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DNA methylation of exercise-responsive genes and PGC1α splice variant expression differs between trained and untrained men

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To my grandmother Hannelotte

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3 List of Abbreviations

AMPK α2	Protein Kinase AMP-Activated Catalytic Subunit Alpha 2
ATF2	Activating Transcription Factor 2
ATP	Adenosine triphosphate
ADP	Adenosine diphosphate
bp	Base pairs
BTM	Beta-2-Microglobulin

C°	Degree Celsius
Ca2+	Calcium
CaMK	Ca2+-calmodulin-dependent family of protein kinases
CAMKII	Ca2+-calmodulin-dependent kinase II
CD144	Vascular Endothelial Cadherin
CFD	Complement Factor D
CG	Control group
CH_3	Methyl group
cm	Centimeter
CpG	Cytosine-phosphate-Guanine
CREB	cAMP response element-binding protein
CREB1	cAMP response element-binding protein 1
CREB5	cAMP response element-binding protein 5
CS	Citrate synthase
DCN	Decorin
DMPs	Differentially methylated positions
DMRs	Differentially methylated regions
DNA	Deoxyribonucleic acid
DNMTs	DNA methyltransferase enzymes
EE	Acute endurance exercise
EG	Endurance group
ERRα	Estrogen Related Receptor Alpha
ESAM	Endothelial Cell Adhesion Molecule
FDR	False Discovery Rate
FOXO	Forkhead box O
FOXO1	Forkhead box O1
FOXO3	Forkhead box O3
GLUT4	Glucose Transporter Type 4, Insulin-Responsive
GSN	Gelsolin
h	Hour
HIF1α	Hypoxia-inducible factor 1-alpha
HIIT	high-intensity interval training
ICAM1	Intercellular Adhesion Molecule 1
IQR	Interquartile range
KDR	Kinase Insert Domain Receptor
kg	Kilogram
MAPKs	Mitogen-activated protein kinases
MEF2A	Myocyte Enhancer Factor 2A
Mg	Milligram
MHC	Myosin Heavy Chain
min	Minutes `
ml	Millilitre
MRFs	Myogenic regulatory factors
mRNA	Messenger RNA
MSTN	Myostatin
MTOR	Mechanistic target of rapamycin
MYH7	Myosin Heavy Chain 7

MYH6	Myosin Heavy Chain 6
MYF5	Myogenic factor 5
MYF6	Myogenic factor 6
MYL3	Myosin Light Chain 3
MYOD1	Myogenic differentiation 1
MYOG	Myogenin
NAD+	Nicotinamide adenine dinucleotide
NDUFA4L2	NDUFA4 Mitochondrial Complex Associated Like 2
Nm	Newton meter
nm	Nanometer
nontruncPGC1a	Nontruncated/Full-length PGC-1α transcripts
OD	Optical density
PAX7	Paired Box 7
PCA	Principal component analysis
PCs	Principal components
PCR	Polymerase chain reaction
PDK4	Pyruvate Dehydrogenase Kinase 4
PECAM-1	Platelet And Endothelial Cell Adhesion Molecule 1
PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PPARGC1A	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PGC-1α -ex1a	PGC-1α proximal promoter transcripts
PGC-1α -ex1b	PGC-1α alternative promoter transcripts
PPAI	Cyclophilin A
QC	Quality control
q-PCR	Real-time quantitative polymerase chain reaction
RE	Acute resistance exercise
RNA	Ribonucleic acid
s	Second
SD	Standard deviation
SG	Strength group
SMAD3	Mothers against decapentaplegic homolog 3
TBP	TATA-Box Binding Protein
TDG	Thymine DNA glycosylase
TEK	TEK Receptor Tyrosine Kinase
TET	Ten-eleven translocation
TFAM	Transcription Factor A
TGFbeta	Transforming Growth Factor Beta
TPM	Transcripts per kilobase million
trunc-PGC-1α	Truncated PGC-1α transcripts
TSS	Transcription start sites
VO_2peak	Peak oxygen uptake
VWF	Von Willebrand Factor
xg	Times gravity
µl	Microliter
5-caC	5-carboxylcytosine
5-mC	5-methylcytosine
5-hmC	5-hydroxymethylcytosine

4 Introduction

Physical activity greatly benefits overall health and reduces the risk of premature death (Booth et al. 2012). According to the World Health Organisation, physical activity includes any skeletal muscle movement that involves energy expenditure, such as activities undertaken while working, playing, travelling, and recreational activities (World Health Organization 5 October 2022). Physical activity improves muscular and cardiorespiratory fitness, bone quality, and enhances functional health. In addition, regular exercise can lower the risk of various diseases such as hypertension, coronary heart disease, stroke, diabetes, several types of cancer, and depression. It also plays a role in preventing falls and reducing the incidence of hip or vertebral fractures while keeping a healthy body weight, acting as a countermeasure of disease and frailty (Egan and Zierath 2013; World Health Organization 5 October 2022).
Public health guidelines emphasise the crucial role of physical activity in preventing, managing and treating a variety of chronic illnesses (World Health Organization 5 October 2022). For instance, in individuals with type 2 diabetes, short-term exercise training can significantly improve insulin-mediated glucose disposal, partially reversing the disease (O'Gorman et al. 2006). Lifestyle changes, including exercise and dietary modifications, have been found to be more effective in reducing the risk of developing diabetes in high-risk individuals compared to pharmacological interventions (Knowler et al. 2002). Physical activity can lower blood pressure, as regular aerobic exercise is strongly recommended as a treatment for patients with hypertension or high-normal blood pressure (Cornelissen and Smart 2013; Williams et al. 2018). Exercise has also been linked to strengthening the immune system, and studies suggest that it can improve immune responses to vaccination (Nieman and Wentz 2019; Pascoe et al. 2014).

Skeletal muscle, known for its remarkable plasticity, can quickly adapt to the varying demands of exercise. However, the complexity of molecular regulation in these adaptations is extensive, and many aspects are yet to be fully understood.

Human development, growth, function, disease, and adaptation are governed by our genome, a unique hardware established at the moment of conception with the fusion of maternal and paternal genetic information. Although the genomic sequence is essentially identical across all of our bodies cells, there's a significant diversity in cell types in the human body. Cell structure and function is dictated by differences in gene expression which also allows cells to respond uniquely to environmental stimuli.

The effect of exercise training on muscle is mediated by the regulation of gene expression. Understanding how the genome operates in skeletal muscle and how its activity is modified during exercise is important, not just from a basic science perpective but also for clinical applications.

4.1 Introduction to skeletal muscle physiology and different exercise types

4.1.1 Skeletal muscle structure and function

Skeletal muscle facilitates all voluntary movements of our body and is therefore often the center of focus in exercise physiology research. Skeletal muscle is an abundant tissue in

healthy humans making up to 40% of total body mass depending on age, sex and size (Janssen et al. 2000). Skeletal muscle cells, also known as muscle fibers, are large, multinucleated cells that form the basic units of skeletal muscle. These muscle fibers are grouped together into bundles called fascicles. Several of these fascicles are then bundled together to make up a skeletal muscle. Inside each muscle cell are long filamentous organelles called myofibrils, which are made up of repeating units called sarcomeres. Sarcomeres are the smallest functional unit of the muscle and contain the two main myofilaments, actin and myosin, which produce mechanical force (Furrer et al. 2023a; Kraemer et al. 2012).

Muscle contraction involves the interaction of actin and myosin filaments within the muscle cells, driven by the energy released from adenosine triphosphate (ATP) hydrolysis. This process enables the myosin heads to form crossbridges with actin, facilitating muscle contraction. Simultaneously, ATP is crucial for maintaining the ion balance necessary for muscle function and powering the sodium-potassium and calcium exchanges essential for muscle contraction and relaxation.

Transitioning from a state of rest to full activity can result in an energy requirement increase of more than 100 times in muscle tissue. In the initial stages of intense, short-duration exercise, the primary energy source for muscle contraction is the direct hydrolysis of ATP. Simultaneously, creatine phosphate contributes to energy production by donating its phosphate group to regenerate ATP. For activities lasting up to about 90 seconds, most of the ATP is supplied through anaerobic lactate production. During longer bouts of exercise, ATP production predominantly occurs in mitochondria. ATP, broken down into adenosine diphosphate (ADP) and a free phosphate molecule, provides energy for almost all chemical reactions of the body. Aerobic ATP production in muscles relies on a steady supply of oxygen and nutrients, such as glucose and fatty acids, delivered via the circulatory system (Furrer et al. 2023a; Kraemer et al. 2012).

In humans, skeletal muscle is composed of three major fiber types: type I, type IIA, and type IIX (Murgia et al. 2021). Those fiber types differ in their performance capabilities based on variations in contractile speed, endurance, and metabolic pathways. Type I fibers, or slow-twitch fibers, excel in endurance and are efficient at using oxygen for long, sustained activities. In contrast type II fibers (type IIA and IIX), are fast-twitch fibers that provide greater power and strength but fatigue more quickly, as they are reliant on anaerobic metabolism. The physical appearance of these fibers also varies: type I fibers generally have a red hue, type IIX are white, and type IIA fibers display an intermediate colour. This colour variation is due to the presence of myoglobin, an oxygen-transporting protein, which correlates with mitochondrial content and the degree of oxidative metabolism, both most prominent in type I fibers. The expression of myosin heavy chain (MHC) isoforms is a key determinant of muscle fiber type, with each isoform corresponding to distinct contractile and energy turnover properties (Egan and Zierath 2013). In slow-twitch fibers, the myosin complex primarily comprises *MYH7* (Myosin Heavy Chain 7), *MYH6* (Myosin Heavy Chain 6), and *MYL3* (Myosin Light Chain 3) (Stuart et al. 2016). Conversely, fast type IIa fibers are typically characterised by the presence of *MYH2* (Myosin Heavy Chain 2) (Murgia et al. 2021). While the common understanding is that of the three major fibre types, studies suggest that muscle fiber composition exists more on a spectrum rather than as strictly separate types. While certain proteins are highly enriched in specific fiber types, others display a gradient of expression across fiber types and fibers can contain multiple myosin heavy chain isoforms making them to hybrid fibers (Medler 2019; Murgia et al. 2021; Plotkin et al. 2021).

4.1.2 Exercise modalities

Exercise is as a disruptor of skeletal muscle homeostasis, challenging the body's equilibrium. Exercise can be performed as different modalities and vary greatly using different frequencies, intensities, and durations. These factors collectively influence the muscle's metabolic and molecular responses to activity. The two ends of the exercise spectrum are aerobic (endurance-based) exercise, characterised by prolonged, repetitive, low-resistance movements, and resistance (strength-based) exercise which involves shorter durations of high-load activities. During, and after exercise, systemic, cellular and molecular mechanisms are activated to supply working muscles with oxygen and nutrients, remove waste products, and preserve essential functions despite disturbances to homeostasis (Egan and Sharples 2023; Hargreaves 2015).

The specific molecular responses depending on exercise modality are linked to functional outcomes and physiological adaptation (Egan and Zierath 2013). For example, aerobic exercise is more effective in modifying cardiovascular risk factors and resistance training is better at maintaining muscle mass, physical function in the elderly and basal metabolic rate. Cellular homeostasis is transiently disrupted during a single bout of exercise, initiating an acute molecular response. With repeated sessions, this response transitions from an unspecific reaction to a more specific adaptation (Reitzner et al. 2023). Over time, these repeated muscle contractions lead to cumulative effects that gradually alter and adapt muscle physiology and structure, enhancing endurance, strength, and efficiency of the muscular system while promoting overall body health (Egan and Sharples 2023; Hargreaves 2015; Hawley 2002).

4.2 Adaptations to exercise

4.2.1 Cellular response to acute exercise and training

The multifaceted process of exercise adaptation is rooted in the communication of homeostatic disturbances within and among cell types. This communication occurs through sophisticated intracellular and neuroendocrine signaling pathways, including the sympathetic neural circuitry and neuroendocrine systems. These systems coordinate whole-body adjustments in respiration, blood flow, fuel selection, and thermoregulation to preserve and/or re-establish homeostasis (Egan and Sharples 2023; Hargreaves 2015; Neufer et al. 2015). Autocrine, paracrine, and endocrine factors facilitate crosstalk between cells, triggering the mobilisation of various progenitor cells and activation of intracellular pathways that contribute to tissue regeneration and recovery. Skeletal muscles and adipose tissue for example can act as endocrine organs, secreting peptides and metabolic intermediates that regulate numerous body functions (Egan and Sharples 2023; Neufer et al. 2015; Severinsen and Pedersen 2020). Exercise training modifies the structure and efficiency of contractile proteins, enhancing muscle strength and power. Mitochondria become more efficient at producing energy, thereby increasing endurance and resistance to fatigue during prolonged activity. Metabolic pathways within muscle cells are fine-tuned to improve energy delivery and utilisation during exercise (Egan and Sharples 2023; Egan and Zierath 2013).

Cellular adaptations to exercise manifest distinctly between endurance and resistance modalities, reflecting the diverse demands these activities place on the body. However, these adaptations are not mutually exclusive, with each modality inducing certain changes that can also be observed, albeit often to a lesser extent, in the other (Hawley et al. 2014; Wang et al. 2011). Endurance exercise enhances mitochondrial density and oxidative function, leading to improved aerobic capacity and endurance (Egan and Zierath 2013; Holloszy 1967; Hughes et al. 2018). There's also an increase in capillarization, which facilitates better oxygen and nutrient delivery as well as waste removal from muscles (Egan and Zierath 2013; Olfert et al. 2016). Endurance athletes typically experience increased lactate tolerance and improved lipid and carbohydrate metabolism. The transport capacity of glucose and fatty acids increases, and they are more abundantly stored in the muscle (Egan and Zierath 2013; Holloszy and Coyle 1984). Resistance exercise promotes adaptations that are particularly beneficial for increasing muscle hypertrophy, strength, power, and anaerobic capacity. This form of training is marked by significant increases in skeletal muscle mass, muscle fiber size and myofibrillar protein synthesis, enhancing basal metabolic rate. Neural adaptations are also prominent, leading to improvements in muscle coordination and strength gains (Egan and Zierath 2013; Hughes et al. 2018). These changes contribute to better performance in activities requiring high-intensity effort and are particularly effective in maintaining physical function and muscle mass in the elderly (Mayer et al. 2011). The degree to which muscle fiber types can transition from one form to another—such as fast-twitch to slow-twitch fibers—is still a subject of debate (Booth et al. 2010; Plotkin et al. 2021). However, in skeletal muscles, great plasticity allows for significant metabolic and morphological changes within fiber types, even if a complete transformation of muscle fiber type may not occur (Egan and Zierath 2013; Plotkin et al. 2021). Interestingly, when combined, aerobic and resistance training can lead to synergistic health benefits. For example, the combination of aerobic and resistance training has been shown to be more effective in reducing insulin resistance and functional limitations associated with obesity and metabolic syndrome (Al-Mhanna et al. 2024; Davidson et al. 2009; Egan and Zierath 2013; Sigal et al. 2007).

4.2.2 Molecular response to acute exercise and training

The translation of acute responses to exercise into long-term training adaptations and health benefits is driven by changes in mRNA abundance leading to differing protein dynamics. The processes not only involve changes in the abundance of proteins governing metabolic and physiological changes but also their activity and stability (Hargreaves 2015; Perry et al. 2010). The adaptations following exercise-induced biochemical and biophysical stimuli perturbate homeostasis and lead to the activation of networks of signalling molecules and downstream cascades that regulate pre- and post-transcriptional and translational processes (Figure 1).

Various signals contribute to and initiate this complex network of responses. These include mechanical forces, changes in calcium (Ca^{2+}) concentration, cellular energy status, levels of creatine phosphate and glycogen, amino acid availability, oxidation-reduction balance, tissue oxygenation, muscle temperature, nicotinamide adenine dinucleotide (NAD^{+}) levels, and hormonal signal fluctuations (Egan and Sharples 2023; Hargreaves 2015). A complex interplay of downstream signalling cascades follows the exercise-induced activation of sensor proteins and receptors.

This leads to alterations in gene expression within skeletal muscle which play a crucial role in facilitating adaptations to exercise (Chapman et al. 2020; Egan and Zierath 2013; Neufer et al. 2015; Pillon et al. 2020). A single bout of acute exercise causes temporary changes in the muscle's gene expression profiles (Amar et al. 2021; Norrbom et al. 2022; Perry et al. 2010; Pillon et al. 2020). The acute response to exercise, in exercise naïve individuals, is a general stress response. However, over time, these acute responses become more specific to the stimulus in which they respond to (Thomas et al. 2024). The gene expression profiles of skeletal muscles in trained individuals differ significantly from those in untrained individuals, even when at rest (Chapman et al. 2020; Stepto et al. 2009). This difference is especially pronounced in individuals who have undergone endurance training (Chapman et al. 2020; Reitzner et al. 2023).

The response to exercise involves changes in transcription of key regulatory, metabolic, and myogenic genes, which mediate subsequent adaptations in skeletal muscle. This thesis focuses on selected genes important in the exercise response and adaptation process, which were shown to be highly exercise-affected in gene expression analysis (Reitzner et al. 2023):

A decoder of changing Ca2+ levels in the cell is, for example the Ca^{2+}-calmodulin-dependent family of protein kinases (*CaMK*) that regulate downstream transcriptional pathways, such as the Ca^{2+}-calmodulin-dependent kinase II (*CAMKII*) (Rose et al. 2006). Another class of kinases involved in mediating signalling cascades as a part of the exercise response are mitogen-activated protein kinases (*MAPKs*), activated by osmotic, oxidative, and mechanical stress (Aronson et al. 1997). Local hypoxia in muscle during exercise stabilises hypoxia-inducible factor 1-alpha (*HIF1α*), a transcription factor that in turn regulates genes important for angiogenesis, erythropoiesis and glycolytic energy metabolism (Egan and Sharples 2023). An important regulator of protein synthesis and myofiber growth is the protein kinase mammalian target of rapamycin (*MTOR*) (Watson and Baar 2014). Myogenesis involves the interplay of transcription factors such as the myogenic regulatory factors (MRFs: *MYF5*, *MYOD1*, *MYOG*, and *MYF6*/*MRF4*) and downstream acting transcriptional regulators like myocyte enhancer factor (*MEF2A*) (Asfour et al. 2018; Schiaffino et al. 2018; Zammit 2017). Members of the Forkhead box class O (FoxOs) protein family *FOXO1* and *FOXO3* are important in preserving muscle energy homeostasis and regulating protein breakdown (Sanchez et al. 2014). Activation of cAMP response element-binding proteins (*CREBs*) regulates gene expression, contributing to metabolic efficiency and muscle growth (Berdeaux and Hutchins 2019). Another exercise-responsive protein involved in balancing protein turnover and muscle mass is *SMAD3* (Sartori et al. 2009).

Lastly, an important regulator in exercise adaptation to be noted is peroxisome proliferator-activated receptor gamma coactivator 1-alpha (*PGC1α*). *PGC1α* expression is highly induced by exercise in skeletal muscle and seems to play a central role in the adaptational process to training by mediating expression of genes involved in mitochondrial biogenesis and energy metabolism but also angiogenesis, neuromuscular junction remodelling and muscle fiber type (Jannig et al. 2022; Martínez-Redondo et al. 2015). Since a specific focus of this thesis is on *PGC1α* it shall be explained in more detail: *PGC1α* is a protein that increases transcription as a transcriptional coactivator. Coactivators don't bind directly to DNA but elicit their effect by interacting with transcription factors. While transcription factors identify the genes to be up- or down-regulated in a sequence-specific manner, the coactivator *PGC1α* influences the rate of transcription. The *PPARGC1A* gene on chromosome 4 encodes for the protein *PGC1α* (Esterbauer et al. 1999). The existence of two promoters in skeletal muscle, the proximal

(exon 1a) and the alternative promotor (exon 1b), coupled with alternative splicing results in several *PGC1α* variants. As primary transcripts, a full-length and a truncated isoform originating from each the proximal and the alternative promoter can be distinguished. The proximal promoter expresses *PGC1α* as the full-length (*PGC1α-α* or *PGC1α-1*) (Arany 2008; Chinsomboon et al. 2009) and the truncated (*NT-PGC1α*) (Zhang et al. 2009) mRNA constitutive at rest and after exercise whereas the alternative promoter plays a dominant role in the exercise-induced expression of *PGC1α* and is inactive at rest. The activation of the alternative promoter produces full-length (*PGC1α-b*) and truncated (*PGC1α-4*) mRNA (Norrbom et al. 2011; Popov et al. 2014; Popov et al. 2015; Ruas et al. 2012; Silvennoinen et al. 2015). Over the last decade, *PGC1α* has been a centrepiece of exercise physiology research revealing its remarkable involvement in many cellular processes. However, the expression patterns of the different isoforms in response to exercise and training are not yet fully understood.

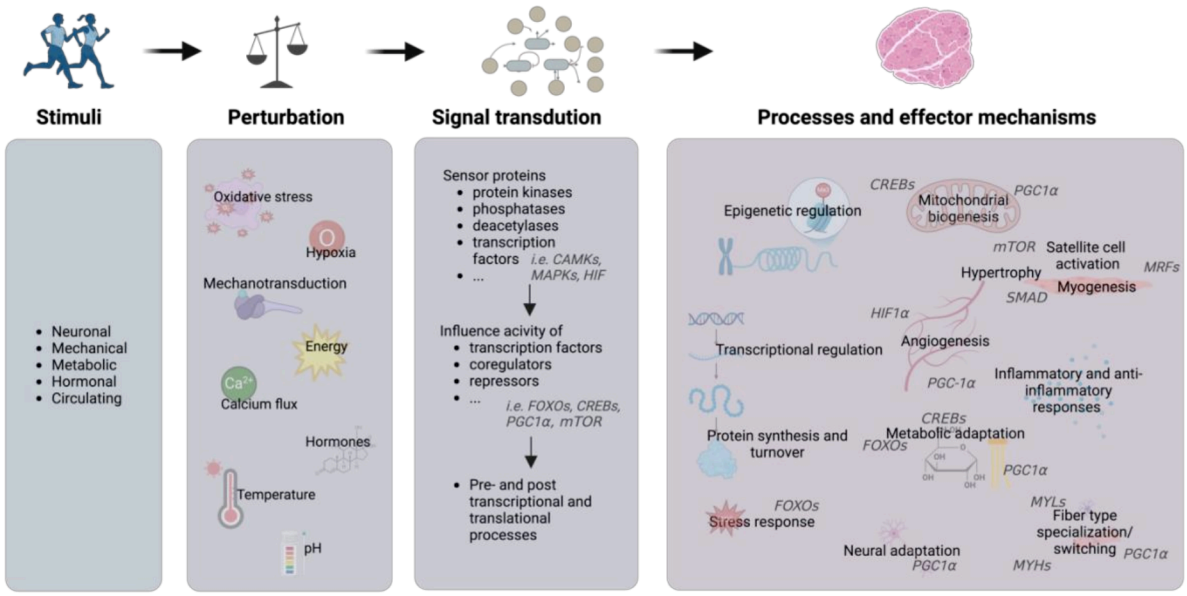

Figure 1: Molecular response to exercise: Various exercise-induced stimuli trigger skeletal muscle adaptation processes. Pertubation of homeostasis activates signal transduction pathways which influence gene expression and protein translation. The culmination of these signaling events results in diverse processes and effector mechanisms. CAMKs, Ca2+-calmodulin-dependent family of protein kinases; MAPKs, Mitogen-activated protein kinases; HIF, Hypoxia-inducible factor 1; FOXOs, Forkhead box O; CREBs, cAMP response element-binding proteins; PGC1α, Peroxisome proliferator-activated receptor gamma coactivator 1-alpha; mTOR, Mechanistic target of rapamycin; MRFs, Myogenic regulatory factors; SMAD, Mothers against decapentaplegic homolog, MYLs, Myosin Light Chains; MYHs, Myosin Heavy Chains. Created with BioRender.com.

4.3 Introduction to Epigenetics in the Context of Exercise

4.3.1 The human genome and epigenetics

The human genome consists of about 3 billion base pairs which hold the information for approximately 20,000 to 25,000 protein-coding genes (Clamp et al. 2007; International Human Genome Sequencing 2004). The double-helical DNA is coiled around histone proteins.

Those individual units are known as nucleosomes and compact together to form chromatin, which is organised into 22 pairs of autosomes and two sex chromosomes (Alberts et al. 2002). As explained by the central dogma of biology, the genetic information in DNA is transcribed into RNA, and this RNA is then translated into proteins (Crick 1970). The interaction between transcription factors and RNA polymerase at the promoter region of a gene initiates transcription. During transcription a messenger RNA (mRNA) template is produced. mRNA carries the genetic code from the DNA for protein biosynthesis during translation. However, the number of proteins that can be produced exceeds the number of genes we have. This phenomenon can be explained by alternative splicing, a mechanism that allows to produce different gene products from the same gene by combining various parts of exons (the coding regions of a gene) to form mRNA or by initiating transcription from alternative promoters (Ayoubi and Van De Ven 1996; Pan et al. 2008). Another mechanism contributing to the diversity of protein abundance is posttranslational modifications. Here, already synthesised proteins are altered by for example adding chemical groups or cleaving them. This diversifies protein functions beyond what is encoded by the original genetic code.

All cells of an individual contain the same genomic sequence, yet cells can differ greatly in appearance and function. The structure and function of a cell, including its response to environmental triggers, are largely influenced by the activation and inactivation of genes. A significant contributor to differential gene expression are epigenetic mechanisms which can regulate the chromatin structure and thereby accessibility to the DNA. Epigenetics, a term derived from the Greek 'epi', meaning 'above', and 'genetics', refers to the study of an important level of gene expression regulation above the genetic code that do not involve alterations in the DNA sequence (Bird 2007). Bird proposed in 2007 the following defintion of epigenetics: “the structural adaptation of chromosomal regions so as to register, signal or perpetuate altered activity states”(Bird 2007). Epigenetics describes mechanisms that can switch genes on or off. Without modifying the underlying genetic code, epigenetics can determine the extent to which genes are expressed. Epigenetic marks are heritable and enable the cell to remember a previous event. While epigenetic regulation encompasses various mechanisms, including histone modifications and non-coding RNAs, this thesis primarily focuses on DNA methylation, a key epigenetic process.

4.3.2 DNA methylation

DNA methylation is a reversible epigenetic mechanism that can be mitotically inherited. It involves the addition of a methyl group (CH_3) primarily to position 5 of a cytosine (C) base within CG dinucleotides (referred to as Cytosine-phosphate-Guanine, CpG) in the genome, catalysed by DNA methyltransferase enzymes (DNMTs). Methylated Cs are referred to as 5-methylcytosine or 5-mC. Not only cytosines can be methylated but also other sequences which is then referred to as non-CpG methylation (Lister et al. 2009). The function of non-CpG methylation is currently not well understood. DNA demethylation can occur passively through the loss of methyl groups during DNA replication or actively via enzymes such as the ten-eleven translocation (TET) enzymes, which convert 5-methylcytosine (5-mC) to 5-hydroxymethylcytosine (5-hmC) and further intermediate forms, with final demethylation steps requiring additional proteins like thymine DNA glycosylase (TDG) and base excision repair mechanisms (Figure 2) (Cai et al. 2017; He et al. 2011; Tahiliani et al. 2009).

In mammalian genomes, CpG dinucleotides are statistically under-represented but can densely cluster in regions known as CpG islands. These islands are defined by high GpC content and are mostly unmethylated, typically located within gene promoter regions. When hypermethylated, CpG islands are generally (but not necessarily) associated with gene silencing. The methylation of a promoter region limits the ability of transcription factors to bind, recruits methyl-binding proteins, leads to chromatin condensation by recruiting, for example, histone-modifying enzymes, and ultimately blocks transcriptional initiation (Du et al. 2015; Jones 2012; Światowy et al. 2021).

Research on DNA methylation has for long mainly concentrated on CpG islands at transcriptional start sites (TSSs), shaping general perceptions about its function. However, genome-wide analyses of the methylome have highlighted that the location of methylation within the transcriptional unit influences the effect on gene regulation. While methylation close to the TSS can inhibit transcriptional initiation, gene body methylation has been positively correlated with transcription activity and might influence alternative splicing and differential promoter usage (Jones 2012). Moreover, distal regulatory regions, including enhancers and insulators, often exhibit methylation changes linked to gene expression alterations in various diseases and have recently received increasing attention for being functionally important.

