Human skeletal muscle transcriptomic analysis of pathways associated with autophagy and mitophagy in response to a single session of high-intensity interval exercise in hypoxia

by

Kangli Cui

Submitted in fulfilment of requirements of the degree of

Master of Research

Victoria University, Australia

Institute for Health and Sport (IHeS)

January 2024

Abstract

Autophagy/mitophagy are cellular processes that play pivotal roles in maintaining mitochondrial function; thus, understanding their responses to high-intensity interval exercise (HIIE) can provide insights into the overall adaptive mechanisms of skeletal muscle. This study aimed to investigate the effects of a single session of HIIE with and without hypoxia on transcriptomic response relating to autophagy/mitophagy. Ten healthy males (age: 26 ± 4 years; BMI: 23.3 ± 2) participated in three HIIE sessions randomly order: hypoxia (HY, simulated altitude of 3200m with an oxygen fraction of 0.14), normoxia matched to the relative intensity of hypoxia (NR), and normoxia matched to the absolute intensity of hypoxia (NA). Skeletal muscle samples were collected at four-time points: before HIIE (B), immediately post-HIIE (P0H), 3 hours post-HIIE (P3H), and 24 hours post-HIIE (P24H). Transcriptomic analysis was employed to identify broad patterns in gene expression related to autophagy/mitophagy in skeletal muscle.

The largest number of differentially expressed genes was observed in hypoxia after exercise 24 hours. PCA analysis did not show any significant difference between exercise conditions. Autophagy-animal and mitophagy-animal pathways (not ranked in the top 20) were significantly enriched only in hypoxia 24 hours post-exercise, and the key signaling pathway (mTOR signaling pathway) did not exhibit pronounced changes at all conditions after HIIE. To focus on autophagy/mitophagy-related genes/pathways, I chose 604 genes related to autophagy/mitophagy as target genes to perform differential expression analysis. Again, the largest number of differentially expressed genes related to autophagy/mitophagy was observed in hypoxia after exercise 24 hours. However, recognized autophagy markers such as *LC3II*, *LC3II/LC3I* ratio, *P62*, and mitophagy receptors (*NIX*, *BNIP3*) did not show significant differences in expression level in all conditions, and mitophagy receptor *FUNDC1* was observed in hypoxia, indicating a lack of robust change in autophagy/mitophagy after a single session of HIIE with and without hypoxia. Similar expression patterns were only observed between different time points under the same exercise condition. Although our result suggests an elevated level of genes related to autophagy/mitophagy pathways under hypoxia exercise, it did not correlate with physiological performance, as a higher percentage of HR_{peak} and $\dot{V}O_{2peak}$, and a higher RPE were observed in

both NR and HY compared to NA. Furthermore, the increased gene expression level in hypoxia is likely affected by time points after exercise. Our result suggests that activation of autophagy/mitophagy pathways is unlikely to be responsible for the difference in observed physiological response.

Student Declaration

"I, Kangli Cui, declare that the Master of Research thesis entitled "Human skeletal muscle transcriptomic analysis of pathways associated with autophagy and mitophagy in response to a single session of high-intensity interval exercise in hypoxia " is no more than 50,000 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."

"I have conducted my research in alignment with the Australian Code for the Responsible Conduct of Research and Victoria University's Higher Degree by Research Policy and Procedures."

Signature: Date: 10 January2024

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: [redacted] Date: 10 January 2024

Acknowledgements

I would like to take this opportunity to extend my heartfelt gratitude to the exceptional individuals who have provided me with invaluable help and unwavering support throughout my master's degree journey. First and foremost, I am deeply grateful to my esteemed supervisors, Dr. Jujiao Kuang, Dr. Sean Yan, and Prof. David Bishop from the Institute for Health and Sport at Victoria University (VU). Their unwavering dedication, invaluable guidance, and continuous provision of advice have been instrumental in shaping the direction and success of my research project. Their passion for scientific exploration and commitment to academic excellence have served as a constant source of inspiration, driving me to strive for excellence as a scientist.

Furthermore, I would like to express my sincere appreciation to Prof. Itamar Levinger from the Institute for Health and Sport at VU, as well as Prof. Li Peng, for their critical insights and valuable contributions to my master's projects. Their expertise and guidance have greatly enriched my research and fostered my academic growth.

In addition, I am deeply grateful to Zecheng Wu, Yuan Xu, Mingzhi Zeng, Liangyu Shi, and Dr. Luyun Ni from the Key Laboratory of Fish Reproduction and Development (Ministry of Education), School of Life Science at Southwest University for their invaluable assistance during the transcriptome analysis. I would also like to express my heartfelt thanks to Dr. Feng Shao for generously providing me with laboratory facilities and creating a conducive workspace. Furthermore, I extend my appreciation to Jia Li, a PhD student from the Institute for Health and Sport at VU, for her support in the preliminary experimental sampling and PCR analysis, as well as her guidance and clarification during the thesis writing process. I am equally grateful to Yimei Hao, a PhD student from the Shanghai Institute of Nutrition and Health, Chinese Academy of Sciences (CAS), for their valuable advice and assistance during the RNA-seq analysis. Their contributions have played a crucial role in the successful completion of my research project, and I am truly indebted to them for their expertise and support.

During my master's degree, I have not only acquired an abundance of knowledge but also undergone personal growth and transformation. The experience has broadened my horizons,

fostered critical thinking, and nurtured a deep passion for scientific inquiry. It is through the guidance and support of my supervisors and mentors that I have been able to navigate the challenges and overcome the obstacles encountered along the way.

Lastly, I would like to express my deepest gratitude to my husband Zheng liu, my son Congyi Liu, my parents and all my friends who have stood by me during this journey. Their unwavering belief in my abilities and their constant encouragement have been instrumental in my achievements.

In conclusion, my master's degree has been a transformative experience, both academically and personally. I am profoundly grateful to all those who have played a part in shaping my journey, and I carry their influence with me.

Conference Abstract:

1. “A single session of high intensity interval exercise in hypoxia modulates changes in mitochondrial biogenesis related gene and protein in human skeletal muscle”, Australia physiology Society Scientific Meeting, 20th-23rd November 2022, Jia Li, Jujiao Kuang, **Kangli Cui**, David Bishop, Xu Yan

Table of Contents

List of Figures

List of Tables

List of Abbreviations

AGR	anterior gradient
AMPK	adenosine monophosphate-activated protein kinase
AKT	v-akt murine thymoma viral oncogene homolog
ARL8B	ADP-ribosylation factor-like 8B
ATF3	Activating Transcription Factor 3
ATG14	autophagy related 14
ATP13A2	ATPase type 13A2
BCL2L1	BCL2-like 1
BNIP3	BCL2/adenovirus E1B 19kDa interacting protein 3
BNIP3L	BCL2/adenovirus E1B 19kDa interacting protein 3-like
CAPN2	calpain 2, (m/II) large subunit
CAST	capitatim
CD68	CD68 Molecule
CLCN6	chloride channel, voltage-sensitive 6
CTNS	cystinosin, lysosomal cystine transporter
CTSB	cathepsin B
CTSL	cathepsin L
DAPK3	death-associated protein kinase 3
DDIT3	DNA-damage-inducible transcript 3
DNASE2	deoxyribonuclease II, lysosomal
DRAM1	DNA Damage Regulated Autophagy Modulator 1
EEF2K	eukaryotic elongation factor-2 kinase
EGF	Epidermal Growth Factor
EGR1	Early Growth Response 1
ERK1/2	extracellular signal-regulated kinases 1/2
FIO2	inspired oxygen fraction
FLCN	folliculin
FNIP2	Folliculin Interacting Protein 2
FOS	FBJ murine osteosarcoma viral oncogene homolog
FOXO1	forkhead box O1
FUNDC1	FUN14 domain containing 1
GABARAPL1	GABA(A) receptor-associated protein like 1
GLYCTK	Glycerate Kinase
HIF1A	hypoxia inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)
HSPA1A	Heat Shock 70 KDa Protein 1A
IRF1	Interferon Regulatory Factor1
JNK	c-Jun NH2-terminal kinases
JUNB	Recombinant Jun B Proto Oncogene
KRAS	Kirsten rat sarcoma viral oncogene homolog
LAMP1	lysosomal-associated membrane protein 1
LC3	microtubule-associated proteins light chain 3（MAP1LC3）

LGALS8	lectin, galactoside-binding, soluble, 8
LIMK1	LIM domain kinase 1
LIR	LC3-interaction region
MAPKAP-K2	mitogen-activated protein kinase-activated protein kinase 2
Mnk1	MAPK interacting serine/threonine kinase 1
MRAS	muscle RAS oncogene homolog
MSK	Mitogen and stress-activated protein kinase
mTORC1	mTOR complex1
MYC	v-myc avian myelocytomatosis viral oncogene homolog
NACC1	nucleus accumbens associated 1, BEN and BTB (POZ) domain containing
NAGLU	N-acetylglucosaminidase, alpha
NFKB1	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1
NIX	BCL2/adenovirus E1B 19kDa interacting protein 3-like，（Bnip3L）
NPC1	NPC Intracellular Cholesterol Transporter 1
NRAS	neuroblastoma RAS viral (v-ras) oncogene homolog
P14K2A	phosphatidylinositol 4-kinase type 2 alpha
P38MAPK	p38 mitogen-activated protein kinases
P90RSK	90 kDa ribosomal s6 kinases (RSKs)
PARKIN	PINK1-Parkin
PCYOX1	prenylcysteine oxidase 1
PIK3C2B	phosphatidylinositol-4-phosphate 3-kinase, catalytic subunit type 2 beta
PINK1	PTEN-induced kinase 1
PPARA	peroxisome proliferator-activated receptor alpha
PPARGC1A	peroxisome proliferator-activated receptor gamma, coactivator 1 alpha
PPARGC1B	PPARG Coactivator 1 Beta
PRKAG2	protein kinase, AMP-activated, gamma 2 non-catalytic subunit
PRKAG3	protein kinase, AMP-activated, gamma 3 non-catalytic subunit
RAB27A	RAB27A, Member RAS Oncogene Family
RAGE	receptor for advanced glycation end products
RAMP2	receptor (G protein-coupled) activity modifying protein 2
RAMP3	receptor (G protein-coupled) activity modifying protein 3
RARA	Retinoic Acid Receptor Alpha
RELA	v-rel avian reticuloendotheliosis viral oncogene homolog A
RELB	RELB proto-oncogene
RICTOR	RPTOR independent companion of MTOR, complex 2
RRAGC	Ras-related GTP binding C
SEH1L	SEH1-like (S. cerevisiae)
SESN2	Sestrin 2
SH3GLB1	SH3-domain GRB2-like endophilin B1
SIRT7	Sirtuin 7
SLC36A1	Solute Carrier Family 36 Member 1
SLC38A9	solute carrier family 38, member 9
STAT3	signal transducer and activator of transcription 3 (acute-phase response factor)
STK39	Serine/Threonine Kinase 39
TBC1D15	TBC1 domain family, member 15

TFE3	Transcription Factor Binding To IGHM Enhancer 3
TGM2	Transglutaminase 2
TP53	tumor protein p53
TPCN1	two pore segment channel 1
WIPI1	WD repeat domain, phosphoinositide interacting 1
XBP1	X-Box Binding Protein 1
$\dot{V}O_2$	oxygen consumption
$\dot{V}O_{2max}$	maximal oxygen consumption
$\dot{V}O_{2peak}$	peak oxygen uptake
W_{max}	peak power output at exhaustion during the incremental exercise

Chapter 1

Review of Literature

1.1 The structure and function of mitochondria

The term “mitochondrion” originates from the Greek words “mito” (thread) and “chondros” (granule), and was introduced by Benda in 1898 [1]. Mitochondria (singular: mitochondrion) are membrane-bound organelles present in the cytoplasm of most eukaryotic cells, including animals, plants, and fungi. Their size ranges from 0.5-1 micrometers in diameter and up to 7 micrometers long [2]. With high plasticity and mobility, mitochondria can assume various shapes, such as spheres, rods, or filamentous bodies, while maintaining a consistent overall architecture [2, 3].

The structural intricacies of mitochondria, including the Outer Mitochondrial Membrane (OMM), Inner Mitochondrial Membrane (IMM), Intermembrane Space (IMS), Cristae, and Matrix [4], lay the foundation for the orchestration of vital cellular processes. The OMM, functioning as the outer boundary, allows the passage of ions and small molecules, setting the stage for interactions within the mitochondrial environment. Meanwhile, the IMM, highly folded into cristae, accommodates the Electron Transport Chain (ETC), a series of five multiprotein enzyme complexes critical for oxidative phosphorylation.

Within the IMM, these ETC complexes, such as ubiquinone oxidoreductase (complex I), succinate dehydrogenase (complex II), ubiquinol-cytochrome c oxidoreductase (complex III or cytochrome bc1 complex), cytochrome oxidase (complex IV), and ATP synthase (complex V), form the core responsible for ATP production [5]. Complex I, the largest enzyme in the ETC, initiates the oxidation of NADH molecules and pumps protons from the matrix to the intermembrane space [6, 7]. Simultaneously, Complex II facilitates the transfer of electrons from succinate to ubiquinone [6, 7]. As the electron transfer progresses through Complexes III and IV, the final complex combines electrons with inspired oxygen (O_2), acting as the ultimate electron acceptor [8]. The ensuing redox energy release is harnessed to transfer protons from the mitochondrial matrix to the intermembrane space, creating a proton-motive force across the inner mitochondrial membrane at complexes I, III, and IV [9]. Capitalizing on this force, Complex V, also known as ATP, synthesizes ATP from ADP and inorganic phosphate, completing the process of Oxidative Phosphorylation (OXPHOS) [5].

This detailed process sets up the essential activity of mitochondria, like a charged battery, keeping life going. The complex structure, explained earlier, is the foundation for smoothly

coordinating these processes, emphasizing how crucial mitochondria are for producing energy and managing metabolism in cells.

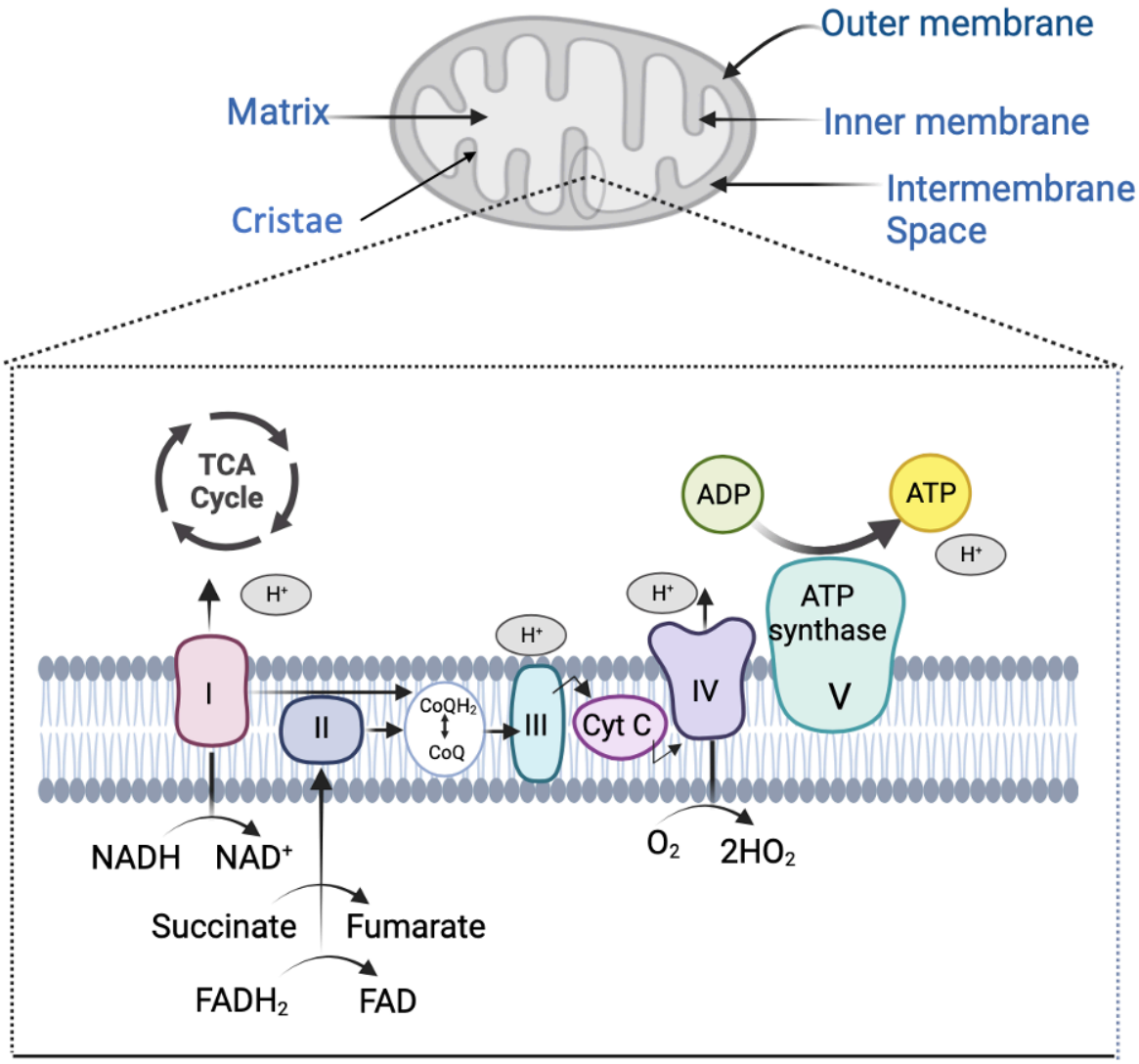


Figure 1. The structure of mitochondrion, the mitochondrial electron transport chain (ETC), and oxidative phosphorylation (OXPHOS). Mitochondria consist of two layers of membranes, including the inner membrane and outer membrane, and are parted by an intermembrane area. The inner membrane has several folds that form a layered structure called cristae. The space within the inner membrane of the mitochondrion is known as a matrix. Electrons are conveyed through Complex I to IV, causing electron leakage and, ultimately, the incorporation of electrons into oxygen (O_2). The proton (H+) gradient generated during electron transfer supplies the energy required for the synthesis of adenosine triphosphate (ATP) by the ultimate Complex V or ATP synthase. Oxidized ubiquinone (CoQ) corresponds to the reduced form of ubiquinone (CoQH2).

1.2 Mitochondria in human exercise performance

The significance of mitochondrial function in exercise performance and overall health has gained growing acknowledgment in recent decades [10]. The exploration of mitochondrial function's impact on exercise performance, as illuminated by Conley KE et al. [11], reveals the potential consequences of age and disease on electron transport chain capacity. However, both acute and chronic interventions [12-16] emerge as effective strategies to rejuvenate mitochondrial function, thereby enhancing ATP production and further impaction on overall muscle [17, 18] and exercise performance [19]. This investigation highlights the critical role of mitochondrial function in bioenergetics, particularly during physical exercise, where skeletal muscle energy turnover can surge significantly.

Pioneering research in the study of Hoppeler et al. found that a higher mitochondrial volume density has consistently been associated with enhanced exercise capacity [20]. The positive relationship between mitochondrial volume density and relative $\dot{V}O_{2peak}$ is evident, particularly when expressed per kilogram of body weight [21]. However, this correlation diminishes when expressed against absolute $\dot{V}O_{2peak}$. Despite the capacity for skeletal muscle oxidative respiration exceeding systemic O_2 transport, mitochondria exhibit adaptive responses to exercise training. Endurance training, characterized by prolonged sessions at 65% of peak power output, results in increased mitochondrial volume density, reflecting an improved capacity to oxidize fat and preserve limited carbohydrate stores [22]. Conversely, a cycling study revealed global improvements in mitochondrial respiratory capacity after examining the effects of repeated 60-second bouts of high-intensity exercise at a workload corresponding to peak power [23].

The dynamic control of ATP synthesis and alterations in mitochondrial function in response to diverse exercise stimuli highlights the complexity of mitochondrial adaptations. Notably, mass-specific skeletal muscle respiratory capacity emerges as a crucial determinant of human endurance performance [24]. These findings emphasize the importance of varying exercise intensity and duration within training programs to optimize skeletal muscle mitochondrial function and overall exercise performance.

1.3 Autophagy

Autophagy is a cellular degradation process by which cytoplasmic components, such as organelles, proteins, and nucleic acids, are delivered and degraded into the lysosomal lumen [25, 26]. In Greek, autophagy literally means “the process of the cell eating itself” [27]. The purpose of autophagy is to maintain cell renovation and homeostasis [25]. Autophagy is a highly conserved cell degradation and cycling process in eukaryotes [28], which can not only eliminate materials but also produce new building blocks and energy as a dynamic recycling system [25]. It is also highly conserved in the evolutionary process, and homologous proteins involved in autophagy can be found in yeast, nematodes, fruit flies, vertebrates, and mammals.

In mammalian cells, autophagy can be divided into three types: macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA), according to the different encapsulation substances and delivery modes [25, 28, 29] (Figure 2). The core of autophagy is the formation of autophagosomes [29]. Macroautophagy refers to the process in which autophagosomes with double-membrane vesicle structures fuse with lysosomes to form autolysosomes and degrade the materials contained in them [25, 30]. In contrast, microautophagy directly engulfs specific organelles or cytoplasmic contents by the inward invagination or deformation of the lysosomal membrane, while chaperone-mediated autophagy misfolds cytoplasm protein into the lysosomal lumen through molecular chaperone [30, 31]. Although each mode of autophagy is morphologically different, all three ultimately transport cargo to lysosomes for degradation and recycling [29]. In the 1990s, a series of autophagy-related genes (ATGs) were identified and analyzed in yeast genetic studies [32, 33], and these study findings greatly improved our understanding of the mechanisms and functions of autophagy [33]. To date, researchers have conducted a wide range of studies on the molecular mechanisms of macroautophagy, microautophagy, and CMA. However, the mechanisms and ways of regulation are diverse due to their different transport materials and forms.

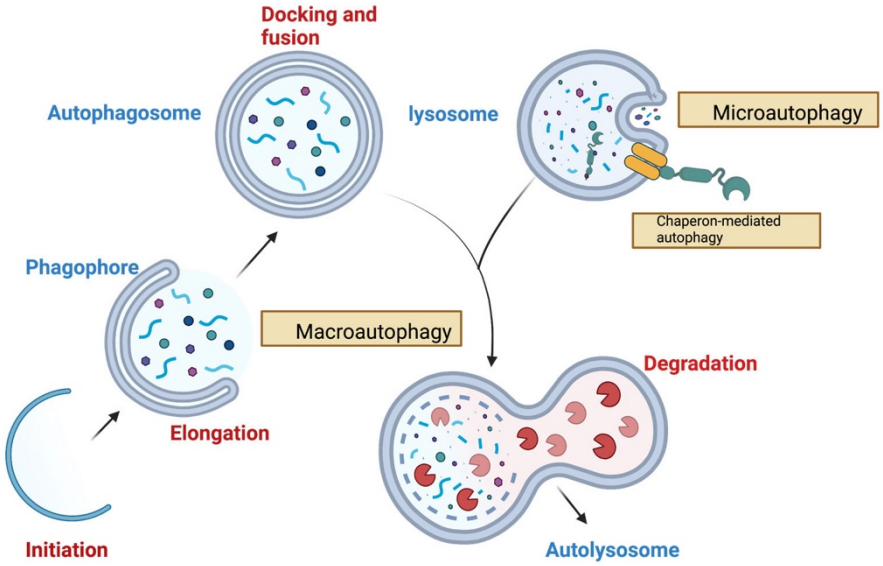


Figure 2. Three types of autophagy: Macroautophagy, Microautophagy, and Chaperone-mediated autophagy (CMA). Macroautophagy initiates with phagophore formation, evolving into autophagosomes that dock and fuse with lysosomes to create autolysosomes for material degradation. Microautophagy involves direct lysosomal engulfment of cargo through membrane invagination, leading to immediate degradation. Chaperone-mediated autophagy (CMA) selectively transports specific proteins recognized by chaperones into lysosomes, where unfolded proteins undergo degradation without vesicle formation.

Macroautophagy is considered the main type of autophagy and has been studied extensively compared to the other two types [25]. The formation of macroautophagy goes through induction, nucleation, elongation, autophagosome completion and fusion [28] (Figure 3).

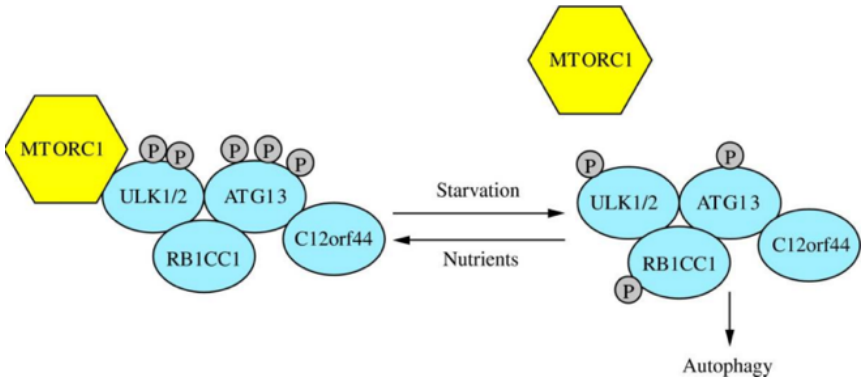


Figure 3. Macroautophagy machinery (This figure is adopted from Parzych and Kilionsky [28].)

Autophagy plays an essential role in human health and diseases, and autophagy dysfunction is related to the pathogenesis of various diseases, such as aging, Parkinson's disease, Alzheimer's disease, cancer, and so on [29, 34]. When cells are exposed to environmental stress, such as pathogen infection, nutrient deficiency, oxidative stress, hypoxia, drug treatment, or radiation, autophagy levels can increase significantly, leading to adaptation and survival as a cellular protective response [29]. Excessive and dysfunctional autophagy may promote cell death and further lead to apoptosis [35]. In short, the highly regulated autophagy process is essential for managing cellular damage [36].

1.4 Mitophagy

Mitochondria are organelles that exist in most cells, such as skeletal muscle, commonly known as the "powerhouses" of the cell, which are involved in cellular functions, including energy production and cell metabolism [37]. The role of mitochondria in mammalian cells is to generate Adenosine triphosphate (ATP), primarily through oxidative phosphorylation (OXPHOS) complexes [5]. Reactive oxygen species (ROS) are produced through the leakage of electrons from the ETC [38]. Excessive accumulation of ROS under stress conditions can damage mitochondrial DNA, protein, and lipids and then result in mitochondrial dysfunction [38]. These damages degrade the quality of mitochondria and further affect human health. At the organelle level, mitochondrial quality control systems are regulated by multiple mechanisms, including mitochondrial fission and fusion, mitochondrial biogenesis, and autophagy/mitophagy [39, 40]. Mitophagy is the core mechanism of mitochondrial quality control and can mediate the clearance of damaged mitochondria [36, 41].

In 1998, Scott and Klionsky proposed the term "mitophagy" as an example of a regulated organelle-specific degradation process [42]. Lemasters first defined mitophagy in 2005, which refers to the selective removal of damaged or abnormal mitochondria by autophagy [43]. However, the documented record of mitochondrial fragments of lysosomes observed by electron microscopy actually dates back to 1962 by Ashford and Porter [44]. Recent studies have also described "mitophagy" as a highly selective process that can promote the elimination of dysfunctional or unnecessary mitochondria [45, 46] or selective autophagy of mitochondria [41]. It was noticed that the term mitochondrial autophagy was commonly used in some literature [47, 48], and the terms

"mitochondrial autophagy" and "mitophagy" are often interchangeable with the same meaning. Tissues such as the nervous system, kidney, heart, liver, and skeletal muscle all show high basal mitophagy activity [49, 50], so some evidence suggests that metabolic and neurodegenerative diseases are associated with poorly functioning mitochondria [27].

To date, some different mechanisms have been proposed for the initiation of mitophagy by exercise (Figure 4). One means by which mitophagy is initiated by 5 'AMP-activated protein kinase (AMPK) is known as AMPK-ULK1 signaling pathway. The phosphatase and tensin homolog-induced putative kinase protein 1 (PINK1) system can also initiate mitophagy, namely the Pink1-Parkin signaling pathway. Endogenous mitochondrial membrane-bound receptor proteins initiate mitophagy, e.g., induction expression of BCL2/adenovirus E1B 19kD interacting protein 3 (BNIP3) and its homolog BNIP3L/NIX [51, 52]. PINK1 and Parkin are currently the most mature mechanisms mediating mitophagy in mammals [53].

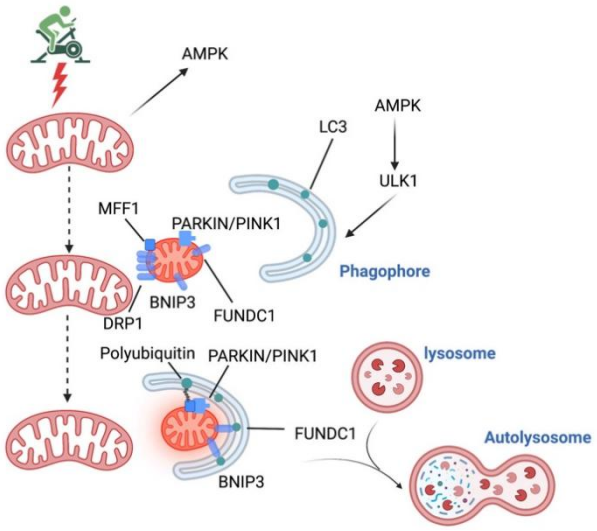


Figure 4. The molecular mechanisms of mitophagy. AMPK-ULK1 signaling pathway and PARKIN-PINK1 signaling pathway are involved in the initiation of mitophagy.

1.5 High-intensity interval exercise

High-intensity interval exercise (HIIE) in competitive sports has a history of more than 100 years [54]. In recent years, some researchers have worked to introduce HIIE to the general population due to its health benefits. In this context, HIIE has been at the forefront of exercise therapy because of its shorter duration benefit [55]. At present, there is no consensus on the

definition of high-intensity interval exercise. Therefore, terms such as high-intensity exercise (HIE), high-intensity training (HIT), and high-intensity interval training (HIIT) are used interchangeably in the literature but refer to different exercise types. The lack of consensus on defining high-intensity exercise is the main obstacle to reconciling some contradictory findings in the literature [56].

For this study, and without causing unnecessary contradictory findings, it first reviewed some definitions of researchers in the previous literature. A previous review study from our research group defined high-intensity exercise as the exercise intensity of more than 75% of the peak power output (PPO) achieved in the graded exercise test (GXT), and it was considered to include high-intensity interval exercise and training (HIIE and HIIT, respectively) and sprint interval exercise and training (SIE and SIT, respectively) [56]. Another review from our research group defined exercise as a single session and training as repetitive exercise sessions performed periodically [57] to better distinguish between exercise and training. Given this, we defined a single bout of high-intensity interval exercise in this study as the exercise intensity of 75% - 95% of the PPO in the GXT, lasting 1-5 min, separated by interval rests or low-intensity exercise. It is worth highlighting "the first bout" effect on molecular response in this study. In the context of HIIT, the term "first bout" refers to the initial exposure of an individual to the intense exercise regimen [58]. According to David Bishop's theory, during this inaugural session of HIIE, there is a unique and pronounced molecular response within the body. This initial bout is considered pivotal, as it sets the stage for subsequent physiological adaptations and influences the overall effectiveness of the training program. Understanding the molecular dynamics during this first bout provides valuable insights into the mechanisms driving the physiological changes associated with HIIT. Consequently, exercise-induced changes in mitochondrial protein synthesis tend to decrease when high-intensity exercise is repeated [59]. These observations indicate that changes in proteins, genes, and mitochondrial protein synthesis in individuals who are naïve to performing high-intensity exercise may represent an atypical response.

1.6 Exercise and mitochondrial adaptations in skeletal muscle

It is well known that exercise can promote human health and prevent chronic diseases. Human movement is highly dependent on skeletal muscle. As a dynamic organ, skeletal muscle is

responsible for performing the exercise required for daily life, physical performance, and metabolic health [60]. Additionally, skeletal muscle is a plastic tissue that responds to external stimuli, including exercise, by altering its phenotype [61]. Literature data showed that exercise adaptations in skeletal muscle can result in many exercise-induced benefits [51]. Skeletal muscle function is critical to human health and physical performance, and its health depends on the optimal function of mitochondria [62]. Mitochondria oxidize nutrient substrates and produce the majority of the Adenosine Triphosphate (ATP), which is used to fuel skeletal muscle contraction and metabolic activity [60, 63]. Mitochondrial adaptation is thought to be a key factor in the beneficial outcomes of exercise and exercise training [63]. The adaptation process involves the regulation of mitochondrial quantity and quality, specifically, the coordinated improvements of mitochondrial content, structure, and function [63].

Currently, our understanding of the molecular mechanism governing mitochondrial adaptation to exercise in the skeletal muscle still needs to be completed. This complexity arises not only from the multifaceted nature of the exercise response but also from the influence of various factors, including exercise type, intensity, volume, frequency, and duration [64, 65]. Most studies focus on measuring mitochondrial biogenesis, a process involving the synthesis of new proteins and mitochondrial DNA in the existing reticulum [66]. This anabolic mechanism has been extensively studied in the context of skeletal muscle. However, with the increasing availability of molecular tools that have allowed us to delve deeper into the intricacies of exercise adaptation, mitophagy has attracted some researchers' attention.

Mitophagy is a multi-step catabolic process that plays a critical role in regulating mitochondrial quality by facilitating the degradation of damaged and dysfunctional mitochondria [51]. While mitochondrial biogenesis and mitophagy are both essential components of mitochondrial adaptations, the delicate balance between these two processes ultimately leads to improved mitochondrial function (Figure 5). Emerging evidence suggests that these processes have a profound impact on multi-organ metabolism and exercise adaptation [64].

It is worth noting that, historically, most attention has been directed toward measuring mitochondrial biogenesis, with numerous studies supporting its significance. However, a growing body of evidence highlights mitophagy's pivotal role in exercise adaptation. For instance, recent research has demonstrated how various types of exercise can influence mitophagy, providing important insights into the regulatory mechanisms [12, 67-69]. In summary, while most of the

focus has centered on mitochondrial biogenesis, it is becoming increasingly evident that achieving a balanced understanding of mitophagy is of paramount importance. This balance between biogenesis and mitophagy holds the key to comprehending the intricate molecular mechanisms behind exercise-induced mitochondrial adaptations.

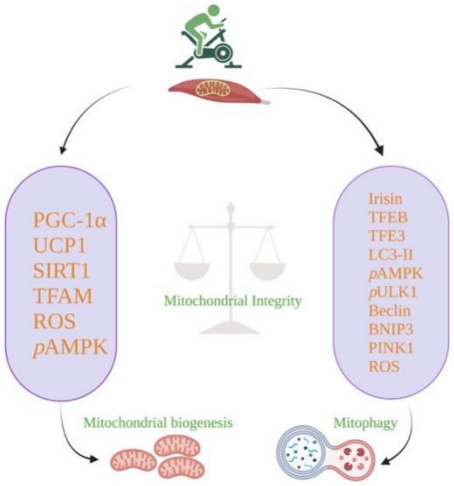


Figure 5. Exercise-induced mitochondrial adaptation at the molecular level. Mitochondrial biogenesis and mitophagy are both essential components of mitochondrial adaptations.

1.7 Molecular mechanisms of autophagy/mitophagy induced by exercise

Autophagy is a cellular process that begins with autophagosome formation and is subsequently fused with lysosomes to form autolysosome. As an adaptive response system, autophagy contributes to maintaining cell homeostasis. It can degrade damaged and dysfunctional organelles and proteins and help maintain cell survival and vitality. Pathological and physiological stimuli induce autophagy, and inducing factors include microbial infection, aging, DNA damage, nutrient deficiency, hypoxia, and physical exercise [70-74]. Exercise and exercise training-induced autophagy is involved in metabolic health and regulation, and it has been observed in multiple tissues and organs, such as live, adipose, pancreas, heart, brain, and muscle [70]. Autophagy induced by exercise in human skeletal muscle is the scope of this study. Currently, the molecular mechanism of exercise-induced autophagy adaptation in skeletal muscle is not completely clear, and limited research has elucidated the molecular mechanism of autophagy induced by HIIE. In retrospect of previous studies, as an activator of exercise-induced autophagy, AMPK can activate autophagy-induced complexes through ULK1 phosphorylation [75-77]. Belin-1 complex mediates

nucleation or expansion of autophagosomes, and LC3 lipidized complex transforms into LC3-II lipids through a series of conjugation reactions [76]. Dysfunctional organelles or protein aggregates are labeled by ubiquitin, recognized by protein P62, and then both bind and interact with LC3 to incorporate the degraded material into the growing autophagosome [76]. The mammalian target of rapamycin (mTOR) as an inhibitor of exercise-induced autophagy has been identified as a major negative regulator of autophagy through AMPK antagonism [75, 77]. Exercise-induced autophagy in skeletal muscle has also been shown to be influenced by multiple transcription factors, including transcription factor EB (TFEB), forkhead box O (FOXO) family, p53, and PGC-1α [75]. These transcription factors mentioned above mediated the transcriptional regulation of autophagy-related genes (ATGs) to increase autophagy capacity [75].

There is increasing evidence that autophagy responses in human and rodent skeletal muscle are related to the adaptation of acute and chronic exercise. In a mouse experiment conducted in 1984, the researcher observed the autophagic response using electron microscopy (EM) [78]. Some autophagic vacuoles are presented in the subsarcolemmal space and myofibrils near necrotic fibers 2-7 days after a single bout of endurance exercise (treadmill running 9 hours with two 10-15 mins rests after 3 hours and 6 hours) [78]. LC3s are highly expressed in skeletal muscle [79], and the number of LC3-II is significantly correlated with the number of autophagosomes [80]. Therefore, LC3-II is often widely used as a marker of autophagosome content [80, 81]. Autophagy flux analysis (i.e., LC3-II/LC3-I ratio) is often considered an alternative method for assessing autophagy levels [82]. An experiment reported a large conversion of LC3-I to LC3-II in the limb muscle of wild-type mice after a single bout of treadmill running [83]. Similar results were shown in several other rodent studies, and an increase in LC3II protein was observed after an acute endurance exercise [84-87]. Studies on human samples presented complex research results. After a single bout of cycling exercise (50% $\dot{V}O_{2max}$ for 1 hour or until fatigued), LC3bII protein expression and LC3bII/LC3bI ratio decreased [88]. Several other studies of acute exercise in human samples support this conclusion [87, 89-92]. In a study of acute ultra-endurance exercise running, data showed a dramatic increase in *LC3b-II* mRNA content in human skeletal muscle [93].

However, after an acute endurance exercise (1-hour continuous cycling at 70% $\dot{V}O_{2peak}$), no changes were found in LC3b-I protein, LC3b-II protein, and LC3b-II/LC3bI ratio [94]. In addition, p62 protein content in mouse and human skeletal muscle has been reported to decrease after acute

endurance exercise [95-98], while no change in p62 has been observed in other studies [93, 94]. Furthermore, evidence suggested that PGC-1α promoted exercise-induced (a single 1-hour treadmill running bout) autophagy in mouse muscle (quadriceps tissue) [86]. However, a contradictory result was observed in this study, suggesting that exercise-induced autophagy seems to be unrelated to AMPK-mediated phosphorylation of ULK1 [86].

