in FiO_2~14.2%) and normoxia group (live in FiO_2 14.5–14.2%, train in FiO_2 20.9%) group. The authors found that HIF-1 α, VEGF and PGC-1α mRNA levels increased at Post-1 in hypoxia (all $p \leq 0.05$) but not in normoxia [300]. This suggests that repeated maximal-intensity hypoxic exercise induces larger short-term post-intervention beneficial changes in muscle transcriptional factors compared to similar normoxic exercise. Previous observations have revealed that a single session of high-intensity exercise initiates rapid cellular signals, leading to the subsequent activation of proteins that increase gene transcription [8].

3.5 *Conclusion and future directions*

I observed comparable increases in gene expression of targets related to mitochondrial biogenesis and other pathways following HIIE in hypoxia and normoxia, with relatively matched exercise intensity compared to normoxia with absolutely matched (lower) intensity. This suggests that the relative exercise intensity is the main factor for the induction of gene expression. However, no changes were observed at the protein level or in mitochondrial function or content after a single HIIE session in normoxia or hypoxia.

One limitation of the current study was that I only implemented an acute study, which might be insufficient to induce changes in mitochondrial respiration and content. However, it has been suggested that the changes in gene expression following acute exercise could be sufficient to predict changes in protein levels after the chronic training period [301, 302]. The observed changes in the expression of genes related to mitochondrial biogenesis added some evidence to the expected adaptations in chronic training studies. Future studies should confirm the changes in mitochondrial function and biogenesis with chronic training in hypoxia.

Chapter 4

The effect of a single HIIE session in hypoxia on the gene expression of skeletal muscle and blood inflammatory markers

Findings in previous chapters have indicated the significance of exercise intensity, rather than hypoxia, in inducing mitochondrial biogenesis. Mitochondria are known to be associated with inflammation and are involved in immune system activation, thereby playing a role in the pathogenesis of inflammatory diseases. However, the specific effects of HIIE in hypoxia on inflammatory signaling in human skeletal muscle, as well as its impact on the inflammatory response in the blood, remain incompletely understood. The objective of this chapter is to investigate the influence of a single HIIE session in hypoxia on the expression of inflammatory genes in human skeletal muscle and in peripheral blood, when compared with HIIE sessions in normoxia. To achieve this, RNA-Seq analysis was employed to identify broad patterns in the expression of inflammatory genes in human skeletal muscle, while RT-PCR was utilized to measure the expression of inflammatory genes in both skeletal muscle and peripheral blood samples. Recognizing the importance of inflammation mechanisms, which are as vital as mitochondrial biogenesis, this chapter aims to provide a more comprehensive understanding of the physiological response to HIIE in hypoxia. By studying both aspects, a deeper insight into the overall adaptation to this type of exercise can be achieved, contributing to the broader understanding of the intricate interplay between exercise, inflammation, and mitochondrial function.

This chapter is preparing as the 4th manuscript for this study and in the final stages of preparation: The effect of a single HIIE session in hypoxia on the gene expression of inflammatory markers in skeletal muscle and blood

4.1 Introduction

Hypoxia and inflammation have been long known as tightly interconnected [143, 185]. The relationship between inflammation and hypoxia has been well documented in studies focusing on the hypoxia signalling pathway [303-306]. Hypoxia is able to trigger a range of rapid physiological adaptations for the maintenance of oxygen homeostasis and increaseing tissue oxygen delivery, such as increasing vasodilation [307], increasing blood flow [308] and producing more red blood cells [309, 310]. On the one hand, when the human body encounters oxygen stress, which initiates tissue protection and repair mechanisms via an inflammatory response [311]. For this reason, hypoxia signalling is potential therapeutic target for the treatment of inflammatory diseases [304]. On the other hand, just as hypoxia can induce inflammation, tissues with inflammation often become hypoxic [143, 312]. For example, levels of circulating pro-inflammatory cytokines increase causing pulmonary edema [304].

Exercise also has long been recognised to have anti-inflammatory effects [172-174]. Regular exercise is a benefit on controlling chronic metabolic and cardiorespiratory diseases, partly in the reason of the anti-inflammatory response occurred upon exercise [172]. And the anti-inflammatory effects of regular exercise may be mediated via the induction of an anti-inflammatory environment with each bout of exercise [172]. Recent findings suggested that an acute bout of exercise [313, 314] induces pro and anti-inflammatory cytokines in the blood [315-317], but regular physical exercise attenuates the inflammatory response promoting anti-inflammation activity [318, 319]. Only a handful of studies have examined the effects of exercise in hypoxia on inflammation. The hypoxic conditions varied across the studies, ranging from 2800 m (FiO_2 of 14.65%) [195] to 4200 m (FiO_2 of 12%) [194] and different exercise intensities were used, including 40%, 60% [195], 70% [194, 320] and 95% $\dot{V}O_{2max}$ [194, 197]. Three of the four studies showed that inflammation marker TNF-α and/or IL-6 stayed unchanged in the blood in both normoxia and hypoxia [194, 195, 197]. Only one study found an increase of TNF-α and IL-6 after 60 min exercise session at 70% of the $\dot{V}O_{2max}$ in normoxia and hypoxia (4200 m, FiO_2 of 12%) [320].

The above-mentioned studies all used qRT-PCR as the main analysis method, however, only a limited number of genes can be analysed at the same time through qRT-PCR. Whole-transcriptome analysis using total RNA sequencing (RNA-Seq) enablesaccurate measurement of transcript abundance. It also allows for the identification of both known and unknown

features of the transcriptome simultaneously [321]. Some exercise studies in normoxia used RNA-Seq technique to measure the enrichment of genes [322] [323]. In an acute hypoxia study without exercise, RNA-Seq was utilized to measure mRNA levels of 250 key inflammation-related genes, and identified the top 20 differentially expressed genes associated with high-altitude exposure (3800m). Only one study utilized whole blood RNA-Seq to assess the immunological and metabolic effects of high-altitude adaptation by combining exercise (1h skating bout) with hypoxia (1850m) [324].

Peripheral blood is an accessible and informative source of sample and has lots of advantages — such as well-established collection, storage, and transportation protocols, relatively minor effects on the subject's well-being, and the presence of many valuable biomarkers [325]. Blood also represents a very diverse mixture of cells [324, 325]. Gene expression profiling of RNA extracted from peripheral blood give access to identify biomarkers, examine disease states, and investigate immune response [325]. Controversially, measurements of plasma cytokine concentrations are also considered a difficult and indirect method due to the low concentrations and rapid clearances from the blood [326]. Furthermore, plasma concentrations are tissue nonspecific and may not reflect the accurate changes in skeletal muscles [325, 326]. To date, there is little known published studies that have documented the cytokine response to exercise directly in both muscle and blood.

In the current study I used RNA-Seq to identify broad patterns in inflammatory gene expression in skeletal muscle, and further compared commonly accepted inflammation markers, such as muscle TNF-α and IL-6 measured by RNA-Seq with the same targets measured by RT- PCR. The gene expression of inflammatory marks in peripheral blood samples was also assessed by RT-PCR. The current study tested the hypothesis that acute HIIE in hypoxia increases cytokine markers of inflammation in blood and muscle. It is expected to reveal the interplay of inflammatory signaling between muscle and blood after HIIE in hypoxia.

4.2 Method

4.2.1 Experimental methods

Please refer to Chapter 2 for the experimental design.

Blood sample collection

Blood samples were collected at the same time points as the muscle biopsies including resting, immediately after, 3h after and 24h after (Fig. 4.1). Samples were collected by a highly experienced phlebotomist using an indwelling venous catheter inserted in an antecubital vein. The entire operating procedure was conducted by current guidelines for blood samples in the Exercise Physiology Laboratory, Institute for Health and Sport, VU. Plasma and serum were separated from the samples and put into liquid Nitrogen immediately, then stored at -80ºC for further analysis. Approximately 3 ml of blood was dispensed directly into the Tempus RNA blood tube. The tubes were shaken vigorously for 10 s to homogenize the blood with the stabilizing agent (6 ml of a guanidine hydrochloride solution), incubated overnight at −20 °C freezer, and then shipped into −80 °C freezer for further analysis [327].

RNA extraction from human muscle and blood

RNA extraction from muscle homogenise tissue has been described in chapter 3.
Blood RNA was extracted via TempusTM Blood RNA Tube and TempusTM Spin RNA Isolation Kit (Cat. No. 4342792 and Cat. No. 4380204). It begins with combining the thawed Tempus tube sample with 3 ml 1× phosphate buffered serum (PBS) in a 50 ml conical tube, vertexing for 30 s, and centrifuged at 3000×*g* at 4 °C for 30 min. This allowed the RNA to pellet on the bottom of the conical tube. Followed by 400 μL of RNA Purification Resuspension Solution into the conical tube, then vortex briefly to resuspend the RNA pellet. 400 μL of the resuspended RNA were transferred into the purification filter, then centrifuge for 30 s at 16,000 × *g*, repeated this step three times for purification. Transfer the purification filter to a new, labelled collection tube to collect the eluate, washed with 100 μL Nucleic Acid Purification Elution Solution and incubate for 2 min at 70°C, then centrifuge for 30 seconds at 16,000 × *g*. 100 μL of the collected RNA eluate were moved back into the purification filter, then centrifuged for 2 min at 16,000–18,000 × *g*. The RNA collection was shipped to −80°C for long-term storage.

RNA quality assessment

The quality and quantity of extracted RNA from muscle samples were evaluated before sequencing, a summary of RNA properties was provided in Table 4.1. RNA concentration and purity were assessed by Nanodrop One (Thermofisher). RNA integrity was tested using the Agilent RNA ScreenTape (Agilent Technologies, USA). The 5 μL RNA sample buffer was mixed with 1 μL RNA for 1 min and then spun down for 1min. The sample was heated at 72ºC for 3 min and placed on the ice for 2 min before being loaded samples into TapeStation instrument. The RNA Integrity Number (RIN) value was 7 or higher for all the samples.

Table 4.1 RNA assessment

	RNA Concentration (ng/μl)	260/280	260/230	RIN
Mean	74.6	1.9	0.7	8.5
SD	14.9	0.1	0.2	0.6
Upper range	133.6	2.0	1.3	9.2
Lower range	38.3	1.2	0.1	3.3

4.2.2 RNA sequencing and bioinfromatic analyses

RNA sequencing

RNA sequencing includes sample preparation, library construction, library quality control and sequencing sections, an overview of a typical workflow is outlined in Figure 4.1. 120 high-quality RNA samples (10 subjects, four time points including baseline, immediately post, 3h-post and 24h-post HIIE in three conditions NA, HY and NR), 1 μg for each sample, were prepared using the NEBNext UltraTM RNA Library Prep Kit for Illumina NEB, USA. According to the manufacturer's protocols, with the following adjustments: 0.8× beads were used during the first purification step after second strand synthesis; the adaptor was diluted 1:15; 0.7× beads were used for purification after adaptor ligand; 13 cycles of enrichment were conducted; and a dual bead size selection (0.5× and 0.7×) was used for size selection of adaptor-ligated RNA. Samples were then pooled and checked for quality via qPCR and Agilent 2100 Bioanalyzer.

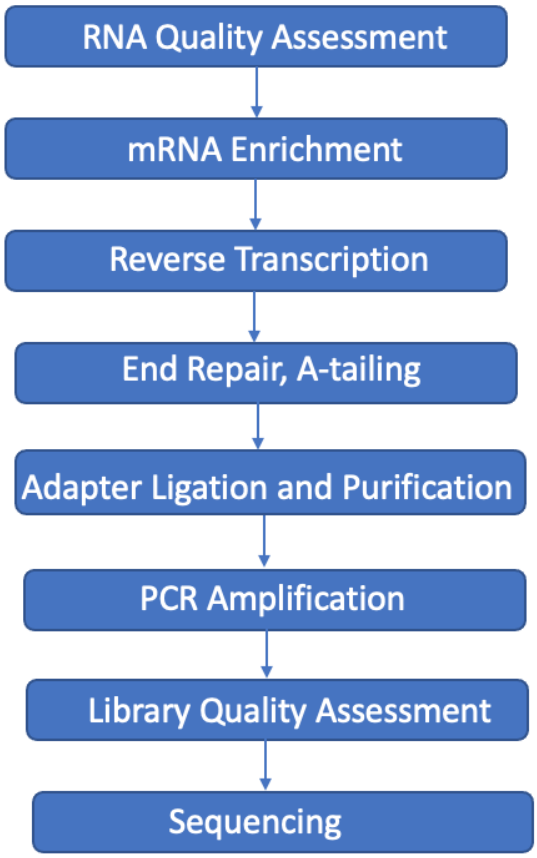


Figure 4.1 RNA-sequencing workflow (According to the manufacturer's report BMK220628-AY185-ZX01-0101)

A systematic bioinformatics pipeline is shown below (Figure 4.2). The cDNA library underwent sequencing using Illumina's high-throughput sequencing platform (Illumina novaseq 6000) which generated highly qualified raw data with Q30 quality scores exceeding the required threshold. These raw data, provided in FASTQ format with end-paired sequencing, were then processed by Perl scripts. This step involved the removal of contaminated reads and nucleotides (N) with low quality-score. As a result, clean data were obtained, with each sample having Q30 bases percentage of no less than 91.49%. Subsequently, the clean data were aligned to the Homo_sapiens reference genome map obtained from the Ensembl database, specifically the GRCh38_release95.genome, using Hisat2 (Version 2.0.4) [328]. After reading alignment and quantification, the dataset was subjected to initial evaluation. From GSEA Human MSigDB v2022.1. database,we selected 200 inflammation-specific genes by searching for "inflammatory response" in the hallmark gene set. Paired contrasts were performed separately for the P0H, P3H and P24H at NA, NR *vs*. HY. Significantly differentially expressed were

determined based on a fold Change (FC) ≥1.5 and p-value < 0.05. To examine gene set enrichment, the differentially expressed genes were analyzed using KEGG (Kyoto Encyclopedia of Genes and Genomes) [329]. The top 10 enriched pathways, determined by the smallest q-value, were visualized using the ClusterProfiler (Version 3.10.1). Additionally, the data of the PCA function were visualized using the "FactoMineR" R package .

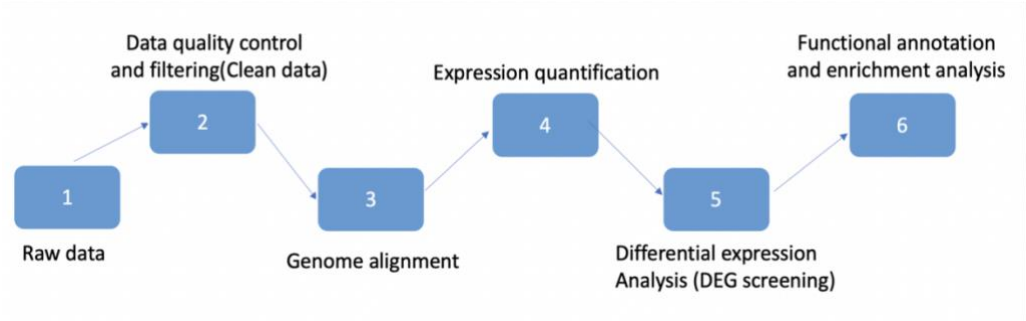


Figure 4.2 Bioinformatics pipeline (According to the manufacturer's report BMK220628-AY185-ZX01-0101)

Statistical analysis

Statistical analyses were performed using either R 4.2.2 (www.r-project.org) or GraphPad Prism 9.0 software (GraphPad Software Inc.). Comparisons were considered statistically significant at $p < 0.05$. Data are presented as box-and-whisker plots unless otherwise specified in figure legends. Normality was tested using the ANOVA and t test before applying appropriate parametric or nonparametric tests. Statistical tests are described in the figure legends. Bioinformatic analyses are described in their respective Materials and Methods sections.

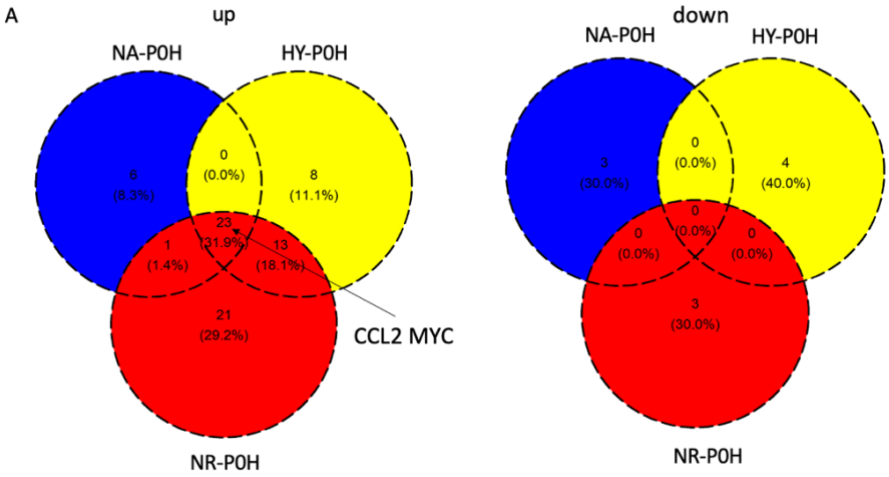
4.3 Results

RNA Sequencing data of muscle-specific inflammation signalling

Comparasion of muscle gene expression levels induced by exercise in different conditions

Overall, 160 up-regulated and 430 down-regulated genes were observed following the intervention of NA, HY and NR. I assessed the genes regulated by conditions at the same time points. Firstly, the results showed that NR exercise led to greatest number of genes at P0H when compared with NA and HY. (Fig. 4.3A). Among three conditions at P0H, 23 genes were upregulated, including two inflammation-related genes, Chemokine (C-C motif) Ligand 2

(CCL2) and MYC Proto-Oncogene (MYC). The number of downregulated genes was similar for NA, HY and NR (Fig. 4.3A) at P0H, without a common gene among the three conditions. There were more inflammation-related genes upregulated at P3H and P24H, including MYC, Cluster of Differentiation 14 (CD14) and pro-inflammatory cytokine Interleukin-1(IL1R1). Of note, there were more upregulated genes in HY than in NA and in NR at P3H (Fig. 4.3B). Out of the 110 upregulated genes among the three conditions at P3H, 15 genes are associated with inflammation. Similar to P0H, there was no common downregulated inflammatory gene at P3H. Interestingly, there were fewer downregulated genes in HY than in NA and in NR at P3H (Fig. 4.3B). Lastly, the number of differentialy expressed genes, including both upregulated and downregulated, is much higher in NA than in HY and NR at P24H (Fig. 4.3C). Among the 322 commonly upregulated genes at P24H from the three conditions, 13 genes are associated with inflammation. The only downregulated inflammation-related gene at P24H among the three conditions was ATP-binding cassette subfamily A member 1 (ABCA1). Through the use of principal components analysis (PCA) plot with the 27317 detected genes, we have assessed the difference among the samples collected at different time points from the three different groups: HY, NA, NR. It appears that HY exhibits slight differences from NA and NR (Fig. 4.3D).



