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DISSERTATION / DOCTORAL THESIS

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„The influence of lifestyle such as exercise, psychological stress, and nutrition on epigenetic regulation via DNA methylation and miRNAs“

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ACE	angiotensin-converting enzyme
ACTN3	alpha-actinin-3
AKT	protein kinase B
AMP	adenosine monophosphate
AMPK	AMP-activated protein kinase
ATP	adenosine triphosphate
BCAA	branched-chain amino acid
BDNF	brain-derived neurotrophic factor
BFM	body fat mass
BMI	body mass index
CAD	coronary artery disease
CFS	chronic fatigue syndrome
ci-miRNA	circulating miRNA
CT	childhood trauma
CV	cardiovascular
CVD	CV disease
DASH	dietary approaches to stop hypertension
DGCR8	DiGeorge syndrome critical region gene 8
DNA	deoxyribonucleic acid
DNMT	DNA methyltransferase
ECW	extracellular water
FAK	focal adhesion kinase
FTO	fat mass and obesity-associated
HDAC	histone deacetylase
HDL	high-density lipoprotein
IGF-1	insulin-like growth factor 1
IMTG	intramuscular triacylglycerols
LBM	lean body mass
lncRNA	long ncRNA
MAPK	mitogen-activated protein kinase
MDD	major depressive disorder
miRNA	microRNA
mRNA	messenger RNA
mtDNA	mitochondrial DNA
mTOR	mammalian target of rapamycin
myomiR	muscle-specific miRNA
NCD	non-communicable disease
ncRNA	noncoding RNA
NRF1	nuclear respiratory factor 1
NRF2	nuclear factor erythroid 2-related factor 2
PGC-1α	PPAR γ coactivator 1α
PPAR	peroxisome proliferator-activated receptor
pre-miRNA	precursor miRNA
pri-miRNA	primary miRNA
RISC	RNA-induced silencing complex
RNA	ribonucleic acid
RNA POL II	RNA polymerase II
ROS	reactive oxygen species
SAM	S-adenosyl-methionine

SCFA	short-chain fatty acid
SERT	serotonin transporter
SIRT	sirtuin
SNP	single nucleotide polymorphism
SRD	stress-related disorder
TFAM	mitochondrial transcription factor A
TG	triacylglycerols
TLR	Toll-like receptor
VO_{2max}	maximal oxygen consumption

1. INTRODUCTION

1.1. Exercise, nutrition, and mental health are the pillars of a long, healthy, and balanced life

To achieve longer, healthier, and more active lives, people of all ages should follow certain recommendations that cover the three main pillars of health: exercise, nutrition, sleep, and mental health, including psychological stress (**Figure 1**).

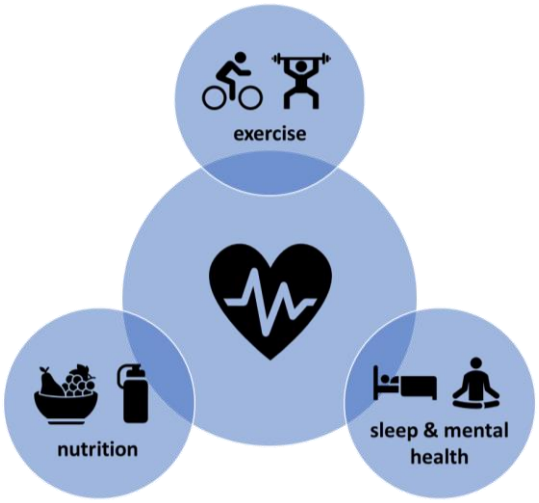

Figure 1. The three main pillars of health and longevity: exercise, nutrition, sleep, and mental health.

Even though life expectancy has increased enormously in the last two centuries, people are still far from living a healthy and long life due to extrinsic (e.g., environmental) and intrinsic (e.g., lifestyle habits) factors, therefore chronic or age-related diseases (e.g., cardiovascular (CV) and metabolic diseases), mental disorders, and premature deaths simultaneously thrive [1, 2]**.**

There is no longer any doubt that daily habits and actions have a major impact on short- and long-term health, quality of life, and longevity [3]. The majority of global deaths can be attributed to non-communicable diseases (NCDs), which are characterized by an unbalanced diet, together with insufficient sleep, psychological stress, an physical inactivity [4, 5]. Coronary heart diseases, strokes, and diabetes account for most of these deaths, and an estimated half of premature death are due to behaviors that can be modified, such as diet and physical activity or exercise. It has also been shown that adopting a healthy lifestyle can reduce the risk of developing diabetes, coronary heart disease, stroke, and cancer by almost 80% [4]. Thus, research on how habits and actions affect both the prevention and treatment of diseases are summarized under the term “lifestyle medicine” and represents an important adjunct to conventional treatment with pharmaceutical or surgical therapies [3].

1.1.1. Exercise and physical activity

Regular exercise or physical activity is one of the most important non-pharmacological intervention that prolongs health and lifespan by delaying the onset of age-related diseases and significantly reducing the risk of obesity, depression, cancer, and CVDs [6]. However, the molecular mechanisms that trigger the protective effects of exercise, such as decreased adiposity, increased cardiorespiratory fitness, decreased levels of circulating lipids, and maintenance of muscle mass, are still relatively unknown. Based on the current state of research, it is believed that they are due to crosstalk between tissues during exercise. During exercise and, to some extent, physical activity, proteins, peptides, metabolites, and enzymes are released from contracting skeletal muscle (myokines), liver (hepatokines), and adipose tissue (adipokines) to affect metabolism in other organs, which are involved, for example, in the regulation of energy homeostasis or insulin sensitivity [7, 8]. **Figure 2** shows an overview of some of the organ-specific acute and chronic adaptions in the human body triggered by exercise and physical activity.

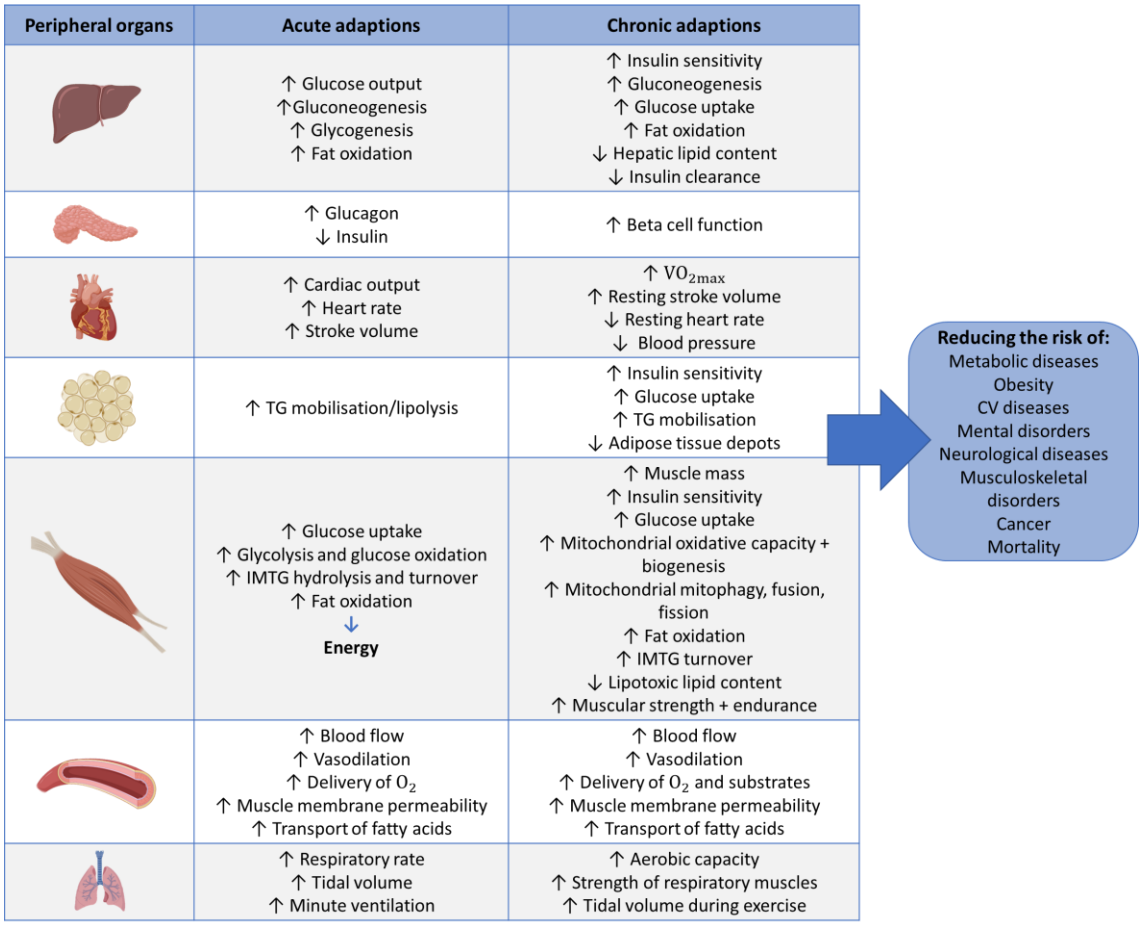

Figure 2. Acute and chronic effects of exercise and physical activity [Inspired by Thyfault and Bergouignan, 2020 [8]; Created with BioRender.com]. Abbreviations: TG = triacylglycerols; IMTG = intramuscular triacylglycerols; CV = cardiovascular.

These exercise-related adjustments depend not only on the duration, volume, intensity, and frequency of training but also on the type of training [9]. Endurance exercise (**Figure 3**) induces changes in the metabolic properties of skeletal muscle by conferring an increased oxidative profile on exercised muscle fibers via the calcium signaling pathways, AMP-activated protein kinase (AMPK), and the peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α). PGC-1α is the main regulator of mitochondrial biogenesis and regulates genes involved in energy metabolism. Healthy mitochondria are essential for maintaining health, extending life expectancy, and improving cardiovascular fitness [10, 11].

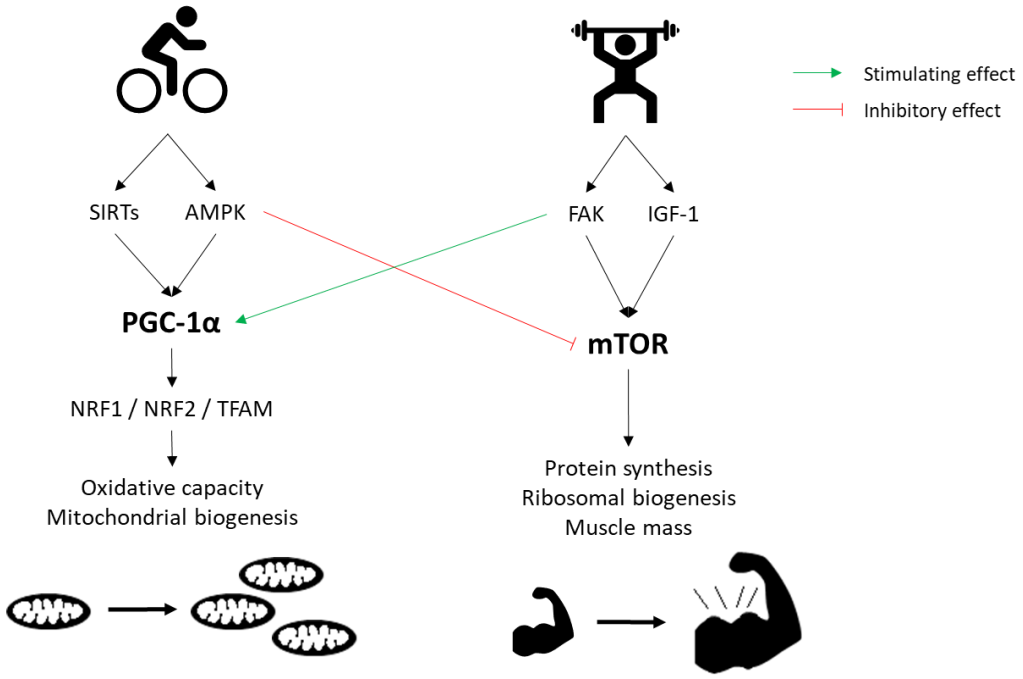

Figure 3. Major signaling pathways in the regulation of hypertrophy and mitochondrial biogenesis [Inspired by Krammer et al., 2022 [11]**]**. Abbreviations: SIRTs = sirtuins; AMPK = AMP-activated protein kinase; PGC-1α = peroxisome proliferator-activated receptor (PPAR)-γ coactivator-1α; NRF1 = nuclear respiratory factor 1; NRF2 = nuclear factor erythroid 2-related factor 2; TFAM = mitochondrial transcription factor A; FAK = focal adhesion kinase; IGF-1 = insulin-like growth factor 1; mTOR = mammalian target of rapamycin.

Resistance training (**Figure 3**), on the other side, leads to an increase in muscle fiber size (hypertrophy) and maximum tension through a positive muscle protein balance and is therefore important for maintaining muscle and bone mass [12]. Activation of mammalian target of rapamycin (mTOR) seems to be important for this increase in muscle protein biosynthesis. Its activation leads to a variety of different reactions, including the translation of proteins, ribosomal biogenesis, and cell growth [10, 11]. Especially in the elderly population, resistance training has a positive effect on muscle mass and strength as well as on physical functions in everyday activities and thus on general health and well-being [13]. Loss of muscle mass and function is also associated with an increased

risk of type 2 diabetes, CVDs, and neurodegenerative diseases such as dementia [14]. Exercise or physical activity in general is therefore essential for a long, healthy, and mobile life. There is strong evidence that regular physical activity or exercise maintains the function of various physiological systems, especially skeletal muscle, during aging, e.g., by affecting blood flow, oxygen, and nutrient supply in the skeletal muscle. Recent studies suggest that optimal protein intake alongside exercise intervention is an auspicious strategy to promote healthy aging of skeletal muscle and prevent muscle mass loss in the elderly [14].

However, most sports are neither exclusively endurance nor solely strength training, but a mixture of both called concurrent exercise. Yet concurrent training leads to less muscle hypertrophy, strength, and power adaptions compared to isolated strength training stimuli. It has been suggested that endurance training negatively affects the adaptions induced by resistance training, presumably by inhibiting the activation of the protein kinase B (AKT)-mTOR signaling pathways through AMPK. Whereas resistance training has no negative impact on the effects of endurance training, it may even stimulate mitochondrial biogenesis in muscle cells via focal adhesion kinase (FAK; **Figure 3**) [9, 11].

1.1.2. Nutrition and bioactive compounds

Lifestyle habits and actions also include diet, which affects almost every chronic disease. Nutrition plays an essential role in the prevention and treatment of CVD, diabetes, obesity, cancer, and many others [3]. These diseases are closely associated with eating foods high in saturated fats, sugars, and salt and low intakes of vegetables, fruits, and omega-3 fatty acids as well as increased alcohol consumption [5]. However, there are many guidelines, recommendations, and advices related to a healthy and balanced diet, such as the dietary approaches to stop hypertension (DASH) diet for high blood pressure therapy or the Mediterranean diet rich in fruits, vegetables, fish, and extra virgin olive oil to prevent NCDs [3, 5]. Apart from vitamins, minerals, and dietary fiber, these beneficial effects are mainly due to the natural bioactive compounds (secondary plant products or phytochemicals) found in fruits, vegetables, legumes, whole grains, and nuts that have antioxidant, anti-inflammatory, antidiabetic, antiviral, anticancer, and antitumor functions [15]. Bioactive compounds (e.g., polyphenols, terpenoids, or glucosinolates) can protect against high concentrations of free radicals and reactive oxygen species (ROS) that could easily react with other molecules, including nucleic acids, lipids, and proteins, and cause cell damage [16]. Therefore, these compounds are considered as a promising therapy for the prevention and treatment of age-related and non-communicable diseases

such as CVDs, Alzheimer's diseases, diabetes, and cancer [15, 17]. Similar to exercise, fasting, or caloric restriction, phytochemicals lead to the activation of AMPK and sirtuin 1 (SIRT1), which in turn influences mTOR, PGC-1α, and nuclear factor erythroid 2-related factor 2 (NRF2) and consequently mitochondrial and ribosomal biogenesis (**Figure 3**) [18].

However, free radicals and ROS are produced by various cellular biochemical reactions occurring in the human body, such as in the mitochondria for oxygen production, in fatty acid metabolism, and in immune system activities, as well as from the skeletal muscle during exercise and physical activity. Low ROS levels or mild oxidative stress are important because, at physiological levels, ROS plays a role in growth, proliferation, and differentiation, and induce adaptive responses that affect short-term (e.g., metabolic), and long-term (e.g., longevity) health [11, 19]. This is also known as hormesis, which states that low exposure leads to adaptive changes and creates an acquired resilience to subsequent exposure to stress [20]. On the other side, excessive ROS production from overtraining/exhaustive exercise or some nutrients (e.g., free fatty acids, glucose, amino acids, or Western diet) leads to oxidative damage, impaired exercise performance (e.g., muscle damage, and impaired muscle functions), premature aging, and further health consequences. Therefore, more and more experts are suggesting the consumption of more antioxidants or bioactive compounds to prevent various diseases and reduce ROS production, in order to prevent oxidative stress [19, 21–23].

1.1.3. Mental health

Mental disorders such as anxiety, depression, and psychological stress are all endemic diseases of the modern world and affect a person's physical and mental well-being and hence overall health [3, 24]. While some mild levels of stress can be protective, excessive stress plays a crucial role in the development and maintenance of many diseases (e.g., cardiovascular, such as hypertension, or metabolic diseases, such as diabetes) and mental or neurodegenerative disorders (such as depression or Alzheimer's disease). In general, a mental disorder is described as a brain dysfunction caused by neurotransmitter imbalance, increased neuronal inflammation, impairments of synaptic transmission, defects in neurogenesis and synaptic plasticity, and mitochondrial dysfunction [25]. However, accumulating literature indicates that excessive inflammation likely contributes directly to the pathophysiology of these stress-related disorders (SRDs) [3, 25, 26]. Depending on the type and intensity of the stressor, both pro- and anti-inflammatory mechanisms are induced. Acute stressors appear to enhance immune functions, while chronic

stressors over-activate the immune system by releasing glucocorticoids, catecholamines, cytokines, and others like C-reactive protein or interleukins (e.g., IL-6), which may contribute to the development of CVDs [26]. Furthermore, it is assumed that stress affects health not only via biological pathways (e.g., neuroendocrine, and autonomic processes) but also via indirect, behavioral pathways, i.e., by influencing health and nutritional behaviors. Stress is associated with a reduced intake of healthy foods and an increased intake of unhealthy foods, and these unhealthy dietary choices can increase the risk of obesity, diabetes, and metabolic syndrome [26, 27]. Lifestyle interventions such as regular physical activity, positive psychology, and adequate amount of sleep have been shown to have a positive effect on anxiety, depression, and stress, and therefore on mental and overall health [3].

1.2. Genetic aspects and single nucleotide polymorphisms (SNPs)

Variations in the human genome (polymorphisms) appear to affect how an individual or a particular group in the population responds to exposure to environmental health hazards and how likely they are to develop a disease after exposure. These relationships are also referred to as gene-environment interactions and include not only genetically determined disease risks but also other factors such as variations in voluntary exercise [28–30]. The most common sequence variation in the human genome is a single base substitution, also known as single nucleotide polymorphisms (SNP), which is heritable and occurs with a frequency of more than 1% in at least one population [31, 32].

For example, 26 different SNPs were identified in six candidate genes that influence behavior during exercise, i.e., intensity, duration, and total dose of exercise. Interestingly, there is also a genetic predisposition for endurance (alpha-actinin-3 (*ACTN3*) gene) and resistance training (angiotensin-converting enzyme (*ACE*) gene) [29]. Certain variations in the *ACE* gene are associated with lower or higher ACE levels. Lower levels lead to improved cardiorespiratory fitness and thus better endurance performance, while higher levels are associated with increased musculoskeletal fitness, which positively influences strength performance [33]. Whereas the *ACTN3* gene influences athletic performance through the expression of alpha-actinin-3, and a deficiency in this protein is associated with lower fast-twitch fiber percentage [33, 34]. However, one of the most debated genes related to obesity and diabetes is the fat mass and obesity-associated (*FTO*) gene. SNPs in this gene are associated with increased body mass index (BMI), waist circumference, weight, and other metabolic characteristics such as increased triglycerides, glucose, and lower HDL cholesterol [35, 36]. In addition to genetic predisposition, environmental

factors also play an important role and some studies have already shown that physical activity can reduce the increased risk of obesity due to genetic variation in the *FTO* gene, which in turn underlines the importance of so-called “lifestyle medicine” [37].

1.3. Epigenetic alterations

In gene-environment interactions, it is assumed that genetic polymorphisms modify the effects of environmental exposure. But the direct effects of environmental influences also include the level of expression or the function of the genes and proteins. This second aspect is known as epigenetics (**Figure 4**) [38]. Epigenetics was originally described as a complex of developmental processes between genotype and phenotype [39]. Today, epigenetics means hereditary changes in gene expression without changing the DNA sequence. Most epigenetic modifications arise from environmental interactions, e.g., inactivity or exercise and nutrition, that cause an increase/decrease in gene expression or gene silencing (e.g., in obesity, diabetes, and hypertension) [40, 41]. Epigenetic mechanisms include DNA methylation, histone modifications, and non-coding RNAs (such as miRNAs), which are involved in the regulation of gene expression, cell differentiation, and genomic imprinting (**Figure 4**) [42].

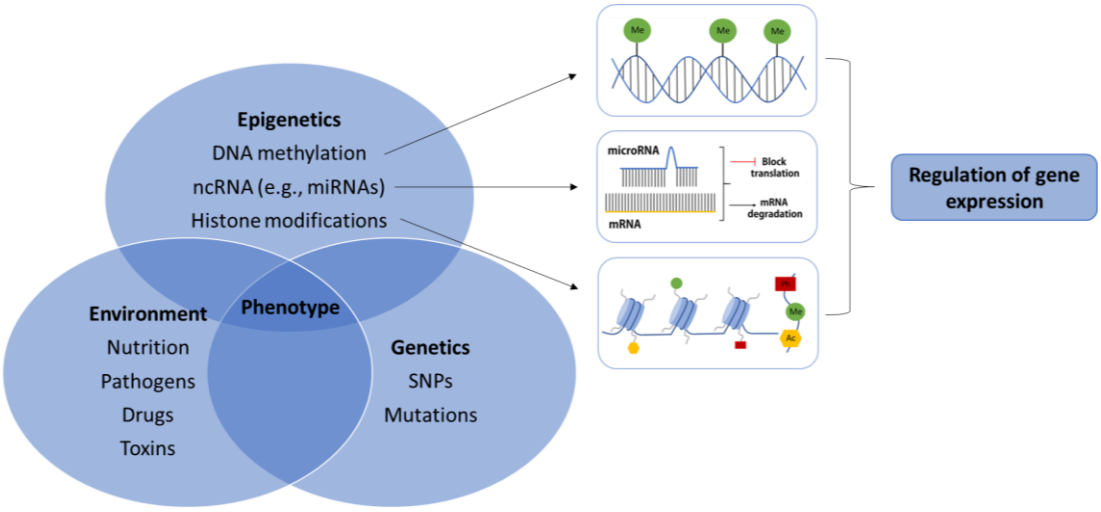

Figure 4. Interactions between genetics, epigenetics, and environmental factors and the epigenetic mechanisms that regulate gene expression [Inspired by Xing and Wong, 2022 [43] and Beetch et al., 2019 [44]]. Abbreviations: ncRNA = noncoding RNA; miRNA = microRNA; SNPs = single nucleotide polymorphisms; mRNA = messenger RNA.

1.3.1. DNA methylation

Every individual has a unique genome found in almost every cell that contains the information needed to build an organism. This information consists of the DNA sequence and a system of chemical modifications (e.g., methylations). Although all cell types contain the same DNA sequence, their methylation patterns are different and determine the cell

phenotype. These modifications play a role in regulating gene expression and have implications for normal physiology, aging, and disease [45]. DNA methylation, especially at CpG methylation, is the best-studied epigenetic mechanism that plays an important role in various biological processes, e.g., X chromosome inactivation, development, and carcinogenesis. DNA methylation is the attachment of a methyl group (CH3) to the 5th position of cytosine induced by DNA methyltransferases (DNMTs) that use S-adenosyl-methionine (SAM) as a methyl donor, leading to modifications of chromatin structure and gene silencing. In humans, DNA methylation occurs almost exclusively in regions with a high frequency of Cytosine-phosphate-Guanine-dinucleotide sites, so-called CpG islands, which are often located in the promoter region of active genes [46–48].

However, the methyl groups necessary for DNA methylation are mainly provided by nutrition or diet via methyl donors and cofactors such as folate or methionine and introduced via the one-carbon metabolism [42]. For example, a diet low in methyl donors can trigger global hypomethylation, and gradual hypomethylation can also be observed during the aging process [49]. This association suggests that nutrition plays an essential role in maintaining DNA methylation patterns by altering substrates and cofactors, that influence the activity of enzymes involved in the one-carbon metabolism, and possibly by altering mechanisms involved in DNA demethylation activity [50].

Alongside the links between nutrition and methylation, it is also believed that some of the beneficial effects of exercise are mediated through DNA methylations, and that exercise affects the methylation status of genes involved in muscle function or exercise metabolism (e.g., *PGC-1α*). Physical activity appears to alter the methylation profile in a dose-dependent, gene- and tissue-specific manner and leads to the formation of a long-lasting favorable expression pattern for improved exercise ability [11, 29, 48].

1.3.2. Small non-coding RNAs

There are two distinct classes of RNAs, the coding RNAs, e.g., messenger RNAs (mRNAs) that are translated into proteins, and the so-called non-coding RNAs (ncRNAs), which have been considered as “evolutionary junk” for years. At the beginning of the 21st century, however, the first sequencing and analysis of the human and mouse genome revealed that this “junk” could very well be transcribed, but not into proteins. To date, numerous ncRNAs with various and important cellular functions, alone or in protein complexes, have been identified [51, 52]. These regulatory ncRNAs can be further classified

based on their size, into the small ncRNAs with transcripts less than 200 nucleotides (nt), and long ncRNAs (lncRNAs) with more than 200 nt [52].

The most common class of small ncRNAs are the microRNAs (miRNAs), which have an average length of 22 nt and regulate gene expression in the cytoplasm and nucleus. They are transcribed from DNA sequences by RNA polymerase II (RNA POL II) into primary miRNAs (pri-miRNAs) with their characteristic hairpin structure and processed by Drosha into precursor miRNAs (pre-miRNAs). Afterward, the pre-miRNAs are transported to the cytoplasm and cleaved by Dicer into mature miRNAs [52, 53]. The mature miRNAs are then integrated into an RNA-induced silencing complex (RISC) which binds to 3-untranslated regions of target mRNAs. This binding induces posttranscriptional silencing by destabilization of the mRNA and inhibiting its translation (**Figure 5**) [53, 54].

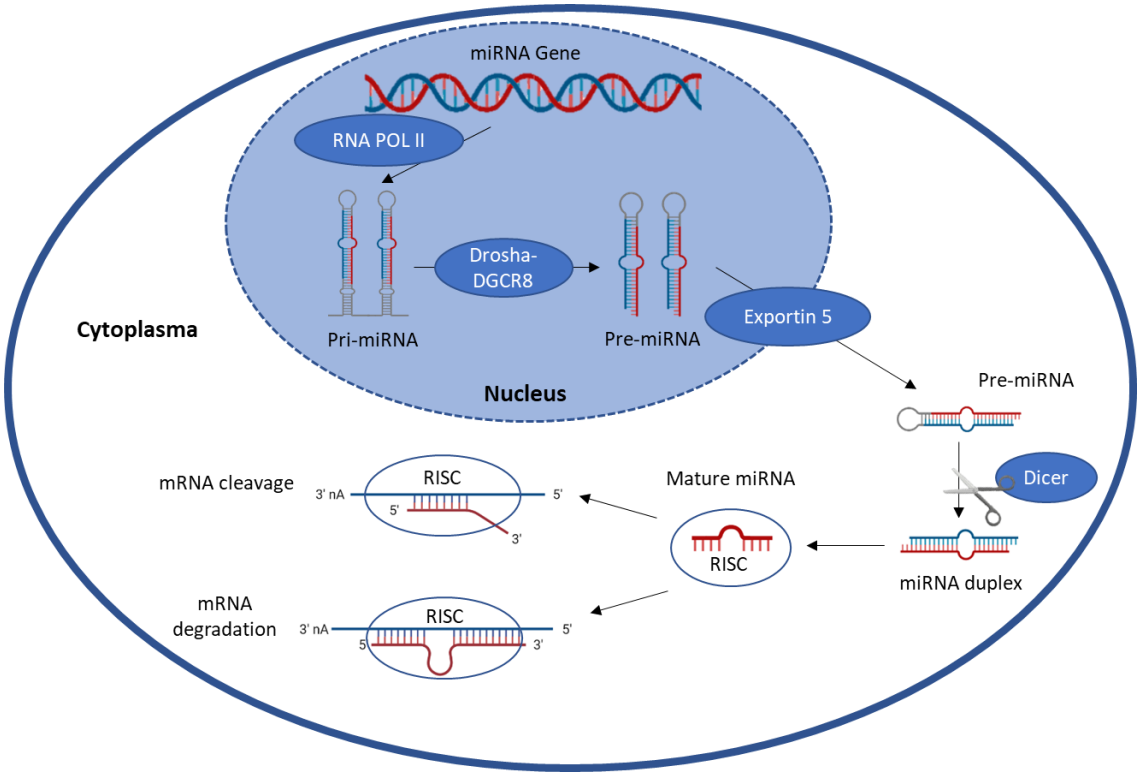

Figure 5. Biosynthesis of miRNAs and their mechanisms [Inspired by O´Brien et al., 2018 [53] and Barca-Mayo and Lu, 2012 [54]; Created with BioRender.com]. Abbreviations: RNA POL II = RNA polymerase II; DGCR8 = DiGeorge syndrome critical region gene 8; RISC = RNA-induced silencing complex.

Although the exact mechanisms of miRNAs and their activities are not fully understood, it is recognized that a single gene can be regulated by multiple miRNAs and conversely, a single miRNA can regulate multiple target genes that are clustered in a specific biological pathway. The miRNAs identified so far are mainly associated with cancer, CVDs, neurological disorders, and inflammation. MiRNAs are involved in the regulation of gene

expression, cell division, cell-cell communication, cell aging, and apoptosis, and also appear to affect metabolic pathways involved in lipid and glucose metabolism, energy homeostasis, and nutrition [55, 56]. Furthermore, these small molecules are ideal biomarkers for the diagnosis of many diseases because they are stable in various body fluids, easily accessible, tissue and cell-specific, and have high sensitivity. The literature provides clear evidence for the suitability of miRNAs as biomarkers for the diagnosis and prognosis of various diseases such as cancer, Alzheimer's disease, epilepsy, CVDs, and infectious diseases [56, 57]. However, not only diseases can influence miRNAs, but also other factors including physical activity, exercise, psychological stress, and nutrition. The level of some miRNAs changes after physical activity and exercise, thereby affecting the expression of genes involved in skeletal muscle regeneration, mitochondrial biogenesis, inflammation, angiogenesis, and remodeling. The miRNA expression varies with the duration and intensity of physical activity or exercise, as well as tissue, and can be further subdivided into cardiac, muscle-dependent, and circulating miRNAs (ci-miRNAs) [11, 29, 55].

1.3.3. Mitochondrial DNA (mtDNA) and biogenesis

Mitochondria, the "power plants" of the cells, are interconnected and highly mobile cell organelles with their own genome, the mitochondrial DNA (mtDNA). Mitochondria are essential for eukaryotic life as they provide the energy and adenosine triphosphate (ATP) needed for cellular metabolism via oxidative phosphorylation [11, 58, 59]. They are also involved in other cell functions, including cell growth, cell signaling, redox status, and regulation of ion homeostasis, and thus play an important role in cell survival and cell death. Furthermore, mitochondria produce ROS, carry out β-oxidation of fatty acids, and are involved in amino acid metabolism as well as in other intracellular signaling cascades [60]. Due to their diverse functions and tasks, mitochondria are involved in the pathogenesis and progression of numerous human diseases such as metabolic disorders, CVDs, cancer, skeletal muscle diseases or neurological disorders, and aging [58]. In order to maintain mitochondrial morphology, homeostasis, and inheritance, i.e. healthy mitochondrial physiology essential for longevity and health, mitochondria continuously undergo fission, fusion, mitophagy, and mitochondrial biogenesis [11, 61].