The long-term adaptive response in skeletal muscle is also stimulated by exercise training, inducing gene expression through transcription factors [99]. Chronic exercise has been suggested to enhance autophagy [100]. Research from Victoria University demonstrated an increased LC3b-II protein level after three weeks of HIIT [87]. Another three-week one-leg knee extensor exercise supported that the LC3bII/LC3bI ratio decreased and p62 protein increased in human both leg muscles [101]. Five weeks of treadmill training increased LC3-II protein level in mouse skeletal muscle, but no change was observed in LC3b-I and p62 protein content [96]. In another study by Brandt et al., data showed LC3I and P62 proteins were elevated, while LC3II remained unchanged after five weeks of wheel-running exercise training [102]. The two studies mentioned above suggest that the adaptation of exercise training-mediated autophagy regulation depends on PGC-1α [96, 102]. By reviewing the protein and gene changes associated with acute exercise and chronic-induced autophagy, we found conflicting results on certain markers, with potential causes that may be related to exercise type, exercise intensity, exercise duration, muscle types, sample sampling time point, and species differences.

Mitophagy is a selective macroautophagy that clears damaged and dysfunctional mitochondria through lysosomal degradation so as to maintain the mitochondrial population in the optimal state [103, 104]. Both excessive and insufficient mitophagy may result in detrimental consequences. Inadequate mitophagy leads to the accumulation of defective mitochondria, which, along with the accumulation of mitochondrial DNA mutations, cause cell death, while excessive and uncontrolled mitophagy disrupts homeostasis [105, 106]. Therefore, it's important for mitophagy to occur in a balanced way in response to various stimuli [104], such as exercise and hypoxia [106]. It has also been suggested that mitophagy is balanced by regulating the new mitochondria biogenesis [107]. Mitophagy, like mitochondrial biogenesis and autophagy, has also been activated and enhanced during exercise [78]. To date, the molecular mechanism of exercise and exercise training-induced mitophagy remains unclear, especially HIIE-induced mitophagy.

1.8 Exercise and autophagy/mitophagy target genes in skeletal muscle

To our best knowledge, very limited human data currently investigates the autophagy/mitophagy response to exercise, notably in the context of HIIE/HIIT. Upon conducting a targeted search with keywords encompassing both "high-intensity interval exercise/training" and "autophagy/mitophagy" on Google Scholar, I found eight methodology studies specifically addressing human skeletal muscle [88, 91, 108-113] (Table 1). Additionally, searches revealed seven methodology studies concentrating on animal models [46, 114-118]. Upon refining our search criteria to include the keywords "high-intensity interval exercise/training," "autophagy/mitophagy," and "hypoxia" or "altitude training" on Google Scholar, I identified only two studies that met the specified criteria [109, 113]. This singular result aligns with the comprehensive exploration of the outlined topics. Across all experimental trials, the evaluated targets encompass LC3, p62, ATG-3, Atg7, Atg4B, Beclin-1, Bnip3, ULK1, p38γ MAPK, PDK4, and AMPK mRNA and protein.

In an acute study similar to mine, ten recreationally active males and females engaged in moderate-intensity continuous training (MICT) and HIIT sessions with treadmill running in a fasted state. Following western blot analysis, significant differences were observed in the expression of LC3II:LC3I and p62 in skeletal muscle post-MICT, indicating an acute impact on autophagy-related markers. However, no notable changes in autophagy markers were identified in peripheral blood mononuclear cells (PBMCs) [108]. However, in another acute study involving well-trained athletes, a significant rise in ULK1(Ser317) phosphorylation and markers of autophagic flux was observed following high-intensity (HI) exercise. This study included reduced levels of LC3bII protein and LC3bII/I ratio, alongside a simultaneous decline in p62/SQSTM1, indicating an amplified activation of autophagy. The heightened autophagic flux post-HI exercise seemed to be associated with increased AMP-activated protein kinase α (AMPKα) activity, highlighting the impact of exercise intensity in triggering autophagy in human skeletal muscle [91].

In addition to acute studies, there was also a chronic study by Brandt et al. in sedentary subjects who underwent an 8-week training regimen, exercising three days per week with HIIT involving moderate cycling interspersed with 30-second sprints at 473 ± 79 W every 10 min. Following the intervention of the training, there was a significant increase in LC3I, LC3II, and BNIP3 proteins, while p62 levels remained unchanged [110].

Two studies examining the autophagic response to exercise in hypoxia conditions were identified—one involving hypoxia exercise (100W under 12% O_2 for 30 min) [109] and the other utilizing a one-repetition maximum (1RM) intervention [113]. In Weng et al.'s study, thirty sedentary males underwent either HIIT or moderate continuous training (MCT) for five weeks, with CD4 lymphocyte responses to hypoxic exercise assessed. Pre-intervention, hypoxic exercise significantly down-regulated autophagy and enhanced apoptosis in CD4 lymphocytes. However, after five weeks of HIIT and MCT, the diminished autophagy and increased apoptosis caused by hypoxic exercise were mitigated. In the hypoxia study by Gnimassou, O. et al., [113] a single session of resistance exercise was conducted in a moderately hypoxic environment (FiO_2 14%). Twenty subjects performed 1-leg knee extensions in either normoxia or hypoxia, with muscle biopsies taken 15 minutes and 4 hours post-exercise. Results showed that hypoxia downregulated the autophagy transcriptional program, evidenced by lower levels of Bnip3 protein and mRNA at 4 hours post-exercise, decreased *LC3B* mRNA at 4 hours post-exercise in hypoxia, and unchanged *Atg12* mRNA.

Furthermore, in animal studies involving rats trained with HIIT for 8-10 weeks, there was an observed increase in LC3-I protein, LC3-II protein, LC3-II/LC3-I ratio, and *LC3* mRNA following the HIIT training [46, 114-118]. It is noteworthy that while several preceding studies have investigated the autophagy/mitophagy responses to HIIT in both normoxic and hypoxic conditions, the majority have primarily relied on the analysis of mRNA and protein through qRT-PCR and Western blot. However, upon investigating studies related to HIIE and mitophagy/autophagy using microarray and RNA-seq assessments, no literature has been identified. It is essential to note that microarray and RNA-seq are advanced techniques for analyzing gene expression, offering a broader and more comprehensive approach compared to traditional methods like qRT-PCR. Microarray allows simultaneous assessment of thousands of genes, while RNA-seq provides a deeper understanding of the transcriptome by sequencing RNA molecules. However, a distinctive research gap persists, as no study to date has undertaken a comprehensive exploration of the autophagy/mitophagy pathway using RNA sequencing. In this context, the current study stands out as a pioneering endeavor, utilizing human skeletal muscle subjected to HIIE in a hypoxic environment. Remarkably, this study employed RNA-seq to examine thousands of genes, providing a novel and detailed perspective on autophagy/mitophagy regulation in response to hypoxic HIIE, thus addressing a significant gap in the existing literature.

Table 1. Overview of autophagy/mitophagy target genes induced by exercise in skeletal muscle with or without hypoxia. ↑ increased, ↓ decreased, ↔ unchanged. WB: Western blot. qPCR: quantitative real-time PCR.

Study	subject	protocol	Targets	Test method
1.Escobar, Kurt A., et.al. (2021). [108]	Ten healthy, active young adults (M=5, F=5)	Acute HIIT: 2-min warm up at 5 mph, 6 bouts of 1 min at $\dot{V}_{max}$ obtained at $\dot{V}O_{2peak}$ at 3% grade and 1 min of 3 mph and 3% grade, and 2 min of cooldown at 3 mph MICT: 2-min warm up at 5 mph, 60 min at 55% $\dot{V}_{max}$ and 3% grade, and 2 min of cooldown at 3 mph	HIIT: LC3II protein ↑(3 h), p62 protein ↑(3 h) MICT: LC3II protein ↓(3 h), LC3II:LC3I ratio ↓(3 h), p62 protein ↑(3 h)	WB
2.Weng, T. P., et al. (2013). [109]	30 sedentary healthy men (HIT 10) (MCT 10) (CTL 10)	HE: 100W under 12% O_2 for 30 min (4460m) HIIT: HIT (3-min intervals at 40% and 80% $\dot{V}O_{2max}$) MCT (sustained 60% $\dot{V}O_{2max}$)	HE: beclin-1 protein, Atg-1 protein, LC3-II protein, Atg-12 protein, and LAMP-2 protein↓; phospho-Bcl-2 and active caspase-9/-3 ↑	WB
3.Brandt, N., et al. (2018). [100]	12 moderately trained male subjects (19-33 years)	8-week, 3 days per week: moderate cycling (MOD) at 157 ± 20 W for 60 min (n=6) MOD + Sprint 30-sec sprints (473 ± 79 W) every 10 min (n=6)	LC3I, LC3II and BNIP3 protein ↑, p62 protein ↔ 2 h after MOD and MOD + Sprint Beclin1 protein ↑ 2 h after MOD	WB

4.Dethlefsen, M.M., et al.(2018). [111]	9 healthy untrained young, 8 untrained aged and 8 lifelong exercise-trained aged men female C57BL/6 whole-body PGC-1α knock out (KO) mice and wild-type (WT) littermate mice produced by intercross breeding of heterozygous parents	Young: $\dot{V}O_{2max}$ (< 45 ml O_2·min-1·kg-1 body weight) Aged: Consisting of cycling at 120 watts for 5 min, increasing by 20 watts every other minute to perceived exertion of 18 on the Borg scale until exhaustion Mice: exhaustive exercise	Human Aged: PGC-1α mRNA, p62 and p21protein ↓ Lifelong: BNIP3 protein ↑, p53 mRNA ↓ Mice Aged: BAX/Bcl-2 ratio, LC3I and BAX protein ↓ lifelong: PGC-1α, p53 and p21 mRNA ↓	qPCR and WB
5.Schwalm et al. (2015). [91]	23 well-trained athletes control (n=8), low-intensity (LI, n=8), high-intensity (HI, n=7).	acute bout of endurance exercise: 2 hours cycling, the LI group at 55% and the HI at 70% of $\dot{V}O_{2peak}$	ULK1 ↑ in LI and HI, LC3-II protein ↓, LC3-II/LC3-I ↓ in LI and HI, p62 protein ↓ in HI	WB
6.Smiles, W. J., et al. (2017). [112]	9 young, healthy but physically inactive males	resistance exercise bout (REX: 4 × 10 leg press repetitions, 70% 1-RM), endurance exercise (END: 30 min cycling, 70% $\dot{V}O_{2peak}$), low-intensity cycling with BFR (15 min, 40% $\dot{V}O_{2peak}$).	$ULK1^{Ser757}$ phosphorylation ↑ $p38\gamma\ MAPK^{Thr180/Tyr182}$ phosphorylation ↑, HK2 and PDK4 mRNA ↑ in BFR	WB
7.Moller, A. B., et al. (2015). [88]	8 healthy, young, recreationally active men	1-h a single bout of cycling exercise at 50% $\dot{V}O_{2max}$ on two occasions: 1) during a 36-h fast, and 2) during continuous	$ULK1^{Ser555}$ phosphorylation ↑ $AMPK^{Thr172}$ phosphorylation ↑ in exercise,	WB

		glucose infusion at 0.2 kg/h.	ULK1 and p62 protein ↑ ULK1 phosphorylation ↔ in fasting	
8.Gnimassou, O., et al. (2018). [113]	Twenty young, physically active males	hypoxia (HYP, FiO_2: 14%, n=10) or normoxia (NOR, n=10), 8 x 8 repetitions at 80% 1-RM, 2-min rest between sets	LC3B-II/I ratio ↑ at 4 h Post in normoxia and hypoxia, Bnip3 protein and mRNA in hypoxia < normoxia at 4h Post, LC3B mRNA ↓ at 4h Post in hypoxia, Atg12 mRNA ↔	WB and qPCR
9.Li, F. H., et al. (2018). [114]	Six-week-old male Sprague-Dawley (SD) rats SED:10 HIIT:12 MICT:12	10wks. HIIT: four cycles (95-99%, 5 min × 18 m/min + 4 min × 42 m/min) + 35-40% cool-down 28 m 10 wks. MICT: 75-80% of $\dot{V}O_{2max,}$ 34 min × 28 m/min + 35-40% cool-down and warm-up of $\dot{V}O_{2max}$, 6 min × 18 m/min	HIIT: LC3-II protein ↑ ATG-3 protein↑, Beclin-1 protein↑, LC3II/LC3-I ratio ↑only in soleus and cardiac muscle MCT: ↔	WB
10.Wan, D. F., et al. (2021). [115]	Healthy male Sprague-Dawley rats (n=120), age 8 weeks	1.Group C: (n=20/each group) 2.Early exercise preconditioning (EEP): 75% $\dot{V}O_{2max}$ 3.Induction of exercise preconditioning (IEP): 75% $\dot{V}O_{2max}$ 4.Exhaustive exercise (EE): 30 m/min until exhausted 5.EEP + EE 6.LEP + EE	After EEP: LC3-I protein, LC3-II protein, LC3-II/LC3-I, Atg7, Atg4B protein, and Atg3 protein ↑, LC3 mRNAs ↑ After LEP: Atg4B protein, Atg3 protein ↑, Atg7, Atg4B, Atg3 and LC3 mRNAs ↑ After EEP+EE: LC3-I protein, LC3-II protein, LC3-II/LC3-I, Atg7 protein, Atg4B protein, and Atg3 protein ↑	WB and qPCR

			After LEP+EE: LC3-I protein, LC3-II protein, Atg7 protein, and Atg4B protein ↑	
11.Jafari M, et al. (2021). [116]	42 elderly female rats	8 weeks: (1) control (C) (2) MICT, 65% $\dot{V}O_{2max}$ intensity and 20-25 m / min speed (3) HIIT, 85-110% $\dot{V}O_{2max}$ intensity and 15-25 m / min speed (4) MICT + citrus aurantium (CA), 300 mg / kg / day (5) HIIT + CA, (6) CA (7) sham (normal saline) groups	LC3II, Bclin1 and MyoD mRNA ↑ in HIIT and MICT LC3II mRNA ↑ in MICT + CA and HIIT + CA	qPCR
12.Botella, J., et al. (2022). [117]	Study1: 28 Wistar rats Study2: 10 healthy males Study3: 9 healthy males Study4: 5 healthy males	Study 1: 80% of top speed achieved during the incremental test (approximately 0.38 $m \cdot s^{-1}$ at a 10-degree incline) for 7x2-min intervals interspersed with 1 min of rest. Study 2: 3 sessions of −18% (43.8 ± 12.1 min), −6% (38.1 ± 10.5 min), or + 6% (33.8 ± 9.2 min) of the MLSS. Study 3: 48 h of rest Study 4: 6 x 30-s 'all-out' cycling bouts against a resistance initially set at 0.075 $kg \cdot kg$ body $mass^{-1}$ (~175% W_{max}),	Rats: LC3B-I protein↑ 0h after exercise, LC3B-II protein ↑ 3 h after, Map1lc3b, SQSTM1/p62 protein and mRNA ↔ Human: LC3BII ↓ 0 h after, LC3B-I protein or MAP1LC3B mRNA, SQSTM1/p62 protein and mRNA ↔	WB and qPCR

13.Giordano, C., et al. (2015). [118]	Male mice (8 weeks old, C57BL/10ScSnJ, Jackson Laboratories)	4d Short-term intermittent hypoxia (8 h per day, 30 episodes per hour to a FiO_2 nadir=6%)	LC3B-II protein and mRNA ↑ after hypoxia	WB and qPCR
14. Ju, J. S., et al. (2016). [46]	16 ten-week-old male wild-type C57BL/6 mice	1h of swimming (45 × 60 × 40 cm)	LC3-II, Bnip3 protein ↑ after exercise, Pink/Parkin protein ↔	WB and qPCR

1.9 Hypoxia and exercise-induced autophagy/mitophagy

1.9.1 Hypoxia

Hypoxia is usually defined as a decrease in oxygen utilization, which refers to the environment or clinical environment that may threaten tissue oxygen homeostasis [119, 120]. Hypoxia responses to a variety of physiological (e.g., physical exercise, high altitude, embryonic development) and pathological conditions (e.g., inflammation, myocardial infarction, solid tumor formation, chronic obstructive pulmonary disease, amenia) and have been observed in many tissues [119, 121]. Hypoxia-inducible factors (HIFs) are key oxygen sensors that sense and coordinate the cell response to hypoxia and mediate the cell's ability to respond to reduced oxygen tension [121]. It is known from the literature review that hypoxia can induce the expression of many genes, for example, erythropoietin (EPO) and vascular endothelial growth factor (VEGF). Still, the molecular mechanisms of hypoxia signal transduction in mammalian cells remain unclear [122]. Hypoxia-inducible factor 1 (HIF-1), a DNA-binding protein, belongs to the basic helix-loop-helix-PAS transcription factor family and has been proven to play an essential role in the transcriptional regulation of the hypoxia response gene [120, 122]. HIF-1 comprises two subunits, HIF-1α and HIF-1β [120, 122]. The former is highly sensitive to oxygen, while the latter is constitutively expressed and extremely insensitive to oxygen levels [120]. HIF-1α is degraded by hydroxylation in normoxic conditions, whereas the degradation of HIF-1α under hypoxia is inhibited by hydroxylation [120].

1.9.2 Hypoxia and autophagy/mitophagy

Both laboratory and clinical evidence have reported that hypoxia as a physiological stimulus can trigger autophagy [123, 124]. However, different definitions of hypoxia result in contradictory conclusions in studies [125]. The research debate focuses on whether autophagy promotes survival or induces death in the process of hypoxia-induced cell adaptation. To date, the molecular mechanism of autophagy in hypoxia is not completely clear. Nevertheless, hypoxia has been shown to modulate autophagy pathways in different ways due to varying severity and duration [126]. A study suggested that autophagy was activated by HIF-1α-dependent pathway in the context of chronic and moderate hypoxia, whereas it was induced by a HIF-independent pathway in acute and severe oxygen fluctuations conditions [126]. Alongside these, it has been reported that the protein kinase C (PKCδ)-c-Jun-N terminal kinase 1 (JNK1) and HIF-1α signals were crucial for cell survival by autophagy activation under well-controlled hypoxia conditions [126]. Conversely, the unfolded protein response (UPR) and mTOR signals played an important role in cell death in severe hypoxia conditions [126]. Another literature review showed similar results, suggesting that HIF-1 was not involved in autophagy in all hypoxia conditions. The mTOR pathway (HIF-independent mechanisms) played a key role in severe hypoxia-induced autophagy [125]. In drastic hypoxia (0.01% oxygen or anoxia), UPR signaling is involved in autophagic induction [125]. Alongside these, it also suggested that the BNIP3s protein is the key to the difference in hypoxia-induced autophagy cell death or survival [125]. In recent years, studies on hypoxia-induced autophagy in tumor cells have increased. The results of a study in tumor cells reveal the positive role of the AMPK pathway in hypoxia-induced autophagy, and it is independent of HIF-1, BNIP3, and BNIP3L [125]. Collectively, our understanding of hypoxia-induced autophagy and signaling pathways is limited due to the complexity of autophagy signaling under different intracellular and extracellular stresses.

It has been suggested that the induction of mitophagy and the inhibition of mitochondrial biogenesis may be the key adaptive mechanisms to maintain oxygen homeostasis under hypoxia [127]. Evidence indicates that hypoxia can also trigger mitophagy [127], which is induced by HIF-1α through receptors BNIP3, BNIP3L(NIX) and FUN14 domain containing 1 (FUNDC1) [128] [106]. Acute hypoxia of cells results in a dramatic increase in ROS produced by ETC complex III [129]. Prolonged elevated levels of ROS can lead to cellular dysfunction or death, and mitophagy

can prevent elevated levels of ROS [127]. It should be noted that there is a correlation between HIF signaling and ROS. HIF signal regulates mitochondrial ROS production at multiple levels, and ROS can regulate HIF-1α stability in hypoxia [130]. HIF-1-mediated mitophagy pathway in cells can adapt to hypoxic stimulation and promote cell survival by changing metabolic manner [127]. A study of mouse embryonic fibroblasts (MEF) demonstrated that hypoxia-induced mitophagy requires HIF-1-dependent expression of *BNIP3* and constitutive expression of Beclin-1 and Atg5 [127]. BNIP3L(NIX), which is closely related to BNIP3, is also regulated by *HIF-1α* to support its role in hypoxia-mediated mitophagy [106]. In hypoxia-induced mitophagy, the FUNDC1 receptor is not only regulated by its own phosphorylation in the LC3-interacting region (LIR) motif [131] but also participates in depolarization-induced mitophagy through interaction with IP3R2 and regulated by direct phosphorylation-of ULK1 [131, 132]. As mentioned in the above discussion, the PINK1/Parkin pathway was considered an essential pathway related to mitophagy, but a study suggested that mitophagy in hypoxia was independent of PINK1 stability [133]. In addition, ROS regulates mitophagy through another hypoxia-inducible factor-2 (HIF-2α). A mice study found that *HIF-2α* can reduce ROS levels by regulating superoxide dismutase 2 (SOD2) activity [134, 135]. Another study also found that hypoxia-induced *HIF-2α* regulates *BNIP3* expression by activating the transcription factor FOXO3 [136].

Currently, little is known about the underlying molecular mechanisms that combine exercise and hypoxia to induce autophagy/mitophagy. Experimental data indicated that hypoxia (10.7% O_2) under resting conditions enhanced autophagy, the markers of autophagy, LC3-II and LC3-II/I ratio increased by 60% and 25%, respectively, P62/SQSTM1 decreased by 25%, whereas autophagic activity decreased by similar to normoxic levels following exercise (20 min submaximal constant load exercise) in hypoxia [137]. *BNIP3* mRNA levels and AMP-activated protein kinase phosphorylation also increased by 34% and 22%, respectively, in hypoxia [137]. A mole rat study revealed species differences in hypoxia-induced *BNIP3* mRNA expression levels in skeletal muscle [138]. In Rattus and S.judaei muscle, *BNIP3* mRNA increased by 5.3 fold and 1.5 fold, respectively, after exposure to 22-hour hypoxia stress and further increased by 6.1 fold and 3.3 fold following 44-hour hypoxia exposure [138]. However, *BNIP3* mRNA decreased significantly to 0.7 fold (after 22-hour hypoxia exposure) and increased by 0.9 fold (after 44-hour hypoxia exposure) in S.galili skeletal muscle [138]. A human study suggested that hypoxia (FiO_2 14%,

3200m for 15 days) decreased HIF-1α protein stability and target gene *BNIP3* mRNA expression in skeletal muscle, while HIF-2α signaling was not affected [139]. So far, there is limited evidence to demonstrate the molecular pathways of mitophagy in skeletal muscle induced by combining exercise and hypoxia. This situation, makes it difficult to understand the interaction between HIIE combined with hypoxia and mitochondrial adaptations.

1.10 Research gap

A growing number of studies have attempted to investigate skeletal muscle mitochondrial adaptation in different exercise regiments, such as endurance exercise, resistance training, long-term training, and acute exercise. However, there is limited data related to mitochondrial adaptation induced by a single session of HIIE in hypoxia. A few studies have reported HIIE-induced mitochondrial biogenesis in combination with HIIE and hypoxia, while no studies have focused on autophagy and mitophagy. The quantitative Real-time PCR (qRT-PCR) method was mostly used in previous studies to investigate the gene expression induced by HIIE, but a genome-wide approach, such as RNA sequencing, has not been reported. In this study, whole genome RNA sequencing will be used to investigate the molecular response of skeletal muscle mitochondrial adaptation (this study focuses on autophagy/mitophagy) induced by a combination of hypoxia and HIIE, providing a more comprehensive perspective to fill the research gap in the literature.

1.11 Research aims

This study aimed to assess the transcriptomic response relating to autophagy/mitophagy induced by a single session of HIIE with and without hypoxia. Another aim was to assess whether hypoxia has an additive effect on transcriptomic response relating to autophagy/mitophagy induced by a single session of HIIE.

1.12 Conclusions

In recent years, HIIE has gained attention as a time-efficient exercise modality with potential health benefits. However, the need for more consensus in defining high-intensity exercise has posed challenges in interpreting conflicting findings. For consistency with our previous study in the lab, we define exercise intensities exceeding 75% of the maximum oxygen uptake ($\dot{V}O_{2max}$) or

peak power output (PPO) as high intensity. Mitochondrial adaptations in skeletal muscle play a crucial role in mediating the positive effects of exercise. These adaptations involve processes such as mitochondrial biogenesis and mitophagy, which are regulated by key molecular mediators, including PGC-1α and HIF1-α, known for their involvement in the hypoxic response.

The effects of hypoxia on autophagy, the cellular process of self-degradation, remain inconsistent due to variations in definitions and durations of hypoxia employed in studies. Despite this, it is evident that hypoxia can trigger autophagy through HIF-1α-dependent or HIF-independent pathways. These pathways involve signaling molecules such as PKCδ, JNK1, and UPR, highlighting the complexity of autophagy regulation under hypoxic conditions. Moreover, hypoxia-induced mitophagy, facilitated by receptors BNIP3, BNIP3L (NIX), and FUNDC1, plays an essential role in maintaining oxygen homeostasis and preventing excessive ROS production. Mitophagy is a selective degradation process that removes damaged mitochondria, thereby promoting cellular health. Notably, the interplay between HIF signaling and ROS further regulates mitochondrial adaptations. HIF signaling influences mitochondrial ROS production at multiple levels, while ROS can also affect HIF-1α stability during hypoxia.

The combined impact of HIIE and hypoxia on autophagy and mitophagy in skeletal muscle remains relatively unexplored, warranting further investigation. Understanding the intricate relationship between HIIE, hypoxia, and mitochondrial adaptations will provide valuable insights into optimizing exercise strategies for health and performance.

Chapter 2

Conceptual Framework and Methodology

2.1 Introduction

Attempting to answer biological questions and explore medical diseases, the rapid development of technology has made omics widely applied. This also helps to elucidate the response of gene regulation to external stimuli such as exercise or hypoxia. Over the past few decades, transcriptomic analysis (RNA sequencing) has gained significant progress, which has been used in a wide range of research fileds, such as biomedical [140], biology [141, 142], and sports science [143, 144]. Nowadays, RNA-seq approaches based on high-throughput sequencing technologies have become a powerful tool for transcriptome studies [145], which has led to the identification of novel genes and transcription factors by its application in small and large animal models [146-150], as well as human tissue and cells [151].

Given the research background discussed, the present research employed RNA-seq to investigate underlying molecular mechanisms of autophagy/mitophagy in response to HIIE in hypoxia. The characteristic of HIIE is basically comprised of repeated bouts of vigorous activity and interspersed by recovery phases of rest or low-intensity exercise [152]. High peak workloads can last for a longer cumulative time in HIIE than in a single session of continuous exercise due to these recovery phases [153, 154]. I combined standardized (Differential expression analysis and Functional enrichment analysis) and personalized analytical methods (Principle components analysis/PCA and Short time-series expression miner analysis/STEM) of transcriptomic to explore the molecular adaptive response activated by a single session of HIIE combined with hypoxia.

The expression of genes is affected by internal and external stimuli, and the genes expressed under different conditions are significantly different, defined as differentially expressed genes (DEGs). Groups under different conditions, such as wild type vs. mutants, treated vs. control, different time points, different exercise intensity, different tissue, etc., were commonly set up in the study. Diverse software tools were used to acquire differentially expressed genes and visualize these results. This study employed edgeR tools (version 3.8.6) to evaluate the differential expression of 604 target genes related to autophagy/mitophagy at different time points (Before, P0H, P3H, P24H) of three conditions (NA, NR, HY) and using ggplot2 to visualize the results.

Functional enrichment analysis is an important method in transcriptomic analysis, whose validity depends on accurate gene functional annotation and rigorous statistical methods [155]. The most frequently used databases in enrichment analysis are Gene Ontology (GO) and Kyoto

Encyclopedia of Genes and Genomes (KEGG) [155]. KEGG is a database for systematic analysis of gene functions and genomic information. Pathway annotation analysis of differentially expressed genes is helpful for further interpretation of gene functions and for researchers to study genes and expression information as a whole network. KEGG annotation analysis was mainly used in this study to find the pathways associated with autophagy/mitophagy, and the significantly differentially expressed genes in the pathway were further analyzed in depth. This study visualized the top 20 enriched pathways using ClusterProfiler (Version 3.10.1).

After obtaining DEG, this study conducted personalized analyses, including PCA, to assess sample reproducibility visually and detect major differences between different conditions. Additionally, this study utilized STEM software for gene expression temporal trend analysis, which can cluster genes with similar expression patterns. This trend analysis aids in observing expression changes in functionally relevant genes under different conditions and identifying genes that may collectively contribute to specific biological processes.

Finally, to ensure the reliability of our RNA-seq data, this study conducted qRT-PCR experiments for validation. Quantitative real-time polymerase chain reaction is commonly called RT-PCR or qRT-PCR, which is a technology used to measure expression levels of target genes, as well as to validate results obtained from other high-throughput techniques, such as microarray analysis and RNA sequencing. The study on the qRT-PCR approach exploring the changes in human skeletal muscle induced by exercise was first published a report in 2000 [156]. qRT-PCR analysis basically involves seven key steps, including acquisition and handling of the experimental samples, RNA extraction, RNA assessment, cDNA synthesis through reverse transcription, qRT-PCR optimization, qRT-PCR assay, and data analysis using appropriate normalization methods [157]. Studies reported that qRT-PCR was fast and easy to conduct compared with other RNA quantification methods [157] and also more sensitive and specific compared to other assays [158]. Consistent expression trends in the validated genes affirm the credibility of our results.

2.2 Ethics approval

This study was approved by the Victoria University Human Research Ethics Committee (Ethics NO. HRE 18-214). The exercise protocol obeyed the Declaration of Helsinki. This study's ethical considerations mainly involved informed consent, confidentiality, and risk management. Before

consent, each potential participant was fully informed of the research purpose, methods, potential benefits and risks. Participants eligible for the study were required to sign the consent form voluntarily. Informed consent was obtained from participants before starting the experimental protocol. Participant's privacy and confidentiality, including private information and relevant research data, were protected.

2.3 Study design overview

The study design was conducted as part of a larger investigation [159]. In this study, skeletal muscle samples were from existing research projects led by Dr. Jia Li as her PhD study. As an offshore student, this master's project started during Covid-19. The human exercise trial, sample collection and preparation were conducted by Dr. Li. Therefore, the current project was focused on transcriptomic data analysis. To be able to discuss the transcriptomic changes in relation to exercise adaptation, the exercise performance data (Cite Jia's paper) was included in this thesis. Data on mRNA content from qPCR (unpublished data from Dr. Li's PhD work) was also included as a validation strategy. It is clearly stated throughout the thesis when the data from Dr. Li's PhD project was present.

This project adopted a randomized crossover experimental method. An overview of the experimental design of this study is shown in Figure 6. Six weeks of exercise trials were divided into three phases: Familiarisation (week 1); Week 2 and 3-Baseline; Week 4 to 6-Exercise trials. The study comprised three sessions with distinct conditions: hypoxia (HY), where participants experienced a simulated altitude of 3200m in a normobaric hypoxic chamber with a FiO_2 of 14%; normoxia with absolute workload matching the exercise intensity of hypoxia (NA); and normoxia with relative workload matching the exercise intensity of hypoxia (NR). Briefly, the experimental protocol included a familiarization procedure, two Graded Exercise tests (GXTs) in normoxia (FiO_2 = 0.209), two GXTs in hypoxia (FiO_2 = 0.140), and three HIIE sessions. Muscle biopsies from the vastus lateralis muscle were obtained before (B), immediately after (P0H), three hours after (P3H), and 24 hours (P24H) after a single session of HIIE. The skeletal muscle biopsy samples have already been collected, and RNA was extracted. The RNA samples were sent to a commercial company (Biomarker Biotechnology Corporation, Beijing, China) for RNA sequencing, which was used for subsequent analysis to reveal the changes in gene expression

induced by a single session of HIIE in normxia and hypoxia. To better understand the molecular response related to mitochondrial adaptation (autophagy/mitophagy) in response to a single session of HIIE in normoxia and hypoxia, transcriptomic analysis was performed on skeletal muscle samples collected from ten healthy males in this study. Additionally, the expression of genes associated with autophagy/mitophagy in skeletal muscle samples was validated using qRT-PCR.

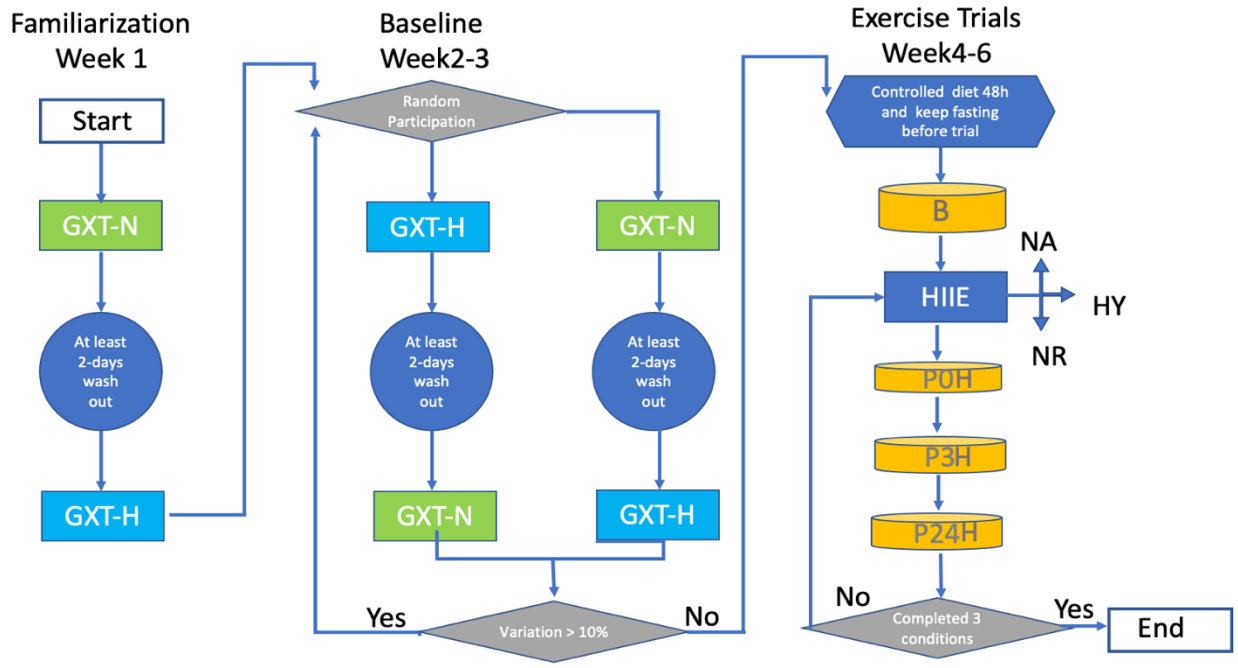


Figure 6. Study design overview. A flowchart of the study's basic structure was conducted on each participant. This includes three phases: Familiarization, Baseline, and Exercise Trials. The flowchart progresses from top to bottom and left to right. Figure legends: GXT-N-Graded exercise test in normoxia; GXT-H-Graded exercise test in hypoxia; before (B), immediate (P0H), 3 (P3H) and 24 (P24H) hours post HIIE, with a number indicating time points after the completion of HIIE.

2.4 Participants

Recruiting qualified volunteers was based on extensive promotion, including campus advertising, newspaper advertising, radio and television, and community contacts [160]. A total of ten healthy young males (aged 18 - 37) were recruited from Victoria University students and residents of nearby communities. Potential participants were first screened over the phone and then extensively screened at the research center. A detailed medical history was evaluated through a questionnaire. Ineligible participants were excluded from the study. The inclusion criteria of individuals include the following several aspects: males aged 18 - 45 years old; body mass index

(BMI) between 20-30 kg / m^2; no hypertension (systolic blood pressure ≥ 140 mmHg and/or diastolic blood pressure ≥ 90 mmHg); no medication was taken before and during the study, no unstable angina pectoris or reversible cardiac ischemia was diagnosed, uncontrolled arrhythmia was diagnosed, accompanied by recurrent or fatigue symptoms, heart failure, and symptomatic aortic stenosis; non-smoker; having not reached an altitude above 1000 meters for more than 24 hours in the past three months (Table 2).

Table 2. Participant characteristics in normoxia (n=10)

Parameter	Mean ± SD
Age (years)	26 ± 4
Body mass (kg)	71.3 ± 2
Height (cm)	175 ± 1
BMI ($kg \cdot m^{-2}$)	23.3 ± 2
$\dot{V}O_{2peak}$ ($mL \cdot min^{-1}$)	3269 ± 708
$\dot{V}O_{2peak}$ ($mL \cdot kg^{-1} \cdot min^{-1}$)	42.8 ± 7.3
Peak power output (W)	259 ± 26
Peak HR ($beat \cdot min^{-1}$)	187 ± 7

BMI, body mass index; $\dot{V}O_{2peak}$, peak oxygen uptake; HR, heart rate; W, watt.

2.5 Exercise testing

2.5.1 Graded Exercise Tests (GXT_S)

Before baseline determination and formal exercise tests, all participants were required to visit the laboratory for initial testing and familiarize themselves with the testing procedures. The exercise test mainly includes GXT and HIIE sections. The GXT was conducted on an electronically braked cycle-ergometer (Excalibur Sport, Lode B·V., The Netherland) and performed under normoxia (FiO_2 = 0.209) and normobaric hypoxia (FiO_2 = 0.140, corresponding to a simulated altitude of ~3200m) conditions. Following the familiarization sessions, participants performed two GXTs in normoxia and hypoxia, respectively, in a randomized, counterbalanced order in the test baseline phase. If the peak power output (PPO) of the two GXTs differs by more than 10% in the same condition (normoxia or hypoxia), a fifth GXT (normoxia and/or hypoxia) was conducted, all tests were performed at least 48 hours apart. The GXTs protocol was modified based on a previous

study, starting at 25% of the PPO and increasing by one-tenth every 4 min, aiming to achieve ten completed stages for each participant [161, 162]. According to a previously published paper, the $\dot{V}O_{2peak}$ was estimated based on age, height, body mass, and physical activity [163]. After each complete stage, capillary blood samples were taken for blood lactate analysis following 30 s rest (YSI 2300 Stat; Yellow Springs Instruments, Yellow Springs, USA) [164], and lactate threshold (LT, expressed in W) was calculated with the modified D-Max formula utilizing the blood lactate data [165]. The test was terminated when the participant reached exhaustion, voluntarily stopped cycling, or/and reported 20 on the RPE scale. During GXTs, exhaled gas from participants was collected and analyzed using the MOXUS Metabolic Cart (AEI Technologies, Bastrop, TX, United States) or a Cosmed system (COSMED, Rome, Italy). To obtain the one-minute mean values of $\dot{V}O_2$, $\dot{V}CO_2$, and ventilation (VE), the $\dot{V}O_2$ data was collected and integrated every 15 s (COSMED, Rome, Italy) or 30 s (Moxus, AEI Technologies Inc., Naperville, IL, USA) interval. There was no significant difference in $\dot{V}O_{2peak}$ values between MOXUS and Cosmed analyzers in our laboratory, which was evidenced by the coefficient of variations (6.4% vs 3.9%) and technical effort of measurements (7.4% vs 7.1%). For GXTs, participants were required to enter the environment chamber for 10-15 min of acclimation testing in hypoxia. To mitigate risks, the Polar was employed to monitor heart rate throughout the test.