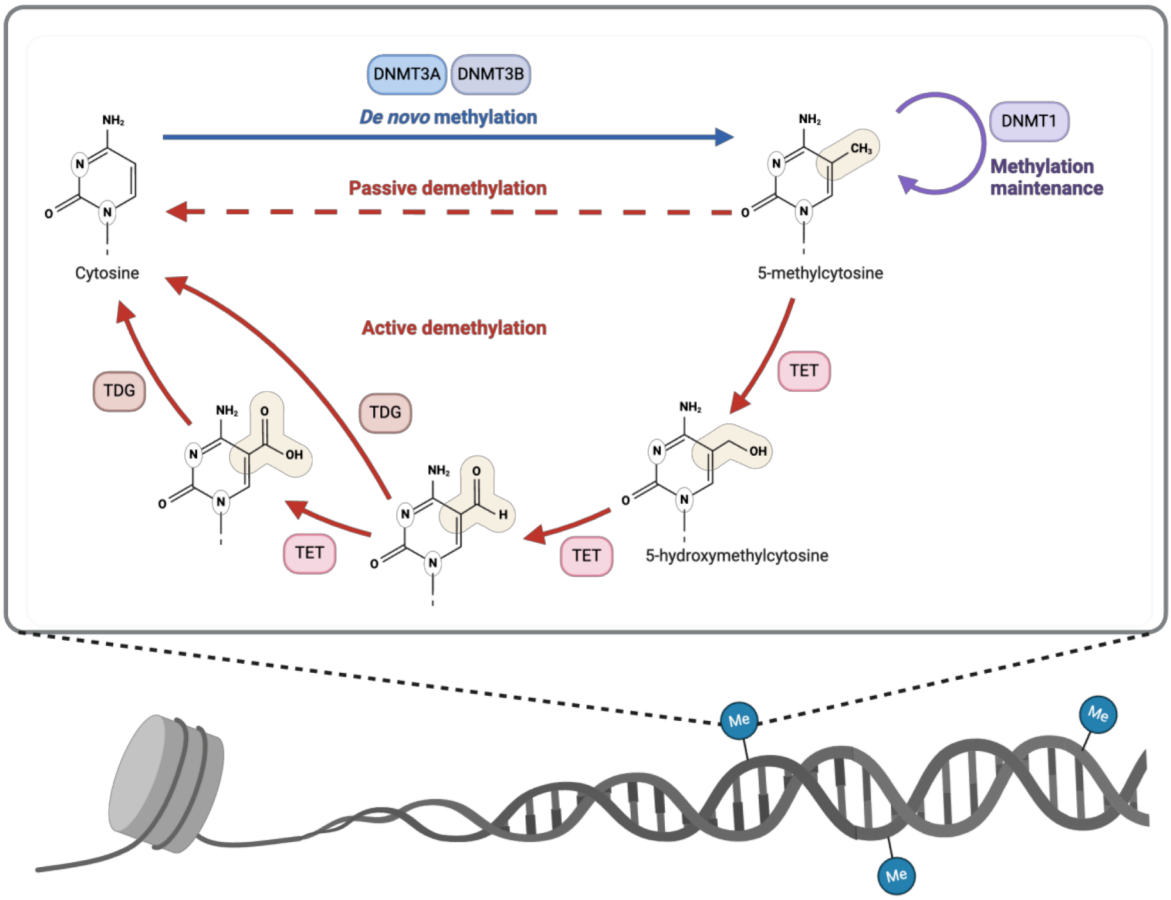

Figure 2: DNA Methylation: Methyl groups (CH3) are added to cytosine (C) bases, catalyzed and maintained by DNA methyltransferase enzymes (DNMTs). Demethylation can occur passively when methylation is not maintained during replication. Active demethylation is driven by ten-eleven translocation (TET) enzyme activity, which converts 5-methylcytosine to 5-hydroxymethylcytosine and

further intermediate forms. These intermediates are subsequently reverted to unmethylated cytosines by enzymes such as TDG (thymine DNA glycosylase). Created with BioRender.com.

4.3.3 DNA methylation and exercise

Previously, DNA methylation was regarded as a stable epigenetic modification set during early development. DNA methylation was recognized for playing an important role in processes such as X-chromosome inactivation and parental imprinting. DNA methylation was thought to change only slowly over time, as seen with ageing and chronic diseases (Jones and Takai 2001; Schaefer et al. 2007; Teschendorff et al. 2013). However, evidence suggests that environmental factors, including physical activity, can induce transient changes in DNA methylation levels (Feil and Fraga 2012; Jacobsen et al. 2012).

It has been demonstrated that exercise induces changes in global DNA methylation profiles (Bittel and Chen 2024; Lindholm et al. 2014b; Nitert et al. 2012; Ntanasis-Stathopoulos et al. 2013; Sailani et al. 2019). This suggests that epigenetic modifications play a role in gene expression and adaptation to exercise and, consequently, exercise-induced health benefits. Findings are however conflicting with some studies reporting DNA hypomethylation and others hypermethylation or no change in global methylation in blood in association with physical activity levels or acute exercise (Denham et al. 2016; Dimauro et al. 2016; Luttropp et al. 2013; Robson-Ansley et al. 2014; White et al. 2013; Zhang et al. 2011). Global methylation levels in blood cells seem to not be a reliable marker for training status, but research should rather focus on specific methylation patterns than overall methylation levels to better understand the epigenetic effects of exercise. While studies primarily investigated blood cells due to the accessibility of blood samples, significant alterations also occur in skeletal muscles. Some studies on the effect of exercise on DNA methylation in muscle have been conducted in the last couple of years reporting mixed results (Bittel and Chen 2024; Ntanasis-Stathopoulos et al. 2013).

A single bout of acute aerobic exercise has been reported to decrease global DNA methylation in previously sedentary young individuals, potentially increasing gene expression to meet the demands of increased physical activity (Barrès et al. 2012) while acute endurance exercise increased methylation of selected genes in competitive endurance-trained cyclists (Lane et al. 2015). Acute resistance exercise has been reported to induce post-exercise methylation changes in previously trained and untrained men (Bagley et al. 2020; Seaborne et al. 2018; Telles et al. 2023; Turner et al. 2019). Altered DNA methylation patterns may underlie the transition from a general stress reaction in training-naïve skeletal muscle to a specific adaptive response with prolonged and consistent exercise (Thomas et al. 2024).

More genes were hypomethylated than hypermethylated after a 6-month endurance exercise intervention in middle-aged men (Nitert et al. 2012). Similarly, older men who maintained an active lifestyle throughout their lives exhibited more demethylation in gene promoters compared to inactive individuals, who had higher body fat, lower muscle mass, and reduced endurance (Sailani et al. 2019). A study on sedentary volunteers highlights the localised impact of exercise on muscle tissue, since three months of one legged exercise induced specific changes in DNA methylation and gene expression in the trained leg only. However, global methylation levels remained unchanged with training, showing no significant difference between trained and untrained legs (Lindholm et al. 2014b). No significant changes

in global CpG methylation were reported by Robinson et al. after a 12-week high-intensity interval training (HIIT), resistance training, or combined training intervention (Robinson et al. 2017).

Chronic resistance exercise over longer periods has been reported to further reprogram muscle cells' methylation profiles. This indicates a form of epigenetic memory that enhances the body's response to subsequent exercise. For instance, two periods of resistance training interrupted by rest resulted in methylation changes at thousands of CpG sites which were maintained during detraining and further enhanced with retraining. Increased frequency of hypomethylation was observed after reloading compared to earlier loading (Seaborne et al. 2018). This pattern illustrates how the body retains a memory of previous exercise through changes in the methylation landscape.

Taken together, these findings highlight the dynamic and complex nature of DNA methylation in muscle cells in response to physical activity and underscore the importance of examining specific methylation changes.

4.3.4 Clinical and scientific significance of epigenetic changes due to exercise

Studying the effect of training and acute exercise on DNA methylation enhances our understanding of basic human biology, particularly the relationship between genotype and phenotype. As an example of this substantial influence, monozygotic twins with the same genetic material can diverge in their DNA methylome and transcriptome as they get older (Fraga et al. 2005). This divergence could be influenced by distinct lifestyle choices, as more epigenetic differences were observed between twins who lived different lifestyles and had spent less of their lives together. Despite having a common genotype, monozygotic twins show different susceptibilities to disease, and epigenetic patterns acquired through lifestyle choices, such as physical activity levels, could be part of the explanation.
Changes in DNA methylation patterns have been linked to the development of various muscle-related diseases, such as muscular dystrophies, myositis, and type 2 diabetes (Coppedè 2020; Nitert et al. 2012). Additionally, cancer cells exhibit a different methylome compared to healthy cells, which might contribute to tumor development by causing genomic and chromosomal instability and affecting gene transcription (Kulis and Esteller 2010; White et al. 2013). Gorski et al. demonstrated that five months of aerobic training could reset a hypermethylated skeletal muscle signature in breast cancer survivors, even years after treatment, to the methylome signature of healthy age-matched individuals in gene regulatory regions (Gorski et al. 2023a). This finding highlights the potential of exercise to reverse adverse epigenetic modifications and improve muscle function and overall health.

Furthermore, exercise-induced epigenetic changes have broader implications for disease prevention and management. By promoting hypo-/hypermethylation in specific gene regions, exercise might enhance the expression of genes involved in muscle function, metabolism, and adaptation. This epigenetic reprogramming may help prevent or mitigate the effects of chronic diseases such as cardiovascular diseases, diabetes and certain cancers.

Understanding how different types of exercise affect the methylome can guide the development of targeted exercise regimens for disease prevention, rehabilitation, and health optimisation, as well as therapeutic approaches for individuals incapable of exercising due to an underlying disease.

4.4 Aim and objectives

The overall aim of this thesis was to investigate molecular patterns associated with high-level exercise training in order to increase our understanding of exercise-induced molecular mechanisms. A focus was put on DNA methylation, the interaction between inherited genetic traits and environmental influences, which can modify gene expression and impact tissue health.

The specific objectives were to:

- Investigate DNA methylation patterns of genes involved in several key aspects of exercise adaptation and its health benefits, such as myogenesis, transcription factors, angiogenesis, and metabolism
- Compare DNA methylation patterns between endurance-trained, strength-trained and untrained men
- Correlate DNA methylation patterns with corresponding gene expression, VO_2peak, leg strength and fiber type distribution
- Assess transient DNA methylation changes after an acute bout of endurance and resistance exercise
- Investigate *PGC1α* isoform expression in trained and untrained skeletal muscle after an acute bout of endurance and resistance exercise

5 Materials and Methods

5.1 Study and methods overview

This thesis is part of a larger study, which aims to understand the effect of long-term training and acute exercise on a systems-biology approach investigating molecular profiles in skeletal muscle, adipose tissue, and blood on a multi-omics level (Reitzner et al. 2023). However, the presented work is independent, utilizing samples and subject characteristics of the study population. This thesis project investigated DNA methylation patterns of genes involved in several key aspects of exercise adaptation and its health benefits, such as myogenesis, transcription factors, angiogenesis, and metabolism (Geiger et al. 2024). Utilising previously analysed global gene expression data of the same muscle samples (Reitzner et al. 2023), the here discovered DNA methylation patterns were linked to transcriptional activity. A particular emphasis was placed on *PGC1α*, analysing the isoform-specific expression of *PGC1α* as a final step for this thesis project. Specific results regarding transcriptomics, proteomics and metabolomics can be found in Reitzner et al. 2023.

5.2 Ethical approval

The study received ethical approval from the Regional Ethical Review Board located in Stockholm, Sweden (record number: 2016/590-31) and was conducted according to the Declaration of Helsinki. Prior to their participation in the study, every individual involved was thoroughly informed about the study's objectives, procedures, potential risks, and benefits. All subjects provided their written informed consent, ensuring their voluntary participation.

5.3 Study protocol and participants

The project employed an intervention study design to investigate molecular differences in skeletal muscle between endurance and strength-trained men compared to untrained men at rest as well as after an acute bout of endurance or resistance exercise.

Twenty-four healthy men between the ages of 35 and 52 years (mean ± SD: 41.6 ± 6 years) were recruited into one of three study groups: 1. endurance group (EG, n=8), 2. strength group (SG, n=8), 3. untrained control subjects (CG, n=8) (Table 1).

Participants were pre-recruited based on their training background, which was assessed by self-reported questionnaires (Reitzner et al. 2023). This self-reported interview ensured a precise pre-selection of participants for physiological testing. Athletes in the EG and SG required at least 15 years of high-level experience (more than 3 times per week) in their respective strength or endurance-based sports while control subjects were required to not be participating in any targeted exercise training. All participants were non-smokers and needed to be in good health without any chronic diseases or medication. Final inclusion into one of the three groups was dependent on physiological testing of participants VO_2peak and maximal isokinetic knee extension (peak torque) (detailed description: Reitzner et al. 2023). For a participant to be included:

- In the EG, participants were required to have a VO_2peak exceeding the 95th percentile for their respective age group (Ekblom-Bak et al. 2011). Moreover, their tested peak torque had to be lower than the lowest recorded value in the SG (Table 1).
- In the SG, participants had to exhibit a peak torque exceeding two standard deviations of the average peak torque observed in the CG. Additionally, their tested VO_2peak had to fall between the 25th and 75th percentile for their age group (Ekblom-Bak et al. 2011).
- For the CG, participants needed to have a VO_2peak between the 25th and 75th percentiles of their age group (Ekblom-Bak et al. 2011). Their peak torque had to be at least two standard deviations below the peak torque of the SG.

All subjects performed one session of acute endurance exercise (EE) and one of acute resistance exercise (RE) separated by a 4-8 week wash-out period (Reitzner et al. 2023). The sessions were performed in a randomised order and subjects were pushed to their maximal performance in both sessions. For both sessions participants warmed up on a stationary bike for 5 minutes. The endurance exercise session consisted of 30 minutes cycling at 75% of their Watt peak reached during the VO_2peak test. For the resistance exercise session participants performed nine sets of eight repetitions on a knee extension machine at 80% of the respective measured one repetition maximum, which was assessed during initial testing. They were instructed to eat the same dinner before each of the intervention days and a standardized breakfast (190g of "ARLA Mild yoghurt grekisk med frön och tranbär", a cheese sandwich, a banana and water and/or decaffeinated tea) three hours before the exercise. Prior to the intervention days, they were not allowed to exercise for 72 hours and instructed to refrain from alcohol and any blood-thinning medications. Only water was allowed to be consumed during the intervention. All interventions started at the same time in the morning to control for circadian rhythm effects.

Blood samples, muscle biopsies and adipose tissue biopsies were taken at baseline, directly after, 1 hour after and 3 hours after the exercise sessions (Reitzner et al. 2023). Since only skeletal muscle tissue was used for the present thesis, blood samples and adipose tissue biopsies will not be further discussed. Skeletal muscle biopsies were taken alternately from the left and right *musculus vastus lateralis* with the first leg randomized based on dominant leg side. Biopsies were collected employing the Bergström needle technique (Bergstrom 1975). To mitigate the potential effects of localised inflammation, subsequent incisions for biopsies on each leg were made at least 2 cm proximal from the site of the initial biopsy. Muscle samples were immediately frozen in liquid nitrogen-cooled 2-methylbutane and stored at -80°C until further analysis.

Table 1: Research subject characteristics. The data are presented as the mean ± SD. The demographic and physiological parameters listed have been previously published and are adopted from Reitzner et al. 2023.

Group	**Sex**	**Sample Size**	**Age (Years)**	**Weight (kg)**	**Height (cm)**	**Leg strength (Nm)**	**Vo_2peak (ml/min/kg)**
Control	Male	8	43.9 ± 6.2	76.0 ± 8.4	181.1 ± 5.6	180.6 ± 30.8	36.2 ± 4.4
Endurance	Male	8	41.8 ± 4,8	73.6 ± 3.9	181.6 ± 4.3	198.3 ± 25.5	67.0 ± 7.2
Strength	Male	8	39.1 ± 5.7	92.0 ± 2.9	180.0 ± 5.1	289.4 ± 28.1	40.2 ± 6.9

Phenotypical characterisation of skeletal muscle, including measurements of citrate synthase activity and fiber typing, was conducted as described in Reitzner et al. (2023). While these analyses were not performed as part of this thesis, their results are mentioned to highlight phenotypical differences among the study subjects. Significantly higher citrate synthase (CS) activity was measured in skeletal muscle biopsies of the EG compared to the other groups (Reitzner et al. 2023). CS activity is an indicator of mitochondrial content and increases with regular endurance training (Holloszy et al. 1970; Larsen et al. 2012; Vigelsø et al. 2014). Muscle fiber typing showed a significantly higher proportion of slow-twitch muscle fibers (type I) in the EG (Reitzner et al. 2023). The CG and SG had more type II fibers, which contain fast-oxidative and fast-glycolytic fibers (Reitzner et al. 2023). Measuring the myofibrillar cross-sectional area to determine the level of muscle hypertrophy, strength-trained athletes mirrored their higher maximal quadriceps torque data by having significantly larger type II fibers compared to the other groups (Reitzner et al. 2023).

Transcriptional profiles of the study subjects' skeletal muscle have been detailed in a separate work, with comprehensive methodological descriptions provided in Reitzner et al. (2023). For context and enhanced interpretation of results, gene expression data from Reitzner et al. (2023) is related to the DNA methylation data explored in this thesis and published by Geiger et al. (2024).

5.4 Targeted DNA methylation analyses

To investigate DNA methylation patterns in skeletal muscle samples a "Targeted DNA Methylation Sequencing" workflow from Twist Bioscience was used (Geiger et al. 2024). This method uses state-of-the-art enzymatic conversion, efficient probe design techniques for post-conversion capture, and fast hybridisation techniques to ensure successful enrichment of target regions and efficient sequencing (Figure 3) (Bioscience 2021). The traditional gold standard to detect DNA methylation uses a sodium bisulfite method to convert unmethylated cytosines to uracils (Clark et al. 2006). After amplification and sequencing methylated cytosines remain intact, while converted unmethylated cytosines, having transformed into uracil, are read as thymines. However, chemical bisulfite treatment can be damaging to DNA, leading to strand breakages, CpG bias, and poor complexity (Morrison et al. 2021; Olova et al. 2018). Addressing these challenges, the Twist workflow incorporates an enzymatic conversion approach developed by New England Biolabs (Hoppers A et al. 2020). This method begins with the oxidation of methylated cytosines to protect them during a subsequent step where unmethylated cytosines are deaminated into uracils. Enzymatic conversion reduces DNA damage and has been shown to offer enhanced resolution, mapping accuracy, and balanced CpG content representation compared to traditional bisulfite-based techniques (Feng et al. 2020; Han et al. 2022; Morrison et al. 2021; Vaisvila et al. 2021).

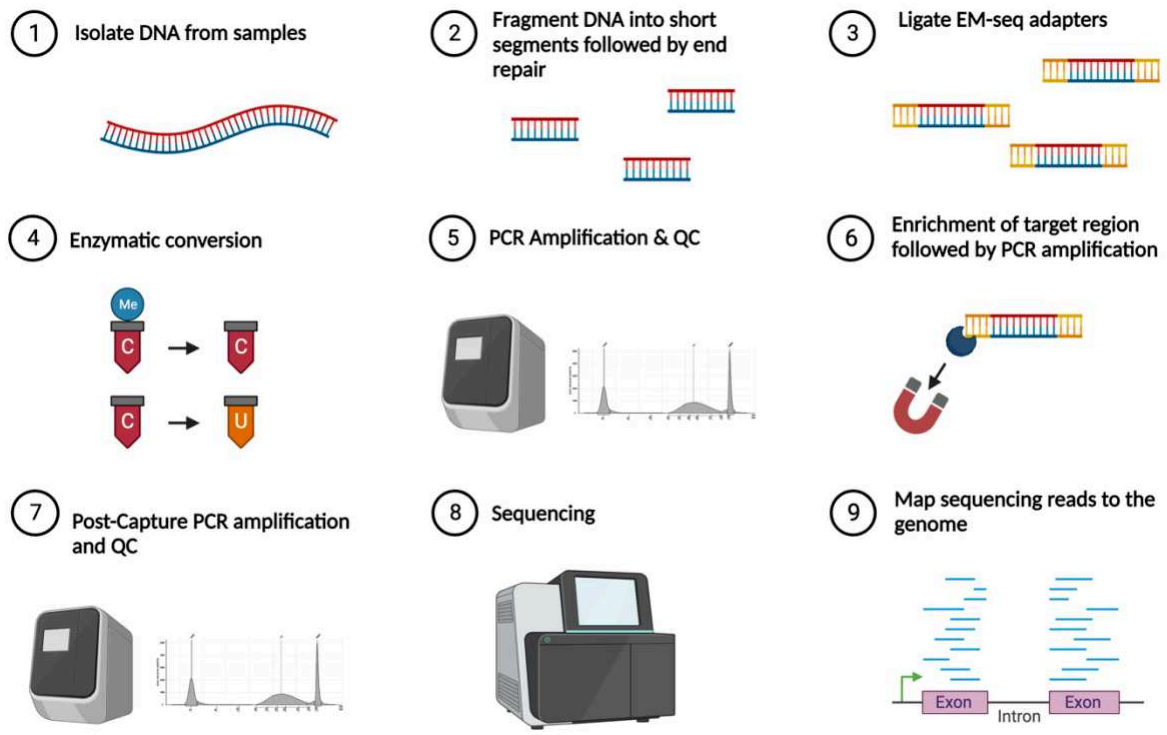

Figure 3: Targeted DNA methylation workflow: After DNA isolation, the DNA is fragmented, and the ends are prepared for sequencing adapter ligation. Enzymatic conversion is performed to differentiate between methylated and unmethylated cytosine bases. The library is then amplified and enriched for target regions. Finally, sequencing is performed, and the resulting data is mapped to the reference genome for analysis. PCR, polymerase chain reaction; EM-seq, Enzymatic Methyl-sequencing; QC, quality control. Created with BioRender.com

5.4.1 Panel selection

The first step involved designing a targeted DNA methylation panel. This panel was specifically designed to investigate regulatory elements of genes that respond to exercise and play roles in training adaptations or the immediate response to a single bout of exercise based on transcriptomic analysis of the same samples in a previous study (Reitzner et al. 2023). The target panel was then subjected to methylation sequencing to identify epigenetic changes.
Promoter regions were selected with the help of the Eukaryotic Promoter Database (Dreos et al. 2014). The Eukaryotic Promoter Database was used to find transcription start sites (TSS) for target genes. The TSS, a minimum of 500 bp upstream of the TSS, and the immediately adjacent downstream base pairs were used for probe design. Enhancer-associated regions were identified with the help of the GeneHancer database (Fishilevich et al. 2017) and chromatin state data from the Roadmap Epigenomics Project (Kundaje et al. 2015). Some enhancers were adopted from the metameth database and a previously performed analysis of the epigenome in human skeletal muscle after training (Lindholm et al. 2014b; Voisin et al. 2021). The final design covered 57 target regions across 37 different genes with a total size of 66,588 bp (Table 2). Using chromosomal locations of the selected targets, Twist Bioscience constructed a custom panel with 561 probes at a length of 120 bp/probe. After removing 14 probes due to repeats, the final design covered 64,959 bp resulting in 97.55% coverage of submitted targets.

Table 2: Targeted DNA methylation panel. Genomic coordinates are based on genome assembly GRCh38/hg38.

Target region	Chromosome	Start	End	Length
MTOR Enhancer	chr1	11157965	11158698	733
MTOR Promoter	chr1	11261784	11262982	1198
PAX7 Promoter	chr1	18630893	18631726	833
AMPK a2 Promoter	chr1	56644815	56645414	599
MYOG Promoter	chr1	203085593	203087076	1483
TFAM Promoter	chr10	58384572	58385550	978
MyoD Core Enhancer	chr11	17699561	17699898	337
MyoD DRR Enhancer	chr11	17713956	17715330	1374
MyoD Promoter	chr11	17718561	17720213	1652
ERRa Promoter	chr11	64305030	64306212	1182
CS Promoter	chr12	56299357	56301150	1793
NDUFA4L2_1 Promoter	chr12	57236323	57237603	1280
NDUFA4L2_2&3 Promoter	chr12	57240621	57241360	739
MYF6 Promoter	chr12	80706525	80707694	1169
MYF5 Promoter	chr12	80716415	80717349	934
FOXO1 Enhancer	chr13	40598208	40599640	1432
FOXO1 Promoter	chr13	40666208	40667577	1369
MYH6 Promoter	chr14	23409253	23409513	260
MYH7 Promoter	chr14	23435560	23436160	600
HIF1A Promoter	chr14	61694712	61695621	909
HIF1A Promoter/Enhancer	chr14	61697314	61698034	720
SMAD3 Promoter	chr15	67065343	67065942	599
SMAD3 Enhancer	chr15	67098033	67098808	775
SMAD3 Enhancer	chr15	67136625	67136886	261
SMAD Enhancer	chr15	67165245	67166183	938
MEF2A Promoter	chr15	99565358	99566091	733
MEF2A Enhancer	chr15	99718988	99719684	696
GLUT4 Promoter	chr17	7279539	7283489	3950
MYH2 Promoter	chr17	10549558	10550157	599
TGFbeta Promoter	chr19	41353376	41354375	999
ATF2 Promoter	chr2	175181611	175182358	747
Myostatin Promoter/Enhancer	chr2	190062542	190066015	3473
CREB1 Promoter	chr2	207529048	207530297	1249
MAPK12 Enhancer	chr22	50248041	50249380	1339
p38 MAPK Promoter	chr22	50261277	50262808	1531
MYL3 Promoter	chr3	46863344	46863943	599
HES1 Promoter	chr3	194135518	194136612	1094
PGC1a Enhancer	chr4	23880612	23883359	2747
PGC1a proximal Promoter	chr4	23887877	23891221	3344

PGC1a alternative Promoter	chr4	23903795	23904977	1182
PGC1a Enhancer	chr4	23934209	23935339	1130
PGC1a Enhancer	chr4	23945150	23946601	1451
AMPK a1 Promoter	chr5	40797940	40798913	973
CAMKIV Promoter	chr5	111223579	111224486	907
CAMKII Enhancer	chr5	150256130	150256868	738
CAMKII Promoter	chr5	150289630	150290353	723
VEGFA Promoter	chr6	43769299	43772782	3483
VEGFA Enhancer	chr6	44057596	44058015	419
FOXO3 Promoter	chr6	108558752	108560683	1931
FOXO3 Enhancer	chr6	108650156	108651861	1705
TBP promoter	chr6	170553777	170554471	694
CREB5 TSS	chr7	28412446	28412669	223
CREB5 Promoter	chr7	28685390	28686760	1370
PDK4 Enhancer	chr7	95592725	95592845	120
PDK4 Promoter	chr7	95596415	95597014	599
NRF1 Promoter	chr7	129610993	129611946	953
cMYC Enhancer	chr8	127898412	127898532	120

5.4.2 DNA extraction

DNA was extracted from frozen skeletal muscle tissue using the Puregene Tissue Kit (Cat. No./ID: 158063) from QIAGEN according to the instructions found in the Puregene Handbook (12/2014). For this method, cells are first lysed with an anionic detergent, followed by the removal of RNA using an RNA-digesting enzyme. Proteins and other contaminations are then removed by salt precipitation. Genomic DNA is recovered by precipitation with alcohol and dissolved in hydration solution (1 mM EDTA, 10 mM Tris·Cl pH 7.5).
Steps:

1) 5-10 mg of tissue were dissected and placed in a 2 ml tube with two ceramic beads.
2) 300 µl Cell Lysis Solution were added and tissue was homogenised using a bead shaker (Vibrating Mill MM 400, Retsch) for 3 cycles of 30 seconds each, with 30-second rests in between.
3) Homogenized tissue was transferred in Cell Lysis Solution into a 1.5 ml tube and 1.5 µl Puregene Proteinase K was added, mixed by inverting 25 times and incubated at 55°C (Block heater Grant QBD2) overnight on a shaking plate (Orbital Platform Shaker, Grant Bio™ PSU-10i).
4) 1.5 µl RNase A Solution was added, mixed by inverting 25 times and incubated at 37°C for 1 h.
5) 1 minute incubation on ice to quickly cool the samples.
6) 100 µl Protein Precipitation Solution were added and then vortexed for 20 s at high speed.
7) Centrifuged for 3 min at 14 400 x g (Centrifuge 5417 R, Eppendorf).
8) 300 µl isopropanol were pipetted into a clean 1.5 ml microcentrifuge tube and supernatant from step 7 was added.
9) Mixed by inverting 50 times.
10) Centrifuged for 1 min at 14 400 x g.