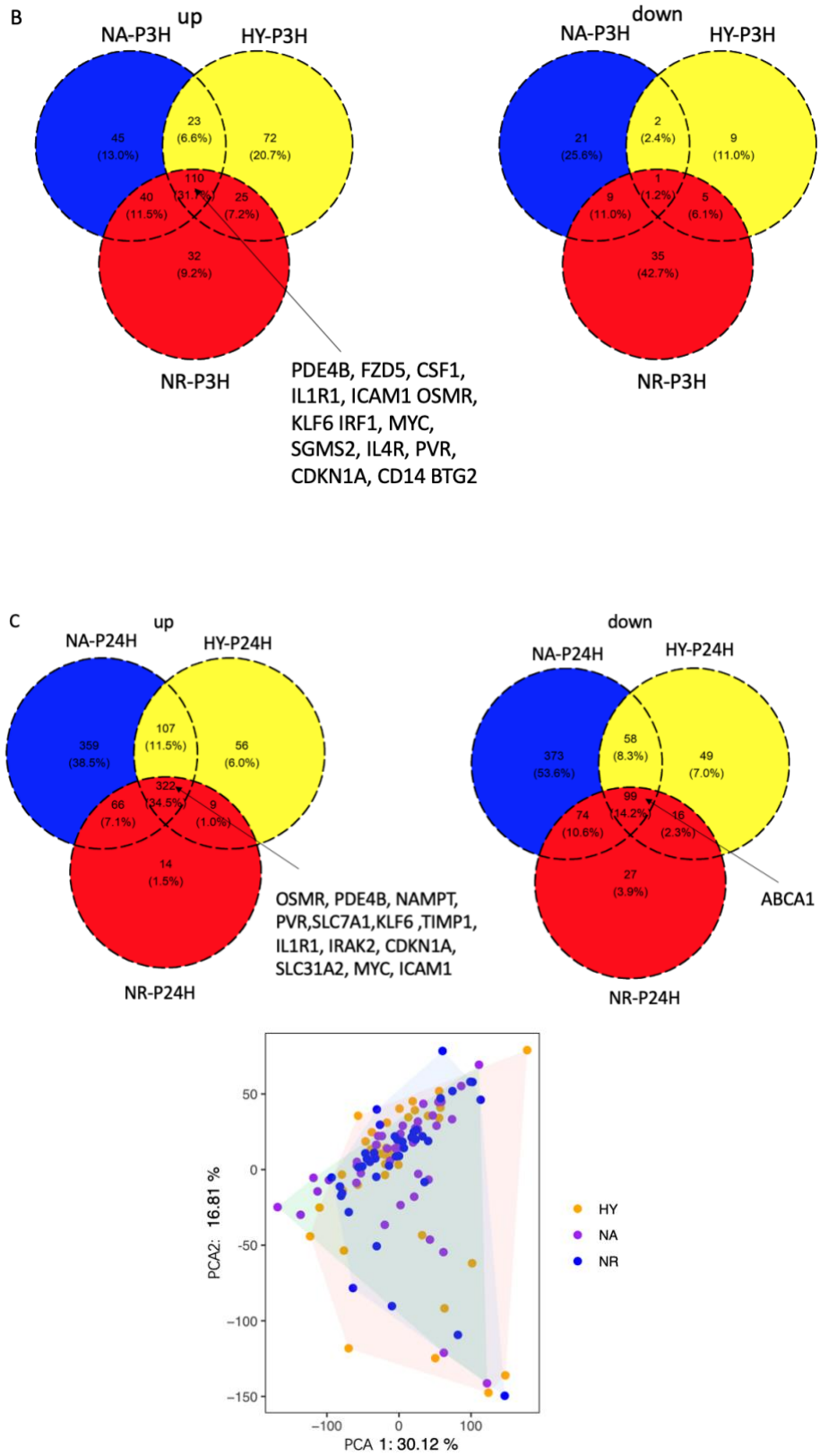
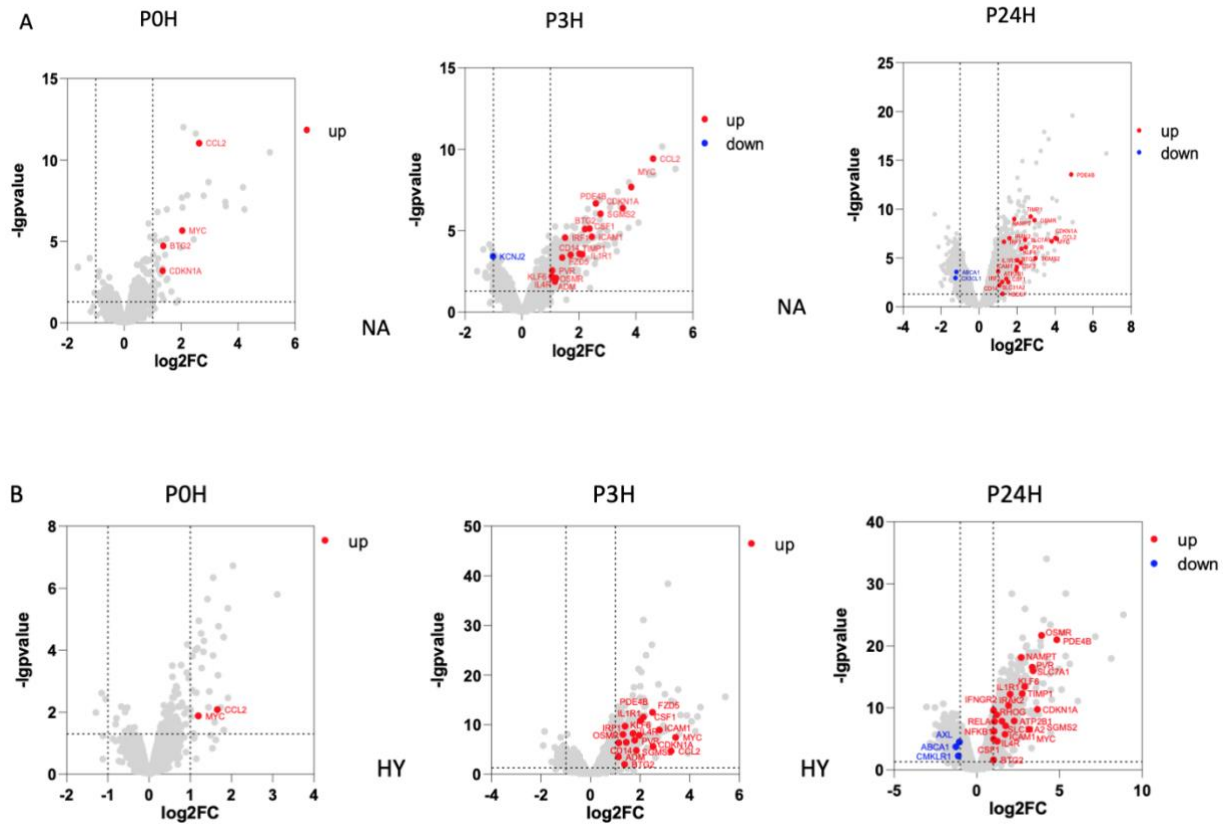


Figure 4.3 Venn diagram of differentially expressed genes identified by RNA-seq A: Proportion of analytes in each condition at P0H. B: Proportion of analytes in each condition at P3H. C: Proportion of analytes in each condition at P24H. Gene overlap indicates the number of genes in the leading edge. D: PCA plot of the skeletal muscle mRNA in response of the intervention of NA, HY and NR. Fold Change (FC) ⩾1.5 and p-value < 0.05 were considered to be significantly differentially expressed.

Significantly changed genes associated with inflammation

I then identified the significantly changed genes at different time points of the NA, HY and NR interventions. Significance was determined based on a fold Change (FC) ≥1.5 and p value < 0.05. In total, I observed 21 upregulated genes and 5 down-regulated genes (Fig.4.4). The volcano plot displayed an increase four inflammation-related genes at P0H, 17 upregulated at P3H, and 23 increased at P24H for NA (Fig.4.4 A). Similarly, two inflammation-related genes increased at P0H, 17 upregulated at P3H, and 22 increased at P24H for HY (Fig.4.4 B). For NR, four genes increased at P0H, 19 increased at P3H, and 15 increased at P24H for NR (Fig.4.4 C). The majority of differentially expressed genes exhibited modest changes in expression, with 18 up-regulated genes displaying a fold change greater than twofold. The number of differentially expressed genes was similar at the same time point between NA, HY and NR (Fig.4.4). However, mRNA showed a higher expression level at P24H in HY compared to all the other time points in NA, HY and NR.



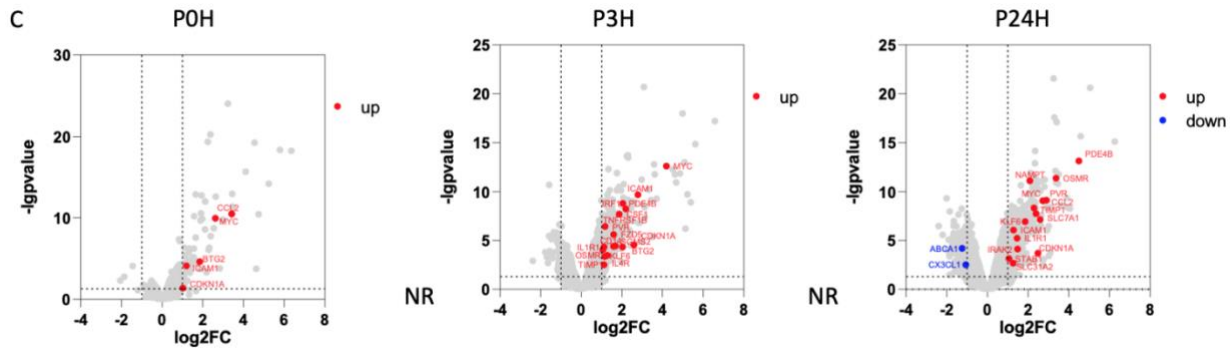


Figure 4.4 Up- and down-regulated skeletal muscle RNA-seq at three time points (P0H, P3H and P24H) after the intervention of NA, HY and NR. A: Volcano plot of differentially expressed genes at P0H, P3H and P24H in NA. B: Volcano plot of differentially expressed genes at P0H, P3H and P24H in HY. C: Volcano plot of differentially expressed genes at P0H, P3H and P24H in NR. Blue points represent significantly down differentially expressed genes-related inflammation and mitobiogenesis while red points represent significantly down differentially expressed genes in inflammation and mitobiogenesis. Values are plotted with raw log10 p-values on the y-axis, with dotted grey lines indicating adjusted log2FC thresholds.

A heatmap was generated based on that the data of the 23 most differently expressed inflammatory-related genes in NA, HY and NR (Fig.4.5). Overall, there was no difference in the transcript response related to inflammation between the three conditions, as indicated by the similar pattern observed in the heatmap. The genes that showed the most differential expression in NR remained consistently differentially expressed throughout NA and HY at P0H. The genes that exhibited the greatest differentially expression in NA and HY at P0H showed slower response compared to NR but increased steadily and remained at a high level at P3H and P24H. On the other hand, NR had most of the inflammatory-related genes upregulated at P0H, followed by downregulated at P3H, and subsequent upregulation to a siminlar high level as NA and HY. Genes with large changes were predominantly observed after HIIE in normoxia (NA or NR), but not in hypoxia. These genes include CCL2, Cyclin-Dependent Kinase Inhibitor 1A (CDKN1A), and Phosphodiesterase 4B (PDE4B). Interestingly, MYC appeared to reach its highest expression at P24H in HY.

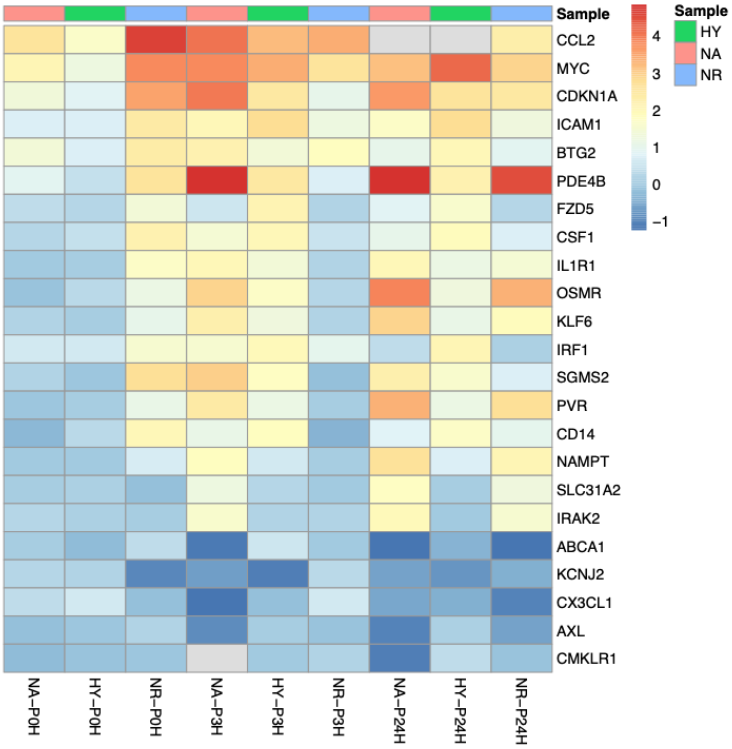


Figure 4.5 Heatmap of the logFC of genes significantly regulated by HIIE in NA, HY and NR at P0H, P3H and P24H. Heat maps with hierarchical clustering of the inflammation and mitobiogenesis-related genes.

The differential expression patterns in NA, HY and NR are visualised by Short Time-series Expression Miner (STEM), a sofeware program designed for the analysis of short time series microarray gene expression data [330]. In Figure 4.6. Profile 4 in each condition contained upregulated genes with a higher expression level. Genes in Profile 1 in each condition showed downregulation, while patterns 0, 2 and 3 exhibited fluctuating trajectories. It was observed that HY induced more genes compared with NA and NR.

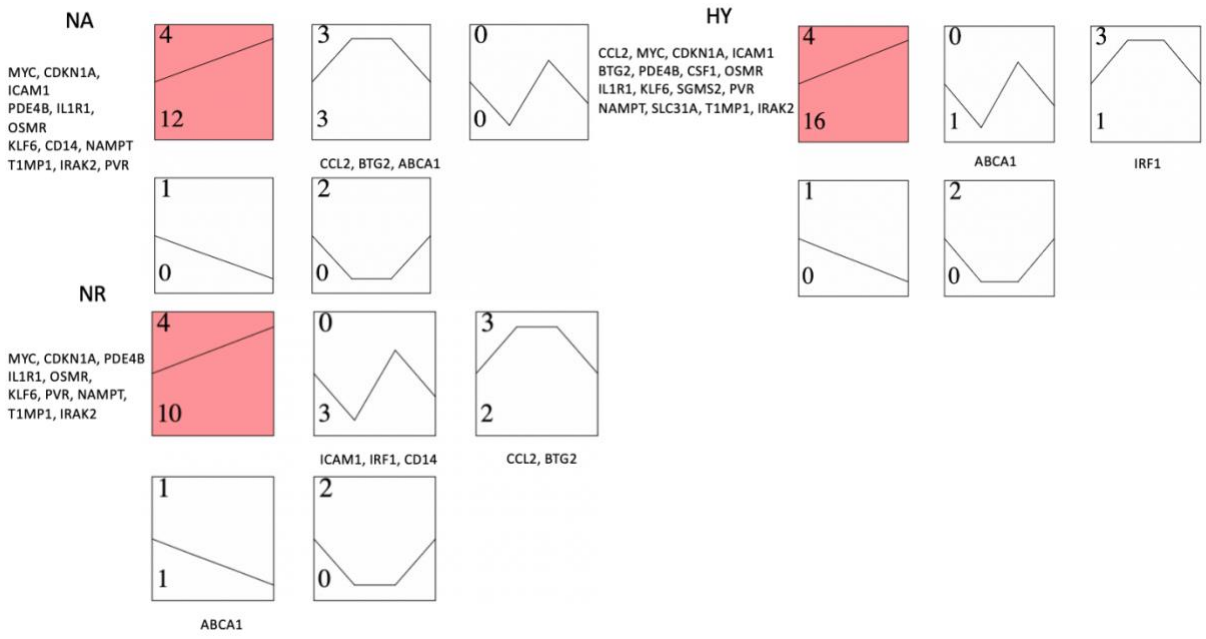


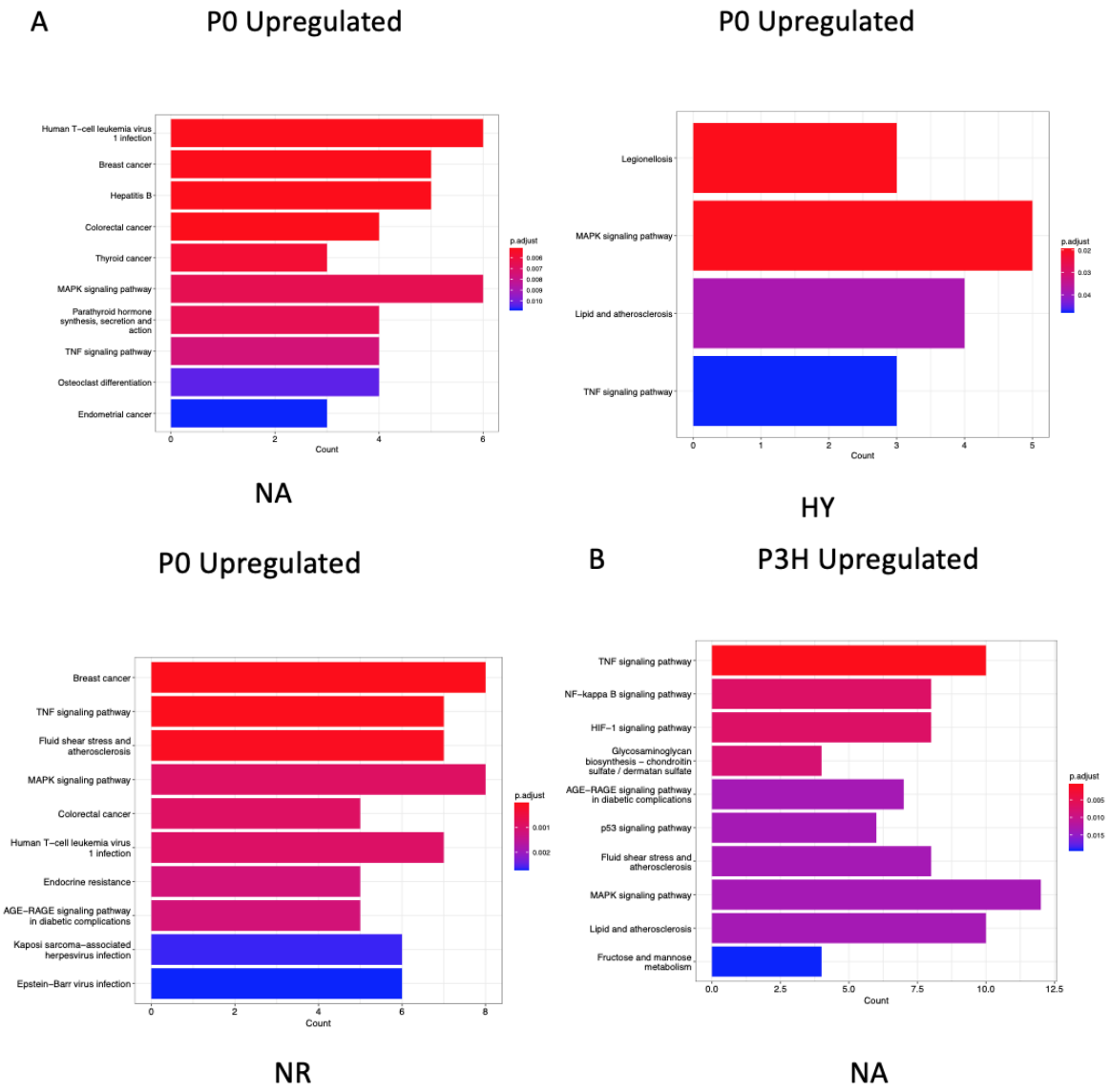
Figure 4.6 Profile plots showing differentially expressed genes in NA, HY and NR. The data was sampled at four time points (Baseline, P0H, P3H, P24H). The data set was filtered to contain 23 most differently expressed inflammatory-related genes. The number in the topleft-hand corner of a profile box is the profile ID number. The colored profiles had a statistically significant number of genes assigned. For each profile the number of inflammation-related genes assigned to it appears in the lower left corner of the profile box.

Pathway and Functional Category Analysis

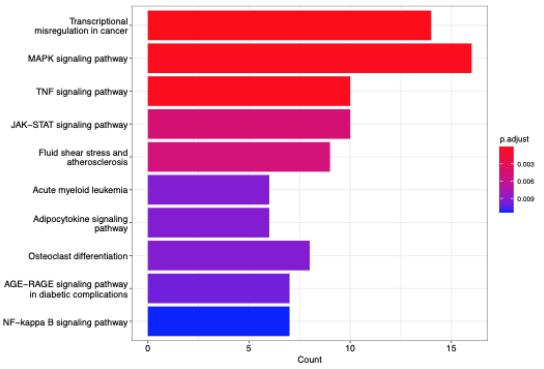
After conducting the initial assessment of differentially expressed genes, I further examined key genes of interest that play significant roles in the top 10 enriched KEGG pathways. Figure 4.7 illustrates the top 10 representative pathways ranked by significance ($p < 0.05$). The differentially expressed genes were found to be enriched for biological processes, including regulation of TNF signalling pathway, MAPK signalling pathway, NF-κB signalling pathway, HIF-1α signalling pathway, p53 signalling pathway, Human-T cell Leukemia virus 1 infection, Age-range signalling pathway, Apoptosis, JAK-STAT signalling and Lipid signalling ranked by significance ($p < 0.05$). Additionally, hypoxia-related genes were significantly upregulated in response to HIIE. The differentially expressed genes at P3H and P24H in three conditions showed enrichment in several inflammatory pathways, including nuclear factor-κB (NF-κB) and the apoptosis pathway.