2.5.2 Acute high-intensity interval exercise (HIIE) session

In this study, participants performed a single session of HIIE on an electronically-braked cycle ergometer (Excalibur Sport, Lode B·V., The Netherland) in normoxia and hypoxia. Each HIIE session consisted of 6 ✖4-min exercise bouts and 2-min rest. Exercise intensity was determined based on the average values of two closest PPO and LT. The exercise workload calculation of three experimental groups during a single session of HIIE is as follows: HY: LT_H+50% (PPO_H-LT_H); NR: LT_N+50% (PPO_N-LT_N); NA: LT_H+50% (PPO_H-LT_H). For the HIIE in hypoxia, participants needed to stay in the simulated hypoxia chamber for 75-85 min, including 30-40 min of environment acclimatization, blood sampling of baseline phase (approximately 30 min after entering the hypoxia chamber), a 5-min warm-up, a 34-min exercise session, and a 5-min post-exercise blood sampling.

2.6 Nutritional and physical activity control

To avoid confounding effects of different dietary habits, participants were asked to consume a controlled diet for 48 h before and 24 h following the HIIE session. Participants were also advised to restrict food intake for two hours before the test and complete a diet and exercise questionnaire before each test. Outside of the dietary-control period, participants were able to maintain a regular diet and physical activities during the study. Following each exercise test, participants were provided a high-carb drink (with 29.7 grams of carbohydrates) to help prevent their blood sugar from dropping. The energy requirement of each participant was calculated by the Mifflin St-Jeor equation and each participant's body mass (BM), height, and age [160]. A controlled diet contained approximately 53-56% of energy from carbohydrates, 22-24% from fat, and 18-21% from protein [159]. Additionally, participants in this study were asked to refrain from strenuous exercise for 24 hours before the test.

2.7 Muscle biopsies

Muscle biopsy samples were taken from the vastus lateralis muscle (single leg) of the participants by medical doctor. First, the skin and fascia were locally anesthetized with 1-2% lidocaine and followed by suction of the vastus lateralis muscle via Bergstrom needles (Bergstrom Muscle Biopsy Cannula). Manual suction was applied to collect muscle samples. Muscle biopsy samples were extracted at four different time points at each exercise session: before (B), immediately (P0H), 3 hours (P3H), and 24 hours (P24H) post HIIE. The muscle samples were snap-frozen in liquid nitrogen and then stored at -80 ^{o}C for subsequent analysis. However, prior to freezing, the muscle samples were immediately dried and removed excess blood, fat and connective tissue.

2.8 RNA extraction

Total RNA was extracted from frozen muscle samples (10-15 mg) using Qiagen AllPrep DNA/RNA Kits, following the manufacturer's instructions. Muscle samples were homogenized for 2 min at 20 Hz using the TissueLyser II. Total RNA was obtained from the aqueous phase after precipitation with 600 µL of 70% ethanol. Before storage, separate aliquots were set aside for RNA assessments. Total RNA was obtained from the aqueous phase after precipitation with 600 µL of 70% ethanol. Before storage, separate aliquots were set aside for RNA assessments. The

concentration and purity of RNA were determined with a Nanodrop spectrophotometer (ND1000, Thermo Fisher Scientific, Wilmington, DE) by measuring specific nucleic acid concentration at 260 nm (A260) and assessing protein and background absorption at 280 nm (A280) and 230 nm (A230) to detect potential contaminants [157]. Agilent RNA ScreenTape (Agilent Technologies, USA) was used to confirm RNA integrity. A mixture of 5 μL RNA sample buffer and 1 μL RNA was vortexed for 1 minute and then centrifuged for 1 minute. Before loading samples into the TapeStation instrument, they were heated at 72°C for 3 min and then placed on ice for 2 min. Samples with an RNA Integrity Number (RIN) of at least 7 were deemed suitable for qRT-PCR preparation.

2.9 RNA sequencing procedure

In an RNA-seq workflow (Figure 7), the process begins with the isolation of high-quality total RNA from biological samples, followed by a thorough assessment of RNA integrity, concentration, and the absence of contaminants. This quality control step, often done using tools like the Agilent Bioanalyzer or TapeStation, ensures that the RNA is suitable for downstream processing. If specific mRNA profiling is desired, enrichment steps like poly(A) selection or rRNA depletion may be employed to reduce sample complexity. The mRNA is then subjected to reverse transcription, converting it into complementary DNA (cDNA). Following this, end repair is performed to clean up cDNA ends, making them amenable to subsequent steps. Sequencing adapters are ligated to the cDNA fragments, and thorough purification is carried out to remove unligated adapters and impurities. PCR amplification enriches the adapter-ligated cDNA fragments, preparing them for sequencing while being mindful not to over-amplify and introduce bias. Library quality and quantity are assessed through qPCR, Bioanalyzer, or TapeStation, ensuring the library has the desired size distribution. Finally, the prepared library is sequenced on a chosen platform, such as Illumina HiSeq or NextSeq, generating raw sequencing data (reads) for subsequent RNA-seq data analysis, enabling gene expression and transcriptome profiling.

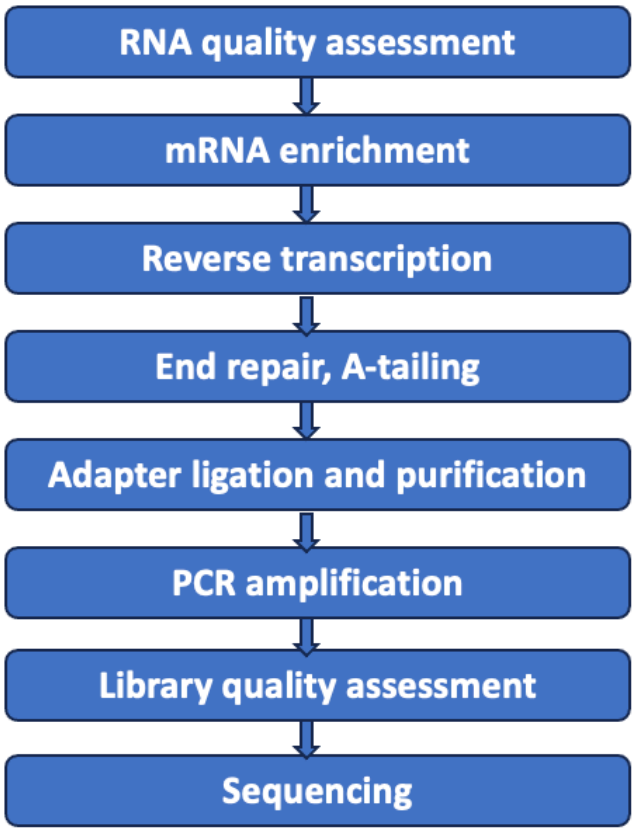


Figure 7. RNA sequencing workflow

2.9.1 RNA quality assessment

The quality and quantity of extracted RNA from muscle samples were evaluated prior to sequencing. RNA concentration and purity were assessed by Nanodrop One (Thermofisher). RNA integrity was tested using the Agilent TapeStation system and 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 1 min. The samples were heated at 72ºC for 3 min and placed on the ice for 2 min before being loaded into TapeStation instrument.

2.9.2 Library construction

1 µg of RNA samples (RIN > 7) were sent to the Biomarker Biotechnology Corporation (Being, China) for RNA-seq library preparation. In accordance with the manufacturer's guidelines, sequencing libraries were produced using NEBNext UltraTM RNA Library Prep Kit for Illumina (NEB, USA). The mRNA was augmented and purified by magnetic beads with poly-T Oligo (dT),

and fragmentation occurred under increased temperature in NEBNext First Strand Synthesis Reaction Buffer (5X). Using these cleaved mRNA fragments as templates, the initial cDNA strand (RNA-DNA hybrid) was generated with a random hexamer primer and M-MuLV Reverse Transcriptase. The second cDNA strand was then synthesized using DNA Polymerase I and RNase H. The remaining overhangs were transformed into blunt ends through exonuclease/polymerase activities. The 3' ends of fragments were subsequently adenylated and connected to the NEBNext Adaptor with a hairpin loop, to prepare for hybridization. For the preferential selection of the 240 bp cDNA fragment, the AMPure XP system (Beckman Coulter, Beverly, USA) was utilized to purify the library fragments. Prior to PCR, 3 μL USER Enzyme (NEB, USA) was employed for size selection. Subsequently, cDNA was ligated to the adaptor at 37 °C for 15 min, followed by 5 min at 95 °C. The PCR was performed with Phusion High-Fidelity DNA polymerase, Index (X) Primer, and Universal PCR primers, which products were purified with the AMPure XP system.

2.9.3 Library quality control and RNA sequencing

In order to guarantee the library's quality and obtain a better output of RNA sequencing in this study, Qubit 2.0 and Agilent Bioanalyzer 2100 were utilized to assess the concentration of cDNA and insert size. qRT-PCR was conducted for a more precise determination of the library concentration. Libraries with concentrations exceeding 2 nM were deemed acceptable. Utilizing sequencing-by-synthesis (SBS) technology, the qualified cDNA libraries were sequenced on an Illumina NovaSeq 6000 platform with a paired-end sequencing length of 150 bp.

2.10 Bioinformatic analysis

Systematic bioinformatics pipeline analysis to investigate the molecular adaptive response related to autophagy/mitophagy induced by a single session of HIIE for this study is summarized in Figure 8. Briefly, clean data was obtained after filtering the raw data. Mapped data was generated by further mapping to the reference genome. Then, the library quality assessment was conducted with inserted fragments length test and randomness test. Differential expression analysis, functional annotation, and functional enrichment of differentially expressed genes were conducted based on the expression levels observed across various samples or sample groups. In this study, a comprehensive annotated gene list related to autophagy/mitophagy from a published

paper was employed as the target genes for our study [166]. Differentially expressed gene analysis and enrichment analysis were conducted for 604 target genes in the gene list. Differentially expressed genes (DEGs) were identified using the $\log_2$FoldChange and *P*-value. KEGG database was mainly used in pathway analyses. Apart from these, principal component analysis (PCA) and short time-series expression miner (STEM) analysis also were used to explore the gene expression induced by a single session of HIIE at different time points in distinct conditions. R packages were the important tool used for visualization in this study.

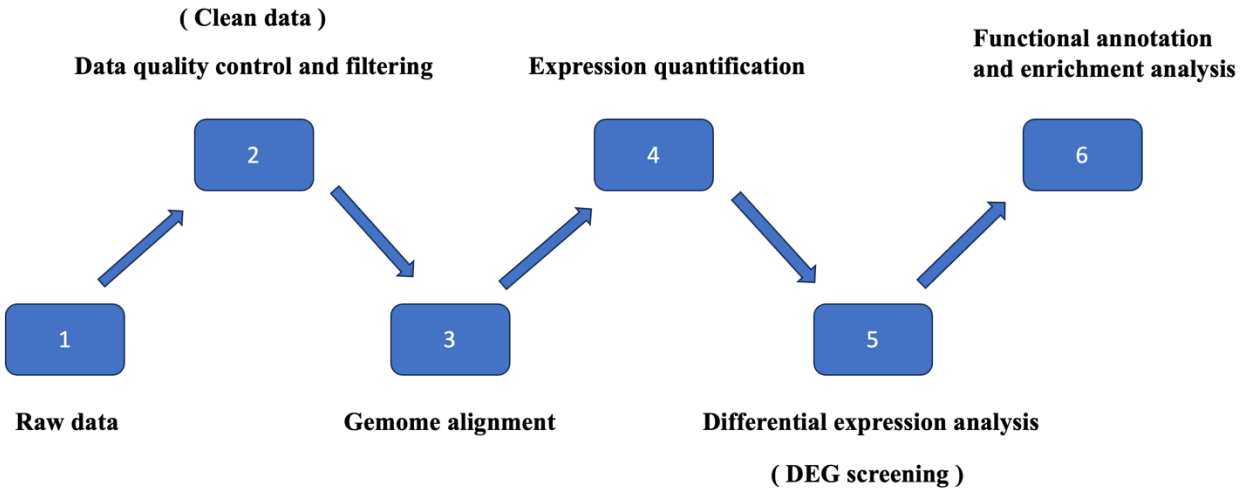


Figure 8. Bioinformatics pipeline

2.10.1 Data quality control and filtering

The cDNA library was sequenced by Illumina high-throughput sequencing platform (Illumina novaseq 6000) and generated a large number of highly qualified raw data, most of its base quality scores can reach or exceed Q30. These raw data were provided in FASTQ format. End-paired sequencing was performed for our study, so the raw data of each sequencing sample includes two FASTQ files containing the reads measured at both ends of all cDNA fragments. Quality control (QC) and sequence data filtering are necessary preprocessing steps in bioinformatics analysis [167], it is crucial to ensure the quality of the reads before moving on to subsequent analysis. For our study, the raw data in FASTQ format was processed by Perl scripts. In this step, clean data saved in FASTQ format was obtained by trimming and removing reads containing adapter contaminations, nucleotides (N) with low-quality score (including reads with N removal ratio

greater than 10% and reads with quality values Q≤10 that accounted for more than 50% of the whole read) from raw data. We also calculated Q20, Q30, GC content, and sequence duplication level of the clean data. 1218Gb of clean data were obtained after sequencing data quality control, and the percentage of Q30 bases in each sample was not less than 91.49%. The following downstream analyses were based on the above filtered, high-quality clean data.

2.10.2 Data alignment to the reference genome

Clean data was obtained by removing adaptor, and low-quality sequence reads from the raw data, and then these clean reads were mapped to the reference genome sequence. In this study, the *Homo sapiens*（GRCh38_release95）genome is the reference genome to align data. The reference genome information was obtained from the Ensembl database. Hisat2 is an efficient alignment system based on the Burrows-Wheeler_transform (BWT) algorithm and Ferragina-Manzini (FM) index, which can quickly align the position of RNA sequences on the genome, through which we can find the original position of sequence in the genome [168]. Thus, Hisat2 (Version 2.0.4) [169] software was employed to map with reference genome, only reads with a perfect match or one mismatch were further analyzed and annotated. From the mapping statistics results, the mapping ratio between reads and reference genome of each sample ranged from 89.25% to 97.11% in this study.

2.10.3 Gene expression quantification

After alignment analysis, StringTie (Version 1.3.4d) [170] was used for assembly and quantification of mapped reads. FPKM (Fragments Per Kilobase of transcript per Million fragments mapped) was employed in our study to measure the expression level of a gene or transcript using the maximum flow algorithm of StringTie. FPKM was specialized for pair-end RNA-seq experiments, standing for fragments per kilobase of exon per million mapped fragments [171]. The FPKM calculation equation is FPKM = cDNA Fragments/Mapped Fragments (Millions) × Transcript Length (kb) [172], in which cDNA Fragments stands for the number of PE reads mapped to the specific transcripts, Mapped Fragments (Millions) represents the all mapped reads number and Transcript Length (kb) is the length of the transcript.

2.10.4 Correlation assessment of biological replicates

This study applied the Pearson correlation coefficient as the evaluation index of 10 biological replicate correlation. The closer the r value was to 1, the better the correlation between the replicate samples was.

2.10.5 PCA analysis

After alignment and quantification, an initial evaluation of the dataset was performed. I used 12984 genes to assess the difference between samples collected between different groups (NA, NR, HY) at different time points after exercise by PCA. The PCA function of the "FactoMineR" and "factoextra" R packages were employed to visualize the data.

2.10.6 Differential expression analysis

The Software edgeR (Version 3.8.6) [173] was used to screen differentially expressed genes based on the count value of genes in each sample. In the process of differentially expressed genes detection, $\log_2$FoldChange ($\log_2$FC) and *P*-value were used as screening criteria. In addition, a comprehensive annotated gene list related to autophagy/mitophagy from a published paper [166] was employed as the target genes for our study. Differentially expressed gene analysis was conducted for 604 target genes associated with autophagy/mitophagy in the gene list. We employed ggplot2 [174] to visualize the results of differentially expressed genes. The volcano plots showed the differences and statistical significance of gene expression between two groups of samples, and the heatmap showed the hierarchical clustering of differentially expressed genes.

2.10.7 Enrichment analysis of DEGs

In this study, I investigated if some pathways were over-presented with DEGs, which are essential biochemical metabolic pathways and signal transduction pathways through enrichment analysis. ClusterProfiler (Version 3.10.1) was used to visualize the top 20 enriched pathways.

2.10.8 Reverse transcription

1 μg of RNA was employed to synthesize cDNA using the iScript™ Reverse Transcription Supermix for qPCR kit (Bio-Rad Laboratories). The components for the cDNA synthesis reaction consist of 4 μl iScript RT supermix, 1 μg RNA template, and nuclear-free water to reach a total

volume of 20 µl. The entire reaction in a thermal cycler comprises priming (5 min at 25°C), reverse transcription (20 min at 46 °C), and RT inactivation (1 min at 95°C) (C1000 Touch Thermal Cycler). Use the same amount (1µg) RT negative control lacking cDNA but with an equivalent level of genomic DNA contamination as in the cDNA sample for the primer specificity test. The cDNA was subsequently diluted 5 to 10 times with nuclease-free water and stored at -20°C for further analysis.

2.10.9 Quantitative Real-Time PCR (qRT-PCR)

To confirm the findings from the transcriptomic analysis, the mRNA content of four selected targets was measured by qPCR (QuantStudio 7 Flex, Applied Biosystems, Foster City, CA) using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad). Using the Primer-BLAST tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi) for primer design and purchased from Sigma-Aldrich. All reactions were carried out 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 comprised of cDNA template, 2.5 µl of 2 × mastermix, and 0.3 µM or 0.9 µM primers and concentration depended on gene targets. All assays were run for 10 min at 95°C, followed by 40 cycles using the C1000 Touch Thermal Cycler seconds at 95°C and 60 seconds at 60°C, respectively. The expression of each target gene was standardized by the mean expression of the two most consistently expressed reference genes (B2M, ACTB, TBP, Cyclophilin, GAPDH), and the expression of each target gene was calculated using the $2^{-\Delta\Delta Ct}$ formula [175]. The list of primers was as follows (Table 3).

Table 3. Primer sequences and amplicon details. F: forward primer; R: reverse Primer

Gene Target	Primer Sequence	Efficiency	Accession no.
TP53	F: GTTCCGAGAGCTGAATGAGG R: TTATGGCGGGAGGTAGACTG	101.8	NM_00546.5
HIF1A	F: CTAGCCGAGGAAGAACTATGAACAT R: CTGAGGTTGGTTACTGTTGGTATCA	90.1	NM_001530.1
PPARGC1A	F: GGCAGAAGGCAATTGAAGAG	103.6	NM_01362

	R: GGCAGAAGGCAATTGAAGAG		
HSPA1A	F: ACCTTCGACGTGTCCATCCTGA R: TCCTCCACGAAGTGGTTCACCA	99.2	NM_005345.5
Housekeeping gene			
B2M	F: TGCTGTCTCCATGTTTGATGTATCT R: TCTCTGCTCCCCACCTCTAAGT	98	NM_004048.2
ACTB	F: GAGCACAGAGCCTCGCCTTT R: TCATCATCCATGGTGAGCTGGC	107	NM_001101.3
TBP	F: CAGTGACCCAGCAGCATCACT R: AGGCCAAGCCCTGAGCGTAA	99	NM_003194.4
Cyclophilin	F: GTCAACCCCACCGTGTTCTTC R: TTTCTGCTGTCTTTGGGACCTTG	100	NM_021130.4
GAPDH	F: AATCCCATCACCATCTTCCA R: TGGACTCCACGACGTACTCA	106	NM_001289746.1

2.11 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.). The reads abundance difference between samples was calculated according to the ratio of Fragments Per Kilobase per Million mapped reads (FPKM), and Fold Change (FC) ≥ 2, and *P*-value < 0.05 was set as the threshold for significantly differential expression. For the convenience of comparison, the logarithm value of the difference multiple was expressed as $\log_2$FC. The larger the absolute value of $\log_2$FC, the smaller the *P*-value, and the more significant the difference change of the genes between the two groups of samples. Enrichment factors and the fisher test were used in the determination of the enrichment degree and significancy of the pathway. Comparisons were considered statistically significant at *P*-value $<$ 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 used are described in the figure legends. One-way ANOVA and Turkey's multiple comparisons test were conducted to compare the differences in physical performance data among three conditions (NA, NR, HY). Two-way ANOVA and Turkey's

multiple comparisons test were used to compare the difference between three conditions and four sampling time points (Validation part). *P*-value < 0.05 was considered statistically significant.

Chapter 3

Transcriptomic Response Associated with Autophagy/Mitophgy in Human Skeletal Muscle Induced by a Single Session of HIIE with and without Hypoxia

3.1 Introduction

HIIE has emerged as a potent strategy for eliciting significant improvements in human skeletal muscle function and metabolic responses [153, 176]. This exercise modality, characterized by brief bursts of high-intensity exercise interspersed with periods of rest or low-intensity exercise, has been widely recognized for its capacity to enhance exercise performance, metabolic efficiency, and cardiovascular health [153]. However, the precise molecular mechanisms underpinning the adaptations within skeletal muscle, particularly those related to autophagy and mitophagy, remain a subject of active investigation.

Autophagy and mitophagy, the cellular processes responsible for degrading and recycling damaged cellular components, have gained prominence in recent years for their roles in maintaining cellular homeostasis and promoting cellular health [177]. Understanding how HIIE influences these crucial processes in human skeletal muscle can provide insights into the intricate molecular adaptations that occur in response to this form of exercise. Furthermore, introducing a hypoxic component to HIIE, which simulates reduced oxygen availability, adding an intriguing dimension to this exploration.

Hypoxia, with its inherent challenges and unique physiological responses, has demonstrated the potential to amplify further the effects of exercise on cellular adaptations, including those related to mitochondria and cellular quality control mechanisms [178]. By subjecting individuals to a single session of HIIE in both normoxia and hypoxia, this study aims to investigate the influence of hypoxia and exercise intensity on mitophagy and autophagy and to identify whether hypoxia or exercise intensity has a greater impact on autophagy and mitophagy. When combining these factors, this study aim to uncover the molecular pathways and gene expressions that underlie the potential benefits of combining HIIE and hypoxia, shedding light on the intricate interplay between exercise, cellular adaptation, and oxygen availability.

3.2 Prescribing exercise intensity and experimental grouping strategy

As an offshore program student during the COVID-19 pandemic, I was unable to perform lab testing or analysis. Thus, I worked collaboratively with Dr. Jia Li, and the exercise trial was conducted by Dr. Jia Li [159]. My master's project focused on data analysis to reveal the

transcriptomic response associated with autophagy/mitophagy induced by a single session of HIIE in hypoxia using skeletal muscle samples collected and processed by Dr. Jia Li.

The study attempted to prescribe exercise intensity of HIIE in hypoxia using peak power output (PPO) and lactate threshold (LT), which are derived from GXTs. More specifically, the experimental protocol in the familiarization phase consisted of two GXTs in normoxia (FiO_2 = 0.209) and two GXTs in hypoxia (FiO_2 = 0.140). The physical performance data showed a lower peak oxygen uptake ($\dot{V}O_{2peak}$), peak heart rate (HR_{peak}), PPO, and LT when compared to those in normoxia, while there was a higher respiratory exchange ratio (RER) in hypoxia. Li, Y. et al. (2022) pointed out that hypoxia increased physiological and perceptual responses when exercising at the same absolute exercise intensity, however, due to hypoxia reducing $\dot{V}O_{2max}$, exercise represented a decrease in mechanical output (power sustained) at a given physiological period intensity [159]. The peak blood lactate level showed no difference between normoxia and hypoxia. The study next proposed the prescription of two HIIE sessions in normoxia to match for absolute and relative intensity in hypoxia based on the data of PPO and LT. This was a novel matching strategy to explore adaptive response induced by a single session of HIIE in hypoxia, as the control groups set up in some studies typically only considered matching relative intensity [178-181] or matching absolute intensity in hypoxia [182-187]. In this study, three HIIE sessions experimental groups were set up: one HIIE session in hypoxia (HY), one matched for the absolute intensity in hypoxia (NA), and one matched for the relative intensity in hypoxia (NR). For the HY session, the exercise workload was calculated as $50\%PPO_H + 50\%LT_H$ based on PPO and LT in hypoxia; for NR, the exercise workload was calculated as $50\%PPO_N + 50\%LT_N$ based on the PPO and LT achieved under normoxic conditions. The exercise workload of NA was the same as HY, equal to $50\%PPO_H + 50\%LT_H$. The study concluded that the strategy of matching intensity between normoxia and hypoxia was effective by comparing the physiological parameters in three different conditions, and the detailed physiological and performance data of HIIE sessions in NA, NR, and HY was presented in Table 4. It was mentioned earlier that NA and HY have the same exercise workload (170.1 ± 48.3 W) (Table 4 and Figure 9A), which was equivalent to 78.2 ± 2.2% and 85.8 ±1.8% of PPO in normoxia and hypoxia, respectively (Table 4 and Figure 9B). The workload for NR was significantly higher (238.3 ± 51.0 W) (Table 4 and Figure 9A), equivalent to 88.5 ± 1.6% of PPO in normoxia (Table 4 and Figure 9B). The data showed a significant difference in PPO% between NA vs. NR ($p < 0.0001$) and between NA vs. HY ($p < 0.0001$), while there was no difference

between NR and HY (p = 0.2056) (Figure 9B). The percentage of HR_{peak} was higher in NR (94.4 ± 4.7%) and HY (92.4 ± 6.2%) compared with NA (85.0 ± 7.1%) (Figure 9C). Both NR (p = 0.0082) and HY (p = 0.0410) showed a significant difference in HR_{peak} % when compared with NA (Figure 9C). However, there was no difference between NR and HY (p = 0.7727). RPE in the NR condition was the highest (19.8 ± 3.1), the lowest was NA (15.3 ± 2.5), and HY was in the middle position (17.3 ± 2.6) (Table 4 and 9D). The RPE data showed a significant difference between NA vs. NR (p = 0.0048) and NA vs. HY (p = 0.0244), and no significant difference was observed between NR vs. HY (p = 0.2351). The percentage of $\dot{V}O_{2peak}$ was higher in HY (92.67 ± 17.07%) compared with NA (74.44 ± 7.44%) and NR (86.87 ± 11.59%), which existed difference between NA and HY (p = 0.0333) (Table 4 and Figure 9E). There was no significant difference for RER in NA (0.94 ± 0.07), NR (0.94 ± 0.06), and HY (1.04 ± 0.12) (Table 4 and Figure 9F).

Table 4. Physiological and performance data of HIIE sessions in NA, NR, HY

Parameter	**NA**	**NR**	**HY**
PO (W)	170.1 ± 48.3	238.3 ± 51.0	170.1 ± 48.3
PPO%	78.2 ± 2.2	88.5 ± 1.6	85.8 ±1.8
HR_{peak}%	85.0 ± 7.1	94.4 ± 4.7	92.4 ± 6.2
RPE	15.3 ± 2.5	19.8 ± 3.1	17.3 ± 2.6
$\dot{V}O_{2peak}$ %	74.44 ± 7.44	86.87 ± 11.59	92.67 ± 17.07
RER	0.94 ± 0.07	0.94 ± 0.06	1.04 ± 0.12

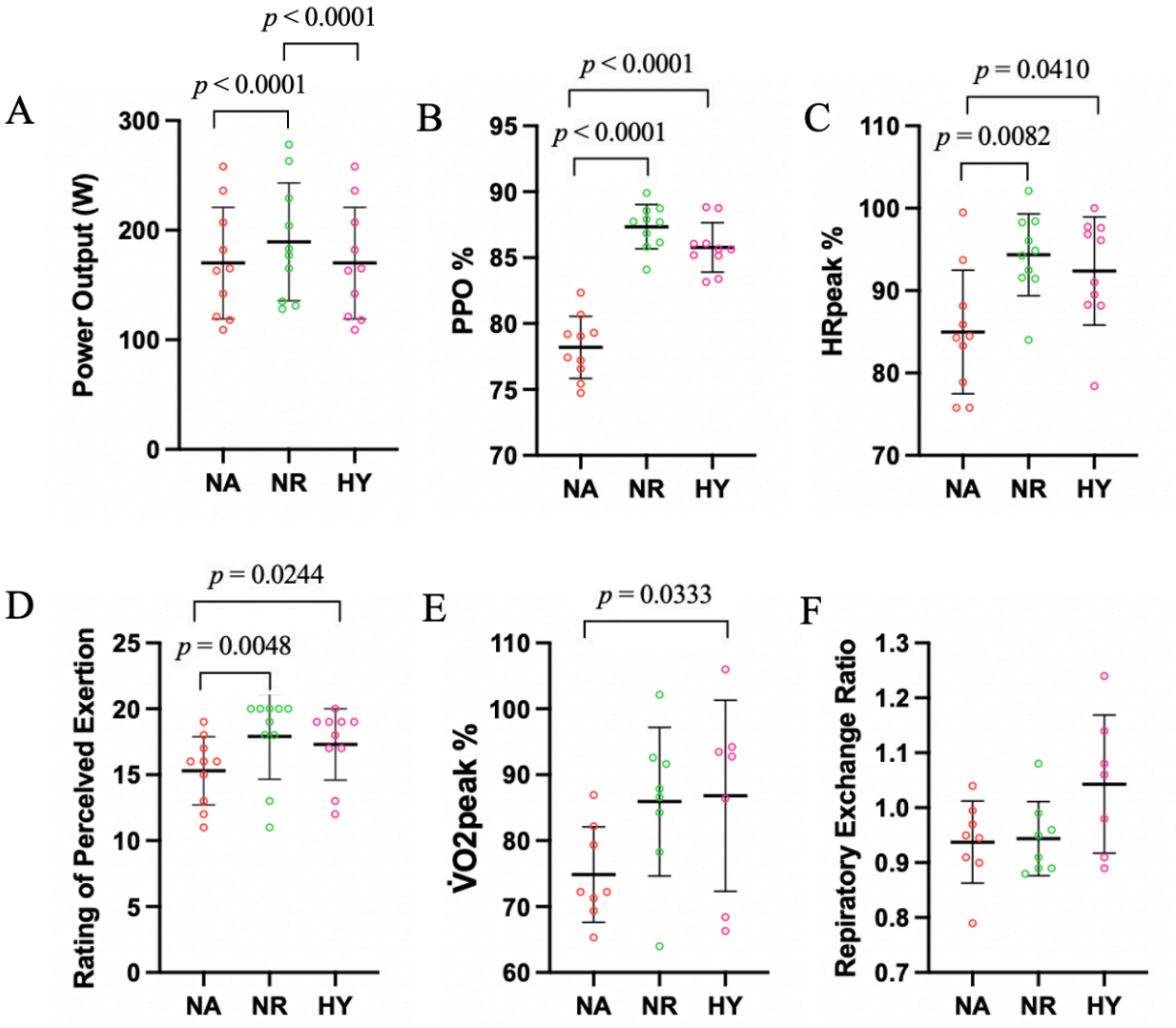


Figure 9. HIIE-induced physiological performance data in normoxia and hypoxia. The red circle represents participants in normoxia matched for absolute intensity to that of hypoxia (NA); the green represents participants in normoxia matched for relative intensity to that of hypoxia (NR); the pink circle represents participants in hypoxia (HY). A: Power output of HIIE sessions (PO); B: the percentage of power out to peak power out (PPO); C: the percentage of heart rate to heart rate peak HRpeak; D: the rating of perceived exertion (RPE); E: the percentage of $\dot{V}O_2$peak; F: the respiratory exchange ratio (RER).

In the present study, methods of prescribing exercise intensity are essential for the experimental design, and it has yet to be reached a consensus on the reliability and validity of determining the exercise intensity methods [159]. Currently, methods for prescribing exercise intensity under normoxic conditions are based on the percentage of various anchor measurements (e.g., maximal anchors and submaximal anchors) derived from a maximal or submaximal graded exercise test or

a series of submaximal or supramaximal exercise bouts [159, 188, 189]. Review articles have summarized the maximal anchors used to determine the exercise intensity, including maximal oxygen uptake ($\dot{V}O_{2max}$), peak oxygen uptake ($\dot{V}O_{2peak}$), maximum work rate/peak power output (PPO), and maximum heart rate (HR_{max}) or peak heart rate (HR_{peak}) [159, 188, 190]. In addition, submaximal anchor approaches have also been employed to prescribe exercise intensity, such as the ventilatory threshold (VT), the first and the second lactate threshold (LT1 and LT2), the maximal lactate steady state (MLSS), the gas exchange threshold (GET), the respiratory compensation point (RCP), critical power (CP) and critical speed (CS) [159, 188, 190-192]. Prescriptions for exercise intensity in hypoxia are commonly based on maximal aerobic speed, PPO, $\dot{V}O_{2max}$, RPE, $HR_{max,}$ and LT [159]. However, it has been demonstrated that the using a fixed percentage of the above-mentioned maximal anchors may result in considerable variability in physiological responses [159, 188]. A study showed that physiological responses exhibited significant variability at a fixed percentage of $\dot{V}O_{2max,}$ and the variability of these responses becomes greater as the percentage of $\dot{V}O_{2max}$ increases [193]. Li and Li's study also verified that it would result in differences in RPE, HR_{peak}%, and $\dot{V}O_{2peak}$% if prescribing exercise intensity only used PPO in normoxia and hypoxia [159]. A review paper indicated that there seemed to be little evidence to support the validity of most commonly used submaximal anchors methods. It remained to be validated in different environments and settings [188]. Moreover, this study also suggested that more homogeneous physiological responses were likely caused if the methods of prescribing exercise intensity inclusive of maximal, submaximal, and resting values [188]. This finding has been examined in other studies of our group, which showed that HIIE prescription in normoxia based on PPO and LT2 can result in comparable physiological adaptations compared with studies based solely on solely maximal or submaximal values [159, 194-196].

In combination, I assert that based on the previous research results of our research team, the experimental grouping design of matching exercise intensity strategy is feasible and effective in our study and worth further exploration. To our knowledge, exercise commonly improves human exercise performance and health by stimulating physiological adaptation [197, 198]. For instance, previous studies have indicated that exercise, especially HIIE, can lead to adaptations such as enhanced cardiovascular function, increased muscular strength, and improved metabolic efficiency [170]. Additionally, it's noteworthy that the specific environmental conditions in which exercise is performed can influence the nature of these adaptations. A previous study [151]

suggested that HIIE matched for absolute and relative intensity in normoxia may lead to distinct adaptations when compared with that in hypoxia. These adaptations may include alterations in oxygen utilization, mitochondrial function, and metabolic responses, all of which warrant further investigation and analysis. Another study has also shown that a single session of exercise likely results in homeostasis perturbations [199]. Some studies have evidenced that exercise-induced autophagy and mitophagy [200, 201]. Thus, it is reasonable to hypothesize that a single session of HIIE may induce divergent transcriptional responses related to autophagy/mitophagy in three different conditions (NA, NR, HY). In recent years, the effects of exercise in hypoxia on physical performance and health improvement have been studied in distinct contexts. A study demonstrated that subjects (33 untrained men) lived at low altitude and training (a cycle ergometer training of 6 weeks, 5d/week, 30 min/d) in hypoxia (corresponding to altitude 3850m) had a greater increase in $\dot{V}O_{2max}$ and peak power, compared to subjects trained in normoxia [202]. It was reported on well-trained cyclists that interval hypoxic training (IHT) elevated exercise performance inclusive of $\dot{V}O_{2max,}$ maximal workload (WR_{max}), $\dot{V}O_2$ at the lactate threshold ($\dot{V}O_{2LT}$), and workload at the lactate threshold (WR_{LT}) [203]. Similar results were observed in overweight and obese individuals, where combining exercise training (constant-load cycling at 75% of maximal heart rate, three sessions per week) and hypoxic exposure (FiO_2 = 0.13, corresponding to altitude 3700m) led to a significant increase in peak oxygen consumption and maximal power output [204]. In addition to these improvements in physical performance, with the number of populations with chronic diseases continuing to rise, hypoxic exercise and training strategies were used to improve health. A previous study reported that combining exercise and hypoxia method had an additive effect on insulin sensitivity in type 2 diabetic patients [205]. Another study has summarized that hypoxic training has many additive and beneficial effects on traditional cardiovascular risk factors [206]. The treatment and intervention effects of intermittent hypoxia on obesity populations have been reported [207, 208]. Nevertheless, I noticed that the adaptive response related to autophagy/mitophagy of exercise in hypoxia to human skeletal muscle has not been reported. Therefore, another aim of this study was to investigate whether hypoxia combined with high-intensity exercise has additive effects on autophagy and mitophagy. Moreover, this study also speculated that it may show a similar transcriptional response between HIIE in hypoxia (HY) and HIIE matched for relative workload in hypoxia (NR) based on similar results of the physiological parameters observed in our study. A narrative literature also reviewed the findings on the effects

of acute exercise and training in hypoxia on health status (glucose metabolism, weight loss, body composition, lipid profile, and blood pressure) [209]. Nevertheless, this study concluded that inconsistent findings were presented in studies on the health effects of combining hypoxia and exercise [209]. Take the study of exercise and training in the hypoxia method on glucose metabolism as an example. De Groote, E. and L. Deldicque noted the reason why studies of hypoxia combined with exercise on glucose metabolism in type 2 diabetes subjects showed divergent results that were due to the intensity of exercise [209]. Furthermore, they also elucidated the effects of absolute and relative intensity on glucose metabolism and highlighted that more research is needed to explore the impact of acute hypoxia exercise on glucose homeostasis [209]. In summary, three HIIE sessions (NA, NR, HY) were designed to differentiate whether exercise intensity or hypoxia had a greater effect on mitochondrial adaptation (autophagy/mitophagy) to human skeletal muscle. The difference in molecular response may be led by distinct exercise intensities when comparing NA with HY, while the comparison of transcriptome response associated with autophagy/mitophagy between NR and HY could help us determine the impact of hypoxia.

3.3 Correlation assessment of biological replicate

To quantify the variability of human skeletal muscle transcriptome among divergent participants and exercise intensity at four different time points, I compared gene identification across all individuals, and correlation coefficients were identified between each variable and other individuals. Most similar samples are reflected by a darker red line (Figure 10). The heatmap showed that no clear separation in NA, NR, and HY at all time points. The reason may be that the intensity of exercise in the three conditions was similar. The similar intensity between three sessions likely results in a high correlation between the biological samples, as our lowest intensity (NA) is still above the lactate threshold. Another reason could also be that individual variations in the human samples were considerable. Variation is greater than adaptation response, therefore, the changes are masked. A 12-week HIIT study evaluated the individual physiological and mitochondria responses that suggested mitochondrial markers were highly variable both between and within individuals [210]. Studies have proved that biological variability is a basic feature of gene expression [211]. Gene expression has biological variability among individuals, and the

degree of expression variability among different genes is different, but sequencing technology cannot eliminate this variability [211]. In order to eliminate the expression differences caused by biological variability and obtain more accurate results, the most commonly used and effective method is to set up biological replicates in experimental design. The reliability of differential expression analysis largely depends on the quality and quantity of biological replicates. Choosing the appropriate number of biological replicates is a trade-off between cost and precision in any experimental design, although it is widely recognized that increasing the number of replicates in RNA-seq experiments generally leads to more reliable results [212, 213]. One study has shown that there should be at least six biological replicates [213]. Assessing the correlation of biological replicates is important for analyzing transcriptome sequencing data, not only to evaluate the reliability of differentially expressed genes but also to assist in the screening of abnormal samples. So, how to accurately evaluate the number of biological replicates and reliable results in human studies needs further exploration.