Mitochondrial homeostasis is maintained through the coordination between two opposing actions, the formation of new mitochondria by mitochondrial biogenesis, and the elimination of damaged mitochondria by mitophagy [16]. Cells initiate the process of mitochondrial biogenesis in response to an oxidative stimulus, by increasing cell's energy

needs, by hormones, by electrical stimulation, in certain mitochondrial diseases, during development, and by physical exercise. Thereby, PGC-1α functions as the main regulator (**Figure 3**) and due to the presence of their own DNA, new mitochondria can be formed from existing ones [11, 60].

Regular physical activity undoubtedly has beneficial or preventive effects on a variety of diseases, such as obesity, type 2 diabetes, and cancer, and it could delay depressive symptoms, neurodegeneration (e.g., Alzheimer's disease), and general aging. Repeated and regular exercise leads to an improvement in mitochondrial volume, function, and biogenesis, improves oxidation capacity, and increases the formation of mitochondrial ROS. Low levels of ROS and/or mild mitochondrial stress induce adaptive responses that can affect health in different ways. This special form of hormesis is called mitohormesis [11, 23, 62]. On the other side, physical inactivity or nutritional overload, overtraining or excessive training inhibits this adaption process, impairs mitochondrial capacity, and thereby leads to incomplete recovery, maladaptation, and possibly an increased risk of disease by increasing mitochondrial respiration and ROS production [63, 64].

2. HYPOTHESIS AND OBJECTIVES

Simultaneous endurance and strength training demonstrated more positive effects on overall health, disease prevention, mitochondrial biogenesis, and healthy aging than either modality alone. Therefore, the effects of concurrent exercise on epigenetic markers, body composition, dietary habits, and possible interaction with genetic markers (SNPs) were examined. In addition, epigenetic biomarkers in the blood (e.g., methylation and miRNAs) can provide information about processes or changes in muscle and general health, since components from tissues such as skeletal muscle, are released into the systemic circulation via extracellular vesicles. Another distinct feature of these biomarkers, especially the miRNAs, is that, in contrast to conventional markers, they can be detected in the circulatory system before consequential damage such as inflammation, injuries or stress-related diseases arise due to overtraining, poor nutrition, or chronic psychological stress. Changes in miRNA expression in body fluids are expected to occur earlier than traditional biomarkers could indicate, making miRNAs more favorable for early detection of potential deficits [11, 29]. Examining the benefits and identifying these epigenetic biomarkers is the main goal of this work and could set the next milestones towards personalized treatments, nutrition, and prevention steps.

The main hypothesis of my Ph. D. thesis was that epigenetic biomarkers, especially miRNAs, in capillary blood can provide diverse information about an individual's overall health such as nutrition, fitness status, and mental health, using a minimally invasive and less expensive method than traditional biomarkers (e.g., electrolytes or proteins).

Main objectives:

1. Investigation and identification of training-, diet-, and stress-related epigenetic mechanisms, especially miRNA expression patterns, in capillary blood and their suitability as biomarkers

2. Investigation of whether concurrent, regular exercise influences mitochondrial biogenesis, athletic ability or performance, and general health through epigenetic mechanisms

3. Investigating relationships between epigenetic mechanisms, particularly miRNA expression profiles and methylation levels, genetic predisposition to endurance or resistance training, and body composition

3. RESULTS AND DISCUSSION

3.1. Effects of concurrent exercise on miRNA expression and their suitability as biomarkers

In our publication **"miRNA-based "fitness score" to assess the individual response to diet, metabolism, and exercise"** (*J. Int. Soc. Sports Nutr., 2022*; Appendix 8.1.2*)*, we analyzed anthropometric and nutritional data and dried capillary blood samples from 95 participants. Through a small pilot study (part 1) with 14 participants, we were able to identify eight miRNAs, miR-19b, -20a, -22, -30e, -101, -146a, -378a, and -505, from a pool of over 340 miRNAs, whose expressions changed through a 10-weeks exercise program. In the second part of the study, these eight miRNAs and one methylation site were further analyzed in 81 participants, of whom 61 completed a 12-weeks training program (66.1% females, 33.9% males) and 20 were assigned to an age- and gender-matched control group without any intervention (65% females, 35% males). 12 weeks of concurrent exercise resulted in a down-regulation of miR-20a, -22, -30e, and -505, whereas in the control group there was an up-regulation of miR-20a, but no change in *Line-1* methylation in either group. However, these miRNAs have already been extensively discussed in the literature (overview in **Table 1**), with sometimes contradictory results. This could be attributed to the fact that the expression of the miRNAs depends on the type, duration, and intensity of the exercise, as well as on the tissue, and partly also on age (e.g., miR-19b, -20a, and -378a) [29, 65, 66]. Furthermore, as can be seen in **Table 1**, the available data is predominantly based on male subjects and a quite small number of study participants. Therefore, a direct comparison of the results we found with the existing literature is rather difficult, but also due to the fact we analyzed dried capillary blood, and not whole blood or muscle biopsy samples like most others, and our intervention consisted of sustained, concurrent exercise.

Table 1. Literature overview of the selected sport-associated miRNAs. The participants of the respective studies are described, what type of exercise was observed, which tissue was used for the expression analysis, and which targets were identified.

miRNA	Krammer et al. 2022 [29]	Author	Year	Sex	Participants	Type of exercise	Tissue	Effects of exercise	Predicted Targets
miR-19b	no sig. change	Margolis et al. [65]	2017	M	n = 18 no regular exercise	acute resistance exercise	Serum	up-regulation (young, 22 yr) down-regulation (old, 74 yr)	*PTEN*
		Massart et al. [67]	2021	M	n = 8 (23 yr) sedentary	14 days endurance exercise	Muscle	up-regulation	*KIF13A* *MAPK6* *RNF11* *VPS37A*
		Rivas et al. [68]	2021	M/F	n = 80 (77,5 yr) community-dwelling	6 mon. resistance training	Serum	up-regulation	*PTEN*
miR-20a	down-regulation	Radom-Aizik et al. [69]	2010	M	n = 11 (22 yr) no elite or competitive athletes	acute exercise (cycle ergometer)	Neutrophils	down-regulation	36 identified: e.g., *PTEN, MAP3K12, PIK3R1, ATP2B4*
		Baggish et al. [70]	2011	M	n = 10 (19 yr) competitive rowers	acute cardiopulmonary exercise	Plasma	up-regulation	*CDKN1A* (p21/WAF1)
		Sawada et al. [71]	2013	M	n = 12 (30 yr) physically active and experience of recreational exercise	acute resistance exercise	Serum	no sig. change	-
		Margolis et al. [65]	2017	M	n = 18 no regular exercise	acute resistance exercise	Serum	up-regulation (young, 22 yr) down-regulation (old, 74 yr)	*PTEN*
		Zhou et al. [72]	2020	M	n = 8 (21 yr)	cardiopulmonary exercise testing	Serum	down-regulation	*TNFSF15*
miR-22	down-regulation	Radom-Aizik et al. [69]	2010	M	n = 11 (22 yr) no elite or competitive athletes	acute exercise (cycle ergometer)	Neutrophils	down-regulation	8 identified: e.g., *H3F3B, PTEN*
		Davidsen et al. [73]	2011	M	n = 56 (24 yr) no active weightlifters	12-week resistance training	Muscle	no sig. change	-
		Kern et al. [74]	2019	M/F	n = 23 previously untrained	8-week endurance training (running)	Whole blood	down-regulation	*WWP1*
miR-30e	down-regulation trend	Radom-Aizik et al. [75]	2013	M	n = 13 (24 yr) no elite athletes	acute exercise (cycle ergometer)	NK cells	up-regulation	-
		Radom-Aizik et al. [76]	2014	M	n = 12 (26 yr) no elite athletes	acute exercise (cycle ergometer)	Monocytes	up-regulation	Jak-STAT

		Backes et al. [77]	2014	M/F	n = 12 (24 yr) elite endurance athl.	exhaustive, stepwise exercise test	Blood	up-regulation	*PTGDR*
		Krammer et al. [11]	2022	M/F	n = 36 (32 yr) sedentary	12-week concurrent training	Capillary blood	down-regulation	*PGC-1α*
miR-101	no sig. change	Keller et al. [78]	2011	M	n = 24 (23 yr) sedentary	6-week training (cycling)	Muscle	down-regulation	*RUNX1*
		Mayr et al. [79]	2019	M/F	n = 20 (healthy, 54 yr) n = 20 (CAD, 58 yr)	all-out cycle ergometry	Plasma	up-regulation (healthy) down-regulation (CAD)	-
miR-146a	no sig. change	Baggish et al. [70]	2011	M	n = 10 (19 yr) competitive rowers	acute cardiopulmonary exercise	Plasma	up-regulation	*IRAK1* *TRAF6*
		Baggish et al. [80]	2014	M	n =24 marathon runners	42-km marathon	Plasma	up-regulation 24 h postexercise: down-regulation	*NF-κB* *IRAK* *TRAF6*
		Sawada et al. [71]	2013	M	n = 12 (30 yr) physically active and recreational exercise	acute resistance exercise	Serum	3 days postexercise: down-regulation	-
		Kangas et al. [66]	2017	M	n = 18 (24 yr) n = 16 (58 yr) n = 15 (84,5 yr)	long-term background in sprint training acute exercise testing	Serum	up-regulation among the oldest participants	*TRAF6* *IRAK1*
miR-378a	no sig. change	McLean et al. [81]	2015	M/F	n = 14 (33 yr) sedentary, normoglycemic	single bout of aerobic exercise	Muscle	up-regulation	*GIMAP8* *NR4A3*
		D'Souza et al. [82]	2018	M	n = 10 (25 yr) no regular aerobic training	10 x 60 sec. intervals of cycling	Plasma Muscle	down-regulation (plasma) no sig. change (muscle)	-
miR-505	down-regulation	Radom-Aizik et al. [69]	2010	M	n = 11 (22 yr) no elite or competitive athletes	acute exercise (cycle ergometer)	Neutrophils	up-regulation	*H3F3B* *SF1*
		Nair et al. [83]	2020	M	n = 5 (68 yr, trained) n = 5 (70 yr, sedentary)	acute aerobic exercise (cycle ergometer)	Plasma	up-regulation (sedentary)	*AKT*

Abbreviations: M = male; F = female; yr = year; CAD = coronary artery disease; PTEN = phosphatase and tensin homolog; KIF13A = kinesin family member 13A; MAPK = mitogen-activated protein kinase; RNF11 = ring finger protein 11; VPS37A = vacuolar protein sorting 37A; PIK3R1 = phosphoinositide-3-kinase regulatory subunit 1; ATP2B4 = ATPase plasma membrane Ca^{2+} transporting 4; CDKN1A = cyclin-dependent kinase inhibitor 1A; TNFSF15 = tumor necrosis factor (TNF) superfamily member 15; H3F3B = H3 histone family 3B; WWP1 = WW domain containing E3 ubiquitin-protein ligase 1; Jak-STAT = Januskinase signal transducers and activators of transcription; PTGDR = prostaglandin D2 receptor; PGC-1α = Peroxisome proliferator-activated receptor gamma coactivator 1-alpha; RUNX1 = RUNX family transcription factor 1; IRAK1 = interleukin 1 receptor-associated kinase 1; TRAF6 = TNF receptor-associated factor 6; NF-κB = Nuclear factor kappa-light-chain-enhancer of activated B cells; GIMAP8 = GTPase, IMAP family member 8; NR4A3 = nuclear receptor subfamily 4 group A member 3; SF1 = splicing factor 1; AKT = protein kinase B.

Nevertheless, we still suspect that some of our miRNAs play a role in the immune response triggered by exercise. Physical exercise stimulates erythropoiesis and leukocytosis and increases erythrocyte mass, leukocyte and neutrophil counts, hematocrit levels, hemoglobin concentration, and plasma volume [84–86]. Although the recruitment of leukocytes by exercise resembles acute inflammation, it is believed that due to the change in hemodynamics in the circulatory system rather than in the tissue, the immune system is not weakened but is actually strengthened by the increased number of immune cells [86]. The majority of miRNAs expressed in whole blood originate from erythrocytes, but some also from other important cell types such as leukocytes and platelets, which contain functional miRNAs as well [87]. Since we could not find any evidence in the literature that our endogenous controls (miR-24 and -93) are involved in erythropoiesis and our results partly overlap with the studies by Radom-Aizik et al., 2010 [69] (e.g., miR-20a and -22; **Table 1**), we assume that some of our miRNAs come from leukocytes or neutrophils, and could play a role in the immune response and possibly reflect the overall inflammatory state. Therefore, miR-20a and -22 could serve as biomarkers for sport-related inflammation.

Some of the miRNAs could also be involved in muscle regeneration, bone health, and injury. In response to muscle damage or injury, activated satellite cells proliferate and undergo myogenic differentiation as part of the muscle regeneration process. As satellite cells proliferate and differentiate, there are also changes in miRNA expressions (e.g., miR-378a) and their target genes, which are important for muscle regeneration [29, 88]. In addition, miR-378a is involved in osteogenesis [89], miR-146a in osteoblastogenesis [90], and miR-30e inhibits osteoblast differentiation [91]. Therefore, we hypothesized that the examined miRNAs, namely miR-378a, -146a, and miR-30e, may also play a role in bone formation, resorption, and regeneration, and could be an indication of muscle damage, bone health, and risk of injury/bone fracture [29]. Furthermore, Baggish et al., 2011 (**Table 1**) also described that miR-146a and -20a could be used as biomarkers for cardiorespiratory fitness and maximal exercise capacity, as both correlated with maximal oxygen consumption (VO_{2max}) [70].

Although some of the miRNAs analyzed in this study have already been widely discussed in the field of exercise and physical activity (**Table 1**) and could be used as possible biomarkers, we believe that a combined analysis of multiple miRNAs promises a more accurate assessment of exercise and health status. Therefore, we have developed a so-called miRNA-based “fitness score”, which could help athletes or coaches to optimize

training and prevent overtraining and its health consequences, e.g., through excessive ROS production [29]. This combined miRNA analysis is based on a minimally invasive sample collection via dried blood spots, comparable to the sample collection for a lactate test which can be taken anywhere and anytime. In addition, it provides a lot of different information, and its compliance with repeated tests (e.g., monitoring of an athlete or training tracking) could be higher than with muscle biopsies or venous blood collection.

3.2. Effects of concurrent exercise on mitochondrial-related epigenetic mechanisms

In our publication **"*PGC-1α* methylation, miR-23a, and miR-30e expression as biomarkers for exercise- and diet-induced mitochondrial biogenesis in capillary blood from healthy individuals: a single-arm intervention"** (*Sports, 2022*; Appendix 8.1.1*)*, we analyzed anthropometric and nutritional data, as well as dried capillary blood samples from 36 participants (58.3% females, 41.7% males), who trained endurance and strength for 12 weeks. The focus was on investigating mitochondrial health, biogenesis, and functions (**Figure 3** shows signaling pathways involved in the regulation of mitochondrial biogenesis) and their possible epigenetic regulation. The methylation of *PGC-1α*, the two miRNAs miR-23a and -30e, that have *PGC-1α* as their predicted target, and the amount of mtDNA were analyzed. After the exercise intervention we could observe changes in *PGC-1α* methylation, miR-23a, and -30e, but none concerning mtDNA levels.

In the literature, it is described that after strenuous training sessions, promotor regions of genes involved in the training metabolism (e.g., pyruvate dehydrogenase kinase 4 (*PDK4*), peroxisome proliferator-activated receptor delta *(PPAR-δ)*, and *PGC-1α*) are hypomethylated and that at the same time mRNA levels increase [92]. We too could observe a hypomethylation of *PGC-1α* up to 32 h after the last training session. However, no hypomethylation was detected in participants who submitted their sample later (up to 48 h after their last training session). It is already known that these epigenetic responses to exercise are dynamic and remethylation can occur post-exercise. Similarly, Barrès et al., 2012 [93] concluded in their work that hypomethylation is a transient mechanism involved in mRNA synthesis. In Barrès' study, also, no methylation change of *PGC-1α* and *TFAM* could be determined 48 h after exercise, but an increased RNA expression of these genes was observed. TFAM increases the transcription of key mitochondrial enzymes that drive mtDNA replication, while PGC-1α also plays a role in cellular energy and lipid metabolism and in determining muscle fiber type [94–96]. This underlines the

importance of regular exercise, not only for overall health, aging, and disease prevention but also to benefit from the positive effects of *PGC-1α* and *TFAM* expression, e.g., activating mitochondrial biogenesis and enhancing endurance performance (**Figure 3**) [11].

However, during exercise and to some extent also during recovery phases, metabolites, mitochondria, and so-called muscle-specific miRNAs (myomiRs) are released from skeletal muscle into the systemic circulation via extracellular vesicles, possibly including miR-23a and -30e [97, 98]. Studies have already shown that endurance exercise downregulated miR-23a expression, which upregulated *PGC-1α* and thereby promoted mitochondrial biogenesis [88]. Regarding concurrent training, we could also measure a downregulation of miR-23a and -30e, as well as a correlation between *PGC-1α* methylation and miR-23a expression. Therefore, we hypothesized that there are distinct regulatory systems and possibly two "fronts" through which personal athletic performance and mitochondrial biogenesis may be affected, either by *PGC-1α* or miR-23a alone and/or through a synergistic effect [11]. In addition, both miRNAs are involved in myogenic differentiation and muscle fiber formation, which is assumed to take place in the regeneration phase and could explain the changes in miRNA expression up to 48 h after the last training session. There is evidence that down-regulation of miR-30e, but possibly also of miR-23a, favors the formation of type 1 muscle fibers, which are particularly beneficial for long-term endurance sessions [88, 99]. Consequently, it can be concluded that individual endurance capacity may be enhanced by downregulating miR-30e and -23a and through their involvement in mitochondrial biogenesis via *PGC-1α*.

3.3. Identification of stress-related miRNA expression pattern

In our observational study "**MiR-10a, miR-15a, let-7a, and let-7g expression as stress-relevant biomarkers to assess acute or chronic psychological stress, stress-related diseases, and mental health in human capillary blood**" (*Molecular biology reports, 2022*; Appendix 8.1.3), we analyzed dried capillary blood samples and questionnaire data on diet and lifestyle habits, with a focus on personal stress levels and stress-related diseases (SRDs, e.g., chronic fatigue syndrome (CFS), sleep disorders, headaches or migraines, mood swings, depression, and anxiety) from 171 participants (63.6% females, 36.4% males). According to the participants' self-assessment of their stress levels, we divided them into two groups to identify stress-related miRNA expression profiles. Participants with low or moderate stress levels and no SRDs were assigned to the "control group" and participants with high or very high-stress levels were assigned to the "stress group". Stress individuals showed higher expression levels of miR-10a, -15a, let-

7a, and -7g. Moreover, miR-15a, let7a, and -7g correlated with SRDs and may indicate long-term consequences of chronic stress.

In the literature, many miRNAs are described and discussed in the context of acute and/or chronic psychological stress and SRDs such as depression, and anxiety. Environmental stressors are believed to affect miRNA expression, leading to changes in neuronal morphology and problems in neuronal circuity, resulting in relevant health issues [100]. In mice, acute compulsive stress resulted in an increase in let-7a expression in serum and brain, which returned to baseline five days later [101]. Something similar was also observed in stressed rats (acute social defeat stress), in which up-regulation of let-7a could be measured, after 1, 4, and 24 h post-exposure [102]. Several members of the let-7 family, including let-7a, are potent activators of Toll-like receptors (TLRs) signaling in neurons and microglia. TLR activation leads to induction of pro-inflammatory cytokines, and activation of mitogen-activated protein kinase (MAPK) family members. Besides, due to its role in neurogenesis, it induces time- and dose-dependent neuronal cell death, which is associated with neurodegeneration and the pathogenesis of neurodegenerative diseases [103]. Acute psychological stress also leads to an up-regulation of miR-10a in whole blood and appears to be associated with stress-induced alcohol consumption [104]. However, up-regulation of this miRNA was also measured in the cerebrospinal fluid of patients with major depressive disorder (MDD). Overexpression of miR-10a appears to downregulate the expression of brain-derived neurotrophic factor (*BDNF*), and low expression levels of *BDNF* appear to play an important role in the pathogenesis of MDD. Furthermore, low expression levels of the *SIRT1* gene also seem to play a role in the pathogenesis, and studies have shown that this is also due to overexpression of miR-10a [105]. Based on our results and the literature, we concluded that these two miRNAs, namely miR-10a and let-7a, are more involved in acute psychological stress and might therefore act as biomarkers in this context [106].

Other studies suggest that miR-15a is involved in chronic stress management mechanisms. Animal models have shown that mice exposed to chronic stress have increased levels of miR-15a. Human studies also provide evidence of an association between elevated miR-15a levels and childhood trauma (CT). Healthy subjects who underwent a CT showed elevated levels of miR-15a in the blood compared to subjects without CT [107, 108]. However, it appears that miR-15a can regulate the expression of the serotonin transporter (*SERT*) by binding to the *SLC6A4* gene. An altered *SERT* expression is associated with a variety of disorders such as depression, and anxiety/obsessive-

compulsive disorders [109]. We also observed an up-regulation of miR-15a and let-7g in participants with CFS compared to the control group. There is further evidence in the literature that let-7g is associated with CT, but also with other neurodegenerative (e.g., Alzheimer's disease) and psychiatric (e.g., depression) diseases [110]. This reinforces the suitability of miR-15a and let-7g as biomarkers for chronic psychological stress [106].

3.4. Correlations between epigenetic mechanisms, genetic predisposition, nutritional habits, and body composition

3.4.1. Correlations between miRNAs and genetic predisposition (e.g., SNPs)

In our publication **"miRNA-based "fitness score" to assess the individual response to diet, metabolism, and exercise"** (*J. Int. Soc. Sports Nutr., 2022*; Appendix 8.1.2*)*, we examined different sport-associated SNPs (*FTO*, *ACE, and ACTN3*) and were able to determine significant differences in the epigenetic phenotypes depending on the genotype (**Figure 6**).

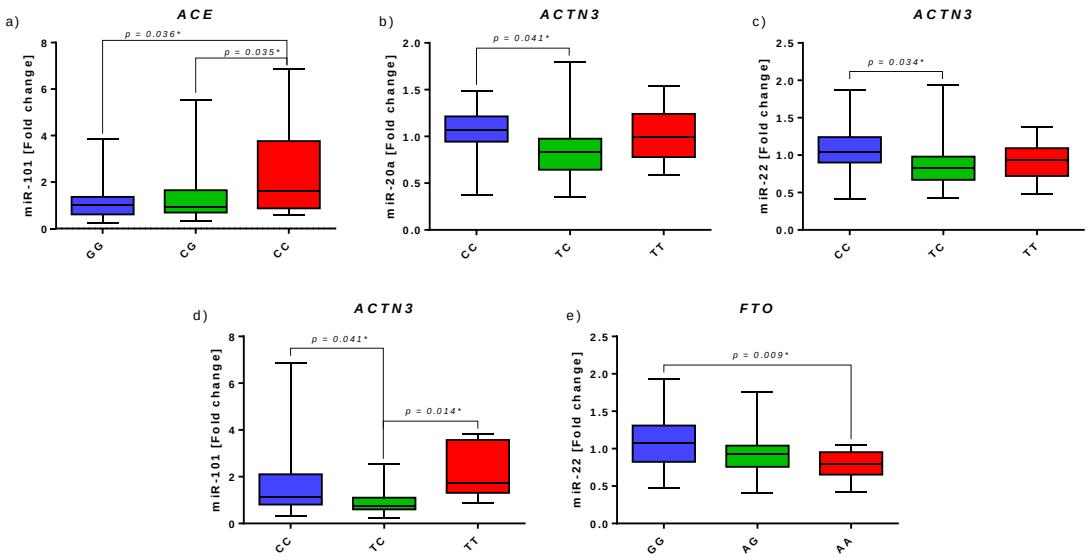

Figure 6. Observed miRNA expression profiles depending on the SNP genotype [29]. Abbreviations: ACTN3 = alpha-actinin-3; ACE = angiotensin-converting enzyme; FTO = fat mass and obesity-associated.

Our results suggest that the *ACTN3* gene, also known as the "speed gene", has an influence on the expression of miR-20a, -22, and -101. The protein ACTN3 is only expressed in the fast type II fibers and is involved in the generation of fast and powerful muscle movements. It is also suspected to play a role in post-exercise adaption and recovery, and in injury risk. Unlike individuals with TC or CC genotype, individuals with homozygous TT cannot express ACTN3, leading to impaired muscle performance and may predispose them to ligament injuries and muscle damage during exercise [111]. Participants with homozygous TT showed higher miR-101 expression after exercise,

while participants with a CC genotype showed higher miR-20a and -22 expression patterns (**Figure 6b-d**). However, animal and human studies provide evidence that the TT genotype is further associated with increased endurance capacity [112–114]. The *ACE* gene also affects aerobic endurance performance. The C allele of this gene is linked to lower ACE levels and higher cardiorespiratory fitness, which is associated with better endurance performance and is reflected by VO_{2max}. Whereas the G allele is associated with higher ACE levels, leading to improved musculoskeletal fitness and higher strength performance [33]. Interestingly, participants with homozygous CC showed higher miR-101 expression than participants with a CT or TT genotype (**Figure 6a**). Therefore, we concluded that miR-101 in particular is related to endurance performance and the ability to perform fast and explosive movements [29]. Furthermore, the expression of miR-22 was downregulated in participants with the *FTO* risk allele (AA) (**Figure 6e**). It is hypothesized that metabolic phenotypes, due to various mutations in the *FTO* gene, are regulated via epigenetic mechanisms [115], possibly also through miR-22.

3.4.2. Correlations between epigenetic mechanisms, and body composition

In our two publications **"*PGC-1α* methylation, miR-23a, and miR-30e expression as biomarkers for exercise- and diet-induced mitochondrial biogenesis in capillary blood from healthy individuals: a single-arm intervention"** (*Sports, 2022*; Appendix 8.1.1) and **"miRNA-based "fitness score" to assess the individual response to diet, metabolism, and exercise"** (*J. Int. Soc. Sports Nutr., 2022*; Appendix 8.1.2) we measured the body composition of the participants before and after the sport intervention using bioelectrical impedance analysis (BIA) and examined it for correlations with the epigenetic markers (**Table 2**).

Table 2. Correlations between anthropometric data and the studied markers.

Anthropometric value	Correlated marker	Comment
LBM	miR-22, miR-101	lower expression led to a higher increase in LBM
BFM	fitness score, miR-505, *PGC-1α* methylation	fitness score: a higher score was associated with a higher loss of BFM miR-505: low expression led to a higher loss of BFM *PGC-1α* methylation: lower methylation was associated with lower BFM
ECW	*Line-1* methylation	higher methylation led to a higher increase in ECW

Abbreviations: LBM = lean body mass [kg]; BFM = body fat mass [kg]; ECW = extracellular water [l]; PGC-1α = peroxisome proliferator-activated receptor gamma coactivator 1-alpha.

In addition to the associations between miR-22 and -101 with *ACE* and *ACTN3* genes, these miRNAs correlated with the LBM (**Table 2**), which further confirms our hypothesis that these miRNAs, especially miR-101, are involved in the ability to perform explosive

and fast movements. Aerobic performance is thought to be affected by body mass and body composition, and the literature provides evidence that there is a positive correlation between LBM and VO_{2max}. That means high muscle mass, the main component of LBM, also leads to increased VO_{2max} and thus probably to a better endurance performance [116]. The correlations between "fitness score", miR-505, and BFM, like those between miR-22 and LBM (**Table 2**), are not surprising since there was a significant change in body composition as a result of the sport intervention. The sport intervention led to a decrease in BFM and an increase in LBM, as well as to a significant change in these epigenetic markers. We concluded that these markers alone or as "fitness score", like measuring body composition, could reflect the personal success in an exercise program or current fitness/health status. Furthermore, the connection between BFM and *PGC-1α* methylation is interesting (**Table 2**). Participants with a higher BFM showed higher *PGC-1α* methylation levels. PGC-1α has regulatory functions in lipid and energy metabolism and it is assumed that PGC-1α, in addition to its role in mitochondrial biogenesis, is also involved in metabolic diseases such as diabetes and obesity [95]. Reduced *PGC-1α* expressions could be observed in the adipose tissue of pathologically obese and insulin-resistant patients [117]. Although our studies did not include severely obese subjects, it is possible that these associations between adipose tissue and PGC-1α also exist in adipose tissue of healthy individuals [11].

3.4.3. Correlations between epigenetic mechanisms and nutritional habits

Table 3. Correlations between nutritional habits and the studied markers.

	Nutritional habits	Influenced marker	Comment	Focus of the study
Supplementation of	B vitamins (e.g., folic acid, cobalamin, and B vitamin complex)	miR-19b, miR-101	up-regulation	exercise
	Iron	miR-378a	up-regulation	exercise
	Magnesium	miR-19b	up-regulation	exercise
	Fluid intake	miR-378a	up-regulation	exercise
	Coffee consumption	miR-15a	down-regulation	psychological stress
Intake of	Red and/or processed meat	*PGC-1α* methylation	higher methylation levels	exercise
	Total meat (red, processed, and white meat)	let-7a	up-regulation	psychological stress
	Cruciferous vegetables (e.g., broccoli, brussels sprouts, and cabbage)	mtDNA	higher mtDNA content	exercise
	Whole grain	miR-101	up-regulation	exercise

Abbreviations: PGC-1α = peroxisome proliferator-activated receptor gamma coactivator 1-alpha; mtDNA = mitochondrial DNA.

As can be seen in **Table 3**, we observed some interesting correlations between the analyzed dried blood markers and the dietary habits reported by the participants in the respective studies [11, 29, 106].

Of particular interest are our observed associations between vitamin and mineral supplementation and miRNAs. Individuals who reported taking B vitamins regularly showed an up-regulation of miR-19b and -101. Furthermore, we observed that participants taking iron supplements had a higher miR-378a expression, and those taking magnesium had a higher miR-19b expression (**Table 3**) [29]. Although there is little evidence of such correlations in the literature, it is known that micronutrients (e.g., vitamins and minerals) are essential for life and maintaining health, growth, and support of physiological functions. B vitamins are essential for synthesizing new cells and repairing damaged cells, while others are needed for bone health, protection against oxidative damage, maintaining immune function (e.g., iron, magnesium), but are also for building and repairing muscle tissue, and maintaining muscle function (e.g., magnesium) [118–120]. Duration, intensity, and type of exercise are believed to determine a person's macro- as well as micronutrient needs. In particular, athletes and physically active people have an increased need not only for macronutrients due to a high overall energy requirement but probably also for micronutrients. Regular physical activity can increase the requirements for certain micronutrients through biochemical adaptions and the metabolic pathways involved in energy production, and their deficit can impair athletic performance and health [118–121]. In addition to an adequate supply of micronutrients, fluid balance also plays an important role in an athlete's performance. We have observed an association between miR-378a and daily fluid intake (**Table 3**) and it is known that sweating and insufficient fluid intake during exercise, especially at high temperatures, lead to dehydration [29, 122]. Even moderate dehydration over short periods trigger impaired cognitive function, headaches, reduced concentration, or fatigue and thus impaired exercise performance [123]. It is therefore important for athletes in particular, but also for physically active people, to maintain an optimal micronutrient status and fluid balance and, if necessary, to recognize a deficit at an early stage before physical signs such as inflammation, impaired performance, and other health consequences appear.

Furthermore, we observed a correlation between regular intake of whole grains and miR-101 (**Table 3**). Participants who reported consuming whole grain products daily or several times a day had higher expression levels of miR-101 than those who consume less [29]. Eating whole grains has some beneficial health effects and can reduce the risk of

NCDs such as diabetes, obesity, coronary artery disease, cancer, and certain gastrointestinal disorders. It is believed that the health benefits of whole grain products can be attributed to their high dietary fiber content [124]. Most of these ingested dietary fibers are degraded and fermented by the gut microbiota. So-called short-chain fatty acids (SCFAs) are formed, which make up the main part of the fermentation products [125]. SCFAs are known to be involved in the regulation of human metabolic pathways and can modulate metabolic responses at various organ sites including skeletal muscle. They appear to affect carbohydrate, protein, and lipid metabolism (i.e., energy metabolism and ATP production), skeletal muscle function, and exercise capacity [126, 127]. It is hypothesized that SCFAs have the potential to increase blood flow, and insulin sensitivity in skeletal muscle, as well as skeletal muscle mass retention by activation of AMPK, PPAR-δ, and PGC-1α and inhibition of histone deacetylases (HDACs), which regulate gene transcription and protein expression. Studies in mice provide evidence that the improvement in exercise capacity may be due to SCFA-mediated increased glycogen synthesis, decreased glycolysis, upregulated fatty acid oxidation, and mitochondrial content [126]. While human studies are still lacking, this demonstrates the importance of diet and gut health, and the link between the gut and the athlete's performance.