Among the down-regulated genes (Fig. 4.7D), only one pathway showed significance at P3H in HY. The majority of the pathways enriched among the down-regulated genes were

associated with cancer and aging-range signalling. An interesting standout is the downregulation of the insulin resistance pathway, observed only at P24H in NA only.

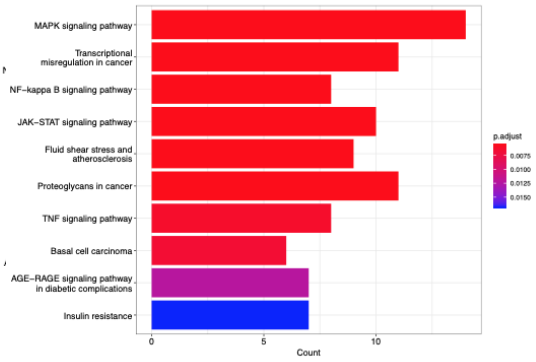


P3H Upregulated



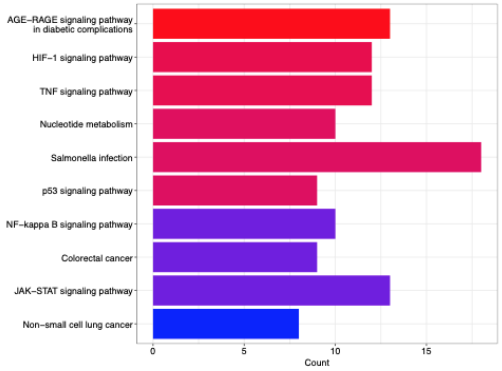
HY

P3H Upregulated



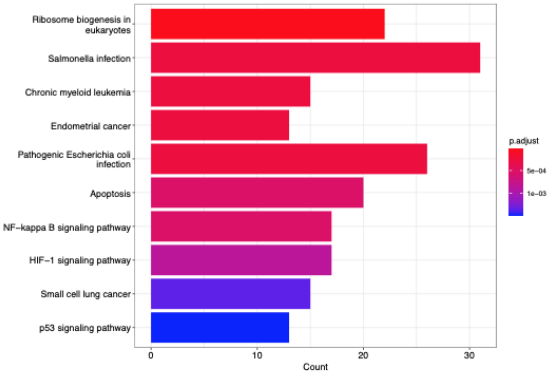
NR

C P24H Upregulated



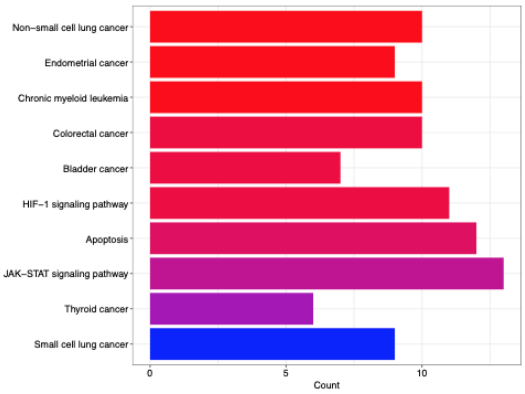
NA

P24H Upregulated



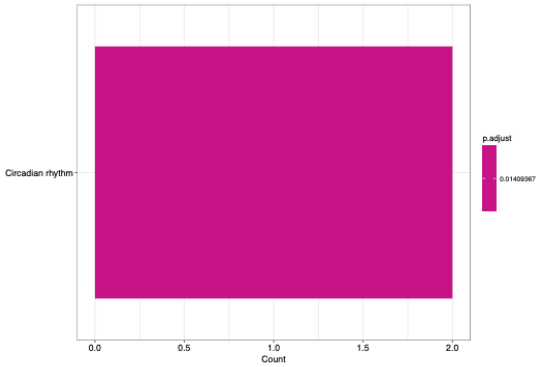
HY

P24H Upregulated



NR

D P3H downregulated



HY

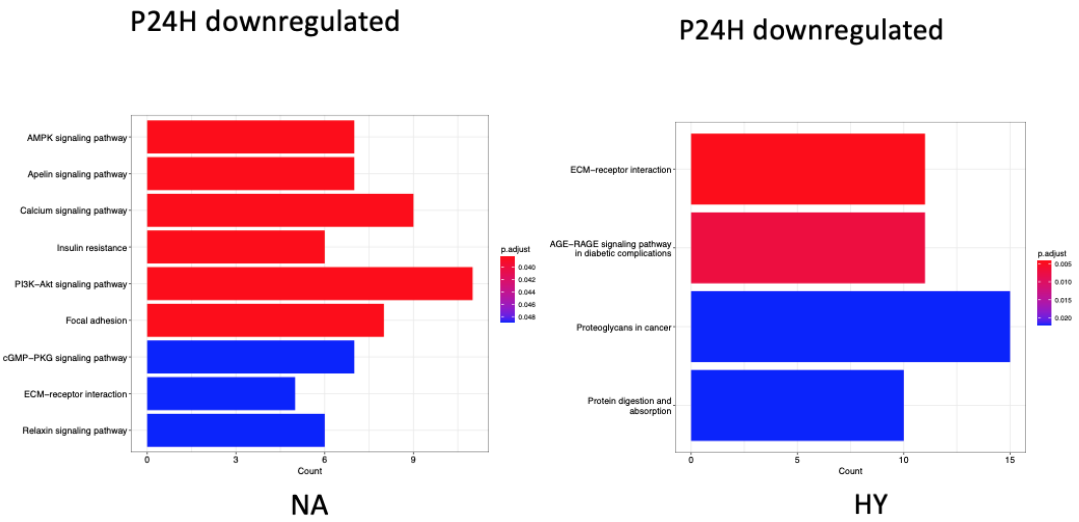


Figure 4.7 Gene overrepresentation and KEGG analysis of the differentially expressed genes. Molecular signature database (MsigDB) H and CP collections were used to functionally characterize expression genes. The letter following the pathway name denotes its source. A: Top 10 significantly up- and down regulated pathways in NA, HY and NR at P0H B: Top 10 significantly up- and down-regulated pathways in NA, HY and NR at P3H C: Top 10 significantly up- and down-regulated pathways in NA, HY and NR at P24H D: Significantly down-regulated pathways in HY at P3H, and in HY and NR at P24H. Down-regulated pathway in the other time points and conditions did not show statistical difference. The differentially expressed genes ($p < 0.05$ and a fold change threshold of one) were used for KEGG enrichment. After the KEGG analysis, we examined key genes of interest that play significant roles in the top 10 enriched KEGG pathways.

Expression of inflammatory genes in muscle measured by qRT-PCR

I performed qRT-PCR to validate the expression of some genes in muscle and compared it to the expression in blood. HIF-1α demonstrated a higher expression at P3H for both NR and HY condition when compared to B ($P < 0.05$), only the fold change of HIF-1α mRNA at P24H in HY is significantly increased ($P < 0.05$), but not in NR (Fig. 4.8A). Muscle p50 (Fig.4.8B) showed a higher fold change at P24H compared to B, P0H and P3H for HY. For other genes such as TNF α (Fig.4.8C) and CCR2 (Fig.4.8D), no significant differences were observed at any time point in the three conditions.

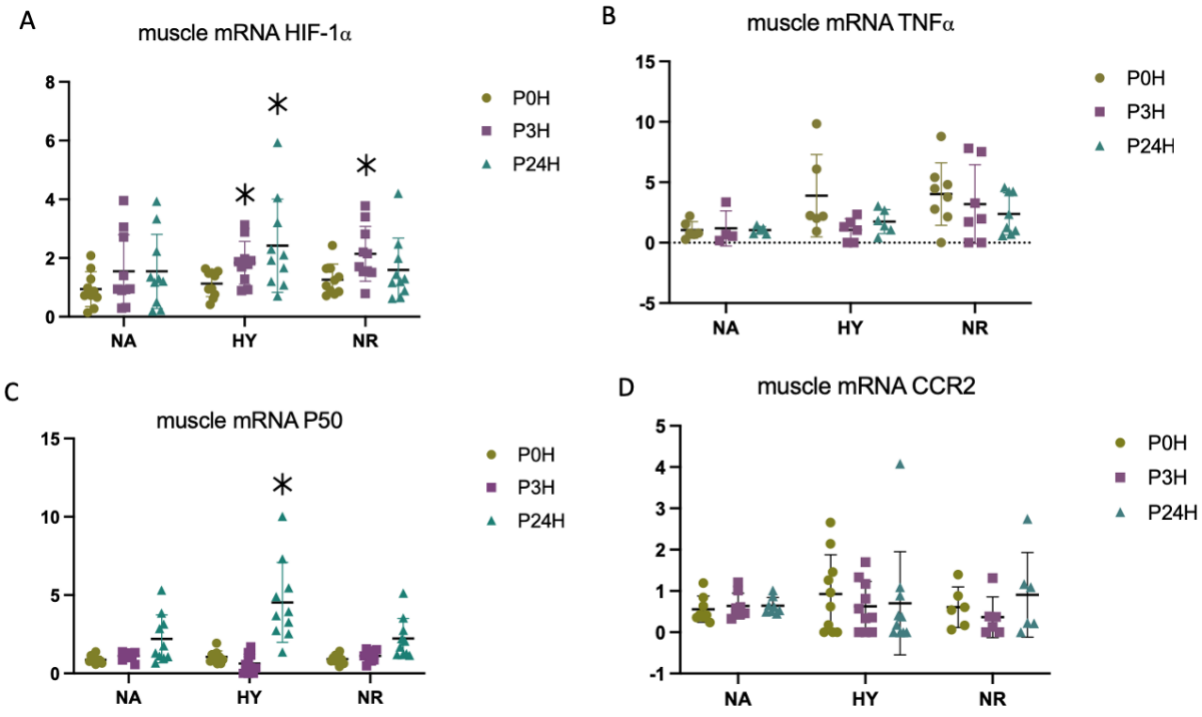


Figure 4.8 Skeletal muscle genes expression in NA/HY/NR at different time points. A: Muscle gene expression of HIF-1α in NA/HY and NR at P0H, P3H and P24H. B: Muscle gene expression of TNFα in NA/HY and NR at P0H, P3H and P24H. C: Muscle gene expression of P50 in NA/HY and NR at P0H, P3H and P24H. D: Muscle gene expression of CCR2 in NA/HY and NR at P0H, P3H and P24H. Muscle samples were obtained at baseline (B) post-exercise immediately (P0H), 3h post-exercise(P3H) and 24h post-exercise (P24H) at each of the conditions: normoxia absolute intensity (NA), hypoxia (HY) and normoxia relative intensity (NR). Mean (horizontal bars) ± standard deviation (SD) is plotted for each graph. * $p < 0.05$.

Expression of inflammatory genes in blood by using qRT-PCR

The relative expression of the inflammatory genes is depicted in Fig.4.9. No significant changes were observed in blood HIF-1α gene expression (Fig.4.9). However, differential expression of certain inflammatory genes was observed in blood, including TNF α, Interleukin β (IL-1β), Interleukin 8 (IL-8), IL-1α, Nuclear factor NF-kappa-B p65 subunit (p65) and CCR2. TNFα gene expression increased at P3H in NA when compared to baseline ($p = 0.04$) (Fig. 4.9A), with no changes in HY or NR. Moreover, the fold chang of IL-1β mRNA showed a significant increase at P0H and P3H in NA when compared to Baseline ($p = 0.002$ for P0H and P3H), with no changes in HY or NR. The fold change of IL-1β mRNA at P0H in NA is significantly higher than that of HY and NR (Fig. 4.9B), ($p = 0.04$ NA vs. HY; $p = 0.05$ NA vs. NR). IL-8 gene expression was elevated at P24H in HY ($P = 0.02$) and increased at P3H in NR ($p = 0.04$), with no changes in NA. IL-1α gene expression increased at P3H for NA($p =$

0.005) and HY (p = 0.04). There was a significant increase of p65 gene expression at P3H in NA (p = 0.01), while lower expression was observed at P0H in NR(p = 0.05). p50 (NF-κB) gene expression decreased at P3H in NR (p = 0.001), the fold change of p50 at P3H was higher in HY when compared with NA and NR(p = 0.03, HY vs. NA; p < 0.001, HY vs. NR). CCR2 gene expression decreased at P3H in HY (p = 0.01), but increased at P3H in NR（p = 0.003）.

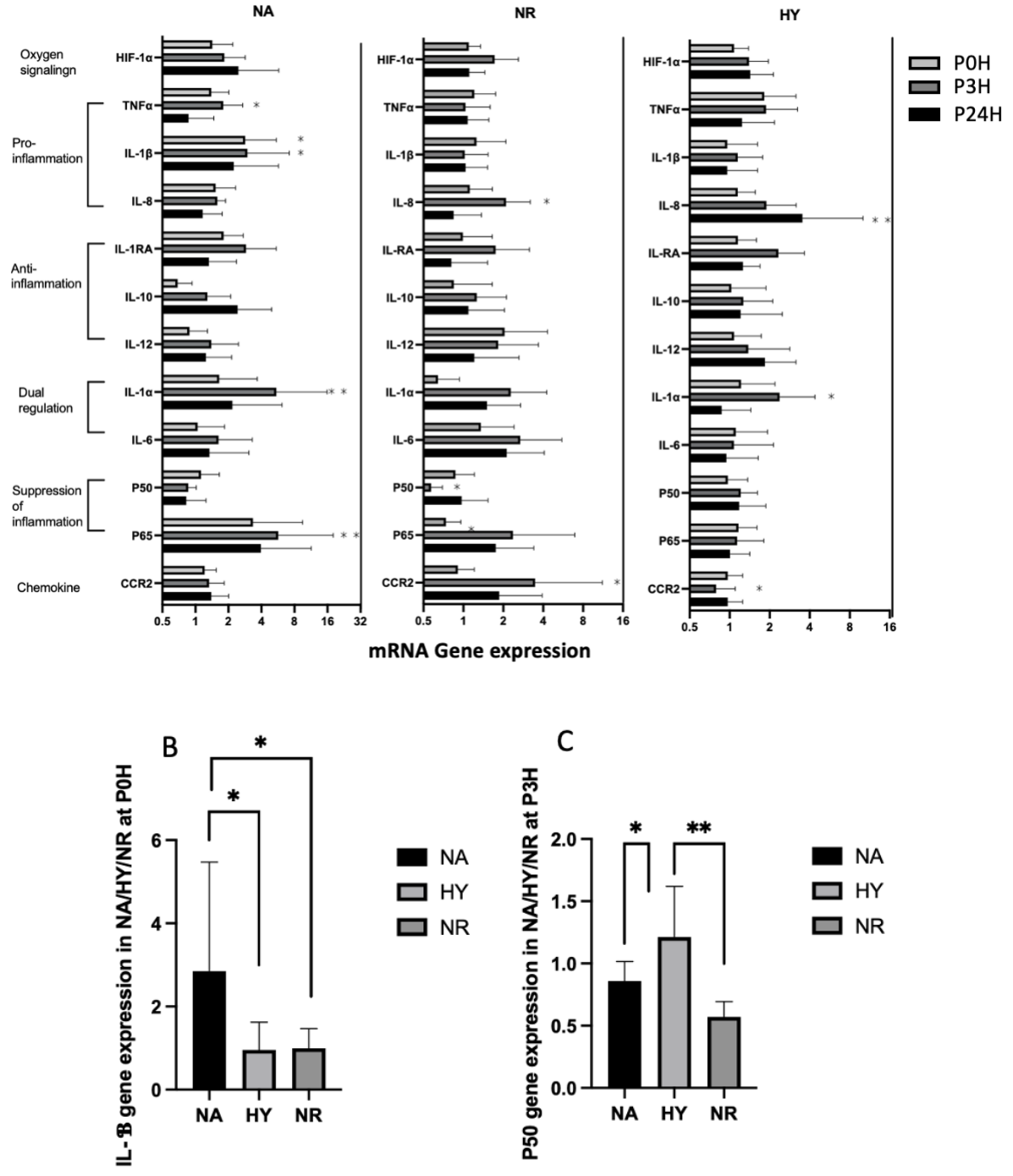


Figure 4.9 A: Fold changes compared to baseline of circulating inflammation-related genes. B-C: Circulating gene expression in NA/HY/NR at different time points. Blood samples were taken at baseline (B) post-exercise immediately (P0H), 3h post-exercise(P3H) and 24h post-exercise (P24H) at

each of the conditions: normoxia absolute intensity (NA), hypoxia (HY) and normoxia relative intensity (NR). Mean (horizontal bars) ± standard deviation (SD) is plotted for each graph. * $p < 0.05$, ** $p < 0.01$.

4.4 *Discussion*

This study aimed to investigate the impact of combining HIIE with hypoxia on inflammatory signalling in the skeletal muscle of healthy participants. RNA sequencing was used to measure the expression of some common inflammatory markers in both muscle samples and blood gene expression was measured using RT-PCR. The results indicate that many inflammation-related genes are significantly upregulated after HIIE in both normoxia and hypoxia, with no significant difference between the two conditions.

Some of the upregulated genes are enriched in pathways involved in the regulation of inflammatory signalling (Fig. 4.6, 4.9). For instance, NF-ĸB and TNF α are associated with the TNF signalling pathway and mitogen-activated protein kinases (MAPK) signalling. TNF α is a master cytokine that mediates inflammatory responses and innate immunity, capable of activating NF- ĸB, and MAPK pathway [331]. Although TNF α and NF-ĸB did not rank in the top 20 up-regulated genes list from RNA-seq, qRT-PCR revealed an increase in blood TNF α gene expression at P3H in NA, while muscle TNF α mRNA did not show significant changes in any of the conditions or time points. Muscle p65 mRNA showed an increase at P24H in HY (Fig. 4.10A). The TNF α /NF-ĸB pathway showed strong upregulation in NA, HY and NR without any notable difference (Fig.4.9).

A commonly upregulated gene in all three conditions is MYC (Fig. 4.7). MYC is a well-known oncogene found in the blood, with significant relevance to lymphomas [332]. It plays a crucial role in cell survival and proliferation. After the HIIE, the MYC gene itself showed significant upregulation in all three conditions, especially at P3H and P24H (Fig. 4.6, 4.7). Additionally, the apoptosis pathway was upregulated at P24H in HY and NR (Fig. 4.9C), MYC is also associated with apoptosis in response to vigorous exercise [333], and as a pro-inflammatory mediator, TNFα can also induce apoptosis [331]. Based on these findings, I, therefore, speculate that skeletal muscle might undergo apoptosis in response to HIIE in both normoxia and hypoxia with the stimulus of relative intensity.

CD14 monocytes are generally considered to have anti-inflammatory properties [334]. A previous study conducted by Glotov, A. S., et al. [324] focused on hypoxia training. In this study, seven elite female speed skaters were profiled on the 18th day of high-altitude adaptation. They underwent whole blood RNA-seq analysis before and after an intense 1 h skating session to evaluate gene expression changes associated with exercise. The study revealed a significant increase in CD14 expression in response to exercise at altitude. Interestingly, in the current study, we observed upregulation of CD14 not only in HY but also in NA and NR (Fig.4.6, 4.7). This result intimates that the acute HIIE can induce the CD14 expression, but hypoxia alone does not provide an additional stimulus for CD14 induction. It is important to note that the previous study mentioned above used HIE rather than HIIE and required the subjects expeirence 18 days of environmental hypoxia exposure (1850m above sea level) prior to exercise. The differences in prolonged hypoxia exposure, exercise intensity, and subjects with different training levels, could all contribute to the disparity in results.