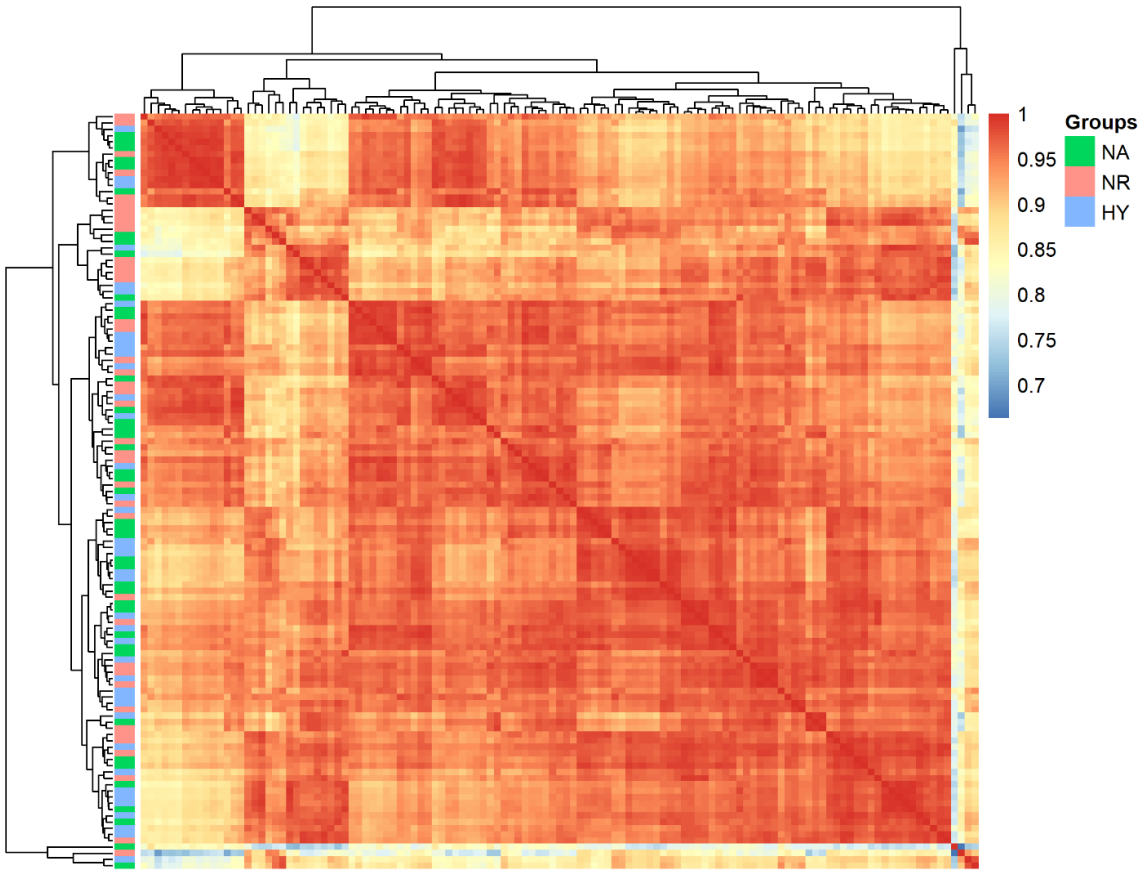


Figure 10. Correlation heatmap between 120 samples in NA, NR, HY

3.4 General transcriptome response to HIIE in different conditions

I conducted RNA-seq analysis on skeletal muscle samples from 120 healthy males, identifying a total of 3698 significantly differential genes. Among these genes, 2546 were up-regulated, while 1152 displayed down-regulation (Table 5). The maximum number of differential expression genes identified under the HY condition was 1742 (Table 5). The overall differential gene number of NA and NR was similar, while NA was slightly higher than NR (1011 and 945, respectively) (Table 5). I also found that the number of up-regulated genes was far more than the down-regulated genes in three different conditions (NA, NR, HY). The largest number of differential genes were observed 24 hours following a single session of HIIE, while the lowest number was identified immediately after a single session of HIIE (Table 5). Bar chart clearly presented the number trend of differentially expressed genes at four time points under different conditions (Figure 11). The largest number of up-regulated and down-regulated genes were detected 24 hours after exercise under hypoxia conditions.

From the number of differentially expressed genes, hypoxia did differ from normoxia, with hypoxia having almost twice as normoxia at 24 hours after HIIE. However, no such differences were found at 0 and 3 hours. These indicated that there were more differentially expressed genes induced by hypoxia in the late stage compared with NA and NR.

Table 5. The number of differentially expressed genes at different conditions and time points after HIIE

DEGs	DEG Number	Up-regulated	Down-regulated
NAB_NAP0	48	44	4
NAB_NAP3	247	230	17
NAB_NAP24	716	494	222
NRB_NRP0	61	58	3
NRB_NRP3	257	207	50
NRB_NRP24	627	411	216
HYB_HYP0	33	30	3
HYB_HYP3	251	218	33
HYB_HYP24	1458	854	604

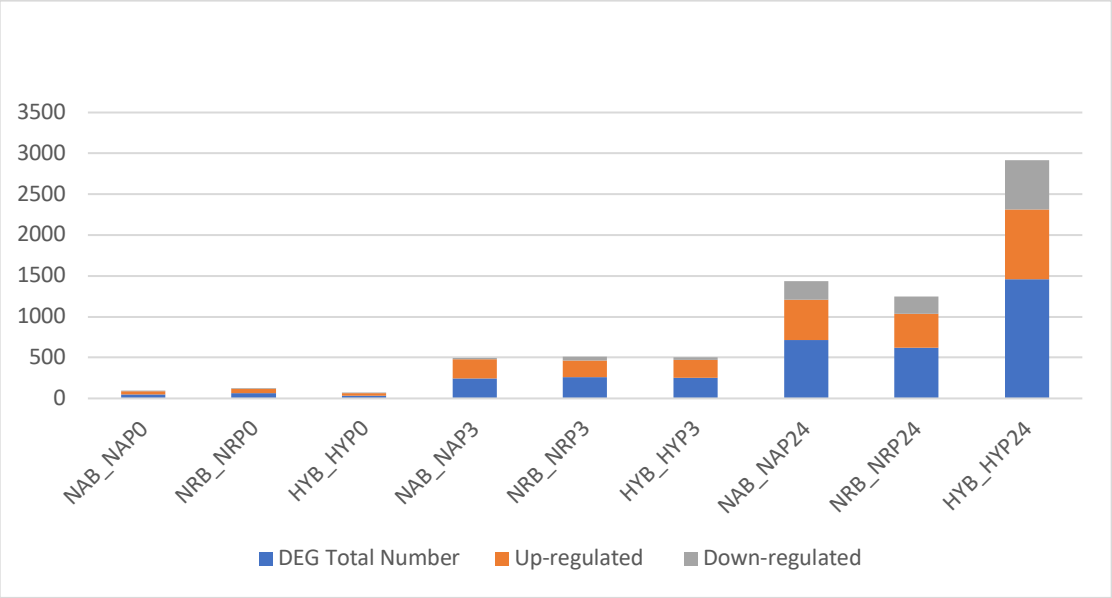


Figure 11. The trend of differential genes that were up-regulated and down-regulated at different conditions and time points after HIIE

3.5 Comparison of the common and unique genes regulated in different conditions at various time points

In this study, the analysis evaluated common and unique genes regulated by different conditions at various time points. At P0H, NR was dominated for up-regulated genes when compared to NA and HY (Figure 12A). Specifically, 23 common genes were increased expression among three different conditions (Figure 12A), with four of them being related to autophagy, including Heat Shock 70 KDa Protein 1A (*HSPA1A*), Recombinant Jun B Proto-Oncogene (*JUNB*), *MYC*, Early Growth Response (*EGR1*) (Figure 12A). Conversely, very few down-regulated genes were detected across these conditions at P0H, with no common genes identified in the three conditions (Figure 12B).

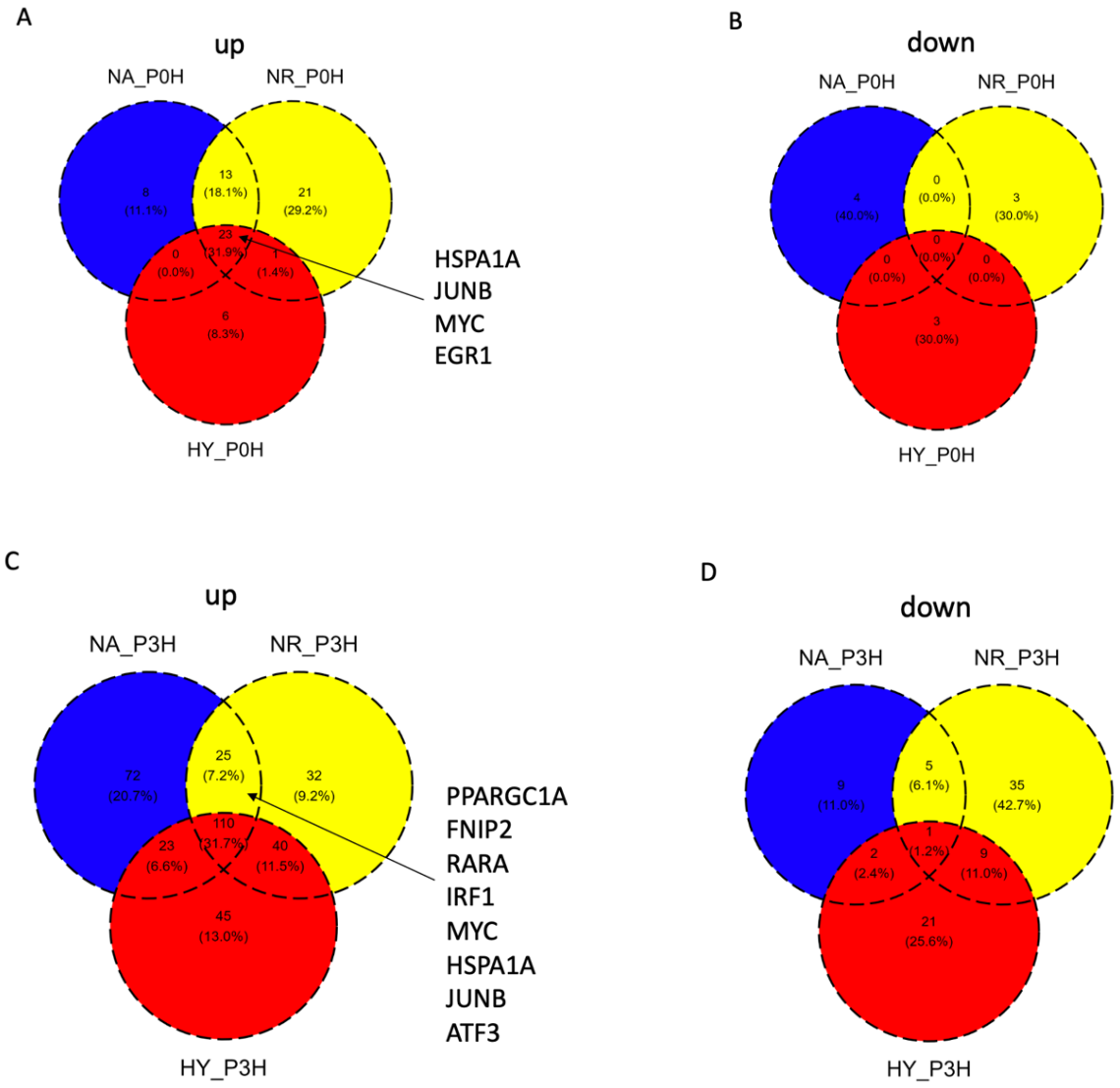
At P3H, there was a notable increase in up-regulated genes, with 110 shared genes across the conditions. Among these, eight genes were associated with autophagy, including PPARGC1A (*PGC-1α*), Folliculin Interacting Protein 2 (*FNIP2*), Retinoic Acid Receptor Alpha (*RARA*), Interferon Regulatory Factor1(*IRF1*), Activating Transcription Factor 3(*ATF3*), as well as *MYC*, *HSPA1A*, *JUNB* mentioned above (Figure 12C). NA had the largest number of unique genes at this time point (Figure 12C). In contrast to P0H, more down-regulated genes were observed, again with

NR contributing significantly (Figure 12D). Only one down-regulated gene was common 3 hours post-exercise (Figure 12D).

Of note, the largest number of up-regulated and down-regulated genes were detected 24 hours following a single session of HIIE, with the number of shared genes reaching 322 and 99, respectively (Figure 12EF). Of the 322 overlapped up-regulated genes, 16 genes were associated with autophagy, they were Transglutaminase 2 (*TGM2*), RELB proto-oncogene (*RELB*), Solute Carrier Family 36 Member 1(*SLC36A1*), FNIP2, X-Box Binding Protein 5 (*XBP1*), Transcription Factor Binding To IGHM Enhancer 3 (*TFE3*), Recombinant human RAB27A protein (*RAB27A*), NPC Intracellular Cholesterol Transporter 1 (*NPC1*), Glycerate Kinase (*GLYCTK*), CD68 Molecule (*CD68*), DNA Damage Regulated Autophagy Modulator 1 (*DRAM1*), Epidermal Growth Factor (*EGF*), Sestrin 2 (*SESN2*), *MYC*, Sirtuin 7 (*SIRT7*), Serine/Threonine Kinase (*STK39*) (Figure 12E) and I also detected *TGM2*, *TFE2*, *SESN2* three genes related to mitophagy. Furthermore, PPARG Coactivator 1 Beta (*PPARGC1B*) was the only one down-regulated autophagy-related gene at 24 h of recovery in different conditions (Figure 12F). Interestingly, this study found that the number of up-regulated and down-regulated genes in the HY condition was much higher than that of NA and NR (Figure 12EF). In addition, the number of unique up-regulated and down-regulated genes in HY condition arrived at 359 and 373, respectively, and the down-regulated genes were slightly higher than that of up-regulated genes (Figure 12EF). At post-exercise 24 h, the number of unique up-regulated and down-regulated genes in NR was much lower than that of HY, which are 14 and 27 (Figure 12EF). There was similar number of unique up-regulated and down-regulated in NA (Figure 12EF).

Venn diagram showed the distinct common and unique genes among NA, NR, and HY across various time points. At P0H, the number of common genes between NR and HY shared only one gene, which is less than NA vs. NR (13) (Figure 12AB). At P3H, there is the largest number of common up-regulated and down-regulated genes between NR and HY (Figure 12CD). At P24H, NR and HY shared the fewest up-regulated genes in common, and the common down-regulated genes also obtained less than NA and NR (Figure 12EF). These results mentioned above indicated that NR and HY did not share more common genes despite the similar results we found with physiological tests. Interestingly, at P24H, there are more unique genes to HY. Specifically, the up-regulated unique genes reached 359 in HY compared with NA (56) and NR (14), and the number of down-regulated unique genes reached 373 compared with NA (49) and NR (27) (Figure 12EF).

The number of unique differentially expressed genes in HY was greater than that in NA and NR, which suggests those genes should be induced by HY only but not increased intensity.



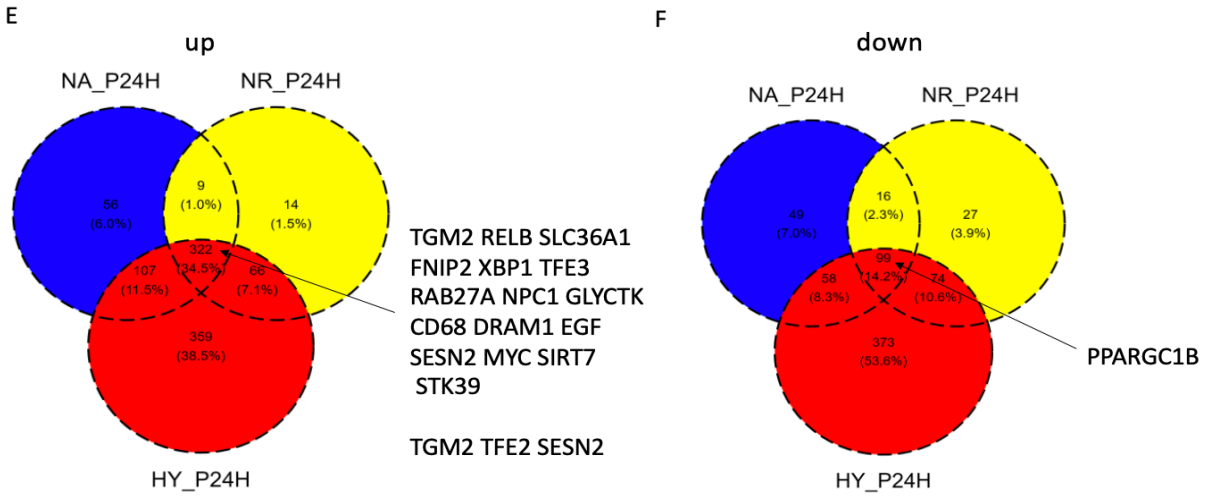


Figure 12. Venn diagram showed the overlap of differentially expressed genes at different time point in different conditions (NA, NR, HY). AB: The number of common and unique genes differentially expressed at P0H in different conditions. CD: The number of common and unique genes differentially expressed at P3H in different conditions. EF: The number of common and unique genes differentially expressed at P24H in different conditions.

3.6 Comparison of exercise-induced skeletal muscle gene expression using PCA analysis in different conditions

The PCA analysis revealed discrete clusters of gene responses associated with different exercise interventions (Figure 13). Specifically, the studies assessing the effects of various conditions exhibited clear clustering patterns. Interestingly, the overall results indicated that the hypoxia condition (HY) did not exhibit a statistically significant difference when compared to the normoxia matched for the absolute intensity of hypoxia (NA) and normoxia matched for the relative intensity of hypoxia (NR) conditions within the scope of the current study. This is consistent with the correlation assessment by heatmap (Figure 10). The reason may be that the intensity design of this exercise protocol is relatively similar, and both belong to high intensity [214]. In general, the PCA plot showed that there was no clear separation under three different conditions, which could suggest that the adaptive response induced by a single session of HIIE was similar in all conditions, just not quite the same degree. We employed two different exercise intensities under normoxia to match for hypoxia exercise sessions. However they are all above the lactate threshold. Thus, it is possible that the adaptive responses with each condition were not distinct to each other.

Furthermore, individual variability between participants could be another possible reason. As suggested by Timmons, J.A., studying the acute molecular response of the human to exercise and attempting to infer the mechanisms driving chronic adaptation, there is also potential danger in the absence of evidence to suggest that each participant has adaptive potential [215]. And participant-to-participant existed in variability in training-induced human skeletal muscle adaptive response [215]. A study has reported that if there was too much within-participant variability, the inter-individual differences in training effects could not be verified [210]. Future studies should consider larger cohorts. In a study with PCA plot showing clear segregation of acute exercise, training, and inactivity, there was no clear difference between acute exercise and training of the same exercise mode, such as acute resistance exercise and acute aerobic exercise or resistance training and aerobic training [216].

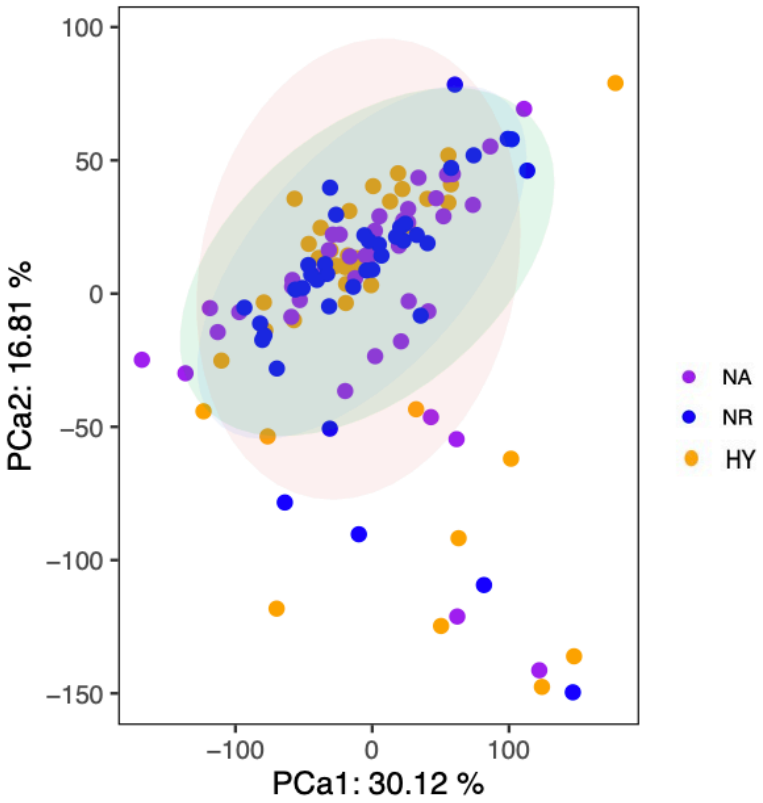


Figure 13. PCA plot of the skeletal muscle mRNA in response to the intervention of NA, NR, and HY

3.7 Pathway and functional enrichment analysis

To investigate the functional and molecular pathways regulated by a single session of HIIE in NA, NR and HY, KEGG enrichment analysis was performed using the differentially expressed genes.

Initially, this study classified these molecular pathways enriched according to the KEGG pathway database (https://www.genome.jp/kegg/pathway.html) to gain a broader perspective on these pathways. The KEGG database pathway categorizes seven categories and many subcategories. In this study, I found significant enrichment in categories such as metabolism, genetic information processing, environment information processing, cellular processes, organismal systems, human diseases, and related subcategories. Notably, the subcategory with a higher proportion of pathways included cancer-specific types, signal transduction, infectious disease: viral, immunes system, and cell growth and death. Details of major categories and subcategories that are significantly enriched are presented in Table 6.

Table 6. Significantly enriched KEGG pathway

KEGG_A_class	KEGG_B_class	Pathway
1.Metabolism	**1.0 Global and overview maps** **1.1 Carbohydrate metabolism** **1.7 Glycan biosynthesis and metabolism**	1.Nucleotide metabolism 2.Biosynthesis of nucleotide sugars 1.Fructose and mannose metabolism 1.Glycosaminoglycan biosynthesis - chondroitin sulfate / dermatan sulfate
2.Genetic Information Processing	**2.2 Transcription** **2.3 Folding, sorting and degradation**	1. Ribosome biogenesis in eukaryotes 1. Protein processing in endoplasmic reticulum
3.Environmental information processing	**3.2 Signal transduction** **3.3 Signaling molecules and interaction**	1.MAPK signaling pathway 2.TNF signaling pathway 3.FoxO signaling pathway 4. HIF-1 signaling pathway 5.AMPK signaling pathway 6.Notch signaling pathway 7.JAK-STAT signaling pathway 8.ErbB signaling pathway 9.NF-kappa B signaling pathway 10.PI3K-Akt signaling pathway 11.Sphingolipid signaling pathway 12.Apelin signaling pathway 13.Calcium signaling pathway 14.cGMP-PKG signaling pathway 1.Cytokine-cytokine receptor interaction 2. ECM-receptor interaction
4.Cellular processes	**4.1 Transport and catabolism** **4.2 Cell growth and death** **4.3 Cellular community – eukaryotes** **4.5 Cell motility**	1. Mitophagy – animal 2. Autophagy – animal 1.Apoptosis 2.P53 signaling pathway 3.Cell cycle 4.Cellular senescence 5.Ferroptosis 1.Gap junction 2.Focal adhesion 1.Regulation of actin cytoskeleton
5.Organismal systems	**5.1Immune system** **5.4 Digestive system** **5.6 Nervous system** **5.9 Aging** **5.10 Environmental adaptation**	1. Th17 cell differentiation 2.Th1 and Th2 cell differentiation 3.C-type lectin receptor signaling pathway 4.Prolactin signaling pathway 5.Oxytocin signaling pathway 6.Insulin signaling pathway 7. Adipocytokine signaling pathway 8.Relaxin signaling pathway 1.Mineral absorption 2.Protein digestion and absorption 1.Neurotrophin signaling pathway 1.Longevity regulating pathway - multiple species 1.Circadian rhythm
6. Human Diseases	**6.1 Cancer: overview** **6.2 Cancer specific types** **6.3 Infectious disease:viral** **6.4 Infectious disease: bacterial** **6.5 Infectious disease: parasitic** **6.6 Immune disease** **6.8 Substance dependence** **6.9 Cardiovascular disease** **6.10 Endocrine and metabolic disease** **6.12 Drug resistance: antineoplastic**	1.Chemical carcinogenesis - receptor activation 2.Transcriptional misregulation in cancer 3.Proteoglycans in cancer 4. MicroRNAs in cancer 1.Breast cancer 2.Colorectal cancer 3.Thyroid cancer 4.Endometrial cancer 5.Chronic myeloid leukemia 6.Bladder cancer 7.Small cell lung cancer 8.Basal cell carcinoma 9.Acute myeloid leukemia 10.Non-small cell lung cancer 11.Prostate cancer 12. Pancreatic cancer 13.Melanoma 14.Glioma 15.Gastric cancer 16.Hepatocellular carcinoma 1.Human T-cell leukemia virus 1 infection 2. Hepatitis B 3.Kaposi sarcoma-associated herpesvirus infection 4.Epstein-Barr virus infection 5. Legionellosis 6. Measles 7. Influenza 8.Hepatitis C 9.Human cytomegalovirus infection 1.Salmonella infection 2.Pathogenic Escherichia coli infection 1.Chagas disease 2. Malaria 1.Rheumatoid arthritis 1.Amphetamine addiction 1.Lipid and atherosclerosis 2. Fluid shear stress and atherosclerosis 1.AGE-RAGE signaling pathway in diabetic complications 2.Insulin resistance 3.Type II diabetes mellitus 4.Non-alcoholic fatty liver disease 1.Endocrine resistance

Subsequently, this study evaluated the signaling pathway induced by a single session of HIIE at different time points under three conditions (NA, NR, HY). I observed that immediately after exercise (P0H), only the up-regulated pathways were significantly enriched under all conditions. NR had the most enriched pathways (34), followed by NA (21), while HY had only four signaling pathways enriched at P0H (Figure 14). To provide a clearer overview of these pathways under different conditions (NA, NR, HY), I visualized the top 20 KEGG pathways. Commonly enriched pathways at P0H, including the MAPK signaling pathway, Lipid and atherosclerosis, and TNF signaling pathway, were detected in all conditions, and Legionellosis only showed in hypoxia (Figure 15ABC).

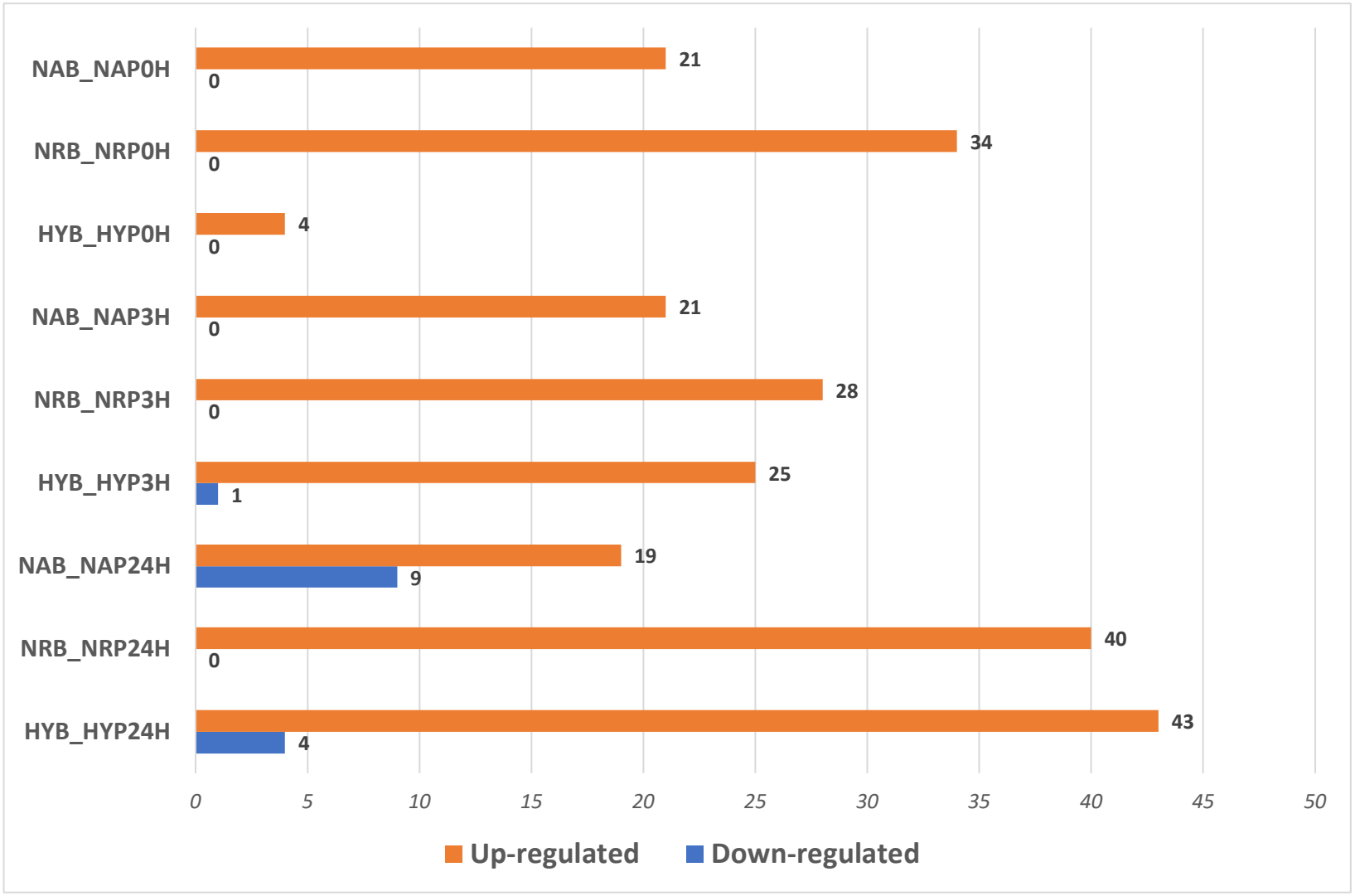
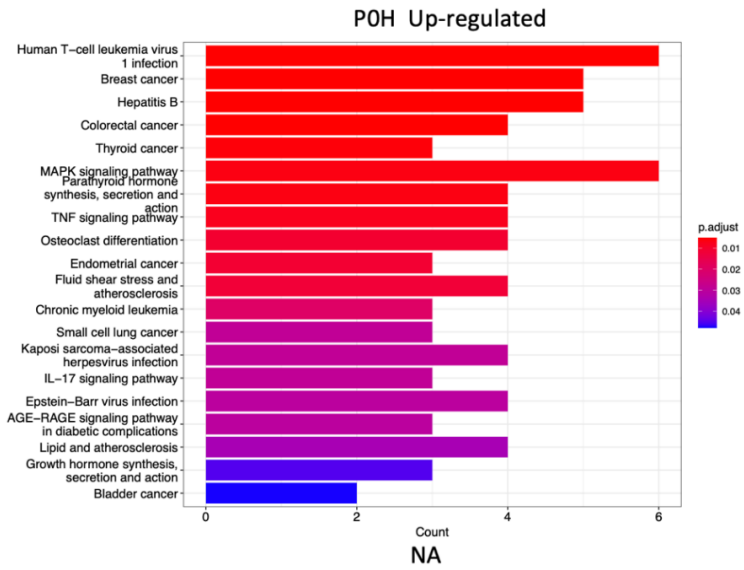
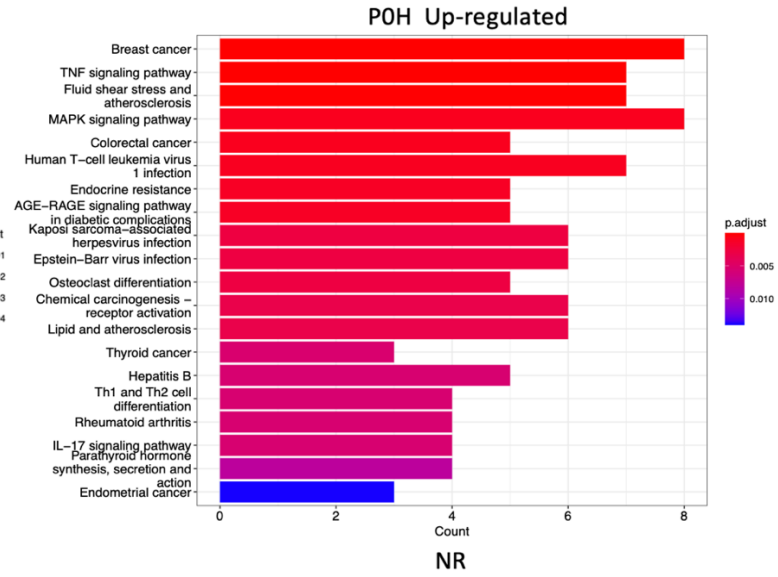


Figure 14. The number of significantly enriched pathways induced by a single session of HIIE at different time points in three conditions (NA, NR, HY)

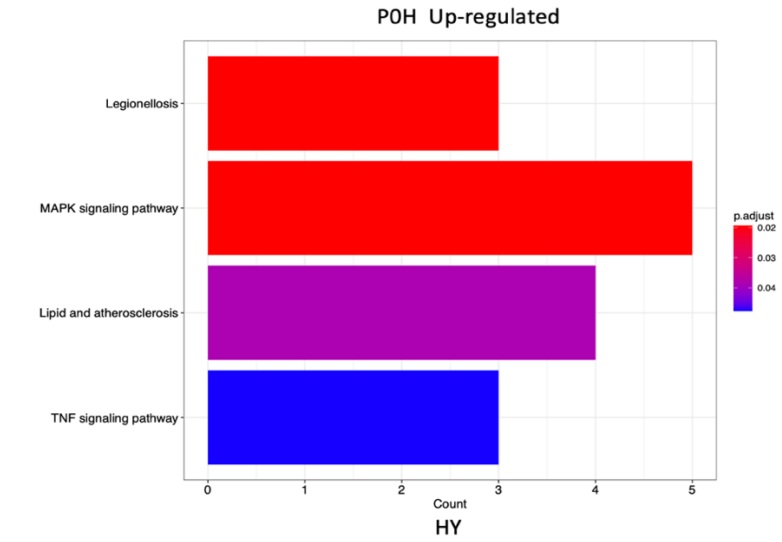
A


P0H Up-regulated
Human T−cell leukemia virus 1 infection
Breast cancer
Hepatitis B
Colorectal cancer
Thyroid cancer
MAPK signaling pathway
Parathyroid hormone synthesis, secretion and action
TNF signaling pathway
Osteoclast differentiation
Endometrial cancer
Fluid shear stress and atherosclerosis
Chronic myeloid leukemia
Small cell lung cancer
Kaposi sarcoma−associated herpesvirus infection
IL−17 signaling pathway
Epstein−Barr virus infection
AGE−RAGE signaling pathway in diabetic complications
Lipid and atherosclerosis
Growth hormone synthesis, secretion and action
Bladder cancer
p.adjust
0.01
0.02
0.03
0.04
0
2
4
6
Count
NA

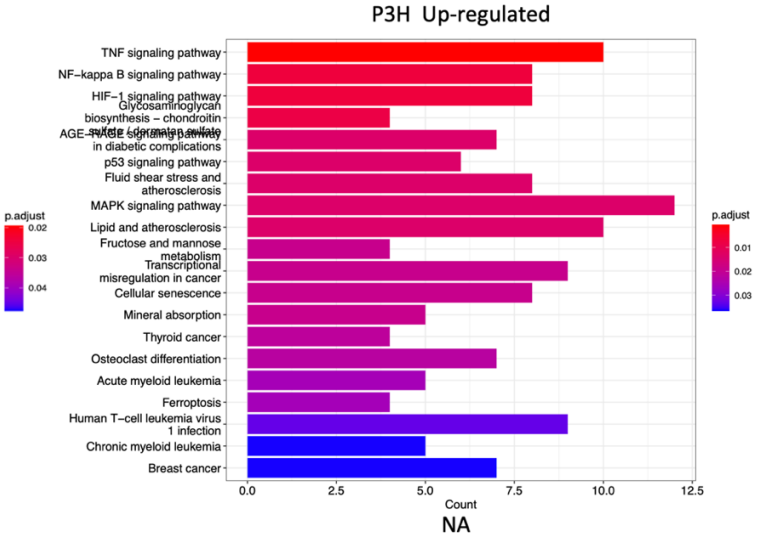
B


P0H Up-regulated
Breast cancer
TNF signaling pathway
Fluid shear stress and atherosclerosis
MAPK signaling pathway
Colorectal cancer
Human T−cell leukemia virus 1 infection
Endocrine resistance
AGE−RAGE signaling pathway in diabetic complications
Kaposi sarcoma−associated herpesvirus infection
Epstein−Barr virus infection
Osteoclast differentiation
Chemical carcinogenesis − receptor activation
Lipid and atherosclerosis
Thyroid cancer
Hepatitis B
Th1 and Th2 cell differentiation
Rheumatoid arthritis
IL−17 signaling pathway
Parathyroid hormone synthesis, secretion and action
Endometrial cancer
p.adjust
0.005
0.010
0
2
4
6
8
Count
NR

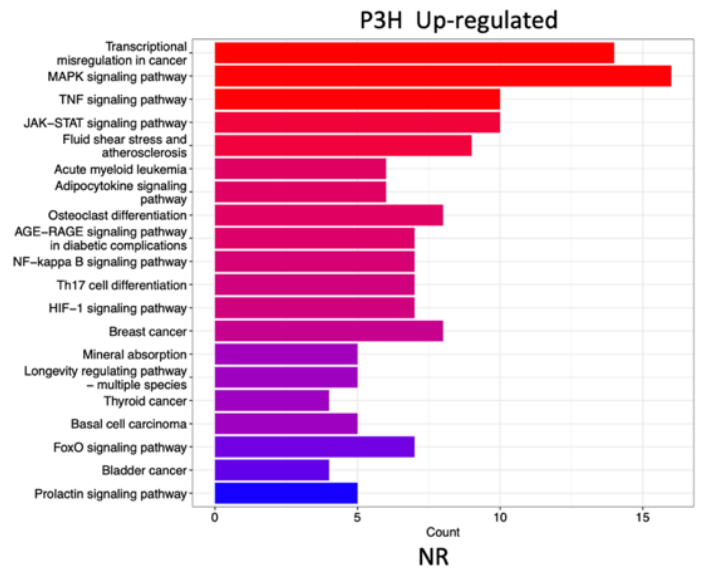
C


P0H Up-regulated
Legionellosis
MAPK signaling pathway
Lipid and atherosclerosis
TNF signaling pathway
p.adjust
0.02
0.03
0.04
0
1
2
3
4
5
Count
HY