11) Supernatant was discarded and the tube was drained while keeping care that the pellet remained in the tube.
12) 300 µl of 70% ethanol were added followed by inverting several times to wash the DNA pellet.
13) Centrifuged for 1 min at 14 400 x g.
14) Supernatant was discarded and the tube drained while keeping care that the pellet remained in the tube. Air-dried for 5 min.
15) 100 µl DNA Hydration Solution was added than vortexed 5s at medium speed.
16) Incubated at 65°C for 1h to dissolve DNA.
17) Incubated at room temperature (15-25°C) overnight with gentle shaking, centrifuged briefly.
18) Final DNA concentration was measured with absorbance at 260/280 nm using NanoDrop 2000 (Thermo Scientific, Goteborg, Sweden). All samples had optical density (OD) 260/280 of 1.8-2.0 which is indicative of pure DNA.
19) Stored at -20°C until further analysis.

5.4.3 Library preparation and sequencing

Library preparation of the Enzymatic Methyl-seq Library, targeted DNA methylation sequencing, pre-processing of sequencing data, alignment of reads and quality control on the results were performed by the SNP&SEQ Technology Platform in Uppsala. The facility is part of the National Genomics Infrastructure (NGI) Sweden and Science for Life Laboratory. The SNP&SEQ Platform is also supported by the Swedish Research Council and the Knut and Alice Wallenberg Foundation.

A total of 500 ng of DNA (see 5.4.2 for DNA extraction method), suspended in 50 µl of DNA hydration solution, was sent to the SNP&SEQ Technology Centre in Uppsala in a 96-well microtiter plate for library preparation and sequencing. The Agilent TapeStation instrument (from Agilent Technologies) was used to confirm sufficient quantity and quality of DNA. DNA concentration was measured with the Invitrogen™ Qubit/Quant-iT test (by Thermo Fisher Scientific). All samples were of sufficient quality. 200 ng of DNA were used for library preparation using the NEBNext Enzymatic Methyl-seq sample preparation kit, specifically the Twist NGS Methylation Detection System (catalog numbers 101977, 101978, and 101979) according to the guidelines provided by the manufacturers in document DOC-001224 REV 3.0. Figure 3 shows an overview of the workflow. First DNA is sheared to an average size of 300 bp, followed by another quality control step, end-repair and dA-tailing. Next, DNA is ligated to the EM-seq adaptor using unique dual indexes. After the ligation process a two-step conversion process follows: 1) TET2 enzyme oxidates 5-mC to 5-caC, providing protection from deamination. 2) APOBEC enzyme deaminates unmethylated cytosines to uracil after denaturation; oxidized forms of 5-mC are not deaminated. After conversion PCR amplification and quality control follow. DNA fragments should have an average fragment length of 350 bp to 450 bp according to manufacturer instructions (Figure 4). Following the instructions detailed in document DOC-001222 REV 2.0 the prepared libraries, ranging from 160 to 187.5 ng, were hybridised using a Twist Methy Custom Panel from Twist Bioscience followed by another round of amplification and quality control. Paired-end sequencing with a read length of 150 base pairs was performed on the NovaSeq 6000 system, utilising an SP flowcell and version 1.5 sequencing chemistry. In the sequencing run, a well-characterised control library from the bacteriophage PhiX was incorporated as a 10% spike-in to ensure base diversity,

calibrate the sequencing machine, and provide a quality control benchmark. All samples fulfilled quality control criteria.

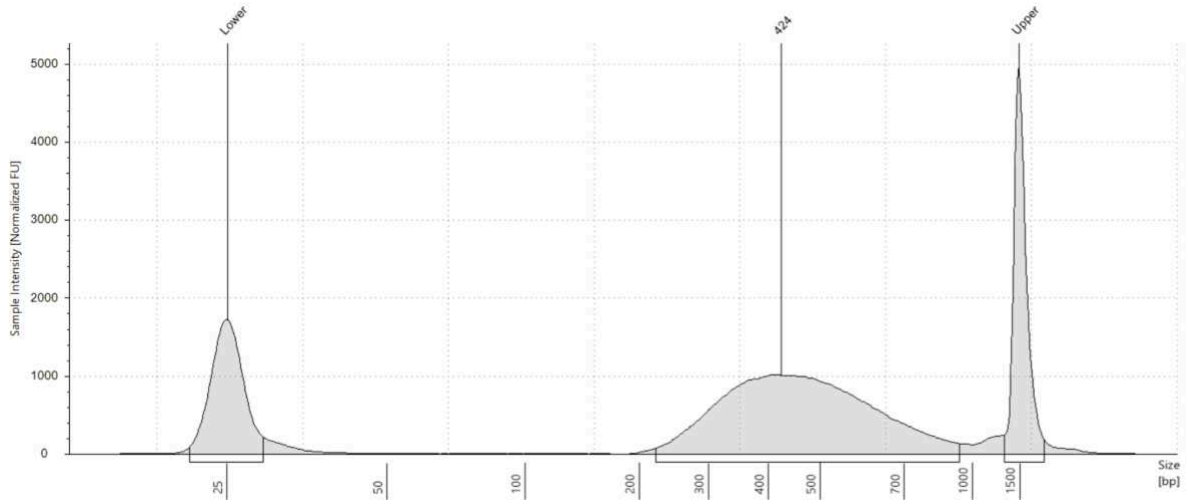

Figure 4: Quality control before enrichment of target regions. As required by the manufacturer's instructions, the fragment length of samples is between 350-450 bp.

5.4.4 Processing of sequencing data

Using the nf-core/methylseq pipeline (version 1.6.1) raw sequencing data in FastQ format was aligned to the human genome (hg38) (Ewels et al. 2020) by the SNP&SEQ Technology Centre in Uppsala. All reads were preprocessed by trimming using TrimmGalore (version 0.6.6) and then aligned using Bismark (version 0.23.0). Quality control for both pre- and post-mapping stages was conducted through the nf-core/methylseq pipeline, utilising MultiQC (version 1.10.1). See Table 3 for all software versions. Base calls exhibited good quality (Figure 5), and the target regions showed high coverage, with a median of 628 reads per base and an interquartile range (IQR) of 374 reads. For subsequent analyses, only CpG sites within the target panel regions that had at least 10X coverage were considered.

Table 3: Software version for processing of sequencing data

Software	**Version**
nf-core/methylseq	v1.6.1
Nextflow	v21.10.6
Bismark genomePrep	v0.23.0
FastQC	v0.11.9
Cutadapt	v3.4
Trim Galore!	v0.6.6
Bismark	v0.23.0
Bismark Deduplication	v0.23.0
Bismark methXtract	v0.23.0
Bismark Report	v0.23.0
Bismark Summary	v0.22.4
Samtools	v1.11

BWA	v0.7.17-r1188
bwa-meth	v0.2.2
Picard MarkDuplicates	v1.6.1
MethylDackel	v0.5.2 (using HTSlib version 1.11)
Qualimap	v2.2.2-dev
Preseq	v2.0.3
MultiQC	v1.10.1
HISAT2	v2.2.1

5.5 Transcriptional activity

5.5.1 RNA extraction and cDNA synthesis

To extract total RNA from skeletal muscle tissue, the TRIzol method (Invitrogen #15596018, Thermo Fisher Scientific, Waltham, MA, USA), a phenol-based extraction technique, was employed. This extraction, initially undertaken for RNA-sequencing in another study (Reitzner et al., 2023), also provided the RNA used in the current study's quantitative PCR analysis (refer to section 5.5.2). Detailed methodology of RNA extraction, RNA sequencing and analysis is available in Reitzner et al. (2023). The RNA quantity was determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Gothenburg, Sweden), measuring absorbance at 260 nm. Subsequently, 200 ng of RNA was converted to single-stranded cDNA in a 20 µl reaction volume using the High-Capacity cDNA Reverse Transcription Kit (Cat. No. 4368814, Applied Biosystems, Foster City, CA).
The cDNA synthesis involved the following steps:

1) Preparation of reaction mixes:
   Reaction Mix 1 (Rx1) per Sample:
   - 2.2 µl RNAse-free H_2O
   - 0.8 µl dNTPs (1mM, providing the nucleotide building blocks)
   - 2 µl Random Primers (to initiate cDNA synthesis)
   Reaction Mix 2 (Rx2) per Sample:
   - 1 µl 10 mM MgCl2 (1 mM, a cofactor for the reverse transcriptase enzyme)
   - 2 µl RT Buffer (to maintain optimal pH and salt conditions)
   - 1 µl RNAse-free H_2O
   - 1 µl Reverse Transcriptase (2.5 U/µl, the enzyme that synthesizes cDNA)
2) Preparation and Denaturation of RNA-Primer Mixture:
   Each well of a 96-well plate was loaded with 10 µl of sample RNA (20 ng/µl concentration) and Rx1. The plate was sealed, vortexed, centrifuged, and placed in a thermocycler with the following settings: 10 min at 65°C, followed by 1 min at 4°C.
3) Reverse transcription and cDNA synthesis:
   Rx2 was added to each well, centrifuged, and the plate was returned to the thermocycler with these settings: 10 min at 25°C, 120 min at 37°C, 5 min at 85°C, and finally 10 min at 4°C.
4) 30 µl of H_2O was added to each sample and the cDNA was stored at -20°C.

5.5.2 Real-time quantitative PCR

Real-time quantitative PCR (q-PCR) was employed to measure the mRNA levels of *PGC1α,* focusing on different splice variants/isoforms, including:

- Total *PGC1α*
- Proximal promoter transcripts (*PGC1α -ex1a*)
- Alternative promoter transcripts (*PGC1α -ex1b*)
- Truncated transcripts (*trunc-PGC1α*)
- Nontruncated transcripts (*nontrunc-PGC1α*)

The primer design and efficiency testing are detailed in Ydfors et al. (2013), with primers synthesized by Cybergene AB, Stockholm, Sweden. Primer sequences are listed in Table 4. For normalization and ensuring data reliability, Beta-2-Microglobulin (*BTM*), TATA-Box Binding Protein (*TBP*), and Cyclophilin A (*PPIA*) were chosen as stable endogenous controls across all time points, based on their proven stability in similar studies (Kuang et al. 2018; Mahoney et al. 2004).

All samples were processed in duplicates using the Applied Biosystems 7500 Fast Real-Time PCR System. The steps involved were:

1) Preparation of Mastermix:
   - 5 µl PowerUp™ SYBR™ Green Master Mix (2X)
   - 1 µl Primermix (with forward and reverse primers at a final concentration of 0.4 µM each)
   - 3 µl H2O.
2) Sample and Control Allocation:
   1 µl cDNA/sample were allocated in duplicates into a 384-well plate, including triplicates of H_2O (negative control) and the negative control from cDNA synthesis (see 5.5.1).
3) Mastermix Addition and Plate Preparation:
   9 µl of the prepared Mastermix were added to each well, the plate was sealed, vortexed, and centrifuged.
4) PCR Cycling:
   The plate was placed on a BIO-Rad C1000 Touch Thermal Cycler. The settings for the thermal cycler were: 3 minutes at 95°C (initiation), followed by 40 cycles of 10 seconds at 95°C (denaturation), and 30 seconds of annealing and elongation at 60°C. A melting curve was used to ensure the quality of the qPCR products.

Table 4: Primer sequences

Amplicon Name	**Forward Primer**	**Reverse Primer**
Total *PGC1α*	GTGGTGCAGTGACCAATGAG	CTGCTAGCAAGTTTGCCTCA
PGC1α-ex1a	TGTATGGAGTGACATCGAGTGT	GCTGGTCTTCACCAACCAGA
PGC1α-ex1b	GACACACATGTTGGGGTTATCA	ACCAACCAGAGCAGCACATTT
trunc-PGC1α	CCACACACAGTCGCAGTCACA	GTCACTGGAAGATATGGCACAT
nontrunc-PGC1α	CCACACACAGTCGCAGTCACA	GGGAACCCTTGGGGTCATTTG
B2M	AAGGACTGGTCTTTCTATCTC	GATCCCACTTAACTATCTTGG
TBP	TGTGCTCACCCACCAACAAT	TCTGCTCTGACTTTAGCACCTG
PPIA	ATGGTTCCCAGTTTTTCATC	CTCCACAATATTCATGCCTTC

5.6 Statistics

Statistical analyses were performed in R (version 4.3.0). See Table 5 for package versions additionally used to R default packages. Figures were plotted in R or created with BioRender.com.

Table 5: R packages used additionally to R default packages

R Package	Version
annotatr	1.26.0
base	4.3.0
biomaRt	2.56.1
BSgenome.Hsapiens.UCSC.hg38	1.4.5
bsseq	1.36.0
dplyr	1.1.2
DSS	2.48.0
factoextra	1.0.7
FactoMineR	1.34
ggplot2	3.4.2
magrittr	2.0.3
org.Hs.eg.db	3.17.0
plotrix	3.8-2
readr	2.1.4
scales	1.2.1
sciplot	1.2-0
stats	4.3.0
tidyverse	2.0.0
tidyr	1.3.0
TxDb.Hsapiens.UCSC.hg38.knownGene	3.17.0

5.6.1 DNA methylation analysis

Methylation coverage files ("_R1_val_1_bismark_bt2_pe.deduplicated.bismark.cov"), sample information and genomic coordinates of target regions were imported into R. Methylation data of target regions were selected and filtered by coverage > 10X. Principal component analysis (PCA) of methylation percentages was performed using R base and factoextra functions to explore the data. A Kruskal-Wallis test was used to assess whether sample characteristics had a significant impact on the proportion of variance explained by different principal components (PCs). For differential methylation analysis the DSS package (Feng et al. 2014; Park and Wu 2016) was used as recommended by Twist Bioscience. The DSS package employs a Wald test, which is based on a beta-binomial distribution model. The test statistics take sequencing depth and biological variation between replicates into account. A shrinkage estimator based on a Bayesian hierarchical model is used to estimate the dispersion parameter, which characterizes biological variation. A p-value of <0.05 was used to call differentially methylated regions (DMRs) and differentially methylated positions (DMPs). For DMRs the package first detects statistically significant CpG sites to then merge nearby loci into regions to form DMRs. Each DMR had a minimum of three CpG sites based on the DSS

package's default configuration. A fixed-effect model was employed to support the paired design in order to compare timepoints within a group and exercises within the control group. For visualisation purposes, sets of DMRs from different comparisons were plotted together if they shared at least one CpG site. The TxDb.Hsapiens.UCSC.hg38.knownGene package in R was used to annotate genes (Maintainer 2023). For functional annotation of DMRs, information from a number of sources, such as the Roadmap Epigenomics Project's analysis of the chromatin states in skeletal muscle (Kundaje et al. 2015), the latest data from the GeneHancer database (Fishilevich et al. 2017), and the Eukaryotic Promoter Database (Dreos et al. 2014) was incorporated. Using base R, Spearman correlation coefficients were calculated to assess the relationship between methylation percentage (beta-values) of DMRs and gene expression (TPM values), VO_2peak, leg strength and fiber type distribution. Benjamini & Hochberg correction was used to correct for multiple testing.

5.6.2 *PGC1α* isoform expression

Using the -ΔCt method, the relative expression of each *PGC1α* transcript at rest was quantified. For each sample, ΔCt was derived by subtracting the Ct value of the endogenous control (housekeeping genes) from the Ct value of the target gene and the logarithm was taken, represented as ΔCtmean(-log). The fold change in gene expression post-exercise compared to pre-exercise was computed by dividing the 2^-ΔCt of post-exercise values by the 2^-ΔCt of pre-exercise values (Livak and Schmittgen 2001). Given that two baseline measurements were available for every subject – one prior to endurance and another before resistance exercise interventions – a median value was computed for individual subjects. This median was used to explore baseline *PGC1α* expression differences among groups. Due to a small sample size (n = 8) and since data did not meet normal distribution criteria, the non-parametric Kruskal-Wallis test was selected. This rank-based test identifies statistically significant differences between two or more groups on a continuous or ordinal dependent variable. To assess differences in fold change of *PGC1α* transcripts across different timepoints after exercise compared to pre-exercise (before exercise) within groups, the non-parametric Friedman test was initially applied. Serving as an alternative to one-way repeated measures ANOVA, the Friedman test determines if statistically significant differences exist between three or more paired measurements. If a significant difference was detected by the Friedman test ($p < 0.05$), pairwise comparisons between different timepoints were conducted using the Wilcoxon signed rank test. This test, a non-parametric alternative to the paired t-test, compares two paired samples to assess if their population mean ranks differ. To compare the two interventions within groups, a series of pairwise Wilcoxon signed-rank tests were employed. The Spearman correlation method was used to assess the relationship between the baseline expression and post-exercise fold change *of PGC1α* transcripts and the methylation percentage of *PGC1α* DMRs. Given the multiple comparisons, all p-values were adjusted employing the Benjamini-Hochberg procedure (Geiger et al. 2024).

5.6.3 Cell type deconvolution

Digital immune cell deconvolution was done with CIBERSORTx (Geiger et al. 2024) , using the signature matrix LM22 (Newman et al., 2019). This method, developed by Newman et al., computationally estimates the proportions of different cell types from bulk transcriptomic

data. It is accessible via an online platform, which applies a signature matrix to analyse cell proportions in an uploaded counts table from bulk RNA sequencing data.
To analyse the CIBERSORTx output and evaluate differences in immune cell proportions between groups, the Kruskal–Wallis test and post hoc Dunn test with Benjamini–Hochberg correction for multiple testing were applied (Geiger et al. 2024). Additionally, RNA sequencing data was examined for specific marker genes to investigate cell heterogeneity. Markers included those for endothelial cells (*VWF, ESAM, KDR, ICAM1, PECAM-1/CD31,* and *CD144*), mesenchymal cells (*DCN, CFD,* and *GSN*), myogenic cells (*PAX7*), and interstitial fibro-adipogenic progenitors (*TEK*) (Geiger et al. 2024). RNA sequencing analysis was initially conducted for a project separate from this thesis (Reitzner et al. 2023). In brief, data were processed using the nfcore/rnaseq analysis pipeline. Statistical analysis of gene expression was performed with the edgeR package in R version 3.6.0 (Reitzner et al. 2023).

6 Results

6.1 Targeted DNA methylation sequencing - quality control

Quality control of targeted DNA methylation sequencing data showed good quality for all base calls (Figure 5), and the target regions showed high coverage (Figure 6), with a median of 628 reads per base and an interquartile range (IQR) of 374 reads. For subsequent analyses, only CpG sites within the target panel regions that had at least 10X coverage were considered. After applying the 10X coverage filter, the average number of cytosine methylation datapoints left per subject was 6260.43 with a median of 6262 and an IQR of 41 (Figure 7) (Geiger et al. 2024).

*Figure 5: **Sequence Quality Histograms**. Mean quality value across each base position. The y-axis displays quality scores. A higher score indicates a superior base call. The graph's background categorizes the y-axis into three quality sections: excellent quality calls (green), acceptable quality calls (orange), and low-quality calls (red). The plot is generated by MultiQC (version 1.10.1).*

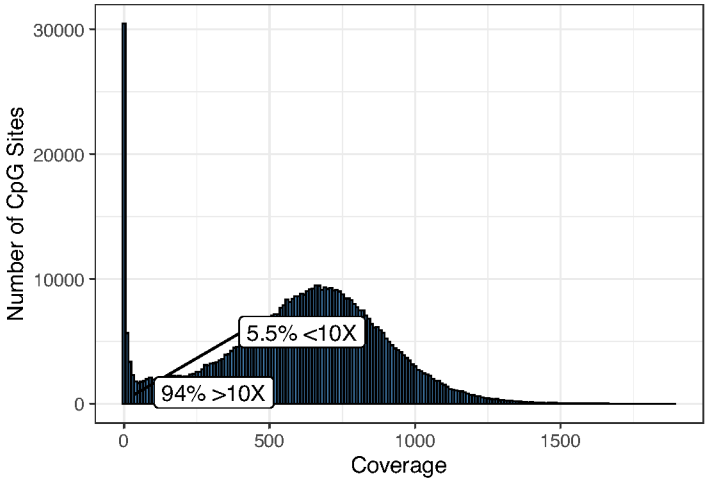

*Figure 6: **Distribution of coverage for target region.** The histogram displays the number of CpG sites versus their respective coverage. 94% of the CpG sites have a coverage greater than 10X, while 5.5% have a coverage less than 10X.*

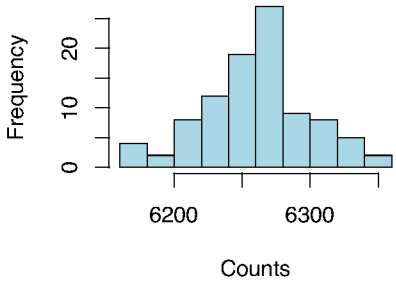

*Figure 7: **Distribution of cytosine methylation datapoints per subject after applying the 10X coverage filter.** The histogram displays the distribution of cytosine methylation datapoints per subject after a 10X coverage filter was applied. The average number of datapoints is 6260.43 with a median of 6262 and an IQR of 41.*

6.2 Data exploration: Principal component analysis of methylation data reveals grouping by training background

To explore the methylation data, an unsupervised principal component analysis (PCA) using all the methylation percentages was performed. It could be observed that the first principal component (PC) explained 48.6% of the variance within the data, while PC2 explained 10.5%. The proportions of explained variance for PC3-96 ranged from 5% to <0.0001%, and were therefore likely irrelevant for further analysis as they did not contribute substantially to the cumulative proportion of variance. Next, a scree plot (Figure 8) was graphed to identify relevant PCs. Using the “elbow criterion”, it was determined that mostly PC1, but also PC2 appropriately add to the explanation of variance (Jolliffe 2002). Therefore, these PCs were examined further (Geiger et al. 2024).

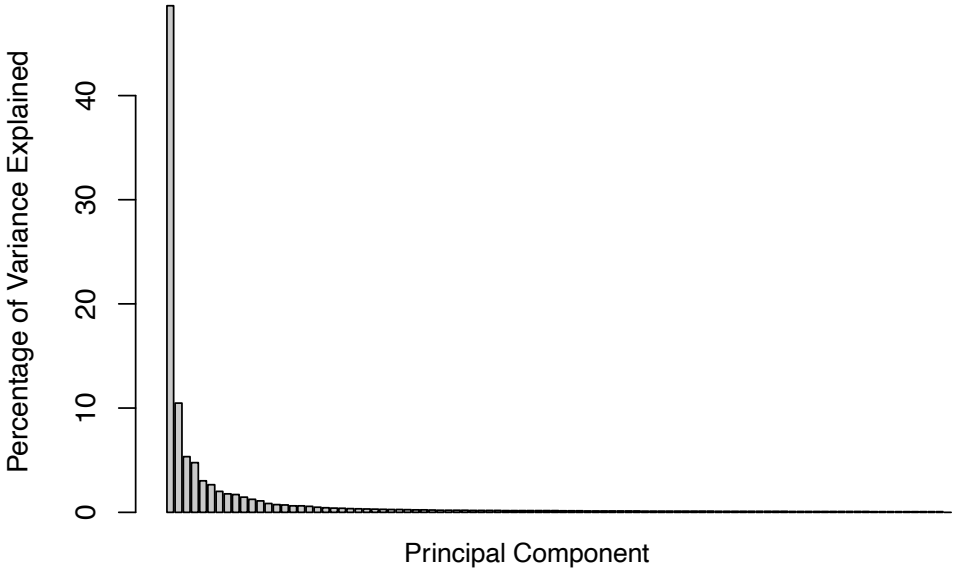

Figure 8: ***Scree Plot of the Explained Variance by Principal Components.*** *The scree plot visualises the percentage of variance explained by each principal component in descending order. The y-axis represents the percentage of variance explained, while each bar on the x-axis corresponds to a specific principal component. The sharp decrease after the first two components, forming an "elbow", suggests that these components capture the most significant variance in the data.*

Individuals from the same group clustered together in both PC1 and PC2 (Figure 9) with the endurance-trained group separating from both the strength-trained and the untrained control groups. A significant difference between the groups for both PC1 ($p < 0.01$) and PC2 ($p < 0.001$) was confirmed by a Kruskal-Wallis test. By study design, the endurance-trained athletes had a significantly higher VO_2peak than the other groups. To further explore the observed group clustering, participants' VO_2peak levels were visualized on another scatterplot. The data points representing higher VO_2 values aligned closely with the distribution of the endurance group as seen in Figure 9 (Figure 10).

In addition to comparing groups with varied training backgrounds, this thesis also sought to understand exercise-induced methylation changes within each group. PCs were therefore investigated for different timepoint measurements. In Figure 11, the overlapping data points in the PC1/PC2 scatterplot, when coloured for different timepoints, suggest that there is little to no variation in methylation percentages between pre- and post-exercise measurements. A

non-significant Kruskal-Wallis test of PCs for timepoint supported this visualisation, indicating that before versus after exercise timepoints aren't important factors explaining the variance of the methylation data. PC scatterplots were utilised to investigate other potential factors that might influence the variation in methylation data. Factors such as acute exercise type (either endurance or resistance), subjects' age, biopsy number, intervention day, the time of year the biopsy was taken, and leg strength were examined. However, none of these factors seemed to significantly explain variation in the methylation data.

Overall, it can be summarised that there is grouping by training background within the PCs and that the clustering of the endurance-trained athletes is potentially linked to their elevated VO_2peak levels (Geiger et al. 2024).