I also measured the gene expression of some classical inflammatory markers such as TNF-α, IL6, IL8, and IL-10 [335], using qRT-PCR in both blood and muscle samples. Blood, being part of the circulatory system, serves as a carrier for inflammatory mediators produced by immune cells and muscle tissue. Therefore, investigating the crosstalk between muscle and blood in the inflammatory response to HIIE in hypoxia can provide insights into the underlying mechanism of the human body's reaction to exercise in hypoxia. Previous studies have suggested that hypoxic exercise can exacerbate immune disturbances, although most of these studies used the absolutely matched exercise intensity. In a study by Svendsen, I. S., et al. (2016) [336], acute exercise in hypoxia (altitude 2000m) was performed with relative intensity (75 min at 70 % of altitude-specific $\dot{V}O_{2max}$). Their findings showed that TNF α and IL-10 remained unchanged, while IL-6 and IL-8 increased after an acute exercise in both normoxia and hypoxia. Our study yielded similar results on TNF α, IL-10, IL 6 and IL-8, aligning with the previous study. Importantly, no significant differences in gene expression of plasma cytokines. The authors suggested that acute hypoxic exercise seems not pose a meaningful additional threat to immune function compared to exercise at sea level [336].

In a well-designed study by Lundy and Steensberg [337], a hypoxia protocol was implemented at 4100m altitude. This study is consist of acute exercise and chronic training, with both absolute intensity (normoxia154 W, 45%$\dot{V}O_{2max}$, acute hypoxia=154 W, 54%$\dot{V}O_{2max}$; chronic

hypoxia =154 W, 59% $\dot{V}O_{2max}$) and relative intensity (acute hypoxia =130 W, 46% $\dot{V}O_{2max}$; chronic hypoxia =120 W, 44% $\dot{V}O_{2max}$). The results showed that post-exercise plasma IL-6 concentration did not differ significantly between acute or chronic hypoxic exposure when participants cycled at relatively matched exercise intensity at both acute and chronic hypoxic exposure. However, the release of IL-6 released increased in hypoxia when compared with exercising at absolutely matched intensity in normoxia, both in acute exercise and chronic training compared with normoxia. In addition, the authors emphasized that IL-6 is exercise-depended during the exercise/training in hypoxia. The findings align with the current study, which showed no effect of hypoxia on post-exercise blood mRNA IL-6. These results highlight the importance of an accurate study design, considering exercise intensity, during hypoxic exercise/training, as hypoxic exercise at the same absolute intensity and relative intensity may have different results.

Exercise is known to induce a robust inflammatory response [172]. This response can be assessed by measuring an increase in circulating inflammatory mediators produced by immune cells and active muscle tissue [338]. After the physical activities of sufficient intensity, pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 are immediately released, followed by the release of anti-inflammatory or regulatory cytokines, such as IL-10 and IL-1RA that attenuate that response [335]. While inflammatory cytokines can be detected in both blood and skeletal muscle, obtaining muscle samples through biopsies can be challenging and invasive, making them less practical outside of medical facilities. In contrast, peripheral blood sampling offers many advantages including well-established collection, storage, and transportation protocols, minimal impact on the subject's well-being, and the presence of many valuable biomarkers [324]. Due to its efficiency and accuracy, studies focusing on inflammation often prefer blood collection instead of muscle biopsy. This could explain the lack of literature comparing the the effects of HIIE in hypoxia on muscle-specific inflammatory responses. As a result, we can only compare the muscle-specific inflammatory response with the response of circulating inflammatory signalling. In our study, I tested the inflammation markers in muscle using RT-PCR and compared them to blood markers. I observed higher levels of HIF-1α mRNA at P0H in HY compared to NA, whereas no changes were observed in blood at any time points across the three conditions. The difference in HIF-1α gene expression between skeletal muscle and blood suggests that acute hypoxia may have more transient effects on skeletal muscles than on the circulating system. Skeletal muscle is the primary site response to exercise [338]. It evidenced by the fact that there was a significant increase in p50 (NF-κB1)

muscle mRNA has been observed at P24H in HY but p50 blood mRNA only slightly increased but did not reach the significance in HY. As for other inflammatory cytokines and regulators, such as TNF-α and CCR2, no change was observed in HY and NR in either muscle or blood.

4.5 *Conclusion*

In this study, I utilised RNA sequencing and detected a significant number (over 4000) gens that were up- or down- regulated in skeletal muscle of heathy males following HIIE under both normoxia and hypoxia conditions. Among these genes, 23 were found to be significantly associated with the inflammatory response after the intervention of HIIE in normoxia and hypoxia. The results showed that a single session of HIIE induced inflammation in skeletal muscle, as evidenced by the upregulated inflammation genes and enrichment of pathway such as TNF α signallings and NF-ƘB pathway. However, hypoxia did not appear to pose any additional effect on inflammtion.

4.6 *Limitation*

One limitation of our study is our moderate sample size (n =10). We acknowledge that a larger sample size would provide stronger power, helping to minimize the individual variation and further validate the potential associations between HIIE in hypoxia and inflammation. Another limitation is the the limited availability of research on muscle-specific inflammatory response, making it challenging to directly compare our findings. Nevertheless, our study is the first one to use muscle mRNA and compared with blood mRNA, shedding light on the unique aspects of muscle-specific inflammation. However, I selected representative inflammation markers to be measured in blood by RT-PCR. In future studies, it would be beneficial to confirm the expression of those the significantly different genes identified from RNA-seq, in the exploration of potential differences between blood and muscle specific inflammatory signalling.

Chapter 5
Conclusions and the future directions

5.1 Overview

Hypoxic exercise studies published in the past 10 years have varied in the fraction of inspired oxygen (10.5-15.2%) (Table 5.1), training duration (a single session to eight weeks), and the fitness of the subjects (Table 5.1). These studies explored physiological adaption, muscle oxidative capacity, muscle glycolytic capacity, mitochondrial biogenesis and inflammation etc, (Table 5.1). Some studies reported the benefits of hypoxic training on performance at altitude, as evidenced by enhanced performance and/or increased physical work capacity [139, 339], for example, increased $\dot{V}O_{2max}$, PPO or W_{max}. Some studies suggested that hypoxia led to several skeletal muscle adaptations, including the modification of mitochondrial biogenesis as the marker of PGC-1 α [56, 151]. However, hypoxia training is still not consistently found to induce molecular adaptions [5, 250]. As shown in the review by Hoppeler, H., et al. [256], four of nine studies on trained subjects reported an additional benefit of hypoxic training. In contrast, studies on untrained subjects reported more positive results of hypoxia training [268].

The overall aim of this thesis is to understand the effect of hypoxia combined with a single session of HIIE on physiological responses and molecular adaptive responses (focusing on mitochondrial adaptation and inflammation) in the skeletal muscle of healthy males. More specifically, this study also aims to determine whether a single session of HIIE in hypoxia induces additional adaptations in mitochondrial respiration and content, and the mRNA and protein content of genes related to mitochondrial biogenesis and inflammation.

This study implemented HIIE in two conditions: hypoxia and normoxia. Ten healthy male participants completed three HIIE sessions (HY, NA and NR) in random orders. During the HIIE interventions, muscle, and blood samples were collected before (B), immediately (P0H), three hours (P3H), and 24 hours post-exercise (P24H). By matching both the absolute and

relative exercise intensity in normoxia, this study was designed to differentiate whether the adaption were mainly due to hypoxia or exercise intensity.

In Chapter 2, I presented the prescription of two HIIE sessions in normoxia (NA and NR) to match for absolute and relative intensity in hypoxia, based on PPO and LT derived from GXTs. The results demonstrated that when matched for relative exercise intensity in normoxia, a similar level of HR_{peak}, $\dot{V}O_{2peak}$ and RPE was observed between hypoxia and normoxia. When the intensity during the HIIE session was matched to the absolute value in hypoxia, a lower HR_{peak} and $\dot{V}O_{2peak}$, and a lower RPE were observed in normoxia. When exercise trials matched for absolute exercise intensity were used to compare the physiological and molecular response between hypoxia and normoxia, the observed difference could be a result of exercise under different intensities. Therefore, to better understand the effect of hypoxia on exercise, it is important to compare the molecular pathway activation between hypoxia and normoxia when similar relative exercise intensity is used. In the followed chapters 3 and 4, I investigated two important molecular pathways (mitochondrial adaptation and inflammation) in response to the three HIIE sessions.

In Chapter 3, I measured the changes in mitochondrial respiration and mitochondrial content, and expression of genes and proteins associated with mitochondrial biogenesis, in response to three HIIE sessions. It was shown that a single session of HIIE, regardless of the exercise condition and intensity, could not induce changes in mitochondrial respiration or content. A significant increase in the expression of mitochondrial biogenesis-related genes was observed only in NR and HY, not in NA, which suggested that exercise intensity rather than hypoxia has a main effect on the induction of mitochondrial biogenesis following acute HIIE.

In Chapter 4, I first utilised RNA-seq to analyse the muscle mRNA samples and screened for inflammatory markers in response to acute HIIE sessions in NA, HY and NR. Based on that, I tested the same genes in the blood sample by using RT-PCR. Then compared the blood mRNA with muscle mRNA, in order to assess the effects of acute HIIE session in hypoxia on inflammation signalling in skeletal muscle and circulating inflammatory markers. HIIE led to an increase in circulating inflammatory mediators originated from the immune cells [324] and could influence skeletal muscle, the primary site for exercise, skeletal muscle [338]. In this context, the crosstalk between inflammation signalling in circulation and muscle could help us to better understand the mechanism of inflammation induced by HIIE in hypoxia at local and

global levels. The results showed that a single session of HIIE induced inflammation in skeletal muscle and blood, however, hypoxia does not appear to pose any additional effect on inflammation compared to exercise intensity.

5.2 Hypothesis and Summary of key findings

The hypothesis of the current study was that the presence of hypoxia would act as an additional stimulus for skeletal muscle mitochondrial biogenesis and inflammation. As such, this study aimed to investigate whether any differences in performance observed between hypoxia and normoxia groups could be attributed to differences in mitochondrial biogenesis and inflammation.

Chapter 2:

- $\dot{V}O_{2peak}$, HR_{peak}, PPO,and LT derived from GXTs in hypoxia werelower, but RER was higher, compared to those from GXTs performed in normoxia.

- Similar HRpeak and $\dot{V}O_{2peak}$, or RPE and RER between HY and NR were achieved when PPO and LT were used to match the relative intensity

Chapter 3:

- A single session of HIIE in all conditions did not change mitochondrial respiratory function and mitochondrial content measured using CS activity as the biomarker.
- A single session of HIIE in HY and NR induced expression of genes related to mitochondria and metabolism, including mitochondrial biogenesis, mitochondrial respiration regulation, glycolytic and fatty acid metabolism. However, very little change was observed in gene expression in NA.
- The observed fold change of gene expression followed by HIIE was not significantly different between HY and NR, suggesting that the exercise intensity, rather than hypoxia, is contributing to the adaptive response.

Chapter 4:

- mRNA showed a very similar trend between NA, HY and NR.
- Inflammatory via TNF signalling and mitochondrial biogenesis via MAPK/p53 signalling ranked by significance.
- The fold change of muscle HIF-1α gene expression at P0H was higher in HY than that of NA, but blood HIF-1α gene expression has no difference at any timepoint between hypoxia and normoxia.

5.3 Discussion

American College of Sports Medicine (ACSM) presented that the essential components of a systematic individualised exercise prescription includes the appropriate mode(s), intensity, duration, frequency, and progression of exercise training, of which exercise intensity is considered the most important of the primary variables [340]. To date, HIIE/HIIT has been combined with hypoxia exposure to improve exercise adaptations. Taking these factors into consideration, the protocol of the current study has some novel factors.

1) methods of exercise prescription to match HIIE intensity

Different methods are employed to prescribe exercise intensity in hypoxia, including PPO [226-230], HR_{max} [232-234], $\dot{V}O_{2max}$ [235, 237], RPE [237], or LT [169, 229, 238]. Among them, HR_{max} and $\dot{V}O_{2max}$ are the most common parameters to determine exercise intensity [340]. However, Hofmann, P., et al. [341]. pointed out that the HR response to incremental exercise was shown to be neither linear nor uniform. So, using HR_{max} to calculate exercise intensity, for example, 85% HR_{max} is a common mode in prescription [342]. The workload of the 85% HR_{max} model is different from the workload determinated by PPO and submaximal power output at the first lactate turn point and at the second lactate turn point. 85% HR_{max} model could result in the overestimation of the individual training heart rate by at least 5% –10% and up to 40% [340]. Brawner, C. A., et al. [343] found that % $\dot{V}O_2$ reserve ($\dot{V}O_2R$) is related to % heart rate reserve (HRR) more than % $\dot{V}O_{2max}$. 10% difference is between % $\dot{V}O_2R$ and % $\dot{V}O_{2max}$ model among healthy untrained subjects, and the difference increases with ages. It has also been reported by Bishop, D. J., et al. that relative exercise intensity calculation could differ by ≥150 W depending on the stage duration of the GXT where from which W_{max} or W_{peak} was from [5, 344]. Thus, exercise intensity needs to be determined better, because it can affect the exercise-induced changes in mitochondrial function, content, gene expression, or protein abundance. Baldwin, J., et al. has suggested that the exercise intensity should be determined by LT instead

of W_{peak} (or W_{max}) if the study aimed to compare exercise-induced changes in individuals under similar physiological conditions [101]. This theory was supported by the evidence that metabolic stress and glycolytic metabolism level [345] were two-fold greater at 70% W_{peak} but were similar during exercise performed at 95% LT. Our previous study also suggested that exercise-induced changes in mitochondrial biogenesis in response to HIIE are more strongly associated with exercise intensity expressed relative to the LT than relative to W_{peak} or W_{max} [5]. Taking this into consideration, this study prescribed three HIIE sessions based on PPO and LT derived from GXTs.

2) study design to distinguish the effect of exercise intensity and hypoxia: match for relative and absolute exercise intensity in normoxia and hypoxia

Many hypoxia studies only used one intensity in normixia as a control, either matched for relative intensity [151, 252, 346, 347] or absolute same intensity [92, 169, 251, 257, 348-352] to that of hypoxia (Table 5.1). Some showed a larger improvement in exercise performance (VO_{2max}, PPO), gene expression, and skeletal muscle oxidative capacity in hypoxia than normoxia with exercising at the same absolute workload in hypoxia than in normoxia [49, 257, 300]. When studies apply the "same relative intensity" approach, the absolute intensity in normoxia is at a lower level than the intensity in hypoxia since low ATP turnover in hypoxia makes exercise in hypoxia more difficult than in normoxia [250]. For example, exercising at similar absolute intensity (same absolute exercise intensity, such as pedalling at 100 W), hypoxia will increase physiological and perceptual responses. However, since hypoxia decreases $\dot{V}O_{2max}$, pedalling at a given physiological intensity (for example, 75% $\dot{V}O_{2max}$) represents a reduced mechanical output (power sustained). To avoid bias, this study set up both the absolutely matched same intensity in normoxia matched to HY, and the relative same intensity matched to HY. After reviewing studies in which exercise sessions were carried out in hypoxia and which included control group training in normoxia (Table 5.1), we could not find a similar study design with have two control conditions to be compared with HY.

Table 5.1 Study design to compare HY with relative intensity or absolute intensity in normoxia.

Study	The absolute same intensity in Normoxia	The relative same intensity in Normoxia	Hypoxia	Key funding
Engfred, K., et al. (1994).[353]	70% of $\dot{V}O_{2max}$	70% of altitude $\dot{V}O_{2max}$	70% of altitude $\dot{V}O_{2max}$, 2500m	$\dot{V}O_{2max}$ ↑in HY, NA and NR, and no

				differences between groups
Vogt, M., et al. (2001).[49]	training blood lactate levels: 4–6 mM		training blood lactate levels: 4–6 mM, 3800m	HIF-1α mRNA↑in HY
Van Thienen, R., et al. (2016). [92]	1.2 W/kg for 20 min, FiO_2=20.9%		1.2 W/kg for 20 min, FiO_2=10.5%	COX-4 and CS ↔ in HY and NOR, HIF-1α mRNA and protein, PDK mRNA↑ in HY
Kong, Z., et al. (2017). [348]	10 X 8-s maximum efforts, FiO_2=21%		10 X 8-s maximum efforts, FiO_2=15%	$\dot{V}O_2$peak ↑ in HY, and 2 folds higher than NOR
Brocherie, F., et al. (2018). [300]	FiO_2=21.0%,4 sets of 5 × 5s maximal sprints interspersed with 25 s		2800-3000 m, FiO2=14.5-14.2%, 4 sets of 5 × 5s maximal sprints interspersed with 25 s	HIF-1α, VEGF, PGC-1α and CS activity ↑ in LHTLH, but not in LHTL or LLTL
Zebrowska, A., et al. (2019).[169]	120% LT, FiO_2=20.9%		120% LT, FiO_2=15.2%	no effect of training conditions for serum angiogenic factors and cytokines HIF-1α↑in HY
Zoll, J., et al. (2006). [151]		Nor: 172 ± 3 beats/min, FiO_2=20.9%	HY: 166 ± 3 beats/min, FiO_2=14.5%	$\dot{V}O_{2max}$ ↑ in HY, HIF-1α, PGC-1α and CS ↑ in HY
Czuba, M., et al. (2013). [346]		90% $\dot{V}O_{2max}$ determinate in NOR	90% $\dot{V}O_{2max}$ determinate in HY	$\dot{V}O_{2max}$↑in HYand NOR
Menz, V., et al. (2016). [252]		4 x 4 min one-legged cycling at 90% HR determinate in NOR, FiO_2=21%	4 x 4 min one-legged cycling at 90% HR determinate in HY, F_iO_2=15%, 4500m	$\dot{V}O_2$max and PPO↑ in HY greater than NOR
Ghaith, A., et al. (2022). [347]		100 W_{peak}, FiO_2=20.9%	80 W_{peak}, FiO_2=12%, 4200m	$\dot{V}O_2$peak and ventilatory thresholds ↑in HY and NOR, no difference between HY and NOR

As shown in Van Thienen, R., et al. [92], 13 pairs of monozygotic twin brothers did cycling in normoxia and hypoxia (FiO_2 of 10.7%, approximately 5300 m altitude). The exercise intensity is corresponding to 50.7 ± 2.3% of $\dot{V}O_{2max}$ in normoxia vs. 81.4 ± 3.2% of $\dot{V}O_{2max}$ in hypoxia. The study aimed to compare responses to an absolute workload rather than a relative workload. The results of this study showed that the hypoxic but not normoxic exercise decreased $\dot{V}O_{2max}$ and HR_{max} [354]. An increase of muscle HIF-1α mRNA content (~ 50% increase) was observed in the hypoxia condition. Neither hypoxia nor exercise altered COX-4 and CS mRNA expressions. The glycolytic metabolism-related gene PDK4 showed a great increase (~ 1200% increase) in only hypoxia. Consistency with the above study, the current study (Figure 5.1) has shown decreased $\dot{V}O_{2max}$ and HR_{max} in HY. HIF-1α and PDK4 mRNA was unchanged after exercise in NA, but significantly increased after the HY and NR interventions (Figure 5.1).