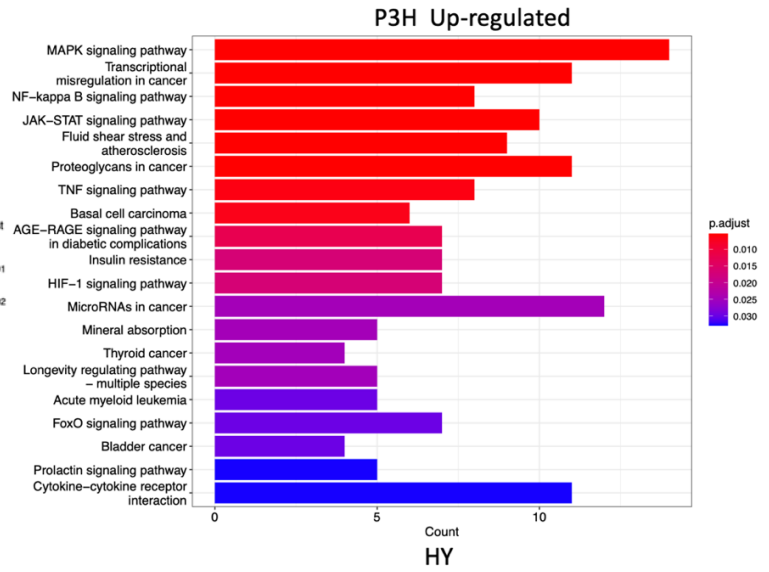
D


P3H Up-regulated
TNF signaling pathway
NF−kappa B signaling pathway
HIF−1 signaling pathway
Glycosaminoglycan biosynthesis − chondroitin
AGE−RAGE signaling pathway in diabetic complications
p53 signaling pathway
Fluid shear stress and atherosclerosis
MAPK signaling pathway
Lipid and atherosclerosis
Fructose and mannose metabolism
Transcriptional misregulation in cancer
Cellular senescence
Mineral absorption
Thyroid cancer
Osteoclast differentiation
Acute myeloid leukemia
Ferroptosis
Human T−cell leukemia virus 1 infection
Chronic myeloid leukemia
Breast cancer
p.adjust
0.01
0.02
0.03
0.0
2.5
5.0
7.5
10.0
12.5
Count
NA

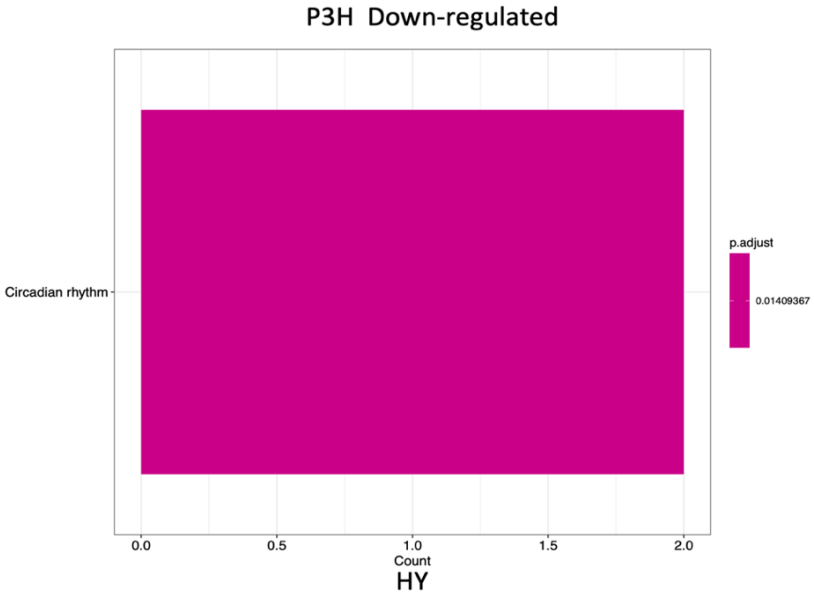
E


P3H Up-regulated
Transcriptional misregulation in cancer
MAPK signaling pathway
TNF signaling pathway
JAK–STAT signaling pathway
Fluid shear stress and atherosclerosis
Acute myeloid leukemia
Adipocytokine signaling pathway
Osteoclast differentiation
AGE–RAGE signaling pathway in diabetic complications
NF–kappa B signaling pathway
Th17 cell differentiation
HIF–1 signaling pathway
Breast cancer
Mineral absorption
Longevity regulating pathway – multiple species
Thyroid cancer
Basal cell carcinoma
FoxO signaling pathway
Bladder cancer
Prolactin signaling pathway
0
5
10
15
Count
p.adjust
0.01
0.02
NR

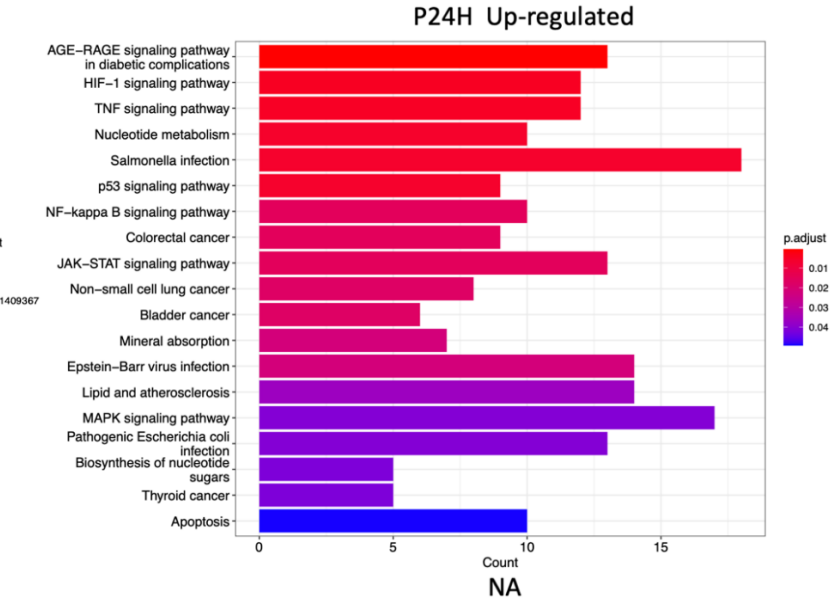
F


P3H Up-regulated
MAPK signaling pathway
Transcriptional misregulation in cancer
NF–kappa B signaling pathway
JAK–STAT signaling pathway
Fluid shear stress and atherosclerosis
Proteoglycans in cancer
TNF signaling pathway
Basal cell carcinoma
AGE–RAGE signaling pathway in diabetic complications
Insulin resistance
HIF–1 signaling pathway
MicroRNAs in cancer
Mineral absorption
Thyroid cancer
Longevity regulating pathway – multiple species
Acute myeloid leukemia
FoxO signaling pathway
Bladder cancer
Prolactin signaling pathway
Cytokine–cytokine receptor interaction
0
5
10
Count
p.adjust
0.010
0.015
0.020
0.025
0.030
HY

G


P3H Down-regulated
Circadian rhythm
0.0
0.5
1.0
1.5
2.0
Count
p.adjust
0.01409367
HY

H


P24H Up-regulated
AGE–RAGE signaling pathway in diabetic complications
HIF–1 signaling pathway
TNF signaling pathway
Nucleotide metabolism
Salmonella infection
p53 signaling pathway
NF–kappa B signaling pathway
Colorectal cancer
JAK–STAT signaling pathway
Non–small cell lung cancer
Bladder cancer
Mineral absorption
Epstein–Barr virus infection
Lipid and atherosclerosis
MAPK signaling pathway
Pathogenic Escherichia coli infection
Biosynthesis of nucleotide sugars
Thyroid cancer
Apoptosis
0
5
10
15
Count
p.adjust
0.01
0.02
0.03
0.04
NA

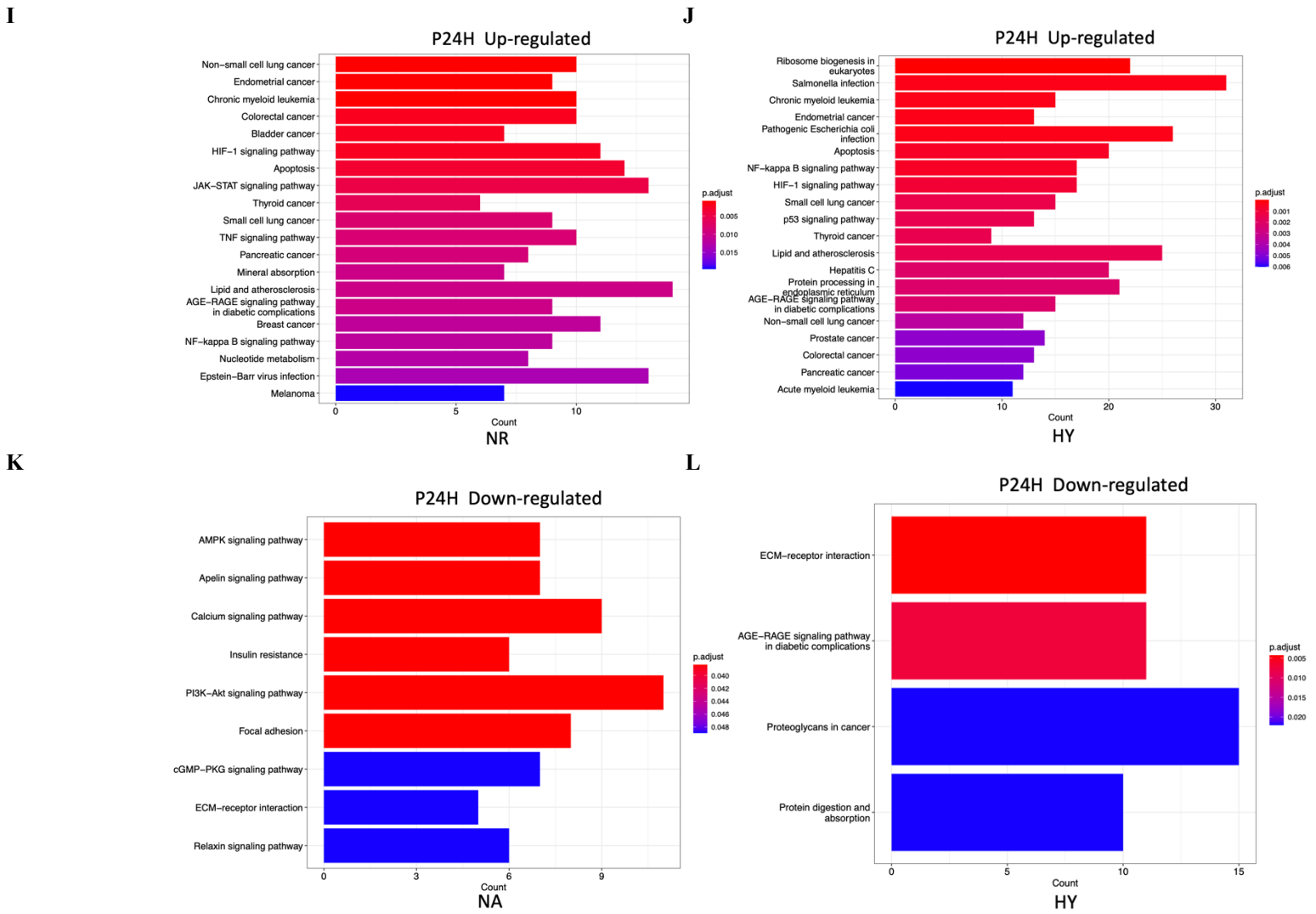


Figure 15. KEGG enrichment analysis of the differentially expressed genes. ABC: Top20 significantly up-regulated pathways in NA, NR, HY at P0H; DEF: Top20 significantly up-regulated in NA, NR, HY at P3H; G: Top20 significantly down-regulated pathways in HY at P3H; HIJ: Top20 significantly up-regulated pathways in NA, NR, HY at P24H; KL: Top20 significantly down-regulated pathways in NA, HY at P24H respectively.

At P3H, a roughly similar number of up-regulated pathways were observed in NA (21), NR (28), and HY (25) (Figure 14). Notably, a greater number of signaling pathways were activated in HY at P3H. Additionally, it was found that more signal transduction pathways were enriched at P3H in all conditions compared with immediately after exercise. These included pathways like the MAPK signaling pathway, JAK-STAT signaling pathway, NF-kappa B signaling pathway, and HIF-1 signaling pathway (Figure 15DEF). FOXO signaling pathway was uniquely found in NR and HY (Figure 15EF).

Apart from the signal transduction pathway, pathways associated with human disease were also identified in three conditions at P3H. These included Transcriptional misregulation in cancer, Fluid shear stress and atherosclerosis, AGE-RAGE signaling pathway, Proteoglycans in cancer in diabetic complications, and Acute myeloid leukemia (Figure 15DEF). Additionally, some pathways were uniquely observed in one condition, such as the Th17 cell differentiation pathway related to the immune system, which was only found at P3H in NR (Figure 15E). Though the PI3K-Akt signaling pathway did not make the top 20 list, it was significantly enriched by up-regulated genes only in hypoxia (Figure 15F). The P53 signaling pathway showed greater significance in NA condition, although it was also observed in HY. Notably, only one down-regulated pathway showed at P3H, which was Circadian rhythm pathway (Figure 15G).

Examining 24 hours after exercise, I noted that NR (40) and HY (43) had more pathways up-regulated genes compared to P0H and P3H, while NA (19) had the fewest (Figure 14). Interestingly, the number of up-regulated pathways enriched under HY showed a sustained upward trend with immediate, 3 hours, and 24 hours after a single session of HIIE (Figure 14). Similar to the 3-hour and the 24-hour post-exercise periods, pathways associated with human disease, metabolism, genetic information processing, organismal systems, cellular processes, and signal transduction accounted for the largest proportion. Notably, pathways associated with cellular processes increased at P24H, especially in hypoxia, such as mitophagy-animal, autophagy-animal, apoptosis, P53 signaling pathway, ferroptosis, gap junction, focal adhesion, cellular senescence. These above pathways were observed at least in one condition, of which the apoptosis pathway was observed in all conditions.

Finally, at P24H, most down-regulation pathways were observed under NA condition, including the AMPK signaling pathway, apelin signaling pathway, calcium signaling pathway, insulin resistance, PI3K-Akt signaling pathway, ECM-receptor interaction and relaxin signaling pathway

(Figure 15K). The ECM-receptor interaction pathway was also be down-regulated in HY. Additionally, the AGR-RAGE signaling pathway in diabetic complications, proteoglycans in cancer, protein digestion and absorption pathway were regulated by down-regulated genes.

Significantly Regulated Pathways Linked to Autophagy and Mitophagy in Response to HIIE in Hypoxia

HIIE induced and activated many significant pathways in both normoxia and hypoxia after exercise immediately, 3 hours, and 24 hours, and the number of enriched up-regulated pathways was significantly higher than that of down-regulated pathways. The majority of pathways observed were implicated in human disease, signal transduction, cellular processes, and the immune system. Reviewing the literature found that the studies using HIIE protocols were relatively limited, particularly the study on activation pathways by a single session of HIIE under hypoxia condition. Subsequently, combining extensive literature research, the partial pathways induced by different exercise protocols have been discussed. I noticed that pathway results activated by exercise were affected by different factors and variables. Although differences in exercise intensity, duration, mode, subject group, and biopsy sampling time may lead to inconsistent results, and it was even difficult to make comparisons due to these discrepancies, I have found that the findings of many exercise protocols studies were similar to those of HIIE intervention. To be specific, other exercise interventions induced pathways, as well as shown in my study. This illustrated that the HIIE protocol was effective in this study. For pathways enrichment in three conditions, at exercise immediately, exercise workload matched for the relative intensity in hypoxia (NR) enriched the highest significant pathways, while the lowest number in hypoxia (HY). Following exercise 3 hours, the number of up-regulated pathways in NR and HY were similar, and exercise workload matched for the absolute intensity in hypoxia (NA) accounted for the lowest number. At post-exercise 24 hours, it was observed that more signals were activated in NR and HY. Especially under hypoxia conditions, the number of induced pathways continuously increased with the sampling time after exercise (P0H, P3H, P24H).

The molecular mechanisms related to autophagy/mitophagy have received more attention from researchers in recent years. A number of studies have shown that many signaling pathways are involved in the regulation of autophagy and mitophagy. However, the precise mechanisms that

HIIE combined hypoxia mediating autophagy/mitophagy responses are not completely understood. In the current study, I performed KEGG functional enrichment analysis to examine whether the core pathways related to autophagy/mitophagy were significantly enriched in three conditions (NA, NR, HY), especially in hypoxia. It is well known that the mammalian target of rapamycin (mTOR) signaling pathway play a crucial role in the regulation of autophagy [217]. mTOR can negatively regulate autophagy [218]. Inhibition of mTOR can lead to Unc-51 kinase 1(ULK1) disinhibition, which phosphorylates $Beclin1^{S14}$, and then enhances the complexes of Atg14L-VPS34 activity and autophagy induction [219, 220]. An experimental study indicated that both acute aerobic exercise (AE, 40 min cycling, 70% HR_{max}) and resistant exercise (RE, 8 sets, 10 repetitions, 65% 1RM) stimulate mTOR signaling in skeletal muscle of untrained individuals during the early post-exercise period (post-exercise 1 hours and 4 hours) [221]. However, mTOR signaling pathway has not been identified significantly in all conditions (NA, NR, HY) in present study. The adenosine monophosphate-activated protein kinase (AMPK) signaling pathway is a classical signaling pathway that regulates autophagy [222]. As a key energy sensor, activation of AMPK can inhibit mTOR complex1(mTORC1) activity, thereby enhancing autophagy [77, 222]. Importantly, AMPK has been shown to be involved in the transcriptional regulation of autophagy by regulating the activity of the transcription factor forkhead box (FOXO) and participating in the post-translational regulation of this pathway through phosphorylation of ULK1 [217]. A mice study demonstrated that acute exercise (a single session of exhaustive treadmill) significantly induced AMPK content and AMPK activity at post-exercise 0 hours, 6 hours, and 12 hours. Moreover, the enhanced AMPK activity was related to the regulation of autophagic marker [223]. A human skeletal muscle study (well-trained athletes) indicated that cycling exercise-induced autophagy relies on the AMPK signaling pathway, and this process seems to rely more on the exercise intensity than the nutrient supply, as high-intensity exercise has been shown to increase autophagic flux [91]. However, a review paper concluded that AMPK alone seems insufficient to increase autophagosome content during exercise [224]. In the current study, the AMPK signaling pathway was significantly enriched by down-regulated genes only at post-exercise 24 hours in NA. The phosphatidylinositol 3 kinase PI3K and kinase AKT (AKT) signaling pathway (PI3K-Akt signaling pathway) was deemed to a main upstream modulator of mTORC1, and its activation can also induce autophagy [225, 226]. Interestingly, the PI3K-Akt signaling pathway was enriched significantly up-regulated 3 hours after exercise in HY (ranked out of top 20), while it was enriched

significantly down-regulated post-exercise 24 hours in NA. MAPK signaling pathway and their upstream and downstream genes were considered to be involved in autophagy regulation [227]. In this study, the MAPK signaling pathway was induced strongly at all time points in three conditions, especially at 3 hours after a single session of HIIE in HY (p = 7.54E-05). Currently, some studies suggest that there were two ways to mediate mitophagy: one was PTEN-induced kinase 1 (PINK1) and PARKIN pathway (PINK1-Parkin pathway), the other was mitophagy receptor mediation (NIX, BNIP3, FUDNC1) [64, 228]. However, these two ways of induction were difficult to assess from the current enriched pathways. Of note, autophagy-animal and mitophagy-animal pathways were significantly enriched by up-regulated genes at 24 hours post-exercise in hypoxia, even though these two pathways were not ranked in the top 20. Collectively, these pathway enrichment analysis findings show that hypoxia combined with HIIE was not observed to make a robust induce on autophagy and mitophagy response. To our knowledge, this is the first study to investigate the early signaling response induced by a single session of HIIE in divergent conditions (normoxia and hypoxia).

MAPK signaling pathway and NF-kappa B signaling pathway

As molecular pathways aforementioned, some of which have been extensively studied, such as the MAPK signaling pathway and NF-kappa B signaling pathway.

In this study, the Mitogen-activated protein kinase (MAPK) signaling pathway was observed to be activated by a single session of HIIE at three time points after exercise (P0H, P3H, P24H) in all conditions (NA, NR, HY). MAPK cascade is a major signal system for cells to transduce extracellular signals into intracellular responses [229, 230], which can be stimulated by external signals, such as growth factors, cytokines, cellular stress, environmental stressors, muscle contraction, and exercise [231-233]. Activation of MAPK has been implicated in a wide array of biological processes and cellular functions, including growth, differentiation, cell proliferation, inflammation, apoptosis, and adaptation [234-237]. MAPK signaling cascades are mainly composed of four branch pathways: extracellular signal-regulated kinases (ERK)1/2 (ERK1/2), p38 MAPK, c-Jun NH_2-terminal kinases (JNK), and ERK5 or big MAPK [231, 237]. A previous study has evidenced that exercise can activate ERK1/2, p38 MAPK, and JNK signaling pathways in rat skeletal muscle [233]. A review paper also reported a similar observation [238]. Some

experimental investigations have supported this conclusion. A single bout of exercise (cycle ergometer exercise) increased the MAPK signaling pathway in human skeletal muscle [229]. A knee extensor resistance exercise session (29 contractions at 70% of max) augmented the phosphorylation of the ERK1/2, P90RSK, and Mnk1 proteins in the sedentary young men [239]. An acute session of intense intermittent cycle exercise (4 × 30 s "all out" exercise sessions interspersed with 4 min of rest) increased phosphorylation of p38 MAPK immediately following exercise [240]. Long-distance running (Marathon running) was also reported to regulate ERK1/2, p38 MAPK, and their downstream substrates (increased p90rsk, MAPKAP-K2, and MSK) in human skeletal muscle [241]. Similar elevated JUN and P38 MAPK phosphorylation were observed in a single cycling session (HIIE, 5 × 4 mins at 75% W_{max}), sprint interval exercise (SIE, 4 × 30 s Wingate sprints), and continuous moderated-intensity exercise work-matched to HIIE (CMIE, 30 min at 50% of W_{max}). It was reported that hypoxia-induced ERK1/2 phosphorylation increased [242]. Overall, these studies suggested that acute exercise can activate and increase the MAPK signaling pathway. Activation of the MAPK signaling modules has been shown to be partially influenced by the type, duration, and intensity of contractile stimuli [237]. Moreover, it has been reported that activating different MAPK pathway genes by exercise provided a candidate mechanism for regulating transcription events in skeletal muscles [241].

Nuclear factor kappa-light-chain-enhancer of activated B cell (NF-kappa B) signals, just like MAPK, was deemed to be fundamental modulators of cellular stress, and which was also activated by exercise [237]. Some studies have demonstrated the crucial role of the NF-kappa B signaling pathway in inflammation, immunity, cell proliferation, apoptosis, and muscle metabolism [243-247]. In this study, the NF-kappa B signaling pathway was enriched significantly at 3 hours and 24 hours after exercise in NA, NR, and HY, while this signaling pathway was not found immediately after exercise in all conditions. The NF-kappa B signaling pathway has been enhanced after some acute exercise in rodents and human skeletal muscle. An acute bout of treadmill exercise increased the NF-kB activity following exercise 2 hours in rats vastus lateralis [248]. Some studies on rats also demonstrated similar observations [249, 250]. A single bout of intense resistance exercise has been testified to activate the NF-kappa B signaling pathway at the following exercise 2 hours in human skeletal muscle [251]. An acute endurance exercise has been reported that can elevate NF-kB activity in human muscles [252]. The above-mentioned studies were conducted under normoxia conditions, but hypoxia has also been reported to activate the NF-

kappa B signaling pathway [242, 253]. In addition to MAPK and NF-kappa B signaling pathways, some other pathways have also been studied. Their induction and activation by a variety of exercises have been reported, such as the TNF signaling pathway [254], HIF-1 signaling pathway [255, 256], P53 signaling pathway [257, 258], JAK-STAT signaling pathway [259, 260], immune system related pathway [261] and soon. However, for the purpose of this study, I would not discuss these pathways too much.

3.8 Differential expression analysis

3.8.1 The source of the target genes list associated with autophagy/mitophagy

With the development of omics technology, transcriptome analysis has been widely used for studying biological samples, and a reliable target gene list is important for research. This study mainly focuses on the autophagy/mitophagy part. To better assess the effects of a single session of HIIE on autophagy/mitophagy in human skeletal muscle with and without hypoxia, I took 604 genes related to autophagy/mitophagy as a target gene list derived from a published paper [166]. The comprehensive gene list was derived from extensive literature analysis. The researcher divided the genes into 6 main categories, including mTOR and upstream pathway (135 genes), autophagy core (197 genes), autophagy regulators (68 genes), mitophagy (80 genes), docking and fusion (22 genes), lysosome (162 genes) and lysosome-related genes (34 genes) [166], in which the study also created subgroups based on whether they belong to the same complex or specific regulatory pathway [166]. The specific classification details of the gene list are shown in the following figure (Figure 16).

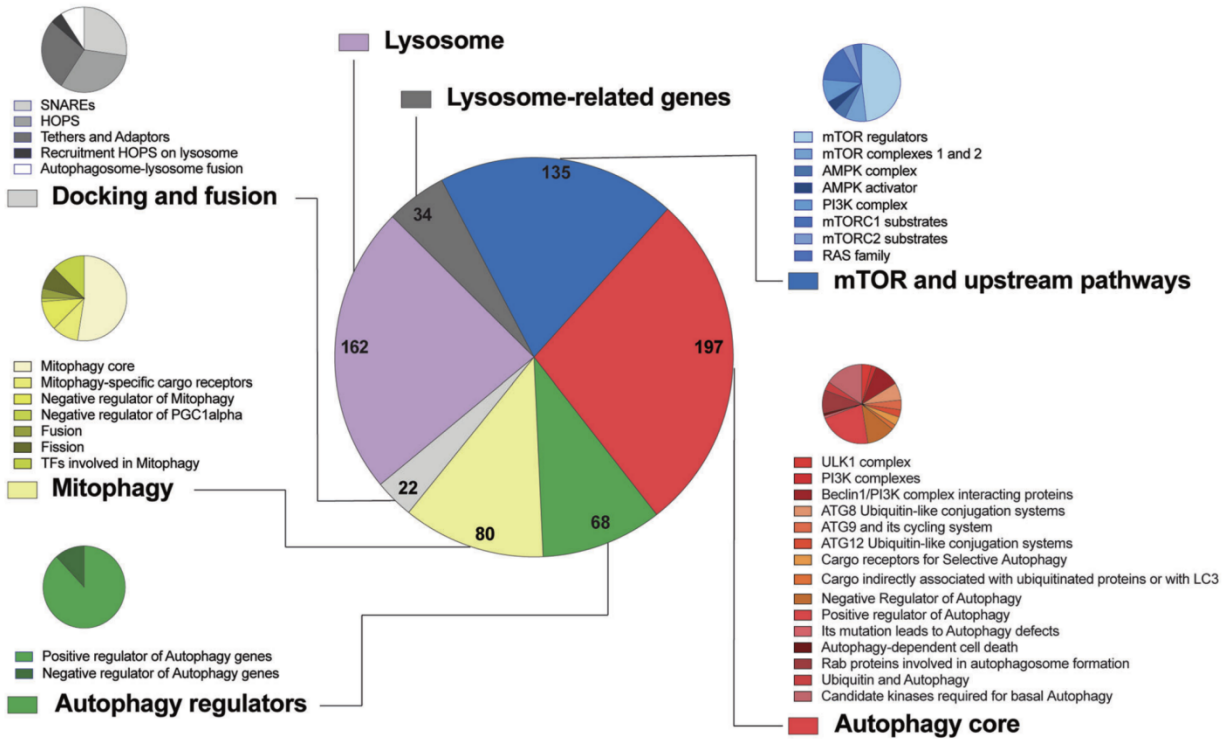


Figure 16. Target gene list categories (This figure is adopted from Matteo and Rossella et al. [166])

3.8.2 Differentially expressed genes related to autophagy/mitophagy in different condition

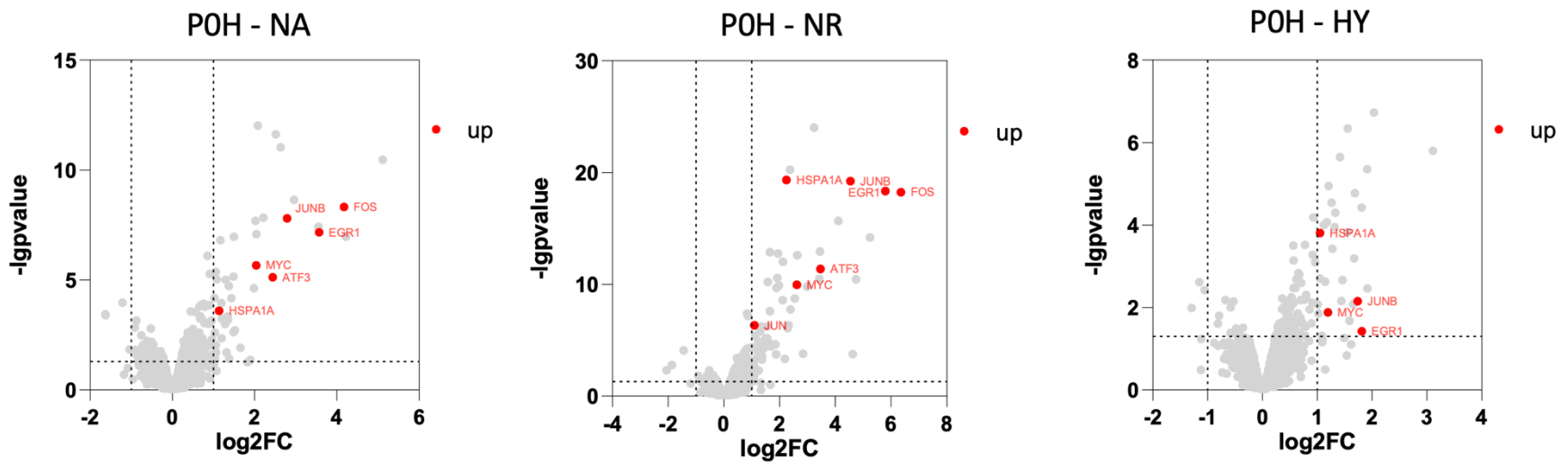
Differential Gene Expression Analysis

A total of 604 genes were selected as the target genes for the current study, and differential expression analysis was performed on them. The criteria for considering a gene as significantly differentially expressed were a Fold Change (FC) equal to or greater than 2 and a *P*-value less than 0.05. Following this stringent analysis, a total of 74 differentially expressed genes were identified, with a noteworthy predominance of 63 genes displaying upregulation and 11 genes exhibiting downregulation. To provide a comprehensive view of the temporal dynamics in gene expression changes, the analysis of this study focused on three distinct time points: immediately after exercise (P0H vs. B), 3 hours post-exercise (P3H vs. B), and 24 hours post-exercise (P24H vs. B). Across all conditions, it was consistently observed that the number of up-regulated genes outweighed down-regulated genes.

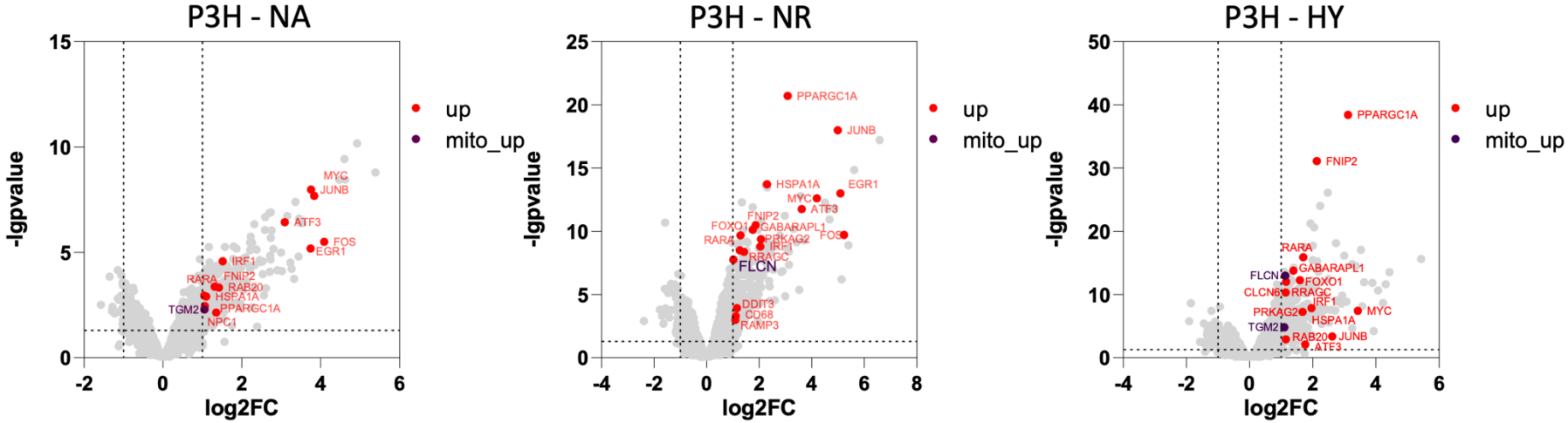
Highlights from Volcano Plot Analysis and discussion on autophagy/mitophagy-related genes

Visualizing the gene expression data using volcano plots unveiled specific patterns of gene regulation immediately after exercise. In normoxia matched for the absolute intensity of hypoxia (NA), 6 genes related to autophagy/mitophagy displayed upregulation, while in normoxia matched for the relative intensity of hypoxia (NR), 7 genes exhibited increased expression. In hypoxia (HY) after exercise, 4 genes showed upregulation compared to baseline (P0H vs. B) (Figure 17A). More up-regulated genes were identified 3 hours after exercise compared to baseline (P03H vs. B), and 13 for NA, 18 for NR, and 16 for HY were induced by a single session of HIIE, respectively (Figure 17B). The largest number of differentially expressed genes were found 24 hours after exercise (P24H vs. B), especially in hypoxia. To be specific, 25 increased and 4 decreased genes in NA, 25 up-regulated and 2 down-regulated genes in NR, and 43 increased, and 11 decreased genes in HY (Figure 17C).

A


P0H - NA
-lgpvalue
log2FC
up
JUNB
FOS
EGR1
MYC
ATF3
HSPA1A
P0H - NR
-lgpvalue
log2FC
up
HSPA1A
JUNB
EGR1
FOS
ATF3
MYC
JUN
P0H - HY
-lgpvalue
log2FC
up
HSPA1A
JUNB
MYC
EGR1

B


P3H - NA
-lgpvalue
log2FC
up
mito_up
MYC
JUNB
ATF3
FOS
EGR1
IRF1
RARA
FNIP2
RAB20
HSPA1A
TGM2
PPARGC1A
NPC1
P3H - NR
-lgpvalue
log2FC
up
mito_up
PPARGC1A
JUNB
HSPA1A
EGR1
MYC
ATF3
FNIP2
FOXO1
GABARAPL1
RARA
PRKAG2
IRF1
FOS
RRAGC
FLCN
DDIT3
CD68
RAMP3
P3H - HY
-lgpvalue
log2FC
up
mito_up
PPARGC1A
FNIP2
RARA
GABARAPL1
FLCN
FOXO1
CLCN6
RRAGC
IRF1
PRKAG2
MYC
HSPA1A
TGM2
RAB20
JUNB
ATF3

C

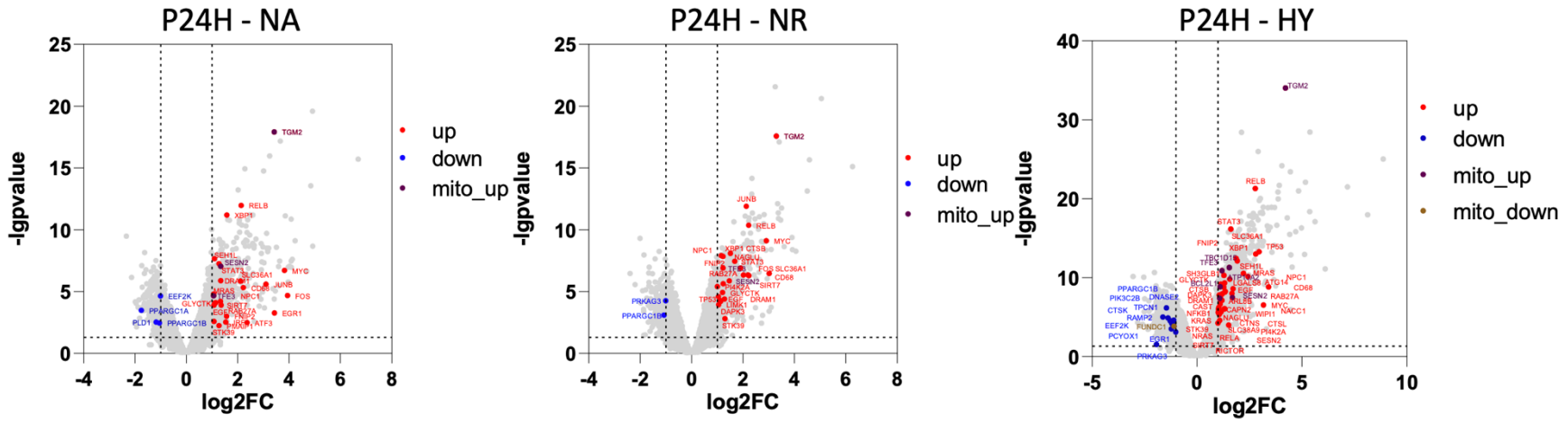


Figure 17. Volcano plot showing the target differentially expressed genes at three time points (P0H, P3H, P24H) after the intervention of NA, NR, and HY. A: Target differentially expressed genes associated with autophagy/mitophagy at P0H in three different conditions. B: Target differentially expressed genes associated autophagy/mitophagy at P3H in three conditions. C: Target differentially expressed genes associated with autophagy/mitophagy at P24H in three conditions. Blue points represent significantly down differentially expressed genes, while red points represent significantly up differentially expressed genes. Purple points represent a significant increase in differentially expressed genes related to mitophagy. Brown points represent significantly down differentially expressed genes related to mitophagy. Each dot represents a gene. X- axis:log2Fold change of expression; Y-axis: -log10(*P*-value).

MYC Regulation and other transcription factors

First, it is important to note that several transcription factors stood out with significant upregulation, characterized by substantial fold change values. These included *MYC*, *JUNB*, *EGR1*, *ATF3*, *FOS*, and *JUN*. *MYC* displayed consistent upregulation at all the examined time points in all three conditions, which aligns with earlier findings in human skeletal muscle (Figure 17ABC). *MYC,* as a super-transcription factor and a proto-oncogene, controls the transcription of nearly 15% of expressed genes [262], and its regulation and transcriptional activity has a wide array of affection in cellular processes, such as cell growth, differentiation, programmed cell death, and apoptosis [263, 264]. *MYC* also plays a critical role in autophagy. A previous study reported that *MYC* knockdown impaired autophagosome formation through decreased phosphorylation of *JNK1* and downstream target *Bcl2* activity [265]. Additionally, the activation of *MYC* can mediate autophagy by triggering the UPR and inducing endoplasmic reticulum (ER) stress [266]. Exercise and hypoxia as stimulus conditions and stressors can trigger autophagy either alone or in combination manner [267]. *MYC* was identified to be highly up-regulated immediately and 3 hours following a single session of HIIE in human skeletal muscle, but the effect was blunted after four weeks of high-intensity interval training (HIIT) [268]. An acute bout of resistance exercise (acute bouts of unilateral leg extension exercise) increased *MYC* mRNA expression 90 min, 3 hours, and 24 hours after exercise in normoxia (FiO_2 21%) and hypoxia (FiO_2 12%), however, no differences between normoxia and hypoxia were observed [269]. These observations were basically in line with the current study.