Besides SCFAs, there are several other dietary components and nutrients, such as certain amino acids or secondary plant products, that can affect metabolism, exercise performance, general health, and lifespan [128]. We observed two interesting correlations between meat consumption, *PGC-1α* methylation, and let-7a (**Table 3**) [11, 106]. Excessive consumption of meat, especially red and processed meat, has some negative health effects and is linked to age-related diseases such as type 2 diabetes and cancer [129, 130]. In particular, the branched-chain amino acids (BCAAs) and methionine found in red meat can regulate metabolism via various mechanisms and influence lifespan and aging. Animal experiments and epidemiological studies have shown that there is an association between elevated BCAA levels, insulin resistance, glucose intolerance, and obesity and that these elevated blood levels can trigger mitochondrial dysfunction [128, 131]. As already mentioned, nutrition plays a role in mental health as well, e.g., brain function and behavior. Some dietary factors affect brain chemistry in a variety of ways and may promote psychological distress by altering brain neurotransmitter levels or their receptor activity. Dietary amino acids, such as those found in meat, make up the major monoamines (e.g., serotonin, dopamine, and norepinephrine) and can affect levels of these neurotransmitters, and therefore mental health [132]. This could explain our correlation between let-7g, meat consumption, and psychological stress. However,

processed meat also contains a relatively high amount of fat, as well as other substances that are harmful to health, such as saturated fatty acids, nitrosamines, sodium nitrate, heme iron, or L-carnitine [133, 134]. The literature provides evidence that the two saturated fatty acids, palmitic and stearic acid, can impair mitochondrial function by reducing mitochondrial hyperpolarization and ATP production, and skeletal muscle cells cultured with palmitic acid showed reduced *PGC-1α* expression, supporting our results [135].

As already mentioned, secondary plant products, or phytochemicals, have numerous positive health effects. In addition, we could observe correlations between cruciferous vegetables and mtDNA content, and between coffee consumption and miR-15a. This could be attributed to their bioactive compounds (**Table 3**) and antioxidant, anti-inflammatory, antidiabetic, antiviral, anticancer, or antitumor properties [11, 15, 106]. For example, polyphenols appear to be involved in the replacement and elimination of damaged mitochondria with newly synthesized ones. The phytochemical sulforaphane, prevalent in cruciferous vegetables, may activate NRF2 and increase the expression of *NRF1* and *TFAM* (**Figure 3**), thereby affecting mitochondrial biogenesis, and exercise performance, which could further explain our correlation with mtDNA content [136]. Although the literature has described coffee as inappropriately activating the sympathetic nervous system, coffee has other health benefits and may improve brain and heart functions [132]. Caffeine from coffee can penetrate the blood-brain barrier, increase dopamine levels, and work synergistically with norepinephrine, reducing feelings of fatigue and stimulating cognition. In addition to caffeine, coffee contains several bioactive components, such as phenolic compounds, and diterpenes, as well as other secondary metabolites, minerals, and vitamins. These could promote the establishment of the gastrointestinal tract and intestinal microbiota and may have beneficial effects on some diseases, including depression and suicidal behavior, presumably due to the phenolic compounds associated with anti-inflammatory activities [25, 137]. Furthermore, phytochemicals are known to affect epigenetic mechanisms via certain regulators (e.g., miRNAs) and thus gene expression, which further explains our link between nutrition, miRNA expression, and methylation patterns [138, 139].

4. CONCLUSION AND OUTLOOK

This study showed that concurrent exercise, nutrition, and mental health, the three pillars of health, alter epigenetic patterns in human capillary blood, resulting in specific DNA methylation and miRNA expression patterns (**Figure 7**). Based on our results and in accordance with previous studies, especially, miR-15a and let-7g could be applied as markers for chronic psychological stress, miR-20a, -146a, and -101 as markers for cardiovascular fitness, miR-19b, -101, and -378a for vitamin or micronutrient status, or miR-23a, -30e and *PGC-1α* methylation for mitochondrial biogenesis/health. Further properties of the analyzed miRNAs as biomarkers are summarized in **Figure 7**.

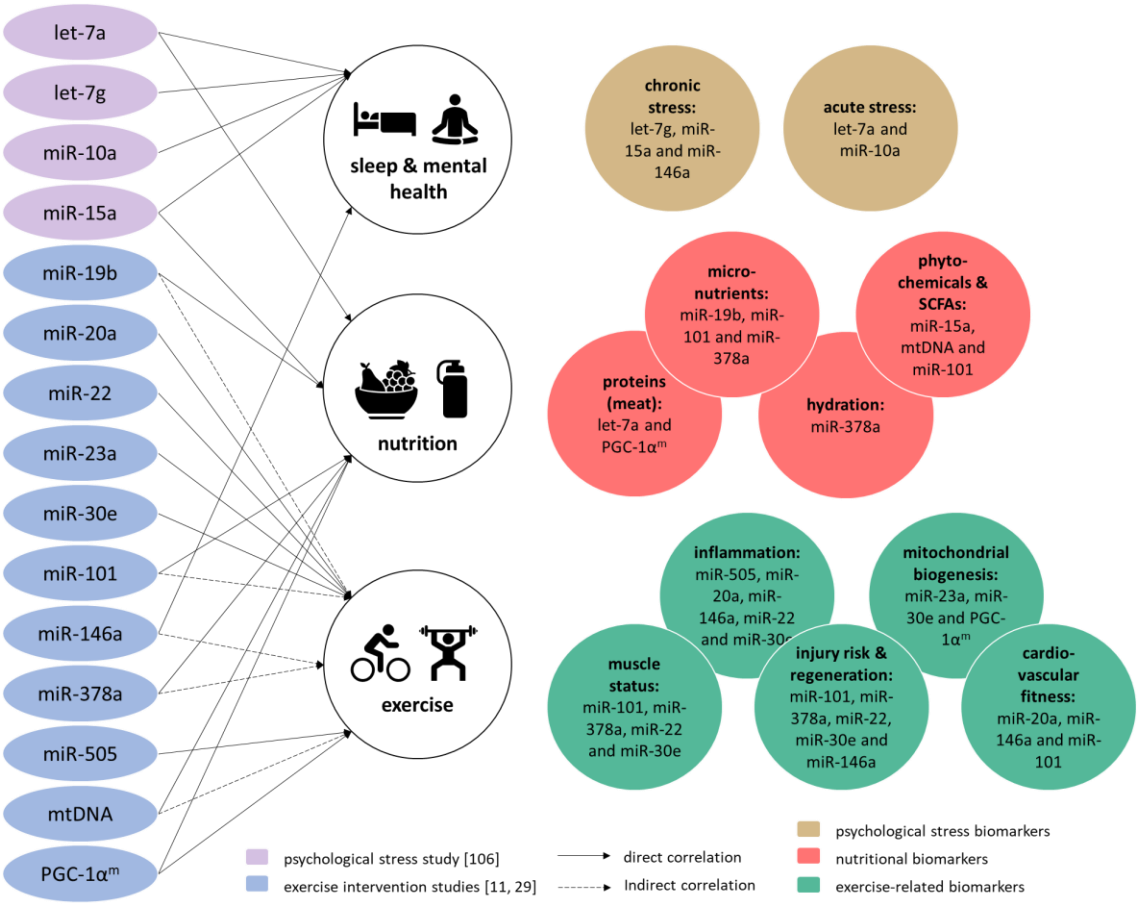

Figure 7. Summary of the analyzed epigenetic biomarkers and their functions in mental health, nutrition, and exercise. Dashed arrows indicate indirect correlations between the biomarkers and exercise, e.g., via body composition measurement or SNP genotype. The solid arrows indicate direct and significant correlations. Abbreviations: mtDNA = mitochondrial DNA; PGC-1α^{m} = *PGC-1α* methylation; PGC-1α = peroxisome proliferator-activated receptor gamma coactivator 1-alpha.

Consequently, miRNAs in particular seem to be promising biomarkers for diagnosis and prognosis, as they can be detected in various body fluids and tissues, have high sensitivity and specificity, and can be detected earlier than conventional biomarkers,

presumably before health consequences occur. Especially in the field of competitive sports, it is important to identify deficits or overtraining of an athlete at an early stage, in order to counteract and thus avoid injuries or dropping out of an athlete. As shown in **Figure 7**, multiple miRNAs could be integrated into multi-marker models to enable more precise diagnosis, treatment, and assessment of health or physical capacity. This fact is also behind the idea of our so-called “fitness score” for individual assessment of an athlete’s health, nutritional needs, and physical performance (e.g., nutrient deficits, endurance performance, and much more).

Regular check-ups and health monitoring are not only important for athletes but also for the general public to recognize and prevent diseases caused by poor lifestyles and/or nutrition at an early stage, e.g., to intercept chronic stress before SRDs such as burnout or CFS occur. Unfortunately, so-called annual health examinations are currently rarely taken up, partly because many medical practices are overcrowded and time-consuming (e.g., long waiting times). The established concept for health maintenance and personalized prevention investigated in this study could develop into a cost-effective alternative that allows taking samples (dried blood spots) anytime and anywhere, i.e., at home after/before the training.

In conclusion, these epigenetic biomarkers or “monitoring tools” for personal health, like lactate or blood glucose measurement, can not only serve as an aid to coaches and athletes but could also support and relieve the current healthcare systems in the future and possibly show better compliance than conventional methods. Especially with increasing age, this could make a significant contribution to the quality of life by being able to intervene at an early stage through personalized “lifestyle medicine”.

5. ABSTRACT

Due to extrinsic environmental factors such as available nutrition or hygiene standards, human life expectancy has increased enormously in the last two decades. Nevertheless, due to other factors (e.g., inactivity and overeating), the number of chronic and age-related diseases is increasing, and we are far from living a fully healthy and long life.

Current treatments are primarily aimed at suppressing symptoms of pre-existing diseases rather than targeting the disease directly. Today there is no doubt that daily habits have a significant impact on short- and long-term health, quality of life, and longevity. Research into alternative treatments and “lifestyle medicine”, is becoming increasingly important and three main pillars of health have been defined: 1. exercise, 2. nutrition, and 3. mental health.

Epigenetic biomarkers play an important role in the early detection and treatment of these diseases and could possibly be used as an “early warning system” for nutritional deficits, chronic psychological stress, or overtraining to counteract them with appropriate nutrition and training. Therefore, the main aim of this work was to examine and identify such biomarkers in dried capillary blood that are related to exercise, nutrition, and psychological stress.

We were able to identify 15 promising biomarkers, some of which could serve as indicators for acute/chronic stress, while others appear to reflect micronutrient status or provide information on inflammation and cardiovascular fitness. Furthermore, a combination of multiple epigenetic biomarkers, especially miRNAs (e.g., our “fitness score”), appears to provide a more accurate diagnosis than traditional single markers.

6. ZUSAMMENFASSUNG

Aufgrund extrinsischer Umweltfaktoren wie verfügbarer Ernährung oder Hygienestandards ist die Lebenserwartung der Menschen in den letzten zwei Jahrzehnten enorm gestiegen. Dennoch nimmt die Zahl chronischer und altersbedingter Krankheiten aufgrund anderer Faktoren (z. B. Inaktivität und übermäßiges Essen) zu, und wir sind weit davon entfernt, ein vollständiges gesundes und langes Leben zu führen.

Gegenwärtige Behandlungen zielen in erster Linie darauf ab, Symptome bereits bestehender Krankheiten zu unterdrücken, anstatt direkt auf die Krankheit abzuzielen. Heute besteht kein Zweifel daran, dass tägliche Gewohnheiten einen erheblichen Einfluss auf kurz- und langfristige Gesundheit, Lebensqualität und Langlebigkeit haben. Die Erforschung alternativer Behandlungsmethoden und der „Lifestyle-Medizin“ wird immer wichtiger, und es wurden drei Hauptsäulen der Gesundheit definiert: 1. Bewegung, 2. Ernährung und 3. psychische Gesundheit.

Epigenetische Biomarker spielen eine immer wichtigere Rolle bei der Früherkennung und Behandlung dieser Erkrankungen und könnten möglicherweise als „Frühwarnsystem“ für Ernährungsdefizite, chronische psychische Belastungen oder Übertraining eingesetzt werden, um diesen mit entsprechender Ernährung und Training entgegenzuwirken. Daher war das Hauptziel dieser Arbeit, solche Biomarker in getrocknetem Kapillarblut zu untersuchen und zu identifizieren, die mit Bewegung, Ernährung und psychischen Belastungen zusammenhängen.

Wir konnten 15 vielversprechende Biomarker identifizieren, von denen einige als Indikatoren für akuten/chronischen Stress dienen könnten, während andere den Mikronährstoffstatus widerzuspiegeln scheinen oder Informationen zu Entzündungen und kardiovaskulärer Fitness liefern. Darüber hinaus scheint eine Kombination mehrerer epigenetischer Biomarker, insbesondere miRNAs (z. B. unser „Fitness-Score“), eine genauere Diagnose zu liefern als herkömmliche Einzelmarker.

7. REFERENCES

1. Migliorelli, C.; Ros-Freixedes, L.; Gómez-Martínez, M.; Orte, S. CarpeDiem: Exploring Health Pillars to Define a Holistic Recommender System to Accomplish a Sutainable Change in Lifestlye Habits. *Preprint* **2022,** 0–22.

2. Strulik, H.; Vollmer, S. Long-Run Trends of Human Aging and Longevity. *J. Popul. Econ.* **2013,** *26*, 1303–1323. https://doi.org/10.1007/s00148-012-0459-z.

3. Rippe, J. M. Lifestyle Medicine: The Health Promoting Power of Daily Habits and Practices. *Am. J. Lifestyle Med.* **2018,** *12*, 499–512. https://doi.org/10.1177/1559827618785554.

4. Phillips, E. M.; Frates, E. P.; Park, D. J. Lifestyle Medicine. *Phys. Med. Rehabil. Clin.* **2020,** *31*, 515–526.

5. Noce, A.; Romani, A.; Bernini, R. Dietary Intake and Chronic Disease Prevention. *Nutrients* **2021,** *13*, 2–5. https://doi.org/10.3390/nu13041358.

6. Sellami, M.; Bragazzi, N.; Prince, M. S.; Denham, J.; Elrayess, M. Regular, Intense Exercise Training as a Healthy Aging Lifestyle Strategy: Preventing DNA Damage, Telomere Shortening and Adverse DNA Methylation Changes Over a Lifetime. *Front. Genet.* **2021,** *12*, 1–12. https://doi.org/10.3389/fgene.2021.652497.

7. Febbraio, M. A. Health Benefits of Exercise - More than Meets the Eye! *Nat. Rev. Endocrinol.* **2017,** *13*,. https://doi.org/10.1038/nrendo.2016.218.

8. Thyfault, J. P.; Bergouignan, A. Exercise and Metabolic Health: Beyond Skeletal Muscle. *Diabetologia* **2020,** *63*, 1464–1474. https://doi.org/10.1007/s00125-020-05177-6.

9. Methenitis, S. A Brief Review on Concurrent Training: From Laboratory to the Field. *Sports* **2018,** *6*, 127. https://doi.org/10.3390/sports6040127.

10. Hawley, J. A.; Hargreaves, M.; Joyner, M. J.; Zierath, J. R. Integrative Biology of Exercise. *Cell* **2014,** *159*, 738–749. https://doi.org/10.1016/j.cell.2014.10.029.

11. Krammer, U. D. B.; Sommer, A.; Tschida, S.; Mayer, A.; Lilja, S. V; Switzeny, O. J.; Hippe, B.; Rust, P.; Haslberger, A. G. PGC- 1α Methylation , MiR-23a , and MiR-30e Expression as Biomarkers for Exercise- and Diet-Induced Mitochondrial Biogenesis in Capillary Blood from Healthy Individuals: A Single-Arm Intervention. *Sports* **2022,** *10*, 73. https://doi.org/doi.org/10.3390/sports10050073.

12. Hong, A. R.; Kim, S. W. Effects of Resistance Exercise on Bone Health. *Endocrinol. Metab.* **2018,** *33*, 435–444. https://doi.org/10.3803/EnM.2018.33.4.435.

13. Solberg, P. A.; Kvamme, N. H.; Raastad, T.; Ommundsen, Y.; Tomten, S. E.; Halvari, H.; Loland, N. W.; Hallén, J. Effects of Different Types of Exercise on Muscle Mass, Strength, Function and Well-Being in Elderly. *Eur. J. Sport Sci.* **2013,** *13*, 112–125. https://doi.org/10.1080/17461391.2011.617391.

14. Franzke, B.; Neubauer, O.; Cameron-Smith, D.; Wagner, K. H. Dietary Protein, Muscle and Physical Function in the Very Old. *Nutrients* **2018,** *10*, 1–14. https://doi.org/10.3390/nu10070935.

15. Banwo, K.; Olojede, A. O.; Adesulu-Dahunsi, A. T.; Verma, D. K.; Thakur, M.; Tripathy, S.; Singh, S.; Patel, A. R.; Gupta, A. K.; Aguilar, C. N.; et al. Functional Importance of Bioactive Compounds of Foods with Potential Health Benefits: A Review on Recent Trends. *Food Biosci.* **2021,** *43*,. https://doi.org/10.1016/j.fbio.2021.101320.

16. Karlic, H.; Krammer, U.; Haslberger, A. Nutritional Supplements for Athletes, Who Benefits? A Review. *Funct. Food Sci.* **2022,** *2*, 224–241.

17. Balsano, C.; Alisi, A. Antioxidant Effects of Natural Bioactive Compounds. *Curr. Pharm. Des.* **2009,** *15*, 3063–3073. https://doi.org/10.2174/138161209789058084.

18. Martel, J.; Ojcius, D. M.; Ko, Y. F.; Ke, P. Y.; Wu, C. Y.; Peng, H. H.; Young, J. D. Hormetic Effects of Phytochemicals on Health and Longevity. *Trends Endocrinol. Metab.* **2019,** *30*, 335–346. https://doi.org/10.1016/j.tem.2019.04.001.

19. D'Angelo, S. Polyphenols: Potential Beneficial Effects of These Phytochemicals in Athletes. *Curr. Sports Med. Rep.* **2020,** *19*, 260–265. https://doi.org/10.1249/jsr.0000000000000729.

20. Peluso, I. Diet and Exercise in Lifestyle Medicine: The Hormetic Effects of Bioactive Compounds on Human Health. *Curr. Opin. Toxicol.* **2022,** *30*, 1–7. https://doi.org/10.1016/j.cotox.2022.03.003.

21. Görlach, A.; Dimova, E. Y.; Petry, A.; Martínez-Ruiz, A.; Hernansanz-Agustín, P.; Rolo, A. P.; Palmeira, C. M.; Kietzmann, T. Reactive Oxygen Species, Nutrition, Hypoxia and Diseases: Problems Solved? *Redox Biol.* **2015,** *6*, 372–385. https://doi.org/10.1016/j.redox.2015.08.016.

22. Mechchate, H.; Allam, A. El; Omari, N. El; Hachlafi, N. El; Shariati, M. A.; Wilairatana, P.; Mubarak, M. S.; Bouyahya, A. Vegetables and Their Bioactive Compounds as Anti-Aging

Drugs. *Molecules* **2022,** *27*,. https://doi.org/10.3390/molecules27072316.

23. Zeng, Z.; Centner, C.; Gollhofer, A.; König, D. Effects of Dietary Strategies on Exercise-Induced Oxidative Stress: A Narrative Review of Human Studies. *Antioxidants* **2021,** *10*, 1–16. https://doi.org/10.3390/antiox10040542.

24. Cohen, J. I. Stress and Mental Health: A Biobehavioral Perspective. *Issues Ment. Health Nurs.* **2000,** *21*, 185–202.

25. de Melo Pereira, G. V.; de Carvalho Neto, D. P.; Magalhães Júnior, A. I.; do Prado, F. G.; Pagnoncelli, M. G. B.; Karp, S. G.; Soccol, C. R. *Chemical Composition and Health Properties of Coffee and Coffee By-Products*, 1st ed.; Elsevier Inc., 2020,; Vol. 91. https://doi.org/10.1016/bs.afnr.2019.10.002.

26. Liu, Y. Z.; Wang, Y. X.; Jiang, C. L. Inflammation: The Common Pathway of Stress-Related Diseases. *Front. Hum. Neurosci.* **2017,** *11*, 1–11. https://doi.org/10.3389/fnhum.2017.00316.

27. Hill, D.; Conner, M.; Clancy, F.; Moss, R.; Wilding, S.; Bristow, M.; O'Connor, D. B. Stress and Eating Behaviours in Healthy Adults: A Systematic Review and Meta-Analysis. *Health Psychol. Rev.* **2021,** 1–25. https://doi.org/10.1080/17437199.2021.1923406.

28. Kelada, S. N.; Eaton, D. L.; Wang, S. S.; Rothman, N. R.; Khoury, M. J. The Role of Genetic Polymorphisms in Environmental Health. *Environ. Health Perspect.* **2003,** *111*, 1055–1064. https://doi.org/10.1289/ehp.6065.

29. Krammer, U. D. B.; Tschida, S.; Berner, J.; Lilja, S.; Switzeny, O. J.; Hippe, B.; Rust, P.; Halsberger, A. G. MiRNA-Based "Fitness Score" to Assess the Individual Response to Diet, Metabolism and Exercise. *J. Int. Soc. Sports Nutr.* **2022,** *19*, 455–473. https://doi.org/10.1080/15502783.2022.2106148.

30. Kelly, S. A.; Pomp, D. Genetic Determinants of Voluntary Exercise. *Trends Genet.* **2013,** *29*, 348–357. https://doi.org/10.1016/j.tig.2012.12.007.

31. Chanock, S. Candidate Genes and Single Nucleotide Polymorphisms (SNPs) in the Study of Human Disease. *Dis. Markers* **2001,** *17*, 89–98. https://doi.org/10.1155/2001/858760.

32. Zhu, H.; Zhou, X. Statistical Methods for SNP Heritability Estimation and Partition: A Review. *Comput. Struct. Biotechnol. J.* **2020,** *18*, 1557–1568. https://doi.org/10.1016/j.csbj.2020.06.011.

33. Vancini, R. L.; Pesquero, J. B.; Fachina, R. J.; Andrade, M. dos S.; Borin, J. P.; Montagner, P.; Barbosa de Lira, C. Genetic Aspects of Athletic Performance: The African Runners Phenomenon. *Open Access J. Sport. Med.* **2014,** *5*, 123. https://doi.org/10.2147/oajsm.s61361.

34. Pickering, C.; Kiely, J. ACTN3: More than Just a Gene for Speed. *Front. Physiol.* **2017,** *8*, 1–9. https://doi.org/10.3389/fphys.2017.01080.

35. Peng, S.; Zhu, Y.; Xu, F.; Ren, X.; Li, X.; Lai, M. FTO Gene Polymorphisms and Obesity Risk: A Meta-Analysis. *BMC Med.* **2011,** *9*, 1–15. https://doi.org/10.1007/s12519-019-00254-2.

36. Candráková, K.; Trakovická, A.; Candrák, J.; Gábor, M.; Miluchová, M. Effect of FTO Rs1121980 to Body Mass Index. *Acta Fytotech. Zootech.* **2016,** *19*, 114–122. https://doi.org/10.15414/afz.2016.19.si.114-122.

37. Vimaleswaran, K. S.; Li, S.; Zhao, J. H.; Luan, J.; Bingham, S. A.; Khaw, K. T.; Ekelund, U.; Wareham, N. J.; Loos, R. J. F. Physical Activity Attenuates the Body Mass Index-Increasing Influence of Genetic Variation in the FTO Gene. *Am. J. Clin. Nutr.* **2009,** *90*, 425–428. https://doi.org/10.3945/ajcn.2009.27652.

38. Bollati, V.; Baccarelli, A. Environmental Epigenetics. *Heredity (Edinb).* **2010,** *105*, 105–112. https://doi.org/10.1038/hdy.2010.2.

39. Deichmann, U. Epigenetics: The Origins and Evolution of a Fashionable Topic. *Dev. Biol.* **2016,** *416*, 249–254. https://doi.org/10.1016/j.ydbio.2016.06.005.

40. Chaturvedi, P.; Tyagi, S. C. Epigenetic Mechanisms Underlying Cardiac Degeneration and Regeneration. *Int. J. Cardiol.* **2014,** *173*, 1–11. https://doi.org/10.1016/j.ijcard.2014.02.008.

41. Martínez, J. A.; Milagro, F. I.; Claycombe, K. J.; Schalinske, K. L. Epigenetics in Adipose Tissue, Obesity, Weight Loss, and Diabetes 1, 2. *Adv. Nutr.* **2013,** *5*, 71–81. https://doi.org/10.3945/an.113.004705.71.

42. Lillycrop, K. A.; Hoile, S. P.; Grenfell, L.; Burdge, G. C. DNA Methylation, Ageing and the Influence of Early Life Nutrition. *Proc Nutr Soc* **2014,** *73*, 413–421. https://doi.org/10.1017/s0029665114000081.

43. Xing, Y.; Wong, G. W. K. Environmental Influences and Allergic Diseases in the Asia-Pacific Region: What Will Happen in Next 30 Years? *Allergy, Asthma Immunol. Res.* **2022,** *14*, 21–39. https://doi.org/10.4168/aair.2022.14.1.21.

44. Beetch, M.; Harandi-Zadeh, S.; Shen, K.; Lubecka, K.; Kitts, D. D.; O'Hagan, H. M.; Stefanska, B. Dietary Antioxidants Remodel DNA Methylation Patterns in Chronic Disease. *Br. J. Pharmacol.* **2020,** *177*, 1382–1408. https://doi.org/10.1111/bph.14888.

45. Dor, Y.; Cedar, H. Principles of DNA Methylation and Their Implications for Biology and Medicine. *Lancet* **2018,** *392*, 777–786. https://doi.org/10.1016/S0140-6736(18)31268-6.

46. Fukushige, S.; Horii, A. DNA Methylation in Cancer : A Gene Silencing Mechanism and the Clinical Potential of Its Biomarkers. *Tohoku J. Exp. Med.* **2013,** *229*, 173–185. https://doi.org/10.1620/tjem.229.173.Correspondence.

47. Ciechomska, M.; Roszkowski, L.; Maslinski, W. DNA Methylation as a Future Therapeutic and Diagnostic Target in Rheumatoid Arthritis. *Cells* **2019,** *8*, 1–16. https://doi.org/10.3390/cells8090953.

48. Voisin, S.; Eynon, N.; Yan, X.; Bishop, D. J. Exercise Training and DNA Methylation in Humans. *Acta Physiol.* **2015,** *213*, 39–59. https://doi.org/10.1111/apha.12414.

49. Jung, M.; Pfeifer, G. P. Aging and DNA Methylation. *BMC Biol.* **2015,** *13*, 1–8. https://doi.org/10.1186/s12915-015-0118-4.

50. Kadayifci, F. Z.; Zheng, S.; Pan, Y. X. Molecular Mechanisms Underlying the Link between Diet and DNA Methylation. *Int. J. Mol. Sci.* **2018,** *19*,. https://doi.org/10.3390/ijms19124055.

51. Hüttenhofer, A.; Schattner, P.; Polacek, N. Non-Coding RNAs: Hope or Hype? *Trends Genet.* **2005,** *21*, 289–297. https://doi.org/10.1016/j.tig.2005.03.007.

52. Zhang, P.; Wu, W.; Chen, Q.; Chen, M. Non-Coding RNAs and Their Integrated Networks. *J. Integr. Bioinform.* **2019,** *16*, 1–12. https://doi.org/10.1515/jib-2019-0027.

53. O'Brien, J.; Hayder, H.; Zayed, Y.; Peng, C. Overview of MicroRNA Biogenesis, Mechanisms of Actions, and Circulation. *Front. Endocrinol. (Lausanne).* **2018,** *9*, 1–12. https://doi.org/10.3389/fendo.2018.00402.

54. Barca-Mayo, O.; Richard Lu, Q. Fine-Tuning Oligodendrocyte Development by Micrornas. *Front. Neurosci.* **2012,** *6*, 13. https://doi.org/10.3389/fnins.2012.00013.

55. Polakovičová, M.; Musil, P.; Laczo, E.; Hamar, D.; Kyselovič, J. Circulating MicroRNAs as Potential Biomarkers of Exercise Response. *Int. J. Mol. Sci.* **2016,** *17*,. https://doi.org/10.3390/ijms17101553.

56. Kuzhandai Velu, V.; Ramesh, R.; Srinivasan, A. R. Circulating MicroRNAs as Biomarkers in Health and Disease. *J. Clin. Diagnostic Res.* **2012,** *6*, 1791–1795. https://doi.org/10.7860/JCDR/2012/4901.2653.

57. Condrat, C. E.; Thompson, D. C.; Barbu, M. G.; Bugnar, O. L.; Boboc, A.; Cretoiu, D.; Suciu, N.; Cretoiu, S. M.; Voinea, S. C. MiRNAs as Biomarkers in Disease: Latest Findings Regarding Their Role in Diagnosis and Prognosis. *Cells* **2020,** *9*, 1–32. https://doi.org/10.3390/cells9020276.

58. Javadov, S.; Kozlov, A. V; Camara, A. K. Mitochondria in Health and Disease. *Cells* **2020,** *9*, 1177. https://doi.org/10.1017/9781139192460.002.

59. Annesley, S. J.; Fischer, P. R. Mitochondria in Health and Disease. *Cells* **2019,** *8*, 680. https://doi.org/10.1017/9781139192460.002.

60. Popov, L. D. Mitochondrial Biogenesis: An Update. *J. Cell. Mol. Med.* **2020,** *24*, 4892–4899. https://doi.org/10.1111/jcmm.15194.

61. Ma, K.; Chen, G.; Li, W.; Kepp, O.; Zhu, Y.; Chen, Q. Mitophagy, Mitochondrial Homeostasis, and Cell Fate. *Front. Cell Dev. Biol.* **2020,** *8*, 1–14. https://doi.org/10.3389/fcell.2020.00467.

62. Ristow, M.; Schmeisser, K. Mitohormesis: Promoting Health and Lifespan by Increased Levels of Reactive Oxygen Species (ROS). *Dose-Response* **2014,** *12*, 288–341. https://doi.org/10.2203/dose-response.13-035.Ristow.

63. Yun, J.; Finkel, T. Mitohormesis. *Cell metabo* **2014,** *19*, 757–766. https://doi.org/10.1016/j.cmet.2014.01.011.Mitohormesis.

64. Cheng, A. J.; Jude, B.; Lanner, J. T. Intramuscular Mechanisms of Overtraining. *Redox Biol.* **2020,** *35*, 101480. https://doi.org/10.1016/j.redox.2020.101480.

65. Margolis, L. M.; Lessard, S. J.; Ezzyat, Y.; Fielding, R. A.; Rivas, D. A. Circulating MicroRNA Are Predictive of Aging and Acute Adaptive Response to Resistance Exercise in Men. *Journals Gerontol. - Ser. A Biol. Sci. Med. Sci.* **2017,** *72*, 1319–1326. https://doi.org/10.1093/gerona/glw243.

66. Kangas, R.; Törmäkangas, T.; Heinonen, A.; Alen, M.; Suominen, H.; Kovanen, V.; Laakkonen, E. K.; Korhonen, M. T. Declining Physical Performance Associates with Serum FasL, MiR-21, and MiR-146a in Aging Sprinters. *Biomed Res. Int.* **2017,** *2017*,. https://doi.org/10.1155/2017/8468469.

67. Massart, J.; Sjögren, R. J. O.; Egan, B.; Garde, C.; Lindgren, M.; Gu, W.; Ferreira, D. M. S.; Katayama, M.; Ruas, J. L.; Barrès, R.; et al. Endurance Exercise Training-Responsive MiR-19b-3p Improves Skeletal Muscle Glucose Metabolism. *Nat. Commun.* **2021,** *12*, 1–13. https://doi.org/10.1038/s41467-021-26095-0.

68. Rivas, D. A.; Peng, F.; Benard, T.; da Silva, A. S. R.; Fielding, R. A.; Margolis, L. M. MiR-19b-3p Is Associated with a Diametric Response to Resistance Exercise in Older Adults and Regulates Skeletal Muscle Anabolism via PTEN Inhibition. *Am. J. Physiol. - Cell Physiol.* **2021,** *321*, C977–C991. https://doi.org/10.1152/ajpcell.00190.2021.