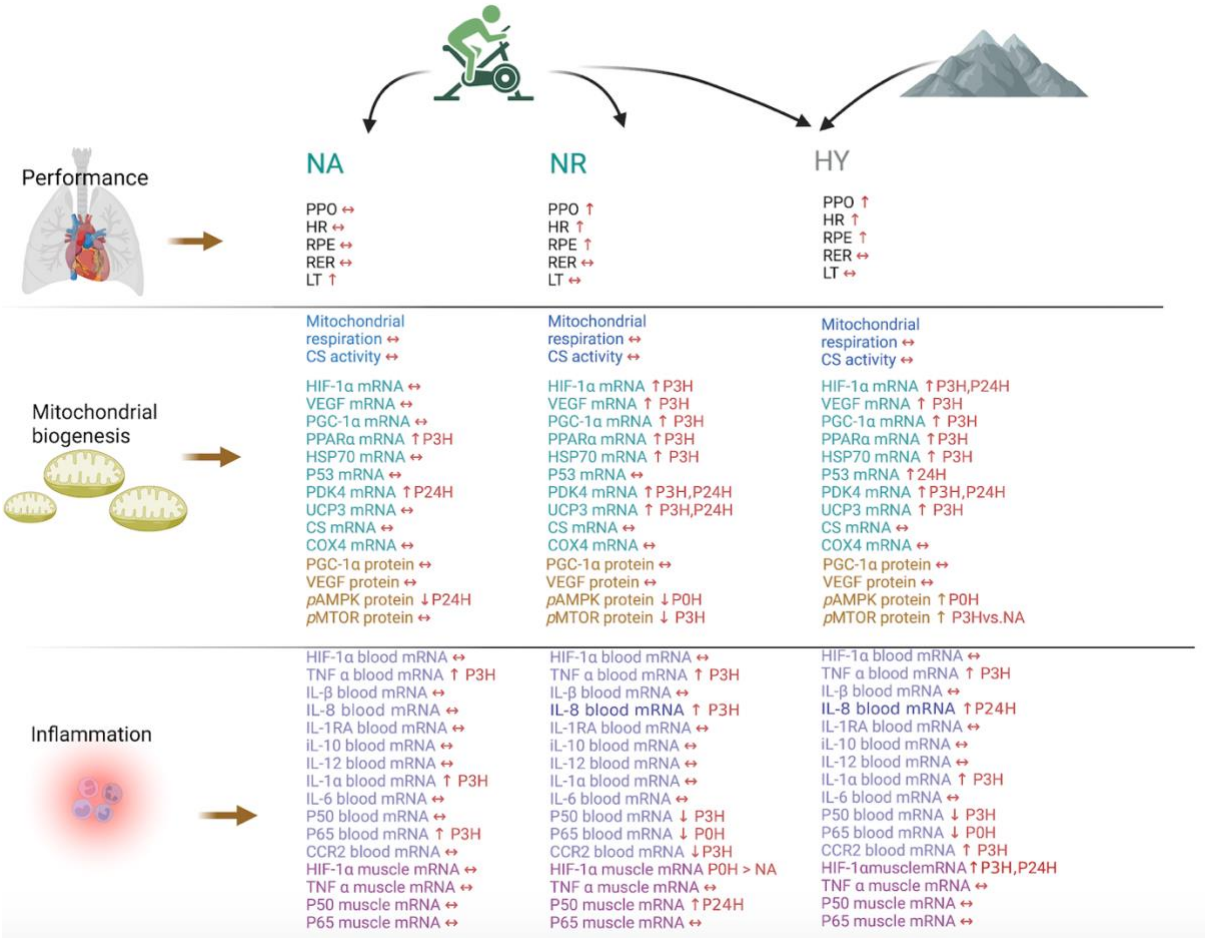


Figure 5.1 Main changes in exercise performance and molecular response

In another example of training in HY and NR, Zoll, J., et al. recruited 15 trained participants, for which six were trainined in six weeks in normoxia, and nine in hypoxia for six weeks. For the normoxia group, the exercise intensity was determined during the incremental test to exhaustion (IET) in normoxia (IET_N; FiO_2 = 20.9%), and, for the group who trained in hypoxia the intensity was determined during the (IET_H; FiO_2 = 14.5%). This study reported improved $\dot{V}O_{2max}$ along with increased PGC-1α, HIF-1α, CS and COX4 in hypoxia but not in normoxia [151]. However, there is no significant difference in the target mRNA (PGC-1α, HIF-1α) between hypoxia and normoxia. No changes in mitochondrial content (measured by CS activity) were observed, which is consistence with my findings (Table 5.1). Nordsborg, N. B., et al. also pointed out that the relative but not the absolute intensity is the major impact of an exercise-induced increase in several mRNAs [259]. Our results suggest that there is not much additional adaptive response induced by hypoxia, with evidenced by the similar induction of mRNA level PGC-1α and HIF-1 α is similar between HY and NR. This might be different from the conclusion of some hypoxia exercise studies [257, 300] with different intensity sets. Those

studies matched for the absolute intensity, but the current study matched for the relative intensity. Therefore, their studies are only comparing the effect of hypoxia and normoxia but the current study is designed to differentiate the influence of exercise intensity and hypoxia.

3) Application of RNA-seq to identify broad patterns of inflammatory gene expression
Many studies used RT-PCR [355, 356], or RNA-seq method solely to detect inflammation signalling under exercise or hypoxia-related interventions. Pham, K., et al. used RNA-seq to study the molecular signalling pathways that regulate inflammation under hypoxia, but exercise was not added to the invention [357]. Glotov, A. S., et al. combined HIE and hypoxia, using the RNA-Seq technique to detect biomarkers of adaptations in whole blood samples [324]. However, this study used only blood samples. To our best knowledge, this current study is the first attempt to apply for RNA-Seq to detect inflammation signalling in skeletal muscle and compared it with circulating inflammatory markers measured by qRT-PCR. Therefore, RNA-Seq allows us to map a broad profile of inflammatory response-related genes so that I could focus on differentiately expressed genes with qRT-PCR, making the target genes more specific and accurate.

5.4 Significance and practical applications

This research has helped define the respective role of exercise intensity and hypoxia in the adaptive responses to HIIE and has provided important mechanistic insights into the cellular processes involved. For a long time, no conclusive recommendations can be made as to which altitude, exposure duration, and exercise intensity might be beneficial to hypoxia training. The above findings provide an important contribution to the prescription of exercise intensity in hypoxia. Practically, matching exercise intensity in hypoxia with that of normoxia is critical for the physiological and molecular adaptive response to HIIE.

The availability of hypoxia is not universal. Natural settings at high altitudes have the advantage that training tasks can be carried out in a sport-specific manner, however, it does come with disadvantages including travel, expenses, and irreversible damage. Artificial hypoxia, for example, hypoxia chamber, allows for exposure to normobaric hypoxia during either rest or exercise, and the level of hypoxia can be precisely adjusted and regulated. This

study used acute HIIE in simulated hypoxia (chamber), it can offer implication for the results of future chronic training and avoid a waste of time, cost, and detraining/overtraining from inappropriate study design, which offered more feasibility and efficiency of "live low train high protocol" in artificial hypoxia environment.

Physical activity is known to alleviate mitochondrial energy metabolism abnormalities in aging [358], type II diabetes [5], stroke and cardiomyopathy [359]. The knowledge gained from this thesis could apply to the prevention and treatment of a growing number of metabolic diseases and even COVID related to systemic hypoxia. Prevention and management of these diseases would undoubtedly help relieve the social and economic burden of the public health sectors.

5.5 Limitation

One limitation of the current study was that we only implemented an acute study, which might not allow me to detect the changes in protein content involved in mitochondrial biogenesis or an overall increase in mitochondrial function and content. It has been known that exercise-induced mitochondrial biogenesis might be resulted from the cumulative effects of transient changes in gene expression that occur in response to each exercise bout [301, 302]. This acute study has shown changes in gene expression. It could be still insufficient to predict changes in protein levels after a chronic training period. Based on the findings, future studies should investigate the effects of a chronic (training) intervention.

Another limitation of the current study is the sample size. As shown by Jacques, M., et al. , individual variations in mitochondrial respiration and performance markers (W_{peak}, lactate threshold [LT], and $\dot{V}O_{2peak}$) did exsit in the exercise studies. If individual variabilities were too large, the differences in subject-by-training interaction could not be verified [360]. When a study is under powered, small differences might not be able to be detected due to statistical variance, and this is one of the most commonly observed sources of variation [361, 362]. Given the limited sample size, more participants might be required to further confirm findings from the current study.

The "first bout" effect is another limitation of this study. Murton, A. J., et al. reported that the transcriptional response after the first bout of resistance exercise represents muscle damage

and differs substantially from a second resistance exercise session performed 48 h later [363]. Perry, C. G., et al. and Wilkinson, D. J., et al. also suggested that increases in PGC-1 α mRNA [268] and mitochondrial protein synthesis [364] in response to HIIE were reduced with every subsequent session, even when the exercise intensity was maintained. These observations indicate that changes in genes, proteins, and even mitochondrial function and content as the outcome of exercise-induced mitochondrial biogenesis in untrained subjects, after the first session of HIIE, may be higher than changes in trained subjects in response to the same intervention [5]. The future study could introduce a control excess period before conducting the experimental (i.e., biopsy) trials. In the study designed by Brocherie, F., et al, a 2-wk lead-in period was performed before the baseline test to pass the most dramatic changes of mRNA [300, 365]. Based on the study by Girard, O., et al. [366], a future study could introduce a control period. The control period will have all participants exercise in the normoxia for about two weeks so that individual variations in gene expression might have already been normolised to similar levels [5, 268]. So, if hypoxia still has additional effects, the changes should be detected under hypoxia compared to normoxia.

References

1. Berg, J.M., J.L. Tymoczko, and S. Lubert, *Biochemistry*. 5th ed. Biochemistry. 2012, New York: WH Freeman.
2. Psilander, N., *The effect of different exercise regiments on mitochondrial biogenesis and performance*, in *The department of physiology and pharmacology*. 2014, Karolinska Institute: Stockholm, Sweden.
3. Sharma, L.K., J. Lu, and Y. Bai, *Mitochondrial respiratory complex I: structure, function and implication in human diseases.* Curr Med Chem, 2009. **16**(10): p. 1266-77.
4. Mairbaurl, H., *Red blood cells in sports: effects of exercise and training on oxygen supply by red blood cells.* Front Physiol, 2013. **4**: p. 332.
5. Bishop, D.J., et al., *High-Intensity Exercise and Mitochondrial Biogenesis: Current Controversies and Future Research Directions.* Physiology (Bethesda), 2019. **34**(1): p. 56-70.
6. Miller, B.F. and K.L. Hamilton, *A perspective on the determination of mitochondrial biogenesis.* Am J Physiol Endocrinol Metab, 2012. **302**(5): p. E496-9.
7. Menshikova, E.V., et al., *Effects of exercise on mitochondrial content and function in aging human skeletal muscle.* J Gerontol A Biol Sci Med Sci, 2006. **61**(6): p. 534-40.
8. Hood, D.A., *Mechanisms of exercise-induced mitochondrial biogenesis in skeletal muscle.* Appl Physiol Nutr Metab, 2009. **34**(3): p. 465-72.
9. Scarpulla, R.C., *Metabolic control of mitochondrial biogenesis through the PGC-1 family regulatory network.* Biochim Biophys Acta, 2011. **1813**(7): p. 1269-78.
10. Marchi, S., S. Patergnani, and P. Pinton, *The endoplasmic reticulum-mitochondria connection: one touch, multiple functions.* Biochim Biophys Acta, 2014. **1837**(4): p. 461-9.
11. Bo, H., Y. Zhang, and L.L. Ji, *Redefining the role of mitochondria in exercise: a dynamic remodeling.* Ann N Y Acad Sci, 2010. **1201**: p. 121-8.
12. Youle, R.J. and A.M. van der Bliek, *Mitochondrial fission, fusion, and stress.* Science, 2012. **337**(6098): p. 1062-5.
13. Short, K.R., *Measuring mitochondrial protein synthesis to assess biogenesis.* Am J Physiol Endocrinol Metab, 2012. **302**(9): p. E1153-4; author reply E1155.
14. Bishop, D.J., C. Granata, and N. Eynon, *Can we optimise the exercise training prescription to maximise improvements in mitochondria function and content?* Biochim Biophys Acta, 2014. **1840**(4): p. 1266-75.
15. Johnson, M.L., M.M. Robinson, and K.S. Nair, *Skeletal muscle aging and the mitochondrion.* Trends Endocrinol Metab, 2013. **24**(5): p. 247-56.
16. Tonkonogi, M. and K. Sahlin, *Physical exercise and mitochondrial function in human skeletal muscle.* Exerc Sport Sci Rev, 2002. **30**(3): p. 129-37.
17. Severinghaus, J.W. and P.B. Astrup, *History of blood gas analysis. IV. Leland Clark's oxygen electrode.* J Clin Monit, 1986. **2**(2): p. 125-39.
18. Veksler, V.I., et al., *Mitochondrial respiratory parameters in cardiac tissue: a novel method of assessment by using saponin-skinned fibers.* Biochim Biophys Acta, 1987. **892**(2): p. 191-6.
19. Picard, M., et al., *Mitochondrial structure and function are disrupted by standard isolation methods.* PLoS One, 2011. **6**(3): p. e18317.
20. Perry, C.G., et al., *Methods for assessing mitochondrial function in diabetes.* Diabetes, 2013. **62**(4): p. 1041-53.

21. Gnaiger, E., *Capacity of oxidative phosphorylation in human skeletal muscle: new perspectives of mitochondrial physiology.* Int J Biochem Cell Biol, 2009. **41**(10): p. 1837-45.
22. Brand, M.D. and D.G. Nicholls, *Assessing mitochondrial dysfunction in cells.* Biochem J, 2011. **435**(2): p. 297-312.
23. Picard, M., et al., *Mitochondria: isolation, structure and function.* J Physiol, 2011. **589**(Pt 18): p. 4413-21.
24. Kuznetsov, A.V., et al., *Analysis of mitochondrial function in situ in permeabilized muscle fibers, tissues and cells.* Nat Protoc, 2008. **3**(6): p. 965-76.
25. Saks, V.A., et al., *Permeabilized cell and skinned fiber techniques in studies of mitochondrial function in vivo.* Mol Cell Biochem, 1998. **184**(1-2): p. 81-100.
26. Krajcova, A., et al., *High resolution respirometry to assess function of mitochondria in native homogenates of human heart muscle.* PLoS One, 2020. **15**(1): p. e0226142.
27. Picard, M., R.T. Hepple, and Y. Burelle, *Mitochondrial functional specialization in glycolytic and oxidative muscle fibers: tailoring the organelle for optimal function.* Am J Physiol Cell Physiol, 2012. **302**(4): p. C629-41.
28. Rostovtseva, T.K., et al., *Tubulin binding blocks mitochondrial voltage-dependent anion channel and regulates respiration.* Proc Natl Acad Sci U S A, 2008. **105**(48): p. 18746-51.
29. Picard, M., K. White, and D.M. Turnbull, *Mitochondrial morphology, topology, and membrane interactions in skeletal muscle: a quantitative three-dimensional electron microscopy study.* J Appl Physiol (1985), 2013. **114**(2): p. 161-71.
30. Larsen, S., et al., *Biomarkers of mitochondrial content in skeletal muscle of healthy young human subjects.* J Physiol, 2012. **590**(14): p. 3349-60.
31. Mogensen, M., et al., *Cycling efficiency in humans is related to low UCP3 content and to type I fibres but not to mitochondrial efficiency.* J Physiol, 2006. **571**(Pt 3): p. 669-81.
32. Vigelso, A., N.B. Andersen, and F. Dela, *The relationship between skeletal muscle mitochondrial citrate synthase activity and whole body oxygen uptake adaptations in response to exercise training.* Int J Physiol Pathophysiol Pharmacol, 2014. **6**(2): p. 84-101.
33. Andreu, A.L., et al., *Quantification of mitochondrial DNA copy number: pre-analytical factors.* Mitochondrion, 2009. **9**(4): p. 242-6.
34. Medeiros, D.M., *Assessing mitochondria biogenesis.* Methods, 2008. **46**(4): p. 288-94.
35. Longchamps, R.J., et al., *Evaluation of mitochondrial DNA copy number estimation techniques.* PLoS One, 2020. **15**(1): p. e0228166.
36. Chabi, B., et al., *Quantification of mitochondrial DNA deletion, depletion, and overreplication: application to diagnosis.* Clin Chem, 2003. **49**(8): p. 1309-17.
37. Kuang, J., et al., *An overview of technical considerations when using quantitative real-time PCR analysis of gene expression in human exercise research.* PLoS One, 2018. **13**(5): p. e0196438.
38. Blijlevens, M., J. Li, and V.W. van Beusechem, *Biology of the mRNA Splicing Machinery and Its Dysregulation in Cancer Providing Therapeutic Opportunities.* Int J Mol Sci, 2021. **22**(10).
39. Latchman, D.S., *Transcription factors: an overview.* Int J Biochem Cell Biol, 1997. **29**(12): p. 1305-12.
40. Gibney, E.R. and C.M. Nolan, *Epigenetics and gene expression.* Heredity (Edinb), 2010. **105**(1): p. 4-13.

41. Pillai-Kastoori, L., A.R. Schutz-Geschwender, and J.A. Harford, *A systematic approach to quantitative Western blot analysis.* Anal Biochem, 2020. **593**: p. 113608.
42. Jensen, E.C., *The basics of western blotting.* Anat Rec (Hoboken), 2012. **295**(3): p. 369-71.
43. Chen, Y., A.T. Lun, and G.K. Smyth, *From reads to genes to pathways: differential expression analysis of RNA-Seq experiments using Rsubread and the edgeR quasi-likelihood pipeline.* F1000Res, 2016. **5**: p. 1438.
44. Stark, R., M. Grzelak, and J. Hadfield, *RNA sequencing: the teenage years.* Nat Rev Genet, 2019. **20**(11): p. 631-656.
45. Han, Y., et al., *Advanced Applications of RNA Sequencing and Challenges.* Bioinform Biol Insights, 2015. **9**(Suppl 1): p. 29-46.
46. Baty, F., et al., *Exploring the transcription factor activity in high-throughput gene expression data using RLQ analysis.* BMC Bioinformatics, 2013. **14**: p. 178.
47. Egan, B. and J.R. Zierath, *Exercise metabolism and the molecular regulation of skeletal muscle adaptation.* Cell Metab, 2013. **17**(2): p. 162-84.
48. Ross, L.M., R.R. Porter, and J.L. Durstine, *High-intensity interval training (HIIT) for patients with chronic diseases.* J Sport Health Sci, 2016. **5**(2): p. 139-144.
49. Vogt, M., et al., *Molecular adaptations in human skeletal muscle to endurance training under simulated hypoxic conditions.* J Appl Physiol (1985), 2001. **91**(1): p. 173-82.
50. MacInnis, M.J. and M.J. Gibala, *Physiological adaptations to interval training and the role of exercise intensity.* J Physiol, 2017. **595**(9): p. 2915-2930.
51. Hoppeler, H., et al., *Response of skeletal muscle mitochondria to hypoxia.* Exp Physiol, 2003. **88**(1): p. 109-19.
52. Mazzeo, R.S., *Physiological responses to exercise at altitude : an update.* Sports Med, 2008. **38**(1): p. 1-8.
53. Bonetti, D.L. and W.G. Hopkins, *Sea-level exercise performance following adaptation to hypoxia: a meta-analysis.* Sports Med, 2009. **39**(2): p. 107-27.
54. Vina, J., et al., *Exercise acts as a drug; the pharmacological benefits of exercise.* Br J Pharmacol, 2012. **167**(1): p. 1-12.
55. Hawley, J.A., et al., *Integrative biology of exercise.* Cell, 2014. **159**(4): p. 738-49.
56. Little, J.P., et al., *An acute bout of high-intensity interval training increases the nuclear abundance of PGC-1alpha and activates mitochondrial biogenesis in human skeletal muscle.* Am J Physiol Regul Integr Comp Physiol, 2011. **300**(6): p. R1303-10.
57. Gibala, M.J., J.B. Gillen, and M.E. Percival, *Physiological and health-related adaptations to low-volume interval training: influences of nutrition and sex.* Sports Med, 2014. **44 Suppl 2**: p. S127-37.
58. Gibala, M.J., et al., *Physiological adaptations to low-volume, high-intensity interval training in health and disease.* J Physiol, 2012. **590**(5): p. 1077-84.
59. Scribbans, T.D., et al., *The Effect of Training Intensity on VO2max in Young Healthy Adults: A Meta-Regression and Meta-Analysis.* Int J Exerc Sci, 2016. **9**(2): p. 230-247.
60. Weston, K.S., U. Wisloff, and J.S. Coombes, *High-intensity interval training in patients with lifestyle-induced cardiometabolic disease: a systematic review and meta-analysis.* Br J Sports Med, 2014. **48**(16): p. 1227-34.
61. Rognmo, O., et al., *Cardiovascular risk of high- versus moderate-intensity aerobic exercise in coronary heart disease patients.* Circulation, 2012. **126**(12): p. 1436-40.