Intriguingly, *JUNB*, *EGR1*, *ATF3*, *FOS*, and *JUN* transcription factors are often found to be investigated simultaneously. This was not surprising that these transcription factors were part of the network of gene expression changes induced by a variety of stress signals and have also been found to be significantly up-regulated rapidly at early exercise time points [268, 270-276]. In addition, part of the reason may be that the JUN family (c-Jun, JunB, and JunD), FOS family (c-Fos, FosB, Fra1, and Fra2), and activating transcription factor (ATF) protein families are all family members of the activator protein-1(AP-1) [277]. The heterodimeric complex AP-1 is a major partner of PPARGC1A (PGC-1α), which also plays a critical role in regulating the PGC-1α-controlled gene program of hypoxia response in muscle cells [278]. Hypoxia as a stress signal can induce *JUNB*, *EGR1*, *ATF3*, *FOS*, and *JUN* expression [271, 279, 280]. It has been suggested that

JUNB, JUN, and FOS can form the dimeric transcription factor AP-1, whose expression was enhanced in all types of cells subjected to hypoxia [280]. However, limited data have been reported on gene expression changes of these transcription factors induced by the combination of exercise and hypoxia. Despite this, a human skeletal muscle study demonstrated disparate results. In normoxia and hypoxia (FiO_2 14%), the *FOS* mRNA levels were lower at 4 hours following a single session of resistance exercise (a 1-leg knee extension session) compared with 15 min post-exercise, and the mRNA levels of *FOS* were higher in the exercised leg at 15 min post-exercise under hypoxia condition compared with normoxia [113]. In the current study, a single session of HIIE session intervention induced a significant elevation for *FOS* at immediately and 3 hours after exercise in normoxia. In contrast, no significant changes were observed in hypoxia. The inconsistency across the literature is probably due to the difference in exercise type. By reviewing the literature, I found that many studies have reported the roles of these transcription factors (JUNB, EGR1, ATF3, FOS, JUN) on autophagy, and a large number of papers have also documented the induction of these transcription factors by exercise. However, so far, there has not been any research yet to reported that the effects of exercise on autophagy after activating these genes, especially in hypoxia.

PPARGC1A (PGC-1α) and Its Role in Exercise-Induced Autophagy and Mitophagy

Likewise, the *PGC-1α* gene, as a key transcriptional regulator, plays a critical role in regulating mitochondrial adaptations in response to exercise, which has been studied extensively. It was reported that acute exercise and training strongly induce *PGC-1α* expression in human and rodent skeletal muscle [281]. Our findings showed that *PGC-1α* was significantly up-regulated in NA (1-fold), NR (3-fold), and HY (3-fold) 3 hours following a single session of HIIE, while it decreased 24 hours after exercise in NA. Increased *PGC-1α* mRNA level has been observed in many exercise-related studies, such as acute bouts of sprint exercise (5-fold) [276], resistance exercise [282, 283], endurance exercise [275, 284], which seem to be a general response to exercise modalities. Many literatures have well documented the role of *PGC-1α* as a master regulator of mitochondrial biogenesis exercise-induced [285, 286], while much less evaluated what role *PGC-1α* played in the process of autophagy/mitophagy. A study has investigated autophagy flux by measuring the change in LC3II and p62 protein in vivo after an acute bout of incremental treadmill

running in the wild-type (WT) counterparts and knockout (KO) animals, and results showed that the LC3II flux tended to elevate immediately after exercise and recovery period, whereas there was no similar trended in KO animals [287]. The research also examined the mitophagy induction and the role of *PGC-1α*. LC3II flux was observed to significantly increase in WT animals, and this signaling was attenuated in KO mice by the lack of *PGC-1α* [287]. Taken together, the evidence above-mentioned supported the idea that an acute bout of exercise enhances autophagy and mitophagy in WT animals, and *PGC-1α* was involved in these processes. However, this effect was attenuated in the absence of *PGC-1α* [287]. Another study has demonstrated that *PGC-1α* was necessary for the induction of LC3 and p62 following exercise [76]. Hypoxia can up-regulate the expression of *PGC-1α,* and the interaction between *PGC-1α* and hypoxia contributes to regulating mitochondrial function [288]. Repeated sprint exercise (total 20 'all-out' 10-s sprints, 4 sets of 5 sprints) increased *PGC-1α* mRNA 3 hours post-exercise in the hypoxia group (hypobaric hypoxia equivalent to altitude 4600m) and control group (equivalent to altitude 1600m) [289]. Overall, *PGC-1α* was observed in response to exercise at both normoxia and hypoxia, and it was transiently activated in the early stages after exercise. These observations [240, 286, 289-291] were generally in line with the current study that the mRNA expression of *PGC-1α* augmented at 3 h after exercise in both normoxia and hypoxia. However, many distinct exercise protocols and sampling time points led to slightly different *PGC-1α* observation times, such as 2 hours [292], 20-300 min [293], and 5 hours [294] after exercise. The current study also observed a decrease in normoxia 24 hours after exercise, yet several studies reported that *PGC-1α* returned to basal levels at 24 hours postexercise [286, 292, 295].

Discussion on Mitophagy-Related Genes

Considering the research's particular focus on mitochondrial adaptation, mitophagy-related genes were meticulously examined. No mitophagy-related genes were observed immediately after exercise. At both 3 hours and 24 hours post-exercise in both normoxia and hypoxia, 8 mitophagy-related genes were found to exhibit significant changes. Specifically, *FLCN* displayed upregulation at 3 hours post-exercise in NR and HY, while *TGM2* was upregulated at P3H in NA and HY. Moreover, *SESN2*, *TFE3*, *TBC1D15*, *ATP13A2*, *BCL2L1*, and *FUNDC1* showed significant alterations 24 hours after exercise. It is worth noting that while *SESN2* and *TFE3* were increased

across all conditions, *TBC1D15*, *ATP13A2*, and *BCL2L1* were upregulated, and *FUNDC1* was uniquely downregulated in hypoxia.

As mentioned earlier, mitophagy was initiated and mediated in two ways, which were the PINK1/Parkin pathway and mitophagy receptor pathways. Known receptors that mediate mitophagy include NIX (BNIP3L), BNIP3 and FUNDC1. Based on the importance of FUNDC1 in inducing mitophagy, I attempted to understand the molecular mechanism by which FUNDC1 mediates mitophagy.

FUNDC1 is an integral mitochondrial outer-membrane protein that interacts with LC3 through a characteristic LC3-interaction region (LIR) at the N-terminal region cytosol-exposed to initiate mitophagy in mammalian cells [296, 297]. A study discovered that FUNDC1-induced mitophagy is highly dependent on its interaction with LC3 through LIR, which was confirmed in the cell experiment of knocking out FUNDC1 [298]. This study also demonstrated that FUNDC1 is a receptor for hypoxia-induced mitophagy, and the dephosphorylation of FUNDC1 can trigger mitophagy in response to hypoxia [298]. Under hypoxic stimulation, Src kinase inactivation and FUNDC1 dephosphorylation lead to increased co-localization and interaction between FUNDC1 and LC3-II, resulting in mitochondria selectively binding to LC3-boud isolation membrane as specific cargo, and then being removed by LAMP1 positive autolysosomes, whereas FUNDC1 mediated mitophagy was inhibited by its phosphorylation at the Tyr 18 position in the LTR motif by Sir kinase in normoxia [298]. Accumulating evidence suggested that Nix (BNIP3L) and BNIP3 also play an essential role in the activation and regulation of hypoxia-induced mitophagy [127, 299-302]. However, the mechanism of FUNDC1-induced mitophagy is distinct from them [298]. *FUNDC1* was observed to have a decreased expression, whereas the expression level of Nix (BNIP3L) or *BNIP3* was increased [298, 303, 304]. The data in the present study showed that *FUNDC1* was significantly downregulated in hypoxia 24 hours after exercise.

Discussion on autophagy/mitophagy marker

Despite expectations, well-documented canonical autophagic markers LC3II, LC3II/LC3I ratio, and p62/SQSTM1 did not exhibit significant expression changes in the current study. This result suggests no robust induction of autophagy/mitophagy pathways by all exercise sessions. These indicators are typically grouped to interpret autophagy/mitophagy. LC3II and LC3II/LC3I ratio is

widely recognized as a marker of autophagy [80, 305], which levels reflect the level of autophagy. p62, also called sequestosome1 (*SQSTM1*), which is an adapter protein related to autophagosome formation, and is commonly used as indicator of autophagy flux as an autophagy substrate [306, 307]. Elevated p62 with exercise may indicate an increased autophagy flux [108]. An experimental study on human skeletal muscle reported that an acute bout of HIIT (12 sessions of 1 min at 100% V_{max} and 1min at 3 miles per hour) increased autophagic flux, and no differences were found in LC3I and LC3II protein content pre-exercise compared with 3 hours post-exercise in an acute bout of HIIT and moderate-intensity continuous training (MICT, 60 min at 55% of max velocity [V_{max}]) [108]. Moreover, the LC3II/LC3I ratio was significantly lower when pre-exercise compared with 3 hours post-exercise in skeletal muscle, whereas p62 was increased [108]. Acute high-intensity endurance exercise in humans (7 trained athletes cycled for 2 hours at 70% $\dot{V}O_{2peak}$) has also been demonstrated not to activate mitophagy regardless of nutritional state, as LC3bII and p62/SQSTM1 were observed no change and LC3bII/LC3bI ratio was showed decreased [308]. Another study on human skeletal muscle demonstrated that acute exercise increased LC3I and LC3II protein [110]. Although a previous acute exercise study has reported similar results [65], there are still some inconsistent (decreased and unchanged) observations [91, 137]. Researchers have different opinions on whether these contradictory results were associated with exercise intensity [91, 110]. A study also reported that the regulation of autophagy during exercise may exist in discrepancies between human skeletal muscles and rodent muscles, as a single endurance exercise increased autophagosome content markers in rodent muscles, but the opposite was found in humans [224].

3.9 Expression level of DEGs

The present study also evaluated the expression pattern of differentially expressed genes associated with autophagy/mitophagy in all conditions. I observed that the expression patterns of the same condition at different time points were similar, and the expression patterns of different conditions at the same time point were significantly different (Figure 18). In order to explore these 74 significantly differentially expressed genes, I classified these genes into six categories (Table 7) according to the study from Matteo and Rossella et al. [166].

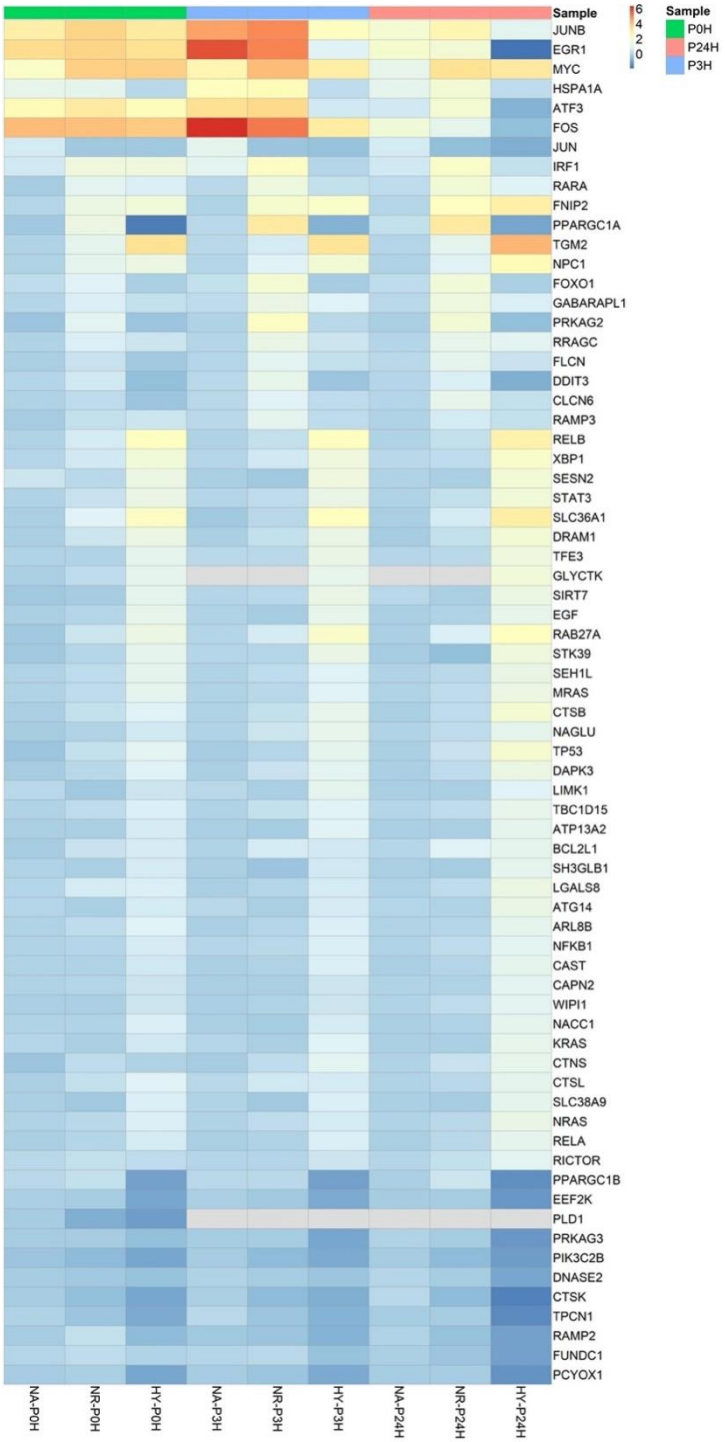


Figure 18. Heatmap shows the expression levels of target differentially expressed genes related to autophagy/mitophagy at P0H, P3H, and P24H in NA, NR, and HY. Different columns represent different samples, and different rows represent different genes. The color represents the level of expression of the gene in the samples.

Table 7. Functional classification of significantly expressed genes. Genes labeled in red represent more than one category.

mTOR and upstream pathways	Autophagy core	Autophagy regulators	Mitophagy	Docking and fusion	lysosome	Lysosome-related
FNIP2	HSPA1A	JUNB	TGM2	RAB20	NPC1	CTSL
NPC1	TGM2	EGR1	FLCN	ATG14	CLCN6	SLC38A9
PRKAG2	GABARAPL1	MYC	SESN2	ARL8B	CD68	
RRAGC	DRAM1	ATF3	TFE3		RAMP3	
FLCN	STK39	FOS	TBC1D15		SLC36A1	
SESN2	DAPK3	JUN	ATP13A2		DRAM1	
STAT3	PMAIP1	IRF1	BCL2L1		GLYCTK	
TFE3	LIMK1	RARA	FUNDC1		EGF	
SEH1L	BCL2L1	PPARGC1A			RAB27A	
MRAS	SH3GLB1	FOXO1			CTSB	
LGALS8	ATG14	DDIT3			NAGLU	
KRAS	CAST	RELB			PI4K2A	
SLC38A9	CAPN2	XBP1			ATP13A2	
NRAS	WIPI1	STAT3			ARL8B	
RICTOR		TFE3			CTNS	
EEF2K		SIRT7			PLD1	
PRKAG3		TP53			DNASE2	
PIK3C2B		NFKB1			CTSK	
		NACC1			TPCN1	
		RELA			RAMP2	
		PPARGC1B			PCYOX1	

Autophagy regulator genes, *EGR1*and *FOS*, exhibited significantly high expression levels at 3 hours post-exercise in NA, and *EGR1* also displayed its lowest expression 24 hours post-exercise in HY (Figure 18). On the other hand, the expression of autophagy regulators gene *PGC-1α* was was notably highest in NR condition and the lowest in HY (Figure 18). Intriguingly, some genes were found to have substantially higher expression in NR compared with NA and HY. These genes encompassed *JUNB*, *MYC*, *ATF3*, *IRF1*, *RARA*, *FOXO1*, *DDIT3* (Autophagy regulators), *HSPA1A*, *GABARAPL1* (Autophagy core), *PRKAG2*, *RRAGC*, *FLCN* (mTOR and upstream pathway), and *CLCN6*, *RAMP3* (Lysosome), respectively (Figure 18 and Table 7). In contrast, most genes displayed varying expression patterns under the HY condition compared to NA and NR. Those genes that exhibited higher expression level in HY included *FNIP2*, *NPC1*, *SESN2*, *STAT3*, *SEH1L*, *MRAS*, *LGALS8*, *KRAS*, *RICTOR* (mTOR and upstream pathways), *TGM2*, *DRAM1*, *STK39*,

LIMK1, *BCL2L1*, *SH3GLB1*, *ATG14*, *CAST*, *CAPN2*, *WIPI1* (Autophagy core), *RELB*, *XBP1*, *STAT3*, *SIRT7*, *TP53*, *NFKB1*, *NACC1*, *RELA* (Autophagy regulators), *TGM2*, *SESN2*, *TFE3*, *TBC1D15*, *ATP13A2*, *BCL2L1* (Mitophagy), *ATG14*, *ARL8B* (Docking and fusion), *SLC36A1*, *DRAM1*, *GLYCTK*, *EGF*, *CTSB*, *NAGLU*, *ARL8B*, *CTNS* (Lysosome), and *CTSL*, *SLC38A9* (Lysosome-related). Nevertheless, *PPARGC1B* (Autophagy regulators), *EEF2K*, *PRKAG3*, *PIK3C2B* ((mTOR and upstream pathways), *FUNDC1* (Mitophagy), *DNASE2*, *CTSK*, *TPCN1*, *RAMP2*, *PCYOX1* (Lysosome) showed significantly low expression under HY condition (Figure 18 and Table7). Additionally, it is important to note that some genes are involved in multiple pathways, as evident from the analysis of genes such as *NPC1*, *FLCN*, *SESN2*, *STAT3*, *SLC38A9*, *TGM2*, *DRAM1*, *BCL2L1*, *ATG14*, *ATP13A2*, *ARL8B* (Table 7).

The heatmap exhibited different expression patterns in three conditions (NA, NR, HY). Under NA conditions, only a few autophagy regulators showed a change in expression level. Partial genes related to autophagy regulators, autophagy core, mTOR and upstream pathway, lysosome showed the highest expression in NR. Most genes with changes in expression levels were detected in hypoxia. Expression of genes involved in mTOR and upstream pathway, autophagy regulator, autophagy core, and lysosome were significantly changed in hypoxia, while the expression levels of mitophagy, docking and fusion, and lysosome-related genes also changed significantly compared with NA and NR. Moreover, under the same exercise condition, the expression patterns of autophagy/mitophagy-related genes among different time points were similar. In summary, the results suggest different patterns between hypoxia and normoxia, as gene expression levels in hypoxia have greater alteration. HY and NR did not show similar expression patterns despite a higher percentage of HR_{peak} and $\dot{V}O_{2peak}$, and a higher RPE observed in both NR and HY compared to NA.

3.10 Investigation on temporal expression patterns of autophagy/mitophagy-related genes in NA, NR, HY (STEM analysis)

This study conducted STEM analysis of significantly changed genes before exercise (B), immediately (P0H), 3 hours (P3H), and 24 hours (P24H) following a single session of HIIE by STEM software aiming to examine the temporal expression patterns in NA, NR, and HY. STEM analysis may cluster genes with similar temporal dynamics patterns, and many different model

profiles will be obtained after clustering. Each box corresponds to a temporal expression model profile. Specifically, the number in the upper left of the box represents the model profile ID, while the lower left refers to the number of clustered genes (Figure 19). Model profiles with significant expression patterns were represented in color. The broken line in the left model profile box showed the temporal expression trends in NA, NR, and HY (Figure 19). In the present study, I found that differentially expressed genes related to autophagy/mitophagy clustered in profile4, profile3, profile2, profile1, profile0. Of all profiles, only profile4 has a significant expression pattern ($P <$ 0.05). The line chart (Figure 19) on the right shows the detailed expression trends of the differential genes clustered in profile 4. Profile 4 in NA, a continuous upward trend was observed in the expression levels of the clustered differential genes from 3 hours to 24 hours after a single session of HIIE. However, no significantly changed genes were observed from 0 hours to 3 hours after exercise (Figure 19). For NR and HY, the expression levels of the clustered differential genes in profile 4 showed a similar expression trend, in which most of the genes also exhibited a constantly increasing trend from post-exercise 3 hours to 24 hours (Figure 19). However, a small number of genes, whether NR or HY, began to rise at P0H and decline gradually after reaching the peak at P3H (Figure 19).

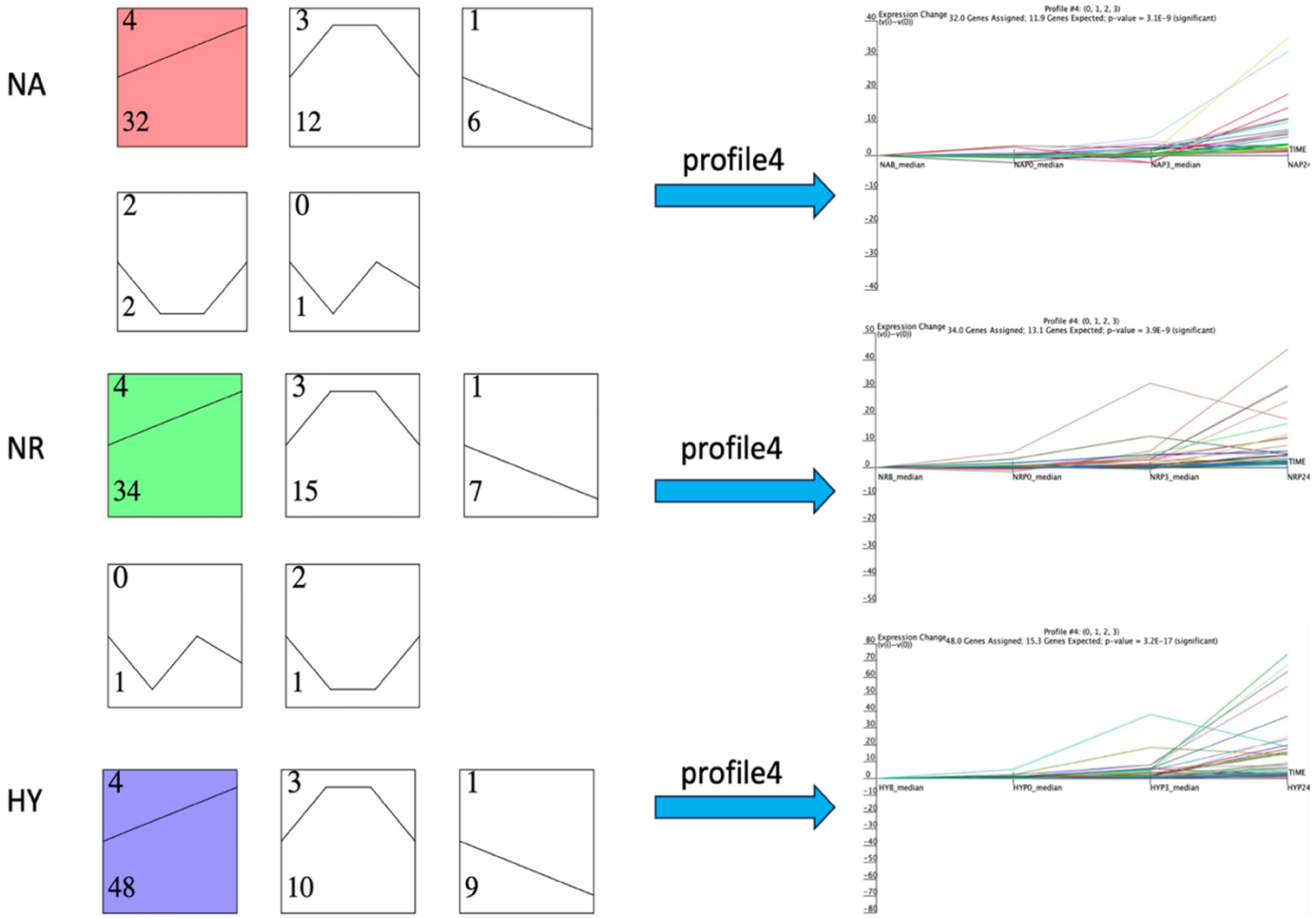


Figure 19. STEM analysis of target differentially expressed genes associated with autophagy/mitophagy. STEM analysis showing the temporal expression patterns of genes before exercise (B), post-exercise immediately (P0H), post-exercise three hours (P3H) and 24 hours (P24H). Genes with the same expression pattern were clustered into a model profile. The top left is the model profile number, and the bottom left is the number of genes clustered. The colored model profiles represent significant differences.

Collectively, I observed similar temporal module profiles in different conditions (NA, NR, HY), including profile 4, profile 3, profile 2, profile 1, profile 0. Profile 4 among different conditions showed a significantly expressed pattern, which meant the mRNA expression for the majority of genes related to autophagy/mitophagy presented a constant upward trend from 0 hours to 24 hours following a single session of HIIE in different conditions (NA, NR, HY).

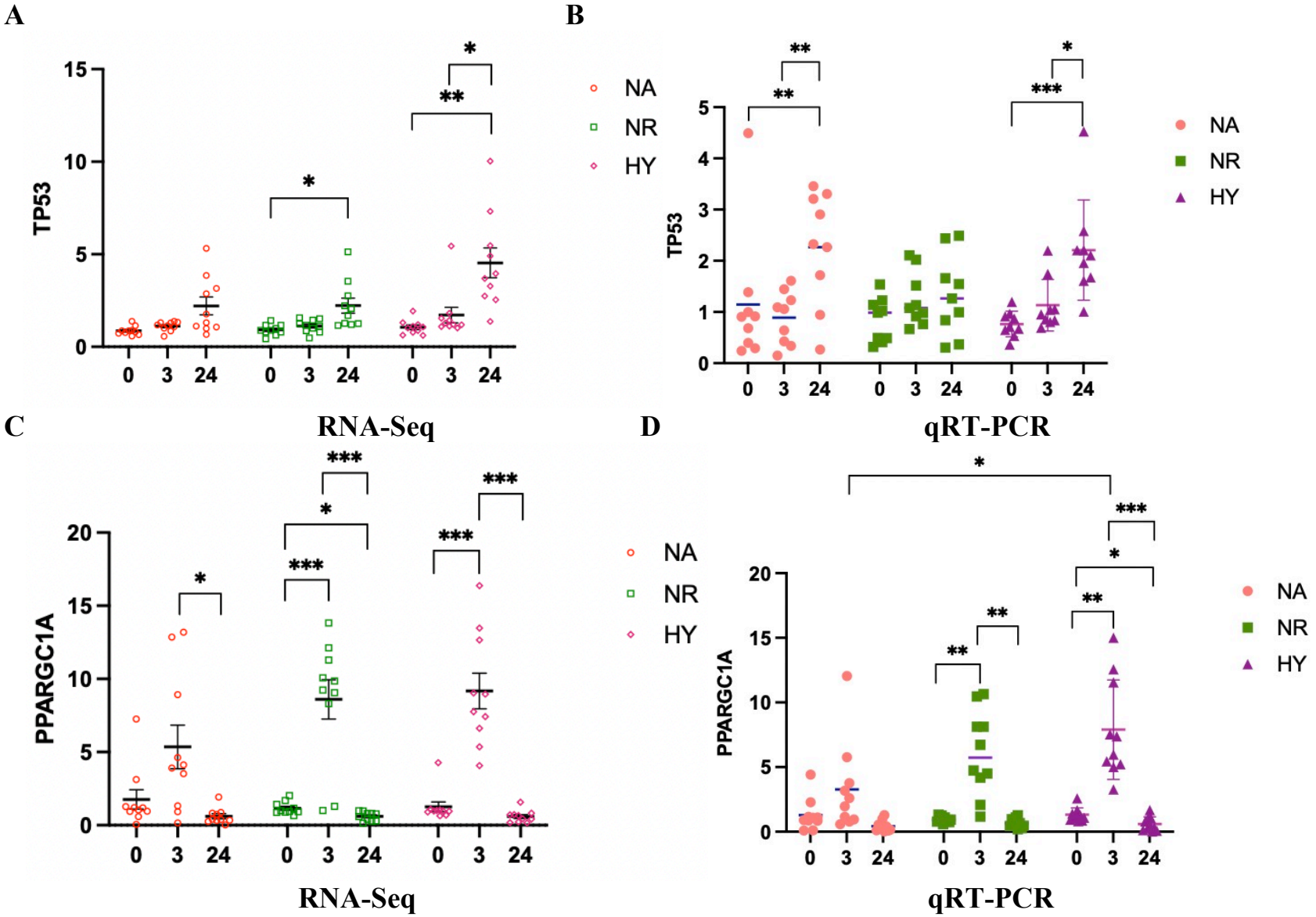
The results were basically consistent with a recent study that the significantly changed genes were observed between 3 hours and 48 hours post-exercise, and the mRNA expression largely depends on the skeletal muscle sampling timing [309]. As for whether the expression levels of these genes continue to increase or decrease 24 hours post-exercise, a more comprehensive biopsies sampling timing is needed to confirm. Otherwise, the study results may be confounded. Based on the aforementioned, the assessment of HIIE-induced genes associated with autophagy/mitophagy in human skeletal muscle is complex, as it is affected by a variety of variables, such as exercise intensity, environmental low oxygen, and biopsies sampling time. The precise changes of individual genes in response to HIIE on autophagy/mitophagy warranted further investigation.

3.11 Validation of RNA-seq data by qRT-PCR and MetaMEx

To validate the accuracy of transcriptome data, a few key genes related to autophagy/mitophagy (*TP53*, *PPARGC1A*, *HIF1A*, *HSPA1A*) were subjected to qRT-PCR analysis.

Validation of TP53 (p53)

RNA-seq data demonstrated similar expression levels of *TP53* (p53) immediately and 3 hours after exercise in NA, NR, and HY conditions but showed higher expression in HY compared to NA and NR at 24 hours post-exercise (Figure 20A). The qRT-PCR results aligned with these trends (Figure 20B). Two-way ANOVA revealed differences for TP53 (p53) in sampling time points (P0H, P3H, P24) but no significant differences among conditions (NA, NR, HY). Both RNA-Seq and qRT-PCR results showed significant differences in P0H compared with p3H and P24H in HY (Figure 20AB). RNA-seq data showed significance between immediately and 24 hours after exercise in NR, while it was not found in qRT-PCR (Figure 20AB). Conversely, under the NA condition, the significance was only observed in qRT-PCR data between P0H compared with p3H and P24H (Figure 20B).


A
B
C
D
TP53
PPARGC1A
NA
NR
HY
RNA-Seq
qRT-PCR
0
3
24

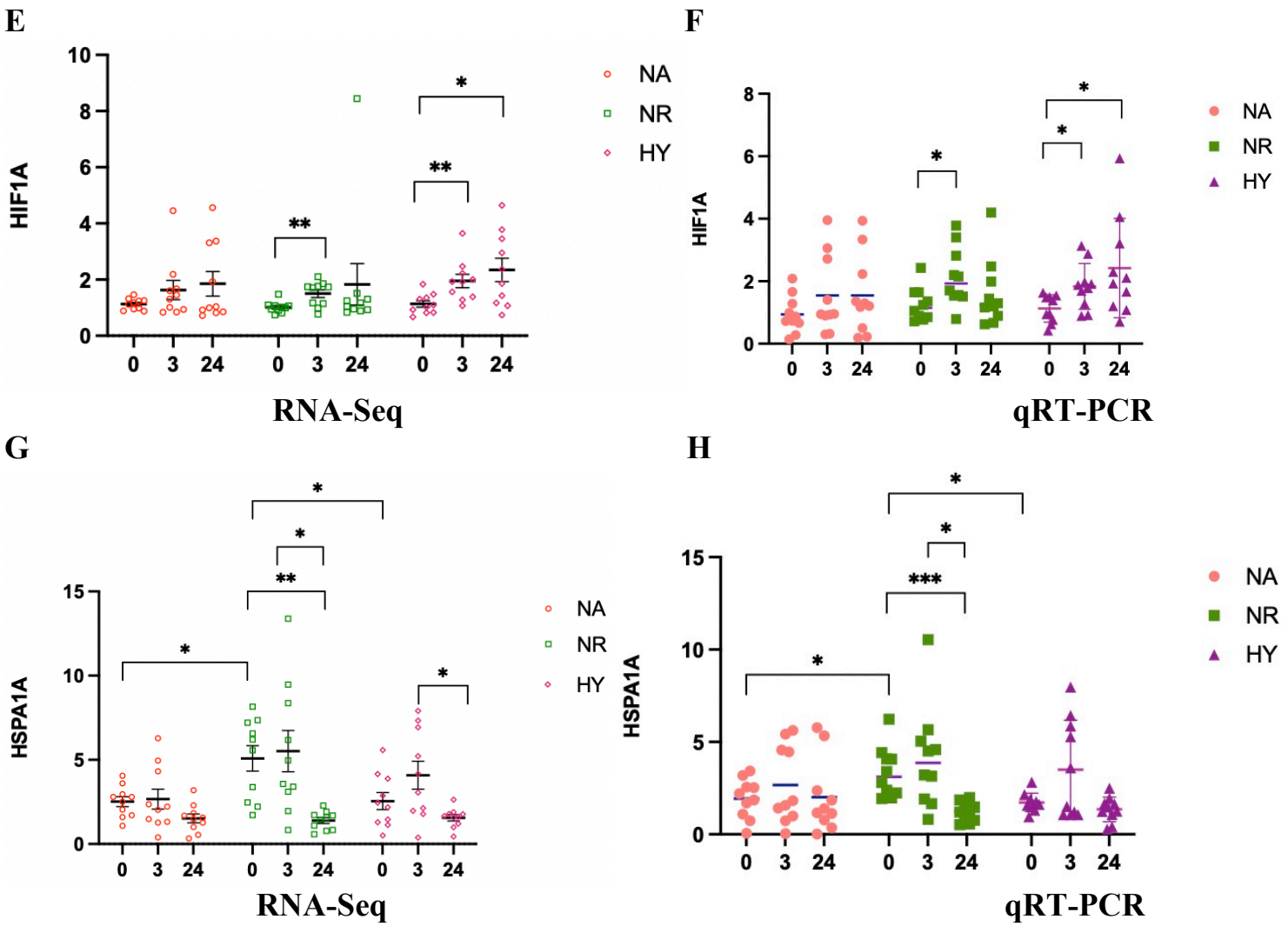


Figure 20. Validation of RNA-seq data by qRT-PCR. Skeletal muscle gene expression in NA, NR, and HY at different time points. On the left is gene expression in RNA sequencing data, and on the right is qRT-PCR data. AB: TP53; CD: PPARGC1A; EF: HIF1A; GH: HSPA1A.

Validation of PGC-1α (PPARGC1A)

RNA-seq and qRT-PCR data illustrated higher expression levels in NR and HY, with the highest mRNA expression recorded at 3 hours post-exercise. Expression levels immediately and 3 hours after exercise were relatively similar in NA, NR, and HY (Figure 20CD). The alignment between RNA-Seq and qRT-PCR results for *PGC-1α* indicated a consistent trend (Figure 20CD). In RNA-Seq data, differences were observed solely among various sampling time points, while qRT-PCR revealed a significant interaction between conditions (NA, NR, HY) and sampling time points (P0H, P3H, P24H) (Figure 20D). In RNA-seq data, Two-way ANOVA revealed differences of *PGC-1α* between 3 hours post-exercise and 24 hours post-exercise in NA ($p = 0.0239$), NR ($p = 0006$) and HY ($p = 0.0002$), with similar findings in qRT-PCR data specifically for NR and HY (Figure 20C). However, in qRT-PCR data, there was a similar observation in NR ($p = 0.0015$) and HY ($p = 0.0005$) (Figure 20D). Both RNA-Seq and qRT-PCR data showed a significant difference for PGC-1α when 3 hours post-exercise compared immediately post-exercise in NR ($p = 0.0008$, $p = 0.0018$) and HY ($p = 0.0005$, $p = 0.0012$) (Figure 20CD). In the RNA-seq data, there was a significant difference immediately after exercise vs. 24 hours after exercise under the NR condition, while a similar result was observed under the HY condition in qRT-PCR data (Figure 20CD). Notably, *PGC-1α* in qRT-PCR data showed a significant difference in conditions (NA vs. HY, $p = 0.0294$) 3 hours after exercise (Figure 20D).

Validation of HIF1A

The expression level of *HIF1A* was lowest immediately after exercise and remained consistent at P0H under NA, NR, and HY conditions in both RNA-seq and qRT-PCR data (Figure 20EF). Furthermore, *HIF1A* expression at 3 hours and 24 hours post-exercise in HY was slightly higher than that of NR in both RNA-seq and qRT-PCR data (Figure 20EF). Multiple comparison tests of the two-way ANOVA revealed a significant difference in *HIF1A* between sampling time points, but no significant differences were observed among conditions (NA, NR, HY). The RNA-seq and qRT-PCR data displayed consistent results, with significant differences between immediate exercise and 3 hours post-exercise identified in both NR and HY conditions, and between immediate exercise and 24 hours post-exercise in the HY condition.

The expression level of *HIF1A* was the lowest immediately after exercise and remained consistent at P0H under NA, NR, and HY conditions in both RNA-Seq and qRT-PCR data (Figure 20EF). In addition, *HIF1A* expression at 3 hours and 24 hours after exercise in HY was slightly higher than that of NR in both RNA-seq and qRT-PCR data (Figure 20EF). Multiple comparison tests of the two-way ANOVA revealed significant differences in *HIF1A* between sampling time points, but no differences were observed among the conditions (NA, NR, HY) compared. The RNA-seq and qRT-PCR data displayed consistent results, with significant differences immediately after exercise vs. 3 hours after exercise found under both NR and HY conditions, and between immediately post-exercise and 24 hours post-exercise in the HY condition.

Validation of HSPA1A

In all sampling time points, the expression level of *HSPA1A* mRNA expression was higher in NR compared to NA and HY. For *HSPA1A*, both RNA-seq and qRT-PCR data showed differences among conditions and sampling time points. In RNA-seq and qRT-PCR data, the expression level of *HAPA1A* exhibited significant differences immediately post-exercise vs. 24 hours post-exercise and 3 hours post-exercise vs. 24 hours post-exercise in NR (Figure 20GH). Moreover, significant differences were observed between NA and NR immediately after exercise, as well as between NR and HY(Figure 20GH). Under the HY condition, only a significant difference was observed in RNA-seq data 3 hours post-exercise vs. 24 hours post-exercise (Figure 20G).

In summary, the results from both qRT-PCR and RNA-seq indicated consistent trends in the up-regulation and down-regulation trends of these selected genes, affirming the reliability of the transcriptome data.

This study also employed a unique validation tool (MetaMEx) to assess the expression of the target gene. This database provides the most extensive datasets on the transcriptomic response of skeletal muscle to acute exercise, exercise training, and inactivity [310]. The MetaMEx database (http://www.metamex.eu) includes 66 published datasets which regarding skeletal muscle transcriptomic responses to different exercise modalities in various populations. Exercise modes contain acute aerobic exercise, acute resistance exercise, aerobic-based exercise training, resistance-based exercise training, and inactivity data. Over 60 studies are annotated by skeletal muscle type, age, sex, fitness level, and disease state. Moreover, the database also displayed the specific exercise type (Concentric, Eccentric, Mixed) and sampling time after exercise (Immediate, 1 hour, 3 hours, 4 hours, 5 hours, 6 hours, 8 hours, 18 hours, 24 hours, 48 hours, 96 hours).

This study randomly selected five differentially expressed genes (*FUNDC1*, *BCL2L1*, *FOXO1*, *GABARAPL1*, *MYC*) and conducted a search in the database. I evaluated the fold change values of these genes. Given the exercise type (a single session of HIIE) of the current study, so only the acute aerobic exercise and acute HIT data were selected as the target in the database, and data on exercise training and inactivity were excluded from the study. In our RNA-seq data, the Fold change values of *FUNDC1*, *BCL2L1*, *FOXO1*, *GABARAPL1*, and *MYC* were basically within these ranges of the MetaMEx database displayed, which meant that our data is relatively reliable. Detailed information is shown in following Table 8.