69. Radom-Aizik, S.; Zaldivar, F.; Oliver, S.; Galassetti, P.; Cooper, D. M. Evidence for MicroRNA Involvement in Exercise-Associated Neutrophil Gene Expression Changes. *J. Appl. Physiol.* **2010,** *109*, 252–261. https://doi.org/10.1152/japplphysiol.01291.2009.

70. Baggish, A. L.; Hale, A.; Weiner, R. B.; Lewis, G. D.; Systrom, D.; Wang, F.; Wang, T. J.; Chan, S. Y. Dynamic Regulation of Circulating MicroRNA during Acute Exhaustive Exercise and Sustained Aerobic Exercise Training. *J. Physiol.* **2011,** *589*, 3983–3994. https://doi.org/10.1113/jphysiol.2011.213363.

71. Sawada, S.; Suzuki, K.; Akimoto, T.; Wada, S.; Kon, M.; Ushida, T. Profiling of Circulating MicroRNAs after a Bout of Acute Resistance Exercise in Humans. *PLoS One* **2013,** *8*, e70823. https://doi.org/10.1371/journal.pone.0070823.

72. Zhou, Q.; Shi, C.; Lv, Y.; Zhao, C.; Jiao, Z.; Wang, T. Circulating MicroRNAs in Response to Exercise Training in Healthy Adults. *Front. Genet.* **2020,** *11*, 1–10. https://doi.org/10.3389/fgene.2020.00256.

73. Davidsen, P. K.; Gallagher, I. J.; Hartman, J. W.; Tarnopolsky, M. A.; Dela, F.; Helge, J. W.; Timmons, J. A.; Phillips, S. M. High Responders to Resistance Exercise Training Demonstrate Differential Regulation of Skeletal Muscle MicroRNA Expression. *J. Appl. Physiol.* **2011,** *110*, 309–317. https://doi.org/10.1152/japplphysiol.00901.2010.

74. Kern, F.; Ludwig, N.; Backes, C.; Maldener, E.; Fehlmann, T.; Suleymanov, A.; Meese, E.; Hecksteden, A.; Keller, A.; Meyer, T. Systematic Assessment of Blood-Borne MicroRNAs Highlights Molecular Profiles of Endurance Sport and Carbohydrate Uptake. *Cells* **2019,** *8*,. https://doi.org/10.3390/cells8091045.

75. Radom-Aizik, S.; Zaldivar, F.; Haddad, F.; Cooper, D. M. Impact of Brief Exercise on Peripheral Blood NK Cell Gene and MicroRNA Expression in Young Adults. *J. Appl. Physiol.* **2013,** *114*, 628–636. https://doi.org/10.1152/japplphysiol.01341.2012.

76. Radom-Aizik, S.; Zaldivar, F. P.; Haddad, F.; Cooper, D. M. Impact of Brief Exercise on

Circulating Monocyte Gene and MicroRNA Expression: Implications for Atherosclerotic Vascular Disease. *Brain. Behav. Immun.* **2014,** *39*, 121–129. https://doi.org/10.1016/j.bbi.2014.01.003.

77. Backes, C.; Leidinger, P.; Keller, A.; Hart, M.; Meyer, T.; Meese, E.; Hecksteden, A. Blood Born MiRNAs Signatures That Can Serve as Disease Specific Biomarkers Are Not Significantly Affected by Overall Fitness and Exercise. *PLoS One* **2014,** *9*,. https://doi.org/10.1371/journal.pone.0102183.

78. Keller, P.; Vollaard, N. B. J.; Gustafsson, T.; Gallagher, I. J.; Sundberg, C. J.; Rankinen, T.; Britton, S. L.; Bouchard, C.; Koch, L. G.; Timmons, J. A. A Transcriptional Map of the Impact of Endurance Exercise Training on Skeletal Muscle Phenotype. *J. Appl. Physiol.* **2011,** *110*, 46–59. https://doi.org/10.1152/japplphysiol.00634.2010.

79. Mayr, B.; Müller, E. E.; Schäfer, C.; Droese, S.; Breitenbach-Koller, H.; Schönfelder, M.; Niebauer, J. Exercise Responsive Micro Ribonucleic Acids Identify Patients with Coronary Artery Disease. *Eur. J. Prev. Cardiol.* **2019,** *26*, 348–355. https://doi.org/10.1177/2047487318808014.

80. Baggish, A. L.; Park, J.; Min, P. K.; Isaacs, S.; Parker, B. A.; Thompson, P. D.; Troyanos, C.; D'Hemecourt, P.; Dyer, S.; Thiel, M.; et al. Rapid Upregulation and Clearance of Distinct Circulating MicroRNAs after Prolonged Aerobic Exercise. *J. Appl. Physiol.* **2014,** *116*, 522–531. https://doi.org/10.1152/japplphysiol.01141.2013.

81. McLean, C. S.; Mielke, C.; Cordova, J. M.; Langlais, P. R.; Bowen, B.; Miranda, D.; Coletta, D. K.; Mandarino, L. J. Gene and MicroRNA Expression Responses to Exercise; Relationship with Insulin Sensitivity. *PLoS One* **2015,** *10*, 1–14. https://doi.org/10.1371/journal.pone.0127089.

82. D'souza, R. F.; Woodhead, J. S. T.; Zeng, N.; Blenkiron, C.; Merry, T. L.; Cameron-Smith, D.; Mitchell, C. J. Circulatory Exosomal MiRNA Following Intense Exercise Is Unrelated to Muscle and Plasma MiRNA Abundances. *Am. J. Physiol. - Endocrinol. Metab.* **2018,** *315*, E723–E733. https://doi.org/10.1152/ajpendo.00138.2018.

83. Nair, V. D.; Ge, Y.; Li, S.; Pincas, H.; Jain, N.; Seenarine, N.; Amper, M. A. S.; Goodpaster, B. H.; Walsh, M. J.; Coen, P. M.; et al. Sedentary and Trained Older Men Have Distinct Circulating Exosomal MicroRNA Profiles at Baseline and in Response to Acute Exercise. *Front. Physiol.* **2020,** *11*, 1–15. https://doi.org/10.3389/fphys.2020.00605.

84. Hu, M.; Lin, W. Effects of Exercise Training on Red Blood Cell Production: Implications

for Anemia. *Acta Haematol.* **2012,** *127*, 156–164. https://doi.org/10.1159/000335620.

85. Belviranli, M.; Okudan, N.; Kabak, B. The Effects of Acute High-Intensity Interval Training on Hematological Parameters in Sedentary Subjects. *Med. Sci.* **2017,** *5*, 15. https://doi.org/10.3390/medsci5030015.

86. Sand, K. L. Effects of Exercise on Leukocytosis and Blood Hemostasis in 800 Healthy Young Females and Males. *World J. Exp. Med.* **2013,** *3*, 11. https://doi.org/10.5493/wjem.v3.i1.11.

87. Sun, L.; Yu, Y.; Niu, B.; Wang, D. Red Blood Cells as Potential Repositories of MicroRNAs in the Circulatory System. *Front. Genet.* **2020,** *11*, 1–8. https://doi.org/10.3389/fgene.2020.00442.

88. Kirby, T. J.; McCarthy, J. J. MicroRNAs in Skeletal Muscle Biology and Exercise Adaptation. *Free Radic. Biol. Med.* **2013,** *64*, 95–105. https://doi.org/10.1016/j.freeradbiomed.2013.07.004.

89. Kang, M.; Huang, C. C.; Lu, Y.; Shirazi, S.; Gajendrareddy, P.; Ravindran, S.; Cooper, L. F. Bone Regeneration Is Mediated by Macrophage Extracellular Vesicles. *Bone* **2020,** *141*,. https://doi.org/10.1016/j.bone.2020.115627.

90. Chang, C. C.; Venø, M. T.; Chen, L.; Ditzel, N.; Le, D. Q. S.; Dillschneider, P.; Kassem, M.; Kjems, J. Global MicroRNA Profiling in Human Bone Marrow Skeletal—Stromal or Mesenchymal–Stem Cells Identified Candidates for Bone Regeneration. *Mol. Ther.* **2018,** *26*, 593–605. https://doi.org/10.1016/j.ymthe.2017.11.018.

91. Suttamanatwong, S. MicroRNAs in Bone Development and Their Diagnostic and Therapeutic Potentials in Osteoporosis. *Connect. Tissue Res.* **2017,** *58*, 90–102. https://doi.org/10.3109/03008207.2016.1139580.

92. Jacques, M.; Hiam, D.; Craig, J.; Barrès, R.; Eynon, N.; Voisin, S. Epigenetic Changes in Healthy Human Skeletal Muscle Following Exercise– a Systematic Review. *Epigenetics* **2019,** *14*, 633–648. https://doi.org/10.1080/15592294.2019.1614416.

93. Barrès, R.; Yan, J.; Egan, B.; Treebak, J. T.; Rasmussen, M.; Fritz, T.; Caidahl, K.; Krook, A.; O'Gorman, D. J.; Zierath, J. R. Acute Exercise Remodels Promoter Methylation in Human Skeletal Muscle. *Cell Metab.* **2012,** *15*, 405–411. https://doi.org/10.1016/j.cmet.2012.01.001.

94. Jornayvaz, F. R.; Shulman, G. I. Regulation of Mitochondrial Biogenesis. *Essays Biochem.* **2010,** *47*, 69–84. https://doi.org/10.1042/BSE0470069.

95. Liang, H.; Ward, W. F. PGC-1α: A Key Regulator of Energy Metabolism. *Am. J. Physiol. - Adv. Physiol. Educ.* **2006,** *30*, 145–151. https://doi.org/10.1152/advan.00052.2006.

96. Lin, J.; Wu, H.; Tarr, P. T.; Zhang, C. Y.; Wu, Z.; Boss, O.; Michael, L. F.; Puigserver, P.; Isotani, E.; Olson, E. N.; et al. Transcriptional Co-Activator PGC-1α Drives the Formation of Slow-Twitch Muscle Fibres. *Nature* **2002,** *418*, 797–801. https://doi.org/10.1038/nature00904.

97. Burtscher, J.; Millet, G. P.; Place, N.; Kayser, B.; Zanou, N. The Muscle-Brain Axis and Neurodegenerative Diseases: The Key Role of Mitochondria in Exercise-Induced Neuroprotection. *Int. J. Mol. Sci.* **2021,** *22*,. https://doi.org/10.3390/ijms22126479.

98. Siracusa, J.; Koulmann, N.; Banzet, S. Circulating MyomiRs: A New Class of Biomarkers to Monitor Skeletal Muscle in Physiology and Medicine. *J. Cachexia. Sarcopenia Muscle* **2018,** *9*, 20–27. https://doi.org/10.1002/jcsm.12227.

99. Jia, H.; Zhao, Y.; Li, T.; Zhang, Y.; Zhu, D. MiR-30e Is Negatively Regulated by Myostatin in Skeletal Muscle and Is Functionally Related to Fiber-Type Composition. *Acta Biochim. Biophys. Sin. (Shanghai).* **2017,** *49*, 392–399. https://doi.org/10.1093/abbs/gmx019.

100. Wiegand, C.; Savelsbergh, A.; Heusser, P. MicroRNAs in Psychological Stress Reactions and Their Use as Stress-Associated Biomarkers, Especially in Human Saliva. *Biomed. Hub* **2017,** *2*, 1–15. https://doi.org/10.1159/000481126.

101. Sung, M.; Sung, S. E.; Kang, K. K.; Choi, J. H.; Lee, S.; Kim, K.; Lim, J. H.; Lee, G. W.; Rim, H. D.; Kim, B. S.; et al. Serum-Derived Neuronal Exosomal Mirnas as Biomarkers of Acute Severe Stress. *Int. J. Mol. Sci.* **2021,** *22*,. https://doi.org/10.3390/ijms22189960.

102. Chen, R. J.; Kelly, G.; Sengupta, A.; Heydendael, W.; Nicholas, B.; Beltrami, S.; Luz, S.; Peixoto, L.; Abel, T.; Bhatnagar, S. MicroRNAs as Biomarkers of Resilience or Vulnerability to Stress. *Neuroscience* **2015,** *305*, 36–48. https://doi.org/10.1016/j.neuroscience.2015.07.045.

103. Hollins, S. L.; Cairns, M. J. MicroRNA: Small RNA Mediators of the Brains Genomic Response to Environmental Stress. *Prog. Neurobiol.* **2016,** *143*, 61–81. https://doi.org/10.1016/j.pneurobio.2016.06.005.

104. Beech, R. D.; Leffert, J. J.; Lin, A.; Hong, K. A.; Hansen, J.; Umlauf, S.; Mane, S.; Zhao, H.; Sinha, R. Stress-Related Alcohol Consumption in Heavy Drinkers Correlates with Expression of MiR-10a, MiR-21, and Components of the TAR-RNA-Binding Protein-Associated Complex. *Alcohol. Clin. Exp. Res.* **2014,** *38*, 2743–2753.

https://doi.org/10.1111/acer.12549.

105. Wan, Y.; Liu, Y.; Wang, X.; Wu, J.; Liu, K.; Zhou, J.; Liu, L.; Zhang, C. Identification of Differential MicroRNAs in Cerebrospinal Fluid and Serum of Patients with Major Depressive Disorder. *PLoS One* **2015,** *10*, 1–12. https://doi.org/10.1371/journal.pone.0121975.

106. Krammer, U. D.; Lerch, M. L.; Haslberger, A. G.; Hippe, B. MiR-10a, MiR-15a, Let-7a, and Let-7g Expression as Stress-Relevant Biomarkers to Assess Acute or Chronic Psychological Stress, Stress-Related Diseases, and Mental Health in Human Capillary Blood. *Submitt. to Mol. Biol. Reports* **2022,**.

107. Maffioletti, E.; Bocchio-Chiavetto, L.; Perusi, G.; Carvalho Silva, R.; Sacco, C.; Bazzanella, R.; Zampieri, E.; Bortolomasi, M.; Gennarelli, M.; Minelli, A. Inflammation-Related MicroRNAs Are Involved in Stressful Life Events Exposure and in Trauma-Focused Psychotherapy in Treatment-Resistant Depressed Patients. *Eur. J. Psychotraumatol.* **2021,** *12*, 1–12. https://doi.org/10.1080/20008198.2021.1987655.

108. Volk, N.; Pape, J. C.; Engel, M.; Zannas, A. S.; Cattane, N.; Cattaneo, A.; Binder, E. B.; Chen, A. Amygdalar MicroRNA-15a Is Essential for Coping with Chronic Stress. *Cell Rep.* **2016,** *17*, 1882–1891. https://doi.org/10.1016/j.celrep.2016.10.038.

109. Moya, P. R.; Wendland, J. R.; Salemme, J.; Fried, R. L.; Murphy, D. L. MiR-15a and MiR-16 Regulate Serotonin Transporter Expression in Human Placental and Rat Brain Raphe Cells. *Int. J. Neuropsychopharmacol.* **2013,** *16*, 621–629. https://doi.org/10.1017/S1461145712000454.

110. Van der Auwera, S.; Ameling, S.; Wittfeld, K.; d'Harcourt Rowold, E.; Nauck, M.; Völzke, H.; Suhre, K.; Najafi-Shoushtari, H.; Methew, J.; Ramachandran, V.; et al. Association of Childhood Traumatization and Neuropsychiatric Outcomes with Altered Plasma Micro RNA-Levels. *Neuropsychopharmacology* **2019,** *44*, 2030–2037. https://doi.org/10.1038/s41386-019-0460-2.

111. Del Coso, J.; Hiam, D.; Houweling, P.; Pérez, L. M.; Eynon, N.; Lucía, A. More than a 'Speed Gene': ACTN3 R577X Genotype, Trainability, Muscle Damage, and the Risk for Injuries. *Eur. J. Appl. Physiol.* **2019,** *119*, 49–60. https://doi.org/10.1007/s00421-018-4010-0.

112. Roth, S. M.; Walsh, S.; Liu, D.; Metter, E. J.; Ferrucci, L.; Hurley, F. The ACTN3 R577X Nonsense Allele Is Under-Represented in Elite-Level Strength Athletes. **2008,** *16*, 391–394. https://doi.org/10.1038/sj.ejhg.5201964.The.

113. Yang, N.; MacArthur, D. G.; Gulbin, J. P.; Hahn, A. G.; Beggs, A. H.; Easteal, S.; North, K. ACTN3 Genotype Is Associated with Human Elite Athletic Performance. *Am. J. Hum. Genet.* **2003,** *73*, 627–631. https://doi.org/10.1086/377590.

114. Yang, R.; Shen, X.; Wang, Y.; Voisin, S.; Cai, G.; Fu, Y.; Xu, W.; Eynon, N.; Bisjop, D. J.; Yan, X. ACTN3 R577X Gene Variant Is Associated with Muscle-Related Phenotypes in Elite Chinese Sprint/Power Athletes. **2017,** *31*, 1107–1115.

115. Franzago, M.; Fraticelli, F.; Marchioni, M.; Di Nicola, M.; Di Sebastiano, F.; Liberati, M.; Stuppia, L.; Vitacolonna, E. Fat Mass and Obesity-Associated (FTO) Gene Epigenetic Modifications in Gestational Diabetes: New Insights and Possible Pathophysiological Connections. *Acta Diabetol.* **2021,** *58*, 997–1007. https://doi.org/10.1007/s00592-020-01668-5.

116. Maciejczyk, M.; Więcek, M.; Szymura, J.; Szyguła, Z.; Wiecha, S.; Cempla, J. The Influence of Increased Body Fat or Lean Body Mass on Aerobic Performance. *PLoS One* **2014,** *9*, 0–5. https://doi.org/10.1371/journal.pone.0095797.

117. Liu, C.; Lin, J. D. PGC-1 Coactivators in the Control of Energy Metabolism. *Acta Biochim. Biophys. Sin. (Shanghai).* **2011,** *43*, 248–257. https://doi.org/10.1093/abbs/gmr007.

118. Woolf, K.; Manore, M. M. B-Vitamins and Exercise: Does Exercise Alter Requirements? *Int. J. Sport Nutr. Exerc. Metab.* **2006,** *16*, 453–484. https://doi.org/10.1123/ijsnem.16.5.453.

119. Laires, M. J.; Monteiro, C. Exercise, Magnesium and Immune Function. *Magnes. Res.* **2008,** *21*, 92–96. https://doi.org/10.1684/mrh.2008.0136.

120. Beard, J.; Tobin, B. Iron Status and Exercise. *Am. J. Clin. Nutr.* **2000,** *72*, 594–597. https://doi.org/10.1093/ajcn/72.2.594s.

121. Beck, K. L.; von Hurst, P. R.; O'Brien, W. J.; Badenhorst, C. E. Micronutrients and Athletic Performance: A Review. *Food Chem. Toxicol.* **2021,** *158*,. https://doi.org/10.1016/j.fct.2021.112618.

122. Lee, E. C.; Fragala, M. S.; Kavouras, S. A.; Queen, R. M.; Pryor, J. L.; Casa, D. J. Biomarkers in Sports and Exercise: Tracking Health, Performance, and Recovery in Athletes. *J. strength Cond. Res.* **2017,** *31*, 2920. https://doi.org/10.1016/j.soncn.2012.03.005.

123. Maughan, R. J. Impact of Mild Dehydration on Wellness and on Exercise Performance.

Eur. J. Clin. Nutr. **2003,** *57*, S19–S23. https://doi.org/10.1038/sj.ejcn.1601897.

124. Nirmala Prasadi, V. P.; Joye, I. J. Dietary Fibre from Whole Grains and Their Benefits on Metabolic Health. *Nutrients* **2020,** *12*, 1–20. https://doi.org/10.3390/nu12103045.

125. Tannock, G. W.; Liu, Y. Guided Dietary Fibre Intake as a Means of Directing Short-Chain Fatty Acid Production by the Gut Microbiota. *J. R. Soc. New Zeal.* **2020,** *50*, 434–455. https://doi.org/10.1080/03036758.2019.1657471.

126. Frampton, J.; Murphy, K. G.; Frost, G.; Chambers, E. S. Short-Chain Fatty Acids as Potential Regulators of Skeletal Muscle Metabolism and Function. *Nat. Metab.* **2020,** *2*, 840–848. https://doi.org/10.1038/s42255-020-0188-7.

127. Carey, R. A.; Montag, D. Exploring the Relationship between Gut Microbiota and Exercise: Short-Chain Fatty Acids and Their Role in Metabolism. *BMJ Open Sport Exerc. Med.* **2021,** *7*, 1–7. https://doi.org/10.1136/bmjsem-2020-000930.

128. Kitada, M.; Ogura, Y.; Monno, I.; Koya, D. The Impact of Dietary Protein Intake on Longevity and Metabolic Health. *EBioMedicine* **2019,** *43*, 632–640. https://doi.org/10.1016/j.ebiom.2019.04.005.

129. Micha, R.; Michas, G.; Mozaffarian, D. Unprocessed Red and Processed Meats and Risk of Coronary Artery Disease and Type 2 Diabetes - An Updated Review of the Evidence. *Curr. Atheroscler. Rep.* **2012,** *14*, 515–524. https://doi.org/10.1007/s11883-012-0282-8.

130. Bellavia, A.; Stilling, F.; Wolk, A. High Red Meat Intake and All-Cause Cardiovascular and Cancer Mortality: Is the Risk Modified by Fruit and Vegetable Intake? *Am. J. Clin. Nutr.* **2016,** *104*, 1137–1143. https://doi.org/10.3945/ajcn.116.135335.

131. Valerio, A.; D'Antona, G.; Nisoli, E. Branched-Chain Amino Acids, Mitochondrial Biogenesis, and Healthspan: An Evolutionary Perspective. *Aging (Albany. NY).* **2011,** *3*, 464–478. https://doi.org/10.18632/aging.100322.

132. Begdache, L.; Chaar, M.; Sabounchi, N.; Kianmehr, H. Assessment of Dietary Factors, Dietary Practices and Exercise on Mental Distress in Young Adults versus Matured Adults: A Cross-Sectional Study. *Nutr. Neurosci.* **2019,** *22*, 488–498. https://doi.org/10.1080/1028415X.2017.1411875.

133. Wolk, A. Potential Health Hazards of Eating Red Meat. *J. Intern. Med.* **2017,** *281*, 106–122. https://doi.org/10.1111/joim.12543.

134. González, N.; Marquès, M.; Nadal, M.; Domingo, J. L. Meat Consumption: Which Are the

Current Global Risks? A Review of Recent (2010–2020) Evidences. *Food Res. Int.* **2020,** *137*, 109341. https://doi.org/10.1016/j.foodres.2020.109341.

135. Hirabara, S. M.; Curi, R.; Maechler, P. Saturated Fatty Acid-Induced Insulin Resistance Is Associated with Mitochondrial Dysfunction in Skeletal Muscle Cells. *J. Cell. Physiol.* **2010,** *222*, 187–194. https://doi.org/10.1002/jcp.21936.

136. Islam, H.; Hood, D. A.; Gurd, B. J. Looking beyond PGC-1α: Emerging Regulators of Exercise-Induced Skeletal Muscle Mitochondrial Biogenesis and Their Activation by Dietary Compounds. *Appl. Physiol. Nutr. Metab.* **2020,** *45*, 11–23.

137. Samsonowicz, M.; Regulska, E.; Karpowicz, D.; Leśniewska, B. Antioxidant Properties of Coffee Substitutes Rich in Polyphenols and Minerals. *Food Chem.* **2019,** *278*, 101–109. https://doi.org/10.1016/j.foodchem.2018.11.057.

138. Shankar, S.; Kumar, D.; Srivastava, R. K. Epigenetic Modifications by Dietary Phytochemicals: Implications for Personalized Nutrition. *Pharmacol. Ther.* **2013,** *138*, 1–17. https://doi.org/10.1016/j.pharmthera.2012.11.002.

139. Milenkovic, D.; Jude, B.; Morand, C. MiRNA as Molecular Target of Polyphenols Underlying Their Biological Effects. *Free Radic. Biol. Med.* **2013,** *64*, 40–51. https://doi.org/10.1016/j.freeradbiomed.2013.05.046.

8. APPENDIX

8.1. Included publications

8.1.1. Original article 1

sports

MDPI

Article

PGC-1α Methylation, miR-23a, and miR-30e Expression as Biomarkers for Exercise- and Diet-Induced Mitochondrial Biogenesis in Capillary Blood from Healthy Individuals: A Single-Arm Intervention

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Abstract: Healthy mitochondria and their epigenetic control are essential to maintaining health, extending life expectancy, and improving cardiovascular performance. Strategies to maintain functional mitochondria during aging include training; cardiovascular exercise has been suggested as the best method, but strength training has also been identified as essential to health and healthy aging. We therefore investigated the effects of concurrent exercise training and dietary habits on epigenetic mechanisms involved in mitochondrial (mt) functions and biogenesis. We analyzed epigenetic biomarkers that directly target the key regulator of mitochondrial biogenesis, *PGC-1α*, and mtDNA content. Thirty-six healthy, sedentary participants completed a 12-week concurrent training program. Before and after the intervention, dried blood spot samples and data on eating habits, lifestyle, and body composition were collected. MiR-23a, miR-30e expression, and mtDNA content were analyzed using real-time quantitative polymerase chain reaction (qPCR) analysis. *PGC-1α* methylation was analyzed using bisulfite pyrosequencing. MiR-23a, miR-30e expression, and *PGC-1α* methylation decreased after the intervention ($p < 0.05$). *PGC-1α* methylation increased with the consumption of red and processed meat, and mtDNA content increased with the ingestion of cruciferous vegetables ($p < 0.05$). Our results indicate that concurrent training could improve mitochondrial biogenesis and functions by altering the epigenetic regulation. These alterations can also be detected outside of the skeletal muscle and could potentially affect athletic performance.

Keywords: mitochondrial biogenesis; epigenetic; PGC-1α; polyphenols; concurrent training

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1. Introduction

In addition to maintaining or improving general health, exercise training aims to improve an individual's physical and conditioning performance by using physical loads as a potential stimulus to induce muscle growth in the human body. These specific reactions or training-related adaptions, triggered by endurance or resistance training, occur via a complex network of several signaling pathways (Figure 1) [1,2].

Endurance training (Figure 1, left side) induces signaling pathways such as peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α), p38 mitogen-activated protein kinase (p38 MAPK), and AMP-activated protein kinase (AMPK), all of which underlie the cellular processes that stimulate mitochondrial biogenesis and angiogenesis. This enables metabolic adaptions that lead to improvement in endurance performance [1,2]. Mitochondrial biogenesis is defined as how existing mitochondria grow and divide to create new mitochondria. This takes place on the one hand by cell division, but also in response to an oxidative

stimulus by increasing the cell's energy requirements, by electrical stimulation, by hormones, in certain mitochondrial diseases, and by physical training [3,4]. Repeated training sessions lead to an improvement in mitochondrial volume and function, thereby improving oxidative capacity. Reactive oxygen species (ROS) or mitochondrial stress occur, and low ROS levels or mild mitochondrial stress are known to induce adaptive responses that affect both short-term (e.g., metabolic) and long-term (e.g., longevity) health—responses that are referred to as mitohormesis [5,6]. Healthy physiology of the mitochondria is essential for health and longevity, whereby dysfunction is related to the pathogenesis of various diseases, such as type 2 diabetes and cardiovascular diseases [7,8]. PGC-1α promotes mitochondrial biogenesis by activating various transcription factors, e.g., nuclear respiratory factor 1 (NRF1) and nuclear factor erythroid 2–related factor 2 (NRF2), which promote the expression of mitochondrial transcription factor A (TFAM). TFAM increases the transcription of critical mitochondrial enzymes, which drives mtDNA replication [3]. Furthermore, PGC-1α plays a major role in regulating cellular energy metabolism and in muscle fiber type determination (e.g., formation of slow-twitch muscle fibers), stimulates mitochondrial biogenesis, and has a regulatory function in lipid metabolism. Therefore it is very likely that PGC-1α is also involved in diseases such as obesity and diabetes [9,10].

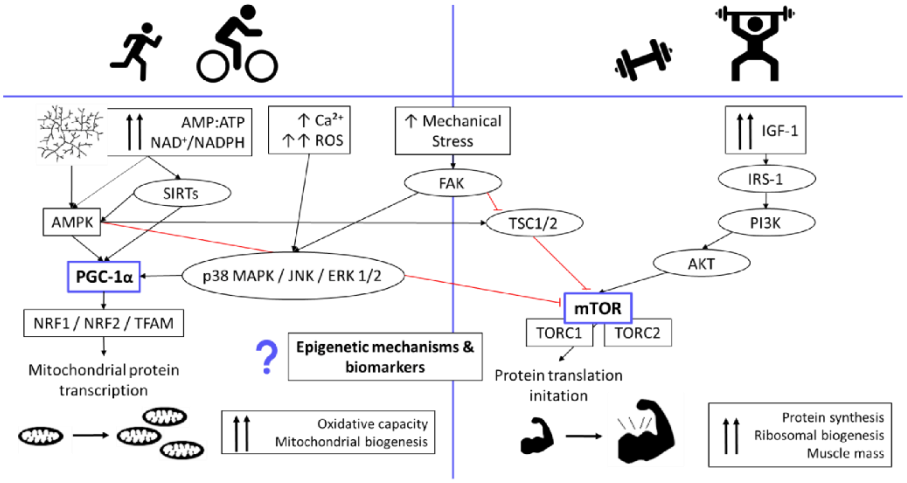

Figure 1. A graphic representation of the main signaling pathways in the regulation of skeletal muscle hypertrophy and mitochondrial biogenesis. Voluntary training alters primary signals such as mechanical stress or muscle energy status (AMP:ATP Ratio) and activates kinases/phosphatases in order to convey a specific exercise-induced signal. These pathways are regulated at several points, with significant crosstalk among the numerous signaling pathways creating a highly sensitive and complex transduction network. (The illustration was inspired by Methenitis [1] and Hawley et al. [11]). AMPK = AMP-activated Protein Kinase, PGC1-α = PPAR-γ Coactivator-1α, NRF1= Nuclear Respiratory Factor 1, NRF2 = Nuclear Factor Erythroid 2-related Factor 2, TFAM = mitochondrial Transcription Factor A, FAK = Focal Adhesion Kinase, TSC = Tuberous Sclerosis Complex, AKT = Protein Kinase B, mTOR = mammalian Target Of Rapamycin, IGF-1 = Insulin-like Growth Factor 1, PI3K = Phosphatidylinositide 3-Kinases, p38 MAPK = p38 Mitogen-activated Protein Kinase, JNK = Jun amino-terminal Kinase, ERK 1/2 = Extracellular signal-regulated Kinases 1/2, IRS-1 = Insulin Receptor Substrate 1, SIRT = Sirtuin, TORC1/2 = mTOR Complex 1/2, ROS = Reactive Oxygen Species, AMP:ATP = Adenosine Monophsphate: Adenosine Triphosphate ratio, NAD = Nicotinamide Adenine Dinucleotide, NADP = Nicotinamide Adenine Dinucleotide Phosphate.

On the other side, resistance training (Figure 1, right side) activates the molecular pathway responsible for muscle hypertrophy and stimulates the myofibrillar protein synthesis via insulin-like growth factor 1 (IGF-1) and the protein kinase B (AKT)-mammalian target of the rapamycin (mTOR) pathway, which leads to an increase in muscle mass and protein synthesis. In addition, it is assumed that high-volume, moderate, repeated, or frequent endurance training can negatively influence the adaptions caused by resistance training, presumably by inhibiting the activation of the AKT-mTOR-signaling pathway through AMPK [1,2]. In contrast, resistance exercise can stimulate mitochondrial biogenesis in the skeletal muscles, possibly via focal adhesion kinase (FAK) [1,12].