62. Beauchamp, M.K., et al., *Interval versus continuous training in individuals with chronic obstructive pulmonary disease--a systematic review.* Thorax, 2010. **65**(2): p. 157-64.
63. Granata, C., N.A. Jamnick, and D.J. Bishop, *Training-Induced Changes in Mitochondrial Content and Respiratory Function in Human Skeletal Muscle.* Sports Med, 2018. **48**(8): p. 1809-1828.
64. Jacobs, R.A. and C. Lundby, *Mitochondria express enhanced quality as well as quantity in association with aerobic fitness across recreationally active individuals up to elite athletes.* J Appl Physiol (1985), 2013. **114**(3): p. 344-50.
65. Zoll, J., et al., *Quantitative and qualitative adaptation of skeletal muscle mitochondria to increased physical activity.* J Cell Physiol, 2003. **194**(2): p. 186-93.
66. Daussin, F.N., et al., *Training at high exercise intensity promotes qualitative adaptations of mitochondrial function in human skeletal muscle.* J Appl Physiol (1985), 2008. **104**(5): p. 1436-41.
67. Drake, J.C., R.J. Wilson, and Z. Yan, *Molecular mechanisms for mitochondrial adaptation to exercise training in skeletal muscle.* FASEB J, 2016. **30**(1): p. 13-22.
68. Vincent, G., et al., *Changes in mitochondrial function and mitochondria associated protein expression in response to 2-weeks of high intensity interval training.* Front Physiol, 2015. **6**: p. 51.
69. Chrois, K.M., et al., *Mitochondrial adaptations to high intensity interval training in older females and males.* Eur J Sport Sci, 2020. **20**(1): p. 135-145.
70. Newsom, S.A., et al., *Substrate-Specific Respiration of Isolated Skeletal Muscle Mitochondria after 1 h of Moderate Cycling in Sedentary Adults.* Med Sci Sports Exerc, 2021. **53**(7): p. 1375-1384.
71. Yoo, S.Z., et al., *Effects of Acute Exercise on Mitochondrial Function, Dynamics, and Mitophagy in Rat Cardiac and Skeletal Muscles.* Int Neurourol J, 2019. **23**(Suppl 1): p. S22-31.
72. Jacobs, R.A., et al., *Improvements in exercise performance with high-intensity interval training coincide with an increase in skeletal muscle mitochondrial content and function.* J Appl Physiol (1985), 2013. **115**(6): p. 785-93.
73. Dohlmann, T.L., et al., *High-intensity interval training changes mitochondrial respiratory capacity differently in adipose tissue and skeletal muscle.* Physiol Rep, 2018. **6**(18): p. e13857.
74. Gillen, J.B., et al., *Twelve Weeks of Sprint Interval Training Improves Indices of Cardiometabolic Health Similar to Traditional Endurance Training despite a Five-Fold Lower Exercise Volume and Time Commitment.* PLoS One, 2016. **11**(4): p. e0154075.
75. Christensen, P.M., et al., *A short period of high-intensity interval training improves skeletal muscle mitochondrial function and pulmonary oxygen uptake kinetics.* J Appl Physiol (1985), 2016. **120**(11): p. 1319-27.
76. Trewin, A.J., et al., *Acute HIIE elicits similar changes in human skeletal muscle mitochondrial H2O2 release, respiration, and cell signaling as endurance exercise even with less work.* Am J Physiol Regul Integr Comp Physiol, 2018. **315**(5): p. R1003-R1016.
77. Lewis, M.T., et al., *Acute high-intensity exercise and skeletal muscle mitochondrial respiratory function: role of metabolic perturbation.* Am J Physiol Regul Integr Comp Physiol, 2021. **321**(5): p. R687-R698.
78. Layec, G., et al., *Acute High-Intensity Exercise Impairs Skeletal Muscle Respiratory Capacity.* Med Sci Sports Exerc, 2018. **50**(12): p. 2409-2417.

79. Flockhart, M., et al., *Excessive exercise training causes mitochondrial functional impairment and decreases glucose tolerance in healthy volunteers.* Cell Metab, 2021. **33**(5): p. 957-970 e6.
80. Ponsot, E., et al., *Exercise training in normobaric hypoxia in endurance runners. II. Improvement of mitochondrial properties in skeletal muscle.* J Appl Physiol (1985), 2006. **100**(4): p. 1249-57.
81. Bakkman, L., et al., *Quantitative and qualitative adaptation of human skeletal muscle mitochondria to hypoxic compared with normoxic training at the same relative work rate.* Acta Physiol (Oxf), 2007. **190**(3): p. 243-51.
82. Salvadego, D., et al., *Separate and combined effects of a 10-d exposure to hypoxia and inactivity on oxidative function in vivo and mitochondrial respiration ex vivo in humans.* J Appl Physiol (1985), 2016. **121**(1): p. 154-63.
83. Meinild Lundby, A.K., et al., *Exercise training increases skeletal muscle mitochondrial volume density by enlargement of existing mitochondria and not de novo biogenesis.* Acta Physiol (Oxf), 2018. **222**(1).
84. Wu, L.H., et al., *High-intensity Interval Training Improves Mitochondrial Function and Suppresses Thrombin Generation in Platelets undergoing Hypoxic Stress.* Sci Rep, 2017. **7**(1): p. 4191.
85. Tsai, H.H., et al., *Exercise Training Alleviates Hypoxia-induced Mitochondrial Dysfunction in the Lymphocytes of Sedentary Males.* Sci Rep, 2016. **6**: p. 35170.
86. Desplanches, D., et al., *Hypoxia refines plasticity of mitochondrial respiration to repeated muscle work.* Eur J Appl Physiol, 2014. **114**(2): p. 405-17.
87. O'Hagan, K.A., et al., *PGC-1alpha is coupled to HIF-1alpha-dependent gene expression by increasing mitochondrial oxygen consumption in skeletal muscle cells.* Proc Natl Acad Sci U S A, 2009. **106**(7): p. 2188-93.
88. Lindholm, M.E. and H. Rundqvist, *Skeletal muscle hypoxia-inducible factor-1 and exercise.* Exp Physiol, 2016. **101**(1): p. 28-32.
89. Puype, J., et al., *Sprint interval training in hypoxia stimulates glycolytic enzyme activity.* Med Sci Sports Exerc, 2013. **45**(11): p. 2166-74.
90. Trewin, A.J., et al., *Acute exercise alters skeletal muscle mitochondrial respiration and H2O2 emission in response to hyperinsulinemic-euglycemic clamp in middle-aged obese men.* PLoS One, 2017. **12**(11): p. e0188421.
91. De Smet, S., et al., *Physiological Adaptations to Hypoxic vs. Normoxic Training during Intermittent Living High.* Front Physiol, 2017. **8**: p. 347.
92. Van Thienen, R., et al., *Twin Resemblance in Muscle HIF-1alpha Responses to Hypoxia and Exercise.* Front Physiol, 2016. **7**: p. 676.
93. Bishop, D.J., et al., *Sodium bicarbonate ingestion prior to training improves mitochondrial adaptations in rats.* Am J Physiol Endocrinol Metab, 2010. **299**(2): p. E225-33.
94. Bizeau, M.E., W.T. Willis, and J.R. Hazel, *Differential responses to endurance training in subsarcolemmal and intermyofibrillar mitochondria.* J Appl Physiol (1985), 1998. **85**(4): p. 1279-84.
95. Burelle, Y. and P.W. Hochachka, *Endurance training induces muscle-specific changes in mitochondrial function in skinned muscle fibers.* J Appl Physiol (1985), 2002. **92**(6): p. 2429-38.
96. MacInnis, M.J., et al., *Superior mitochondrial adaptations in human skeletal muscle after interval compared to continuous single-leg cycling matched for total work.* J Physiol, 2017. **595**(9): p. 2955-2968.
97. Baekkerud, F.H., et al., *Comparison of Three Popular Exercise Modalities on V O2max in Overweight and Obese.* Med Sci Sports Exerc, 2016. **48**(3): p. 491-8.

98. Henriksson, J. and J.S. Reitman, *Quantitative measures of enzyme activities in type I and type II muscle fibres of man after training.* Acta Physiol Scand, 1976. **97**(3): p. 392-7.
99. Granata, C., et al., *Training intensity modulates changes in PGC-1alpha and p53 protein content and mitochondrial respiration, but not markers of mitochondrial content in human skeletal muscle.* FASEB J, 2016. **30**(2): p. 959-70.
100. Helgerud, J., et al., *Aerobic high-intensity intervals improve VO2max more than moderate training.* Med Sci Sports Exerc, 2007. **39**(4): p. 665-71.
101. Baldwin, J., R.J. Snow, and M.A. Febbraio, *Effect of training status and relative exercise intensity on physiological responses in men.* Med Sci Sports Exerc, 2000. **32**(9): p. 1648-54.
102. Yu, M., et al., *Metabolic and mitogenic signal transduction in human skeletal muscle after intense cycling exercise.* J Physiol, 2003. **546**(Pt 2): p. 327-35.
103. Jacobs, R.A., et al., *Twenty-eight days at 3454-m altitude diminishes respiratory capacity but enhances efficiency in human skeletal muscle mitochondria.* FASEB J, 2012. **26**(12): p. 5192-200.
104. Salvadego, D., et al., *Skeletal muscle oxidative function in vivo and ex vivo in athletes with marked hypertrophy from resistance training.* J Appl Physiol (1985), 2013. **114**(11): p. 1527-35.
105. Wyckelsma, V.L., et al., *Preservation of skeletal muscle mitochondrial content in older adults: relationship between mitochondria, fibre type and high-intensity exercise training.* J Physiol, 2017. **595**(11): p. 3345-3359.
106. Hedges, C.P., et al., *Peripheral blood mononuclear cells do not reflect skeletal muscle mitochondrial function or adaptation to high-intensity interval training in healthy young men.* J Appl Physiol (1985), 2019. **126**(2): p. 454-461.
107. Vaccari, F., et al., *Effects of 3-month high-intensity interval training vs. moderate endurance training and 4-month follow-up on fat metabolism, cardiorespiratory function and mitochondrial respiration in obese adults.* Eur J Appl Physiol, 2020. **120**(8): p. 1787-1803.
108. Levinger, I., et al., *The influence of alpha-actinin-3 deficiency on bone remodelling markers in young men.* Bone, 2017. **98**: p. 26-30.
109. Little, J.P., et al., *A practical model of low-volume high-intensity interval training induces mitochondrial biogenesis in human skeletal muscle: potential mechanisms.* J Physiol, 2010. **588**(Pt 6): p. 1011-22.
110. Atakan, M.M., et al., *Six high-intensity interval training sessions over 5 days increases maximal oxygen uptake, endurance capacity, and sub-maximal exercise fat oxidation as much as 6 high-intensity interval training sessions over 2 weeks.* J Sport Health Sci, 2021. **10**(4): p. 478-487.
111. Talanian, J.L., et al., *Two weeks of high-intensity aerobic interval training increases the capacity for fat oxidation during exercise in women.* J Appl Physiol (1985), 2007. **102**(4): p. 1439-47.
112. Safdar, A., et al., *Exercise increases mitochondrial PGC-1 alpha content and promotes nuclear-mitochondrial cross-talk to coordinate mitochondrial biogenesis.* J Biol Chem, 2018. **293**(13): p. 4953.
113. Baar, K., et al., *Adaptations of skeletal muscle to exercise: rapid increase in the transcriptional coactivator PGC-1.* FASEB J, 2002. **16**(14): p. 1879-86.
114. Combes, A., et al., *Exercise-induced metabolic fluctuations influence AMPK, p38-MAPK and CaMKII phosphorylation in human skeletal muscle.* Physiol Rep, 2015. **3**(9).

115. Mathai, A.S., et al., *Rapid exercise-induced changes in PGC-1alpha mRNA and protein in human skeletal muscle.* J Appl Physiol (1985), 2008. **105**(4): p. 1098-105.
116. Papadimitriou, I.D., et al., *A "human knockout" model to investigate the influence of the alpha-actinin-3 protein on exercise-induced mitochondrial adaptations.* Sci Rep, 2019. **9**(1): p. 12688.
117. Yan, X., et al., *ACE I/D gene variant predicts ACE enzyme content in blood but not the ACE, UCP2, and UCP3 protein content in human skeletal muscle in the Gene SMART study.* J Appl Physiol (1985), 2018. **125**(3): p. 923-930.
118. Granata, C., et al., *Forty high-intensity interval training sessions blunt exercise-induced changes in the nuclear protein content of PGC-1alpha and p53 in human skeletal muscle.* Am J Physiol Endocrinol Metab, 2020. **318**(2): p. E224-E236.
119. Granata, C., et al., *Sprint-interval but not continuous exercise increases PGC-1alpha protein content and p53 phosphorylation in nuclear fractions of human skeletal muscle.* Sci Rep, 2017. **7**: p. 44227.
120. Cochran, A.J., et al., *Intermittent and continuous high-intensity exercise training induce similar acute but different chronic muscle adaptations.* Exp Physiol, 2014. **99**(5): p. 782-91.
121. Holmes, B.F., E.J. Kurth-Kraczek, and W.W. Winder, *Chronic activation of 5'-AMP-activated protein kinase increases GLUT-4, hexokinase, and glycogen in muscle.* J Appl Physiol (1985), 1999. **87**(5): p. 1990-5.
122. Lehman, J.J., et al., *Peroxisome proliferator-activated receptor gamma coactivator-1 promotes cardiac mitochondrial biogenesis.* J Clin Invest, 2000. **106**(7): p. 847-56.
123. Malek, M.H., et al., *Similar skeletal muscle angiogenic and mitochondrial signalling following 8 weeks of endurance exercise in mice: discontinuous versus continuous training.* Exp Physiol, 2013. **98**(3): p. 807-18.
124. Folkman, J., *Seminars in Medicine of the Beth Israel Hospital, Boston. Clinical applications of research on angiogenesis.* N Engl J Med, 1995. **333**(26): p. 1757-63.
125. Banting, L.K., et al., *Elite athletes' genetic predisposition for altered risk of complex metabolic traits.* BMC Genomics, 2015. **16**: p. 25.
126. Lin, J., et al., *Transcriptional co-activator PGC-1 alpha drives the formation of slow-twitch muscle fibres.* Nature, 2002. **418**(6899): p. 797-801.
127. Handschin, C., et al., *Skeletal muscle fiber-type switching, exercise intolerance, and myopathy in PGC-1alpha muscle-specific knock-out animals.* J Biol Chem, 2007. **282**(41): p. 30014-21.
128. Lahteenvuo, J. and A. Rosenzweig, *Effects of aging on angiogenesis.* Circ Res, 2012. **110**(9): p. 1252-64.
129. Tan, R., et al., *Skeletal muscle fiber-type-specific changes in markers of capillary and mitochondrial content after low-volume interval training in overweight women.* Physiol Rep, 2018. **6**(5).
130. Kwak, S.E., et al., *Angiogenesis: focusing on the effects of exercise in aging and cancer.* J Exerc Nutrition Biochem, 2018. **22**(3): p. 21-26.
131. Ucuzian, A.A., et al., *Molecular mediators of angiogenesis.* J Burn Care Res, 2010. **31**(1): p. 158-75.
132. Thurston, G., et al., *Angiopoietin-1 protects the adult vasculature against plasma leakage.* Nat Med, 2000. **6**(4): p. 460-3.
133. Maisonpierre, P.C., et al., *Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis.* Science, 1997. **277**(5322): p. 55-60.
134. Guo, D., et al., *VEGF stimulated the angiogenesis by promoting the mitochondrial functions.* Oncotarget, 2017. **8**(44): p. 77020-77027.

135. Shin, K.O., et al., *The effect of exercise on expression of myokine and angiogenesis mRNA in skeletal muscle of high fat diet induced obese rat.* J Exerc Nutrition Biochem, 2015. **19**(2): p. 91-8.
136. Hoier, B., et al., *Intense intermittent exercise provides weak stimulus for vascular endothelial growth factor secretion and capillary growth in skeletal muscle.* Exp Physiol, 2013. **98**(2): p. 585-97.
137. Linthorne, N.P., *Improvement in 100-m Sprint Performance at an Altitude of 2250 m.* Sports (Basel), 2016. **4**(2).
138. Favier, R., et al., *Maximal exercise performance in chronic hypoxia and acute normoxia in high-altitude natives.* J Appl Physiol (1985), 1995. **78**(5): p. 1868-74.
139. Brutsaert, T., *Why Are High Altitude Natives So Strong at High Altitude? Nature vs. Nurture: Genetic Factors vs. Growth and Development.* Adv Exp Med Biol, 2016. **903**: p. 101-12.
140. Marconi, C., M. Marzorati, and P. Cerretelli, *Work capacity of permanent residents of high altitude.* High Alt Med Biol, 2006. **7**(2): p. 105-15.
141. Wilber, R.L., *Application of altitude/hypoxic training by elite athletes.* Med Sci Sports Exerc, 2007. **39**(9): p. 1610-24.
142. Bailey, D.M. and B. Davies, *Physiological implications of altitude training for endurance performance at sea level: a review.* Br J Sports Med, 1997. **31**(3): p. 183-90.
143. Fagenholz, P.J. and N.S. Harris, *Hypoxia and inflammation.* N Engl J Med, 2011. **364**(20): p. 1976; author reply 1977.
144. Semenza, G.L., *Regulation of mammalian O2 homeostasis by hypoxia-inducible factor 1.* Annu Rev Cell Dev Biol, 1999. **15**: p. 551-78.
145. Semenza, G.L., et al., *Hypoxia-inducible nuclear factors bind to an enhancer element located 3' to the human erythropoietin gene.* Proc Natl Acad Sci U S A, 1991. **88**(13): p. 5680-4.
146. Wang, G.L. and G.L. Semenza, *General involvement of hypoxia-inducible factor 1 in transcriptional response to hypoxia.* Proc Natl Acad Sci U S A, 1993. **90**(9): p. 4304-8.
147. Thomas, L.W. and M. Ashcroft, *Exploring the molecular interface between hypoxia-inducible factor signalling and mitochondria.* Cell Mol Life Sci, 2019. **76**(9): p. 1759-1777.
148. Semenza, G.L., et al., *Hypoxia response elements in the aldolase A, enolase 1, and lactate dehydrogenase A gene promoters contain essential binding sites for hypoxia-inducible factor 1.* J Biol Chem, 1996. **271**(51): p. 32529-37.
149. Wenger, R.H., *Cellular adaptation to hypoxia: O2-sensing protein hydroxylases, hypoxia-inducible transcription factors, and O2-regulated gene expression.* FASEB J, 2002. **16**(10): p. 1151-62.
150. Doria, C., et al., *Improved VO2 uptake kinetics and shift in muscle fiber type in high-altitude trekkers.* J Appl Physiol (1985), 2011. **111**(6): p. 1597-605.
151. Zoll, J., et al., *Exercise training in normobaric hypoxia in endurance runners. III. Muscular adjustments of selected gene transcripts.* J Appl Physiol (1985), 2006. **100**(4): p. 1258-66.
152. Zimna, A. and M. Kurpisz, *Hypoxia-Inducible Factor-1 in Physiological and Pathophysiological Angiogenesis: Applications and Therapies.* Biomed Res Int, 2015. **2015**: p. 549412.
153. Kelly, B.D., et al., *Cell type-specific regulation of angiogenic growth factor gene expression and induction of angiogenesis in nonischemic tissue by a constitutively active form of hypoxia-inducible factor 1.* Circ Res, 2003. **93**(11): p. 1074-81.