Table 8. The logFC value of five differentially expressed genes in the MetaMEx database and RNA-seq data

	logFC range MetaMEx		P0H			P3H			P24H		
	Acute aerobic	Acute HIT	NA	NR	HY	NA	NR	HY	NA	NR	HY
FUNDC1	-0.90 - 1.76	-0.27 - 0.26	0.08	0.05	-0.05	0.24	0.17	0.13	0.10	-0.43	-1.10
BCL2L1	-0.43 – 1.16	0.05 – 1.24	-0.12	0.01	0.13	0.47	0.66	0.88	0.66	0.56	1.10
FOXO1	-0.66 – 2.31	-0.14 – 1.87	0.28	0.34	0.27	0.95	1.75	1.59	-0.04	-0.14	-0.09
GABARAPL1	-0.83 – 1.88	-0.03 – 1.38	0.13	0.25	0.18	0.76	1.29	1.39	0.33	0.85	0.78
MYC	-0.08 – 5.50	0.14 – 3.81	2.04	2.62	1.95	3.83	4.20	3.43	3.82	2.90	3.16

3.12 Physiological performance data and Autophagy/Mitophagy Responses to HIIE in Hypoxia

HIIE can deliver significant fitness and health benefits in a shorter time frame and is gaining recognition as an effective approach, particularly in the context of our increasingly sedentary and aging population. Exercise prescription involves several key components, with exercise intensity being paramount among them, according to the American College of Sports Medicine (ACSM) [311]. Exercise intensity is pivotal in the physiological response to HIIE [312]. Our study introduced a novel approach by prescribing HIIE intensity in hypoxia based on both maximal and submaximal values, which deserves further exploration. Precise adjustment of exercise intensity in hypoxia is crucial for achieving specific physiological adaptations during HIIE. This study has demonstrated that our method of prescribing HIIE intensity in hypoxia, based on both maximal and submaximal values, has led to meaningful and robust results. In our data, we observed intriguing differences when exercise intensity was matched between normoxia and hypoxia, and the parameters we assessed, including percentage of peak heart rate (HR_{peak}), percentage of peak oxygen consumption ($\dot{V}O_{2peak}$), ratings of perceived exertion (RPE), and respiratory exchange ratio (RER). These physiological performance data showed more similar results between NR and HY, as a higher percentage of HR_{peak} and $\dot{V}O_{2peak}$ and a higher RPE were observed in both NR and HY compared to NA.

Based on the performance data results, we hypothesize that transcription responses on autophagy/mitophagy induced by HIIE likely have similar results between NR and HY compared to NA if induction of autophagy/mitophagy is responsible for the observed differences from physiological tests. However, at the molecular level, only a moderate level of induction of autophagy/mitophagy by hypoxia was observed. There was no clear separation between the three different exercise interventions in the PCA plot, meaning that the adaptive response of human skeletal muscle induced by a single session of HIIE was very similar in NA, NR, and HY. Venn diagram revealed that comparison of the common and unique genes regulated in different conditions at various time points. No more common genes were found between NR and HY at all time points after exercise, whereas hypoxia-induced more common and unique genes compared with NA and NR 24 hours after exercise. Exercise in hypoxia has a greater induction on the autophagy/mitophagy pathway compared to NA and NR. Although many upstream pathways were

found in NA and NR, autophagy-animal and mitophagy-animal pathways were significantly enriched only in hypoxia post-exercise 24 hours. Volcano plot (differential expression analysis on autophagy/mitophagy-related genes) showed similar observations to pathway analysis. The number of autophagy/mitophagy-related differentially expressed genes in hypoxia post-exercise 24 hours were more than NA and NR. However, the heatmap revealed that different expression patterns were observed in three different exercise conditions (NA, NR, HY) at the same time points. In summary, no causal relationship was found between physiological performance data and transcriptional responses. The results suggest that autophagy and mitophagy are unlikely to be linked with the adaptative response to exercise in hypoxia.

Chapter 4

Conclusions and Future Directions

4.1 Summary of key findings

- The key findings of the thesis include: Utilizing the RNA-seq on human skeletal muscle samples obtained a total of 3698 significantly differential genes, including 2546 up-regulated genes and 1152 down-regulated genes. The number of DEGs in NA, NR, and HY were similar after exercise immediately and 3 hours. However, the number of DEGs in hypoxia was significantly higher than in nomoxia post-exercise 24 hours. These results indicated that a larger number of genes were activated by a single session of HIIE in hypoxia.
- Venn diagram showed that the largest number of common and unique genes was identified 24 hours after a single session of HIIE in hypoxia (HY), suggesting unique transcriptomic changes in response to hypoxia rather than increased relative exercise intensity. There was a similar number of common genes between NR vs. HY and NA vs. HY, which was not correlated with observed physiological performance results. This could indicate that the observed difference from physiological tests was not due to the activation of unique pathways but to the different magnitude of activation of common pathways.
- The PCA plot showed no clear separation of three different conditions, which suggested that the adaptive response induced by a single session of HIIE was similar in all conditions, just at different magnitudes.
- Upstream pathways involved in activating and mediating autophagy/mitophagy were significantly enriched in all conditions, including the AMPK signaling pathway, MAPK signaling pathway, and PI3K-Akt signaling pathway. Moreover, autophagy-animal and mitophagy animal pathways (not ranked in the top 20) were significantly enriched only in HY. The mTOR signaling pathway was not significantly enriched. Collectively, these pathway enrichment analysis findings showed that HIIE in hypoxia and normoxia did not make robust induction on autophagy and mitophagy response.
- Differential expression analysis was performed on 604 target genes (derived from a published paper), and the highest number of up-regulated and down-regulated genes related to autophagy/mitophagy was observed following exercise 24 hours in HY. Gene expression of autophagy regulators (*MYC*, *JUNB*, *EGR1*, *ATF3*, *FOS*, *JUN*, *PPARGC1A*) were significantly changed. However, recognized autophagy markers such as LC3II,

LC3II/LC3I ratio, P62, and mitophagy receptors (*NIX*, *BNIP3*) did not show significant differences in expression level in all conditions, with only mitophagy receptor *FUNDC1* showing the change in expression in hypoxia, indicating a lack of robust change in autophagy/mitophagy signaling after a single session of HIIE with and without hypoxia.

- Heatmap showed that the expression pattern of the same condition at different time points (P0H, P3H, P24H) was similar, while the expression of NA, NR, and HY at the same time point was different. The results suggest a greater induction of gene expression in hypoxia than in normoxia. HY and NR did not show similar expression patterns, which did not correlate with the observed higher percentage of HR_{peak} and $\dot{V}O_{2peak}$, and a higher RPE in both NR and HY compared to NA.
- This research has also used STEM analysis to assess the temporal module profile, and profile 4 showed a significantly expressed pattern, which represented the mRNA expression for the majority of genes related to autophagy/mitophagy displaying a continuous upward trend from immediately to 24 hours after HIIE in three conditions.

4.2 Conclusions and future directions

The attraction of high-intensity interval exercise (HIIE) compared to other forms of exercise is that it can achieve the same fitness and health effects in a shorter period. With the increase of sedentary people and the acceleration of the aging population, researchers have valued HIIE intervention strategies for human health [313-315]. Incorporating “adaptive” high intensity (relative to the subject’s current physical ability) into the exercise protocol is a key component of exercise being more effective as a “medicine” [314]. Exercising in hypoxic conditions was commonly used to enhance athletes’ physical performance, while it was also considered a promising therapeutic approach for some chronic diseases. Autophagy and mitophagy play an important role in maintaining cellular homeostasis and improving human health. Exercise and hypoxia as external stressors can stimulate and activate autophagy and mitophagy, contributing to skeletal muscle cellular adaptation, mitochondrial quality control, and overall human cellular health. Despite considerable research that has reported the molecular mechanisms related to autophagy/mitophagy induced by different exercise protocols, little is known about the mechanisms by which HIIE intervention protocol stimulated autophagy /mitophagy, especially in

hypoxia. The increasing application of the 'omics' approach in multiple research fields (Medicine, Biology, and Sports science) has laid the foundation for our understanding of mRNA levels that regulate skeletal muscle mitochondrial adaptations in human subjects.

Given the research background discussed, the present research employed transcriptomic analysis to investigate underlying molecular mechanisms of mitochondrial adaptation (autophagy/ mitophagy) in response to HIIE in hypoxia. I combined standardized (Differential expression analysis and Functional enrichment analysis) and personalized analytical methods (PCA analysis and STEM analysis) of transcriptomic to explore the molecular response activated by a single session of HIIE. This provided an updated and innovative perspective to explore the cellular health of human skeletal muscle. The largest number of differentially expressed genes was observed in hypoxia after exercise 24 hours. PCA analysis did not show any significant difference between exercise conditions. Autophagy-animal and mitophagy-animal pathways (not ranked in the top 20) were significantly enriched only in hypoxia 24 hours post-exercise, and the key signaling pathway (mTOR signaling pathway) did not exhibit pronounced changes at all conditions after HIIE. To focus on autophagy/mitophagy-related genes/pathways, I chose 604 genes related to autophagy and mitophagy as target genes to perform differential expression analysis. Again, the largest number of differentially expressed genes related to autophagy/mitophagy was observed in hypoxia 24 hours after exercise. However, recognized autophagy markers such as *LC3II*, *LC3II*/*LC3I* ratio, *P62*, and mitophagy receptors (*NIX*, *BNIP3*) did not show significant differences in expression level in all conditions, and mitophagy receptor *FUNDC1* was observed in hypoxia, indicating a lack of robust change in autophagy/mitophagy after a single session of HIIE with and without hypoxia. Similar expression patterns were only observed between different time points under the same exercise condition. Although our result suggests a moderately elevated level of genes related to autophagy/mitophagy pathways under hypoxia exercise, it did not correlate with physiological performance, as a higher percentage of HR_{peak} and $\dot{V}O_{2peak}$, and a higher RPE were observed in both NR and HY compared to NA. Furthermore, the increased genes expression level in hypoxia is affected by time points after exercise. Our result suggests that activation of autophagy and mitophagy pathways is unlikely to be responsible for the difference in observed physiological response.

In order to better understand how autophagy/mitophagy mechanisms HIIE induced impact health benefits and physical performance, further training (HIIT) intervention studies are

warranted. Acute exercise induces autophagy/mitophagy in human skeletal muscle, which seems to be an integral part of beneficial adaptations by long-term exercise [67]. The present study only conducted an acute exercise protocol, which has its limitation that it can only observe short-term changes in mRNA levels after exercise. This resulted in a strongly high requirement for the selection of biopsies sampling time points, as the reasonable sampling time contributes to capturing the key gene information. A review study also concluded that the timing of muscle sampling was an essential methodological issue helping to address the controversial questions of molecular response to exercise, and this study also suggested that a comprehensive time course was needed to establish [56]. However, a single session of HIIE did not replace training (HIIT)-induced observation on protein levels, although an acute of HIIE could likely partially predict the cumulative effects of transient changes in gene expression, as it has been suggested that training-induced adaptive response to exercise performance and substrate metabolism are due to the cumulative effects of each individual exercise bout [316]. Based on the present study's findings, further investigation on autophagy/mitophagy induced by training intervention should be required. Another important point of concern is sampling size. In this study, neither biological replicates correlation analysis nor PCA analysis showed a clear separation between NA, NR, and HY. It is possible that the analysis is under power to detect the molecular changes due to large individual variability. Jacques,M., et al. reported that individual variations exist in performance marker (W_{peak}, $\dot{V}O_{2peak}$, LT) and mitochondria marker (Citrate synthase activity, COX activity, mtCN, SDH) induced by 12 weeks of HIIT intervention over time, whether between participants or within participants [210]. This study also suggested, for other measures (fiber type and mitochondria marker), that inter-individual differences in training effects cannot be verified if there is too much within-participant variability [210]. In further research, the sample size should be increased to achieve the statistical power to detect small differences due to high individual variability in human samples.

In future research, it is suggested to include other auxiliary research methods, in combination of transcriptomic analysis, such as transmission electron microscopy (TEM), immunoblotting and cell culture study to validate the outcomes from transcriptomic analysis. TEM technology is a powerful and valuable tool for studying autophagy and mitophagy, which can clearly visualize mitochondria undergoing autophagic engulfment and degradation [317, 318], which was a key to the visualization and quantification of autophagosomes and lysosomes [319]. Immunoblotting or

immunofluorescence has become a reliable method for detecting LC3, thereby monitoring autophagy and autophagy-related processes [320]. CRISPR tool has revolutionized genome editing in cell culture models, and it allows genome editing in primary human cells or established human muscle cell lines. For example, despite the challenges in editing primary human cells, CRISPR/Cas9 technology has been used in human skeletal muscle cell lines to edit the Duchene muscular dystrophy (DMD) genes [321]. CRISPR/Cas9 system has also been employed in studies related to autophagy/mitophagy [322] and mitochondrial function [323, 324] in skeletal muscle. CRISPR tool can be used to help validate the function of target genes involved in autophagy/mitophagy in future research since the current study has conducted the screening of the differential genes. By overexpression or knocking out specific genes, the research can elucidate the role of these genes in response to hypoxia and simulate exercise-like conditions in cell culture, such as, exposure to hypoxia and using an "exercise in a dish" approach by electric pulse stimulation. Future studies may also consider biological sex's influence on autophagy/mitophagy. Female participants are significantly under-represented in sports and exercise science research (the average percentage of women represented ranged from 35% - 37%) [325]. An experimental study on mice suggested that both young and old female mice have more mitochondrial proteins than male mice of the same age, and young female mice have a greater abundance of autophagy, mitophagy, and lysosome proteins than young male mice [326]. In addition, some studies have reported the influence of sex differecnces on mitochondrial biogenesis [327] and fiber type [328]. Therefore, to avoid biological sex bias, adding female cohorts is necessary in future studies.

4.3 Statement of significance

The study will contribute to a better understanding of the molecular pathways underneath the synergistic beneficial effect of combining HIIE and hypoxia. This study's findings help to build the theoretical support to prescribe hypoxia exercise training to populations that are normally unable to perform HIIE in normoxic condition and, therefore, unable to gain the health benefit of HIIE. The study outcomes will also be able to provide theoretical support for improving athlete's exercise performance.

References

1. Ernster, L. and G. Schatz, *Mitochondria: a historical review.* The Journal of cell biology, 1981. **91**(3): p. 227s-255s.
2. Krauss, S., *Mitochondria: Structure and role in respiration.* e LS, 2001.
3. Petriz, B.A., et al., *Mitochondrial Proteomics: From Structure to Function*, in *Proteomics-Human Diseases and Protein Functions*. 2012, IntechOpen.
4. Ježek, P., et al., *Mitochondrial cristae morphology reflecting metabolism, superoxide formation, redox homeostasis, and pathology.* Antioxidants & Redox Signaling, 2023. **39**(10-12): p. 635-683.
5. Sharma, L.K., J. Lu, and Y. Bai, *Mitochondrial respiratory complex I: structure, function and implication in human diseases.* Current medicinal chemistry, 2009. **16**(10): p. 1266-1277.
6. Sousa, J.S., E. D'Imprima, and J. Vonck, *Mitochondrial respiratory chain complexes.* Membrane protein complexes: structure and function, 2018: p. 167-227.
7. DURING, P.C., *Structural and computational analysis of the quinone-binding site of complex II (succinate-ubiquinone oxidoreductase).* THE JOURNAL OF BIOLOGICAL CHEMISTRY, 2006. **281**(11): p. 7309-7316.
8. Ramsay, R.R., *Electron carriers and energy conservation in mitochondrial respiration.* ChemTexts, 2019. **5**(2): p. 9.
9. Kakkar, P. and B. Singh, *Mitochondria: a hub of redox activities and cellular distress control.* Molecular and cellular biochemistry, 2007. **305**: p. 235-253.
10. San-Millán, I., *The Key Role of Mitochondrial Function in Health and Disease.* Antioxidants, 2023. **12**(4): p. 782.
11. Conley, K.E., *Mitochondria to motion: optimizing oxidative phosphorylation to improve exercise performance.* Journal of Experimental Biology, 2016. **219**(2): p. 243-249.
12. Yoo, S.-Z., et al., *Effects of acute exercise on mitochondrial function, dynamics, and mitophagy in rat cardiac and skeletal muscles.* International Neurourology Journal, 2019. **23**(Suppl 1): p. S22.
13. 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.
14. Matta, L., et al., *The effect of acute aerobic exercise on redox homeostasis and mitochondrial function of rat white adipose tissue.* Oxidative Medicine and Cellular Longevity, 2021. **2021**.
15. Fernström, M., M. Tonkonogi, and K. Sahlin, *Effects of acute and chronic endurance exercise on mitochondrial uncoupling in human skeletal muscle.* The Journal of physiology, 2004. **554**(3): p. 755-763.
16. De las Heras, N., et al., *Chronic exercise improves mitochondrial function and insulin sensitivity in brown adipose tissue.* Frontiers in physiology, 2018. **9**: p. 1122.

17. Bangsbo, J., et al., *ATP production and efficiency of human skeletal muscle during intense exercise: effect of previous exercise.* American Journal of Physiology-Endocrinology and Metabolism, 2001. **280**(6): p. E956-E964.
18. Hargreaves, M. and L.L. Spriet, *Skeletal muscle energy metabolism during exercise.* Nature metabolism, 2020. **2**(9): p. 817-828.
19. Freitas, M.C., et al., *A single dose of oral ATP supplementation improves performance and physiological response during lower body resistance exercise in recreational resistance-trained males.* The Journal of Strength & Conditioning Research, 2019. **33**(12): p. 3345-3352.
20. Hoppeler, H., et al., *Endurance training in humans: aerobic capacity and structure of skeletal muscle.* Journal of applied physiology, 1985. **59**(2): p. 320-327.
21. Boushel, R., et al., *Muscle mitochondrial capacity exceeds maximal oxygen delivery in humans.* Mitochondrion, 2011. **11**(2): p. 303-307.
22. Montero, D., et al., *Haematological rather than skeletal muscle adaptations contribute to the increase in peak oxygen uptake induced by moderate endurance training.* The Journal of physiology, 2015. **593**(20): p. 4677-4688.
23. 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.* Journal of applied physiology, 2013. **115**(6): p. 785-793.
24. Jacobs, R.A., et al., *Determinants of time trial performance and maximal incremental exercise in highly trained endurance athletes.* Journal of Applied Physiology, 2011. **111**(5): p. 1422-1430.
25. Mizushima, N. and M. Komatsu, *Autophagy: renovation of cells and tissues.* Cell, 2011. **147**(4): p. 728-41.
26. Nakatogawa, H., *Mechanisms governing autophagosome biogenesis.* Nature Reviews Molecular Cell Biology, 2020. **21**(8): p. 439-458.
27. Doblado, L., et al., *Mitophagy in Human Diseases.* International Journal of Molecular Sciences, 2021. **22**(8): p. 3903.
28. Parzych, K.R. and D.J. Klionsky, *An overview of autophagy: morphology, mechanism, and regulation.* Antioxid Redox Signal, 2014. **20**(3): p. 460-73.
29. Yang, Z. and D.J. Klionsky, *Mammalian autophagy: core molecular machinery and signaling regulation.* Current opinion in cell biology, 2010. **22**(2): p. 124-131.
30. Parzych, K.R. and D.J. Klionsky, *An overview of autophagy: morphology, mechanism, and regulation.* Antioxidants & redox signaling, 2014. **20**(3): p. 460-473.
31. Marzella, L., J. Ahlberg, and H. Glaumann, *Autophagy, heterophagy, microautophagy and crinophagy as the means for intracellular degradation.* Virchows Archiv B, 1981. **36**(1): p. 219-234.
32. Sandoval, I., et al., *A unified nomenclature for yeast autophagy-related genes.* Developmental Cell, 2003. **5**(4): p. 539545.
33. Nakatogawa, H., et al., *Dynamics and diversity in autophagy mechanisms: lessons from yeast.* Nature reviews Molecular cell biology, 2009. **10**(7): p. 458-467.
34. Mizushima, N., et al., *Autophagy fights disease through cellular self-digestion.* Nature, 2008. **451**(7182): p. 1069-1075.

35. Coto-Montes, A., et al., *Role of melatonin in the regulation of autophagy and mitophagy: A review.* Molecular and Cellular Endocrinology, 2012. **361**(1): p. 12-23.
36. Zhang, J., *Autophagy and Mitophagy in Cellular Damage Control.* Redox biology, 2013. **1**(1): p. 19-23.
37. William, M., *Evolutionary biology: essence of mitochondria [J].* Nature, 2003. **426**(6963): p. 127-128.
38. Saito, T. and J. Sadoshima, *Molecular mechanisms of mitochondrial autophagy/mitophagy in the heart.* Circ Res, 2015. **116**(8): p. 1477-90.
39. Sugiura, A., et al., *A new pathway for mitochondrial quality control: mitochondrial-derived vesicles.* The EMBO journal, 2014. **33**(19): p. 2142-2156.
40. Yoo, S.-M. and Y.-K. Jung, *A Molecular Approach to Mitophagy and Mitochondrial Dynamics.* Molecules and cells, 2018. **41**(1): p. 18-26.
41. Ashrafi, G. and T.L. Schwarz, *The pathways of mitophagy for quality control and clearance of mitochondria.* Cell death and differentiation, 2013. **20**(1): p. 31-42.
42. Scott, S.V. and D.J. Klionsky, *Delivery of proteins and organelles to the vacuole from the cytoplasm.* Current Opinion in Cell Biology, 1998. **10**(4): p. 523-529.
43. Lemasters, J.J., *Selective mitochondrial autophagy, or mitophagy, as a targeted defense against oxidative stress, mitochondrial dysfunction, and aging.* Rejuvenation research, 2005. **8**(1): p. 3-5.
44. Ashford, T.P. and K.R. Porter, *Cytoplasmic components in hepatic cell lysosomes.* J Cell Biol, 1962. **12**(1): p. 198-202.
45. Sanchez, A.M., et al., *Autophagy is essential to support skeletal muscle plasticity in response to endurance exercise.* Am J Physiol Regul Integr Comp Physiol, 2014. **307**(8): p. R956-69.
46. Ju, J.-s., et al., *Autophagy plays a role in skeletal muscle mitochondrial biogenesis in an endurance exercise-trained condition.* The Journal of Physiological Sciences, 2016. **66**(5): p. 417-430.
47. Ney, P.A., *Mitochondrial autophagy: Origins, significance, and role of BNIP3 and NIX.* Biochimica et Biophysica Acta (BBA) - Molecular Cell Research, 2015. **1853**(10, Part B): p. 2775-2783.
48. Jimenez, R.E., D.A. Kubli, and Å.B. Gustafsson, *Autophagy and mitophagy in the myocardium: therapeutic potential and concerns.* British journal of pharmacology, 2014. **171**(8): p. 1907-1916.
49. Sun, N., et al., *Measuring In Vivo Mitophagy.* Molecular Cell, 2015. **60**(4): p. 685-696.
50. Cummins, N. and J. Götz, *Shedding light on mitophagy in neurons: what is the evidence for PINK1/Parkin mitophagy in vivo?* Cellular and Molecular Life Sciences, 2018. **75**(7): p. 1151-1162.
51. Guan, Y., J.C. Drake, and Z. Yan, *Exercise-Induced Mitophagy in Skeletal Muscle and Heart.* Exercise and sport sciences reviews, 2019. **47**(3): p. 151-156.
52. Hanna, R.A., et al., *Microtubule-associated protein 1 light chain 3 (LC3) interacts with Bnip3 protein to selectively remove endoplasmic reticulum and mitochondria via autophagy.* Journal of Biological Chemistry, 2012. **287**(23): p. 19094-19104.

53. Youle, R.J. and D.P. Narendra, *Mechanisms of mitophagy.* Nature Reviews Molecular Cell Biology, 2011. **12**(1): p. 9-14.
54. Tschakert, G. and P. Hofmann, *High-intensity intermittent exercise: methodological and physiological aspects.* Int J Sports Physiol Perform, 2013. **8**(6): p. 600-10.
55. Whitaker, A.A., et al., *Effects of high intensity interval exercise on cerebrovascular function: A systematic review.* PloS one, 2020. **15**(10): p. e0241248.
56. 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.
57. Li, J., et al., *The Molecular Adaptive Responses of Skeletal Muscle to High-Intensity Exercise/Training and Hypoxia.* Antioxidants (Basel), 2020. **9**(8).
58. Bishop, D.J., et al., *High-intensity exercise and mitochondrial biogenesis: current controversies and future research directions.* Physiology, 2019. **34**(1): p. 56-70.
59. 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.
60. Drake, J.C. and Z. Yan, *Mitophagy in maintaining skeletal muscle mitochondrial proteostasis and metabolic health with ageing.* The Journal of physiology, 2017. **595**(20): p. 6391-6399.
61. Camera, D.M., W.J. Smiles, and J.A. Hawley, *Exercise-induced skeletal muscle signaling pathways and human athletic performance.* Free Radic Biol Med, 2016. **98**: p. 131-143.
62. Carter, H.N., C.C. Chen, and D.A. Hood, *Mitochondria, muscle health, and exercise with advancing age.* Physiology (Bethesda), 2015. **30**(3): p. 208-23.
63. Drake, J.C., R.J. Wilson, and Z. Yan, *Molecular mechanisms for mitochondrial adaptation to exercise training in skeletal muscle.* FASEB journal : official publication of the Federation of American Societies for Experimental Biology, 2016. **30**(1): p. 13-22.
64. Roberts, F.L. and G.R. Markby, *New insights into molecular mechanisms mediating adaptation to exercise; A review focusing on mitochondrial biogenesis, mitochondrial function, mitophagy and autophagy.* Cells, 2021. **10**(10): p. 2639.
65. Camera, D.M., W.J. Smiles, and J.A. Hawley, *Exercise-induced skeletal muscle signaling pathways and human athletic performance.* Free Radical Biology and Medicine, 2016. **98**: p. 131-143.
66. Miller, B.F. and K.L. Hamilton, *A perspective on the determination of mitochondrial biogenesis.* American journal of physiology. Endocrinology and metabolism, 2012. **302**(5): p. E496-E499.
67. Guan, Y., J.C. Drake, and Z. Yan, *Exercise-induced mitophagy in skeletal muscle and heart.* Exercise and sport sciences reviews, 2019. **47**(3): p. 151.
68. Chen, C.C.W., et al., *Parkin is required for exercise-induced mitophagy in muscle: Impact of aging.* American Journal of Physiology-Endocrinology and Metabolism, 2018. **315**(3): p. E404-E415.
69. Arribat, Y., et al., *Distinct patterns of skeletal muscle mitochondria fusion, fission and mitophagy upon duration of exercise training.* Acta Physiologica, 2019. **225**(2): p. e13179.

70. He, C., J. Sumpter, Rhea, and B. Levine, *Exercise induces autophagy in peripheral tissues and in the brain.* Autophagy, 2012. **8**(10): p. 1548-1551.
71. Lira, V.A., et al., *Autophagy is required for exercise training-induced skeletal muscle adaptation and improvement of physical performance.* The FASEB Journal, 2013. **27**(10): p. 4184-4193.
72. Galluzzi, L., et al., *Metabolic control of autophagy.* Cell, 2014. **159**(6): p. 1263-1276.
73. Cheng, Y., et al., *Therapeutic targeting of autophagy in disease: biology and pharmacology.* Pharmacological reviews, 2013. **65**(4): p. 1162-1197.
74. Zhang, Y., J.R. Sowers, and J. Ren, *Targeting autophagy in obesity: from pathophysiology to management.* Nature Reviews Endocrinology, 2018. **14**(6): p. 356-376.
75. Halling, J.F. and H. Pilegaard, *Autophagy-dependent beneficial effects of exercise.* Cold Spring Harbor perspectives in medicine, 2017. **7**(8): p. a029777.
76. Vainshtein, A. and D.A. Hood, *The regulation of autophagy during exercise in skeletal muscle.* Journal of Applied Physiology, 2016. **120**(6): p. 664-673.
77. Kim, J., et al., *AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1.* Nature cell biology, 2011. **13**(2): p. 132-141.
78. Salminen, A. and V. Vihko, *Autophagic response to strenuous exercise in mouse skeletal muscle fibers.* Virchows Archiv B, 1984. **45**(1): p. 97-106.
79. Tanida, I., T. Ueno, and E. Kominami, *LC3 conjugation system in mammalian autophagy.* The international journal of biochemistry & cell biology, 2004. **36**(12): p. 2503-2518.
80. Mizushima, N. and T. Yoshimori, *How to interpret LC3 immunoblotting.* Autophagy, 2007. **3**(6): p. 542-545.
81. Klionsky, D., et al., *Guidelines for the use and interpretation of assays for monitoring autophagy.* Autophagy, 2016. **12**(1): p. 1-222.
82. Yoshii, S.R. and N. Mizushima, *Monitoring and measuring autophagy.* International journal of molecular sciences, 2017. **18**(9): p. 1865.
83. Grumati, P., et al., *Physical exercise stimulates autophagy in normal skeletal muscles but is detrimental for collagen VI-deficient muscles.* Autophagy, 2011. **7**(12): p. 1415-1423.
84. He, C., et al., *Exercise-induced BCL2-regulated autophagy is required for muscle glucose homeostasis.* Nature, 2012. **481**(7382): p. 511-515.
85. Pagano, A.F., et al., *Autophagy and protein turnover signaling in slow-twitch muscle during exercise.* Med Sci Sports Exerc, 2014. **46**(7): p. 1314-1325.
86. Halling, J.F., et al., *PGC-1α promotes exercise-induced autophagy in mouse skeletal muscle.* Physiological reports, 2016. **4**(3): p. e12698.
87. Botella, J., et al., *Exercise and training regulation of autophagy markers in human and rodent skeletal muscle.* bioRxiv, 2021.
88. Møller, A.B., et al., *Physical exercise increases autophagic signaling through ULK1 in human skeletal muscle.* Journal of Applied Physiology, 2015. **118**(8): p. 971-979.
89. Fritzen, A.M., et al., *Regulation of autophagy in human skeletal muscle: effects of exercise, exercise training and insulin stimulation.* The Journal of physiology, 2016. **594**(3): p. 745-761.

90. Kruse, R., et al., *Intact initiation of autophagy and mitochondrial fission by acute exercise in skeletal muscle of patients with Type 2 diabetes.* Clinical Science, 2017. **131**(1): p. 37-47.
91. Schwalm, C., et al., *Activation of autophagy in human skeletal muscle is dependent on exercise intensity and AMPK activation.* The FASEB Journal, 2015. **29**(8): p. 3515-3526.
92. Fry, C.S., et al., *Skeletal muscle autophagy and protein breakdown following resistance exercise are similar in younger and older adults.* J Gerontol A Biol Sci Med Sci, 2013. **68**(5): p. 599-607.
93. Jamart, C., et al., *Modulation of autophagy and ubiquitin-proteasome pathways during ultra-endurance running.* Journal of applied physiology, 2012. **112**(9): p. 1529-1537.
94. Tachtsis, B., et al., *Acute Endurance Exercise Induces Nuclear p53 Abundance in Human Skeletal Muscle.* Front Physiol, 2016. **7**: p. 144.
95. Schwalm, C., et al., *Activation of autophagy in human skeletal muscle is dependent on exercise intensity and AMPK activation.* Faseb j, 2015. **29**(8): p. 3515-26.
96. Brandt, N., et al., *PGC-1α and exercise intensity dependent adaptations in mouse skeletal muscle.* PloS one, 2017. **12**(10): p. e0185993.
97. Brandt, N., et al., *PGC-1α and exercise intensity dependent adaptations in mouse skeletal muscle.* PloS one, 2017. **12**(10): p. e0185993-e0185993.
98. He, C., et al., *Exercise-induced BCL2-regulated autophagy is required for muscle glucose homeostasis.* Nature, 2012. **481**(7382): p. 511-515.
99. Roberts, F.L. and G.R. Markby, *New Insights into Molecular Mechanisms Mediating Adaptation to Exercise; A Review Focusing on Mitochondrial Biogenesis, Mitochondrial Function, Mitophagy and Autophagy.* Cells, 2021. **10**(10).
100. Brandt, N., et al., *Exercise and exercise training-induced increase in autophagy markers in human skeletal muscle.* Physiol Rep, 2018. **6**(7): p. e13651.
101. Fritzen, A.M., et al., *Regulation of autophagy in human skeletal muscle: effects of exercise, exercise training and insulin stimulation.* The Journal of physiology, 2016. **594**(3): p. 745-761.
102. Brandt, N., et al., *Impact of β-adrenergic signaling in PGC-1α-mediated adaptations in mouse skeletal muscle.* American Journal of Physiology-Endocrinology and Metabolism, 2018. **314**(1): p. E1-E20.
103. Rodriguez-Enriquez, S., et al., *Tracker dyes to probe mitochondrial autophagy (mitophagy) in rat hepatocytes.* Autophagy, 2006. **2**(1): p. 39-46.
104. Palikaras, K., E. Lionaki, and N. Tavernarakis, *Mechanisms of mitophagy in cellular homeostasis, physiology and pathology.* Nature cell biology, 2018. **20**(9): p. 1013-1022.
105. Youle, R.J., *Mitochondria—Striking a balance between host and endosymbiont.* Science, 2019. **365**(6454).
106. Killackey, S.A., D.J. Philpott, and S.E. Girardin, *Mitophagy pathways in health and disease.* Journal of Cell Biology, 2020. **219**(11): p. e202004029.
107. Brown, G.C., et al., *Regulation of mitochondrial biogenesis.* Essays in biochemistry, 2010. **47**: p. 69-84.
108. Escobar, K.A., et al., *Autophagy response to acute high-intensity interval training and moderate-intensity continuous training is dissimilar in skeletal muscle and peripheral*

blood mononuclear cells and is influenced by sex. Human Nutrition & Metabolism, 2021. **23**: p. 200118.
109. Weng, T.-P., et al., *Effects of interval and continuous exercise training on CD4 lymphocyte apoptotic and autophagic responses to hypoxic stress in sedentary men.* PloS one, 2013. **8**(11): p. e80248.
110. Brandt, N., et al., *Exercise and exercise training‐induced increase in autophagy markers in human skeletal muscle.* Physiological reports, 2018. **6**(7): p. e13651.
111. Dethlefsen, M.M., et al., *Regulation of apoptosis and autophagy in mouse and human skeletal muscle with aging and lifelong exercise training.* Experimental Gerontology, 2018. **111**: p. 141-153.
112. Smiles, W.J., et al., *Acute low-intensity cycling with blood-flow restriction has no effect on metabolic signaling in human skeletal muscle compared to traditional exercise.* European journal of applied physiology, 2017. **117**: p. 345-358.
113. Gnimassou, O., et al., *Environmental hypoxia favors myoblast differentiation and fast phenotype but blunts activation of protein synthesis after resistance exercise in human skeletal muscle.* The FASEB Journal, 2018. **32**.
114. Li, F.-H., et al., *Beneficial autophagic activities, mitochondrial function, and metabolic phenotype adaptations promoted by high-intensity interval training in a rat model.* Frontiers in Physiology, 2018. **9**: p. 571.
115. Wan, D.-F., et al., *Exercise preconditioning promotes autophagy to cooperate for cardioprotection by increasing LC3 lipidation-associated proteins.* Frontiers in Physiology, 2021. **12**: p. 599892.
116. Jafari, M., et al., *The Effect of Eight Weeks of Continuous and Interval Training with Citrus Aurantium Consumption on Autophagy Markers and MyoD Activation in the Muscle Tissue of Elderly Rats.* 2021.
117. Botella, J., et al., *Exercise and training regulation of autophagy markers in human and rat skeletal muscle.* International Journal of Molecular Sciences, 2022. **23**(5): p. 2619.
118. Giordano, C., et al., *Autophagy-associated atrophy and metabolic remodeling of the mouse diaphragm after short-term intermittent hypoxia.* PLoS One, 2015. **10**(6): p. e0131068.
119. Lemieux, P. and O. Birot, *Altitude, Exercise, and Skeletal Muscle Angio-Adaptive Responses to Hypoxia: A Complex Story.* Front Physiol, 2021. **12**: p. 735557.
120. 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.
121. Kumar, H. and D.-K. Choi, *Hypoxia inducible factor pathway and physiological adaptation: a cell survival pathway?* Mediators of inflammation, 2015. **2015**.
122. Wang, G.L. and G.L. Semenza, *Purification and Characterization of Hypoxia-inducible Factor 1 (∗).* Journal of biological chemistry, 1995. **270**(3): p. 1230-1237.
123. Bursch, W., et al., *Cell death and autophagy: Cytokines, drugs, and nutritional factors.* Toxicology, 2008. **254**(3): p. 147-157.
124. Schaaf, M.B.E., et al., *The autophagy associated gene, ULK1, promotes tolerance to chronic and acute hypoxia.* Radiotherapy and Oncology, 2013. **108**(3): p. 529-534.

125. Mazure, N.M. and J. Pouysségur, *Hypoxia-induced autophagy: cell death or cell survival?* Current Opinion in Cell Biology, 2010. **22**(2): p. 177-180.
126. Fang, Y., J. Tan, and Q. Zhang, *Signaling pathways and mechanisms of hypoxia-induced autophagy in the animal cells.* Cell Biol Int, 2015. **39**(8): p. 891-8.
127. Zhang, H., et al., *Mitochondrial autophagy is an HIF-1-dependent adaptive metabolic response to hypoxia.* Journal of Biological Chemistry, 2008. **283**(16): p. 10892-10903.
128. Sun, Y., et al., *Mitophagy Protects the Retina Against Anti-Vascular Endothelial Growth Factor Therapy-Driven Hypoxia via Hypoxia-Inducible Factor-1α Signaling.* Frontiers in Cell and Developmental Biology, 2021: p. 3048.
129. Chandel, N.S., et al., *Reactive oxygen species generated at mitochondrial complex III stabilize hypoxia-inducible factor-1α during hypoxia: a mechanism of O2 sensing.* Journal of Biological Chemistry, 2000. **275**(33): p. 25130-25138.
130. Thomas, L.W. and M. Ashcroft, *Exploring the molecular interface between hypoxia-inducible factor signalling and mitochondria.* Cellular and molecular life sciences : CMLS, 2019. **76**(9): p. 1759-1777.
131. Wu, W., et al., *ULK 1 translocates to mitochondria and phosphorylates FUNDC 1 to regulate mitophagy.* EMBO reports, 2014. **15**(5): p. 566-575.
132. Wu, S., et al., *Binding of FUN14 domain containing 1 with inositol 1, 4, 5-trisphosphate receptor in mitochondria-associated endoplasmic reticulum membranes maintains mitochondrial dynamics and function in hearts in vivo.* Circulation, 2017. **136**(23): p. 2248-2266.
133. Wei, H., L. Liu, and Q. Chen, *Selective removal of mitochondria via mitophagy: distinct pathways for different mitochondrial stresses.* Biochimica et Biophysica Acta (BBA) - Molecular Cell Research, 2015. **1853**(10, Part B): p. 2784-2790.
134. Semenza, G.L., *HIF-1: upstream and downstream of cancer metabolism.* Current opinion in genetics & development, 2010. **20**(1): p. 51-56.
135. Scortegagna, M., et al., *Multiple organ pathology, metabolic abnormalities and impaired homeostasis of reactive oxygen species in Epas1−/− mice.* Nature genetics, 2003. **35**(4): p. 331-340.
136. Kume, S., et al., *Calorie restriction enhances cell adaptation to hypoxia through Sirt1-dependent mitochondrial autophagy in mouse aged kidney.* The Journal of clinical investigation, 2010. **120**(4): p. 1043-1055.
137. Masschelein, E., et al., *Acute environmental hypoxia induces LC3 lipidation in a genotype‐dependent manner.* The FASEB Journal, 2014. **28**(2): p. 1022-1034.
138. Band, M., et al., *Hypoxia‐induced BNIP3 expression and mitophagy: in vivo comparison of the rat and the hypoxia‐tolerant mole rat, Spalax ehrenbergi.* The FASEB Journal, 2009. **23**(7): p. 2327-2335.
139. D'Hulst, G., et al., *Fifteen days of 3,200 m simulated hypoxia marginally regulates markers for protein synthesis and degradation in human skeletal muscle.* Hypoxia (Auckland, N.Z.), 2016. **4**: p. 1-14.
140. Costa, V., et al., *RNA-Seq and human complex diseases: recent accomplishments and future perspectives.* European Journal of Human Genetics, 2013. **21**(2): p. 134-142.