Training also leads to transcriptional, translational, and post-translational changes. Even if these molecular mechanisms are not yet fully understood, it is assumed that epigenetic mechanisms play a role in training-related adaptions [13]. Through epigenetic mechanisms, cells can react to various environmental stimuli, adapt gene expression to new circumstances, and regulate mitochondrial functions. Epigenetic mechanisms include DNA modifications (i.e., methylation), the regulation of gene expression by non-coding RNAs (e.g., miRNAs), and post-translational modifications of histones [14]. It has already been described that, after a strenuous training session, the promoter regions of genes that are important for training metabolism (e.g., *PGC-1α*) are hypomethylated, with a simultaneous increase in the mRNA level. Furthermore, it has been shown that the expression of some miRNAs differs after exercise, which could affect skeletal muscle regeneration, gene transcription, and mitochondrial biogenesis [13,15]. Often-discussed examples are miR-23, miR-696, and miR-30e, which have *PGC-1α* as a predicted target [16–18]. Endurance training seems to downregulate miR-23a expression, which promotes mitochondrial biogenesis by the upregulation of *PGC-1α*. In mice, it is also reported that overexpression of miR-23a downregulates the protein abundance of PGC-1α in skeletal muscles. However, the exact role and function of miR-23a in the mitochondria is currently unknown, but miR-23a appears to inhibit myogenic differentiation and consequently regulates the contractile function of mature myofibers [16,17]. Also, miR-30e seems to be involved in myogenic differentiation. In vivo studies have shown that overexpression of miR-30e reduces *PGC-1α* expression and increases the formation of glycolytic myotubes [19].

The main purpose of this work was to investigate whether a medium-term sports intervention focusing on endurance training and resistance training causes long-term epigenetic modifications which, according to the literature, have an influence on mitochondrial biogenesis or play a role in the metabolic and signaling pathways described above (Figure 1). Since endurance and resistance training at the same time are believed to have more beneficial effects on general health, mitochondrial biogenesis, disease prevention, and healthy aging than each modality alone [20,21], we considered the effects of endurance and resistance training. Furthermore, we hypothesized that epigenetic biomarkers in the blood (e.g., miRNAs, CpG methylations) reflect processes or changes in the muscles and the general state of health, as components from the skeletal muscles are released into the systemic circulation via extracellular vesicles [22]. However, it is not only physical activity that can influence health, physical performance, and mitochondrial biogenesis; nutrients, e.g., bioactive food components like phytochemicals, or diets such as fasting or caloric restriction [23,24] can do this as well. For example, the intake of phytochemicals, as in exercise or fasting, leads to the activation of AMKP and SIRT1 and thus ultimately influences mitochondrial biogenesis (Figure 1) [25,26]. Furthermore, there is an association between body composition and mitochondrial energy metabolism. Mitochondria regulate maximum oxygen consumption and control several important functions in adipose tissue. Total body fat is also correlated with impaired mitochondrial function [27,28]. Therefore, we additionally investigated whether dietary habits or body composition influence health or the selected biomarkers.

2. Materials and Methods

2.1. Subjects and Experimental Design

This study includes the data of 36 healthy, sedentary women and men, who completed a 12-week concurrent training intervention, including 60 min of endurance training twice a week and total-body resistance training three times a week (Table 1). The subjects were on average 31.86 ± 8.02 years old. The mean body mass index (BMI) was 24.17 ± 2.95 kg/m^2, and over 58% of the participants had a BMI in the normal range (18.5–24.9 kg/m^2). Twenty-one participants were female (58.3%) and fifteen participants were male (41.7%). Exclusion criteria were elite athletes, individuals who have participated extensively (for the past 2 years) in competitive sports, and individuals with a history of chronic illness or medication use. All participants were advised to maintain their lifestyle and dietary habits throughout the intervention. Before [T0] and after [T1] the intervention, food frequency questionnaires, health records, dried blood spots (DBS), and body composition data (e.g., lean body mass (LBM), body fat mass (BFM), using bioelectrical impedance analysis (BIA, Nutriguard-MS)) were collected and basal metabolic rate (the number of calories the body needs for basic life-support functions) calculated. Participants were instructed to report for sample collection and body composition measurement between 24 and 48 h after their last training session. In addition, it was precisely noted when the respective participants came to their appointment after the last training session. Most participants (75%) came within 32 h after their last training session. Only nine participants (25%) came between 32 and 48 h after their last training. This was also considered in the statistical evaluation of the data and considered separately. In accordance with the declaration of the Viennese Human Ethics Committee, all the human subjects gave their written approval for the use of the data.

Table 1. Training Plan. During strength training, there should be 60–120 s breaks between the sets. Training B would start the following Monday, followed by endurance training on Tuesday.

Monday Training A	Tuesday Endurance	Wednesday Training B	Thursday Break	Friday Training A	Saturday Endurance	Sunday Break
Warm up 5–10 min. stepper	30 to at least 60 min. in the last weeks of intervention	Warm up 5–10 min. stepper	-	Warm up 5–10 min. stepper	30 to at least 60 min. in the last weeks of intervention	-
Working sets Squats: 3 × 8–12 rep. Gluteus 3 × 8–12 rep. Chest press: 3 × 8–12 rep. Dumbbell row: 3 × 8–12 rep. Plank 3 × 8–12 rep.		Working sets Leg press: 3 × 8–12 rep. Lunges: 3 × 8–12 rep. Push-ups: 3 × 8–12 rep. Inverted row: 3 × 8–12 rep. Abdominal crunches 3 × 8–12 rep.		Working sets Squats: 3 × 8–12 rep. Gluteus 3 × 8–12 rep. Chest press: 3 × 8–12 rep. Dumbbell row: 3 × 8–12 rep. Plank 3 × 8–12 rep.		
Cool down 10 min stretching		Cool down 10 min stretching		Cool down 10 min stretching		

Rep = Repetition.

2.2. Training Plan

Strength Training: In the first two to four weeks the participants were accustomed to the equipment/strength exercises with no or little additional weight, so as to learn the correct execution. After the "familiarization phase", the training was carried out with additional weight, which was increased by 1.25 to 5 kg (depending on the exercise machine) as soon as the participants managed 3 sets with 12 repetitions. The training alternated between plans A and B so that there was a break of approximately 48 h between the different strength exercises (Table 1).

Endurance Training: The endurance training units (treadmill or ergometer) should initially be carried out for 30 min at an intensity of 60–85% maximum heart rate. After an adjustment period of three to four weeks, the duration was extended by 5 to 10 min per week over the next weeks, up to a duration of at least 60 min in the last three to four weeks (Table 1).

2.3. Dried Blood Spot Sample Preparation (RNA and DNA Extraction)

The total RNA was isolated from the dried blood spots using the *miRNeasy Micro Kit* (Qiagen, Hilden, Germany) and the total DNA using the *QIAamp® DNA Mini Kit* (Qiagen, Hilden, Germany), according to the manufacturer's protocol. Quantity and quality were assessed using a *NanoDropTM One/OneC Microvolume UV-Vis Spectrophotometer* (ThermoFisher Scientific, Waltham, MA, USA), and the samples were then stored at −20 °C.

2.4. Bisulfite Conversion and Pyrosequencing

The gDNA samples were bisulfite-converted using the *EpiTect Bisulfite Kit* (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The bisulfite-converted DNA (bisDNA) samples were diluted to 20 ng/µL. The polymerase chain reaction (PCR) of the diluted bisDNA samples was performed with the *PyroMark® Kit* (Qiagen, Hilden, Germany). According to our established protocol, there was an initial activation period of 15 min. at 95 °C, a 3-step cycle of denaturation (94 °C for 30 s), annealing (58 °C for 5 s), and extension (72 °C for 15 s) for 45 cycles. The PCR process was terminated with a final extension period of 72 °C for 10 min. PyroMark assays (biomers.net, Ulm, Germany), primer sequences, and the sequence to analyze, based on the work by Hunter et al. 2019 [15], are listed in Table 2.

Table 2. Details of pyrosequencing assays and primers used to measure CpG methylation.

Assay ID	Primer	Sequence	No. of CpG Sites
PGC1A	fw:	5′-TAT AGT TAT TTT GTT ATG AAA TAG GGA GTT TTG -3′	1
	rev:	5′- biotin-CCA ATC ACA TAA CAA AAC TAT TAA AAA ATA A -3′	
	seq:	5′-GGA TTT TGG TTA TTA TAT GGT TAG G -3′	
Sequence to analyze:		GTT TYG TTT AGA GTT TG	

Fw = forward, rev = reverse, seq = sequence, biotin = biotinylating.

Gel electrophoresis was performed on a 2% agarose gel using *GeneRuler 100 bp DNA Ladder* (ThermoFisher Scientific, Waltham, MA, USA) to check the PCR products. CpG methylation was determined using *PyroMark Q24* (Qiagen, Hilden, Germany) and *PyroMark Gold Q24 Reagents* (Qiagen, Hilden, Germany). The nucleotide dispensing order was generated by entering the sequence to be analyzed into the *PyroMark Q24 software version 2.0.8* (Qiagen, Hilden, Germany). The methylation at each CpG site was evaluated using the *PyroMark Q24 software* in the CpG mode.

2.5. Real-Time Quantitaive PCR (qPCR)

The mtDNA content was measured in the gDNA samples. For the qPCR, a *StepOne Plus real-time PCR Detection System* (Applied Biosystems, Waltham, MA, USA), a single-copy gene primer (fw: GCT TCT GAC ACA ACT GTG TTC ACT AGC, rev: CAC CAA CTT CAT CCA CGT), mtDNA primer (fw: CAT CTG GTT CCT ACT TCA GGG, rev: TGA GTG GTT AAT AGG GTG ATA GA; Biomers, Ulm, Germany) [29] and a *LightCycler® 480 Sybr® Green I Master Mix* (Roche, Munich, Germany) were used. For the miRNA analyses, we used *the TaqMan™ Advanced microRNA cDNA Synthesis Kit* and *TaqMan™ Advanced microRNA assays* (miR-23a-3p, -30e-3p, and endogenous controls miR-24-3p and -93-5p) under the default settings on *QuantStudio™ 3* from ThermoFisher Scientific, Waltham, MA, USA. Fold changes were calculated with the formula $2^{-\Delta\Delta Ct}$ [30].

$$\Delta Ct = Ct^{target} - Ct^{endogenous\ control}$$
$$\Delta\Delta Ct = \Delta Ct^{T1} - \Delta Ct^{T0}$$

2.6. Statistical Analysis

The statistical analysis was carried out using IBM SPSS Statistics 20 and GraphPad Prism 6. All data are represented as mean ± standard deviation (SD). Paired t-tests

were used to analyze the different time points for parametric and Wilcoxon tests for nonparametric values. Correlations between methylation, miRNA expression, and body composition were tested using Pearson correlation and linear regression. Correlations between dietary habits, methylation, and mtDNA were tested using Spearman's Rho correlation and Kendall's tau. A p-value less than or equal to 0.05 was defined as significant for all tests.

3. Results

3.1. Molecular and Physiological Changes Following 12 Weeks of Concurrent Training

Body weight (kg, $p = 0.009$), BMI (kg/m^2, $p = 0.009$), basal metabolic rate (kcal, $p = 0.003$), LBM (kg, $p = 0.017$), and BFM (kg, $p = 0.000$) or body fat percentage (BFP; %, $p = 0.000$) improved significantly through the 12-week concurrent training. Body weight, BMI, and BFM could be reduced, and basal metabolic rate and LBM increased (Table 3).

Table 3. Participant characteristics.

	Total (n = 36)	**Female (n = 21)**	**Male (n = 15)**
Age ± SD [years]	31.86 ± 8.02	30.14 ± 8.51	34.27 ± 6.82
BMI ± SD [T0, kg/m^2]	24.17 ± 2.95	23.78 ± 3.18	24.71 ± 2.61
BMI ± SD [T1, kg/m^2]	23.88 ± 2.71	23.51 ± 2.95	24.40 ± 2.33
BMI classes			
Normal weight 18.5–24.9 kg/m^2	58.3%	61.9%	53.3%
Overweight, 25–29.9 kg/m^2	38.9%	33.3%	46.7%
Obesity grad I, 30–34.9 kg/m^2	2.8%	4.8%	-
Basal metabolic rate ± SD [T0, kcal]	1481.11 ± 178.41	1355.71 ± 73.46	1656.67 ± 123.56
Basal metabolic rate ± SD [T1, kcal]	1495.81 ± 175.74	1369.05 ± 61.72	1673.27 ± 118.03
LBM ± SD [T0, kg]	51.93 ± 9.74	45.02 ± 4.96	61.60 ± 5.47
LBM ± SD [T1, kg]	52.44 ± 9.87	45.47 ± 4.72	62.20 ± 6.07
BFM ± SD [T0, kg]	20.06 ± 5.67	21.21 ± 6.08	18.44 ± 4.77
BFM ± SD [T1, kg]	18.53 ± 5.62	19.99 ± 5.84	16.47 ± 4.75
BFP ± SD [T0, %]	27.75 ± 6.21	31.24 ± 4.88	22.86 ± 4.31
BFP ± SD [T1, %]	26.04 ± 6.77	29.76 ± 5.27	20.83 ± 5.02
Intake frequencies			
Red/processed meat			
Rarely or never	44.4%	57.2%	26.6%
Once/week	25.0%	23.8%	26.6%
2–3×/week	22.2%	9.5%	40.0%
>4×/week or daily	8.4%	9.5%	6.8%
Cruciferous vegetables			
Rarely or never	58.3%	57.1%	60.0%
≥once/week	41.7%	42.9%	40.0%

SD = Standard Deviation, BMI = Body Mass Index, LBM = Lean Body Mass, BFM = Body Fat Mass, BFP = Body Fat Percentage.

Furthermore, the 12-week intervention changed the miR-23a ($n = 36$, $p = 0.023$, 0.92-fold, Figure 2B) and miR-30e ($n = 36$, $p = 0.047$, 0.94-fold, Figure 2C) expression significantly. Participants whose capillary blood samples were collected 24 to 32 h after their last training session showed a change in *PGC-1α* methylation ($n = 27$, $p = 0.024$, Figure 2A) and expression of miR-23a ($n = 27$, $p = 0.028$, Table 4). Furthermore, there were no differences in *PGC-1α* methylation, mtDNA, miR-23a, and -30e expression between sex, smoker ($n = 11$, 30.6%) or non-smoker ($n = 25$, 69.4%) status, or age, so we included the entire study population for further analysis.

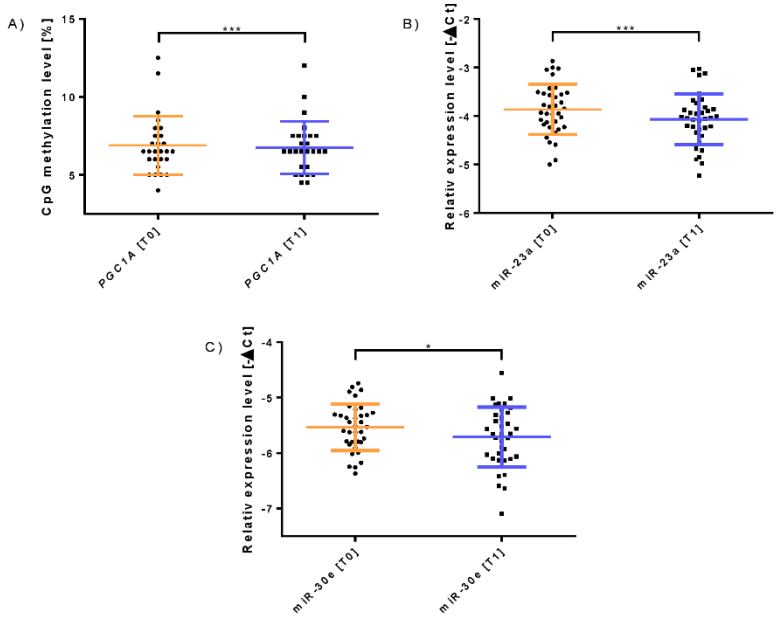

Figure 2. Molecular changes following 12 weeks of concurrent training. (**A**) Change in *PGC-1α* methylation during the intervention (n = 27). (**B**) Change in miR-23a expression during the intervention (n = 36). (**C**) Change in miR-30e expression during the intervention (n = 36). The results are expressed as mean ± SD. * Shows significant p-values below 0.05 and *** p-values below 0.03 (paired t-test).

Table 4. Main results of the analyzed marker.

Marker	Total (n = 36) 24–48 h Post-Exercise		Reduced Group (n = 27) 24–32 h Post-Exercise	
	Fold Change ± SD	p Value	Fold Change ± SD	p Value
PGC-1α Methylation	1.49 ± 1.42	0.826	1.88 ± 1.45	0.024 *
mtDNA	1.09 ± 0.37	0.426	1.10 ± 0.39	0.583
miR-23a-3p	0.92 ± 0.35	0.023 *	0.91 ± 0.36	0.028 *
miR-30e-3p	0.94 ± 0.34	0.047 *	0.94 ± 0.37	0.088

PGC-1α = Peroxisome Proliferator-activated Receptor Gamma Coactivator 1-Alpha, mtDNA = mitochondrial DNA, SD = Standard Deviation. * Shows significant p-values (paired t-test).

3.2. Correlations between Eating and Lifestyle Habits and Anthropometric Data with Selected Mitochondria- and Exercise-Related Markers Independent of the Intervention

A positive correlation between *PGC-1α* methylation and body fat mass (BFM, kg) was obtained (p = 0.008, Figure 3A). As described in the literature, increasing *PGC-1α* methylation negatively correlated with miR-23a expression (p = 0.003, Figure 3B).

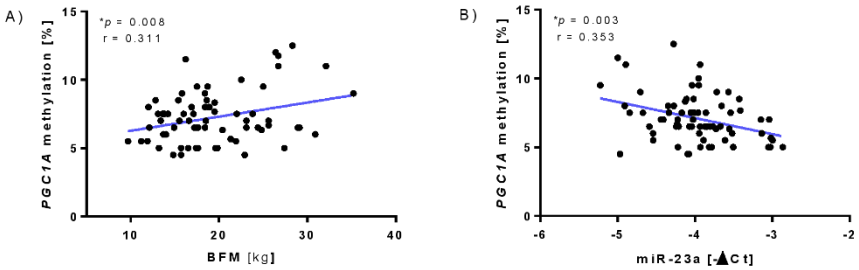

Figure 3. Correlations, independent of the intervention. (**A**) Linear regression between body fat mass (BFM, kg) and *PGC1A* methylation. (**B**) Linear regression between *PGC1A* methylation and miR-23a expression. * Shows significant *p*-values.

The participants who reported consuming red or processed meat at least 4 times a week (above the limit of the recommendation) or daily had higher levels of *PGC-1α* methylation (mean methylation 8.292 ± 2.147, $p = 0.022$) than those who reported consuming less or no red or processed meat (mean methylation 6.758 ± 1.686). Also, intake of cruciferous vegetables (e.g., broccoli, cabbage, brussels sprouts) and mtDNA content ($p = 0.043$) correlated positively (see Table 3 for intake frequencies). Participants who reported regularly, at least once a week, consuming cruciferous vegetables had higher mtDNA content (mean mtDNA 6.095 ± 0.408) than those who reported rarely or never consuming cruciferous vegetables (mean mtDNA 5.868 ± 0.327).

4. Discussion

The main aim of this study was to examine whether a medium-term concurrent exercise intervention induces measurable epigenetic changes in dried capillary blood samples (DBS), which allow conclusions to be drawn about a person's fitness or health and would be suitable as biomarkers for exercise- and diet-induced mitochondrial biogenesis. Additionally, we analyzed the influence of lifestyle and eating habits on these biomarkers and the amount of mitochondrial DNA (mtDNA).

Chronic sustained concurrent exercise training decreased *PGC-1α* methylation levels and led to a downregulation of miR-23a and -30e in the capillary blood samples (DBS) for up to 32 h after a training session. These results are confirmed by the literature, since downregulation of miR-23a leads to an upregulation of *PGC-1α*, promoting mitochondrial biogenesis and improving endurance performance [16]. After more than 32 h, however, we could no longer detect a significant change in the methylation of *PGC-1α*. This is in accordance with the results of Barrès et al. [31], who also discovered no difference in methylation 48 h after a three-week training program. This suggests that hypomethylation is a transient mechanism that needs to be repeatedly stimulated, underlining the importance of regular exercise to benefit from the positive effects of *PGC-1α* expression, e.g., activation of mitochondrial biogenesis with subsequent improvement in endurance performance.

Furthermore, it is described that exercise-related changes in DNA methylation and miRNA expression patterns can be detected in skeletal muscles [32] and in leukocytes or neutrophils [15,33]. The currently available literature does not provide information on how long these changes last or can be detected after exercise, as explanatory studies are not yet available, and samples are usually taken directly or a few hours after exercise. But during exercise and partly during recovery periods, components such as mitochondria, metabolites, and muscle-enriched miRNAs (myomiRs) are "flushed" from the skeletal muscles into the systemic circulation [22,34]. This could explain why we observed changes in miR-23a and -30e expression but not in *PGC-1α* methylation in our DBS samples more than 32 h after the intervention. Like miR-23a, miR-30e is also involved in myogenic differentiation or muscle

fiber formation, which presumably takes place in the regeneration phase [17,19]. It can be assumed that downregulation of miR-30e favors the formation of type 1 muscle fibers, which are particularly advantageous for long-term endurance units, as overexpression increases the formation of glycolytic or type 2 muscle fibers, so exercise possibly has an impact on type of muscle fiber formation [19,35]. Therefore, our results suggest that endurance capacity is improved by concurrent exercise training and by downregulating miR-30e and can possibly influence mitochondrial biogenesis. This is further reinforced by the correlation between the methylation of *PGC-1α* and the miR-23a expression. We suspect that there are two "fronts" through which personal athletic performance can be influenced, either through miR-23a or *PGC-1α* alone or through a synergistic effect, or both. This indicates that individuals respond differently to the different regulation systems.

Mitochondria are interconnected and highly mobile cell organelles that undergo dynamic changes in terms of physiological and pathological modifications. During the life of a cell, there is a relatively stable equilibrium in a multitude of processes: fission, fusion, and biogenesis [36,37]. Maintaining this balance, and the fact that mitochondria can fuse with each other, causing the mtDNA to mix, could be a possible explanation for why we did not see any change in the mtDNA content. As mentioned, the timing of sampling and the intensity (effort and duration) of the training seem to be of great concern, including how many mitochondria are released from the muscle into the bloodstream to detect changes of mtDNA [38]. Further studies as well as a comparison between muscle biopsy samples and DBS could clarify this.

However, we discovered an association between body fat mass (BFM) and *PGC-1α* methylation. The participants who had a higher BFM had an increased methylation level of *PGC-1α*. Due to its regulatory function in lipid and energy metabolism, it is likely that PGC-1α, in addition to its function in mitochondrial biogenesis, is also involved in metabolic diseases, such as obesity and diabetes [9]. A reduced *PGC-1α* expression could be observed in the skeletal muscle of patients with type 2 diabetes and in the adipose tissue of insulin-resistant and pathologically obese people [39]. It is possible that these are also found in the fatty tissue of healthy people with a slightly increased body fat percentage. This and the fact that the mRNA expression of *PGC-1α* correlates with the methylation highlight our results, even though our study did not include heavily overweight people.

Furthermore, we observed an association between *PGC-1α* methylation and the consumption of red and/or processed meat. Metabolic health as well as lifespan can be influenced by various nutrients, such as certain amino acids. Above all, the branched-chain amino acids (BCAAs) and methionine, which are particularly found in red meat, can regulate lifespan, aging, and metabolism through various mechanisms [40]. An excessive intake of red meat is also associated with age-related diseases such as cardiovascular diseases, type 2 diabetes, and cancer [41,42]. Animal studies as well as epidemiological studies have shown that reducing dietary BCAAs improves the Western diet–induced prevalence of obesity and glucose intolerance, and there is also an association between elevated BCAA levels and insulin resistance in obese and diabetic patients [40]. These elevated blood levels can trigger mitochondrial dysfunction. On the other hand, it should also be mentioned that supplementing BCAAs, especially in older people, can also have positive effects on health and mitochondrial biogenesis via mTOR, as shown in Figure 1 [40,43]. However, in addition to BCAAs, red or processed meat also contains other substances such as saturated fatty acids, heme iron, nitrosamines, sodium nitrite, L-carnitine, etc., which can have negative health consequences if consumed in excess [44]. These substances may be also responsible for the correlation between red meat consumption and *PGC-1α* methylation observed in this study. At minimum, there is evidence that the intake of the saturated fatty acids palmitic and stearic acid can impair mitochondrial function by reducing mitochondrial hyperpolarization and production of adenosine triphosphate (ATP). The treatment of cultured skeletal muscle cells with palmitic acid reduced *PGC-1α* expression [45]. However, clarifying data on the mechanisms in humans are still needed.

In addition to exercise, phytochemicals such as polyphenols in plant-based foods also seem to regulate the intracellular pathways of mitochondrial biogenesis [8]. We were able to point out an association between the consumption of cruciferous vegetables and mtDNA content. Cruciferous vegetables (e.g., broccoli, cabbage, or cauliflower) contain several nutrients, phytochemicals, and polyphenols such as carotenoids, chlorophyll, tocopherols, glucosinolates, flavonoids, sulforaphane, fibers, and a few others with health-promoting properties, such as potassium and folate [46,47]. The literature describes that polyphenols play a key role in replacing damaged mitochondria with newly synthesized mitochondria [48], and oxidative stress, e.g., induced by exercise, can modify mtDNA and lead to mitochondrial dysfunction. The consumption of cruciferous vegetables may maintain mtDNA levels via their antioxidant properties by fighting against oxidative stress and thereby affecting athletic performance [40,49]. Furthermore, mitochondria provide a significant portion of the energy of a muscle cell, and the more efficient the mitochondria are, the better an athlete's performance [48]. In vivo and ex vivo studies have shown that isothiocyanate sulforaphane, from cruciferous vegetables, can activate NRF2 and increases the expression of *NRF1* and *TFAM* (Figure 1), supporting the model that sulforaphane influences mitochondrial biogenesis [50]. Although the effects of sulforaphane on mitochondrial biogenesis are largely uncharted, administration to rodents improved training performance and increased AMPK phosphorylation of skeletal muscles [50]. Our findings provide new evidence for the influence of sulforaphane on mitochondria via mtDNA content in humans, but further studies are needed to confirm study results, including a precise supplementation dose.

There are some potential limitations in this study that could be addressed in future research. Initially, this study focused on exercise- and diet-induced mitochondrial biomarkers; however, the functional aspects of mitochondria were not directly measured. This should be considered in future work. Second, unfortunately, no control group was studied, and the number of participants was relatively small. Nevertheless, we were able to identify exercise- and diet-related changes in both women and men using a minimally invasive method instead of muscle biopsy, which can be used for the general population and can be collected from anyone at any time.

5. Conclusions

In conclusion, our data support the model that concurrent exercise training affects different areas of athletic performance, e.g., mitochondrial biogenesis, and leads to positive effects on health. These are confirmed in particular by the decrease in body fat, increase in fat-free mass, and basal metabolic rate, which in turn leads us to expect better physical performance. In contrast to most previous scientific work using skeletal muscle biopsies, we measured the effects of exercise using a less invasive method (DBS) and epigenetic biomarkers such as miR-23a, miR-30e, and *PGC-1α* methylation, which may provide insight into exercise- and diet-induced mitochondrial biogenesis. Furthermore, our data showed that the consumption of red and/or processed meat has a negative impact on our measured biomarkers, whereas the intake of cruciferous vegetables has a positive effect. Taking this into account, eating habits may have an influence on athletic performance.

Author Contributions: U.D.B.K., O.J.S., B.H., P.R. and A.G.H. made significant contributions to the conception and design of the manuscript, while U.D.B.K., A.S., S.T., A.M. and S.V.L. assisted in conducting the study and collecting, analyzing, and interpreting the data. All authors were involved in the preparation of the manuscript, and U.D.B.K., O.J.S., B.H., P.R. and A.G.H. provided critical revisions. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and the guidelines of the Vienna Ethics Committee. According to this commission, no specific ethical approval was required (MA 15-EK/22-083-VK_NZ).

Informed Consent Statement: Informed approval was obtained from all subjects involved in the study.

Data Availability Statement: Not applicable.

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Conflicts of Interest: All authors contributed appropriately to the design, planning, implementation, and preparation of this work and have declared no conflict of interest regarding the publication of this manuscript.