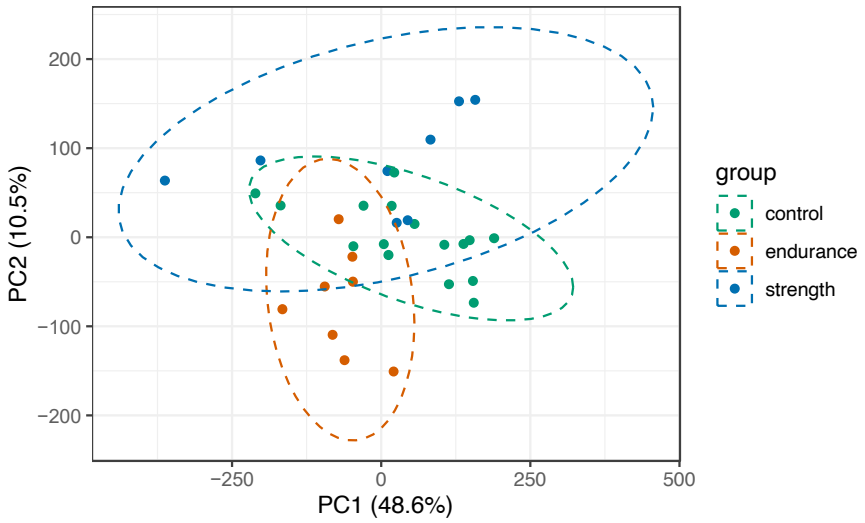

*Figure 9: **Unsupervised Principal Component Analysis by Group**. Beta-values (methylation percentage) of all CpG sites with 10X coverage across the target probes were used. Datapoints are coloured by group (green = CG, orange = EG, blue = SG). The dashed ellipses represent the 95% confidence regions for the multivariate mean of each group in the PC1 vs PC2 space, providing a visual representation of the dispersion and overlap of the groups. Adopted from Geiger et al. 2024.*

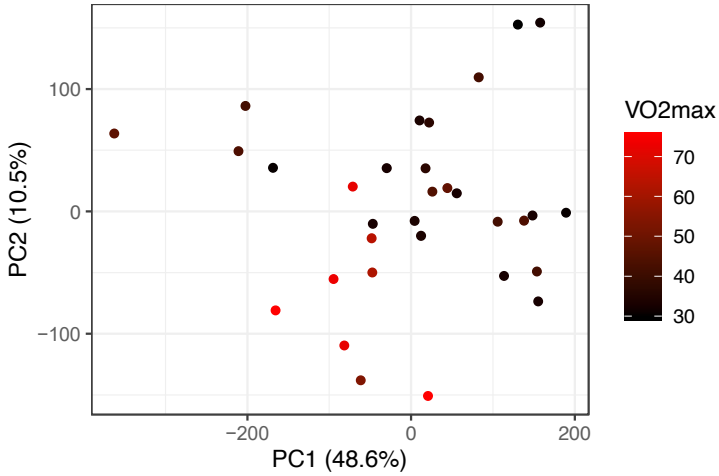

*Figure 10: **Unsupervised Principal Component Analysis by VO_2peak**. Beta-values (methylation percentage) of all CpG sites with 10X coverage across the target probes were used. Datapoints are coloured by VO_2max (VO_2peak) of subjects. VO_2max in ml/kg.*

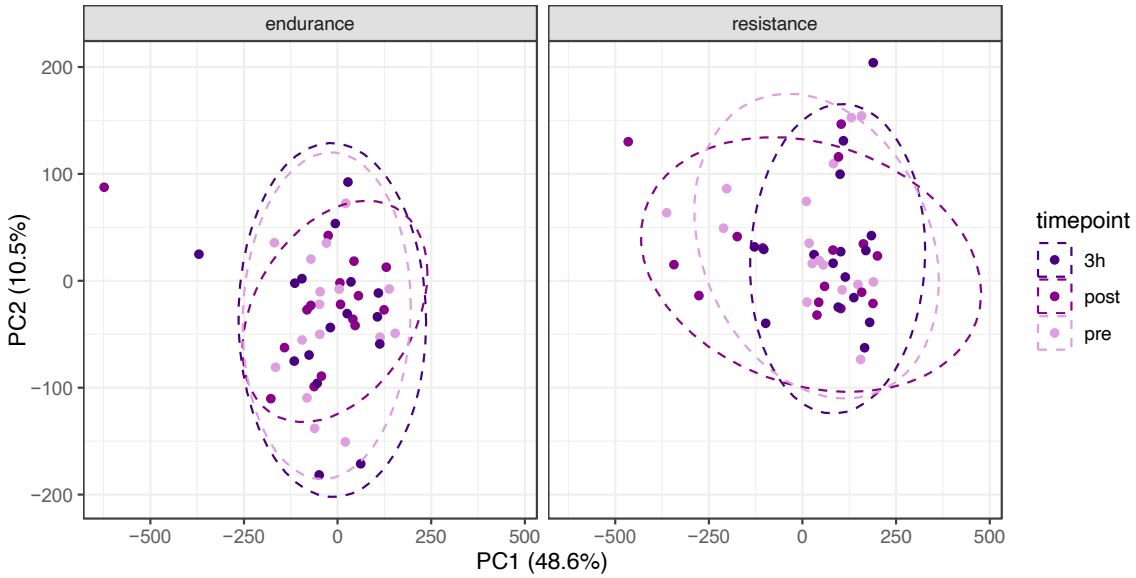

Figure 11: ***Unsupervised Principal Component Analysis by Timepoint****. Beta-values (methylation percentage) of all CpG sites with 10X coverage across the target probes were used. Datapoints are coloured by timepoint (dark purple = pre exercise, purple = directly post exercise , rose = 3h post exercise). The dashed ellipses represent the 95% confidence regions for the multivariate mean of each timepoint in the PC1 vs PC2 space, providing a visual representation of the dispersion and overlap of the timepoint. Left panel: acute endurance exercise (endurance), right panel: acute resistance exercise (resistance).*

6.3 Baseline methylation differences between groups

Analysis of differential methylation, comparing the three groups with varying training backgrounds at rest, affirmed the patterns highlighted by the PCA (Geiger et al. 2024). Notably, most DMRs and DMPs were detected when the EG was compared to the CG or SG (Figure 12, Table 6, Appendix 1). Specifically, 16 DMRs and 7 DMPs were identified between the EG and CG, with the DMPs found within 3 out of the 16 DMRs. The strength and control groups showed fewer differences: 8 DMRs and no individual DMPs. Comparing the two trained groups resulted in 13 DMRs and 17 DMPs. Three of the DMPs were not associated with a DMR while the rest of the DMPs were found within 4 of the 13 DMRs. By design more promoter regions than enhancer regions were part of the targeted DNA methylation panel (Table 2). Of the detected DMRs and DMPs the majority were within promoters (Geiger et al. 2024). In Table 6 all DMRs between groups at baseline are listed. No DMRs or DMPs were found for the following probes of the target methylation panel: *MTOR* Promoter (chr1:11261784-11262982), *PAX7* Promoter (chr1:18630893-18631726), *AMPK α2* Promoter (chr1:56644815-56645414), *TFAM* Promoter (chr10:58384572-58385550), *MYOD1* Promoter (chr11:17718561-17720213), *ERRα* Promoter (chr11:64305030-64306212), *CS* Promoter (chr12:56299357-56301150), *NDUFA4L2*_1 Promoter (chr12:57236323-57237603), *NDUFA4L2*_2&3 Promoter (chr12:57240621-57241360), *MYF6* Promoter (chr12:80706525-80707694), *FOXO1* Promoter (chr13:40666208-40667577), *MYH6* Promoter (chr14:23409253-23409513), *HIF1A* Promoter (chr14:61694712-61695621), *SMAD3* Promoter (chr15:67065343-67065942), *SMAD3* Enhancer (chr15:67136625-67136886), *MEF2A* Promoter (chr15:99565358-99566091), *GLUT4* Promoter (chr17:7279539-7283489),

TGFbeta Promoter (chr19:41353376-41354375), *ATF2* Promoter (chr2:175181611-175182358) and *MSTN* Promoter/Enhancer (chr2:190062542-190066015).

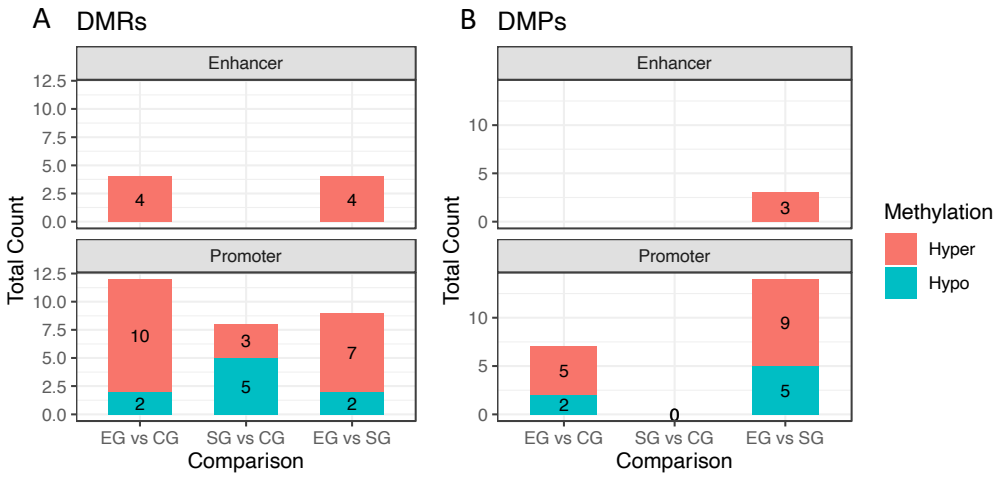

Figure 12: ***(A) Number of differentially methylated regions (DMRs) and (B) number of differentially methylated positions (DMPs) comparing the control group (CG), endurance-trained group (EG) and strength-trained group (SG) in skeletal muscle at rest.*** *The red part of the bars represents the number of DMRs or DMPs with higher methylation and the blue part of the bars represents the number with lower methylation in the EG or SG compared to the CG or the EG to SG.*

Table 6: ***Differentially methylated regions (DMRs) at baseline.*** *A p-value threshold of <0.05 was set to identify DMRs. nGC = methylated Cytosine-phosphate-Guanine site; EG = endurance group; CG = control group; SG = strength group; areaStat, sum of the test statistics of all CpG sites within a DMR (larger areaStat is more likely to be a DMR).*

DMRs endurance group versus control group at baseline							
Gene	**Feature**	**Coordinates GRCh38/hg38**	**nCG**	**EG-DNA methylation**	**CG-DNA methylation**	**Methylation difference**	**areaStat**
MTOR	Enhancer (Intron)	chr1:11158468 -11158562	6	66%	57%	9%	20
MYOG	Promoter	chr1:203085967 -203087070	46	36%	29%	7%	129
MYF5	Promoter	chr12:80717111 -80717317	32	39%	34%	5%	67
FOXO1	Enhancer (Intron)	chr13:40598853 -40599581	14	76%	70%	7%	42
MYH7	Promoter	chr14:23435722 -23435895	9	18%	23%	-5%	-24
SMAD3	Intron	chr15:67098355 -67098798	23	30%	22%	8%	95
SMAD3	Intron	chr15:67165938 -67166065	7	27%	22%	5%	16
MEF2A	Enhancer (Intron)	chr15:99719115 -99719305	6	58%	47%	11%	20
MYH2	Promoter	chr17:10549780 -10550002	10	35%	29%	6%	28
MAPK12	Promoter	chr22:50262507 -50262805	14	42%	34%	8%	51
MYL3	Promoter	chr3:46863453 -46863927	14	56%	69%	-13%	-62

PPARGC1A	Proximal Promoter (ex1a_1)	chr4:23887966 -23888152	12	32%	25%	7%	36
PPARGC1A	Proximal Promoter (ex1a)	chr4:23890728 -23890998	9	12%	9%	3%	19
PPARGC1A	Alternative Promoter (ex1b)	chr4:23904902 -23904977	6	53%	42%	11%	27
FOXO3	Enhancer (Intron)	chr6:108650294 -108651584	19	56%	50%	6%	53
CREB5	Promoter (Intron)	chr7:28685405 -28685505	7	26%	21%	5%	20
DMRs strength group versus control group at baseline							
Gene	**Feature**	**Coordinates GRCh38/hg38**	**nCG**	**SG-DNA methylation**	**CG-DNA methylation**	**Methylation difference**	**areaStat**
MYOG	Promoter	chr1:203085687 -203085787	19	56%	46%	10%	54
MYOG	Promoter	chr1:203085852 -203085918	7	51%	42%	9%	15
MYOD1	Promoter	chr11:17718827 -17718883	7	16%	11%	5%	22
MYF5	Promoter	chr12:80716429 -80717047	48	31%	37%	-6%	-134
HIF1A	Promoter	chr14:61697813 -61697982	6	62%	69%	-7%	-17
SMAD3	Exon	chr15:67165276 -67165335	9	71%	75%	-4%	-15
SMAD3	Intron	chr15:67165398 -67165484	9	79%	84%	-5%	-18
CREB1	Promoter	chr2:207529068 -207529144	6	14%	19%	-4%	-13
DMRs endurance group versus strength group at baseline							
Gene	**Feature**	**Coordinates GRCh38/hg38**	**nCG**	**EG-DNA methylation**	**SG-DNA methylation**	**Methylation difference**	**areaStat**
MTOR	Enhancer (Intron)	chr1:11158468 -11158562	6	66%	50%	16%	26
MYF5	Promoter	chr12:80716429 -80717241	78	39%	30%	9%	319
FOXO1	Enhancer (Intron)	chr13:40599492 -40599582	6	86%	80%	5%	15
MYH7	Promoter	chr14:23435722 -23435895	9	18%	22%	-4%	-21
SMAD3	Intron	chr15:67098355 -67098799	24	32%	24%	8%	97
SMAD3	Intron	chr15:67165537 -67165630	8	47%	39%	8%	19
SMAD3	Intron	chr15:67165686 -67165773	7	55%	48%	7%	17
SMAD3	Intron	chr15:67165888 -67166060	7	29%	24%	5%	16
CREB1	Promoter	chr2:207529068 -207529144	6	19%	14%	4%	15
MAPK12	Enhancer	chr22:50248042 -50248138	6	58%	53%	4%	13

MYL3	Promoter	chr3:46863453 -46863927	14	56%	70%	-14%	-63
PPARGC1A	Proximal Promoter (ex1a_1)	chr4:23888049 -23888151	9	31%	26%	5%	18
FOXO3	Enhancer (Intron)	chr6:108650254 -108650370	6	78%	70%	7%	20

6.3.1 Baseline methylation patterns of myogenic regulatory factors

6.3.1.1 MYF5

The endurance group exhibited greater methylation of DMRs associated with the *MYF5* promoter than both the strength-trained and untrained participants (Figure 13A, Table 6), which positively correlated with type I fiber percentage (R = 0.50, FDR = 0.04, Appendix 2). The strength-trained group had the lowest methylation level, which was also significantly lower than that of the control group. Correlation analysis indicated no significant relationship between *MYF5* baseline expression and promoter DMR methylation (R = -0.23, FDR = 0.39, Figure 13B) (Geiger et al. 2024).

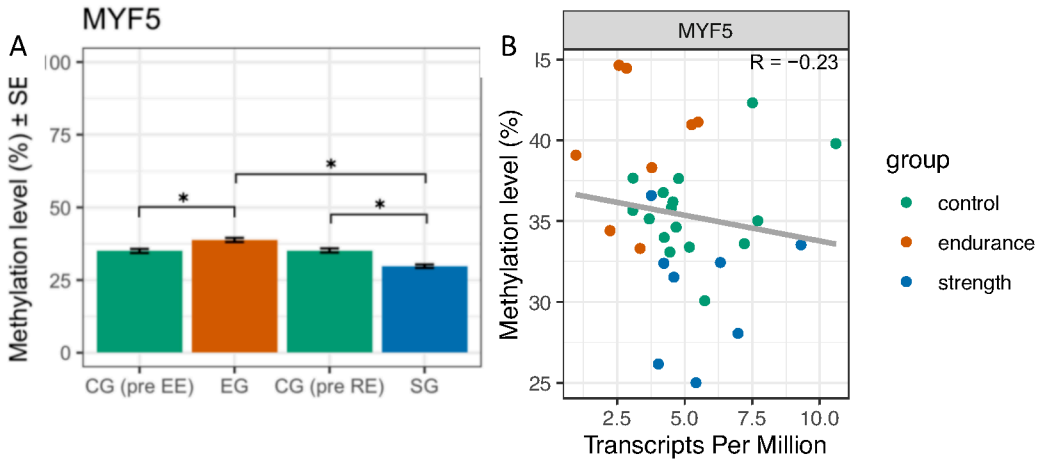

*Figure 13: **(A) Bar plot of MYF5 promoter DMRs at baseline comparing EG, SG and CG.** Overlapping DMRs from different group comparisons are plotted in the same figure (plotted area chr12:80716429-80717241). Results are mean ± SEM for n = 8 subjects. ∗ = p < 0.05. CG = control group, EG = endurance group, SG = strength group, EE = acute endurance exercise, RE = acute resistance exercise. See Table 6 for more details on DMRs. **(B) Scatter plot showing correlation between mean methylation of MYF5 promoter DMRs and MYF5 gene expression at baseline**. R = Spearman correlation coefficient. FDR = 0.39. Grey line = linear regression model including subjects from all groups. Geiger et al. 2024.*

6.3.1.2 MYOD1

Strength athletes exhibited higher methylation of a DMR associated with the *MYOD1* promoter compared to the control group (Figure 14A, Table 6), which correlated positively with leg strength (R = 0.66, FDR <0.01, Appendix 2). There was no significant correlation observed between *MYOD1* gene expression and promoter DMR methylation (R = 0.12, FDR = 0.63, Figure 14B) (Geiger et al. 2024).

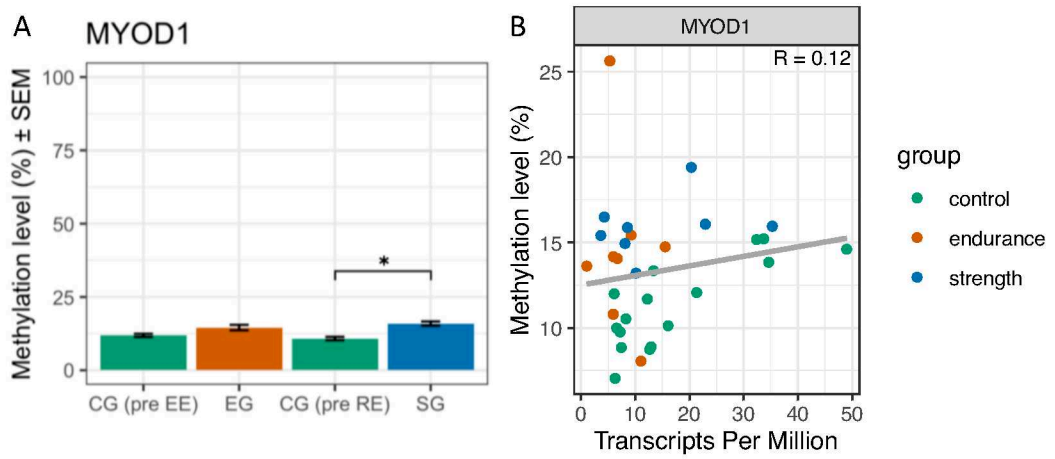

*Figure 14: (**A**) **Bar plot of MYOD1 promoter DMRs at baseline comparing EG, SG and CG.** Overlapping DMRs from different group comparisons are plotted in the same figure (plotted area chr11:17718827-17718883). Results are mean ± SEM for n = 8 subjects. ∗ = p < 0.05. CG = control group, EG = endurance group, SG = strength group, EE = acute endurance exercise, RE = acute resistance exercise. See Table 6 for more details on DMRs. (**B**) **Scatter plot showing correlation between mean methylation of MYOD1 promoter DMRs and MYOD1 gene expression at baseline**. R = Spearman correlation coefficient. FDR = 0.63). Grey line = linear regression model including subjects from all groups.*

6.3.1.3 MYOG

One DMR comparing EG and CG and two DMRs comparing SG and CG were identified around the *MYOG* promoter (Figure 15A, Table 6). Both endurance and strength-trained individuals exhibited higher methylation compared to the control group. Correlation analysis between *MYOG* baseline expression and methylation of the DMRs did not indicate a relationship between the two (R = 0.01, FDR = 0.98, Figure 15B). VO_2peak correlated positively with *MYOG* methylation (R = 0.42, FDR = 0.02, Appendix 2) (Geiger et al. 2024).

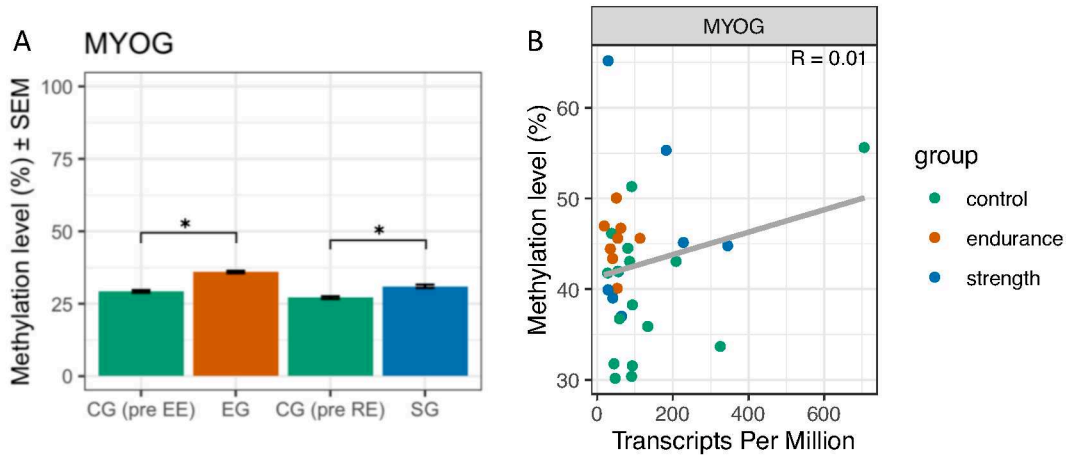

*Figure 15: (**A**) **Bar plot of MYOG promoter DMRs at baseline comparing EG, SG and CG.** Overlapping DMRs from different group comparisons are plotted in the same figure (plotted area chr1: 203085852-203087070). Results are mean ± SEM for n = 8 subjects. ∗ = p < 0.05. CG = control group, EG = endurance group, SG = strength group, EE = acute endurance exercise, RE = acute resistance exercise. See Table 6 for more details. (**B**) **Scatter plot showing correlation between mean methylation of MYOG promoter DMRs and MYOG gene expression at baseline**. R = Spearman correlation coefficient. FDR = 0.98. Grey line = linear regression model including subjects from all groups*

6.3.1.4 *MEF2A*

For *MEF2A* an intronic DMR with an enhancer associated chromatin state (Kundaje et al. 2015) was detected, showing an increased methylation state in endurance trained athletes compared to the control group (Figure 16A, Table 6). *MEF2A* expression at baseline showed no significant correlation with DMR methylation (R = -0.14, FDR = 0.58, Figure 16B) (Geiger et al. 2024).

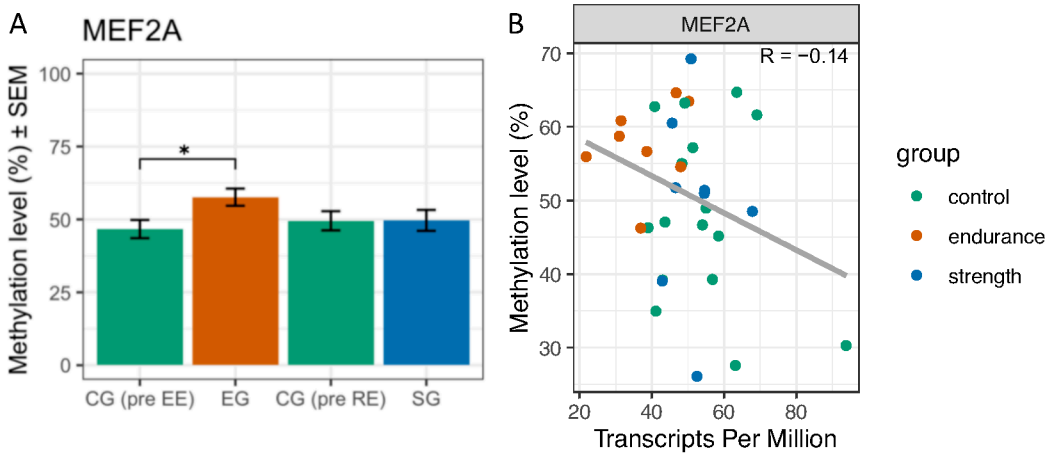

*Figure 16: (**A**) **Bar plot of intronic MEF2A DMR at baseline comparing EG, SG and CG** (plotted area chr15:99719115-99719305). Results are mean ± SEM for n = 8 subjects. ∗ = p < 0.05. CG = control group, EG = endurance group, SG = strength group, EE = acute endurance exercise, RE = acute resistance exercise. See Table 6 for more details. (**B**) **Scatter plots showing correlation between mean methylation of intronic MEF2A DMR and MEF2A gene expression at baseline**. R = Spearman correlation coefficient. FDR = 0.58. Grey line = linear regression model including subjects from all groups.*

6.3.2 Endurance-trained skeletal muscle exhibits differential DNA methylation of fiber-type specific genes

Endurance-trained athletes exhibited a hypomethylated DMR by the *MYH7* promoter and another by the *MYL3* promoter when compared to the strength and control groups (Figure 17A & 17B, Table 6). Within the *MYL3* DMR, individual DMPs were identified showing lower methylation in the EG (Appendix 1). Conversely, for *MYH2*, a DMR was observed with higher methylation levels in the EG relative to the CG (Figure 17C, Table 6). Spearman correlation analyses revealed a moderate negative association between promoter DMR methylation levels and baseline gene expression for *MYL3* (R = -0.53, FDR = 0.04; Figure 18). The associations between the expression of *MYH7* and *MYH2* and the methylation of their respective promoter DMRs were non-significant (*MYH7*: R = -0.18, FDR = 0.50; *MYH2*: R = -0.39, FDR = 0.11; Figure 18). A negative correlation between *MYH7* and *MYL3* promoter methylation with VO_2peak and type I fiber percentage was observed (*MYH7*/ VO_2peak: R = -0.45, FDR = 0.02; *MYH7*/ type I fiber percentage: R = -0.62, FDR <0.01; *MYL3*/ VO_2peak: R = -0.51, FDR = 0.01; *MYL3*/ type I fiber percentage: R = -0.83, FDR <0.01; Appendix 2), while *MYH2* methylation correlated positively with VO_2peak (*MYH2*/ VO_2peak: R = 0.54, FDR <0.01; Appendix 2). There was no significant correlation between leg strength and *MYL3*, *MYH7* and *MYH2* promoter methylation (Appendix 2) (Geiger et al. 2024).

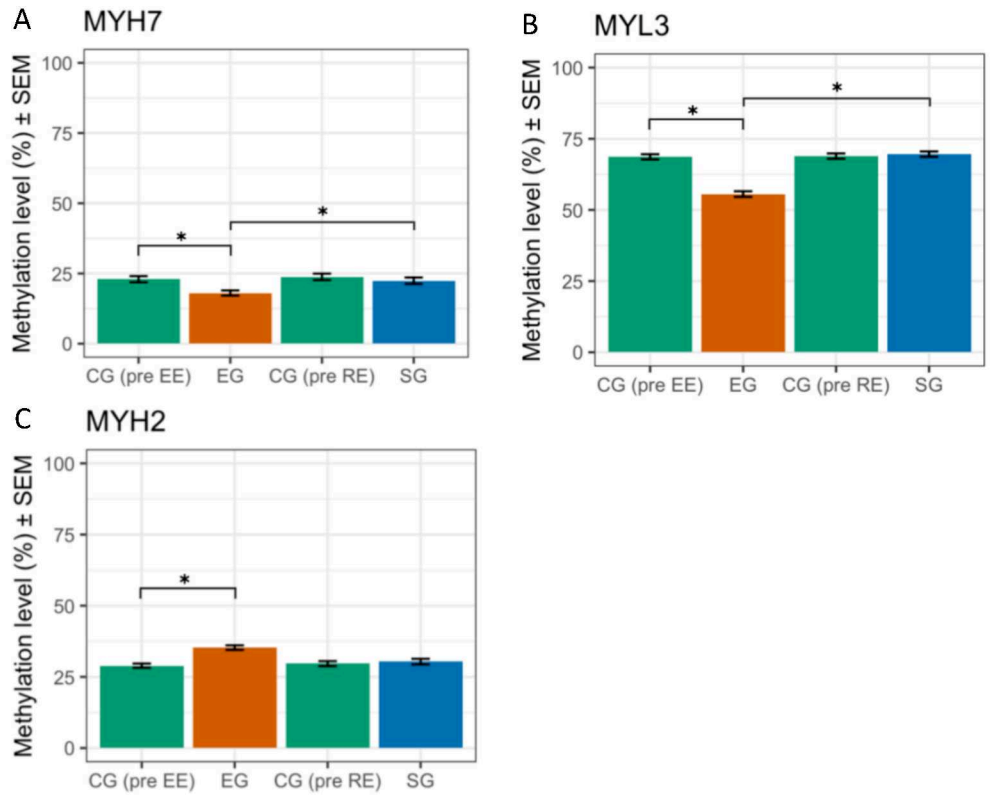

Figure 17: ***Bar plots of fiber-type specific gene DMRs at baseline comparing EG, SG and CG.*** *(**A**) MYH7 promoter (plotted area chr14:23435722-23435895) (**B**) MYL3 promoter (plotted area chr3:46863453-46863927) (**C**) MYH2 promoter (plotted area chr17:10549780-10550002). Overlapping DMRs from different group comparisons are plotted in the same figure. Results are mean ± SEM for n = 8 subjects. ∗ = p < 0.05. CG = control group, EG = endurance group, SG = strength group, EE = acute endurance exercise, RE = acute resistance exercise. See Table 6 for more details on DMRs. Geiger et al. 2024.*

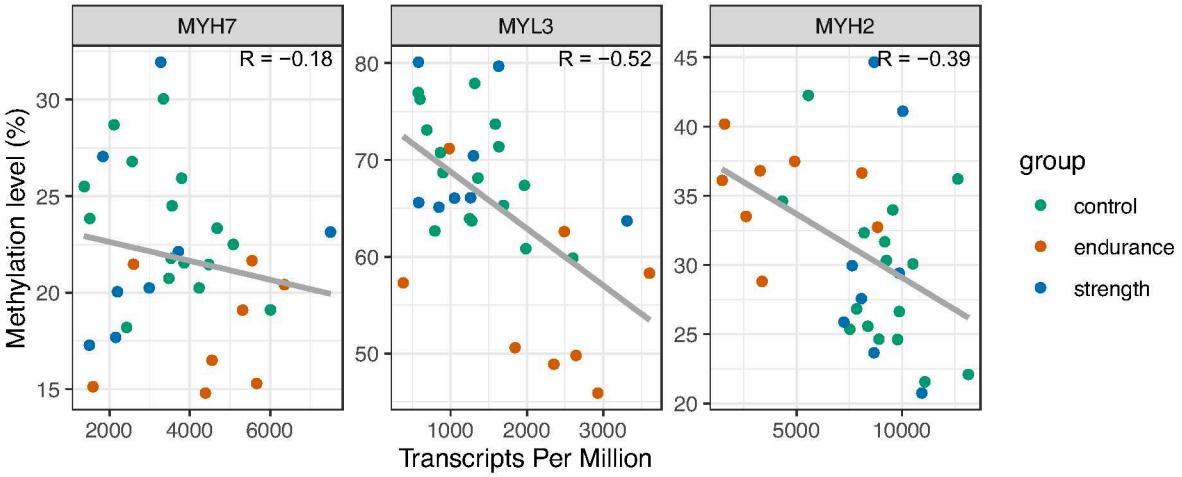

Figure 18: ***Scatter plots showing correlation between mean methylation of MYH7, MYL3 and MYH2 promoter DMRs and their respective gene expression at baseline****. R = Spearman correlation coefficient. FDR/MYH7 = 0.5; FDR/MYL3 = 0.39; FDR/MYH2 = 0.11. Grey line = linear regression model including subjects from all groups. Geiger et al. 2024.*

6.3.3 Differential DNA methylation of signalling molecules and pathways regulating protein turnover and muscle adaptation

6.3.3.1 MTOR

Differential methylation was identified for an intron of the *MTOR* gene, marked by histone modifications indicative of regulatory enhancer-associated properties (Kundaje et al. 2015). The EG exhibited higher methylation levels compared to both the CG and SG (Figure 19A, Table 6). Baseline MTOR gene expression did not correlate with methylation status of the DMR (R = -0.09, FDR = 0.7, Figure 19B). Methylation status correlated however positively with VO_2peak (R = 0.43, FDR = 0.02, Appendix 2) (Geiger et al. 2024).