141. Marguerat, S. and J. Bähler, *RNA-seq: from technology to biology.* Cellular and molecular life sciences, 2010. **67**: p. 569-579.
142. Ayturk, U., *RNA-seq in skeletal biology.* Current Osteoporosis Reports, 2019. **17**: p. 178-185.
143. Popov, D.V., et al., *Intensity-dependent gene expression after aerobic exercise in endurance-trained skeletal muscle.* Biology of sport, 2018. **35**(3): p. 277-289.
144. Dickinson, J.M., et al., *Transcriptome response of human skeletal muscle to divergent exercise stimuli.* Journal of Applied Physiology, 2018. **124**(6): p. 1529-1540.
145. Maza, E., et al., *Comparison of normalization methods for differential gene expression analysis in RNA-Seq experiments: a matter of relative size of studied transcriptomes.* Communicative & integrative biology, 2013. **6**(6): p. e25849.
146. Cardoso, T.F., et al., *RNA-seq based detection of differentially expressed genes in the skeletal muscle of Duroc pigs with distinct lipid profiles.* Scientific reports, 2017. **7**(1): p. 40005.
147. Hu, Z., et al., *Skeletal muscle transcriptome analysis of Hanzhong Ma Duck at different growth stages using RNA-Seq.* Biomolecules, 2021. **11**(2): p. 315.
148. Qian, X., et al., *RNA-Seq technology and its application in fish transcriptomics.* Omics: a journal of integrative biology, 2014. **18**(2): p. 98-110.
149. Park, K.-D., et al., *Whole transcriptome analyses of six thoroughbred horses before and after exercise using RNA-Seq.* BMC genomics, 2012. **13**: p. 1-8.
150. Söllner, J.F., et al., *An RNA-Seq atlas of gene expression in mouse and rat normal tissues.* Scientific data, 2017. **4**(1): p. 1-11.
151. Brown, A.A., et al., *Predicting causal variants affecting expression by using whole-genome sequencing and RNA-seq from multiple human tissues.* Nature genetics, 2017. **49**(12): p. 1747-1751.
152. Tschakert, G., et al., *How to regulate the acute physiological response to "aerobic" high-intensity interval exercise.* J Sports Sci Med, 2015. **14**(1): p. 29-36.
153. Gibala, M.J., et al., *Physiological adaptations to low‐volume, high‐intensity interval training in health and disease.* The Journal of physiology, 2012. **590**(5): p. 1077-1084.
154. Saltin, B., B. Essén, and P.K. Pedersen, *Intermittent exercise: its physiology and some practical applications*, in *Advances in exercise physiology*. 1976, Karger Publishers. p. 23-51.
155. Wijesooriya, K., et al., *Urgent need for consistent standards in functional enrichment analysis.* PLoS Comput Biol, 2022. **18**(3): p. e1009935.
156. Febbraio, M. and I. Koukoulas, *HSP72 gene expression progressively increases in human skeletal muscle during prolonged, exhaustive exercise.* Journal of Applied Physiology, 2000. **89**(3): p. 1055-1060.
157. 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.
158. O'Driscoll, L., et al., *The use of reverse transcriptase-polymerase chain reaction (RT-PCR) to investigate specific gene expression in multidrug-resistant cells.* Multiple Drug Resistance in Cancer: Cellular, Molecular and Clinical Approaches, 1994: p. 289-314.

159. 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.
160. Yan, X., et al., *The gene SMART study: method, study design, and preliminary findings.* BMC genomics, 2017. **18**(8): p. 15-28.
161. Jamnick, N.A., et al., *An Examination and Critique of Current Methods to Determine Exercise Intensity.* Sports Medicine, 2020. **50**(10): p. 1729-1756.
162. 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.
163. Jackson, A., et al., *557 ACCURACY OF VO2 MAX PREDICTION MODELS TO MEASURE CHANGE.* Medicine & Science in Sports & Exercise, 1990. **22**(2): p. S96.
164. Yan, X., et al., *The gene SMART study: method, study design, and preliminary findings.* BMC genomics, 2017. **18**(Suppl 8): p. 821-821.
165. Bishop, D., D.G. Jenkins, and L.T. Mackinnon, *The relationship between plasma lactate parameters, Wpeak and 1-h cycling performance in women.* Medicine and science in sports and exercise, 1998. **30**(8): p. 1270-1275.
166. Bordi, M., et al., *A gene toolbox for monitoring autophagy transcription.* Cell death & disease, 2021. **12**(11): p. 1044.
167. Pérez-Rubio, P., C. Lottaz, and J.C. Engelmann, *FastqPuri: high-performance preprocessing of RNA-seq data.* BMC bioinformatics, 2019. **20**(1): p. 1-11.
168. Wen, G. *A simple process of RNA-sequence analyses by Hisat2, Htseq and DESeq2*. in *Proceedings of the 2017 International Conference on Biomedical Engineering and Bioinformatics*. 2017.
169. Musich, R.J., *A Recent (2020) Comparative Analysis of Genome Aligners Shows HISAT2 and BWA are Among the Best Tools*. 2020: Rochester Institute of Technology.
170. Shumate, A., et al., *Improved transcriptome assembly using a hybrid of long and short reads with StringTie.* PLOS Computational Biology, 2022. **18**(6): p. e1009730.
171. Zhao, Y., et al., *TPM, FPKM, or Normalized Counts? A Comparative Study of Quantification Measures for the Analysis of RNA-seq Data from the NCI Patient-Derived Models Repository.* Journal of Translational Medicine, 2021. **19**(1): p. 269.
172. Li, B., et al., *Integrated mRNA-seq and miRNA-seq analysis of goat fibroblasts response to Brucella Melitensis strain M5-90.* PeerJ, 2021. **9**: p. e11679.
173. Robinson, M.D., D.J. McCarthy, and G.K. Smyth, *edgeR: a Bioconductor package for differential expression analysis of digital gene expression data.* bioinformatics, 2010. **26**(1): p. 139-140.
174. Wickham, H., W. Chang, and M.H. Wickham, *Package 'ggplot2'.* Create elegant data visualisations using the grammar of graphics. Version, 2016. **2**(1): p. 1-189.
175. Livak, K.J. and T.D. Schmittgen, *Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method.* methods, 2001. **25**(4): p. 402-408.
176. Pescatello, L.S., *ACSM's guidelines for exercise testing and prescription*. 2014: Lippincott Williams & Wilkins.
177. Mizushima, N. and M. Komatsu, *Autophagy: renovation of cells and tissues.* Cell, 2011. **147**(4): p. 728-741.

178. Zoll, J., et al., *Exercise training in normobaric hypoxia in endurance runners. III. Muscular adjustments of selected gene transcripts.* Journal of applied physiology, 2006. **100**(4): p. 1258-1266.
179. Menz, V., et al., *Cardiorespiratory effects of one-legged high-intensity interval training in normoxia and hypoxia: A pilot study.* Journal of Sports Science & Medicine, 2016. **15**(2): p. 208.
180. Czuba, M., et al., *The effects of high intensity interval training in normobaric hypoxia on aerobic capacity in basketball players.* Journal of human kinetics, 2013. **39**: p. 103.
181. Ghaith, A., et al., *Hypoxic high-intensity interval training in individuals with overweight and obesity.* American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, 2022. **323**(5): p. R700-R709.
182. Van Thienen, R., et al., *Twin resemblance in muscle HIF-1α responses to hypoxia and exercise.* Frontiers in Physiology, 2017. **7**: p. 676.
183. Żebrowska, A., et al., *Comparison of the effectiveness of high-intensity interval training in hypoxia and normoxia in healthy male volunteers: a pilot study.* BioMed research international, 2019. **2019**.
184. Camacho-Cardenosa, A., et al., *Effects of high-intensity interval training under normobaric hypoxia on cardiometabolic risk markers in overweight/obese women.* High Altitude Medicine & Biology, 2018. **19**(4): p. 356-366.
185. Mackenzie, R., et al., *Intermittent exercise with and without hypoxia improves insulin sensitivity in individuals with type 2 diabetes.* The Journal of Clinical Endocrinology & Metabolism, 2012. **97**(4): p. E546-E555.
186. Kong, Z., et al., *High-intensity interval training in normobaric hypoxia improves cardiorespiratory fitness in overweight Chinese young women.* Frontiers in physiology, 2017. **8**: p. 175.
187. 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. 237-245.
188. Jamnick, N.A., et al., *An Examination and Critique of Current Methods to Determine Exercise Intensity.* Sports Med, 2020. **50**(10): p. 1729-1756.
189. Bok, D., M. Rakovac, and C. Foster, *An Examination and Critique of Subjective Methods to Determine Exercise Intensity: The Talk Test, Feeling Scale, and Rating of Perceived Exertion.* Sports Med, 2022. **52**(9): p. 2085-2109.
190. 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.
191. 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.
192. 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.
193. Scharhag-Rosenberger, F., et al., *Exercise at given percentages of VO2max: heterogeneous metabolic responses between individuals.* J Sci Med Sport, 2010. **13**(1): p. 74-9.

194. Granata, C., et al., *Training intensity modulates changes in PGC-1α 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.
195. 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.
196. 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.
197. McGee, S.L. and M. Hargreaves, *Exercise adaptations: molecular mechanisms and potential targets for therapeutic benefit.* Nat Rev Endocrinol, 2020. **16**(9): p. 495-505.
198. 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.
199. Egan, B. and A.P. Sharples, *Molecular responses to acute exercise and their relevance for adaptations in skeletal muscle to exercise training.* Physiological Reviews, 2023. **103**(3): p. 2057-2170.
200. Lira, V.A., et al., *Autophagy is required for exercise training-induced skeletal muscle adaptation and improvement of physical performance.* The FASEB Journal, 2013. **27**(10): p. 4184.
201. Ju, J.-s., et al., *Autophagy plays a role in skeletal muscle mitochondrial biogenesis in an endurance exercise-trained condition.* The Journal of Physiological Sciences, 2016. **66**: p. 417-430.
202. Geiser, J., et al., *Training high-living low: changes of aerobic performance and muscle structure with training at simulated altitude.* International journal of sports medicine, 2001. **22**(08): p. 579-585.
203. Czuba, M., et al., *The effects of intermittent hypoxic training on aerobic capacity and endurance performance in cyclists.* Journal of sports science & medicine, 2011. **10**(1): p. 175.
204. Chacaroun, S., et al., *Hypoxic exercise training to improve exercise capacity in obese individuals.* Medicine and Science in Sports and Exercise, 2020. **52**(8): p. 1641-1649.
205. Mackenzie, R., et al., *Acute hypoxia and exercise improve insulin sensitivity (SI2*) in individuals with type 2 diabetes.* Diabetes/metabolism research and reviews, 2011. **27**(1): p. 94-101.
206. Heinonen, I.H., R. Boushel, and K.K. Kalliokoski, *The circulatory and metabolic responses to hypoxia in humans–with special reference to adipose tissue physiology and obesity.* Frontiers in endocrinology, 2016. **7**: p. 116.
207. Urdampilleta, A., et al., *Usefulness of combining intermittent hypoxia and physical exercise in the treatment of obesity.* Journal of physiology and biochemistry, 2012. **68**: p. 289-304.
208. Kayser, B. and S. Verges, *Hypoxia, energy balance and obesity: from pathophysiological mechanisms to new treatment strategies.* Obesity reviews, 2013. **14**(7): p. 579-592.
209. De Groote, E. and L. Deldicque, *Is physical exercise in hypoxia an interesting strategy to prevent the development of type 2 diabetes? A narrative review.* Diabetes, Metabolic Syndrome and Obesity, 2021: p. 3603-3616.

210. Jacques, M., et al., *Individual physiological and mitochondrial responses during 12 weeks of intensified exercise.* Physiological Reports, 2021. **9**(15): p. e14962.
211. Hansen, K.D., et al., *Sequencing technology does not eliminate biological variability.* Nature biotechnology, 2011. **29**(7): p. 572-573.
212. Molinari, M.D.C., et al., *Transcriptome analysis using RNA-Seq fromexperiments with and without biological replicates: areview.* 2021.
213. Schurch, N.J., et al., *How many biological replicates are needed in an RNA-seq experiment and which differential expression tool should you use?* Rna, 2016. **22**(6): p. 839-851.
214. Li, J., et al., *The molecular adaptive responses of skeletal muscle to high-intensity exercise/training and hypoxia.* Antioxidants, 2020. **9**(8): p. 656.
215. Timmons, J.A., *Variability in training-induced skeletal muscle adaptation.* Journal of applied physiology, 2011. **110**(3): p. 846-853.
216. !!! INVALID CITATION !!! [183].
217. Sanchez, A.M., et al., *Autophagy is essential to support skeletal muscle plasticity in response to endurance exercise.* American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, 2014. **307**(8): p. R956-R969.
218. Proud, C., *Amino acids and mTOR signalling in anabolic function.* Biochemical Society Transactions, 2007. **35**(5): p. 1187-1190.
219. Zeng, Z., et al., *Exercise-induced autophagy suppresses sarcopenia through Akt/mTOR and Akt/FoxO3a signal pathways and AMPK-mediated mitochondrial quality control.* Frontiers in Physiology, 2020. **11**: p. 583478.
220. Russell, R.C., et al., *ULK1 induces autophagy by phosphorylating Beclin-1 and activating VPS34 lipid kinase.* Nature cell biology, 2013. **15**(7): p. 741-750.
221. Mazo, C.E., et al., *The effects of acute aerobic and resistance exercise on mTOR signaling and autophagy markers in untrained human skeletal muscle.* European journal of applied physiology, 2021. **121**(10): p. 2913-2924.
222. Wang, L., et al., *Exercise-mediated regulation of autophagy in the cardiovascular system.* Journal of Sport and Health Science, 2020. **9**(3): p. 203-210.
223. Wang, P., et al., *A single bout of exhaustive treadmill exercise increased AMPK activation associated with enhanced autophagy in mice skeletal muscle.* Clinical and Experimental Pharmacology and Physiology, 2022. **49**(4): p. 536-543.
224. Sanchez, A.M., *Autophagy regulation in human skeletal muscle during exercise.* The Journal of physiology, 2016. **594**(18): p. 5053.
225. Heras-Sandoval, D., et al., *The role of PI3K/AKT/mTOR pathway in the modulation of autophagy and the clearance of protein aggregates in neurodegeneration.* Cellular signalling, 2014. **26**(12): p. 2694-2701.
226. Zhai, C., et al., *Selective inhibition of PI3K/Akt/mTOR signaling pathway regulates autophagy of macrophage and vulnerability of atherosclerotic plaque.* PloS one, 2014. **9**(3): p. e90563.
227. Chen, C., H. Gao, and X. Su, *Autophagy-related signaling pathways are involved in cancer.* Experimental and Therapeutic Medicine, 2021. **22**(1): p. 1-13.

228. Wei, H., L. Liu, and Q. Chen, *Selective removal of mitochondria via mitophagy: distinct pathways for different mitochondrial stresses.* Biochimica et Biophysica Acta (BBA)-Molecular Cell Research, 2015. **1853**(10): p. 2784-2790.
229. Aronson, D., et al., *Exercise stimulates the mitogen-activated protein kinase pathway in human skeletal muscle.* The Journal of clinical investigation, 1997. **99**(6): p. 1251-1257.
230. Seger, R. and E.G. Krebs, *The MAPK signaling cascade.* The FASEB journal, 1995. **9**(9): p. 726-735.
231. Long, Y.C., U. Widegren, and J.R. Zierath, *Exercise-induced mitogen-activated protein kinase signalling in skeletal muscle.* Proceedings of the Nutrition Society, 2004. **63**(2): p. 227-232.
232. Force, T. and J.V. Bonventre, *Growth factors and mitogen-activated protein kinases.* Hypertension, 1998. **31**(1): p. 152-161.
233. Goodyear, L., et al., *Effects of exercise and insulin on mitogen-activated protein kinase signaling pathways in rat skeletal muscle.* American Journal of Physiology-Endocrinology and Metabolism, 1996. **271**(2): p. E403-E408.
234. Ji, L.L., M.C. GOMEZ‐CABRERA, and J. Vina, *Exercise and hormesis: activation of cellular antioxidant signaling pathway.* Annals of the New York Academy of Sciences, 2006. **1067**(1): p. 425-435.
235. Roux, P.P. and J. Blenis, *ERK and p38 MAPK-activated protein kinases: a family of protein kinases with diverse biological functions.* Microbiology and molecular biology reviews, 2004. **68**(2): p. 320-344.
236. Zhang, W. and H.T. Liu, *MAPK signal pathways in the regulation of cell proliferation in mammalian cells.* Cell research, 2002. **12**(1): p. 9-18.
237. Kramer, H.F. and L.J. Goodyear, *Exercise, MAPK, and NF-κB signaling in skeletal muscle.* Journal of applied physiology, 2007. **103**(1): p. 388-395.
238. Zierath, J.R., *Invited review: exercise training-induced changes in insulin signaling in skeletal muscle.* Journal of applied physiology, 2002. **93**(2): p. 773-781.
239. Williamson, D., et al., *Mitogen‐activated protein kinase (MAPK) pathway activation: effects of age and acute exercise on human skeletal muscle.* The Journal of physiology, 2003. **547**(3): p. 977-987.
240. Gibala, M.J., et al., *Brief intense interval exercise activates AMPK and p38 MAPK signaling and increases the expression of PGC-1α in human skeletal muscle.* Journal of applied physiology, 2009. **106**(3): p. 929-934.
241. Yu, M., et al., *Marathon running increases ERK1/2 and p38 MAP kinase signalling to downstream targets in human skeletal muscle.* The Journal of physiology, 2001. **536**(1): p. 273-282.
242. Osorio-Fuentealba, C., et al., *Hypoxia stimulates via separate pathways ERK phosphorylation and NF-κB activation in skeletal muscle cells in primary culture.* Journal of Applied Physiology, 2009. **106**(4): p. 1301-1310.
243. Gallego-Selles, A., et al., *Fast regulation of the NF-κB signalling pathway in human skeletal muscle revealed by high-intensity exercise and ischaemia at exhaustion: Role of oxygenation and metabolite accumulation.* Redox Biology, 2022. **55**: p. 102398.

244. Sen, R. and D. Baltimore, *Inducibility of K immunoglobulin enhancer-binding protein NF-KB by a posttranslational mechanism.* Cell, 1986. **47**(6): p. 921-928.
245. Hayden, M.S. and S. Ghosh, *Shared principles in NF-κB signaling.* Cell, 2008. **132**(3): p. 344-362.
246. Doyle, S.L. and L.A. O'Neill, *Toll-like receptors: from the discovery of NFκB to new insights into transcriptional regulations in innate immunity.* Biochemical pharmacology, 2006. **72**(9): p. 1102-1113.
247. Remels, A., et al., *TNF-α-induced NF-κB activation stimulates skeletal muscle glycolytic metabolism through activation of HIF-1α.* Endocrinology, 2015. **156**(5): p. 1770-1781.
248. Hollander, J., et al., *Superoxide dismutase gene expression is activated by a single bout of exercise in rat skeletal muscle.* Pflügers Archiv, 2001. **442**: p. 426-434.
249. Ji, L., et al., *Acute exercise activates nuclear factor (NF)-κB signaling pathway in rat skeletal muscle.* The FASEB Journal, 2004. **18**(13): p. 1499-1506.
250. Ho, R.C., et al., *Regulation of IκB kinase and NF-κB in contracting adult rat skeletal muscle.* American Journal of Physiology-Cell Physiology, 2005. **289**(4): p. C794-C801.
251. Vella, L., et al., *Resistance exercise increases NF-κB activity in human skeletal muscle.* American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, 2012. **302**(6): p. R667-R673.
252. Tantiwong, P., et al., *NF-κB activity in muscle from obese and type 2 diabetic subjects under basal and exercise-stimulated conditions.* American Journal of Physiology-Endocrinology and Metabolism, 2010. **299**(5): p. E794-E801.
253. Oliver, K.M., et al., *Hypoxia activates NF-κB–dependent gene expression through the canonical signaling pathway.* Antioxidants & redox signaling, 2009. **11**(9): p. 2057-2064.
254. Keller, C., et al., *Exercise normalises overexpression of TNF-α in knockout mice.* Biochemical and biophysical research communications, 2004. **321**(1): p. 179-182.
255. Ameln, H., et al., *Physiological activation of hypoxia inducible factor-1 in human skeletal muscle.* The FASEB journal, 2005. **19**(8): p. 1009-1011.
256. Lundby, C., J.A. Calbet, and P. Robach, *The response of human skeletal muscle tissue to hypoxia.* Cellular and molecular life sciences, 2009. **66**: p. 3615-3623.
257. Beyfuss, K., et al., *The role of p53 in determining mitochondrial adaptations to endurance training in skeletal muscle.* Scientific reports, 2018. **8**(1): p. 14710.
258. Tachtsis, B., et al., *Acute endurance exercise induces nuclear p53 abundance in human skeletal muscle.* Frontiers in physiology, 2016. **7**: p. 144.
259. Moresi, V., S. Adamo, and L. Berghella, *The JAK/STAT pathway in skeletal muscle pathophysiology.* Frontiers in physiology, 2019. **10**: p. 500.
260. Trenerry, M.K., et al., *Impact of resistance exercise training on interleukin-6 and JAK/STAT in young men.* Muscle & nerve, 2011. **43**(3): p. 385-392.
261. Terra, R., et al., *Effect of exercise on immune system: response, adaptation and cell signaling.* Revista brasileira de medicina do esporte, 2012. **18**: p. 208-214.
262. Dang, C.V., et al. *The c-Myc target gene network*. in *Seminars in cancer biology*. 2006. Elsevier.
263. Ahmadi, S.E., et al., *MYC: a multipurpose oncogene with prognostic and therapeutic implications in blood malignancies.* Journal of hematology & oncology, 2021. **14**: p. 1-49.

264. Meyer, N. and L.Z. Penn, *Reflecting on 25 years with MYC.* Nature Reviews Cancer, 2008. **8**(12): p. 976-990.
265. Toh, P.P., et al., *Myc inhibition impairs autophagosome formation.* Human molecular genetics, 2013. **22**(25): p. 5237-5248.
266. Dey, S., F. Tameire, and C. Koumenis, *PERK-ing up autophagy during MYC-induced tumorigenesis.* Autophagy, 2013. **9**(4): p. 612-614.
267. Zhang, Y. and N. Chen, *Autophagy is a promoter for aerobic exercise performance during high altitude training.* Oxidative medicine and cellular longevity, 2018. **2018**.
268. Harvey, N.R., *Identifying the Genomic Predictors of Exercise-induced Adaptation*. 2020, Bond University.
269. Horwath, O., et al., *Acute hypoxia attenuates resistance exercise-induced ribosome signaling but does not impact satellite cell pool expansion in human skeletal muscle.* The FASEB Journal, 2023. **37**(3).
270. McLean, C.S., et al., *Gene and microRNA expression responses to exercise; relationship with insulin sensitivity.* PloS one, 2015. **10**(5): p. e0127089.
271. Ku, H.-C. and C.-F. Cheng, *Master regulator activating transcription factor 3 (ATF3) in metabolic homeostasis and cancer.* Frontiers in Endocrinology, 2020. **11**: p. 556.
272. Koltsova, S.V., et al., *Ubiquitous [Na+] i/[K+] i-sensitive transcriptome in mammalian cells: evidence for Ca2+ i-independent excitation-transcription coupling.* PLoS One, 2012. **7**(5): p. e38032.
273. Simonsen, M.L., et al., *Acute physical activity effects on cardiac gene expression.* Experimental physiology, 2010. **95**(11): p. 1071-1080.
274. Woodson, C.M. and K. Kehn-Hall, *Examining the role of EGR1 during viral infections.* Frontiers in Microbiology, 2022. **13**: p. 1020220.
275. Neubauer, O., et al., *Time course-dependent changes in the transcriptome of human skeletal muscle during recovery from endurance exercise: from inflammation to adaptive remodeling.* Journal of Applied Physiology, 2014. **116**(3): p. 274-287.
276. Rundqvist, H.C., et al., *Acute sprint exercise transcriptome in human skeletal muscle.* PLoS One, 2019. **14**(10): p. e0223024.
277. Papoudou-Bai, A., et al., *Expression patterns of the activator protein-1 (AP-1) family members in lymphoid neoplasms.* Clinical and experimental medicine, 2017. **17**: p. 291-304.
278. Baresic, M., et al., *Transcriptional network analysis in muscle reveals AP-1 as a partner of PGC-1α in the regulation of the hypoxic gene program.* Molecular and cellular biology, 2014. **34**(16): p. 2996-3012.
279. Wu, J., et al., *ATF3 and its emerging role in atherosclerosis: a narrative review.* Cardiovascular Diagnosis and Therapy, 2022. **12**(6): p. 926.
280. Koltsova, S.V., et al., *Transcriptomic changes triggered by hypoxia: Evidence for HIF-1α-independent,[Na+] i/[K+] i-mediated, excitation-transcription coupling.* PloS one, 2014. **9**(11): p. e110597.
281. Arany, Z., *PGC-1 coactivators and skeletal muscle adaptations in health and disease.* Curr Opin Genet Dev, 2008. **18**(5): p. 426-34.

282. Raue, U., et al., *Transcriptome signature of resistance exercise adaptations: mixed muscle and fiber type specific profiles in young and old adults.* Journal of applied physiology, 2012. **112**(10): p. 1625-1636.
283. Vissing, K. and P. Schjerling, *Simplified data access on human skeletal muscle transcriptome responses to differentiated exercise.* Scientific data, 2014. **1**(1): p. 1-9.
284. Baar, K., et al., *Adaptations of skeletal muscle to exercise: rapid increase in the transcriptional coactivator PGC-1.* The FASEB journal, 2002. **16**(14): p. 1879-1886.
285. Carter, H.N., et al., *Effect of contractile activity on PGC-1α transcription in young and aged skeletal muscle.* Journal of Applied Physiology, 2018. **124**(6): p. 1605-1615.
286. Little, J.P., et al., *An acute bout of high-intensity interval training increases the nuclear abundance of PGC-1α and activates mitochondrial biogenesis in human skeletal muscle.* American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, 2011.
287. Vainshtein, A., et al., *Role of PGC-1α during acute exercise-induced autophagy and mitophagy in skeletal muscle.* American Journal of Physiology-Cell Physiology, 2015. **308**(9): p. C710-C719.
288. Yun, C.W., J.H. Lee, and S.H. Lee, *Hypoxia-induced PGC-1α regulates mitochondrial function and tumorigenesis of colorectal cancer cells.* Anticancer research, 2019. **39**(9): p. 4865-4876.
289. Nava, R.C., et al., *Repeated sprint exercise in hypoxia stimulates HIF-1-dependent gene expression in skeletal muscle.* European Journal of Applied Physiology, 2022. **122**(4): p. 1097-1107.
290. Granata, C., et al., *Forty high-intensity interval training sessions blunt exercise-induced changes in the nuclear protein content of PGC-1α and p53 in human skeletal muscle.* American Journal of Physiology-Endocrinology and Metabolism, 2020. **318**(2): p. E224-E236.
291. Bartlett, J.D., et al., *Matched work high-intensity interval and continuous running induce similar increases in PGC-1α mRNA, AMPK, p38, and p53 phosphorylation in human skeletal muscle.* Journal of applied physiology, 2012. **112**(7): p. 1135-1143.
292. Pilegaard, H., B. Saltin, and P.D. Neufer, *Exercise induces transient transcriptional activation of the PGC-1α gene in human skeletal muscle*. 2003, Wiley Online Library.
293. Terada, S., et al., *Effects of high-intensity intermittent swimming on PGC-1α protein expression in rat skeletal muscle.* Acta physiologica scandinavica, 2005. **184**(1): p. 59-65.
294. De Filippis, E., et al., *Insulin-resistant muscle is exercise resistant: evidence for reduced response of nuclear-encoded mitochondrial genes to exercise.* American Journal of Physiology-Endocrinology and Metabolism, 2008. **294**(3): p. E607-E614.
295. Tripp, T.R., et al., *Time course and fibre type-dependent nature of calcium-handling protein responses to sprint interval exercise in human skeletal muscle.* The Journal of Physiology, 2022. **600**(12): p. 2897-2917.
296. Chen, M., et al., *Mitophagy receptor FUNDC1 regulates mitochondrial dynamics and mitophagy.* Autophagy, 2016. **12**(4): p. 689-702.

297. Li, G., et al., *FUNDC1: a promising mitophagy regulator at the mitochondria-associated membrane for cardiovascular diseases.* Frontiers in cell and developmental biology, 2021. **9**: p. 788634.
298. Liu, L., et al., *Mitochondrial outer-membrane protein FUNDC1 mediates hypoxia-induced mitophagy in mammalian cells.* Nat Cell Biol, 2012. **14**(2): p. 177-85.
299. Zhang, J. and P.A. Ney, *Role of BNIP3 and NIX in cell death, autophagy, and mitophagy.* Cell Death Differ, 2009. **16**(7): p. 939-46.
300. Novak, I. and I. Dikic, *Autophagy receptors in developmental clearance of mitochondria.* Autophagy, 2011. **7**(3): p. 301-303.
301. Sandoval, H., et al., *Essential role for Nix in autophagic maturation of erythroid cells.* Nature, 2008. **454**(7201): p. 232-235.
302. Bellot, G., et al., *Hypoxia-induced autophagy is mediated through hypoxia-inducible factor induction of BNIP3 and BNIP3L via their BH3 domains.* Molecular and cellular biology, 2009. **29**(10): p. 2570-2581.
303. Lee, Y., et al., *Potential signaling pathways of acute endurance exercise-induced cardiac autophagy and mitophagy and its possible role in cardioprotection.* The journal of physiological sciences, 2017. **67**(6): p. 639-654.
304. Li, H., et al., *Acute exercise-induced mitochondrial stress triggers an inflammatory response in the myocardium via NLRP3 inflammasome activation with mitophagy.* Oxidative Medicine and Cellular Longevity, 2016. **2016**.
305. Menghini, R., et al., *MiR-216a: a link between endothelial dysfunction and autophagy.* Cell death & disease, 2014. **5**(1): p. e1029-e1029.
306. Liu, H., et al., *From autophagy to mitophagy: the roles of P62 in neurodegenerative diseases.* Journal of bioenergetics and biomembranes, 2017. **49**: p. 413-422.
307. Feng, Y., et al., *The machinery of macroautophagy.* Cell research, 2014. **24**(1): p. 24-41.
308. Schwalm, C., L. Deldicque, and M. Francaux, *Lack of activation of mitophagy during endurance exercise in human.* Medicine & Science in Sports & Exercise, 2017. **49**(8): p. 1552-1561.
309. 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.
310. Pillon, N.J., et al., *Transcriptomic profiling of skeletal muscle adaptations to exercise and inactivity.* Nature communications, 2020. **11**(1): p. 470.
311. Hofmann, P. and G. Tschakert, *Special needs to prescribe exercise intensity for scientific studies.* Cardiology research and practice, 2011. **2011**.
312. Tschakert, G. and P. Hofmann, *High-intensity intermittent exercise: methodological and physiological aspects.* International journal of sports physiology and performance, 2013. **8**(6): p. 600-610.
313. Maturana, F.M., et al., *Effectiveness of HIIE versus MICT in improving cardiometabolic risk factors in health and disease: a meta-analysis.* Medicine & Science in Sports & Exercise, 2021. **53**(3): p. 559-573.
314. Ito, S., *High-intensity interval training for health benefits and care of cardiac diseases-the key to an efficient exercise protocol.* World journal of cardiology, 2019. **11**(7): p. 171.

315. Teles, G.d.O., et al., *HIIE Protocols promote better acute effects on blood glucose and pressure control in people with type 2 diabetes than continuous exercise.* International Journal of Environmental Research and Public Health, 2022. **19**(5): p. 2601.
316. Egan, B., J.A. Hawley, and J.R. Zierath, *SnapShot: exercise metabolism.* Cell Metabolism, 2016. **24**(2): p. 342-342. e1.
317. Chakraborty, J., et al., *Investigating mitochondrial autophagy by routine transmission electron microscopy: Seeing is believing?* Pharmacological Research, 2020. **160**: p. 105097.
318. Jung, M., et al., *Correlative light and transmission electron microscopy showed details of mitophagy by mitochondria quality control in propionic acid treated SH-SY5Y cell.* Materials, 2020. **13**(19): p. 4336.
319. Collins, H.E., et al., *Mitochondrial morphology and mitophagy in heart diseases: qualitative and quantitative analyses using transmission electron microscopy.* Frontiers in Aging, 2021. **2**: p. 670267.
320. Tanida, I., T. Ueno, and E. Kominami, *LC3 and Autophagy.* Autophagosome and phagosome, 2008: p. 77-88.
321. Dara, M., et al., *Dystrophin gene editing by CRISPR/Cas9 system in human skeletal muscle cell line (HSkMC).* Iranian Journal of Basic Medical Sciences, 2021. **24**(8): p. 1153.
322. Poyatos-García, J., et al., *CRISPR-Cas9 editing of a TNPO3 mutation in a muscle cell model of limb-girdle muscular dystrophy type D2.* Molecular Therapy-Nucleic Acids, 2023. **31**: p. 324-338.
323. Matre, P.R., et al., *CRISPR/Cas9-based dystrophin restoration reveals a novel role for dystrophin in bioenergetics and stress resistance of muscle progenitors.* Stem Cells, 2019. **37**(12): p. 1615-1628.
324. Wang, L., et al., *CRISPR/Cas9-mediated MSTN gene editing induced mitochondrial alterations in C2C12 myoblast cells.* Electronic Journal of Biotechnology, 2019. **40**: p. 30-39.
325. Costello, J.T., F. Bieuzen, and C.M. Bleakley, *Where are all the female participants in sports and exercise medicine research?* European journal of sport science, 2014. **14**(8): p. 847-851.
326. Triolo, M., et al., *The influence of age, sex, and exercise on autophagy, mitophagy, and lysosome biogenesis in skeletal muscle.* Skeletal muscle, 2022. **12**(1): p. 13.
327. Montero, D., et al., *Sexual dimorphism of substrate utilization: differences in skeletal muscle mitochondrial volume density and function.* Experimental physiology, 2018. **103**(6): p. 851-859.
328. Maher, A.C., et al., *Sex differences in global mRNA content of human skeletal muscle.* PLoS One, 2009. **4**(7): p. e6335.

APPENDIX

1 - **RNA assessment**

2 - **KEGG functional enrichment table – HYB_P24H_UP**

Table1.RNA assessment

	RNA Concentration (ng/µl)	A260/280	A260/230	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

Table2. KEGG functional enrichment table – HYB_P24H_UP

ID	Description	Gene Ratio	Bg Ratio	pvalue	qvalue	Count
hsa03008	Ribosome biogenesis in eukaryotes	22/409	119/8219	0.000	0.000	22
hsa05132	Salmonella infection	31/409	249/8219	0.000	0.000	31
hsa05220	Chronic myeloid leukemia	15/409	76/8219	0.000	0.000	15
hsa05213	Endometrial cancer	13/409	58/8219	0.000	0.000	13
hsa05130	Pathogenic Escherichia coli infection	26/409	197/8219	0.000	0.000	26
hsa04210	Apoptosis	20/409	136/8219	0.000	0.000	20
hsa04064	NF-kappa B signaling pathway	17/409	104/8219	0.000	0.000	17
hsa04066	HIF-1 signaling pathway	17/409	109/8219	0.000	0.001	17
hsa05222	Small cell lung cancer	15/409	92/8219	0.000	0.001	15
hsa04115	p53 signaling pathway	13/409	73/8219	0.000	0.001	13
hsa05216	Thyroid cancer	9/409	37/8219	0.000	0.001	9
hsa05417	Lipid and atherosclerosis	25/409	215/8219	0.000	0.001	25
hsa05160	Hepatitis C	20/409	157/8219	0.000	0.002	20
hsa04141	Protein processing in endoplasmic reticulum	21/409	171/8219	0.000	0.002	21
hsa04933	AGE-RAGE signaling pathway in diabetic complications	15/409	100/8219	0.000	0.002	15
hsa05223	Non-small cell lung cancer	12/409	72/8219	0.000	0.003	12
hsa05215	Prostate cancer	14/409	97/8219	0.000	0.004	14
hsa05210	Colorectal cancer	13/409	86/8219	0.000	0.004	13
hsa05212	Pancreatic cancer	12/409	76/8219	0.000	0.004	12
hsa05221	Acute myeloid leukemia	11/409	67/8219	0.000	0.005	11
hsa04630	JAK-STAT signaling pathway	19/409	166/8219	0.001	0.006	19
hsa05167	Kaposi sarcoma-associated herpesvirus infection	21/409	194/8219	0.001	0.006	21
hsa04722	Neurotrophin signaling pathway	15/409	119/8219	0.001	0.007	15
hsa05219	Bladder cancer	8/409	41/8219	0.001	0.007	8
hsa04540	Gap junction	12/409	88/8219	0.001	0.012	12
hsa04010	MAPK signaling pathway	27/409	294/8219	0.001	0.012	27
hsa04668	TNF signaling pathway	14/409	114/8219	0.002	0.012	14
hsa04218	Cellular senescence	17/409	156/8219	0.002	0.015	17
hsa04917	Prolactin signaling pathway	10/409	70/8219	0.002	0.017	10

hsa04810	Regulation of actin cytoskeleton	22/409	229/8219	0.002	0.017	22
hsa05169	Epstein-Barr virus infection	20/409	202/8219	0.002	0.018	20
hsa04978	Mineral absorption	9/409	60/8219	0.003	0.018	9
hsa04137	Mitophagy - animal	10/409	72/8219	0.003	0.018	10
hsa05218	Melanoma	10/409	72/8219	0.003	0.018	10
hsa05161	Hepatitis B	17/409	162/8219	0.003	0.018	17
hsa01232	Nucleotide metabolism	11/409	85/8219	0.003	0.019	11
hsa05166	Human T-cell leukemia virus 1 infection	21/409	222/8219	0.003	0.021	21
hsa04216	Ferroptosis	7/409	41/8219	0.004	0.022	7
hsa05214	Glioma	10/409	75/8219	0.004	0.022	10
hsa04140	Autophagy - animal	15/409	141/8219	0.004	0.024	15
hsa04625	C-type lectin receptor signaling pathway	12/409	104/8219	0.005	0.029	12
hsa04071	Sphingolipid signaling pathway	13/409	119/8219	0.006	0.032	13
hsa05226	Gastric cancer	15/409	149/8219	0.007	0.037	15