References

1. Methenitis, S. A Brief Review on Concurrent Training: From Laboratory to the Field. *Sports* **2018**, *6*, 127. [CrossRef]
2. Simoes, D.C.M.; Vogiatzis, I. Can Muscle Protein Metabolism Be Specifically Targeted by Exercise Training in COPD? *J. Thorac. Dis.* **2018**, *10*, 1367–1376. [CrossRef] [PubMed]
3. Jornayvaz, F.R.; Shulman, G.I. Regulation of Mitochondrial Biogenesis. *Essays Biochem.* **2010**, *47*, 69–84. [CrossRef]
4. Valero, T. Mitochondrial Biogenesis: Pharmacological Approaches. *Curr. Pharm. Des.* **2014**, *20*, 5507–5509. [CrossRef] [PubMed]
5. Ristow, M.; Schmeisser, K. Mitohormesis: Promoting Health and Lifespan by Increased Levels of Reactive Oxygen Species (ROS). *Dose-Response* **2014**, *12*, 288–341. [CrossRef] [PubMed]
6. Yun, J.; Finkel, T. Mitohermesis. *Cell Metab.* **2014**, *19*, 757–766. [CrossRef] [PubMed]
7. Irrcher, I.; Adhihetty, P.J.; Joseph, A.M.; Ljubicic, V.; Hood, D.A. Regulation of Mitochondrial Biogenesis in Muscle by Endurance Exercise. *Sport. Med.* **2003**, *33*, 783–793. [CrossRef]
8. Chodari, L.; Aytemir, M.D.; Vahedi, P.; Alipour, M.; Vahed, S.Z.; Khatibi, S.M.H.; Ahmadian, E.; Ardalan, M.; Eftekhari, A. Targeting Mitochondrial Biogenesis with Polyphenol Compounds. *Oxid. Med. Cell. Longev.* **2021**, *2021*, 4946711. [CrossRef]
9. Liang, H.; Ward, W.F. PGC-1α: A Key Regulator of Energy Metabolism. *Am. J. Physiol.-Adv. Physiol. Educ.* **2006**, *30*, 145–151. [CrossRef]
10. Lin, J.; Wu, H.; Tarr, P.T.; Zhang, C.Y.; Wu, Z.; Boss, O.; Michael, L.F.; Puigserver, P.; Isotani, E.; Olson, E.N.; et al. Transcriptional Co-Activator PGC-1α Drives the Formation of Slow-Twitch Muscle Fibres. *Nature* **2002**, *418*, 797–801. [CrossRef]
11. Hawley, J.A.; Hargreaves, M.; Joyner, M.J.; Zierath, J.R. Integrative Biology of Exercise. *Cell* **2014**, *159*, 738–749. [CrossRef] [PubMed]
12. Groennebaek, T.; Vissing, K. Impact of Resistance Training on Skeletal Muscle Mitochondrial Biogenesis, Content, and Function. *Front. Physiol.* **2017**, *8*, 713. [CrossRef] [PubMed]
13. Jacques, M.; Hiam, D.; Craig, J.; Barrès, R.; Eynon, N.; Voisin, S. Epigenetic Changes in Healthy Human Skeletal Muscle Following Exercise– a Systematic Review. *Epigenetics* **2019**, *14*, 633–648. [CrossRef] [PubMed]
14. Mohammed, S.A.; Ambrosini, S.; Lüscher, T.; Paneni, F.; Costantino, S. Epigenetic Control of Mitochondrial Function in the Vasculature. *Front. Cardiovasc. Med.* **2020**, *7*, 1–16. [CrossRef]
15. Hunter, D.J.; James, L.; Hussey, B.; Wadley, A.J.; Lindley, M.R.; Mastana, S.S. Impact of Aerobic Exercise and Fatty Acid Supplementation on Global and Gene-Specific DNA Methylation. *Epigenetics* **2019**, *14*, 294–309. [CrossRef]
16. Silver, J.; Wadley, G.; Lamon, S. Mitochondrial Regulation in Skeletal Muscle: A Role for Non-Coding RNAs? *Exp. Physiol.* **2018**, *103*, 1132–1144. [CrossRef]
17. Kirby, T.J.; McCarthy, J.J. MicroRNAs in Skeletal Muscle Biology and Exercise Adaptation. *Free Radic. Biol. Med.* **2013**, *64*, 95–105. [CrossRef]
18. Xu, M.; Chen, X.; Chen, D.; Yu, B.; Li, M.; He, J.; Huang, Z. Regulation of Skeletal Myogenesis by MicroRNAs. *J. Cell. Physiol.* **2020**, *235*, 87–104. [CrossRef]
19. Jia, H.; Zhao, Y.; Li, T.; Zhang, Y.; Zhu, D. MiR-30e Is Negatively Regulated by Myostatin in Skeletal Muscle and Is Functionally Related to Fiber-Type Composition. *Acta Biochim. Biophys. Sin.* **2017**, *49*, 392–399. [CrossRef]
20. Murlasits, Z.; Kneffel, Z.; Thalib, L. The Physiological Effects of Concurrent Strength and Endurance Training Sequence: A Systematic Review and Meta-Analysis. *J. Sports Sci.* **2018**, *36*, 1212–1219. [CrossRef]
21. Cadore, E.L.; Izquierdo, M. New Strategies for the Concurrent Strength-, Power-, and Endurance-Training Prescription in Elderly Individuals. *J. Am. Med. Dir. Assoc.* **2013**, *14*, 623–624. [CrossRef] [PubMed]
22. Burtscher, J.; Millet, G.P.; Place, N.; Kayser, B.; Zanou, N. The Muscle-Brain Axis and Neurodegenerative Diseases: The Key Role of Mitochondria in Exercise-Induced Neuroprotection. *Int. J. Mol. Sci.* **2021**, *22*, 6479. [CrossRef] [PubMed]
23. Pons, V.; Riera, J.; Capó, X.; Martorell, M.; Sureda, A.; Tur, J.A.; Drobnic, F.; Pons, A. Calorie Restriction Regime Enhances Physical Performance of Trained Athletes. *J. Int. Soc. Sports Nutr.* **2018**, *15*, 1–10. [CrossRef] [PubMed]
24. Duivenvoorde, L.P.M.; van Schothorst, E.M.; Bunschoten, A.; Keijer, J. Dietary Restriction of Mice on a High-Fat Diet Induces Substrate Efficiency and Improves Metabolic Health. *J. Mol. Endocrinol.* **2011**, *47*, 81–97. [CrossRef] [PubMed]

25. Martel, J.; Ojcius, D.M.; Ko, Y.F.; Ke, P.Y.; Wu, C.Y.; Peng, H.H.; Young, J.D. Hormetic Effects of Phytochemicals on Health and Longevity. *Trends Endocrinol. Metab.* **2019**, *30*, 335–346. [CrossRef]
26. Giampieri, F.; Alvarez-Suarez, J.M.; Cordero, M.D.; Gasparrini, M.; Forbes-Hernandez, T.Y.; Afrin, S.; Santos-Buelga, C.; González-Paramás, A.M.; Astolfi, P.; Rubini, C.; et al. Strawberry Consumption Improves Aging-Associated Impairments, Mitochondrial Biogenesis and Functionality through the AMP-Activated Protein Kinase Signaling Cascade. *Food Chem.* **2017**, *234*, 464–471. [CrossRef]
27. Heinonen, S.; Jokinen, R.; Rissanen, A.; Pietiläinen, K.H. White Adipose Tissue Mitochondrial Metabolism in Health and in Obesity. *Obes. Rev.* **2020**, *21*, 1–23. [CrossRef]
28. Sanayei, M.; Hajizadeh-Sharafabad, F.; Amirsasan, R.; Barzegar, A. High Intensity Interval Training with or without Chlorella Vulgaris Supplementation in Obese and Overweight Women: Effects on Mitochondrial Biogenesis, Performance, and Body Composition. *Br. J. Nutr.* **2021**, 1–11. [CrossRef]
29. Tyrka, A.R.; Parade, S.H.; Price, L.H.; Kao, H.-T.; Porton, B.; Philip, N.S.; Welch, E.S.; Carpenter, L.L. Alterations of Mitochondrial DNA Copy Number and Telomere Length with Early Adversity and Psychopathology. *Biol. Psychiatry* **2016**, *79*, 78–86. [CrossRef]
30. Lilja, S.; Bäck, H.; Stoll, C.; Mayer, A.; Pointner, A.; Hippe, B.; Krammer, U.; Haslberger, A.G. Increased Sirtuin Expression, Senescence Regulating MiRNAs, MtDNA, and Bifidobacteria Correlate with Wellbeing and Skin Appearance after Sirtuin-Activating Drink. *Bioact. Compd. Health Dis.* **2021**, *4*, 45–62. [CrossRef]
31. Barrès, R.; Yan, J.; Egan, B.; Treebak, J.T.; Rasmussen, M.; Fritz, T.; Caidahl, K.; Krook, A.; O'Gorman, D.J.; Zierath, J.R. Acute Exercise Remodels Promoter Methylation in Human Skeletal Muscle. *Cell Metab.* **2012**, *15*, 405–411. [CrossRef] [PubMed]
32. Voisin, S.; Eynon, N.; Yan, X.; Bishop, D.J. Exercise Training and DNA Methylation in Humans. *Acta Physiol.* **2015**, *213*, 39–59. [CrossRef] [PubMed]
33. Radom-Aizik, S.; Zaldivar, F.; Oliver, S.; Galassetti, P.; Cooper, D.M. Evidence for MicroRNA Involvement in Exercise-Associated Neutrophil Gene Expression Changes. *J. Appl. Physiol.* **2010**, *109*, 252–261. [CrossRef] [PubMed]
34. Siracusa, J.; Koulmann, N.; Banzet, S. Circulating MyomiRs: A New Class of Biomarkers to Monitor Skeletal Muscle in Physiology and Medicine. *J. Cachexia. Sarcopenia Muscle* **2018**, *9*, 20–27. [CrossRef]
35. Scott, W.; Stevens, J.; Binder-Macleod, S.A. Human Skeletal Muscle Fiber Type Classifications. *Phys. Ther.* **2001**, *81*, 1810–1816. [CrossRef]
36. Xie, L.L.; Shi, F.; Tan, Z.; Li, Y.; Bode, A.M.; Cao, Y. Mitochondrial Network Structure Homeostasis and Cell Death. *Cancer Sci.* **2018**, *109*, 3686–3694. [CrossRef]
37. Youle, R.J.; Van Der Bliek, A.M. Mitochondrial Fission, Fusion and Stress. *Science* **2012**, *337*, 1062–1065. [CrossRef]
38. Bishop, D.J.; Botella, J.; Genders, A.J.; Lee, M.J.C.; Saner, N.J.; Kuang, J.; Yan, X.; Granata, C. High-Intensity Exercise and Mitochondrial Biogenesis: Current Controversies and Future Research Directions. *Physiology* **2019**, *34*, 56–70. [CrossRef]
39. Liu, C.; Lin, J.D. PGC-1 Coactivators in the Control of Energy Metabolism. *Acta Biochim. Biophys. Sin.* **2011**, *43*, 248–257. [CrossRef]
40. Kitada, M.; Ogura, Y.; Monno, I.; Koya, D. The Impact of Dietary Protein Intake on Longevity and Metabolic Health. *EBioMedicine* **2019**, *43*, 632–640. [CrossRef]
41. Micha, R.; Michas, G.; Mozaffarian, D. Unprocessed Red and Processed Meats and Risk of Coronary Artery Disease and Type 2 Diabetes-An Updated Review of the Evidence. *Curr. Atheroscler. Rep.* **2012**, *14*, 515–524. [CrossRef] [PubMed]
42. Bellavia, A.; Stilling, F.; Wolk, A. High Red Meat Intake and All-Cause Cardiovascular and Cancer Mortality: Is the Risk Modified by Fruit and Vegetable Intake? *Am. J. Clin. Nutr.* **2016**, *104*, 1137–1143. [CrossRef] [PubMed]
43. Valerio, A.; D'Antona, G.; Nisoli, E. Branched-Chain Amino Acids, Mitochondrial Biogenesis, and Healthspan: An Evolutionary Perspective. *Aging* **2011**, *3*, 464–478. [CrossRef] [PubMed]
44. Wolk, A. Potential Health Hazards of Eating Red Meat. *J. Intern. Med.* **2017**, *281*, 106–122. [CrossRef]
45. Hirabara, S.M.; Curi, R.; Maechler, P. Saturated Fatty Acid-Induced Insulin Resistance Is Associated with Mitochondrial Dysfunction in Skeletal Muscle Cells. *J. Cell. Physiol.* **2010**, *222*, 187–194. [CrossRef]
46. Niedzwiecki, A.; Roomi, M.W.; Kalinovsky, T.; Rath, M. Anticancer Efficacy of Polyphenols and Their Combinations. *Nutrients* **2016**, *8*, 552. [CrossRef]
47. Mahn, A.; Reyes, A. An Overview of Health-Promoting Compounds of Broccoli (Brassica Oleracea Var. Italica) and the Effect of Processing. *Food Sci. Technol. Int.* **2012**, *18*, 503–514. [CrossRef]
48. D'Angelo, S. Polyphenols: Potential Beneficial Effects of These Phytochemicals in Athletes. *Curr. Sports Med. Rep.* **2020**, *19*, 260–265. [CrossRef]
49. Eluamai, A.; Brooks, K. Effect of Aerobic Exercise on Mitochondrial DNA and Aging. *J. Exerc. Sci. Fit.* **2013**, *11*, 1–5. [CrossRef]
50. Islam, H.; Hood, D.A.; Gurd, B.J. Looking beyond PGC-1α: Emerging Regulators of Exercise-Induced Skeletal Muscle Mitochondrial Biogenesis and Their Activation by Dietary Compounds. *Appl. Physiol. Nutr. Metab.* **2020**, *45*, 11–23. [CrossRef]

8.1.2. Original article 2

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RESEARCH ARTICLE

MiRNA-based "fitness score" to assess the individual response to diet, metabolism, and exercise

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ABSTRACT

Background: Regular, especially sustained exercise plays an important role in the prevention and treatment of multiple chronic diseases. Some of the underlying molecular and cellular mechanisms behind the adaptive response to physical activity are still unclear, but recent findings suggest a possible role of epigenetic mechanisms, especially miRNAs, in the progression and management of exercise-related changes. Due to the combination of the analysis of epigenetic biomarkers (miRNAs), the intake of food and supplements, and genetic dispositions, a "fitness score" was evaluated to assess the individual response to nutrition, exercise, and metabolic influence.

Methods: In response to a 12-week sports intervention, we analyzed genetic and epigenetic biomarkers in capillary blood from 61 sedentary, healthy participants (66.1% females, 33.9% males, mean age 33 years), including *Line-1* methylation, three SNPs, and ten miRNAs using HRM and qPCR analysis. These biomarkers were also analyzed in a healthy, age- and sex-matched control group (n, 20) without intervention. Food frequency intake, including dietary supplement intake, and general health questionnaires were surveyed under the supervision of trained staff.

Results: Exercise training decreased the expression of miR-20a-5p, −22-5p, and −505-3p ($p < 0.02$) and improved the "fitness score," which estimates eight different lifestyle factors to assess, nutrition, inflammation, cardiovascular fitness, injury risk, regeneration, muscle and hydration status, as well as stress level. In addition, we were able to determine correlations between individual miRNAs, miR-20a-5p, −22-5p, and −101-3p ($p < 0.04$), and the genetic predisposition for endurance and/or strength and obesity risk (*ACE, ACTN3*, and *FTO*), as well as between miRNAs and the body composition ($p < 0.05$). MiR-19b-3p and −101-3p correlated with the intake of B vitamins. Further, miR-19b-3p correlated with magnesium and miR-378a-3p with iron intake ($p < 0.05$).

Conclusions: In summary, our results indicate that a combined analysis of several biomarkers (miRNAs) can provide information about an individual's training adaptions/fitness, body composition, nutritional needs, and possible recovery. In contrast to most studies using muscle biopsies, we were able to show that these biomarkers can also be measured using a minimally invasive method.

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Introduction

Regular, especially sustained, exercise plays an important role in the prevention and treatment of multiple chronic diseases, such as cardiovascular diseases, metabolic disorders, neurological/cognitive diseases, musculoskeletal disorders, and cancer, but also for the maintenance of health and well-being [1]. In addition, aging conditions, such as a decline in cognitive/motor functions, and loss of muscle mass and strength, can be improved through regular exercise and age-related deterioration can be delayed [2,3]. Moreover, our genetic architecture and epigenetic manifestations play an essential role in this big area, for example, adherence to exercise, a person's response to exercise, and metabolic adaptions through exercise [4].

Variations in voluntary physical activity are an important determinant of long-term human health. The predisposition to voluntary activity is heritable and leads to protective metabolic changes [5]. From animal and human research, however, it is known that neural signaling and pleasure/reward systems in the brain to a large extent promote the tendency to be physically active and to adhere to a training program. Based on a large epidemiologic study (TIGER study), 26 single nucleotide polymorphisms (SNPs) in six candidate genes (*BDNF, BDNFOS, DRD2, DRD4, HTR2A*, and *HTR2C*) were identified that are related to behavior during physical activity, that is, compliance, duration, intensity, and total dose of exercise in young men and women [6]. On the other hand, it is assumed that there is also a genetic predisposition to the type of sport (endurance *ACTN3* or strength *ACE*) a human is better at. One example is the *Alpha-actinin-3* (*ACTN3*) genotype, which affects human performance in various ways through the absence or presence of α-actinin-3 in skeletal muscle [7]. In addition, variants of the *Fat mass- and obesity-associated* (*FTO*) gene show a strong association with obesity and fat mass but are also described to influence the skeletal muscle phenotypes of athletes [8]. A study by Almén et al., 2012 [9] suggests that the effect of the *FTO* obesity risk allele may be mediated through epigenetic changes.

On the other hand, genetic dispositions alone are insufficient to explain the individual response to diet, nutrition, and exercise. It has already been shown that exercise can alter gene expression [10] and that an early response to physical activity triggers increased transcription of regulatory, metabolic, and myogenic genes, which are important for mediating subsequent adaptations in skeletal muscle and contribute to improved fitness [4]. Through increased transcription, exercise can change the methylation status of certain genes that are involved in muscle function. And this can lead to the formation of a long-lasting favorable expression pattern for improved training ability. In response to physical activity, the methylation profile changes in a dose-dependent, gene-specific, and tissue-specific manner [11,12]. MiRNAs are small (approx. 22 nucleotides in length), highly conserved noncoding RNA molecules, which are also altered by physical activity [13] and they can influence gene expression post-transcriptional. MiRNAs bind to their miRNA response elements (MREs) situated on RNAs and upon binding initiate the degradation of the RNA and inhibit their translation, that is, miRNAs silence-specific genes, so the expression of miRNAs depends on the exercise type, duration, intensity, and tissue. Therefore, miRNAs are subdivided into cardiac-, and muscle depending, and circulated miRNAs (ci-miRNAs) [14,15].

In addition, to understand the various pathways that physical activity triggers in the human body, there is growing interest and the use of so-called biomarkers, which individually reflect and evaluate the health, performance, and recovery of every human. This information could help athletes and/or coaches to improve performance while reducing the risk of overtraining and injury. At the moment, mainly proteins or electrolytes are used as biomarkers, which are measured in blood, urine, or saliva, but also genetic tests [16]. By the time, these markers are detectable in the circulation; however, consequential damage, such as inflammation and injuries may already have occurred. It is assumed that changes in miRNA expressions in body fluids will occur earlier than with conventional biomarkers, which is why miRNAs are more favorable for the early detection of possible deficits [17].

The main goal of our work was to identify miRNAs that are influenced by endurance and strength training, and are suitable as biomarkers to provide information about the current fitness and health status of a person. This can even be achieved through a noninvasive method using capillary blood [13], which makes it easier for athletes and trainers to take samples and counteract possible deficits immediately. Additionally, there is increasing evidence that finger capillary blood sampling can provide a reliable alternative to venous blood sampling in clinical and field settings [18]. Furthermore, another aim was to identify possible interactions between selected epigenetic and genetic markers, the diet, and the anthropometric data to be able to make personal recommendations for athletes in the future. Since the literature almost exclusively contains sports intervention studies with men, with this study we also wanted to create a certain basis for the interactions and effects of exercise and epigenetics in women.

Materials and methods

Marker selection

In a pilot study from 2018, we analyzed over 340 different miRNAs and two methylation sites from capillary blood from 14 healthy women, aged between 36 and 59 years, with an average BMI of 32 kg/m^2. The results of this study as well as literature data were used to set up and select the markers for the intervention of the following sport study (Figure 1 A and B).

Study design of the sports intervention study

For the sports intervention study at the University of Vienna, Department of Nutritional Science, over 90 participants, women, and men, aged between 19 and 55 years, who were physically inactive, and had a BMI between 20 and 35 kg/m^2 (Table S1, for further details, for example, body composition) were recruited but were generally healthy. All study participants have given their written consent to the use of their data. Furthermore, elite athletes, individuals who participated vigorously in competitive sports (for the past 2 years), and anyone with a history of chronic illness or medication use have been excluded from participation. The participants received a 12-week training intervention that included both endurance and strength training, as previously published by Krammer et al. 2022 [55]. During their training sessions,

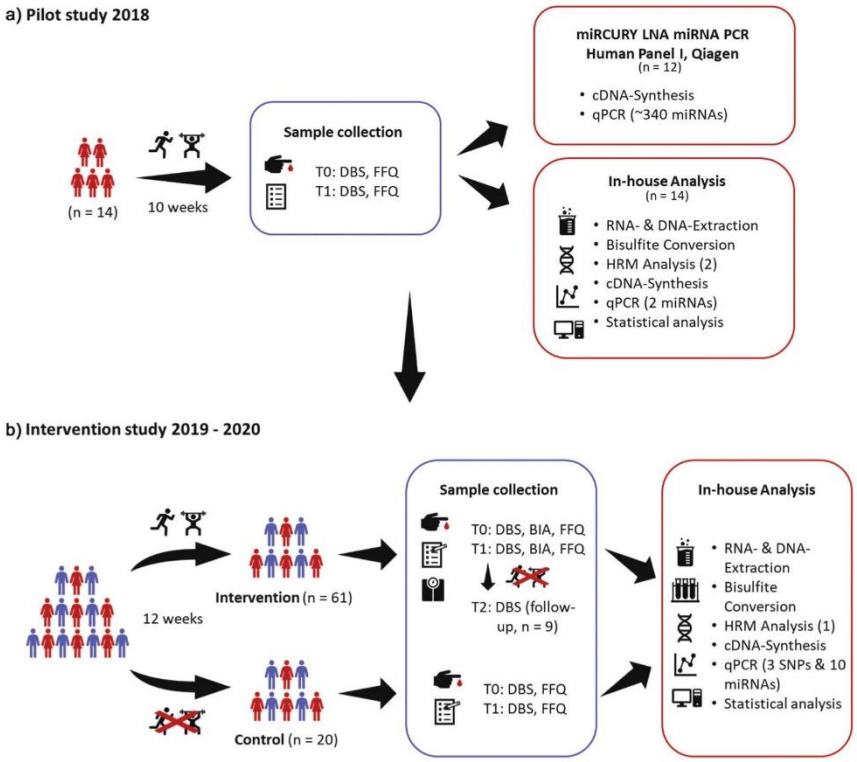

Figure 1. Study design of the pilot study 2018 (**A**) and the intervention study 2019 – 2020 (**B**). T0, begin, T1, after 10-/12-weeks, T2, after 10-month, DBS, dried blood spot, BIA, bioelectrical impedance analysis, FFQ, food frequency questionnaire, HRM, high-resolution melting, SNP, single nucleotide polymorphism.

they were supervised by the gym trainers and our staff and had to keep a training log. The participants were contacted weekly by phone and asked about their progress or motivation when needed. Before (T0) and after (24–48 hours after the last training session, T1) the intervention, capillary blood samples (DBS) were collected, the body composition was measured (using bioelectrical impedance analysis (BIA) to measure basal metabolic rate, phase angle, LBM, BFM, etc.), and a questionnaire regarding diet and lifestyle was queried. Sixty-one participants (20 men and 41 women) completed the intervention and in addition, capillary blood samples were collected from nine participants 10 months after the intervention (T2, Table S2) to see whether the expressions returned to baseline (Figure 1B).

To exclude seasonal or diet-related changes in expression patterns, 20 participants were examined as a control group, women and men, aged between 23 and 65 years, and with a BMI between 20 and 30 kg/m^2, without any sports intervention or lifestyle change (Figure 1B and Table S3).

Bioelectrical impedance analysis (BIA)

Body composition was assessed using a multi-frequency (5, 50, and 100 kHz) and phase-sensitive BIA. The measurement device Nutriguard-MS (Data Input GmbH, Germany) measures resistance R and reactance Xc. According to the manufacturers' protocol, study participants were instructed not to eat for 4–5 h, not to consume alcoholic beverages for 24 h, and to refrain from strenuous exercise for 12 h before the analysis. The subjects had to lie down and rest for 5 minutes before measurement. Extremities should have the temperature of regular skin circulation. The participant's legs should lie apart at approximately 45°, arm should be spread at approximately 30°. They should not touch the rest of the body. One pair of adhesive gel electrodes were placed on the hand and the other pair on the foot. The BIA analysis shows excellent reliability with repeat measurements [19].

Sample collection, RNA- and DNA extraction

The capillary blood was collected on *Whatman® protein saver cards* (Sigma-Aldrich, Austria) using the *safety Lancet Extra 18 G* (Sarstedt, Germany) from the fingertip of the non-dominant hand and dried overnight. Two points with a diameter of 10 mm were punched out of each DBS. Then, the RNA was extracted using the *miRNeasy Micro Kit* (Qiagen, Germany) and the DNA using *the QIAamp® DNA Mini Kit* (Qiagen, Germany). Samples were stored at −20 °C. Twelve RNA samples from the pilot study were sent to Qiagen in Germany for further analysis (*miRCURY LNA miRNA PCR Human Panel I*, Cat. No./ ID, 339,322 and YAHS-301).

SNP genotyping

We used the *TaqMan™ Genotyping Assays* from Thermofisher, Netherlands for the analysis of the SNPs (*ACTN3* (rs1815739), *ACE* (rs4341), and *FTO* (rs1121980)) under the default settings on *QuantStudio™ 3*. The SNPs were then evaluated using the *TaqMan Genotyper Software v1.6*.

Bisulfite conversion and high-resolution melting (HRM) analysis

The DNA samples were prepared with the *EpiTect Bisulfite Kit* (Qiagen, Germany) for the qPCR and HRM analysis using Rotor Gene® Q. For the *Line-1* methylation analysis, we used the primers from Marques-Rocha et al., 2016 [20]. The methylation percentage was then calculated using the AUC method.

cDNA-Synthesis and real-time PCR (qPCR)

We used the *TaqMan™ Advanced microRNA cDNA Synthesis Kit* and *TaqMan™ Advanced microRNA assays* under the default settings on *QuantStudio™ 3* from Thermofisher, Netherlands. The cDNA samples were stored at −20 °C. In the pilot study, miR-21-5p and miR-155-3p were analyzed in addition to the Qiagen panel. Based on this study, eight miRNAs (miR-19b-3p, −20a-5p, −22-5p, −30e-3p, −101-3p, −146a-5p, −378a-3p and −505-

3p) were then included and analyzed in the sports intervention study 2019 – 2020. Furthermore, two reference genes (miR-24-3p and –93-5p) were examined for the evaluation of the expression patterns (using the ΔΔCt method) of the eight selected miRNAs. The reference genes were selected based on the pilot study and through the literature.

Statistical analysis

The statistical evaluation was carried out using *IBM SPSS statistics 20* and *GraphPad Prism 6*. Paired t-tests and ANCOVA (adjusted to baseline) were used to compare the different time points. Correlations between miRNA expressions and genotypes were tested using one-way ANOVA (Bonferroni and Scheffé). Correlations between miRNA expressions, *Line-1* methylation, and body composition were tested using Pearson correlation and linear regression, while those between nutritional habits and miRNA expressions were tested using Spearman's Rho correlation and Kendall's Tau. For all tests, a p-value equal to or less than 0.05 was assumed to be significant.

Results

Pilot study 2018

In total, 12 out of the 14 samples were sent to Qiagen for miRNA analysis (Figure 1A, red box "miRCURY LNA miRNA PCR Human Panel I, Qiagen") revealing eight significant altered miRNAs (miR-494-3p, –369-5p, –122-5p, –135a-5p, –146a-5p, –30e-3p, –22-5p, and –20a-5p; $p < 0.05$) after the intervention. Four of these miRNAs (miR-146a-5p, –30e-3p, –22-5p, and –20a-5p) were altered uniformly in all 12 samples. These miRNAs and four others (miR-19b-3p, –101-3p, –505-3p, and –378a-3p, $p < 0.07$), which also changed in all samples, but not significantly, were then analyzed in the sports intervention study. Furthermore, *Line-1* methylation (n, 14, p, 0.003, Figure 1A, red box "In-house Analysis") increased significantly, which was also analyzed in the following study.

Intervention study 2019 – 2020

The selected miRNAs and DNA methylation sites were analyzed in the control group and intervention group before (T0) and after (T1) 12 weeks. Since training adjustments require several weeks, even up to 3 months for muscle building [21], and to provide a "buffer", for example, if a participant has a cold for a week, which could affect or interrupt their training, we decided to extend the study duration of the following study by 2 weeks. To assess the persistent consequence of the intervention, analysis was also done 10 months after the intervention (T2) in the follow-up group. SNP genotyping was carried out in the intervention and control group.

Results of the selected miRNAs and *Line-1* methylation

Intervention group. Five of the selected miRNAs showed an altered expression level after the sports intervention. Three miRNAs (miR-20a-5p, p, 0.017, 0.95-fold, miR-22-5p, p, 0.012, 0.95-fold and miR-505-3p, p, 0.006, 0.95-fold) were significantly downregulated and two (miR-30e-3p, p, 0.075, 0.97-fold and miR-146a-5p, p, 0.066, 0.97-fold) showed

a downregulation trend. Adjusting to baseline supports the results for miR-20a-5p, −22-5p, −30e-3p, and −505-3p. Additional adjustment for age did not change the effects of sports on these miRNAs and we could exclude age as a confounder (Table 1). However, to quantify the effect of the intervention and because a single molecular biomarker alone is not as meaningful as a combined analysis, we evaluated a "fitness score" that depends on the strength/p-values of the measured miRNAs in the intervention and control group, and determined using the following formula,

$$\Omega = \sum_{i=1}^{8}\left(\Delta Ct_i * ln\frac{p_i^C}{p_i^I}\right)$$

[22] where ΔCt_i is the respective miRNA, p_i^C is the p-value of the control group and p_i^I is the p-value of the intervention group. Where a high score indicates a very small p-value in the intervention group and means better fitness, and as can be seen in Figure 2, the "fitness score" increased significantly in the entire intervention group (p, 0.000, effect size, 0.521),

Table 1. Results of the analyzed markers of the participants of the sports intervention group. Results were given in mean ± SD.

	Total (n, 61)			Male (n, 20)		Female (n, 41)	
	Fold Change ± SD	Paired t-test	ANCOVA	Fold Change ± SD	Paired t-test	Fold Change ± SD	Paired t-test
miR-19b-3p	1.212 ± 0.764	0.463	0.192	1.119 ± 0.619	0.641	1.221 ± 0,832	0.576
miR-20a-5p	0.948 ± 0.291	**0.017***	**0.002***	0.917 ± 0.265	0.083	0.963 ± 0.305	0.095
miR-22-5p	0.945 ± 0.315	**0.012***	**0.010***	0.891 ± 0.311	**0.034***	0.971 ± 0.318	0.123
miR-30e-3p	0.973 ± 0.290	0.075	0.059	1.010 ± 0.330	0.568	0.954 ± 0.271	0.072
miR-101-3p	1.424 ± 1.241	0.256	0.265	1.744 ± 1.537	0.280	1.268 ± 1.054	0.621
miR-146a-5p	0.967 ± 0.297	0.066	0.258	0.905 ± 0.290	0.114	0.998 ± 0.298	0.345
miR-378a-3p	0.985 ± 0.248	0.172	0.397	0.974 ± 0.213	0.315	0.991 ± 0.266	0.313
miR-505-3p	0.948 ± 0.177	**0.006***	**0.016***	0.923 ± 0.127	**0.011***	0.961 ± 0.197	0.075
Line-1 methylation	−0.306 ± 4.166	0.568	0.851	−0.448 ± 3.818	0.606	−0.238 ± 4.369	0.729

Abbreviations: SD, standard deviation, *Shows significant p-values.

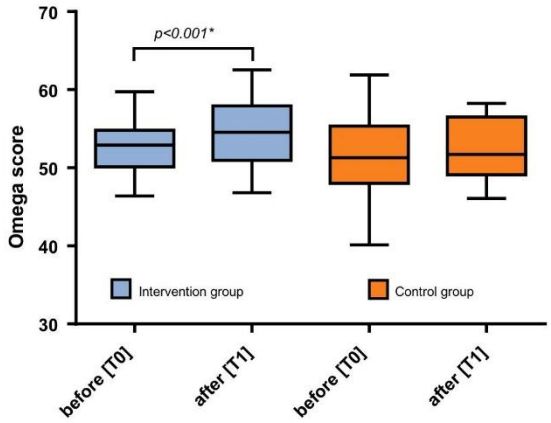

Figure 2. Fitness/Ω score of the intervention group (n, 61) and the control group (n, 20) before [T0] and after [T1]. *Shows significant p-values (paired t-test).

in men (p, 0.005) and women (p, 0.009), while it did not change in the control group (p, 0.740). Furthermore, the methylation of *Line-1* showed no change after the 12-week intervention.

Control group. In the control group, only one miRNA had a changed expression after 12 weeks. The miR-20a-5p showed a significant upregulation (p, 0.020, 1.48-fold), whereas in the intervention group a significant downregulation was observed. When the intervention and the control group are compared, they showed a significantly different expression of miR-20a-5p (p, 0.042) before the intervention (T0), and significantly different expressions of miR-22-5p (p, 0.000), −30e-3p (p, 0.015), and −146a-5p (p, 0.047) after 12 weeks (T1). If considering the fold changes of the two groups, they differ significantly in miR-20a-5p (p, 0.012) and miR-22-5p (p, 0.046).

Follow-up group. In the follow-up group, we observed an upregulated miR-378a-3p 10 months after the sports intervention (T2), compared to immediately after the intervention (T1) (p, 0.050). When compared before intervention (T0) and 10 months after the intervention (T2), there were no differences in the expression levels of these miRNAs.

Results of the SNP genotyping

Table 2 shows the distribution of the selected single nucleotide polymorphisms (SNPs) in the respective genes of our study participants in the intervention (n, 61) and control group (n, 20).

Table 2. Distribution of the SNPs in the *FTO* (rs1121980), *ACE* (rs4341), and *ACTN3* (rs1815739) gene. Genotyping values are given in total numbers and as a percentage.

Gene	SNP ID			
FTO	rs1121980	Homozygotes (GG)	Homozygotes (AA)	Heterozygotes (AG)
Intervention	Total, % (n)	29.5 % (18)	24.6 % (15)	45.9 % (28)
	Male, % (n)	20.0 % (4)	30.0 % (6)	50.0 % (10)
	Female, % (n)	34.1 % (14)	22.0 % (9)	43.9 % (18)
Control	Total, % (n)	30.0 % (6)	25.0 % (5)	45.0 % (9)
	Male, % (n)	57.1 % (4)	0 % (0)	42.9 % (3)
	Female, % (n)	15.4 % (2)	38.5 % (5)	46.2 % (6)
ACE	**rs4341**	**Homozygotes (CC)**	**Homozygotes (GG)**	**Heterozygotes (CG)**
Intervention	Total, % (n)	19.7 % (12)	31.1 % (19)	49.2 % (30)
	Male, % (n)	20.0 % (4)	30.0 % (6)	50.0 % (10)
	Female, % (n)	19.5 % (8)	31.7 % (13)	48.8 % (20)
Control	Total, % (n)	5.0 % (1)	25.0 % (5)	70.0 % (14)
	Male, % (n)	14.3 % (1)	14.3 % (1)	71.4 % (5)
	Female, % (n)	0 % (0)	30.8 % (4)	69.2 % (9)
ACTN3	**rs1815739**	**Homozygotes (CC)**	**Homozygotes (TT)**	**Heterozygotes (TC)**
Intervention	Total, % (n)	32.8 % (20)	18.0 % (11)	49.2 % (30)
	Male, % (n)	20.0 % (4)	30.0 % (6)	50.0 % (10)
	Female, % (n)	39.0 % (16)	12.2 % (5)	48.8 % (20)
Control	Total, % (n)	35.0 % (7)	30.0 % (6)	35.0 % (7)
	Male, % (n)	57.1 % (4)	14.3 % (1)	28.6 % (2)
	Female, % (n)	23.1 % (3)	38.5% (5)	38.5 % (5)

Abbreviations: FTO, Fat mass and obesity-associated protein, ACE, Angiotensin-converting enzyme, ACTN3, alpha-Actinin 3.