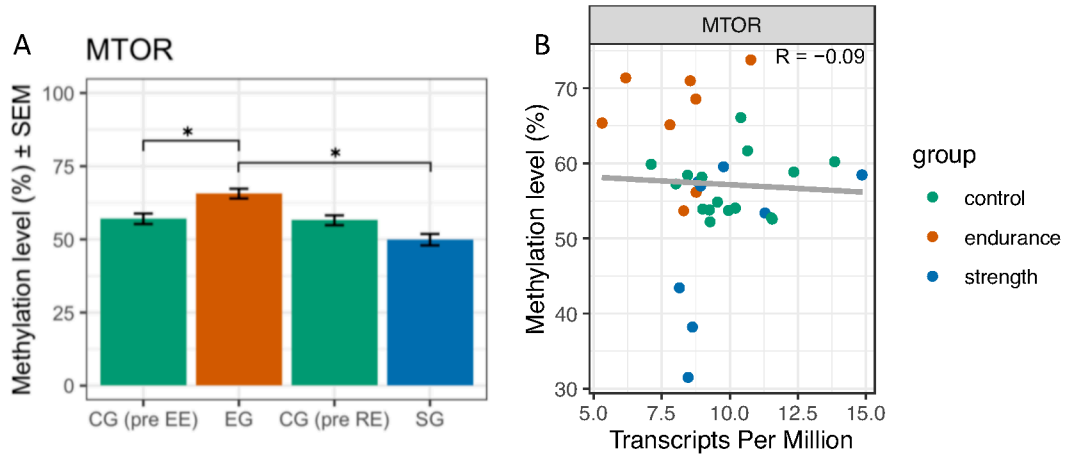

*Figure 19: (**A**) **Bar plot of intronic MTOR DMRs at baseline comparing EG, SG and CG.** Overlapping DMRs from different group comparisons are plotted in the same figure (plotted area chr1:11158468-11158562). Results are mean ± SEM for n = 8 subjects. ∗ = p < 0.05. CG = control group, EG = endurance group, SG = strength group, EE = acute endurance exercise, RE = acute resistance exercise. See Table 6 for more details. (**B**) **Scatter plot showing correlation between mean methylation of intronic MTOR DMRs and MTOR gene expression at baseline**. R = Spearman correlation coefficient. FDR = 0.7. Grey line = linear regression model including subjects from all groups.*

6.3.3.2 SMAD3

Six distinct intragenic DMRs were identified within the *SMAD3* gene. Of these, two DMRs demonstrated higher mean methylation levels in EG compared to both the CG and SG. Another two regions exhibited higher mean methylation in the EG compared to the SG, with the CG showing intermediate methylation values. The last two DMRs showed significant differences in methylation only between the CG and SG, with the SG displaying lower methylation. Within these DMRs, four individual DMPs were identified when comparing the EG and SG, and three DMPs were found when comparing the EG and CG (Appendix 1). Figure 20 illustrates DMRs for two intragenic *SMAD3* regions (see Table 6 for additional details on all DMRs). VO_2peak correlated positively with methylation status (R = 0.45, FDR = 0.02, Appendix 2) while no significant correlation was observed between DMR methylation and gene expression (R = -0.37, FDR = 0.11, Figure 21) (Geiger et al. 2024).

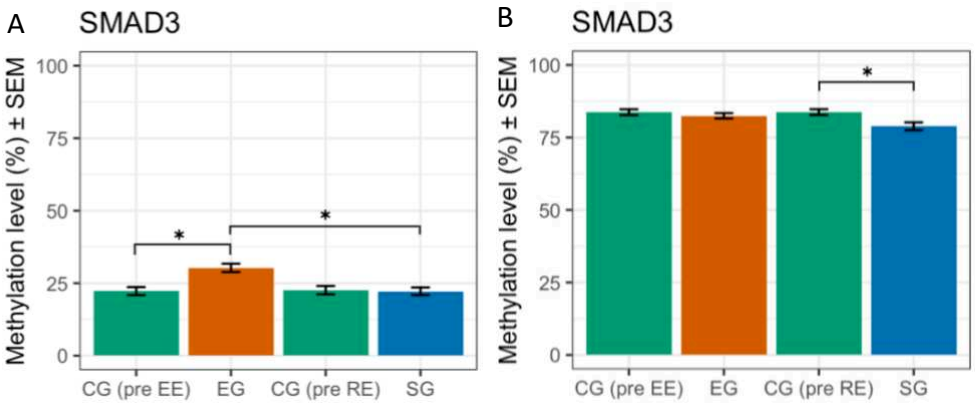

*Figure 20: **Bar plots of SMAD3 DMRs at baseline comparing EG, SG and CG.** (**A**) SMAD3 (plotted area chr15:67098355-67098798) (**B**) SMAD3 (plotted chr15:67165398-67165484). Overlapping DMRs from different group comparisons are plotted in the same figure. Results are mean ± SEM for n = 8 subjects. ∗ = p < 0.05. CG = control group, EG = endurance group, SG = strength group, EE = acute endurance exercise, RE = acute resistance exercise. See Table 6 for more details on DMRs.*

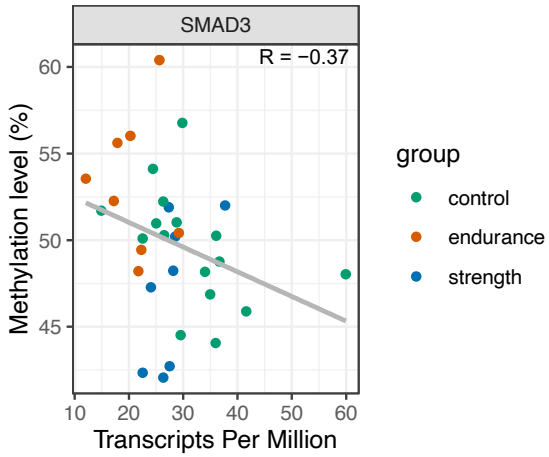

*Figure 21: **Scatter plot showing correlation between mean methylation of intragenic SMAD3 DMRs and SMAD3 gene expression at baseline**. R = Spearman correlation coefficient. FDR = 0.11. Grey line = linear regression model including subjects from all groups.*

6.3.3.3 MAPK12

A DMR about 780 bp upstream of the *MAPK12* promoter showed higher methylation in endurance-trained men compared to untrained men (Figure 22A, Table 6). There was no significant relationship between DMR methylation and *MAPK12* gene expression at baseline (R = -0.39, FDR = 0.11, Figure 22B). Methylation levels positively correlated with VO_2peak (R = 0.69, FDR < 0.01, Appendix 2).

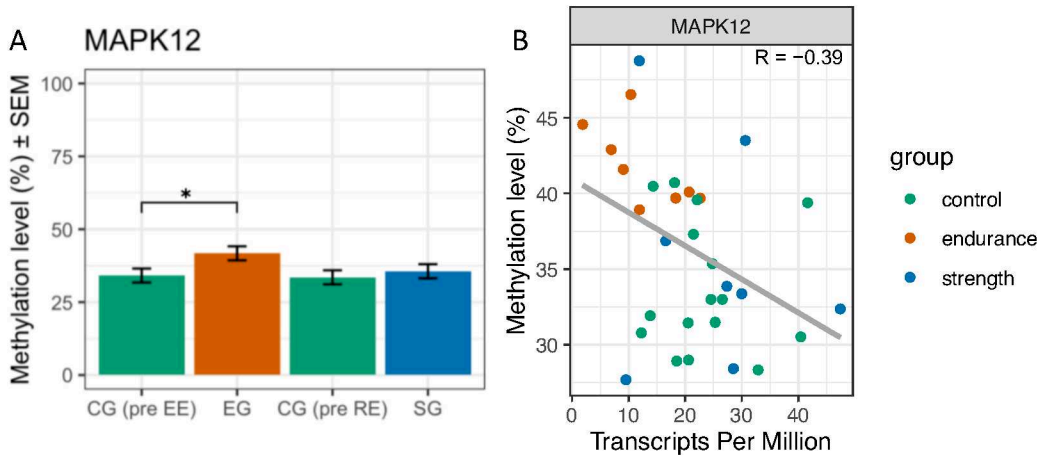

Figure 22: ***Bar plots of MAPK12 associated DMR at baseline comparing EG, SG and CG.*** *(**A**) MAPK12 associated DMR upstream if MAPK12 promoter (plotted area chr22:50262507-50262805). Results are mean ± SEM for n = 8 subjects. ∗ = p < 0.05. CG = control group, EG = endurance group, SG = strength group, EE = acute endurance exercise, RE = acute resistance exercise. See Table 6 for more details on DMRs. (**B**) **Scatter plot showing correlation between mean methylation of MAPK12 gene expression and DMR at baseline**. R = Spearman correlation coefficient. FDR = 0.11. Grey line = linear regression model including subjects from all groups.*

A second DMR with potential enhancer activity, associated with the *MAPK12* locus, was found with lower methylation in strength-trained men compared to endurance-trained men (Figure 23A, Table 6). The methylation status of this DMR did not show a significant correlation with *MAPK12* gene expression levels (R = -0.09, FDR = 0.64, Figure 23B). Additionally, this DMR is situated within an exon of the *HDAC10* gene; however, the correlation analysis revealed a negligible association between the methylation of this DMR and the expression of the *HDAC10* gene (R = 0.02, FDR = 0.98) (Geiger et al. 2024).

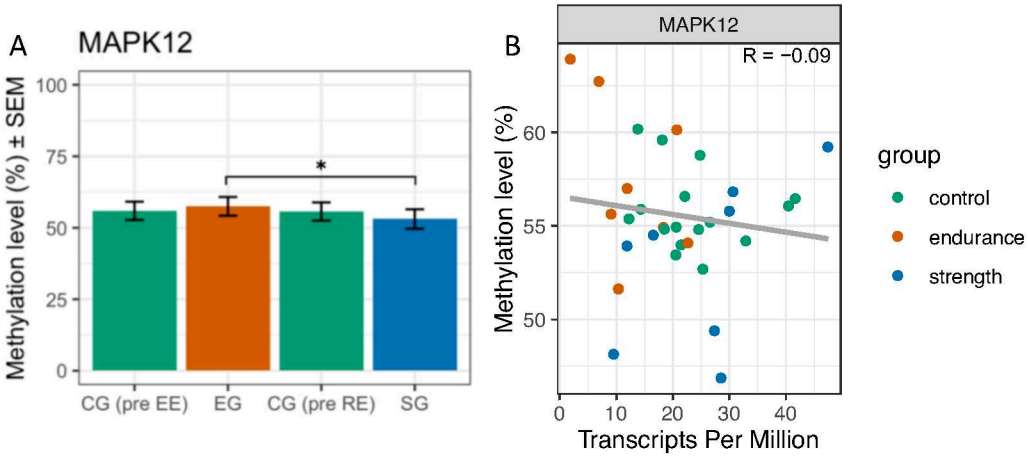

Figure 23: ***Bar plots of MAPK12 associated DMR at baseline comparing EG, SG and CG.*** *(**A**) MAPK12 associated DMR with potential enhancer activity (plotted area chr22:50248042-50248138). Results are mean ± SEM for n = 8 subjects. ∗ = p < 0.05. CG = control group, EG = endurance group, SG = strength group, EE = acute endurance exercise, RE = acute resistance exercise. See Table 6 for more details on DMRs. (**B**) **Scatter plot showing correlation between mean methylation of MAPK12 gene expression and DMR at baseline**. R = Spearman correlation coefficient. FDR = 0.64. Grey line = linear regression model including subjects from all groups.*

6.3.4 Transcriptional regulators are differentially methylated in trained and untrained skeletal muscle

6.3.4.1 HIF1A

An intronic DMR associated with an alternative promoter of the *HIF1A* gene displayed lower methylation levels in strength-trained athletes compared to untrained men (Figure 24A, Table 6) and no significant correlation to *HIF1A* gene expression at baseline (R = 0.17, FDR = 0.5, Figure 24B) (Geiger et al. 2024).

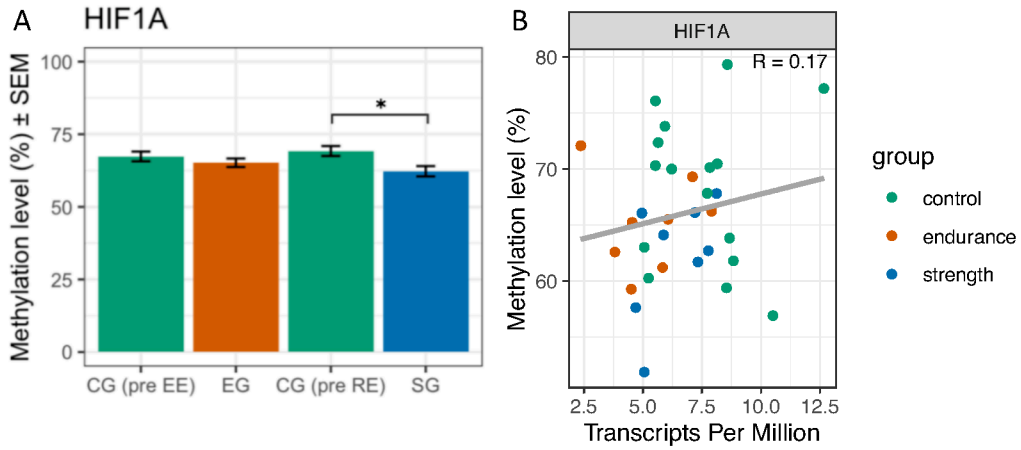

*Figure 24: (**A**) **Bar plot of intronic HIF1A DMR at baseline comparing EG, SG and CG.** Plotted area chr14:61697813-61697982. Results are mean ± SEM for n = 8 subjects. ∗ = p < 0.05. CG = control group, EG = endurance group, SG = strength group, EE = acute endurance exercise, RE = acute resistance exercise. See Table 6 for more details. (**B**) **Scatter plot showing correlation between mean methylation of HIF1A DMR and gene expression at baseline**. R = Spearman correlation coefficient. FDR = 0.5. Grey line = linear regression model including subjects from all groups.*

6.3.4.2 CREB1 and CREB5

The SG exhibited lower methylation levels for a DMR located within a *CREB1* promoter in comparison to both the CG and EG (Figure 25A, Table 6). Hypomethylation correlated with lower type I fiber percentage (R = 0.49, FDR = 0.04, Appendix 2). No significant correlation was observed between the mean methylation of this promoter DMR and *CREB1* gene expression at baseline (R = 0.21, FDR = 0.43, Figure 25C).
An intronic DMR near the *CREB5* promoter with higher methylation in the EG compared to the CG was identified (Figure 25B, Table 6). There was no significant correlation between mean methylation of this DMR and *CREB5* baseline expression (R = -0.38, FDR = 0.11, Figure 25C). However, a positive correlation between *CREB5* methylation and VO_2peak was detected (R = 0.59, FDR < 0.01, Appendix 2) (Geiger et al. 2024).

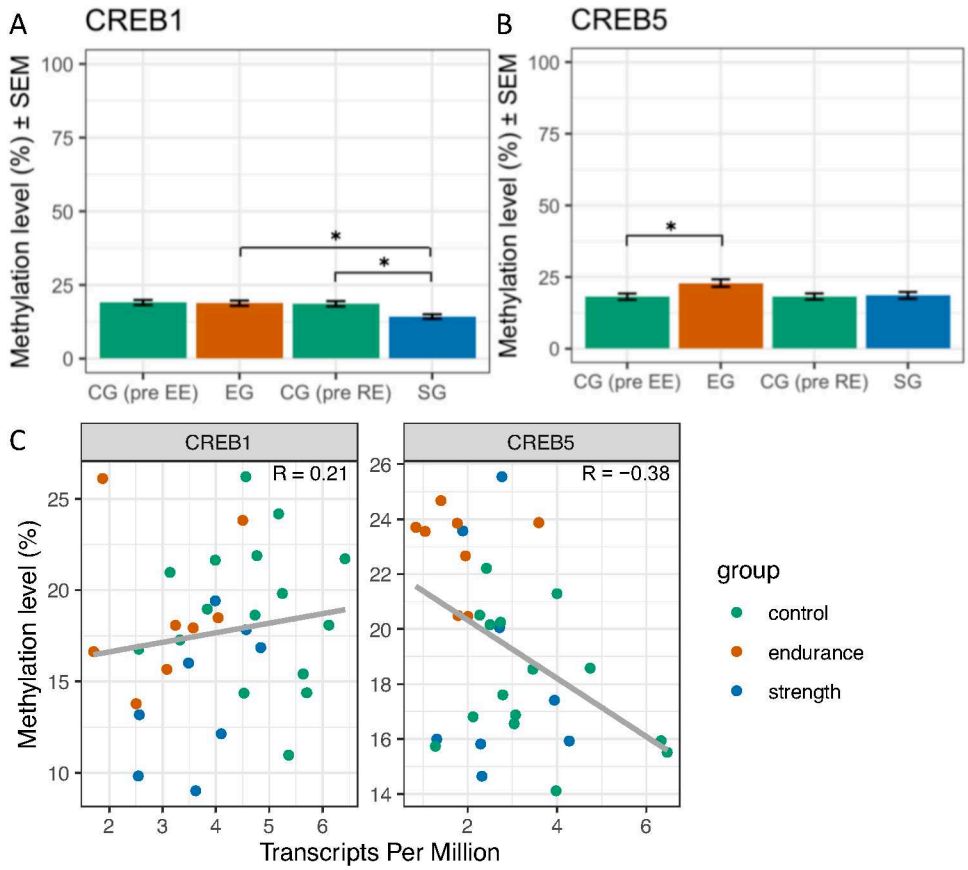

Figure 25: ***Bar plots of CREB1 and CREB5 DMRs at baseline comparing EG, SG and CG.*** *(**A**) CREB1 promoter DMRs (plotted area chr2:207529068-207529144) (**B**) intronic CREB5 DMR (plotted area chr7:28685405-28685505). Overlapping DMRs from different group comparisons are plotted in the same figure. Results are mean ± SEM for n = 8 subjects. ∗ = p < 0.05. CG = control group, EG = endurance group, SG = strength group, EE = acute endurance exercise, RE = acute resistance exercise. See Table 6 for more details on DMRs. (**C**) **Scatter plot showing correlation between mean methylation of CREB1 and CREB5 DMRs and respective gene expression at baseline**. R = Spearman correlation coefficient. FDR/CREB1 = 0.43. FDR/CREB5 = 0.11. Grey line = linear regression model including subjects from all groups. Geiger et al. 2024.*

6.3.4.3 *FOXO1 and FOXO3*

For both *FOXO1* and *FOXO3*, DMRs situated within an intron were found (Figure 26A and 24B, Table 6). The EG displayed higher methylation levels for these DMRs compared to both the CG and SG. Mean methylation for *FOXO1* and *FOXO3* didn't show a strong correlation with gene expression (*FOXO1*: R = -0.25, FDR = 0.36; *FOXO3*: R = -0.30, FDR = 0.22, Figure 26C). However, both *FOXO1* and *FOXO3* correlated with VO_2peak (*FOXO1*: R = 0.70, FDR < 0.01; *FOXO3*: R = 0.71, FDR < 0.01, Appendix 2) and *FOXO3* methylation also with type I fiber percentage (R = 0.63, FDR = 0.01, Appendix 2) (Geiger et al. 2024).

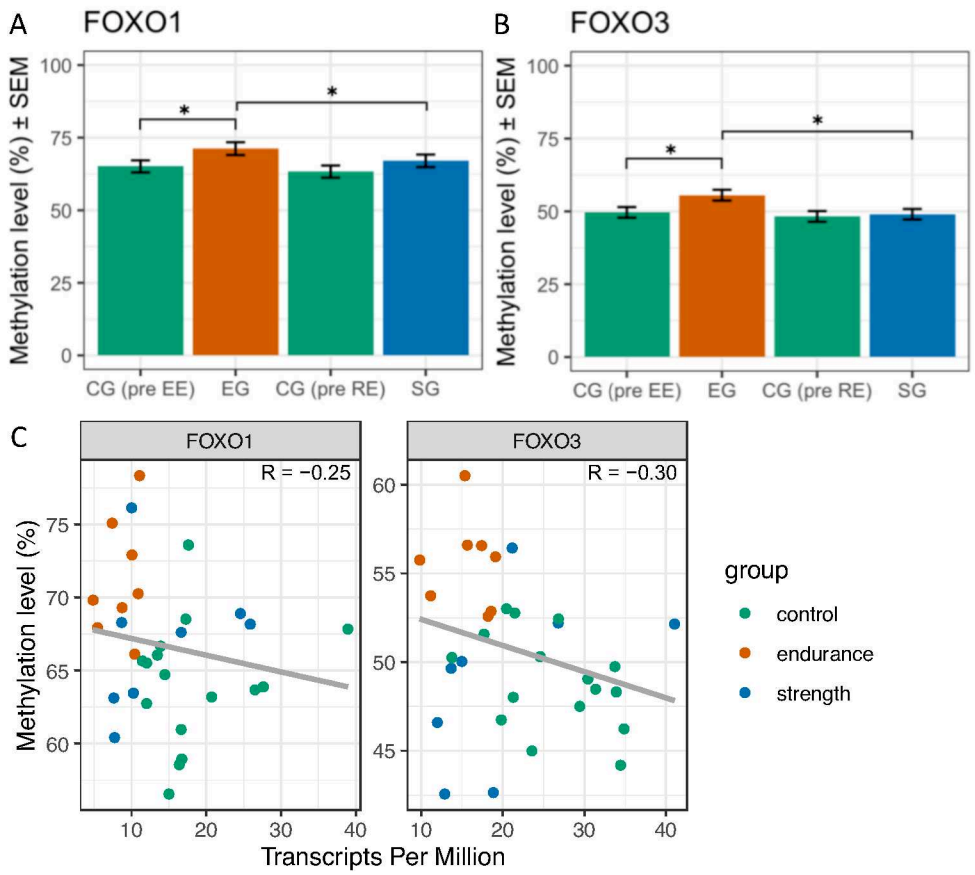

Figure 26: ***Bar plots of intragenic FOXO1 and FOXO3 DMRs at baseline comparing EG, SG and CG.*** *(**A**) FOXO1 (plotted area chr13:40598853-40599581) (**B**) FOXO3 (plotted area chr6:108650294-108651584). Overlapping DMRs from different group comparisons are plotted in the same figure. Results are mean ± SEM for n = 8 subjects. ∗ = p < 0.05. CG = control group, EG = endurance group, SG = strength group, EE = acute endurance exercise, RE = acute resistance exercise. See Table 6 for more details on DMRs. (**C**) **Scatter plot showing correlation between mean methylation of intragenic FOXO1 and FOXO3 DMRs and respective gene expression at baseline**. R = Spearman correlation coefficient. FDR/FOXO1 = 0.36. FDR/FOXO3 = 0.22. Grey line = linear regression model including subjects from all groups. Geiger et al. 2024.*

6.3.5 PGC1α promoter methylation and associated isoform expression

Endurance-trained athletes had a significantly higher methylated DMR in the *PGC1α (PPARGC1A)* proximal promoter region compared to the CG (Figure 27A, Table 6) and another one being significantly higher methylated compared to the SG and CG (Figure 27B, Table 6). The EG also exhibited a significantly higher methylated DMR in the *PGC1α* alternative promoter region compared to the CG (Figure 27C, Table 6). Correlation analysis showed a non-significant moderate negative relationship between *PGC1α* expression at baseline and mean methylation across the *PGC1α* promoter DMRs (R = -0.42, FDR = 0.11, Figure 27D). Methylation levels correlated positively with VO_2peak (R = 0.64, FDR < 0.01, Appendix 2) (Geiger et al. 2024).

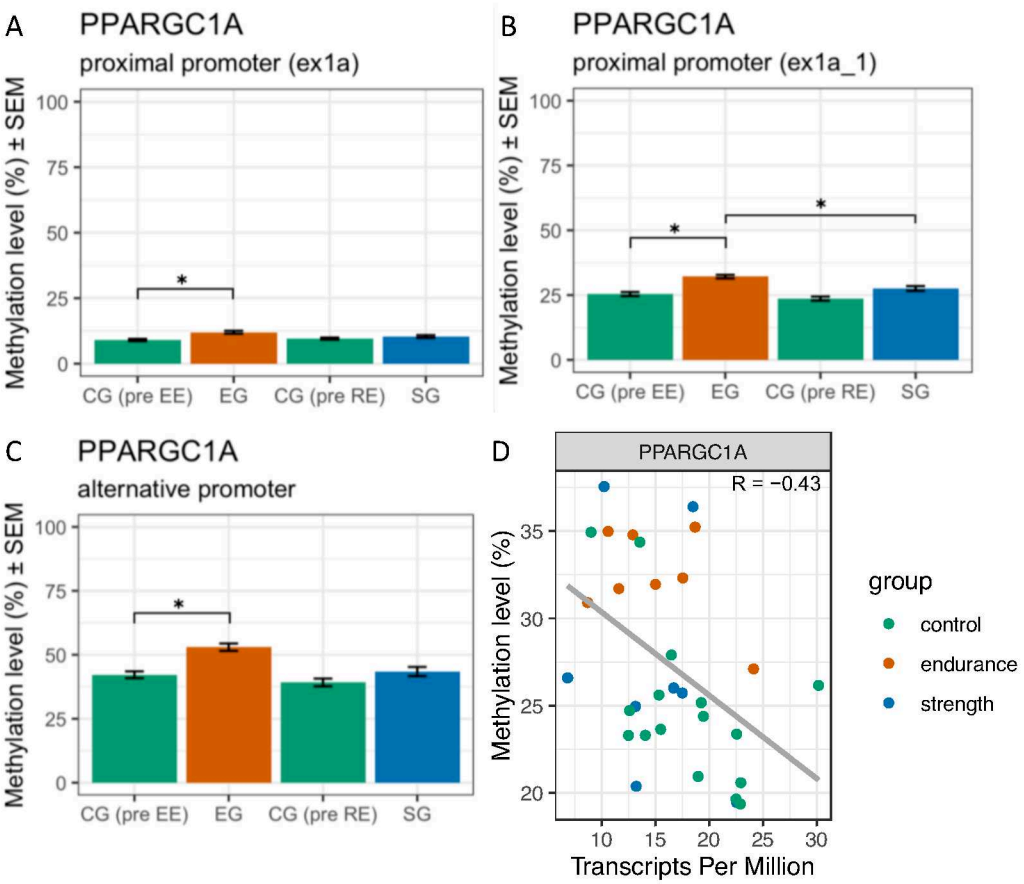

Figure 27: ***Bar plots of PGC1a promoter DMRs at baseline comparing EG, SG and CG.*** *(**A**) proximal promoter DMR (ex1a) (area plotted chr4:23890728-23890998) (**B**) Proximal promoter DMR (ex1a_1) (area plotted chr4:23887966-23888152) (**C**) alternative promoter DMR (area plotted chr4:23904902-23904977). Overlapping DMRs from different group comparisons are plotted in the same figure. Results are mean ± SEM for n = 8 subjects. ∗ = p < 0.05. CG = control group, EG = endurance group, SG = strength group, EE = acute endurance exercise, RE = acute resistance exercise. See Table 6 for more details on DMRs. (**D**)* ***Scatter plot showing correlation between mean methylation of PGC1a promoter DMRs and PGC1a gene expression at baseline****. R = Spearman correlation coefficient. FDR = 0.11. Grey line = linear regression model including subjects from all groups. Geiger et al. 2024.*

6.3.5.1 *Baseline expression of PGC-1α transcripts in skeletal muscle*

Baseline expression of *PGC1α* mRNA in skeletal muscle was comparable between trained and untrained men at rest (Figure 28, Table 7, Appendix 3). Distinguishing between different *PGC1α* isoforms revealed that the baseline transcription of *PGC1α* mostly happened from the proximal promoter and produced nontruncated *PGC1α* forms. *PGC1α* mRNA from the alternative promoter was nondetectable at rest. The mRNA levels of truncated *PGC1α* variants were minimal in rested muscle of all three groups (Geiger et al. 2024).