154. Carroll, V.A. and M. Ashcroft, *Role of hypoxia-inducible factor (HIF)-1alpha versus HIF-2alpha in the regulation of HIF target genes in response to hypoxia, insulin-like growth factor-I, or loss of von Hippel-Lindau function: implications for targeting the HIF pathway.* Cancer Res, 2006. **66**(12): p. 6264-70.
155. Greijer, A.E., et al., *Up-regulation of gene expression by hypoxia is mediated predominantly by hypoxia-inducible factor 1 (HIF-1).* J Pathol, 2005. **206**(3): p. 291-304.
156. Ceradini, D.J., et al., *Progenitor cell trafficking is regulated by hypoxic gradients through HIF-1 induction of SDF-1.* Nat Med, 2004. **10**(8): p. 858-64.
157. Manalo, D.J., et al., *Transcriptional regulation of vascular endothelial cell responses to hypoxia by HIF-1.* Blood, 2005. **105**(2): p. 659-69.
158. Zhang, K., et al., *Baicalin increases VEGF expression and angiogenesis by activating the ERR{alpha}/PGC-1{alpha} pathway.* Cardiovasc Res, 2011. **89**(2): p. 426-35.
159. Arany, Z., et al., *HIF-independent regulation of VEGF and angiogenesis by the transcriptional coactivator PGC-1alpha.* Nature, 2008. **451**(7181): p. 1008-12.
160. Lee, P., N.S. Chandel, and M.C. Simon, *Cellular adaptation to hypoxia through hypoxia inducible factors and beyond.* Nat Rev Mol Cell Biol, 2020. **21**(5): p. 268-283.
161. Lee, S.H., et al., *Early expression of angiogenesis factors in acute myocardial ischemia and infarction.* N Engl J Med, 2000. **342**(9): p. 626-33.
162. Chen, L., A. Endler, and F. Shibasaki, *Hypoxia and angiogenesis: regulation of hypoxia-inducible factors via novel binding factors.* Exp Mol Med, 2009. **41**(12): p. 849-57.
163. Fraser, J., et al., *Hypoxia-inducible myoglobin expression in nonmuscle tissues.* Proc Natl Acad Sci U S A, 2006. **103**(8): p. 2977-81.
164. Lundby, C., J.A. Calbet, and P. Robach, *The response of human skeletal muscle tissue to hypoxia.* Cell Mol Life Sci, 2009. **66**(22): p. 3615-23.
165. Disanzo, B.L. and T. You, *Effects of exercise training on indicators of adipose tissue angiogenesis and hypoxia in obese rats.* Metabolism, 2014. **63**(4): p. 452-5.
166. Ameln, H., et al., *Physiological activation of hypoxia inducible factor-1 in human skeletal muscle.* FASEB J, 2005. **19**(8): p. 1009-11.
167. Nagahisa, H., et al., *Effect of High-Intensity Training in Normobaric Hypoxia on Thoroughbred Skeletal Muscle.* Oxid Med Cell Longev, 2016. **2016**: p. 1535367.
168. Kon, M., et al., *Effects of systemic hypoxia on human muscular adaptations to resistance exercise training.* Physiol Rep, 2015. **3**(1).
169. Zebrowska, A., et al., *Comparison of the Effectiveness of High-Intensity Interval Training in Hypoxia and Normoxia in Healthy Male Volunteers: A Pilot Study.* Biomed Res Int, 2019. **2019**: p. 7315714.
170. Biddlestone, J., D. Bandarra, and S. Rocha, *The role of hypoxia in inflammatory disease (review).* Int J Mol Med, 2015. **35**(4): p. 859-69.
171. Yan, X., et al., *Maternal obesity-impaired insulin signaling in sheep and induced lipid accumulation and fibrosis in skeletal muscle of offspring.* Biol Reprod, 2011. **85**(1): p. 172-8.
172. Gleeson, M., et al., *The anti-inflammatory effects of exercise: mechanisms and implications for the prevention and treatment of disease.* Nat Rev Immunol, 2011. **11**(9): p. 607-15.
173. Petersen, A.M. and B.K. Pedersen, *The anti-inflammatory effect of exercise.* J Appl Physiol (1985), 2005. **98**(4): p. 1154-62.
174. Pedersen, B.K., *Anti-inflammatory effects of exercise: role in diabetes and cardiovascular disease.* Eur J Clin Invest, 2017. **47**(8): p. 600-611.

175. Kaisho, T. and S. Akira, *Toll-like receptor function and signaling.* J Allergy Clin Immunol, 2006. **117**(5): p. 979-87; quiz 988.
176. Takeda, K., T. Kaisho, and S. Akira, *Toll-like receptors.* Annu Rev Immunol, 2003. **21**: p. 335-76.
177. Kolmychkova, K.I., et al., *Mitochondria and inflammation.* Patol Fiziol Eksp Ter, 2016. **60**(4): p. 114-21.
178. Missiroli, S., et al., *Mitochondria-associated membranes (MAMs) and inflammation.* Cell Death Dis, 2018. **9**(3): p. 329.
179. Meyer, A., et al., *Mitochondria: An Organelle of Bacterial Origin Controlling Inflammation.* Front Immunol, 2018. **9**: p. 536.
180. Green, D.R., L. Galluzzi, and G. Kroemer, *Mitochondria and the autophagy-inflammation-cell death axis in organismal aging.* Science, 2011. **333**(6046): p. 1109-12.
181. Zimowska, M., et al., *Inflammatory response during slow- and fast-twitch muscle regeneration.* Muscle Nerve, 2017. **55**(3): p. 400-409.
182. da Rocha, A.L., et al., *Treadmill Slope Modulates Inflammation, Fiber Type Composition, Androgen, and Glucocorticoid Receptors in the Skeletal Muscle of Overtrained Mice.* Front Immunol, 2017. **8**: p. 1378.
183. Bloise, F.F., et al., *Differential Effects of Sepsis and Chronic Inflammation on Diaphragm Muscle Fiber Type, Thyroid Hormone Metabolism, and Mitochondrial Function.* Thyroid, 2016. **26**(4): p. 600-9.
184. Szade, A., et al., *Cellular and molecular mechanisms of inflammation-induced angiogenesis.* IUBMB Life, 2015. **67**(3): p. 145-59.
185. Bartels, K., A. Grenz, and H.K. Eltzschig, *Hypoxia and inflammation are two sides of the same coin.* Proc Natl Acad Sci U S A, 2013. **110**(46): p. 18351-2.
186. Hackett, P.H. and R.C. Roach, *High-altitude illness.* N Engl J Med, 2001. **345**(2): p. 107-14.
187. Hartmann, G., et al., *High altitude increases circulating interleukin-6, interleukin-1 receptor antagonist and C-reactive protein.* Cytokine, 2000. **12**(3): p. 246-52.
188. Rosenberger, P., et al., *Hypoxia-inducible factor-dependent induction of netrin-1 dampens inflammation caused by hypoxia.* Nat Immunol, 2009. **10**(2): p. 195-202.
189. Ye, J., *Emerging role of adipose tissue hypoxia in obesity and insulin resistance.* Int J Obes (Lond), 2009. **33**(1): p. 54-66.
190. Karhausen, J., et al., *Epithelial hypoxia-inducible factor-1 is protective in murine experimental colitis.* J Clin Invest, 2004. **114**(8): p. 1098-106.
191. Giatromanolaki, A., et al., *Hypoxia inducible factor 1alpha and 2alpha overexpression in inflammatory bowel disease.* J Clin Pathol, 2003. **56**(3): p. 209-13.
192. Buchheit, M. and P.B. Laursen, *High-intensity interval training, solutions to the programming puzzle. Part II: anaerobic energy, neuromuscular load and practical applications.* Sports Med, 2013. **43**(10): p. 927-54.
193. Clambey, E.T., et al., *Hypoxia-inducible factor-1 alpha-dependent induction of FoxP3 drives regulatory T-cell abundance and function during inflammatory hypoxia of the mucosa.* Proc Natl Acad Sci U S A, 2012. **109**(41): p. E2784-93.
194. Caris, A.V., et al., *Carbohydrate and glutamine supplementation modulates the Th1/Th2 balance after exercise performed at a simulated altitude of 4500 m.* Nutrition, 2014. **30**(11-12): p. 1331-6.
195. Blegen, M., et al., *The immunological and metabolic responses to exercise of varying intensities in normoxic and hypoxic environments.* J Strength Cond Res, 2008. **22**(5): p. 1638-44.

196. Richardson, A.J., et al., *Similar Inflammatory Responses following Sprint Interval Training Performed in Hypoxia and Normoxia.* Front Physiol, 2016. **7**: p. 332.
197. Sumi, D., C. Kojima, and K. Goto, *Impact of Endurance Exercise in Hypoxia on Muscle Damage, Inflammatory and Performance Responses.* J Strength Cond Res, 2018. **32**(4): p. 1053-1062.
198. Morrison, J., et al., *The Post-Exercise Inflammatory Response to Repeated-Sprint Running in Hypoxia.* J Sports Sci Med, 2018. **17**(4): p. 533-538.
199. Kasai, N., et al., *Inflammatory, Oxidative Stress, and Angiogenic Growth Factor Responses to Repeated-Sprint Exercise in Hypoxia.* Front Physiol, 2019. **10**: p. 844.
200. Britto, F.A., et al., *Acute environmental hypoxia potentiates satellite cell-dependent myogenesis in response to resistance exercise through the inflammation pathway in human.* FASEB J, 2020. **34**(1): p. 1885-1900.
201. Kammerer, T., et al., *Hypoxic-inflammatory responses under acute hypoxia: In Vitro experiments and prospective observational expedition trial.* Int J Mol Sci, 2020. **21**(3).
202. Woo, J., et al., *Effects of Hyperbaric Oxygen Therapy on Inflammation, Oxidative/Antioxidant Balance, and Muscle Damage after Acute Exercise in Normobaric, Normoxic and Hypobaric, Hypoxic Environments: A Pilot Study.* Int J Environ Res Public Health, 2020. **17**(20).
203. Baygutalp, F., et al., *Impacts of different intensities of exercise on inflammation and hypoxia markers in low altitude.* BMC Sports Sci Med Rehabil, 2021. **13**(1): p. 145.
204. Maciejczyk, M., et al., *Effect of Normobaric Hypoxia on Alterations in Redox Homeostasis, Nitrosative Stress, Inflammation, and Lysosomal Function following Acute Physical Exercise.* Oxid Med Cell Longev, 2022. **2022**: p. 4048543.
205. Davison, G., et al., *Metabolomic Response to Acute Hypoxic Exercise and Recovery in Adult Males.* Front Physiol, 2018. **9**: p. 1682.
206. Gyntelberg, F., et al., *Effect of training on the response of plasma glucagon to exercise.* J Appl Physiol Respir Environ Exerc Physiol, 1977. **43**(2): p. 302-5.
207. Little, J.P., et al., *Low-volume high-intensity interval training reduces hyperglycemia and increases muscle mitochondrial capacity in patients with type 2 diabetes.* J Appl Physiol (1985), 2011. **111**(6): p. 1554-60.
208. Larsen, J.J., et al., *The effect of altitude hypoxia on glucose homeostasis in men.* J Physiol, 1997. **504 (Pt 1)**(Pt 1): p. 241-9.
209. Mackenzie, R., et al., *Acute hypoxia and exercise improve insulin sensitivity (S(I) (2*)) in individuals with type 2 diabetes.* Diabetes Metab Res Rev, 2011. **27**(1): p. 94-101.
210. Cartee, G.D., et al., *Stimulation of glucose transport in skeletal muscle by hypoxia.* J Appl Physiol (1985), 1991. **70**(4): p. 1593-600.
211. Cooper, D.M., et al., *Glucose turnover in response to exercise during high- and low-FIO2 breathing in man.* Am J Physiol, 1986. **251**(2 Pt 1): p. E209-14.
212. De Groote, E., et al., *Effect of hypoxic exercise on glucose tolerance in healthy and prediabetic adults.* Am J Physiol Endocrinol Metab, 2021. **320**(1): p. E43-E54.
213. Scheffer, D.D.L. and A. Latini, *Exercise-induced immune system response: Anti-inflammatory status on peripheral and central organs.* Biochim Biophys Acta Mol Basis Dis, 2020. **1866**(10): p. 165823.
214. Jamnick, N.A., et al., *An Examination and Critique of Current Methods to Determine Exercise Intensity.* Sports Med, 2020. **50**(10): p. 1729-1756.
215. Mann, T., R.P. Lamberts, and M.I. Lambert, *Methods of prescribing relative exercise intensity: physiological and practical considerations.* Sports Med, 2013. **43**(7): p. 613-25.

216. Pettitt, R.W., et al., *Gas exchange threshold and VO2max testing for athletes: an update.* J Strength Cond Res, 2013. **27**(2): p. 549-55.
217. Beneke, R., *Methodological aspects of maximal lactate steady state-implications for performance testing.* Eur J Appl Physiol, 2003. **89**(1): p. 95-9.
218. Jones, A.M., et al., *Critical power: implications for determination of V O2max and exercise tolerance.* Med Sci Sports Exerc, 2010. **42**(10): p. 1876-90.
219. Sarkar, M., N. Niranjan, and P.K. Banyal, *Mechanisms of hypoxemia.* Lung India, 2017. **34**(1): p. 47-60.
220. Hughson, R.L., H.J. Green, and M.T. Sharratt, *Gas exchange, blood lactate, and plasma catecholamines during incremental exercise in hypoxia and normoxia.* J Appl Physiol (1985), 1995. **79**(4): p. 1134-41.
221. Ozcelik, O. and H. Kelestimur, *Effects of acute hypoxia on the estimation of lactate threshold from ventilatory gas exchange indices during an incremental exercise test.* Physiol Res, 2004. **53**(6): p. 653-9.
222. Osawa, T., T. Arimitsu, and H. Takahashi, *Hypoxia affects tissue oxygenation differently in the thigh and calf muscles during incremental running.* Eur J Appl Physiol, 2017. **117**(10): p. 2057-2064.
223. Girard, O., et al., *An Updated Panorama of "Living Low-Training High" Altitude/Hypoxic Methods.* Front Sports Act Living, 2020. **2**: p. 26.
224. Li, J., et al., *The Molecular Adaptive Responses of Skeletal Muscle to High-Intensity Exercise/Training and Hypoxia.* Antioxidants (Basel), 2020. **9**(8).
225. Atakan, M.M., et al., *Evidence-Based Effects of High-Intensity Interval Training on Exercise Capacity and Health: A Review with Historical Perspective.* Int J Environ Res Public Health, 2021. **18**(13).
226. Morton, J.P. and N.T. Cable, *Effects of intermittent hypoxic training on aerobic and anaerobic performance.* Ergonomics, 2005. **48**(11-14): p. 1535-46.
227. Roels, B., et al., *Effects of hypoxic interval training on cycling performance.* Med Sci Sports Exerc, 2005. **37**(1): p. 138-46.
228. Roels, B., et al., *Effects of intermittent hypoxic training on cycling performance in well-trained athletes.* Eur J Appl Physiol, 2007. **101**(3): p. 359-68.
229. Lecoultre, V., et al., *Training in hypoxia fails to further enhance endurance performance and lactate clearance in well-trained men and impairs glucose metabolism during prolonged exercise.* Exp Physiol, 2010. **95**(2): p. 315-30.
230. Chacaroun, S., et al., *Physiological responses to hypoxic constant-load and high-intensity interval exercise sessions in healthy subjects.* Eur J Appl Physiol, 2019. **119**(1): p. 123-134.
231. Sanchez, A.M.J. and F. Borrani, *Effects of intermittent hypoxic training performed at high hypoxia level on exercise performance in highly trained runners.* J Sports Sci, 2018. **36**(18): p. 2045-2052.
232. Park, H.Y. and K. Lim, *Effects of Hypoxic Training versus Normoxic Training on Exercise Performance in Competitive Swimmers.* J Sports Sci Med, 2017. **16**(4): p. 480-488.
233. Jung, W.S., S.W. Kim, and H.Y. Park, *Interval Hypoxic Training Enhances Athletic Performance and Does Not Adversely Affect Immune Function in Middle- and Long-Distance Runners.* Int J Environ Res Public Health, 2020. **17**(6).
234. Geiser, J., et al., *Training high--living low: changes of aerobic performance and muscle structure with training at simulated altitude.* Int J Sports Med, 2001. **22**(8): p. 579-85.
235. Truijens, M.J., et al., *Effect of high-intensity hypoxic training on sea-level swimming performances.* J Appl Physiol (1985), 2003. **94**(2): p. 733-43.

236. Dufour, S.P., et al., *Exercise training in normobaric hypoxia in endurance runners. I. Improvement in aerobic performance capacity.* J Appl Physiol (1985), 2006. **100**(4): p. 1238-48.
237. Ventura, N., et al., *The response of trained athletes to six weeks of endurance training in hypoxia or normoxia.* Int J Sports Med, 2003. **24**(3): p. 166-72.
238. Czuba, M., et al., *Intermittent Hypoxic Training at Lactate Threshold Intensity Improves Aiming Performance in Well-Trained Biathletes with Little Change of Cardiovascular Variables.* Biomed Res Int, 2019. **2019**: p. 1287506.
239. Yan, X., et al., *The gene SMART study: method, study design, and preliminary findings.* BMC Genomics, 2017. **18**(Suppl 8): p. 821.
240. Granata, C., et al., *Mitochondrial adaptations to high-volume exercise training are rapidly reversed after a reduction in training volume in human skeletal muscle.* FASEB J, 2016. **30**(10): p. 3413-3423.
241. Ferri, A., et al., *Fifteen days of moderate normobaric hypoxia does not affect mitochondrial function, and related genes and proteins, in healthy men.* Eur J Appl Physiol, 2021. **121**(8): p. 2323-2336.
242. Wehrlin, J.P. and J. Hallen, *Linear decrease in .VO2max and performance with increasing altitude in endurance athletes.* Eur J Appl Physiol, 2006. **96**(4): p. 404-12.
243. Cerretelli, P., P. Mognoni, and C. Marconi, *Aerobic and anaerobic metabolism in health and disease: the role of training.* Ann Clin Res, 1982. **14 Suppl 34**: p. 12-9.
244. Kayser, B., *Lactate during exercise at high altitude.* Eur J Appl Physiol Occup Physiol, 1996. **74**(3): p. 195-205.
245. Daussin, F.N., et al., *Effect of interval versus continuous training on cardiorespiratory and mitochondrial functions: relationship to aerobic performance improvements in sedentary subjects.* Am J Physiol Regul Integr Comp Physiol, 2008. **295**(1): p. R264-72.
246. Wright, D.C., et al., *Exercise-induced mitochondrial biogenesis begins before the increase in muscle PGC-1alpha expression.* J Biol Chem, 2007. **282**(1): p. 194-9.
247. Barres, R., et al., *Acute exercise remodels promoter methylation in human skeletal muscle.* Cell Metab, 2012. **15**(3): p. 405-11.
248. Egan, B., J.A. Hawley, and J.R. Zierath, *SnapShot: Exercise Metabolism.* Cell Metab, 2016. **24**(2): p. 342-342 e1.
249. Kuang, J., et al., *Interpretation of exercise-induced changes in human skeletal muscle mRNA expression depends on the timing of the post-exercise biopsies.* PeerJ, 2022. **10**: p. e12856.
250. Vogt, M. and H. Hoppeler, *Is hypoxia training good for muscles and exercise performance?* Prog Cardiovasc Dis, 2010. **52**(6): p. 525-33.
251. Camacho-Cardenosa, A., et al., *Effects of High-Intensity Interval Training Under Normobaric Hypoxia on Cardiometabolic Risk Markers in Overweight/Obese Women.* High Alt Med Biol, 2018. **19**(4): p. 356-366.
252. Menz, V., et al., *Cardiorespiratory Effects of One-Legged High-Intensity Interval Training in Normoxia and Hypoxia: A Pilot Study.* J Sports Sci Med, 2016. **15**(2): p. 208-13.
253. Papandreou, I., et al., *HIF-1 mediates adaptation to hypoxia by actively downregulating mitochondrial oxygen consumption.* Cell Metab, 2006. **3**(3): p. 187-97.
254. Brahimi-Horn, M.C., J. Chiche, and J. Pouyssegur, *Hypoxia signalling controls metabolic demand.* Curr Opin Cell Biol, 2007. **19**(2): p. 223-9.