Differences between genotypes and selected miRNAs. Polymorphisms in the *ACE* gene seem to influence the expression of miR-101-3p (p, 0.019). Participants with a CC genotype had a significantly higher expression after the intervention compared to participants with a CG or GG phenotype, with no differences between CG and GG (Figure 3A). Furthermore, polymorphisms in the *ACTN3* gene appear to have an influence on the

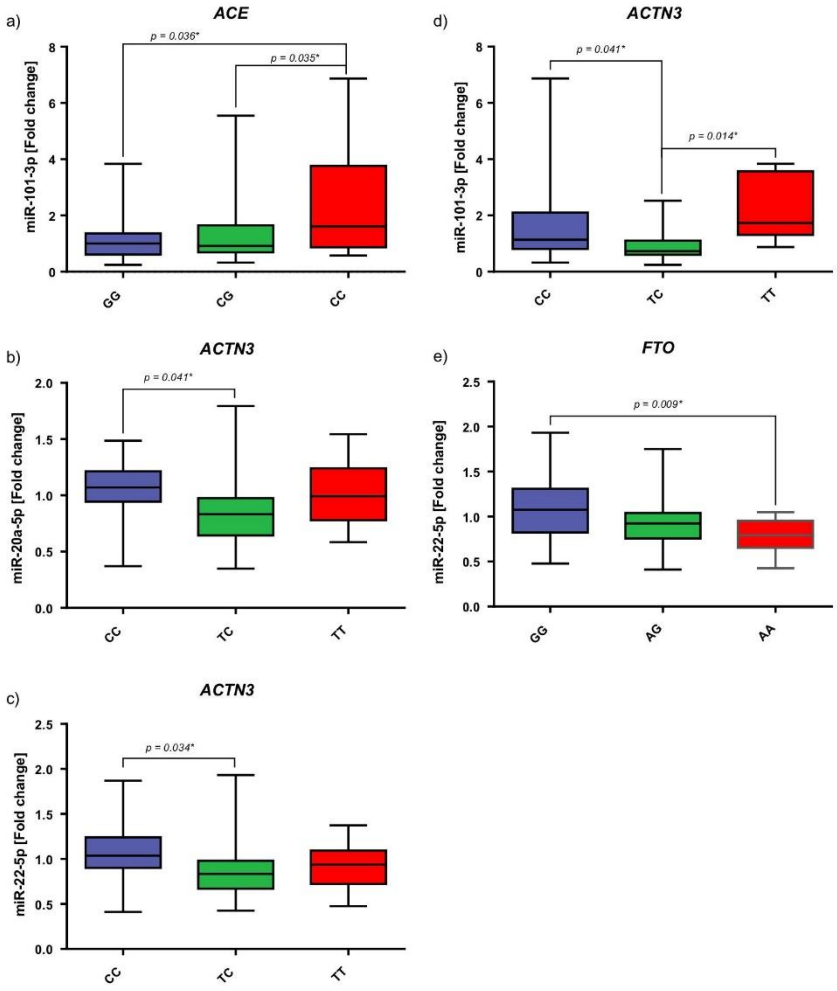

Figure 3. Fold changes of the selected miRNAs by genotype. **A)** Fold change of miR-101-3p by *ACE* genotype (GG strength, CC endurance). **B)** Fold change of miR-20a-5p by *ACTN3* genotype (CC strength, TT endurance). **C)** Fold change of miR-22-5p by *ACTN3* genotype (CC strength, TT endurance). **D)** Fold change of miR-101-3p by *ACTN3* genotype (CC strength, TT endurance). **E)** Fold change of miR-22-5p by *FTO* genotype (AA risk variant). The p-values given here are the results of the post hoc tests (Scheffé). * Shows significant p-values.

expression of miR-20a-5p (p, 0.027), miR-22-5p (p, 0.033), and miR-101-3p (p, 0.004), see Figure 3B-D. Participants with a CC genotype had a significantly higher expression of these miRNAs after 12 weeks than those with a TC genotype. Whereas the participants with a TT genotype showed a higher expression of miR-101-3p than TC genotypes. Furthermore, the polymorphism in the *FTO* gene also seems to influence the expression of miR-22-5p (p, 0.008). However, only the GG genotype differed from the AA genotype. Participants with a GG genotype showed a higher expression after the intervention than those with an AA genotype (Figure 3E). In the control group, we could not detect any significant correlations between the different SNP genotypes and the individual miRNAs ($p > 0.05$).

Results of the body composition measurement (BIA)

The Intervention resulted in significant changes in the anthropometric data. There was an increase in basal metabolic rate (p, 0.000), phase angle (p, 0.004), LBM (p, 0.000), BCM (p, 0.007), as well as ICW (p, 0.007), and ECW (p, 0.015), and a decrease in BFM (p, 0.000), see Table 3.

Epigenetic and genetic markers and anthropometric data. We were also able to determine significant correlations between miRNAs, *Line-1* methylation, and anthropometric data. Between miR-22-5p (linear regression, −0.277, p, 0.031), miR-101-3p, and LBM (linear regression, −0.267, p, 0.038). Among miR-505-3p and BFM (linear regression, 0.290, p, 0.024), between *Line-1* methylation and ECW (linear regression, −0.267, p, 0.037), and among the "fitness score" and BFM (linear regression, −0.343, p, 0.007), see Figure 4. For example, the participants who gained more LBM had a lower expression of miR-22-5p (Figure 4A) and miR-101-3p (Figure 4C), and participants who lost more BFM had a lower expression of miR-505-3p and a higher "fitness score" (Figure 4 B and D).

Furthermore, we could observe correlations between *ACTN3* gene, LBM (p, 0.039), ICW (p, 0.040), and BMI (p, 0.072). The participants with a TC genotype showed a higher gain in LBM and ICW compared to participants with a TT genotype.

Table 3. Body composition changes through the 12-week sports intervention. Anthropometric measurements were given in mean ± SD.

	Total (n, 61)		Male (n, 20)		Female (n, 41)	
	Change ± SD	p-Value	Change ± SD	p-Value	Change ± SD	p-Value
Basal metabolic rate [kcal]	23.10 ± 41.74	**0.000***	20.45 ± 25.67	**0.002***	24.39 ± 47.91	**0.002***
Phase angel [°]	0.16 ± 0.42	**0.004***	0.07 ± 0.25	0.232	0.21 ± 0.48	**0.009***
LBM [kg]	0.68 ± 1.39	**0.000***	0.84 ± 1.32	**0.010***	0.60 ± 1.43	**0.010***
BFM [kg]	−1.14 ± 1.87	**0.000***	−1.33 ± 2.19	**0.014***	−1.04 ± 1.72	**0.000***
BCM [kg]	0.77 ± 2.16	**0.007***	0.64 ± 3.17	0.378	0.83 ± 1.48	**0.001***
ICW [l]	0.31 ± 0.46	**0.000***	0.33 ± 0.43	**0.003***	0.30 ± 0.48	**0.000***
ECW [l]	0.20 ± 0.63	**0.015***	0.29 ± 0.64	**0.056**	0.16 ± 0.63	0.112

Abbreviations: LBM, lean body mass, BFM, body fat mass, BCM, body cell mass, ICW, intracellular water, ECW, extracellular water. Fold Change, mean difference between the 2 timepoints, SD, standard deviation. *Shows significant p-values (paired t-test).

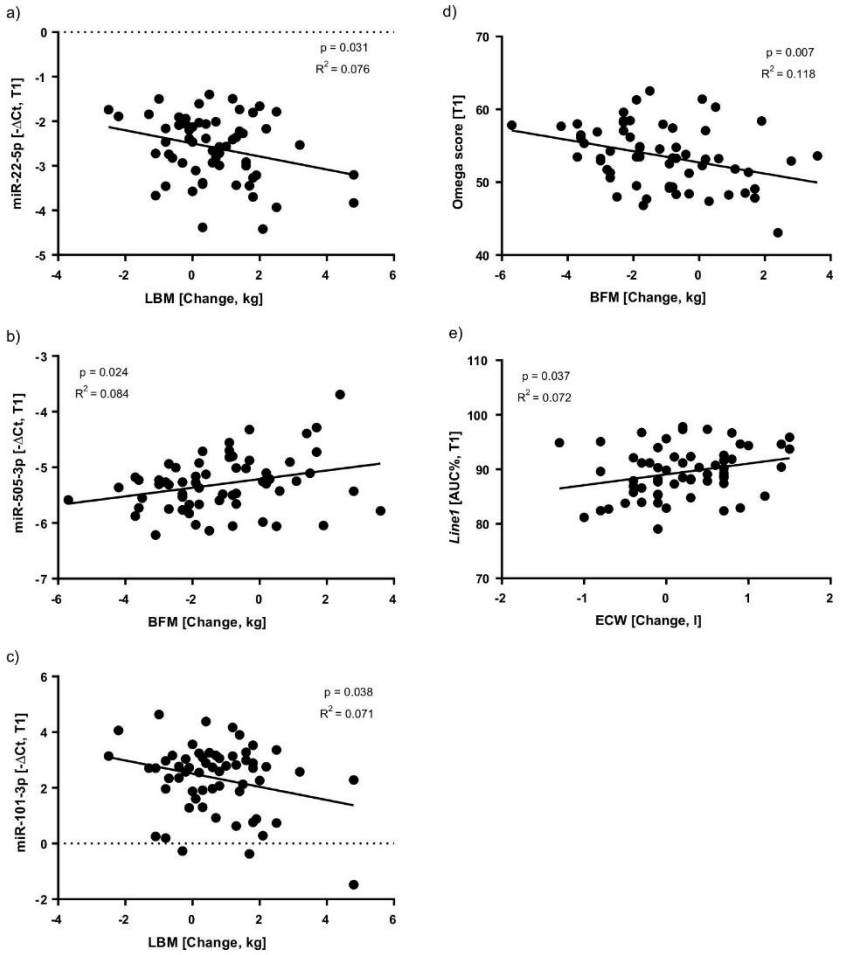

Figure 4. Significant correlations between the change of the anthropometric data and the expression of selected miRNAs, the fitness/Ω score, and *Line-1* methylation after the Intervention [T1]. **A)** Correlation between the change of LBM and the expression of miR-22-5p at T1. **B)** Correlation between the change of BFM and the expression of miR-505-3p at T1. **C)** Correlation between the change of LBM and the expression of miR-101-3p at T1. **D)** Correlation between the change of BFM and the fitness/Ω score at T1. **E)** Correlation between the change of ECW and the *Line-1* methylation at T1. Statistical significance was defined as a p-value below 0.05.

Results of epigenetic markers, nutrition, and lifestyle

In the intervention group, we were able to determine a significant correlation between the intake of B vitamins (B vitamin complex, cobalamin, and folic acid), miR-19b-3p (p, 0.033), and miR-101-3p (p, 0.047). Participants who supplemented with B vitamins had a higher expression of these miRNAs compared to participants who did not supplement.

A similar correlation could be established between the intake of magnesium and miR-19b-3p (p, 0.052). Furthermore, we were able to observe an association between miR-378a-3p and iron supplementation (p, 0.017). The participants who supplemented iron had a significantly higher expression of miR-378a-3p. In addition, the participants who had a higher fluid intake (2–3 liters a day) showed an upregulation of miR-378a-3p (p, 0.003). Furthermore, the participants who reported consuming whole grain products daily or several times a day showed a higher expression level of miR-101-3p (p, 0.002) than those who consumed less. Whereas miR-146a-5p correlated with stress (p, 0.029). The participants who reported having an increased stress level had a significantly lower expression of miR-146a-5p.

Discussion

The main aim of this study was to measure training effects via miRNAs from dried blood spots (DBS) and to determine how miRNAs are regulated by medium-term exercise (strength and endurance training), according to Ashton et al. 2020 [23], in sedentary, healthy individuals. But also, what role genetic predisposition or nutrition plays, and the possible interactions with the selected miRNAs, as well as the development of a biomarker algorithm that provides information about the performance, health, and recovery of an athlete.

As far as we know, we are among the first to describe a so-called miRNA-based "fitness score" biomarker in capillary blood, which can describe a person's current fitness level regardless of age, sex, and genetic predisposition. And even if some of the miRNAs analyzed in this study have already been described in combination with exercise, and could be used as biomarkers, such as miR-20a and −146a for cardiorespiratory fitness and peak exercise capacity [24], a combination of several miRNAs promises a more precise assessment of health and training levels. Also, with the current commonly used biomarkers, it was found that a single maker alone is too meaningless and that a combined analysis of several parameters provides much more accurate information and thus helps athletes or coaches to optimize training and prevent overtraining [16]. Furthermore, using a miRNA-based biomarker algorithm would have the further advantage that the miRNA expression patterns change much earlier than markers from proteins and/or electrolytes [17]. For this purpose, it should also be considered which information our miRNAs provide, which sport-relevant physiological functions they may reflect or have and where they come from.

It is known that exercise training leads to an increased rate of red blood cell (RBC) breakdown due to oxidative stress. However, as a result, exercise training stimulates erythropoiesis and increases RBC mass, the number of leukocytes, hematocrit values, and hemoglobin concentrations, as well as the plasma volume. And, an increased rate of RBC turnover can be beneficial as young cells are more efficient at transporting oxygen [25–27]. Moreover, it was found that RBCs are the leading cause of miRNA expression in whole blood and that the amount of erythrocyte-derived miRNAs reflects the majority of miRNAs expressed in the whole blood. It is described that these RBC-derived miRNAs show high-expression patterns, and some are delivered to recipient cells via extracellular erythrocyte vesicles and are thus involved in the regulation of gene expression. However, the amount of the endogenous controls

(miR-24-3p and −93-5p) in our DBS samples did not change during the intervention, and we could not find any indications in the literature that they are involved in the regulation of erythropoiesis in healthy humans. Moreover, there are other important cell types in the whole blood, such as leukocytes and platelets, which contain functional miRNAs [28].

As already indicated, exercise also affects leukocytosis and increases the number of leukocytes and neutrophils. It is believed that these changes in the hemodynamics of blood cells take place in the circulatory system and may not weaken the immune system but instead strengthen it due to the increased number of immune cells. Although exercise can resemble acute inflammation by recruiting leukocytes, this could be beneficial as most cells are recruited into the bloodstream rather than into inflamed tissues, as in infection [29]. The analyzed miRNAs may come from leukocytes or neutrophils, since exercise can also alter the miRNA expression patterns in neutrophils in the circulating blood and thus possibly influence the function of neutrophils [30]. Furthermore, it was shown that miR-20a and −22 were downregulated in neutrophils after endurance training, while miR-505 was upregulated, and miR-30e in natural killer cells and monocytes. Some of the neutrophil-derived miRNAs, including miR-20a, are also known to regulate genes involved in immune processes and apoptosis, such as Jak-STAT signaling pathways [15,30–32]. Also, miR-146a plays an essential role in the inflammatory signaling in several cell types, and it was concluded that changes in the circulating miR-146a could reflect the overall inflammatory state that is seen in athletes who engage in prolonged aerobic exercise [33]. Moreover, this miRNA also correlates with the inflammation marker high-sensitivity C-reactive protein [34]. In summary, this suggests that some of our miRNAs play a role in the immune response that is triggered by exercise.

However, we were able to determine differences in the expression of miR-20a-5p, −22-5p, and −101-3p depending on the *α-actinin-3* gene (*ACTN3)*, often also referred to as the "speed gene". Subjects with a CC (or RR) genotype had a higher expression of miR-20a-5p and −22-5p after 12 weeks than those with a TC (or XR) genotype, while participants with a TT (or XX) genotype had a higher expression of miR-101-3p than those with a TC or CC genotype. The protein ACTN3 is only expressed in the fast type II fibers and is related to the generation of fast and powerful muscle movements. It also appears to play a role in adaption and recovery after exercise and in the risk of injury. In contrast to individuals with TC or CC, those with homozygous TT cannot express ACTN3, which can lead to impaired muscle performance and make them predisposed to muscle damage and ligament injuries during exercise [7]. In animal studies, but also in some human studies, especially in women, this phenotype has been also associated with increased endurance capacity [35–37]. Our results indicate that there may be an association between the expression of miR-20a-5p, −22-5p, and −101-3p, the genotype of *ACTN3*, and the ability to perform fast and explosive movements and/or the injury risk. Additionally, we were able to observe a correlation between miR-22-5p, −101-3p and the increase in lean body mass, which further confirms a possible connection to jumping power or explosive movements. It is also discussed that these miRNAs are involved in cardiac hypertrophy triggered by endurance exercise, and we suspected that they may also have pro-/anti-hypertrophic effects in other tissues [38]. Furthermore, for participants with

the *FTO* risk allele (AA), the expression of miR-22-5p was downregulated. One known target of miR-22 is the *histone deacetylase 4* (*HDAC4*) [39], supporting the theory that metabolic phenotypes are probably mediated by epigenetic mechanisms due to the different *FTO* mutations [40]. These results highlight the importance of personalized analysis and interventions, as the epigenetic phenotype varies significantly depending on the genotype.

Anyway, it is known that exercise-induced muscle injuries and damage often occur after unusual or intense training, especially if the training involves many eccentric contractions [41,42]. In response to damage, activated satellite cells begin to multiply and undergo myogenic differentiation as part of the muscle regeneration process. The proliferation and differentiation of satellite cells are associated with changes in miRNA expression, which leads to an altered expression of target genes that are important for muscle regeneration. These muscle-specific miRNAs also include miR-378a-3p, which has been identified as a promising marker for acute muscle damage [43,44]. Furthermore, miR-378a-3p is a positive regulator of osteogenesis and is therefore involved in bone formation and resorption, making miR-378a-3p a marker of bone health and injury/bone fracture risk [45,46]. Other miRNAs are also involved in the regulation of bone remodeling and regeneration, such as miR-146a-5p, which, in contrast to miR-378a-3p, suppresses osteoblastogenesis [47], and miR-30e, which inhibits osteoblast differentiation, whereby its expression level maintains the balance between osteoblast and adipocyte differentiation [48]. Bone marrow adipocytes influence bone remodeling and play a role in bone loss disorders [49]. However, exercise can suppress bone marrow adipocyte expansion [50], maybe through miR-30e.

Besides these findings, in our study, we were also able to observe a correlation between the self-reported supplementation of B vitamins, which include thiamine, riboflavin, niacin, pantothenic acid, pyridoxine, biotin, and folic acid or folate, and miR-19b-3p expression. An intake of B vitamins leads to an upregulation of miR-19b-3p. Furthermore, we could also observe that the participants who supplemented magnesium had a higher expression of miR-19b-3p, and those who consumed iron supplements had a higher expression of miR-378a-3p. Very little evidence of such correlations can be found in the literature. But it is known that micronutrients are essential for maintaining the health of physically active people and that especially B vitamins are essential for energy production, synthesis of new cells, and repair of damaged cells. Whereas others are necessary for maintaining immune function, bone health, and protecting against oxidative damage, but also for building and repairing muscle tissue. Both biochemical adaptions to exercise and the metabolic pathways for energy production can increase the need for certain micronutrients during regular physical activity, and athletes with a poor B vitamin status may have a decreased ability to exercise at a high-intensity level [51–53]. Furthermore, it is important particularly for athletes to maintain an optimal micronutrient status and, if necessary, to recognize a deficiency at an early stage before physical signs appear. Additionally, the miR-378a-3p expression correlated with the amount of average fluid intake. During exercise, especially at high temperatures, sweating and inadequate hydration can lead to dehydration [16]. And there are indications that moderate hypohydration, even in short phases, can lead to disturbance of cognitive functions, a reduction in the ability to concentrate as well as headaches or fatigue, and thus impair the performance of

an athlete [54]. In summary, the correlations between micronutrients, fluid intake, and miRNAs observed in our study indicate that some miRNAs are suitable for reflecting the current micronutrient and hydration status of an athlete, as predictive biomarkers.

The results of our study reported here should be considered in light of some limitations. First, the effects of concurrent strength and endurance exercise were examined since the general population tends to engage in both types of exercise. However, future studies should also consider the individual training methods alone, with each person completing three intervention cycles, strength, endurance, and concurrent. Second, the study focused on a wide age range. The main goal compared to most previous studies was to develop a minimally invasive method with biomarkers that are suitable for the general population, not just for young people or elite athletes. Although we could exclude age as a confounder, this should be considered in future studies. Nevertheless, our minimally invasive method offers the advantage that anyone, amateurs, and elite athletes, can easily take samples anywhere and at any time to monitor their molecular response to exercise.

Conclusions

In conclusion, our "fitness score" not only reflects the effect of the intervention, moreover, but the individual miRNAs also provide information to assess the health, performance, and recovery of an athlete/person. Based on our findings, correlations between miRNAs, SNPs, body composition, diet, and the literature, we have divided the miRNAs, according to the work by Lee et al., 2017 [16], into the following subgroups (Figure 5), nutrition, inflammation, cardiovascular fitness, injury risk, regeneration, muscle- and hydration status, as well as stress level. As a result, our biomarker enables easier training tracking, as samples can be taken anytime and anywhere (DBS), and athletes and coaches can make possible adjustments at an early stage to optimize performance or prevent deficits.

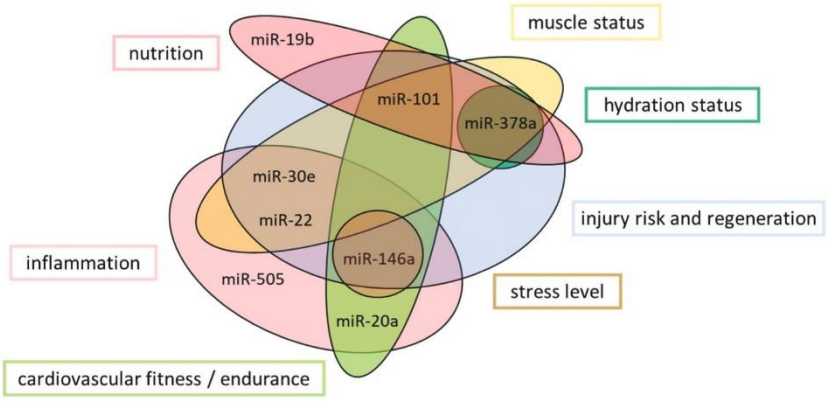

Figure 5. The properties of the measured miRNAs and their importance and classification as sports-relevant biomarkers.

Abbreviations

ACE, Angiotensin-converting enzyme, ACTN3, Alpha-actin-3, AUC, Area under the curve, BCM, Body cell mass, BFM, Body fat mass, BIA, Bioelectrical impedance analysis, BMI, Body mass index, DBS, Dried blood spot, ECW, Extracellular water, FFQ, Food frequency questionnaire, FTO, Fat mass and obesity-associated gene, HRM, High-resolution melting, ICW, Intracellular water, LBM, Lean body mass, miRNA, microRNA, MREs, miRNA response elements, qPCR, Real-time polymerase chain reaction, RBC, Red blood cell, SNP, Single nucleotide polymorphism

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Ethics approval and consent to participate

No ethics approval was required for this minimally invasive intervention study. Written consent was obtained from the participants for the publication of this study.

Consent for publication

All authors have consented to the contents of this paper and the submission guidelines of the Journal of the International Society of Sports Nutrition.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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References

[1] Mikkelsen K, Stojanovska L, Polenakovic M, et al. Exercise and mental health. Maturitas. 2017;106:48–56.
[2] Radak Z, Torma F, Berkes I, et al. Exercise effects on physiological function during aging. Free Radic Biol Med. 2019;132:33–41.

[3] Distefano G, Goodpaster BH. Effects of exercise and aging on skeletal muscle. Cold Spring Harb Perspect Med. 2018;8(3):a029785.
[4] Ling C, Rönn T. Epigenetic adaptation to regular exercise in humans. Drug Discov Today. 2014;19(7):1015–1018.
[5] Kelly SA, Pomp D. Genetic determinants of voluntary exercise. Trends Genet. 2013;29 (6):348–357.
[6] Herring MP, Sailors MH, Bray MS. Genetic factors in exercise adoption, adherence and obesity. Obes Rev. 2014;15(1):29–39.
[7] Del Coso J, Hiam D, Houweling P, et al. More than a 'speed gene', ACTN3 R577X genotype, trainability, muscle damage, and the risk for injuries. Eur J Appl Physiol. 2019;119(1):49–60.
[8] Heffernan SM, Stebbings GK, Kilduff LP, et al. Fat mass and obesity associated (FTO) gene influences skeletal muscle phenotypes in non-resistance trained males and elite rugby playing position. BMC Genet. 2017;18(1):1–9.
[9] Almén MS, Jacobsson JA, Moschonis G, et al. Genome wide analysis reveals association of a FTO gene variant with epigenetic changes. Genomics. 2012;99(3):132–137.
[10] Grazioli E, Dimauro I, Mercatelli N, et al. Physical activity in the prevention of human diseases, role of epigenetic modifications. BMC Genomics. 2017;18(S8). DOI:10.1186/s12864-017-4193-5.
[11] Voisin S, Eynon N, Yan X, et al. Exercise training and DNA methylation in humans. Acta Physiol. 2015;213(1):39–59.
[12] McGee SL, Hargreaves M. Epigenetics and exercise. Trends Endocrinol Metab. 2019;30 (9):636–645.
[13] Kern F, Ludwig N, Backes C, et al. Systematic assessment of blood-borne MicroRNAs highlights molecular profiles of endurance sport and carbohydrate uptake. Cells. 2019;8(9):1045.
[14] Wang H, Liang Y, Li Y. Non-coding RNAs in exercise. Non-coding RNA Investig. 2017;1:10.
[15] Silva GJJ, Bye A, El Azzouzi H, et al. MicroRNAs as important regulators of exercise adaptation. Prog Cardiovasc Dis. 2017;60(1):130–151.
[16] Lee EC, Fragala MS, Kavouras SA, et al. Biomarkers in sports and exercise, tracking health, performance, and recovery in athletes. J Strength Cond Res. 2017;31(10):2920.
[17] Moldovan L, Batte KE, Trgovcich J, et al. Methodological challenges in utilizing mi RNA s as circulating biomarkers. J Cell Mol Med. 2014;18(3):371–390.
[18] Simmonds MJ, Baskurt OK, Meiselman HJ, et al. A comparison of capillary and venous blood sampling methods for the use in haemorheology studies. Clin Hemorheol Microcirc. 2011;47 (2):111–119.
[19] Von Hurst PR, Walsh DCI, Conlon CA, et al. Validity and reliability of bioelectrical impedance analysis to estimate body fat percentage against air displacement plethysmography and dual-energy X-ray absorptiometry. Nutr Diet. 2016;73(2):197–204.
[20] Marques-Rocha JL, Milagro FI, Mansego ML, et al. LINE-1 methylation is positively associated with healthier lifestyle but inversely related to body fat mass in healthy young individuals. Epigenetics. 2016;11(1):49–60.
[21] Abe T, DeHoyos D V, Pollock ML, et al. Time course for strength and muscle thickness changes following upper and lower body resistance training in men and women. Eur J Appl Physiol Occup Physiol. 2000;81(3):174–180.
[22] Cohen JD, Li L, Wang Y, et al. Detection and localization of surgically resectable cancers with a multi-analyte blood test. Science. 2018;359(6378):926–930.
[23] Ashton RE, Tew GA, Aning JJ, et al. Effects of short-term, medium-term and long-term resistance exercise training on cardiometabolic health outcomes in adults, systematic review with meta-analysis. Br. J. Sports Med. 2020;54(6):341–348.
[24] Baggish AL, Hale A, Weiner RB, et al. Dynamic regulation of circulating microRNA during acute exhaustive exercise and sustained aerobic exercise training. J Physiol. 2011;589 (16):3983–3994.
[25] Smith JA. Exercise, training and red blood cell turnover. Sport Med. 1995;19(1):9–31.

[26] Hu M, Lin W. Effects of exercise training on red blood cell production, Implications for anemia. Acta Haematol. 2012;127(3):156–164.
[27] Belviranli M, Okudan N, Kabak B. The effects of acute high-intensity interval training on hematological parameters in sedentary subjects. Med. Sci. 2017;5:15.
[28] Sun L, Yu Y, Niu B, et al. Red blood cells as potential repositories of MicroRNAs in the circulatory system. Front Genet. 2020;11:1–8.
[29] Sand KL. Effects of exercise on leukocytosis and blood hemostasis in 800 healthy young females and males. World J Exp Med. 2013;3(1):11.
[30] Radom-Aizik S, Zaldivar F, Oliver S, et al. Evidence for microRNA involvement in exercise-associated neutrophil gene expression changes. J Appl Physiol. 2010;109 (1):252–261.
[31] Radom-Aizik S, Zaldivar F, Haddad F, et al. Impact of brief exercise on peripheral blood NK cell gene and microRNA expression in young adults. J Appl Physiol. 2013;114(5):628–636.
[32] Radom-Aizik S, Zaldivar FP, Haddad F, et al. Impact of brief exercise on circulating monocyte gene and microRNA expression, implications for atherosclerotic vascular disease. Brain Behav Immun. 2014;39:121–129.
[33] Baggish AL, Park J, Min PK, et al. Rapid upregulation and clearance of distinct circulating microRNAs after prolonged aerobic exercise. J Appl Physiol. 2014;116(5):522–531.
[34] Li Y, Yao M, Zhou Q, et al. Dynamic regulation of circulating microRNAs during acute exercise and long-term exercise training in basketball athletes. Front Physiol. 2018;9:1–11.
[35] Roth SM, Walsh S, Liu D, et al. The ACTN3 R577X nonsense allele is under-represented in elite-level strength athletes. Eur J Hum Genet. 2008;16(3):391–394.
[36] Yang N, MacArthur DG, Gulbin JP, et al. ACTN3 genotype is associated with human elite athletic performance. Am J Hum Genet. 2003;73(3):627–631.
[37] Yang R, Shen X, Wang Y, et al. ACTN3 R577X gene variant is associated with muscle-related phenotypes in elite Chinese sprint/power athletes. J Strength Cond Res. 2017;31 (4):1107–1115.
[38] Soplinska A, Zareba L, Wicik Z, et al. MicroRNAs as biomarkers of systemic changes in response to endurance exercise⇔a comprehensive review. Diagnostics. 2020;10(10):1–17.
[39] Lu W, You R, Yuan X, et al. The microRNA miR-22 inhibits the histone deacetylase HDAC4 to promote TH17 cell-dependent emphysema. Nat Immunol. 2015;16(11):1185–1194.
[40] Franzago M, Fraticelli F, Marchioni M, et al. Fat mass and obesity-associated (FTO) gene epigenetic modifications in gestational diabetes, new insights and possible pathophysiological connections. Acta Diabetol. 2021;58(8):997–1007.
[41] Clarkson PM, Hubal MJ. Exercise-induced muscle damage in humans. Int. J. Sports Med. 2002;15:132–135.
[42] Siracusa J, Koulmann N, Sourdrille A, et al. Phenotype-specific response of circulating miRNAs provides new biomarkers of slow or fast muscle damage. Front Physiol. 2018;9:1–9.
[43] Kirby TJ, McCarthy JJ. MicroRNAs in skeletal muscle biology and exercise adaptation. Free Radic Biol Med. 2013;64:95–105.
[44] Siracusa J, Koulmann N, Bourdon S, et al. Circulating miRNAs as Biomarkers of Acute Muscle Damage in Rats. Am J Pathol. 2016;186:1313–1327.
[45] Kang M, Huang CC, Lu Y, et al. Bone regeneration is mediated by macrophage extracellular vesicles. Bone. 2020;141:115627.
[46] Amir LR, Everts V, Bronckers ALJJ. Bone regeneration during distraction osteogenesis. Odontology. 2009;97(2):63–75.
[47] Chang CC, Venø MT, Chen L, et al. Global MicroRNA profiling in human bone marrow skeletal —stromal or mesenchymal–stem cells identified candidates for bone regeneration. Mol Ther. 2018;26(2):593–605.
[48] Suttamanatwong S. MicroRNAs in bone development and their diagnostic and therapeutic potentials in osteoporosis. Connect. Tissue Res. 2017;58(1):90–102
[49] Muruganandan S, Govindarajan R, Sinal CJ. Bone marrow adipose tissue and skeletal health. Curr. Osteoporos. Rep. 2018;16(4):434–442

[50] Styner M, Pagnotti GM, McGrath C, et al. Exercise decreases marrow adipose tissue through ß-oxidation in obese running mice. J Bone Miner Res. 2017;32(8):1692–1702.
[51] Woolf K, Manore MM. B-vitamins and exercise, does exercise alter requirements? Int. J. Sport Nutr. Exerc. Metab. 2006;16(5):453–484
[52] Laires MJ, Monteiro C. Exercise, magnesium and immune function. Magnes Res. 2008;21 (2):92–96.
[53] Beard J, Tobin B. Iron status and exercise. Am J Clin Nutr. 2000;72(2):594–597.
[54] Maughan RJ. Impact of mild dehydration on wellness and on exercise performance. Eur J Clin Nutr. 2003;57(S2):S19–S23.
[55] Krammer UDB, Sommer A, Tschida S, Mayer A, Lilja SV, Switzeny OJ, Hippe B, Rust P and Haslberger A G. (2022). PGC-1α Methylation, miR-23a, and miR-30e Expression as Biomarkers for Exercise- and Diet-Induced Mitochondrial Biogenesis in Capillary Blood from Healthy Individuals: A Single-Arm Intervention. Sports, 10(5), 73 10.3390/sports10050073

8.1.3. Original manuscript 1

MiR-10a, miR-15a, let-7a, and let-7g expression as stress-relevant biomarkers to assess acute or chronic psychological stress, stress-related diseases, and mental health in human capillary blood

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Abstract

Background: Psychological stress, as an important cofactor in the development of many acute and chronic diseases, is crucial for general health or well-being, and improved markers are needed to distinguish situations of progressive pathological development, such as depression, anxiety, or burnout, to be recognized at an early stage. Epigenetic biomarkers play an important role in the early detection and treatment of complex diseases such as cancer, and metabolic or mental disorders. Therefore, this study aimed to identify so-called miRNAs, which would be suitable as stress-related biomarkers.