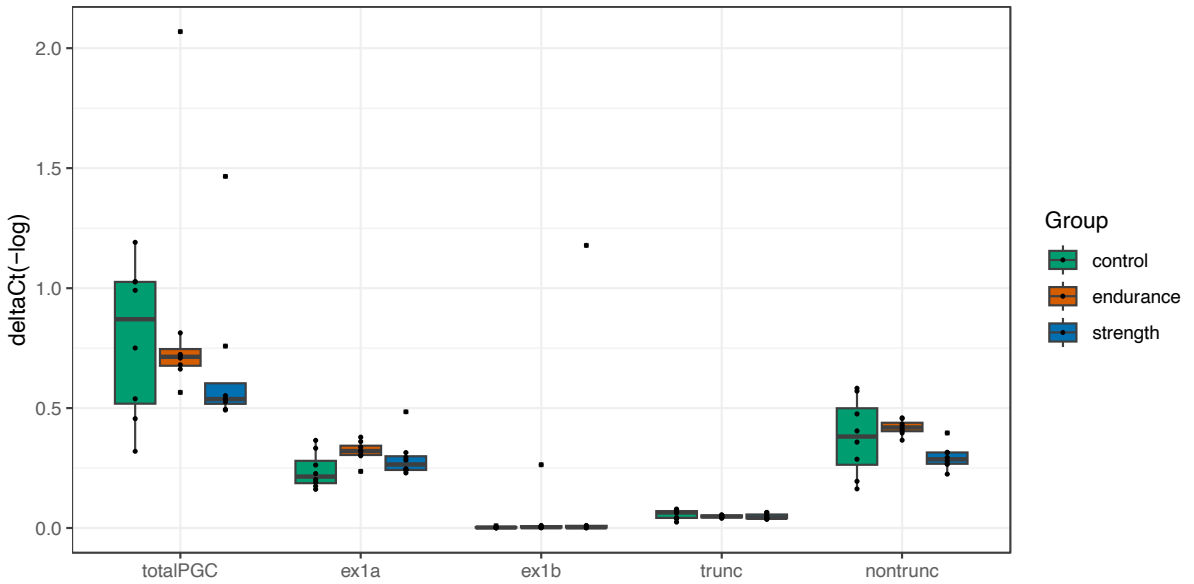

Figure 28: ***Baseline PGC1α Isoform expression PGC1α Isoform expression in skeletal muscle of trained endurance and resistance athletes and healthy untrained control individuals****. No significant differences could be detected. totalPGC = all transcripts, ex1a = proximal promoter transcripts, ex1b = alternative promoter transcripts, trunc = truncated transcripts, non-trunc = full-length transcripts.*

Table 7: ***Baseline Expression of PGC1α Transcripts****. Summary of baseline expression levels of PGC1α transcripts for each group, with values calculated as negative log-transformed delta CT relative to the endogenous control*

Group	Transcript	Median	IQR	Mean	SEM
control	totalPGC	0.87	0.51	0.79	0.11
control	ex1a	0.21	0.09	0.24	0.03
control	ex1b	0	0	0	0
control	trunc	0.07	0.03	0.06	0.01
control	nontrunc	0.38	0.24	0.38	0.06
endurance	totalPGC	0.71	0.07	0.87	0.17
endurance	ex1a	0.32	0.04	0.32	0.02
endurance	ex1b	0	0.01	0.04	0.03
endurance	trunc	0.05	0.01	0.05	0
endurance	nontrunc	0.42	0.04	0.42	0.01
strength	totalPGC	0.54	0.09	0.67	0.12
strength	ex1a	0.27	0.06	0.29	0.03
strength	ex1b	0	0.01	0.15	0.15
strength	trunc	0.04	0.02	0.05	0
strength	nontrunc	0.29	0.05	0.29	0.02

6.3.5.2 Expression of PGC1α splice variants after acute endurance and resistance exercise

After both endurance and resistance exercises, all study groups, including trained and untrained men, showed an increase in the gene expression of *PGC1α* transcripts compared to resting levels (Figure 29, Table 8, Appendix 4) (Geiger et al. 2024).

Specifically, an increase in total *PGC1α* mRNA was recorded one and three hours after endurance exercises across all groups. Following resistance exercise, a significant increase in expression was exclusive to the strength-trained group at the one-hour mark but extended to all groups by the three-hour mark. The strength-trained individuals demonstrated a significantly greater gene expression fold change at the one-hour and three-hour post-resistance exercise timepoints compared to the endurance and control groups (Figure 29, Appendix 5).

Focusing on the *PGC1α-ex1α* transcripts, originating from the proximal promoter, an elevation at one- and three-hours post-endurance exercise in all groups was observed. In contrast, after resistance exercise, only the strength group and control group had significant increases at both timepoints. The endurance group did not show a significant rise in *PGC1α-ex1α* levels after resistance exercise (Figure 29, Appendix 4). Additionally, a direct comparison of the responses to exercises highlighted a comparatively lower fold increase for the endurance group at one-hour post-resistance exercise, particularly against the strength group's response (Figure 29, Appendix 5).

For the alternative promoter transcripts, *PGC1α*-ex1b, which were barely detectable at rest and immediately after exercise, gene expression induction at one and three hours post-exercise in all forms of training was observed (Figure 29, Appendix 4). While the expression changes were modest directly post exercise, they were significant when contrasting the control and strength groups for endurance and the endurance and strength groups for resistance exercises. Notably, three hours after endurance exercise, the control group exhibited a significantly greater fold increase than the endurance group (Figure 29, Appendix 5).

The exercise-induced changes in truncated and non-truncated *PGC1α* variants largely mirrored each other, with significant upregulation occurring three hours post-exercise for all groups and both exercise types (Figure 29, Appendix 4). Remarkably, the strength group showed an early response with a significant increase in the truncated variants one hour after resistance and the non-truncated variants one hour after endurance exercises. This group also had a significantly greater increase in both transcript variants three hours after resistance exercise compared to the endurance and control groups (Figure 29, Appendix 5).

When comparing the responses to endurance versus resistance exercises within each group, the only statistically significant finding was in the control group for non-truncated transcripts at the three-hour mark, where a greater response was induced after endurance compared to resistance exercise (Figure 29, Appendix 6) .

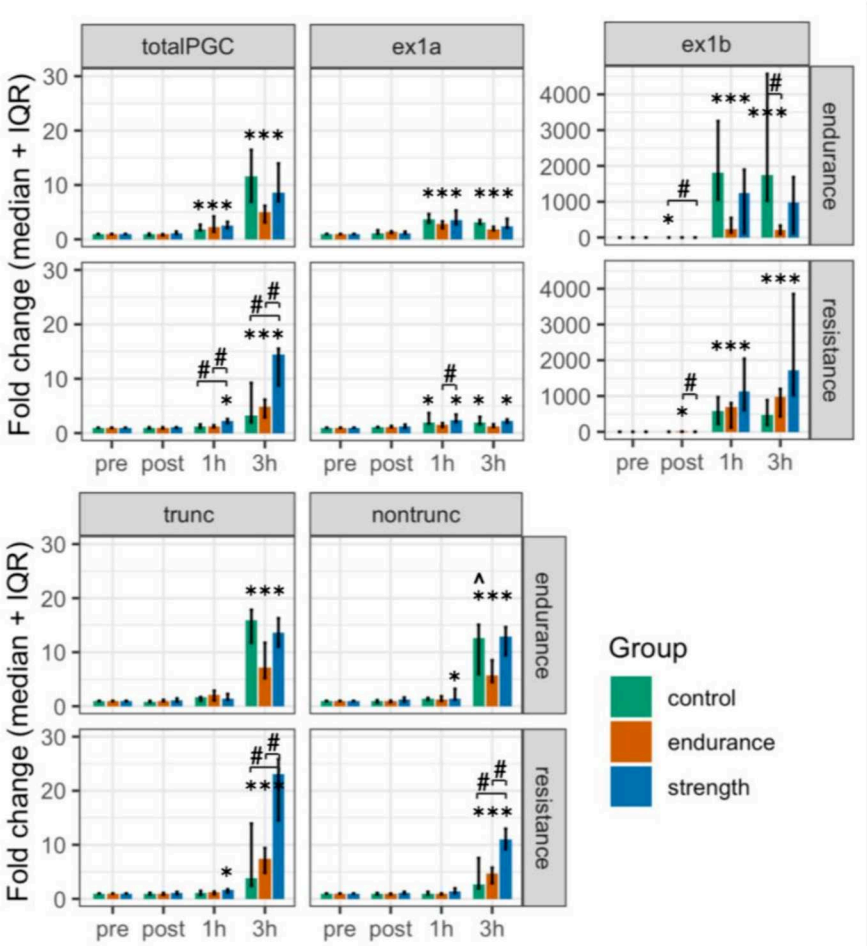

Figure 29: ***Fold change of PGC1α expression in skeletal muscle after endurance and resistance exercise compared to expression levels before the exercise session****. * = timepoint vs pre within group, # = difference of fold change between groups doing the same form of exercise, ^. = endurance exercise vs resistance exercise within the group; FDR < 0.05*

Table 8: ***Fold Change in PGC1α Transcript Expression After Exercise.*** *Summary data showing the fold change in PGC1α transcript expression levels for each group immediately post-exercise and at 1 hour and 3 hours after exercise, relative to pre-exercise levels. IQR = Interquartile range; SEM = Standard error of the mean.*

Timepoint	Group	Intervention	Gene	Median	IQR	Mean	SEM
post	control	endurance	*totalPGC*	0.99	0.31	0.94	0.12
post	control	endurance	*ex1a*	1.07	0.64	1.31	0.14
post	control	endurance	*ex1b*	1.85	0.99	2.59	0.74
post	control	endurance	*trunc*	0.87	0.23	0.86	0.08
post	control	endurance	*nontrunc*	0.99	0.44	0.95	0.13
post	control	resistance	*totalPGC*	0.93	0.24	1.17	0.28
post	control	resistance	*ex1a*	1.07	0.08	1.44	0.38
post	control	resistance	*ex1b*	1.88	0.77	1.72	0.3
post	control	resistance	*trunc*	1	0.31	1.37	0.46
post	control	resistance	*nontrunc*	0.91	0.3	1.13	0.24
post	endurance	endurance	*totalPGC*	0.95	0.21	0.93	0.16
post	endurance	endurance	*ex1a*	1.39	0.32	1.32	0.11

post	endurance	endurance	*ex1b*	1.03	0.53	2.49	1.42
post	endurance	endurance	*trunc*	0.99	0.28	1.09	0.1
post	endurance	endurance	*nontrunc*	0.92	0.29	0.93	0.08
post	endurance	resistance	*totalPGC*	0.94	0.23	1.02	0.11
post	endurance	resistance	*ex1a*	1.13	0.25	1.25	0.11
post	endurance	resistance	*ex1b*	3.16	3.86	52.52	48.61
post	endurance	resistance	*trunc*	0.93	0.34	0.94	0.08
post	endurance	resistance	*nontrunc*	0.9	0.18	0.91	0.08
post	strength	endurance	*totalPGC*	1.07	0.49	1.19	0.13
post	strength	endurance	*ex1a*	1.19	0.42	1.22	0.09
post	strength	endurance	*ex1b*	1.22	0.68	1.33	0.23
post	strength	endurance	*trunc*	1.15	0.6	1.52	0.44
post	strength	endurance	*nontrunc*	1.2	0.76	1.46	0.3
post	strength	resistance	*totalPGC*	1.04	0.13	1.11	0.11
post	strength	resistance	*ex1a*	1.2	0.6	1.34	0.19
post	strength	resistance	*ex1b*	0.9	1.4	2.87	1.72
post	strength	resistance	*trunc*	1.12	0.45	1.08	0.13
post	strength	resistance	*nontrunc*	1.11	0.39	1.18	0.12
1h	control	endurance	*totalPGC*	1.89	1.04	2.48	0.54
1h	control	endurance	*ex1a*	3.77	1.62	3.86	0.48
1h	control	endurance	*ex1b*	1809.21	2202.66	2360.74	721.57
1h	control	endurance	*trunc*	1.69	0.69	1.66	0.31
1h	control	endurance	*nontrunc*	1.34	0.39	1.44	0.21
1h	control	resistance	*totalPGC*	1.18	0.66	1.49	0.32
1h	control	resistance	*ex1a*	1.97	2	2.44	0.48
1h	control	resistance	*ex1b*	574.94	754.97	822.77	322.33
1h	control	resistance	*trunc*	1.03	0.75	1.48	0.45
1h	control	resistance	*nontrunc*	0.92	0.52	1.12	0.19
1h	endurance	endurance	*totalPGC*	2.21	2.82	2.64	0.66
1h	endurance	endurance	*ex1a*	2.8	1.27	2.62	0.44
1h	endurance	endurance	*ex1b*	239.69	416.26	510.24	238.78
1h	endurance	endurance	*trunc*	2.18	1.8	2.13	0.45
1h	endurance	endurance	*nontrunc*	1.28	0.84	1.36	0.22
1h	endurance	resistance	*totalPGC*	1.19	0.41	1.26	0.12
1h	endurance	resistance	*ex1a*	1.51	0.73	1.43	0.17
1h	endurance	resistance	*ex1b*	685.35	686.55	574.3	161.62
1h	endurance	resistance	*trunc*	1.22	0.54	1.21	0.16
1h	endurance	resistance	*nontrunc*	0.92	0.22	0.92	0.05
1h	strength	endurance	*totalPGC*	2.54	1.17	2.85	0.4
1h	strength	endurance	*ex1a*	3.49	2.56	3.75	0.57
1h	strength	endurance	*ex1b*	1251.71	1784.66	1360.19	484.37
1h	strength	endurance	*trunc*	1.45	1.05	1.87	0.39
1h	strength	endurance	*nontrunc*	1.45	2.03	2.27	0.54
1h	strength	resistance	*totalPGC*	2.27	0.75	2.18	0.19

1h	strength	resistance	*ex1a*	2.39	1.39	2.94	0.54
1h	strength	resistance	*ex1b*	1141.71	1439.28	2308.22	1150.73
1h	strength	resistance	*trunc*	1.62	0.59	1.67	0.22
1h	strength	resistance	*nontrunc*	1.31	0.91	1.47	0.19
3h	control	endurance	*totalPGC*	11.55	9.54	12.77	2.79
3h	control	endurance	*ex1a*	3.18	0.74	3.23	0.2
3h	control	endurance	*ex1b*	1758.72	3546.01	2657.74	827.48
3h	control	endurance	*trunc*	15.98	6.12	18.49	4.97
3h	control	endurance	*nontrunc*	12.58	9.16	13.29	3.52
3h	control	resistance	*totalPGC*	3.25	7.28	7.14	3.09
3h	control	resistance	*ex1a*	1.95	1.37	2.63	0.69
3h	control	resistance	*ex1b*	484.02	696.83	1158.04	678.93
3h	control	resistance	*trunc*	3.81	11.55	12.22	6.42
3h	control	resistance	*nontrunc*	2.63	5.63	6.64	3.1
3h	endurance	endurance	*totalPGC*	4.93	3.06	7.24	3.14
3h	endurance	endurance	*ex1a*	1.87	0.65	2.12	0.35
3h	endurance	endurance	*ex1b*	215.33	232.18	601.87	412.13
3h	endurance	endurance	*trunc*	7.18	6.56	13.28	5.71
3h	endurance	endurance	*nontrunc*	5.68	4.04	7.57	2.25
3h	endurance	resistance	*totalPGC*	4.86	3.29	4.68	0.72
3h	endurance	resistance	*ex1a*	1.25	0.54	3.93	2.67
3h	endurance	resistance	*ex1b*	986.24	768.78	1101.86	376.29
3h	endurance	resistance	*trunc*	7.37	4.6	7.31	1.3
3h	endurance	resistance	*nontrunc*	4.64	2.91	4.39	0.61
3h	strength	endurance	*totalPGC*	8.58	6.95	9.75	1.49
3h	strength	endurance	*ex1a*	2.38	1.68	2.91	0.5
3h	strength	endurance	*ex1b*	973.29	1577.18	1024.33	330.53
3h	strength	endurance	*trunc*	13.65	5.25	14.42	1.52
3h	strength	endurance	*nontrunc*	12.83	5.17	12.84	1.73
3h	strength	resistance	*totalPGC*	14.43	6.78	12.37	1.61
3h	strength	resistance	*ex1a*	2.29	0.62	2.36	0.23
3h	strength	resistance	*ex1b*	1729.23	2827.4	5445.44	3117.67
3h	strength	resistance	*trunc*	23.08	11.34	19.74	2.91
3h	strength	resistance	*nontrunc*	11.01	3.81	11.49	1.57

6.4 Time course methylation differences

6.4.1 One bout of exercise induces few DNA methylation changes in selected targets

In Chapter 6.2, DNA methylation at different timepoints using PCA was examined. The PCA did not reveal a clear separation between pre and post-exercise measurements (Figure 11) (Geiger et al. 2024). Consistent with this, a differential methylation analysis aimed at exploring dynamic changes in response to both resistance and endurance exercise identified only a few DMRs and DMPs in the selected targets (Figure 30, Table 9). The detected changes

were small, with the maximum change being a 6-percentage point difference between timepoints (Table 9, Appendix 7). For endurance-trained athletes who engaged in endurance exercise, one DMR and five DMPs within the *MYL3* promoter were hypermethylated immediately after compared to before exercise (Figure 31, Table 9, Appendix 7). In contrast, strength-trained athletes showed a decrease in methylation within the same DMR on the *MYL3* promoter 3 hours post-resistance exercise (Figure 31, Table 9). Additionally, the strength-trained group displayed increased methylation of a DMR in the *CAMKII* promoter (Figure 31, Table 9) 3 hours after resistance exercise, whereas one DMP located farther from the transcription start site exhibited decreased methylation (Appendix 7). Only one DMP was detected in the CG. This DMP was located on the *MYH2* promoter and showed decreased methylation 3h after acute resistance exercise compared to before exercise (Appendix 3).

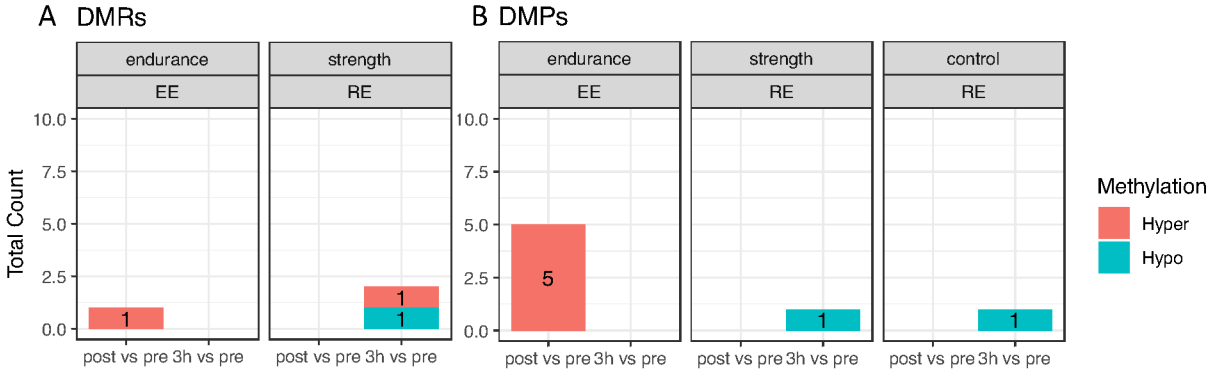

*Figure 30: (**A**) **Number of differentially methylated regions (DMRs)** and (**B**) **number of differentially methylated positions (DMPs) directly after (post) and 3 hours after (3h) acute exercise compared to before the exercise (pre)**. All DMRs and DMPs are within promoters. Interventions/groups not depicted did not show acute DNA methylation changes with exercise. endurance = acute endurance exercise and endurance group, resistance = acute resistance exercise, strength = strength group. The red part of the bars represents the number of DMRs or DMPs with higher methylation (Hyper) and the blue part of the bars represents the number with lower methylation (Hypo) after exercise. Geiger et al. 2024.*

*Table 9: **Differentially methylated regions with acute exercise.** A p-value threshold of <0.05 was set to identify DMRs. nGC = methylated Cytosine-phosphate-Guanine site; areaStat = sum of the test statistics of all CpG sites within a DMR (larger areaStat is more likely to be a DMR)*

DMR: Endurance Group doing acute endurance exercise							
Gene	**Feature**	**Coordinates GRCh38/hg38**	**nCG**	**Pre: DNA methylation**	**Post: DNA methylation**	**Methylation difference**	**area-Stat**
MYL3	Promoter	chr3:46863810-46863927	6	60%	66%	6%	-24
DMRs: Strength Group doing acute resistance exercise							
Gene	**Feature**	**Coordinates GRCh38/hg38**	**nCG**	**Pre: DNA methylation**	**3h: DNA methylation**	**Methylation difference**	**area-Stat**
MYL3	Promoter	chr3:46863810-46863927	6	75%	70%	-5%	-15
CAMK2A	Promoter	chr5:150289804-150289871	11	38%	40%	2%	-22

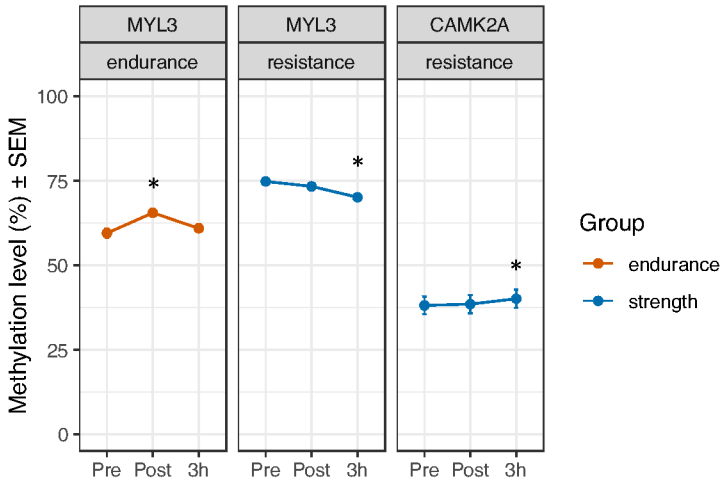

Figure 31: ***DMRs after acute exercise.*** *Endurance = acute endurance exercise and endurance group, resistance = acute resistance exercise, strength = strength group. Results are mean ± SEM for n = 8 subjects. ∗ (post or 3h compared to pre) = p < 0.05. Geiger et al. 2024.*

6.5 Cell type deconvolution

To deconvolute the cell heterogeneity between bulk muscle tissue samples, gene expression of specific marker genes for endothelial cells (*VWF, ESAM, KDR, ICAM1, PECAM-1/CD31*, and *CD144*), mesenchymal cells (*DCN, CFD*, and *GSN*), myogenic cells (*PAX7*), and interstitial fibro-adipogenic progenitors (*TEK*) was analysed showing no significant differences between groups (Geiger et al. 2024). Immune cell type composition was investigated with CIBERSORTx showing slightly lower M2 macrophages (FDR = 0.04) in the endurance group compared to the strength group and higher resting NK cells (FDR < 0.01) comparing the endurance to the control group (Appendix 8) (Geiger et al. 2024).

7 Discussion

This thesis project investigated DNA methylation in skeletal muscle of well-specialised endurance and strength-trained athletes compared with healthy untrained men, both at rest and after an acute exercise session. Exercise training background was linked to unique baseline DNA methylation patterns in skeletal muscle. A single exercise session had minimal impact on acute DNA methylation alterations in the areas under investigation (Geiger et al. 2024).

The observed differences in methylation between trained and untrained men suggest that epigenetic mechanisms might serve as a muscle memory mechanism, as supported by current literature (Seaborne et al. 2018). Chronic exercise affects genes related to muscle metabolism and structural dynamics. For example, lifelong physical activity has been shown to be associated with hypomethylation in genes related to energy metabolism, myogenesis, and oxidative stress response (Sailani et al. 2019). Differentially methylated positions after 3-month endurance exercise training were reported to be functionally relevant to muscle biology, with increased methylation associated with myogenic regulatory factors and myocyte enhancer factor 2 binding motifs and decreased methylation enriched for ETS (Erythroblast Transformation Specific) family binding domains involved in processes like angiogenesis, crucial for endurance exercise adaptation (Lindholm et al., 2014). According to this, information from previous exercise sessions might be retained as a specific DNA methylation signature in cells. This allows information induced by the exercise stress stimuli to be kept beyond the immediate time of exercise.

7.1 Baseline methylation differences between groups

7.1.1 Baseline methylation patterns of myogenic regulatory factors

Differences in DNA methylation between groups at baseline were present for myogenic regulatory factors (MRFs: *MYF5*, *MYOD1*, *MYOG*, *MRF6*) and the downstream acting myocyte enhancer factor (*MEF2A*) (Geiger et al. 2024). These key transcription factors are essential for muscle cell proliferation, differentiation, and growth, forming the foundation for subsequent adaptations during exercise (Asfour et al. 2018; Zammit 2017), and are known to be impaired in chronic diseases such as diabetes (Espino-Gonzalez et al. 2024). The association between DNA methylation and transcription levels was weak, suggesting a complex interplay between methylation and gene expression and other potential regulatory processes in response to training (Figure 13-16). An increased methylation state of MRFs in endurance-trained athletes compared to untrained men aligns with the findings of Lindholm et al. who reported hypermethylation of *MRFs* and *MEF2* classes after a 3-month endurance training period (Lindholm et al. 2014b). Increased *MYF5, MYOD1* and *MYOG* methylation has been described with older age, while the *MYOD1* promoter was hypomethylated in active individuals in another study (Sailani et al. 2019; Voisin et al. 2021). Interestingly, the differentially methylated region associated with the *MYOD1* promoter in the here presented study was hypermethylated in strength-trained compared to untrained men, which also correlated with leg strength (Figure 14, Appendix 2) (Geiger et al. 2024). Older age was associated with decreased methylation for the *MEF2A* enhancer, showing increased methylation in the here

presented endurance-trained men (Figure 16) (Geiger et al. 2024; Voisin et al. 2021). Nitert et al. reported a decrease in *MEF2A* methylation after a 6-month endurance training period (Nitert et al. 2012).
In summary, the findings, in conjunction with previous research, indicate an intricate relationship between training status, exercise type and the methylation and expression patterns of myogenic regulatory factors, underscoring the complexity of epigenetic responses to physical activity. The observed methylation differences could be part of training adaptation processes and indicate improved ability of trained skeletal muscle to regenerate.