255. Saxena, S., D. Shukla, and A. Bansal, *Augmentation of aerobic respiration and mitochondrial biogenesis in skeletal muscle by hypoxia preconditioning with cobalt chloride.* Toxicol Appl Pharmacol, 2012. **264**(3): p. 324-34.
256. Hoppeler, H., S. Klossner, and M. Vogt, *Training in hypoxia and its effects on skeletal muscle tissue.* Scand J Med Sci Sports, 2008. **18 Suppl 1**: p. 38-49.
257. Mackenzie, R., et al., *Intermittent exercise with and without hypoxia improves insulin sensitivity in individuals with type 2 diabetes.* J Clin Endocrinol Metab, 2012. **97**(4): p. E546-55.
258. Torres-Peralta, R., et al., *Muscle activation during exercise in severe acute hypoxia: role of absolute and relative intensity.* High Alt Med Biol, 2014. **15**(4): p. 472-82.
259. Nordsborg, N.B., et al., *Relative workload determines exercise-induced increases in PGC-1alpha mRNA.* Med Sci Sports Exerc, 2010. **42**(8): p. 1477-84.
260. Saner, N.J., et al., *The effect of sleep restriction, with or without high-intensity interval exercise, on myofibrillar protein synthesis in healthy young men.* J Physiol, 2020. **598**(8): p. 1523-1536.
261. Kuang, J., et al., *Assessing mitochondrial respiration in permeabilized fibres and biomarkers for mitochondrial content in human skeletal muscle.* Acta Physiol (Oxf), 2022. **234**(2): p. e13772.
262. Jacques, M., et al., *Mitochondrial respiration variability and simulations in human skeletal muscle: The Gene SMART study.* FASEB J, 2020. **34**(2): p. 2978-2986.
263. Livak, K.J. and T.D. Schmittgen, *Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method.* Methods, 2001. **25**(4): p. 402-8.
264. Xie, F., et al., *miRDeepFinder: a miRNA analysis tool for deep sequencing of plant small RNAs.* Plant Mol Biol, 2012.
265. Deshmukh, A.S., et al., *Deep muscle-proteomic analysis of freeze-dried human muscle biopsies reveals fiber type-specific adaptations to exercise training.* Nat Commun, 2021. **12**(1): p. 304.
266. Li, Y., et al., *Methods to match high-intensity interval exercise intensity in hypoxia and normoxia - A pilot study.* J Exerc Sci Fit, 2022. **20**(1): p. 70-76.
267. Murray, A.J. and J.A. Horscroft, *Mitochondrial function at extreme high altitude.* J Physiol, 2016. **594**(5): p. 1137-49.
268. Perry, C.G., et al., *Repeated transient mRNA bursts precede increases in transcriptional and mitochondrial proteins during training in human skeletal muscle.* J Physiol, 2010. **588**(Pt 23): p. 4795-810.
269. Daussin, F.N., et al., *Different timing of changes in mitochondrial functions following endurance training.* Med Sci Sports Exerc, 2012. **44**(2): p. 217-24.
270. Semenza, G.L., *Hypoxia-inducible factor 1 (HIF-1) pathway.* Sci STKE, 2007. **2007**(407): p. cm8.
271. Forsythe, J.A., et al., *Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1.* Mol Cell Biol, 1996. **16**(9): p. 4604-13.
272. Wenger, R.H. and M. Gassmann, *Oxygen(es) and the hypoxia-inducible factor-1.* Biol Chem, 1997. **378**(7): p. 609-16.
273. Shoag, J. and Z. Arany, *Regulation of hypoxia-inducible genes by PGC-1 alpha.* Arterioscler Thromb Vasc Biol, 2010. **30**(4): p. 662-6.
274. Jung, S. and K. Kim, *Exercise-induced PGC-1alpha transcriptional factors in skeletal muscle.* Integr Med Res, 2014. **3**(4): p. 155-160.
275. Lin, J., C. Handschin, and B.M. Spiegelman, *Metabolic control through the PGC-1 family of transcription coactivators.* Cell Metab, 2005. **1**(6): p. 361-70.

276. Wu, Z., et al., *Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1.* Cell, 1999. **98**(1): p. 115-24.
277. Puigserver, P. and B.M. Spiegelman, *Peroxisome proliferator-activated receptor-gamma coactivator 1 alpha (PGC-1 alpha): transcriptional coactivator and metabolic regulator.* Endocr Rev, 2003. **24**(1): p. 78-90.
278. Kelly, D.P. and R.C. Scarpulla, *Transcriptional regulatory circuits controlling mitochondrial biogenesis and function.* Genes Dev, 2004. **18**(4): p. 357-68.
279. Beyfuss, K. and D.A. Hood, *A systematic review of p53 regulation of oxidative stress in skeletal muscle.* Redox Rep, 2018. **23**(1): p. 100-117.
280. Ljubicic, V., et al., *Transcriptional and post-transcriptional regulation of mitochondrial biogenesis in skeletal muscle: effects of exercise and aging.* Biochim Biophys Acta, 2010. **1800**(3): p. 223-34.
281. Matoba, S., et al., *p53 regulates mitochondrial respiration.* Science, 2006. **312**(5780): p. 1650-3.
282. Chen, Y.W., et al., *Response of rat muscle to acute resistance exercise defined by transcriptional and translational profiling.* J Physiol, 2002. **545**(1): p. 27-41.
283. Wang, C., et al., *Hypoxia Inhibits Myogenic Differentiation through p53 Protein-dependent Induction of Bhlhe40 Protein.* J Biol Chem, 2015. **290**(50): p. 29707-16.
284. Zhou, M., et al., *UCP-3 expression in skeletal muscle: effects of exercise, hypoxia, and AMP-activated protein kinase.* Am J Physiol Endocrinol Metab, 2000. **279**(3): p. E622-9.
285. Tsuboyama-Kasaoka, N., et al., *Up-regulation of uncoupling protein 3 (UCP3) mRNA by exercise training and down-regulation of UCP3 by denervation in skeletal muscles.* Biochem Biophys Res Commun, 1998. **247**(2): p. 498-503.
286. Boss, O., et al., *Effect of endurance training on mRNA expression of uncoupling proteins 1, 2, and 3 in the rat.* FASEB J, 1998. **12**(3): p. 335-9.
287. Cortright, R.N., et al., *Regulation of skeletal muscle UCP-2 and UCP-3 gene expression by exercise and denervation.* Am J Physiol, 1999. **276**(1): p. E217-21.
288. Fiorenza, M., et al., *Metabolic stress-dependent regulation of the mitochondrial biogenic molecular response to high-intensity exercise in human skeletal muscle.* J Physiol, 2018. **596**(14): p. 2823-2840.
289. Wang, L. and K. Sahlin, *The effect of continuous and interval exercise on PGC-1alpha and PDK4 mRNA in type I and type II fibres of human skeletal muscle.* Acta Physiol (Oxf), 2012. **204**(4): p. 525-32.
290. Edgett, B.A., et al., *Dissociation of increases in PGC-1alpha and its regulators from exercise intensity and muscle activation following acute exercise.* PLoS One, 2013. **8**(8): p. e71623.
291. Taylor, C.W., et al., *Exercise duration-matched interval and continuous sprint cycling induce similar increases in AMPK phosphorylation, PGC-1alpha and VEGF mRNA expression in trained individuals.* Eur J Appl Physiol, 2016. **116**(8): p. 1445-54.
292. Boyd, J.C., et al., *Reducing the intensity and volume of interval training diminishes cardiovascular adaptation but not mitochondrial biogenesis in overweight/obese men.* PLoS One, 2013. **8**(7): p. e68091.
293. Terada, S., et al., *Effects of low-intensity prolonged exercise on PGC-1 mRNA expression in rat epitrochlearis muscle.* Biochem Biophys Res Commun, 2002. **296**(2): p. 350-4.
294. Terada, S., et al., *Effects of high-intensity intermittent swimming on PGC-1alpha protein expression in rat skeletal muscle.* Acta Physiol Scand, 2005. **184**(1): p. 59-65.

295. Lira, V.A., et al., *PGC-1alpha regulation by exercise training and its influences on muscle function and insulin sensitivity.* Am J Physiol Endocrinol Metab, 2010. **299**(2): p. E145-61.
296. Wright, D.C., et al., *Calcium induces increases in peroxisome proliferator-activated receptor gamma coactivator-1alpha and mitochondrial biogenesis by a pathway leading to p38 mitogen-activated protein kinase activation.* J Biol Chem, 2007. **282**(26): p. 18793-9.
297. Jager, S., et al., *AMP-activated protein kinase (AMPK) action in skeletal muscle via direct phosphorylation of PGC-1alpha.* Proc Natl Acad Sci U S A, 2007. **104**(29): p. 12017-22.
298. Chen, J., et al., *Hypoxia exacerbates nonalcoholic fatty liver disease via the HIF-2alpha/PPARalpha pathway.* Am J Physiol Endocrinol Metab, 2019. **317**(4): p. E710-E722.
299. Alvarez-Herms, J., et al., *Anaerobic training in hypoxia: A new approach to stimulate the rating of effort perception.* Physiol Behav, 2016. **163**: p. 37-42.
300. Brocherie, F., et al., *Repeated maximal-intensity hypoxic exercise superimposed to hypoxic residence boosts skeletal muscle transcriptional responses in elite team-sport athletes.* Acta Physiol (Oxf), 2018. **222**(1).
301. Pilegaard, H., B. Saltin, and P.D. Neufer, *Exercise induces transient transcriptional activation of the PGC-1alpha gene in human skeletal muscle.* J Physiol, 2003. **546**(Pt 3): p. 851-8.
302. Hood, D.A., *Invited Review: contractile activity-induced mitochondrial biogenesis in skeletal muscle.* J Appl Physiol (1985), 2001. **90**(3): p. 1137-57.
303. D'Ignazio, L., D. Bandarra, and S. Rocha, *NF-kappaB and HIF crosstalk in immune responses.* FEBS J, 2016. **283**(3): p. 413-24.
304. Semenza, G.L., *Life with oxygen.* Science, 2007. **318**(5847): p. 62-4.
305. Corcoran, S.E. and L.A. O'Neill, *HIF1alpha and metabolic reprogramming in inflammation.* J Clin Invest, 2016. **126**(10): p. 3699-3707.
306. Gorlach, A. and S. Bonello, *The cross-talk between NF-kappaB and HIF-1: further evidence for a significant liaison.* Biochem J, 2008. **412**(3): p. e17-9.
307. Zhi, X., et al., *Physiological Hypoxia Enhances Stemness Preservation, Proliferation, and Bidifferentiation of Induced Hepatic Stem Cells.* Oxid Med Cell Longev, 2018. **2018**: p. 7618704.
308. Hockel, M. and P. Vaupel, *Tumor hypoxia: definitions and current clinical, biologic, and molecular aspects.* J Natl Cancer Inst, 2001. **93**(4): p. 266-76.
309. Moore, L.G., *Human Genetic Adaptation to High Altitudes: Current Status and Future Prospects.* Quat Int, 2017. **461**: p. 4-13.
310. Simonson, T.S., *Altitude Adaptation: A Glimpse Through Various Lenses.* High Alt Med Biol, 2015. **16**(2): p. 125-37.
311. Harris, A.J., et al., *HIF-mediated innate immune responses: cell signaling and therapeutic implications.* Hypoxia (Auckl), 2014. **2**: p. 47-58.
312. Eltzschig, H.K. and P. Carmeliet, *Hypoxia and inflammation.* N Engl J Med, 2011. **364**(7): p. 656-65.
313. Cruzat, V.F., M. Krause, and P. Newsholme, *Amino acid supplementation and impact on immune function in the context of exercise.* J Int Soc Sports Nutr, 2014. **11**(1): p. 61.
314. Bruunsgaard, H., et al., *Exercise-induced increase in serum interleukin-6 in humans is related to muscle damage.* J Physiol, 1997. **499 (Pt 3)**(Pt 3): p. 833-41.
315. Pinto, A., et al., *Effects of physical exercise on inflammatory markers of atherosclerosis.* Curr Pharm Des, 2012. **18**(28): p. 4326-49.

316. Karalaki, M., et al., *Muscle regeneration: cellular and molecular events.* In Vivo, 2009. **23**(5): p. 779-96.
317. Smith, L.R., G. Meyer, and R.L. Lieber, *Systems analysis of biological networks in skeletal muscle function.* Wiley Interdiscip Rev Syst Biol Med, 2013. **5**(1): p. 55-71.
318. Ahmed, H.M., et al., *Effects of physical activity on cardiovascular disease.* Am J Cardiol, 2012. **109**(2): p. 288-95.
319. Radom-Aizik, S., et al., *Brief bout of exercise alters gene expression in peripheral blood mononuclear cells of early- and late-pubertal males.* Pediatr Res, 2009. **65**(4): p. 447-52.
320. Santos, S.A., et al., *Vitamin E supplementation inhibits muscle damage and inflammation after moderate exercise in hypoxia.* J Hum Nutr Diet, 2016. **29**(4): p. 516-22.
321. Gjevestad, G.O., K.B. Holven, and S.M. Ulven, *Effects of Exercise on Gene Expression of Inflammatory Markers in Human Peripheral Blood Cells: A Systematic Review.* Curr Cardiovasc Risk Rep, 2015. **9**(7): p. 34.
322. Xiang, L., K.E. Rehm, and G.D. Marshall, Jr., *Effects of strenuous exercise on Th1/Th2 gene expression from human peripheral blood mononuclear cells of marathon participants.* Mol Immunol, 2014. **60**(2): p. 129-34.
323. Kimsa, M.C., et al., *Differential expression of inflammation-related genes after intense exercise.* Prague Med Rep, 2014. **115**(1-2): p. 24-32.
324. Glotov, A.S., et al., *RNA Sequencing of Whole Blood Defines the Signature of High Intensity Exercise at Altitude in Elite Speed Skaters.* Genes (Basel), 2022. **13**(4).
325. Vartanian, K., et al., *Gene expression profiling of whole blood: comparison of target preparation methods for accurate and reproducible microarray analysis.* BMC Genomics, 2009. **10**: p. 2.
326. Liburt, N.R., et al., *Exercise-induced increases in inflammatory cytokines in muscle and blood of horses.* Equine Vet J Suppl, 2010(38): p. 280-8.
327. Ferrante, J.A., et al., *A novel technique for isolating DNA from Tempus blood RNA tubes after RNA isolation.* BMC Res Notes, 2018. **11**(1): p. 563.
328. Kim, D., et al., *Graph-based genome alignment and genotyping with HISAT2 and HISAT-genotype.* Nat Biotechnol, 2019. **37**(8): p. 907-915.
329. Wijesooriya, K., et al., *Urgent need for consistent standards in functional enrichment analysis.* PLoS Comput Biol, 2022. **18**(3): p. e1009935.
330. Ernst, J. and Z. Bar-Joseph, *STEM: a tool for the analysis of short time series gene expression data.* BMC Bioinformatics, 2006. **7**: p. 191.
331. Sabio, G. and R.J. Davis, *TNF and MAP kinase signalling pathways.* Semin Immunol, 2014. **26**(3): p. 237-45.
332. Cai, Q., et al., *MYC-driven aggressive B-cell lymphomas: biology, entity, differential diagnosis and clinical management.* Oncotarget, 2015. **6**(36): p. 38591-616.
333. Mooren, F.C., et al., *Lymphocyte apoptosis after exhaustive and moderate exercise.* J Appl Physiol (1985), 2002. **93**(1): p. 147-53.
334. Durrer, C., et al., *Acute high-intensity interval exercise reduces human monocyte Toll-like receptor 2 expression in type 2 diabetes.* Am J Physiol Regul Integr Comp Physiol, 2017. **312**(4): p. R529-R538.
335. Moldoveanu, A.I., R.J. Shephard, and P.N. Shek, *The cytokine response to physical activity and training.* Sports Med, 2001. **31**(2): p. 115-44.
336. Svendsen, I.S., E. Hem, and M. Gleeson, *Effect of acute exercise and hypoxia on markers of systemic and mucosal immunity.* Eur J Appl Physiol, 2016. **116**(6): p. 1219-29.

337. Lundby, C. and A. Steensberg, *Interleukin-6 response to exercise during acute and chronic hypoxia.* Eur J Appl Physiol, 2004. **91**(1): p. 88-93.
338. Cerqueira, E., et al., *Inflammatory Effects of High and Moderate Intensity Exercise-A Systematic Review.* Front Physiol, 2019. **10**: p. 1550.
339. Brutsaert, T.D., *Do high-altitude natives have enhanced exercise performance at altitude?* Applied Physiology Nutrition and Metabolism, 2008. **33**(3): p. 582-592.
340. Hofmann, P. and G. Tschakert, *Special needs to prescribe exercise intensity for scientific studies.* Cardiol Res Pract, 2010. **2011**: p. 209302.
341. Hofmann, P., et al., *%HRmax target heart rate is dependent on heart rate performance curve deflection.* Med Sci Sports Exerc, 2001. **33**(10): p. 1726-31.
342. Thompson, P.D., et al., *ACSM's new preparticipation health screening recommendations from ACSM's guidelines for exercise testing and prescription, ninth edition.* Curr Sports Med Rep, 2013. **12**(4): p. 215-7.
343. Brawner, C.A., S.J. Keteyian, and J.K. Ehrman, *The relationship of heart rate reserve to VO2 reserve in patients with heart disease.* Med Sci Sports Exerc, 2002. **34**(3): p. 418-22.
344. Granata, C., N.A. Jamnick, and D.J. Bishop, *Principles of Exercise Prescription, and How They Influence Exercise-Induced Changes of Transcription Factors and Other Regulators of Mitochondrial Biogenesis.* Sports Med, 2018. **48**(7): p. 1541-1559.
345. Coyle, E.F., et al., *Determinants of endurance in well-trained cyclists.* J Appl Physiol (1985), 1988. **64**(6): p. 2622-30.
346. Czuba, M., et al., *The effects of high intensity interval training in normobaric hypoxia on aerobic capacity in basketball players.* J Hum Kinet, 2013. **39**: p. 103-14.
347. Ghaith, A., et al., *Hypoxic high-intensity interval training in individuals with overweight and obesity.* Am J Physiol Regul Integr Comp Physiol, 2022. **323**(5): p. R700-R709.
348. Kong, Z., et al., *High-Intensity Interval Training in Normobaric Hypoxia Improves Cardiorespiratory Fitness in Overweight Chinese Young Women.* Front Physiol, 2017. **8**: p. 175.
349. Huang, Y.C., et al., *A randomized controlled trial of enhancing hypoxia-mediated right cardiac mechanics and reducing afterload after high intensity interval training in sedentary men.* Sci Rep, 2021. **11**(1): p. 12564.
350. Hobbins, L., et al., *Psycho-physiological responses to perceptually-regulated interval runs in hypoxia and normoxia.* Physiol Behav, 2019. **209**: p. 112611.
351. Sousa, A., et al., *Effect of hypoxia and nitrate supplementation on different high-intensity interval-training sessions.* Eur J Appl Physiol, 2021. **121**(9): p. 2585-2594.
352. Bailey, D.P., et al., *Appetite and gut hormone responses to moderate-intensity continuous exercise versus high-intensity interval exercise, in normoxic and hypoxic conditions.* Appetite, 2015. **89**: p. 237-45.
353. Engfred, K., et al., *Hypoxia and training-induced adaptation of hormonal responses to exercise in humans.* Eur J Appl Physiol Occup Physiol, 1994. **68**(4): p. 303-9.
354. Masschelein, E., et al., *High twin resemblance for sensitivity to hypoxia.* Med Sci Sports Exerc, 2015. **47**(1): p. 74-81.
355. Pillon, N.J., et al., *Distinctive exercise-induced inflammatory response and exerkine induction in skeletal muscle of people with type 2 diabetes.* Sci Adv, 2022. **8**(36): p. eabo3192.
356. Eaton, M., et al., *Impact of a single bout of high-intensity interval exercise and short-term interval training on interleukin-6, FNDC5, and METRNL mRNA expression in human skeletal muscle.* J Sport Health Sci, 2018. **7**(2): p. 191-196.

357. Pham, K., et al., *Inflammatory gene expression during acute high-altitude exposure.* J Physiol, 2022. **600**(18): p. 4169-4186.
358. Zeviani, M. and S. Di Donato, *Mitochondrial disorders.* Brain, 2004. **127**(Pt 10): p. 2153-72.
359. Schaefer, A.M., et al., *The epidemiology of mitochondrial disorders--past, present and future.* Biochim Biophys Acta, 2004. **1659**(2-3): p. 115-20.
360. Jacques, M., et al., *Individual physiological and mitochondrial responses during 12 weeks of intensified exercise.* Physiol Rep, 2021. **9**(15): p. e14962.
361. Hecksteden, A., et al., *Repeated testing for the assessment of individual response to exercise training.* J Appl Physiol (1985), 2018. **124**(6): p. 1567-1579.
362. Ross, R., et al., *Precision exercise medicine: understanding exercise response variability.* Br J Sports Med, 2019. **53**(18): p. 1141-1153.
363. Murton, A.J., et al., *Transient transcriptional events in human skeletal muscle at the outset of concentric resistance exercise training.* J Appl Physiol (1985), 2014. **116**(1): p. 113-25.
364. Wilkinson, D.J., et al., *A validation of the application of D(2)O stable isotope tracer techniques for monitoring day-to-day changes in muscle protein subfraction synthesis in humans.* Am J Physiol Endocrinol Metab, 2014. **306**(5): p. E571-9.
365. Brocherie, F., et al., *High-intensity intermittent training in hypoxia: a double-blinded, placebo-controlled field study in youth football players.* J Strength Cond Res, 2015. **29**(1): p. 226-37.
366. Girard, O., J.P. Micallef, and G.P. Millet, *Changes in spring-mass model characteristics during repeated running sprints.* Eur J Appl Physiol, 2011. **111**(1): p. 125-34.