7.1.2 Endurance-trained skeletal muscle exhibits differential DNA methylation of fiber-type specific genes

Distinct epigenetic signatures were observed when investigating the relationships between DNA methylation patterns at the promoter regions of myosin heavy and light chain genes (*MYH7, MYH6, MYL3,* and *MYH2,* Figure 17). These signatures varied among endurance-trained, strength-trained, and untrained individuals and were consistent with their respective phenotypic traits (Geiger et al. 2024).

The contractile properties of skeletal muscle are largely determined by the expression of particular myosin heavy and light chain proteins. Type I fibers, known as slow-twitch fibers specialised in long-duration contractile activities, predominantly contain *MYH7*, *MYH6*, and *MYL3* proteins (Holloszy and Coyle 1984; Schiaffino and Reggiani 2011). These proteins are almost absent in type IIx fibers, which are fast-twitch fibers (Stuart et al. 2016). Fast-twitch type IIx and IIa fibers are essential for short-duration anaerobic activities. Fast type IIa fibers are generally discriminated by the presence of *MYH2*.

Endurance training has been shown to increase the proportion of slow-twitch fibers, thereby enhancing aerobic capacity (Wilson et al. 2012). In line with this, the here investigated endurance-trained group exhibited a significantly higher proportion of slow-twitch fibers compared to the strength-trained and control groups, coupled with elevated gene expression of *MYH7* and *MYL3* (Reitzner et al. 2023). The higher gene expression was associated with lower promoter methylation of *MYH7* and *MYL3* in the endurance athletes (Figure 18). Conversely, the *MYH2* promoter showed higher methylation in endurance-trained compared to untrained men (Geiger et al. 2024). This suggests that methylation patterns may influence gene expression and fiber type specification, as promoter methylation generally suppresses transcription while demethylation promotes transcription.

The findings align with previous research that identified fiber type-specific methylation profiles, with fast-twitch fibers displaying increased intronic methylation of *MYH7* and slow-twitch fibers showing higher *MYH2* promoter methylation which was associated with reduced gene expression (Begue et al. 2017).

Epigenetic regulation is implicated in fiber type regulation (Baar 2010). Cultivated muscle fibers derived from either slow or fast muscle continued to express the respective myosin chains even when extrinsic factors such as hormonal cues and neural stimulation were eliminated. Human satellite cells have been shown to express both slow and fast myosin chains in culture, enabling them to fuse with any fiber type (Bonavaud et al., 2001).

The distribution of different muscle fiber types is not only important in the exercise context but also in understanding disease progression and when exploring therapeutic potential. For

instance, at the onset of Duchenne Muscular Dystrophy, type I muscle fibers are less affected than type II fibers, suggesting that increasing type I fibers could alleviate the disease's severity and progression (Webster et al. 1988). The significance of muscle fiber composition is also evident in other muscular dystrophies, sarcopenia, and ageing (Jennifer and Masatoshi 2018; Murgia et al. 2017; Talbot and Maves 2016). Slow-twitch muscle fibers, known for their superior quality control mechanisms and heightened protein synthesis and turnover rates, may provide increased resistance to age-related sarcopenia (Murgia et al. 2017). In summary, examining the interplay between skeletal muscle DNA methylation and fiber type identity provides important insights for potential therapeutic strategies.

7.1.3 Differential DNA methylation of signalling molecules and pathways regulating protein turnover and muscle adaptation

Genes encoding kinases that orchestrate distinct processes of skeletal muscle adaptation, such as *MTOR* and *MAPK12*, were found to be differentially methylated in endurance-trained men compared to strength-trained and untrained men (Geiger et al. 2024). Endurance-trained men exhibited higher levels of methylation within an intronic *MTOR* DMR compared to control and strength-trained groups (Figure 19) which positively correlated with VO_2peak. This aligns with findings by Lindholm et al. (2014), who reported hypermethylation at a differentially methylated position within this DMR following a three-month period of endurance training. No difference in *MTOR* promoter methylation between sedentary and resistance-trained individuals was found by Bagley et al. (2020). Known as a central regulator of protein turnover in muscle cells, *MTOR* is essential for myofibrillar protein synthesis (Watson and Baar 2014; Yoon 2017). The differential methylation of the *MTOR* DMR might reflect the distinct adaptive focuses between resistance and endurance training. These observations suggest that there could be differential epigenetic regulation of *MTOR* in endurance-trained men, possibly favouring metabolic efficiency over muscle hypertrophy.
Complementing this finding, higher *SMAD3* methylation was observed in endurance athletes compared to the other groups (Figue 20). *SMAD3* is part of the transforming growth factor beta (*TGF-β*) pathway, known to interact with *MTOR* signalling and influence muscle cell differentiation and atrophy, and to help balance protein turnover (Ge et al. 2011; Ge et al. 2012). Currently, this pathway is investigated as a metabolic therapeutic agent to preserve muscle mass and improve metabolic health in obese adults (Heymsfield et al. 2021). The concomitant hypermethylation within these key regulatory pathways may reflect a multifaceted epigenetic response and complex regulatory mechanism leading to distinct muscle adaptation between strength and endurance-trained men.
Another important class of signalling molecules mediating the molecular exercise response are MAPKs. Sensitivity of the MAPK pathway methylome to exercise has been shown previously after running exercises (Maasar et al. 2021). The p38 *MAPK* subfamily has different isoforms. Especially deletion of the p38*γ* isoform *MAPK12* in a mouse model mitigated endurance exercise-induced mitochondrial biogenesis and angiogenesis (Pogozelski et al. 2009). Here, *MAPK12*-associated DMRs were positively correlated with VO_2peak and showed higher methylation in endurance-trained athletes compared to untrained men (Figure 22 and 23) (Geiger et al. 2024), which could play a part in endurance adaptation processes. However, due to the observational nature of the data, definitive conclusions cannot be drawn, and these findings should be interpreted with caution. Further studies are needed to clarify the biological implications of these methylation changes in the training context.

7.1.4 Transcriptional regulators are differentially methylated in trained and untrained skeletal muscle

Differential methylation of key transcriptional regulators, including *HIF1A, FOXOs*, and *CREBs*, was observed between trained and untrained individuals (Geiger et al. 2024).
HIF1A gene-associated intronic methylation was lower in strength-trained men compared to untrained men (Figure 24). Interestingly, *HIF1A* expression is reported to increase after acute endurance exercise and the response is blunted in trained individuals (Lundby et al. 2006). Reitzner et al. (2023) found a significant increase of *HIF1A* transcripts in untrained men after acute endurance and resistance exercise. While no significant change in *HIF1A* transcripts was seen in endurance-trained athletes, strength-trained athletes increased *HIF1A* transcription only after resistance exercise but not after endurance exercise (Reitzner et al. 2023). There seems to be an effect of training on exercise-induced *HIF1A* expression. The exact function of the here observed methylation difference in strength-trained men could be an interesting target to investigate further to fully understand a potential relevance during strength training adaptation.

DNA methylation was higher for intronic regions of *FOXO1* and *FOXO3* in endurance-trained athletes compared to strength-trained and untrained men (Figure 26). This finding is consistent with previous research that reported higher methylation in these regions following a three-month period of endurance exercise (Lindholm et al. 2014b). Notably, while all participant groups demonstrated a rise in *FOXO1* expression post-exercise (Reitzner et al. 2023a), only the endurance-trained group showed a significant increase in *FOXO3* expression. A prior study has identified the *FOXO3* DMR as an enhancer, which is dynamic in its methylation patterns (Pan et al. 2022). It's worth mentioning that, particularly in oncology studies, enhancer methylation has been closely linked to gene expression changes, more so than promoter methylation (Aran et al. 2013; Cho et al. 2022). The impact on DNA methylation of environmental stimuli may be more pronounced around enhancers and intragenic regions. For example, intragenic regions contain the majority of the methylation variations linked to child abuse (Yang et al. 2013). Similarly, exercise and cardiovascular fitness levels have been reported to have minimal impact on promoter regions while influencing DNA methylation in enhancers, gene bodies, and intergenic regions to a greater extent (Landen et al. 2023; Lindholm et al. 2014b). Ageing-related DNA methylation changes in skeletal muscle primarily occur around active transcription start sites and enhancers (Voisin et al. 2021). While promoter methylation is generally associated with blocking the access of RNA polymerase and thereby leading to gene silencing (Bogdanović and Veenstra 2009), the implications of intragenic methylation on gene transcription vary greatly (Baar 2010; Lindholm et al. 2014b; Lister et al. 2009; Sharples et al. 2016; Voisin et al. 2021). For example, gene body methylation has been linked to highly expressed genes across the human genome (Ball et al. 2009).

The roles of *FOXO3* and *FOXO1* are crucial in maintaining cellular homeostasis and responding to the metabolic stress induced by exercise, optimising protein turnover during recovery (Sanchez et al. 2014). They are recognised as a potential target for combating muscle ageing through their cytoprotective effects (Jing et al. 2022). Interestingly, *FOXO1* and *FOXO3* show reduced methylation with age in skeletal muscle (Voisin et al. 2021). Gender differences in *FOXO3* DNA methylation and expression in skeletal muscle might be attributed to variations in muscle fiber type proportions (Landen et al. 2021). The identified *FOXO3* DMR in

endurance-trained men correlated with type I fiber percentage and VO_2peak. The methylation and expression patterns of *FOXO3* observed in the here presented study suggest enhanced capability in managing metabolic disruptions post-exercise in endurance-trained men and might have an impact on muscle ageing.

The CREB family did also show differential methylation between groups. For *CREB1*, methylation was lower in resistance-trained men (Figure 25), correlating with a lower type I percentage. Interestingly, while all three groups, strength-trained, endurance-trained and untrained men, showed a significant decrease of *CREB1* mRNA transcripts after acute resistance exercise, the endurance group had a blunted response after acute endurance exercise while being significant in the two other groups after endurance exercise (Reitzner et al. 2023). This finding could indicate training-specific reprogramming. While hypomethylated *CREB1* could guarantee greater transcriptional plasticity needed in strength-trained men the blunted transcriptional response might reflect transcriptional stability after long-term endurance training. An intronic DMR of *CREB5* had higher methylation in the endurance group compared to controls, with no significant difference between the control and strength groups (Figure 25). *CREB5* transcription was only upregulated following acute resistance exercise (Reitzner et al. 2023). Past studies have shown higher *CREB5* methylation in female versus male myoblasts and myotubes, correlating with lower expression levels (Davegårdh et al. 2017). A moderate non-significant inverse relationship between *CREB5* gene expression at baseline and methylation was found in the here-presented work, implying that long-term endurance training might heighten *CREB5* methylation, thereby dampening its expression or induction after endurance exercise.

These methylation patterns suggest a 'primed' state of gene regulation where baseline methylation might affect exercise-induced transcription. Research on mice has proposed that methylation changes induced by hypertrophic stimuli may prepare muscle cells for a more efficient response to future stimulation (Von Walden et al. 2020). In trained muscles, altered methylation of regulatory genes could impact gene expression following acute exercise events (Furrer et al. 2023b). Studies have shown that epigenetic changes induced by initial resistance training phases endure through subsequent rest and retraining periods, hinting at an epigenetic memory of resistance training (Seaborne et al. 2018; Turner et al. 2019). This could lead to further gene upregulation upon retraining in genes with retained methylation states. The here presented findings support the concept that training modifies DNA methylation in specific genes, impacting the gene expression response to acute exercise in trained individuals.

7.1.5 PGC1α promoter methylation and associated isoform expression

Analysis of *PGC1α* DNA methylation and isoform expression revealed nuanced dynamics differing between trained and untrained men. *PGC1α* is a highly versatile transcriptional coactivator that plays a role in regulating various aspects of skeletal muscle adaptation to exercise and training (Jannig et al. 2022; Martínez-Redondo et al. 2015).

Investigating *PGC1α* proximal and alternative promoter methylation, higher methylation was detected in endurance-trained men compared to strength-trained and untrained men (Figure 27). This might appear contradictory to what is known from literature so far where elevated *PGC1α* promoter methylation has previously been linked to type II diabetes and physical inactivity (Alibegovic et al. 2010; Barrès et al. 2009). Demethylation across all CpG sites of the

PGC1α gene has been reported following prolonged electrical stimulation in spinal cord injury patients (Petrie et al. 2020). Barrès et al. showed demethylation of the *PGC1α* promoter after high-intensity exercise in sedentary individuals. No acute methylation changes were detected in any group after acute exercise for *PGC1α* promoters for the here reported data. Similarly Lochman et al. didn't find significant methylation differences for the proximal or alternative promoter after acute exercise in mice (Lochmann et al. 2015).

While *PGC1α* expression at rest did not differ between trained and untrained groups, *PGC1α* expression following acute exercise varied among the different transcript variants (Figure 28 and 29) (Geiger et al. 2024). The overall trend showed a blunted transcriptional response in endurance-trained men after acute exercise while they had higher promoter methylation at rest. These observations leads to the intriguing hypothesis that promoter DNA hypermethylation in endurance trained men might modulate the gene's responsiveness to acute exercise stimuli. Comparing the expression of *PGC1α* transcripts to previous literature, expression levels at rest match those seen in other studies (Norrbom et al. 2011; Popov 2018; Silvennoinen et al. 2015). Baseline *PGC1α* expression did not change in healthy men after 5 weeks of resistance and aerobic exercise training (Lundberg et al., 2014). Lundberg et al. observed a similar steady state expression pattern in healthy men after 5 weeks of resistance and aerobic exercise training as the here presented data proposes, with dominant expression of total *PGC1α*, transcripts from the proximal promoter and full-length transcripts whereas the alternative promoter was inactive at baseline and alternative splicing leading to truncated mRNA transcripts was minimal. Interestingly, Krämer et. al found higher *PGC1α* baseline expression in cycling athletes compared to normally active individuals, correlating with higher type I fiber percentage (Krämer et al. 2006). This finding was not reproduced in the here presented endurance athletes.

Despite similar expression levels at baseline, the magnitude of mRNA foldchange after acute exercise differed between groups. Endurance trained athletes had a blunted increase in total *PGC1α* and proximal promoter transcripts after resistance exercise and of alternative promoter transcripts after endurance exercise compared to the strength and control group respectively. Blunted exercise-induced *PGC1α* transcription in endurance trained muscle has been reported previously (Perry et al. 2010; Popov et al. 2014; Popov et al. 2017; Popov et al. 2015). Strength-trained athletes, in contrast, had a significantly greater foldchange after resistance exercise compared to the other groups, especially when looking at truncated *PGC1α* and nontruncated *PGC1α* transcripts individually. Although exercise intensities matched in relative terms, differences in muscle fiber composition and size may account for variability in transcriptional responses. Type II fibers undergo greater metabolic alterations during exercise and have been shown to exhibit a more pronounced *PGC1α* transcriptional response to endurance exercise compared to type I fibers (Popov 2018; Russell et al. 2003). Strength-trained and untrained participants had a higher proportion of type II fibers which might drive the observed differences and suggest specific adaptational processes in strength-trained men. The role of different *PGC1α* isoforms in response to resistance exercise, for example as pro-hypertrophic factor, is still conflicit and more mechanistic research of this complex transcriptional coactivator is warranted (Schiaffino et al. 2021).

7.2 Time course methylation differences

7.2.1 One bout of exercise induces few DNA methylation changes in selected targets

Literature regarding gene-specific methylation changes after acute execise reports that endurance exercise can acutely modulate DNA methylation within one hour, for example in genes associated with mitochondrial functions and fuel usage, such as *PGC1α*, *PDK4*, *CS*, *TFAM1* and *MEF2A* (Barrès et al. 2012). Demethylation of myogenic regulatory factors *MYOD1*, *MYF5* and *MYF6* has been reported after one bout of resistance and/or high-intensity interval exercise in muscle biopsies taken 4 and 8 hours after exercise (Telles et al. 2023). Temporal changes in DNA methylation after exercise have also been reported in trained men and in connection with dietary state (Gorski et al. 2023b; Lane et al. 2015; Maasar et al. 2021). Only very few changes for the selected targets were found in this investigation (Geiger et al. 2024). The lack of significant results could be due to the relatively small sample size, which reduces statistical power. This issue is particularly pronounced when combined with the small effect sizes typically observed in DNA methylation changes in response to environmental stimuli, increasing the risk of Type II errors (Breton et al. 2017; Nitert et al. 2012; Telles et al. 2023; Tsai and Bell 2015). Samples were not collected beyond the 3-hour post-exercise time point. Methylation changes occurring at other time points might have been missed. Furthermore, the here-analysed samples stemmed from bulk tissue which could have diluted methylation signals from specific cell types or loci. Lastly, the lack of significant changes after acute exercise could be a result of the narrow genomic focus which might not have encompassed loci most responsive to exercise-induced methylation changes.

7.3 Limitations

Analyses were performed on bulk muscle tissue which consists of various cell types, including muscle fibers, immune cells, endothelial cells, erythrocytes, and fibroblasts (Rubenstein et al. 2020). This cellular heterogeneity and associated cell type-specific epigenetic marks must be kept in mind when interpreting results (Houseman et al. 2012; Von Walden et al. 2020). Since each autosomal chromosome has two copies (alleles) of each gene CpG sites are typically either unmethylated (0%), hemimethylated (50%), or fully methylated (100%). Therefore, when analysing bulk tissue samples, detecting subtle variations in methylation levels suggests that only a small percentage of cells exhibit differential methylation at specific loci, which can have a major effect on how those specific cells function. Analysing bulk tissue can dilute cell type-specific methylation signals and thereby potential significant results might not be detected. In an attempt to deconvolute the cell heterogeneity between samples, cell-specific marker genes were analysed, showing no significant differences between groups (Reitzner et al. 2023). Furthermore, digital immune cell deconvolution was performed, revealing minimal differences between the groups (Geiger et al. 2024). However, a significant difference in fiber type composition was observed between endurance-trained, strength-trained, and untrained muscles, which likely influenced the results. Ideally, future studies should be conducted using single-cell techniques.

As mentioned before, the sample size was relatively small, with eight subjects per group, which limited the statistical power to detect small changes in DNA methylation. Despite the

study design and number of subjects being guided by a power analysis, this could limit the effects being measurable in this study. In combination with the small effect sizes generally seen for alterations in methylation in reaction to environmental stimuli, this increases the risk of type II errors. Sample collection was limited to three timepoints and important findings could have been missed occurring at other time points. Another limitation of this work is that it focuses only on male subjects. Even though Landen et al. reported no sex-specific training effect on the skeletal muscle methylome, sex differences in methylation in general exist, and future studies should include both sexes (Landen et al. 2021; Landen et al. 2023; Lindholm et al. 2014a). Various intrinsic and extrinsic factors, including age, genetic background, nutrition, stress, and other lifestyle choices, affect DNA methylation. To control for some of those variables, all subjects exercised at the same time in the morning in a rested state while the dietary intake the evening before and the morning off the intervention day were controlled. However, multiple confounders remain such as individual genetic differences. To investigate if there were intraindividual differences caused by sample handling or other environmental triggers, two baseline samples from each subject in the control group were compared. No significant methylation differences could be found between those samples taken of the same individual at different days. Similarly, no differentially expressed genes were found when analysing the transcriptomic data of two baseline samples from the same subject (Reitzner et al. 2023).

When investigating DNA methylation, there is growing recognition of the roles of 5-hmC and non-CpG methylation. This study focused on CpG methylation and did not examine the regulation of 5-hmC or non-CpG methylation after exercise. 5-hmC, an intermediate in the demethylation process, may influence gene activity differently than 5-mC. As a limitation, this study did not distinguish between 5-mC and 5-hmC, which could result in inaccurate assumptions about gene activation and silencing. Non-CpG methylation, which occurs in 5’-cytosine-phosphate-cytosine-3’ (CpC), 5’-cytosine-phosphate-thymine-3’ (CpT), or 5’-cytosine-phosphate-adenine-3’ (CpA) contexts, has been implicated in the exercise response following both endurance and resistance exercise (Barrès et al. 2012; Rowlands et al. 2014). For example, non-CpG methylation and 5-hmC are suggested to play critical roles in the epigenetic regulation of the *PGC1α* promoter (Barrès et al. 2009; Lochmann et al. 2015). Future studies should distinguish between 5-mC and 5-hmC and measure non-CpG methylation, as these may contribute significantly to the epigenetic remodelling process following exercise.

The results presented in this thesis offer a snapshot of the DNA methylation profiles of selected genes in differently trained individuals at various time points in relation to an acute exercise bout. However, the observational nature of this study does not permit definitive conclusions or mechanistic insights. Despite these limitations, the data provide valuable findings that can inform future research and expand our understanding of differential methylation signatures in trained versus untrained men, and between men specialised in resistance and endurance exercise. These results should be interpreted within the broader context of gene regulation, where the relationship between DNA methylation and gene expression is highly complex. Even subtle changes in methylation, depending on cell type and genomic context, can influence a larger network of regulatory interactions.

7.4 Conclusion

This thesis showed a differing DNA methylation signature of genes central during exercise adaptation in skeletal muscle of trained and untrained men, particularly in endurance-trained individuals. DNA methylation patterns and muscle fiber type appear closely intertwined. The results suggest a training-background-dependent process where the training-induced baseline DNA methylation landscape influences the transcriptional response of certain genes to exercise. This could be part of an epigenetic 'muscle memory' where DNA methylation might facilitate a more efficient and organised transcriptional response to future exercise bouts.

This thesis concludes that:

- DNA methylation of regulatory regions for genes that are central to exercise adaptation differs between trained and untrained men
- It is possible that DNA methylation in trained men modulates an adaptive and potentially more efficient transcriptional response to acute exercise stimuli for example for transcription factors and transcriptional co-activators
- Fiber type distribution needs to be considered when comparing DNA methylation in skeletal muscle
- Transient DNA methylation changes with acute exercise are subtle and might be limited to specific genes
- *PGC1α* expression is blunted in endurance-trained men after acute exercise
- In contrast, strength-trained men seem to highly induce *PGC1α* expression after their familiar form of exercise, namely resistance exercise

8 Summary

8.1 How exercise shapes our genes – a potential muscle memory mechanism

Regular physical activity has many positive health benefits: It strengthens the cardiovascular system, improves metabolism, and maintains muscle health. But how does our body adapt to exercise? Changes in gene expression play an important role in driving exercise adaptations. Epigenetic mechanisms influence gene activity without altering the genetic information itself and are believed to influence physiological adaptations and health benefits from exercise. A key epigenetic mechanism is DNA methylation. In this process, small chemical groups attach to the DNA and influence whether a gene is active or inactive. But how does long-term training affect DNA methylation in muscles? And does the DNA methylation of 'exercise-relevant' genes change after a single exercise session? The literature suggests that lifestyle factors, such as physical activity, can influence DNA methylation. The role of DNA methylation in long-term training adaptation is not only interesting from a basic human biology perspective but carries therapeutic potential. To investigate this in more detail, the present thesis compared DNA methylation patterns in the thigh muscle (musculus vastus lateralis) of eight endurance-trained athletes, eight strength-trained athletes, and eight untrained individuals before and after a single bout of endurance and resistance exercise. The endurance athletes reported at least 15 years of endurance training in form of running, biking or a combination on a high level while the strength-trained athletes were involved in heavy strength training regimes for the past 15 years (powerlifting and Olympic lifting). The control group consisted of untrained men who were not regularly physical active. All participants completed an acute exercise bout – 30 minutes of cycling or an intense leg workout on a strength machine. To explore DNA methylation patterns that occur in a trained state and immediate alterations following exercise, muscle samples were taken before, immediately after, and three hours after the exercise bouts. The methylation patterns of selected 'training-relevant' genes such as genes involved in myogenesis, gene regulation, angiogenesis, and metabolism were analysed and associated with the gene activity of the corresponding genes. Additionally, methylation levels were correlated with the participants' maximum oxygen uptake capacity, leg strength, and differential muscle fiber type distribution.

The results indicate that long-term training is associated with a specific methylation signature in the DNA of muscle cells. Genes characteristic for slow-twitch muscle fibers showed lower methylation in endurance-trained athletes than strength-trained and untrained men. Slow-twitch muscle fibers, also known as type I fibers, are particularly enduring and efficiently use energy during prolonged activities. The lower methylation of these genes was associated with increased gene activity and a higher prevalence of type I fibers. Particularly striking were the differences in genes that act as transcription factors to influence the activity of other genes. Regulatory regions of *FOXO3, CREB5,* and *PGC1α*, which moderate important genes in muscle metabolism and cellular homeostasis, were higher methylated in endurance athletes compared to the other groups. Interestingly, the gene expression of the corresponding genes was less responsive to an acute exercise bout in endurance-trained individuals. This observation suggests that methylation patterns, associated with long-term endurance training, might affect gene transcription after an acute exercise bout. It appears that trained muscles might possess a 'memory', allowing them to respond more efficiently to exercise. This has also been suggested in other studies. Furthermore, the DNA methylation of genes

regulating muscle growth and muscle repair differed between endurance-trained athletes, strength-trained athletes, and untrained participants. A single exercise bout – both endurance and resistance – led to only few immediate DNA methylation changes of the examined genes. Notably, this study only targeted selected genes, so it cannot be ruled out that DNA methylation in other gene regions may have changed temporarily. Such short-term methylation changes might play a potentially important role in the molecular exercise response.

Overall, the study provides important insights into how long-term physical activity might influence DNA methylation in muscle cells. These findings are not only interesting in a sports science context but also have significant implications for medicine. A better understanding of how epigenetic processes affect muscle function could help develop targeted therapies. This could potentially enable better treatment of conditions such as muscle atrophy and preserve muscle health during physical impairments by mimicking the molecular effects of exercise. In the long term, this could help maintain muscle function into old age and improve quality of life. Exercise appears to affect our genes, and DNA methylation is a potential mechanism through which muscle cells can ‘remember’ previous training.

8.2 Wie Bewegung unsere Gene prägt – Ein Blick ins Muskelgedächtnis

Regelmäßige Bewegung hat viele positive Effekte auf unseren Körper: Sie stärkt das Herz-Kreislauf-System, verbessert den Stoffwechsel und erhält die Gesundheit der Muskeln. Doch wie passt sich unser Körper dem Sport an? Eine wichtige Rolle spielt dabei die Aktivität verschiedener Gene. Epigenetische Veränderungen beeinflussen die Aktivität von Genen, ohne die Erbinformation selbst zu verändern. Ein wichtiger Mechanismus hierbei ist die DNA-Methylierung. Dabei heften sich kleine chemische Gruppen an die DNA und beeinflussen, ob ein Gen aktiv oder inaktiv ist. Wie verändert Langzeittraining die DNA-Methylierung in der Muskulatur? Und verändert sich die DNA-Methylierung ’trainingsrelevanter’ Gene nach einem einzelnen Training? Die Datenlage zeigt, dass Lebensstilfaktoren wie Bewegung die DNA-Methylierung beeinflussen können.

Um dies genauer zu erforschen, wurde in der vorliegenden Arbeit das DNA-Methylierungsmuster im Oberschenkelmuskel (Musculus vastus lateralis) von acht Ausdauersportlern, acht Kraftsportlern und acht untrainierten Personen vor und nach einem einmaligen Training verglichen. Die Ausdauersportler hatten über 15 Jahre lang regelmäßig Sportarten wie Laufen oder Radfahren betrieben, während die Kraftsportler seit Jahren Krafttraining absolvierten. Die dritte Gruppe bestand aus untrainierten Männern, die körperlich nicht regelmäßig aktiv waren. Alle Probanden absolvierten eine Trainingseinheit – entweder ein 30-minütiges Radfahren oder ein intensives Beintraining an der Kraftmaschine. Um die Veränderungen in den Muskeln zu messen, wurden vor, direkt nach und drei Stunden nach dem Training Muskelproben entnommen. Das Methylierungsmuster selektierter ’trainingsrelevanter’ Gene wurde analysiert und mit der Genaktivität der entsprechenden Gene assoziiert. Zusätzlich wurde der Methylierungsgrad mit der maximalen Sauerstoffaufnahmekapazität, der Beinstärke und dem Anteil von verschiedenen Muskelfasern der Probanden korreliert.

Die Ergebnisse zeigen, dass langjähriges Training Spuren im Erbgut der Muskelzellen zu hinterlassen scheint. In den Muskeln der Ausdauersportler waren Gene, die für die langsamen

Muskelfasern charakteristisch sind, weniger stark methyliert. Diese Fasern, die auch als Typ-I-Fasern bezeichnet werden, sind besonders ausdauernd und verbrauchen bei langen Belastungen effizient Energie. Die geringere Methylierung dieser Gene war mit erhöhter Genaktivität und einem erhöhten Vorkommen der Typ-I-Fasern bei Ausdauertrainierten assoziiert. Besonders auffällig waren auch die Unterschiede bei Genen, die als Transkriptionsfaktoren die Aktivität anderer Gene beeinflussen. Regulatorische Regionen von *FOXO3, CREB5* und *PGC1α*, die unter anderem wichtige Gene im Muskelstoffwechsel und zellulären Gleichgewicht steuern, waren bei den Ausdauersportlern stärker methyliert im Vergleich zu den anderen Gruppen. Interessanterweise reagierte der Genausdruck der entsprechenden Gene weniger stark auf ein einzelnes Training bei den Ausdauersportlern. Diese Beobachtung lässt vermuten, dass ein verändertes Methylierungsmuster durch langjährigen Ausdauersport die Genantwort bei erneuter Belastung beeinflussen könnte. Es scheint also, als hätten trainierte Muskeln ein ‘Gedächtnis’, das sie gezielter auf körperliche Anstrengungen reagieren lässt. Diese Beobachtung wurde auch in anderen Studien vermutet. Auch die DNA-Methylierung von Genen, die Muskelwachstum und Muskelreparatur regulieren, unterschied sich zwischen Ausdauersportlern, Kraftsportlern und untrainierten Probanden. Die einzelnen Trainingseinheiten – sowohl das Ausdauertraining als auch das Krafttraining – führten zu nur minimalen kurzfristigen Veränderungen in der DNA-Methylierung der untersuchten Gene. Es muss hierbei berücksichtigt werden, dass die vorliegende Arbeit nur gezielt bestimmte Gene untersuchte. Daher kann nicht ausgeschlossen werden, dass sich die DNA-Methylierung anderer Genregionen vorübergehend änderte und kurzfristige Methylierungsveränderungen einen potenziell wichtigen Mechanismus in der molekularen Belastungsantwort darstellen.

Insgesamt liefert die Arbeit wichtige Beobachtungen darüber, wie langjährige Bewegung die DNA in unseren Muskelzellen beeinflussen könnte. Diese Erkenntnisse sind nicht nur für Sportler spannend, sondern haben auch Bedeutung für die Medizin. Ein besseres Verständnis davon, wie epigenetische Prozesse die Muskelfunktion beeinflussen, könnte dabei helfen, gezielte Therapien zu entwickeln. So ließen sich möglicherweise Erkrankungen wie Muskelschwund und der Erhalt der Muskelgesundheit bei Bewegungseinschränkungen besser behandeln, indem die molekularen Effekte von Training nachgeahmt werden. Langfristig könnte dies dazu beitragen, die Muskelfunktion zum Beispiel im Alter zu erhalten und die Lebensqualität zu verbessern. Training wirkt bis tief in unsere Gene, und DNA-Methylierung ist ein potenzieller Mechanismus, mit dem sich Muskelzellen Belastung ‘merken’ können.

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10 Appendix

Appendix 1: Differentially methylated positions (DMPs) at baseline
Appendix 2: Correlations between methylation percentage at baseline and gene expression, VO2peak, leg Strength and type I fiber percentage
Appendix 3: Comparative Analysis of *PGC1α* Expression at rest (baseline, before exercise).
Appendix 4: Comparative Analysis of *PGC1α* Expression Over Time Following Exercise
Appendix 5: Group comparison of *PGC1α* Expression at different timepoints post exercise
Appendix 6: Exercise comparison within a group of *PGC1α* Expression at different timepoints post exercise
Appendix 7: Differentially methylated positions after acute exercise
Appendix 8: Proportions of different immune cells estimated by CIBERSORTx

Appendix 1: ***Differentially methylated positions (DMPs) at baseline.*** *EG = endurance group; CG = control group; SG = strength group; FDR = false discovery rate.*

Gene	**Feature**	**Coordinates GRCh38/hg38**	**EG-DNA methylation**	**CG-DNA methylation**	**Methylation difference**	**FDR**
MAPK12	Promoter	chr22:50262698/50262699	61%	44%	17%	<0.001
MYL3	Promoter	chr3:46863810/46863811	56%	73%	-16%	<0.001
SMAD3	Intron	chr15:67098355	35%	21%	14%	<0.001
SMAD3	Intron	chr15:67098570-67098569	39%	25%	14%	<0.001
				SG-DNA methylation		
MYOG	Promoter	chr1:203086955/203086954	61%	43%	18%	<0.001
SMAD3	Intron	chr15:67098570/67098569	39%	23%	16%	<0.001
SMAD3	Intron	chr15:67098355/67098356	36%	21%	15%	<0.001
MYL3	Promoter	chr3:46863927/46863926	65%	81%	-16%	<0.001
MYL3	Promoter	chr3:46863810/46863811	56%	73%	-17%	<0.001
MYL3	Promoter	chr3: 46863453	50%	66%	-16%	<0.001
MTOR	Enhancer (Intron)	chr1:11158548/11158547	67%	50%	17%	<0.001
MTOR	Enhancer (Intron)	chr1: 11158561	75%	59%	16%	<0.001
MYF5	Promoter	chr12:80716521/80716522	70%	56%	14%	<0.001
MAPK12	Promoter	chr22:50262698	61%	46%	15%	<0.001

Appendix 2: ***Spearman correlations between methylation percentage at baseline and gene expression, VO_2peak, leg Strength and type I fiber percentage****. R = Spearman correlation coefficient; FDR = false discovery rate.*

	Gene expression		**VO_2peak**		**Leg Strength**		**Type I Fiber %**	
Gene	R	FDR	R	FDR	R	FDR	R	FDR
CREB1	0.21	0.43	-0.01	0.94	-0.26	0.66	0.49	0.04
CREB5	-0.38	0.11	0.59	<0.01	-0.10	0.84	0.26	0.31
FOXO1	-0.25	0.36	0.70	<0.01	0.07	0.89	0.46	0.06
FOXO3	-0.30	0.22	0.71	<0.01	-0.12	0.84	0.63	0.01
HIF1A	0.17	0.50	-0.18	0.35	-0.30	0.66	0.30	0.25
MAPK12	-0.39	0.11	0.69	<0.01	0.04	0.89	0.31	0.24
MEF2A	-0.14	0.59	0.23	0.23	-0.19	0.70	0.20	0.42
MTOR	-0.09	0.70	0.43	0.02	-0.04	0.89	0.51	0.05
MYF5	-0.23	0.39	0.32	0.09	-0.27	0.66	0.50	0.04
MYH2	-0.39	0.11	0.54	<0.01	-0.02	0.89	0.09	0.77
MYH7	-0.18	0.50	-0.45	0.02	-0.24	0.66	-0.62	<0.01
MYL3	-0.52	0.04	-0.51	0.01	-0.05	0.89	-0.83	<0.01
MYOD1	0.12	0.62	0.33	0.08	0.66	<0.01	0.001	0.99
MYOG	0.01	0.98	0.42	0.02	0.10	0.84	0.002	0.99
PPARGC1A	-0.43	0.11	0.64	<0.01	0.10	0.84	0.24	0.33
SMAD3	-0.37	0.11	0.45	0.02	-0.14	0.84	0.34	0.20

Appendix 3: ***Comparative Analysis of PGC1α Expression at rest*** *(baseline, before exercise) between groups, showing no significant baseline difference. FDR = false discovery rate.*

Transcript	**FDR**
totalPGC	0.451
ex1a	0.138
ex1b	0.633
trunc	0.522
nontrunc	0.117

Appendix 4: ***Comparative Analysis of PGC1α Expression Over Time Following Exercise.*** *FDR derived from comparisons of PGC1α expression fold change at different time points (post-exercise, 1 hour post-exercise, and 3 hours post-exercise) relative to baseline (pre-exercise levels). The exercise modalities considered in the study included Endurance Exercise (EE) and Resistance Exercise (RE). FDR = false discovery rate.*

Transcript	**Group**	**Test**	**FDR (EE)**	**FDR (RE)**
totalPGC	control	Friedman	0.003	0.013
totalPGC	control	pre vs post	0.673	0.705
totalPGC	control	pre vs 1h	0.013	0.313
totalPGC	control	pre vs 3h	0.013	0.013
totalPGC	endurance	Friedman	0.005	0.012
totalPGC	endurance	pre vs post	0.223	0.870

totalPGC	endurance	pre vs 1h	0.050	0.078
totalPGC	endurance	pre vs 3h	0.013	0.013
totalPGC	strength	Friedman	0.003	0.004
totalPGC	strength	pre vs post	0.273	0.630
totalPGC	strength	pre vs 1h	0.013	0.013
totalPGC	strength	pre vs 3h	0.013	0.013
ex1a	control	Friedman	0.003	0.020
ex1a	control	pre vs post	0.050	0.144
ex1a	control	pre vs 1h	0.013	0.036
ex1a	control	pre vs 3h	0.013	0.036
ex1a	endurance	Friedman	0.017	0.123
ex1a	endurance	pre vs post	0.050	-
ex1a	endurance	pre vs 1h	0.035	-
ex1a	endurance	pre vs 3h	0.024	-
ex1a	strength	Friedman	0.003	0.004
ex1a	strength	pre vs post	0.093	0.106
ex1a	strength	pre vs 1h	0.013	0.013
ex1a	strength	pre vs 3h	0.013	0.013
ex1b	control	Friedman	0.003	0.004
ex1b	control	pre vs post	0.013	0.106
ex1b	control	pre vs 1h	0.013	0.013
ex1b	control	pre vs 3h	0.013	0.013
ex1b	endurance	Friedman	0.003	0.004
ex1b	endurance	pre vs post	0.757	0.013
ex1b	endurance	pre vs 1h	0.013	0.013
ex1b	endurance	pre vs 3h	0.013	0.013
ex1b	strength	Friedman	0.003	0.004
ex1b	strength	pre vs post	0.273	0.799
ex1b	strength	pre vs 1h	0.013	0.013
ex1b	strength	pre vs 3h	0.013	0.013
trunc	control	Friedman	0.005	0.013
trunc	control	pre vs post	0.129	0.945
trunc	control	pre vs 1h	0.093	0.705
trunc	control	pre vs 3h	0.013	0.013
trunc	endurance	Friedman	0.010	0.013
trunc	endurance	pre vs post	0.852	0.537
trunc	endurance	pre vs 1h	0.068	0.377
trunc	endurance	pre vs 3h	0.013	0.013
trunc	strength	Friedman	0.011	0.004
trunc	strength	pre vs post	0.338	0.705
trunc	strength	pre vs 1h	0.050	0.013
trunc	strength	pre vs 3h	0.013	0.013
nontrunc	control	Friedman	0.005	0.013
nontrunc	control	pre vs post	0.757	0.799

nontrunc	control	pre vs 1h	0.068	0.945
nontrunc	control	pre vs 3h	0.013	0.013
nontrunc	endurance	Friedman	0.005	0.013
nontrunc	endurance	pre vs post	0.489	0.451
nontrunc	endurance	pre vs 1h	0.173	0.251
nontrunc	endurance	pre vs 3h	0.013	0.013
nontrunc	strength	Friedman	0.003	0.009
nontrunc	strength	pre vs post	0.273	0.313
nontrunc	strength	pre vs 1h	0.013	0.078
nontrunc	strength	pre vs 3h	0.013	0.013

Appendix 5: ***Group comparison of PGC1α Expression at different timepoints post exercise.*** *FDR = False discovery rate; ns = not significant.*

Isoform	Timepoint	Intervention	group1	group2	p-value	FDR	signif
ex1a	post	endurance	control	endurance	0.860	0.860	ns
ex1a	post	endurance	control	strength	0.548	0.822	ns
ex1a	post	endurance	endurance	strength	0.437	0.822	ns
ex1a	post	resistance	control	endurance	0.437	0.822	ns
ex1a	post	resistance	control	strength	0.548	0.822	ns
ex1a	post	resistance	endurance	strength	0.860	0.860	ns
ex1a	1h	endurance	control	endurance	0.104	0.221	ns
ex1a	1h	endurance	control	strength	0.860	0.860	ns
ex1a	1h	endurance	endurance	strength	0.147	0.221	ns
ex1a	1h	resistance	control	endurance	0.066	0.099	ns
ex1a	1h	resistance	control	strength	0.437	0.437	ns
ex1a	1h	resistance	endurance	strength	0.009	0.027	*
ex1a	3h	endurance	control	endurance	0.018	0.054	ns
ex1a	3h	endurance	control	strength	0.305	0.305	ns
ex1a	3h	endurance	endurance	strength	0.179	0.269	ns
ex1a	3h	resistance	control	endurance	0.112	0.167	ns
ex1a	3h	resistance	control	strength	0.525	0.525	ns
ex1a	3h	resistance	endurance	strength	0.026	0.078	ns
nontrunc	post	endurance	control	endurance	0.888	0.888	ns
nontrunc	post	endurance	control	strength	0.229	0.344	ns
nontrunc	post	endurance	endurance	strength	0.179	0.344	ns
nontrunc	post	resistance	control	endurance	0.572	0.572	ns
nontrunc	post	resistance	control	strength	0.273	0.410	ns
nontrunc	post	resistance	endurance	strength	0.097	0.290	ns
nontrunc	1h	endurance	control	endurance	0.805	0.805	ns
nontrunc	1h	endurance	control	strength	0.377	0.565	ns
nontrunc	1h	endurance	endurance	strength	0.258	0.565	ns
nontrunc	1h	resistance	control	endurance	0.671	0.671	ns
nontrunc	1h	resistance	control	strength	0.071	0.107	ns

nontrunc	1h	resistance	endurance	strength	0.026	0.078	ns
nontrunc	3h	endurance	control	endurance	0.157	0.236	ns
nontrunc	3h	endurance	control	strength	0.437	0.437	ns
nontrunc	3h	endurance	endurance	strength	0.028	0.085	ns
nontrunc	3h	resistance	control	endurance	0.860	0.860	ns
nontrunc	3h	resistance	control	strength	0.009	0.013	*
nontrunc	3h	resistance	endurance	strength	0.005	0.013	*
ex1b	post	endurance	control	endurance	0.061	0.125	ns
ex1b	post	endurance	control	strength	0.083	0.125	ns
ex1b	post	endurance	endurance	strength	0.888	0.888	ns
ex1b	post	resistance	control	endurance	0.028	0.043	*
ex1b	post	resistance	control	strength	0.777	0.777	ns
ex1b	post	resistance	endurance	strength	0.013	0.040	*
ex1b	1h	endurance	control	endurance	0.028	0.085	ns
ex1b	1h	endurance	control	strength	0.322	0.322	ns
ex1b	1h	endurance	endurance	strength	0.229	0.322	ns
ex1b	1h	resistance	control	endurance	0.671	0.671	ns
ex1b	1h	resistance	control	strength	0.243	0.365	ns
ex1b	1h	resistance	endurance	strength	0.112	0.335	ns
ex1b	3h	endurance	control	endurance	0.016	0.049	*
ex1b	3h	endurance	control	strength	0.128	0.193	ns
ex1b	3h	endurance	endurance	strength	0.377	0.377	ns
ex1b	3h	resistance	control	endurance	0.646	0.646	ns
ex1b	3h	resistance	control	strength	0.028	0.085	ns
ex1b	3h	resistance	endurance	strength	0.083	0.125	ns
totalPGC	post	endurance	control	endurance	0.525	0.525	ns
totalPGC	post	endurance	control	strength	0.289	0.433	ns
totalPGC	post	endurance	endurance	strength	0.090	0.269	ns
totalPGC	post	resistance	control	endurance	1.000	1.000	ns
totalPGC	post	resistance	control	strength	0.396	0.594	ns
totalPGC	post	resistance	endurance	strength	0.396	0.594	ns
totalPGC	1h	endurance	control	endurance	0.750	0.750	ns
totalPGC	1h	endurance	control	strength	0.340	0.750	ns
totalPGC	1h	endurance	endurance	strength	0.525	0.750	ns
totalPGC	1h	resistance	control	endurance	0.832	0.832	ns
totalPGC	1h	resistance	control	strength	0.020	0.029	*
totalPGC	1h	resistance	endurance	strength	0.011	0.029	*
totalPGC	3h	endurance	control	endurance	0.048	0.125	ns
totalPGC	3h	endurance	control	strength	0.805	0.805	ns
totalPGC	3h	endurance	endurance	strength	0.083	0.125	ns
totalPGC	3h	resistance	control	endurance	0.888	0.888	ns
totalPGC	3h	resistance	control	strength	0.018	0.027	*
totalPGC	3h	resistance	endurance	strength	0.012	0.027	*
trunc	post	endurance	control	endurance	0.168	0.252	ns

trunc	post	endurance	control	strength	0.138	0.252	ns
trunc	post	endurance	endurance	strength	0.916	0.916	ns
trunc	post	resistance	control	endurance	0.777	0.777	ns
trunc	post	resistance	control	strength	0.480	0.719	ns
trunc	post	resistance	endurance	strength	0.322	0.719	ns
trunc	1h	endurance	control	endurance	0.458	0.750	ns
trunc	1h	endurance	control	strength	0.750	0.750	ns
trunc	1h	endurance	endurance	strength	0.671	0.750	ns
trunc	1h	resistance	control	endurance	0.805	0.805	ns
trunc	1h	resistance	control	strength	0.120	0.286	ns
trunc	1h	resistance	endurance	strength	0.191	0.286	ns
trunc	3h	endurance	control	endurance	0.077	0.193	ns
trunc	3h	endurance	control	strength	0.805	0.805	ns
trunc	3h	endurance	endurance	strength	0.128	0.193	ns
trunc	3h	resistance	control	endurance	0.944	0.944	ns
trunc	3h	resistance	control	strength	0.018	0.032	*
trunc	3h	resistance	endurance	strength	0.022	0.032	*

Appendix 6: ***Exercise comparison within a group of PGC1α Expression at different timepoints post exercise.*** *FDR = False discovery rate.*

Transcript	Timepoint	Group	p-value	FDR
ex1a	post	control	0.547	0.742
ex1a	post	endurance	0.742	0.742
ex1a	post	strength	0.742	0.742
ex1a	1h	control	0.250	0.375
ex1a	1h	endurance	0.039	0.117
ex1a	1h	strength	0.383	0.383
ex1a	3h	control	0.250	0.461
ex1a	3h	endurance	0.461	0.461
ex1a	3h	strength	0.383	0.461
nontrunc	post	control	0.945	1.000
nontrunc	post	endurance	1.000	1.000
nontrunc	post	strength	0.641	1.000
nontrunc	1h	control	0.195	0.293
nontrunc	1h	endurance	0.055	0.164
nontrunc	1h	strength	0.313	0.313
nontrunc	3h	control	0.016	0.047
nontrunc	3h	endurance	0.109	0.164
nontrunc	3h	strength	0.547	0.547
ex1b	post	control	0.742	0.844
ex1b	post	endurance	0.148	0.445
ex1b	post	strength	0.844	0.844
ex1b	1h	control	0.109	0.328
ex1b	1h	endurance	0.945	0.945

ex1b	1h	strength	0.945	0.945
ex1b	3h	control	0.148	0.148
ex1b	3h	endurance	0.078	0.148
ex1b	3h	strength	0.148	0.148
totalPGC	post	control	0.945	0.945
totalPGC	post	endurance	0.461	0.820
totalPGC	post	strength	0.547	0.820
totalPGC	1h	control	0.109	0.164
totalPGC	1h	endurance	0.078	0.164
totalPGC	1h	strength	0.742	0.742
totalPGC	3h	control	0.039	0.117
totalPGC	3h	endurance	0.742	0.742
totalPGC	3h	strength	0.148	0.223
trunc	post	control	0.461	0.820
trunc	post	endurance	0.844	0.844
trunc	post	strength	0.547	0.820
trunc	1h	control	0.195	0.293
trunc	1h	endurance	0.078	0.234
trunc	1h	strength	0.742	0.742
trunc	3h	control	0.109	0.164
trunc	3h	endurance	0.742	0.742
trunc	3h	strength	0.078	0.164

Appendix 7: ***Differentially methylated positions after acute exercise.*** *FDR = False discovery rate.*

DMPs: Endurance Group doing acute endurance exercise			
Gene	**Feature**	**Coordinates GRCh38/hg38**	**FDR**
MYL3	Promoter	chr3:46863811	<0.01
MYL3	Promoter	chr3:46863810	0.02
MYL3	Promoter	chr3:46863726	0.01
MYL3	Promoter	chr3:46863927	<0.01
MYL3	Promoter	chr3:46863768	<0.01
DMPs: Control Group doing acute resistance exercise			
Gene	**Feature**	**Coordinates GRCh38/hg38**	**FDR**
MYH2	Promoter	chr17:10550012	0.03
DMRs: Strength Group doing acute resistance exercise			
Gene	**Feature**	**Coordinates GRCh38/hg38**	**FDRS**
CAMKII	Promoter	chr5: 150290251	0.04

Appendix 8: Proportions of different immune cells estimated by CIBERSORTx. CG = control group; EG = endurance group; SG = strength group; NK cells = natural killer cells.

CellType	CG	EG	SG	Significant difference
B.cells.memory	0.001	0	0.001	
B.cells.naive	0.133	0.125	0.114	
Dendritic.cells.activated	0.006	0.002	0.006	
Dendritic.cells.resting	0.001	0.001	0.006	
Eosinophils	0.025	0.014	0.044	
Macrophages.M0	0.007	0.001	0	
Macrophages.M1	0.07	0.055	0.07	
Macrophages.M2	0.469	0.375	0.509	* EGvsSG
Mast.cells.activated	0.011	0.005	0.003	
Mast.cells.resting	0.269	0.248	0.275	
Monocytes	0.061	0.058	0.075	
Neutrophils	0.068	0.018	0.034	
NK.cells.activated	0.065	0.058	0.062	
NK.cells.resting	0.096	0.167	0.119	*EGvsCG
Plasma.cells	0.059	0.015	0.017	
T.cells.CD4.memory.activated	0	0	0	
T.cells.CD4.memory.resting	0.296	0.302	0.377	
T.cells.CD4.naive	0.003	0.01	0.015	
T.cells.CD8	0.058	0.04	0.017	
T.cells.follicular.helper	0.071	0.075	0.106	
T.cells.gamma.delta	0	0	0	
T.cells.regulatory..Tregs.	0.002	0.001	0.005	

11 Own Contributions and personal publications

This project was conducted as part of a larger study—the CrossEX project. The CrossEX project aims to understand the effects of long-term training and acute exercise through a systems biology approach by investigating molecular profiles in skeletal muscle, adipose tissue, and blood at a multi-omics level (Reitzner et al., 2023). Sample collection was led by my co-supervisor, Stefan Reitzner. The research question addressed here, focusing on DNA methylation, which serves as the main result of this dissertation, was conceived by me. The conceptualization was led by me and carried out together with my supervisors. The selection of methods and the design of the target panel were based on my own literature review, followed by discussions with my supervisors and an investigation of RNA-sequencing data from the same samples (Reitzner et al., 2023). I prepared the muscle samples for EM-seq, which was performed by the SNP&SEQ Technology Platform in Uppsala. This facility is part of the National Genomics Infrastructure (NGI) Sweden and the Science for Life Laboratory.
Additionally, I conducted the *PGC1α* isoform analysis and independently carried out all statistical analysis.

I published the findings of this dissertation in the following article:

Geiger C, Needhamsen M, Emanuelsson EB, et al. DNA methylation of exercise-responsive genes differs between trained and untrained men. *BMC Biol*. 2024;22(1):147. Published 2024 Jul 4. doi:10.1186/s12915-024-01938-6

My personal contribution to this publication was conceptualisation and generation of the findings and writing the first draft which was then reviewed and edited by my co-authors.

I also presented my findings to the research community:

Oral presentation: „Difference in DNA methylation of muscle fiber type genes in trained and untrained men“, SFAIM Vårmöte May 2024 (Sports Medicine Spring Meeting of the Swedish Association for Sports Medicine), Stockholm, Sweden

Poster presentation “DNA methylation of exercise responsive genes differs between trained and untrained men”, Cell Symposia: Exercise metabolism. May 2024, Lisbon, Portugal

Oral presentation „PGC-1α splice variant expression after acute resistance exercise positively correlates with leg strength“, SFAIM Vårmöte May 2022 (Sports Medicine Spring Meeting of the Swedish Association for Sports Medicine), Stockholm, Sweden

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13 Eidesstattliche Versicherung

1. Bei der eingereichten Dissertation zu dem Thema

“***DNA methylation of exercise-responsive genes and PGC1a splice variant expression differs between trained and untrained men”***

handelt es sich um meine eigenständig erbrachte Leistung.

2. Ich habe nur die angegebenen Quellen und Hilfsmittel benutzt und mich keiner unzulässigen Hilfe Dritter bedient. Insbesondere habe ich wörtlich oder sinngemäß aus anderen Werken übernommene Inhalte als solche kenntlich gemacht.

3. Die Arbeit oder Teile davon habe ich bislang nicht an einer Hochschule des In- oder Auslands als Bestandteil einer Prüfungs- oder Qualifikationsleistung vorgelegt.

4. Die Richtigkeit der vorstehenden Erklärungen bestätige ich.

5. Die Bedeutung der eidesstattlichen Versicherung und die strafrechtlichen Folgen einer unrichtigen oder unvollständigen eidesstattlichen Versicherung sind mir bekannt. Ich versichere an Eides statt, dass ich nach bestem Wissen die reine Wahrheit erklärt und nichts verschwiegen habe.

Heidelberg, 06.02.2025 Carla Geiger

14 Angaben zu verwendeten KI-basierter elekrtonischer Hilfsmittel

Zur Dokumentation der verwendeten Hilfsmittel ist der schriftlichen Ausarbeitung ein besonderer Anhang hinzugefügt, der eine Liste und Beschreibung aller verwendeter KI-basierter Hilfsmittel enthält. Der besondere Anhang zur Dokumentation der verwendeten Hilfsmittel erfüllt folgende Kriterien: 1. Auflistung der Ziele, für die die KI-basierten Hilfsmittel in der vorliegenden Arbeit eingesetzt wurden. 2. Dokumentation der Verwendungsweise der KI-basierten Hilfsmittel. 3. Nennung der Kapitel und Abschnitte der vorliegenden Arbeit, in denen die KI-basierten Hilfsmittel eingesetzt wurden, um Inhalte zu erzeugen.

ChatGPT wurde zur Hilfe beim Programmieren eingesetzt. Genauer wurde ChatGPT verwendet um Fehler im eigenständig generierten Code zu finden. Scribbr KI-Text-Korrektur und ChatGPT wurden zur Korrektur von Rechtschreibung und Grammatik angewandt.

Der Gebrauch dieser Hilfsmittel inklusive Art, Ziel und Umfang des Gebrauchs wurde mit meinem offiziellen Betreuer Prof. Dr. Karen Steindorf abgesprochen.

Mir ist bewusst, dass insbesondere der Versuch einer nicht dokumentierten Nutzung KI-basierter Hilfsmittel als Täuschungsversuch zu werten ist:

Gem. § 16 Abs. 2 der Promotionsordnung „Dr. med. / dent.“:

„Ergibt sich vor Aushändigung der Promotionsurkunde, dass der Kandidat/die Kandidatin bei einer Promotionsleistung getäuscht hat, so können einzelne oder alle Promotionsleistungen für ungültig erklärt werden. In schweren Fällen kann die Zulassung zum Promotionsverfahren zurückgenommen werden.“

Und § 16 Abs. 2 der Promotionsordnung „Dr. sc. hum.“:

„Ergibt sich vor Aushändigung der Promotionsurkunde, dass der Doktorand / Doktorandin bei einer Promotionsleistung getäuscht hat, so kann der Promotionsausschuss diese Promotionsleistung oder alle bisher erbrachten Promotionsleistungen für ungültig erklären. In besonders schweren Fällen kann der Promotionsausschuss die Annahme als Doktorand / Doktorandin endgültig widerrufen.“

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