Methods and Results: In this study, 173 participants (36.4% males, and 63.6% females) were interviewed about stress, stress-related diseases, lifestyle, and diet to assess their acute and chronic psychological stress status. Using qPCR analysis, 13 different miRNAs were analyzed in dried capillary blood samples. Four miRNAs were identified, miR-10a-5p, miR-15a-5p, let-7a-5p, and let-7g-5p ($p < 0.05$), which could be used as possible candidates for measuring pathological forms of acute or chronic stress. Let-7a-5p, let-7g-5p, and miR-15a-5p ($p < 0.05$) were also significantly higher in subjects with at least one stress-related disease. Further, correlations were identified between let-7a-5p and meat consumption ($p < 0.05$) and between miR-15a-5p and coffee consumption ($p < 0.05$).

Conclusion: The examination of these four miRNAs as biomarkers using a minimally invasive method offers the possibility of detecting health problems at an early stage and counteracting them to maintain general and mental health.

Keywords: psychological distress, mental health, biomarkers, epigenetic, miRNAs

Introduction

Acute and chronic psychological stress leads to complex changes in physiological systems that, besides behavior, also affects inflammatory, cellular, and metabolic processes in the human body. It has been shown that excessive prolonged exposure to stressors plays a critical role in the development and maintenance of many diseases (e.g., cardiovascular or metabolic diseases) and mental or neurodegenerative disorders such as depression, anxiety, or chronic fatigue syndrome (CFS) (Kemeny, 2003; Liu et al., 2017).

Stress is a state of threatened homeostasis that is caused by internal or external adverse forces, so-called stressors (e.g., psychological, environmental, or physiological factors). Stressful events result in multiple neurochemical, neurotransmitter, and hormonal changes by primarily activating the sympathetic nervous system (SNS) and hypothalamic-pituitary-adrenal (HPA) axis (Chrousos, 2009; Liu et al., 2017). In reaction to stress, there is an increased release of hormones such as glucocorticoids, catecholamines, growth hormones, and prolactin, mobilizing energy sources and adapting the individual to his new circumstances. The most important targets of these hormones include the cognitive, reward, and fear system, as well as the gastrointestinal, cardiovascular, metabolic, and immune systems. Insufficient functionality of this stress system, dysregulation, or chronic activity, can harm the organism, affecting development, growth, and body composition, and lead to a variety of behavioral and somatic pathological conditions (Chrousos, 2009; Ranabir and Reetu, 2011).

In addition to these inflammatory and metabolic processes, mechanisms are activated in response to stress at the cellular level, which leads to the repair or elimination of stress-damaged macromolecules such as proteins, lipids but also DNA, and RNA, and these can trigger apoptosis (Olejniczak et al., 2018). Stress reactions are also epigenetically regulated and thus affect gene expression and post-transcriptional mechanisms. Besides, an altered stress-induced gene expression can lead to premature aging, inflammation, oxidative damage to various organs, or, in the long term, to burnout, depression, or CFS (Park et al., 2019; Wiegand et al., 2017). Furthermore, there is growing evidence that non-coding RNAs, especially miRNAs, play an important role in physiological and psychological stress responses. These short RNAs with a length of 22-23 nucleotides control the expression of many protein-coding genes, which are responsible for cellular processes such as cell growth, proliferation, differentiation, and apoptosis. MiRNAs can affect multiple targets by binding to messenger RNAs (mRNAs) and inducing their cleavage, degradation, or translation repression (Olejniczak et al., 2018; Wiegand et al., 2017). MiRNAs can be identified in several body fluids and can provide information about various processes throughout the body, because miRNAs are mobile and can travel from one tissue to another, e.g., via extracellular vesicles (Mori et al., 2019). This is one of the main reasons that miRNAs could make ideal

candidates for biomarkers in a variety of diseases. Compared to traditional biomarkers, e.g., proteins, miRNAs would have the advantage that the development of new assays takes less time and is less expensive than the generation of new antibodies for protein markers. They can be easily extracted from blood, urine, and other body fluids, and can be used as multimarker panels, improving the reliability of a diagnosis, while testing many proteins is more expensive and time-consuming (Condrat et al., 2020).

Since stress is an important cofactor in the development and maintenance of many acute and chronic diseases, stress is of high epidemiological importance. It is of relevance to health economics and should be recognized at an early stage before health consequences occur. Since miRNAs are involved in the epigenetic regulation of stress reactions, they can be used as biomarkers in the diagnosis, prevention, and therapy evaluation of stress-associated diseases and mental health issues (Wiegand et al., 2017). The main aim of this study was to identify miRNAs in dried capillary blood spot (DBS) samples that are associated with stress-related diseases or the stress of a person and could be used as minimally invasive biomarkers. We also examined the role of diet and other lifestyle factors, such as exercise, as it is known that mood or mental health is closely related to diet (Begdache et al., 2019).

Materials and Methods

Study design

For this observational study, we interviewed 173 individuals, 36.4% males, and 63.6% females, about their lifestyle and eating habits, with a special focus on their stress levels and stress-related diseases (SRD) or symptoms, such as chronic fatigue syndrome (CFS), sleep disorders, mood swings, headaches/migraines (H/M), depression, and anxiety. From all study participants, capillary blood (DBS) and a standardized questionnaire were collected. The conduct of the study was approved by the Vienna Ethics Committee (MA 15 – EK/22-091-VK_NZ) and all participants gave their written consent following the Declaration of Helsinki to the use of their data. The information provided by the study participants was treated confidentially throughout the process and the general data protection regulations were complied with. **Table 1** gives an overview of all participants and their stress levels or stress-related diseases.

58 **Table 1**. Overview of the participants including their self-reported current stress levels and stress-related
59 symptoms or diseases (SRD). Participants could indicate several SRDs and a total of 83 reported having one or
60 more SRDs. Age and BMI data were given as mean ± standard deviation (SD).

	Male	**Female**	**Total**
n	63	110	173
Age [years]	48.30 ± 13.08	50.47 ± 15.11	49.68 ± 14.41
Age range [years]	22 - 77	18 - 84	18 - 84
BMI [kg/m^2]	27.18 ± 4.74	24.53 ± 5.10	25.43 ± 5.12
BMI range [kg/m^2]	20.45 - 46.75	17.26 - 43.51	17.26 - 46.75
Stress level distribution:			
Low % (*n*)	25.4% (16)	26.4% (29)	26.0% (45)
Moderate % (*n*)	15.9% (10)	7.3% (8)	10.4% (18)
High % (*n*)	42.9% (27)	44.5% (49)	43.9% (76)
Very high % (*n*)	15.9% (10)	21.8% (24)	19.7% (34)
Stress-related diseases (SRD):			
Depression % (*n*)	9.5% (6)	9.1% (10)	9.2% (16)
Anxiety % (*n*)	23.8% (15)	26.4% (29)	25.4% (44)
Chronic fatigue syndrome (CFS) % (*n*)	30.2% (19)	26.4% (29)	27.7% (48)
Mood swings % (*n*)	3.2% (2)	3.6% (4)	3.5% (6)
Headaches or migraines % (*n*)	3.2% (2)	9.1% (10)	6.9% (12)
Sleep disorder % (*n*)	1.6% (1)	3.6% (4)	2.9% (5)
Total participants with at least one SRD % (*n*)	46.0% (29)	49.1% (54)	48.0% (83)

61 ***Sample collection and RNA-extraction***

62 Capillary blood was collected on *Whatman® protein saver cards* (Sigma-Aldrich, Vienna, Austria) using *Safety*
63 *Lancet Extra 18G* (Sarstedt, Nümbrecht, Germany), dried, and stored at room temperature. For the RNA
64 extraction, the *MagMAX™ FFPE DNA/RNA Ultra Kit* (ThermoFisher Scientific, Waltham, MA, USA) was used.
65 Four 4 mm points were punched out of each card, which were then incubated in protease solution at 55°C
66 overnight. The further extraction steps were carried out according to the manufacturer's protocol. The RNA
67 samples were stored at -20°C.

cDNA synthesis and quantitative real-time quantitative PCR (qPCR)

TaqMan™ Advanced microRNA cDNA Synthesis Kit and *TaqMan™ Advanced microRNA assays* were used under the default settings on *QuantStudio™ 3* from ThermoFisher Scientific, Waltham, MA, USA. The cDNA samples were stored at -20°C. The following 13 miRNAs were analyzed: miR-10a-5p, miR-15a-5p, miR-16-5p, miR-19b-3p, miR-26b-5p, miR-29c-3p, miR-106b-5p, miR-126-3p, miR-142-3p, let-7a-5p, let-7g-5p, miR-21-5p and miR-877-5p, as well as two endogenous controls (miR-24-3p and miR-93-5p). ΔCt was used to evaluate the expression pattern and calculated as follows $\Delta Ct = Ct^{target} - Ct^{endogenous\ control}$ (Krammer et al., 2022).

Statistical analysis

The programs *IBM SPSS statistics 20* and *GraphPad Prism 6* were used for statistical analysis. All data are represented as mean ± standard deviation (SD). To test whether there is a difference between stress and no stress or between SRDs and no SRDs the independent t-test was used, ANCOVA to account for covariates for parametric and the Quade's ANCOVA and Mann-Whitney-U test for nonparametric values. Correlations between nutritional or lifestyle habits and miRNA expressions were tested using Pearson's or Spearman's Rho correlation and Kendall's Tau. A p-value ≤ 0.05 was assumed to be significant for all tests.

Results

Evaluation of stress-related miRNA expression profiles

To identify stress-related miRNA expression profiles, we divided the participants into two groups. All participants reporting high or very high-stress levels were assigned to the "stress group" (n = 110) and all participants reporting low or moderate stress levels and no SRDs were assigned to the "control group" (n = 47; **Table 2**). Participants who did not indicate their stress level or reported that they had low-stress levels but had an SRD were not assigned to either group (n = 16).

Table 2. Overview of the participants of the stress and control group. Age and BMI data were given as mean ± standard deviation (SD).

	Stress group	**Control group**
n (%)	110 (63.6%)	47 (27.2%)
Male *n* (%)	37 (33.6%)	20 (42.6%)
Female *n* (%)	73 (66.4%)	27 (57.4%)
Age [years]	47.81	53.62
Age range [years]	18 - 84	19 - 79
BMI [kg/m^2]	24.52	25.20
BMI range [kg/m^2]	17.26 - 37.13	18.82 - 39.26

Four of the analyzed miRNAs were expressed significantly differently between the two groups (independent t-test, **Table 3**). Stress group participants had a significantly higher expression of miR-15a, let-7a, let-7g, and miR-877 than those in the control group (**Fig 1**).

Table 3. Overview of the results of stress-related miRNA expression profiling. Expression data were presented as mean ± standard deviation (SD).

Marker	**independent t-test** *p*-value	**ANCOVA** *p*-value (covariate = age)	**Hedges' *g***	**Stress group** *n* = 110 mean ΔCt ± SD	**Control group** *n* = 47 mean ΔCt ± SD
miR-10a-5p	0.067	0.048*	0.406	11.417 ± 1.031	11.840 ± 1.081
miR-15a-5p	0.025*	0.028*	0.396	-0.078 ± 0.754	0.205 ± 0.609
let-7a-5p	0.000*	0.000*	1.073	1.348 ± 0.599	2.038 ± 0.734
let-7g-5p	0.000*	0.003*	0.626	3.459 ± 0.753	3.891 ± 0.518
miR-877-5p	0.018*	0.079	0.343	9.619 ± 1.278	10.008 ± 0.704

*Significant at $p \leq 0.05$

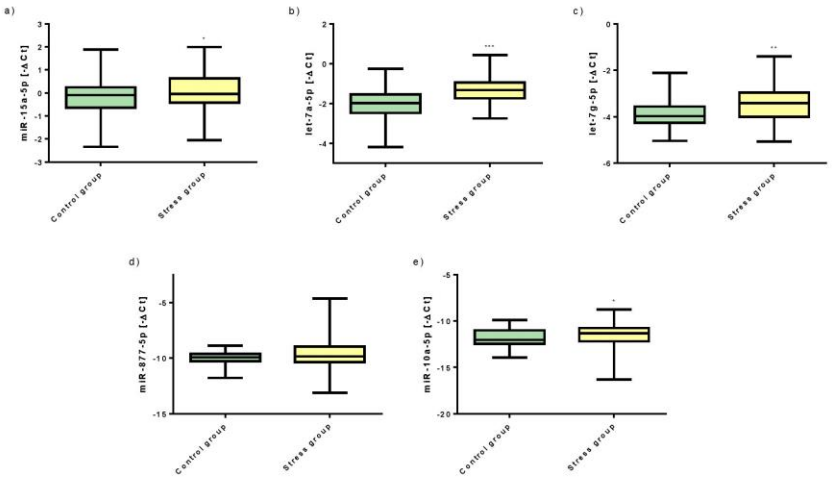

Fig 1. Stress-related miRNA expression profiles. (**a-e**) Boxplots of miRNA expression for **a**) miR-15a-5p, **b**) let-7a-5p, **c**) let-7g-5p, **d**) miR-877-5p, and **e**) miR-10a-5p. The results are expressed as mean ± standard deviation (SD). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ (ANCOVA).

However, since the two groups differ in terms of age (p = 0.021), we additionally analyzed the measured miRNAs using ANCOVA and age as covariates. The ANCOVA showed significant differences between the groups in the expression of miR-10a, miR-15a, let-7a, and let-7g (**Table 3** and **Fig 1**).

MiRNAs associated with stress-related diseases (SRDs)

83 participants reported having one or more SRDs (**Table 1**). Participants who reported suffering from depression had significantly higher let-7a expression levels compared to the control group (**Fig 2a**). Participants reporting anxiety had significantly higher expression levels of let-7a (**Fig 2a**) and let-7g (**Fig 2b**). Participants who stated suffering from chronic fatigue syndrome (CFS) and participants with headaches/migraines (H/M) also had higher expression levels of let-7a (**Fig 2a**), let-7g (**Fig 2b**), and miR-15a (**Fig 2c**). The mean miRNA expressions of the respective SRDs can be found in the supplementary material **Table S1**.

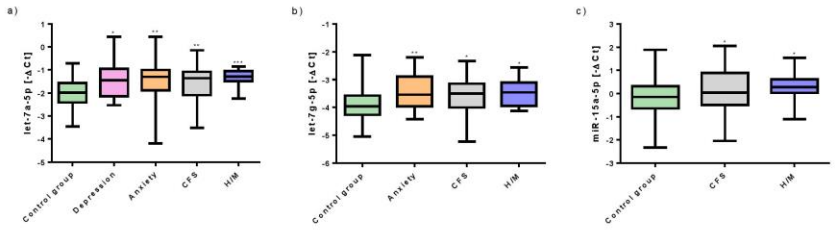

Fig 2. Specific miRNA expression levels for stress-related diseases (SRDs). (**a-c**) Boxplots of miRNA expression for **a**) let-7a-5p, **b**) let-7g-5p, and **c**) miR-15a-5p. The results are expressed as mean ± standard deviation (SD). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ (independent t-test or ANCOVA).

Correlations between miRNAs and nutritional or lifestyle habits

We discovered a negative correlation between the number of SRDs mentioned by a participant and the BMI (one n = 50, mean BMI = 26.82 kg/m^2; two n = 20, mean BMI = 23.95 kg/m^2; ≥ three n = 13, mean BMI = 22.38 kg/m^2; p = 0.024). The BMI reduced significantly the more SRDs a participant reported. Additionally, an association between meat consumption and let-7a was observed. The more meat consumed per week, the higher the expression of let-7a (rarely/never n = 43, mean ΔCt = 1.822; 1-6 portions/week n = 114, mean ΔCt = 1.524; ≥ everyday n = 7, mean ΔCt = 1.484; p = 0.031). Furthermore, participants who reported that they regularly consumed coffee (≥ 1 cup/day, n = 137, mean ΔCt = 0.027) had a lower expression of miR-15a than those who stated that they never consume coffee (n = 33, mean ΔCt = -0.257; p = 0.028).

Discussion

The identification of new biomarkers to assess acute or chronic psychological stress, vulnerability, and stress-related diseases (SRDs) could not only provide helpful information for the prevention and treatment of stress-related disorders but also support overall health or well-being and healthy aging. Therefore, this study aimed to determine miRNAs that are suitable for assessing personal stress levels and detecting stress-related consequences before physical impairments occur. We were able to identify four possible candidates, namely miR-10a, miR-15a, let-7a, and let-7g. These miRNAs showed higher expression levels in stressed individuals than in non-stressed, and three (let-7a, let-7g, and miR-15a) of them correlated with SRDs, indicating long-term consequences of chronic stress.

Many miRNAs are discussed in the context of psychological stress, anxiety, depression, and other SRDs (Wiegand et al., 2017). It is hypothesized that changes in miRNA expression induced by environmental stressors

result in altered neuronal morphology and problems with neuronal circuitry leading to relevant health problems. An increase of let-7a expression in the brain and serum of mice could be observed after exposure to acute obsessive-compulsive stress, which only returned to its initial level five days later (Sung et al., 2021; Wiegand et al., 2017). Upregulation of this miRNA could also be measured in rats after an acute social defeat (Chen et al., 2015). These results and our findings in human capillary blood support the hypothesis that let-7a would be suitable as a biomarker to detect acute stress. Furthermore, it was discovered that several members of the let-7 family, including let-7a, are potent activators of toll-like receptors (TLRs) signaling in microglia and neurons, and that TLR activation induces dose- and time-dependent neuronal cell death, which is associated with neurodegeneration and pathogenesis of neurodegenerative diseases (Hollins and Cairns, 2016).

MiR-10a is also considered a possible biomarker for acute psychological stress (Wiegand et al., 2017). In line with our observations, acute stress induction resulted in the upregulation of miR-10a in whole blood and appears to correlate with stress-induced alcohol consumption (Beech et al., 2014). Additionally, overexpression of miR-10a downregulates the expression of Brain-derived neurotrophic factor (*BDNF*) in neurons and low expression levels of *BDNF* are hypothesized to play an important role in the pathogenesis of major depression disorder (MDD) (Wan et al., 2015). Another link between miR-10a and stress is SIRT1. Overexpression of miR-10a leads to downregulation of *SIRT1* expression, which is also associated with MDD (Wan et al., 2015).

Studies suggest that miR-15a plays a role in chronic stress management mechanisms. Animal models have shown elevated levels of miR-15a in the blood of healthy subjects who suffered childhood trauma (CT) compared to subjects without CT (Maffioletti et al., 2021). Moreover, let-7g could be linked to CT (Van der Auwera et al., 2019). MiR-15a appears to regulate serotonin transporter (*SERT*) expression through the binding to the gene *SLC6A4*. Altered *SERT* expression is associated with a variety of diseases such as anxiety/obsessive-compulsive disorders, depression, or autism (Moya et al., 2013). In this study, we were able to identify connections between miR-15a, let-7g, and SRDs (e.g., CFS or anxiety), which could further confirm the suitability of these miRNAs as new biomarkers for chronic stress.

However, mental disorders are brain dysfunctions caused by, among other things, increased neuronal inflammation, and current evidence strongly suggests that neurodevelopment and physiology, and brain protection can be influenced through diet and exercise, presumably through influencing inflammation (de Melo Pereira et al., 2020). Our data indicated possible effects of lifestyle and dietary habits on these miRNAs. Participants who regularly consumed meat showed higher let-7a expression than those who rarely or never consumed meat. Interestingly, studies in rats showed that a high-fat diet resulted in let-7a upregulation (Sangiao-

171 Alvarellos et al., 2014). Meat, especially red or processed meat, when consumed in excess, has many negative
172 health effects (e.g., metabolic, and cardiovascular diseases or cancer) and contains a relatively high amount of fat
173 (González et al., 2020), which could explain our observation between let-7a and meat consumption in humans.
174 Another explanation is that dietary amino acids, such as those in meat, form the major monoamines (e.g.,
175 norepinephrine, dopamine, and serotonin), affecting their levels and, in turn, mental health (Begdache et al.,
176 2019). It can be assumed that let-7a is also involved in this. Furthermore, we could observe a correlation between
177 miR-15a expression and coffee consumption. While the literature describes coffee as inappropriately activating
178 the sympathetic nervous system, particularly via caffeine, and is associated with psychological distress, coffee
179 has other health benefits, such as improved heart and brain functions (Begdache et al., 2019). Coffee contains
180 many bioactive compounds such as polyphenols, polysaccharides, vitamins, and minerals that have health-
181 promoting (e.g., antioxidant, and anti-inflammatory) properties (de Melo Pereira et al., 2020; Samsonowicz et
182 al., 2019). Polyphenols are known to have certain regulators, e.g., miRNAs, that affect gene expression
183 (Milenkovic et al., 2013), which reinforces our findings regarding miR-15a.

184 The findings of this study should be considered in light of some limitations. Since this is an observational study
185 based on participants' self-reports on stress levels and lifestyle and dietary habits, we could only report
186 correlational relationships and speculate on the pathways behind potential biomarkers. However, our results are
187 based on a relatively large number of study participants and thus offer valuable insights into the possibility of
188 using a minimally invasive method to monitor a person's health.

189 **Conclusion**

190 Taken together, our results indicate that miR-10a and let-7a are pointing towards acute stress, whereas miR-15a,
191 and let-7g towards chronic stress and may be suitable as new biomarkers for assessing mental health.
192 Furthermore, our study provides some evidence that miRNAs from human capillary blood could be used as a
193 cost-effective monitoring tool for the prevention of stress-related diseases in the future. However, some
194 nutritional factors (e.g., meat or coffee) also play an important role in mental health and should be considered to
195 maintain overall health.

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199

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Competing Interests

The authors have no conflicts of interest to report. The sponsors had no role in the collection, analysis or interpretation of the data.

Author Contributions

All authors contributed to the conception and design of the study. Data collection, laboratory implementation, and analysis, as well as statistical evaluation, were carried out by UDBK and MLL. The first draft of the manuscript was written by UDBK, and MLL and BH have commented on previous versions of the manuscript. All authors have read and approved the final manuscript.

Ethics approval

The conduct of the study was approved by the Vienna Ethics Committee (MA 15 – EK/22-091-VK_NZ).

Consent to participate and publish

Written informed consent was obtained from all participants.

References

Beech R.D., Leffert J.J., Lin A., Hong K.A., Hansen J., Umlauf S., Mane S., Zhao H., Sinha R. (2014). Stress-related alcohol consumption in heavy drinkers correlates with expression of miR-10a, miR-21, and components of the TAR-RNA-binding protein-associated complex. *Alcoholism: Clinical and Experimental Research*, *38*, 2743–2753. https://doi.org/10.1111/acer.12549

Begdache L., Chaar M., Sabounchi N., Kianmehr H. (2019). Assessment of dietary factors, dietary practices and exercise on mental distress in young adults versus matured adults: A cross-sectional study. *Nutritional Neuroscience*, *22*, 488–498.

Chen R.J., Kelly G., Sengupta A., Heydendael W., Nicholas B., Beltrami S., Luz S., Peixoto L., Abel T., Bhatnagar S. (2015). MicroRNAs as biomarkers of resilience or vulnerability to stress. *Neuroscience*, *305*, 36–48. https://doi.org/10.1016/j.neuroscience.2015.07.045

Chrousos G.P. (2009). Stress and disorders of the stress system. *Nature Reviews Endocrinology*, *5*, 374–381.

https://doi.org/10.1038/nrendo.2009.106

Condrat C.E., Thompson D.C., Barbu M.G., Bugnar O.L., Boboc A., Cretoiu D., Suciu N., Cretoiu S.M., Voinea S.C. (2020). miRNAs as Biomarkers in Disease: Latest Findings Regarding Their Role in Diagnosis and Prognosis. *Cells*, *9*, 1–32.

de Melo Pereira G. V., de Carvalho Neto D.P., Magalhães Júnior A.I., do Prado F.G., Pagnoncelli M.G.B., Karp S.G., Soccol C.R. (2020). Chemical composition and health properties of coffee and coffee by-products, 1st ed, Advances in Food and Nutrition Research *91*, https://doi.org/10.1016/bs.afnr.2019.10.002

González N., Marquès M., Nadal M., Domingo J.L. (2020). Meat consumption: Which are the current global risks? A review of recent (2010–2020) evidences. *Food Research International*, *137*, 109341. https://doi.org/10.1016/j.foodres.2020.109341

Hollins S.L., Cairns M.J. (2016). MicroRNA: Small RNA mediators of the brains genomic response to environmental stress. *Progress in Neurobiology*, *143*, 61–81.

Kemeny M.E. (2003). The Psychobiology of Stress. *Current Directions in Psychological Science*, *12*, 124–129.

Krammer U.D.B., Sommer A., Tschida S., Mayer A., Lilja S. V, Switzeny O.J., Hippe B., Rust P., Haslberger A.G. (2022). PGC- 1α Methylation , miR-23a , and miR-30e Expression as Biomarkers for Exercise- and Diet-Induced Mitochondrial Biogenesis in Capillary Blood from Healthy Individuals: A Single-Arm Intervention. *Sports*, *10*, 73. https://doi.org/10.3390/sports10050073

Liu Y.Z., Wang Y.X., Jiang C.L. (2017). Inflammation: The common pathway of stress-related diseases. *Frontiers in Human Neuroscience*, *11*, 1–11. https://doi.org/10.3389/fnhum.2017.00316

Maffioletti E., Bocchio-Chiavetto L., Perusi G., Carvalho Silva R., Sacco C., Bazzanella R., Zampieri E., Bortolomasi M., Gennarelli M., Minelli A. (2021). Inflammation-related microRNAs are involved in stressful life events exposure and in trauma-focused psychotherapy in treatment-resistant depressed patients. *European Journal of Psychotraumatology*, *12*, 1–12. https://doi.org/10.1080/20008198.2021.1987655

Milenkovic D., Jude B., Morand C. (2013). miRNA as molecular target of polyphenols underlying their biological effects. *Free Radical Biology and Medicine*, *64*, 40–51.

https://doi.org/10.1016/j.freeradbiomed.2013.05.046

Mori M.A., Ludwig R.G., Garcia-Martin R., Brandão B.B., Kahn C.R. (2019). Extracellular miRNAs: From Biomarkers to Mediators of Physiology and Disease. *Cell Metabolism*, *30*, 656–673. https://doi.org/10.1016/j.cmet.2019.07.011

Moya P.R., Wendland J.R., Salemme J., Fried R.L., Murphy D.L. (2013). MiR-15a and miR-16 regulate serotonin transporter expression in human placental and rat brain raphe cells. *International Journal of Neuropsychopharmacology*, *16*, 621–629. https://doi.org/10.1210/en.2013-1770

Olejniczak M., Kotowska-Zimmer A., Krzyzosiak W. (2018). Stress-induced changes in miRNA biogenesis and functioning. *Cellular and Molecular Life Sciences*, *75*, 177–191. https://doi.org/10.1007/s00018-017-2591-0

Park C., Rosenblat J.D., Brietzke E., Pan Z., Lee Y., Cao B., Zuckerman H., Kalantarova A., McIntyre R.S. (2019). Stress, epigenetics and depression: A systematic review. *Neuroscience and Biobehavioral Reviews*, *102*, 139–152. https://doi.org/10.1016/j.neubiorev.2019.04.010

Ranabir S., Reetu K. (2011). Stress and hormones. *Indian Journal of Endocrinology and Metabolism*, *15*, 18. https://doi.org/10.4103/2230-8210.77573

Samsonowicz M., Regulska E., Karpowicz D., Leśniewska B. (2019). Antioxidant properties of coffee substitutes rich in polyphenols and minerals. *Food Chemistry*, *278*, 101–109. https://doi.org/10.1016/j.foodchem.2018.11.057

Sangiao-Alvarellos S., Pena-Bello L., Manfredi-Lozano M., Tena-Sempere M., Cordido F. (2014). Perturbation of hypothalamic microrna expression patterns in male rats after metabolic distress: Impact of obesity and conditions of negative energy balance. *Endocrinology*, *155*, 1838–1850. https://doi.org/10.1210/en.2013-1770

Sung M., Sung S.E., Kang K.K., Choi J.H., Lee S., Kim Kilsoo, Lim J.H., Lee G.W., Rim H.D., Kim B.S., Won S., Kim Kyungmin, Jang S., Seo M.S., Woo J. (2021). Serum-derived neuronal exosomal mirnas as biomarkers of acute severe stress. *International Journal of Molecular Sciences*, *22*, https://doi.org/10.3390/ijms22189960

279 Van der Auwera S., Ameling S., Wittfeld K., d'Harcourt Rowold E., Nauck M., Völzke H., Suhre K., Najafi-
280 Shoushtari H., Methew J., Ramachandran V., Bülow R., Völker U., Grabe H.J. (2019). Association of
281 childhood traumatization and neuropsychiatric outcomes with altered plasma micro RNA-levels.
282 *Neuropsychopharmacology*, *44*, 2030–2037. https://doi.org/10.1038/s41386-019-0460-2

283 Wan Y., Liu Y., Wang X., Wu J., Liu K., Zhou J., Liu L., Zhang C. (2015). Identification of differential
284 microRNAs in cerebrospinal fluid and serum of patients with major depressive disorder. *PLoS ONE*, *10*,
285 1–12. https://doi.org/10.1371/journal.pone.0121975

286 Wiegand C., Savelsbergh A., Heusser P. (2017). MicroRNAs in Psychological Stress Reactions and Their Use as
287 Stress-Associated Biomarkers, Especially in Human Saliva. *Biomedicine Hub*, *2*, 1–15.
288 https://doi.org/10.1159/000481126

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8.2. Further publications and conference proceedings

8.2.1. Publications

Lilja S, Gessner D, Krammer U, Hippe B and Haslberger AG. Mit epigenetischen Markern Zellschäden früh aufspüren. *Ärzte Woche*, 2019 Mar.

Lilja S, Stoll C, Krammer U, Hippe B, Duszka K, Debebe T, Höfinger I, König J, Pointner A and Haslberger A. Five Days Periodic Fasting Elevates Levels of Longevity Related *Christensenella* and Sirtuin Expression in Humans. *International Journal of Molecular Sciences*, 2021 Feb.

Pointner A, Mölzer C, Magnet U, Zappe K, Hippe B, Tosevska A, Tomeva E, Dum E, Lilja S, Krammer U, Haslberger A. The green tea polyphenol EGCG is differentially associated with telomeric regulation in normal human fibroblasts versus cancer cells. *Functional Foods in Health and Disease*, 2021 Mar.

Lilja S, Bäck H, Stoll C, Mayer A, Pointner A, Hippe B, Krammer U, Haslberger A. Increased Sirtuin expression, senescence regulating miRNAs, mtDNA, and bifidobacteria correlate with wellbeing and skin appearance after Sirtuin-activating drink. *Bioactive Compounds in Health and Disease*, 2021 Apr.

Krammer U, Hippe B, Rust P, Haslberger A. Körperliches Training: Auswirkungen von Ausdauer- und Kraftsport auf epigenetische Mechanismen und mitochondriale Biogenese. *VEÖ Einblicke*, 2022 July.

Hippe B, Krammer U, Mödder F, Mayer A, Speich S, Gruber U, Jacob U, Haslberger A. Epigenetic active phytoceuticals activate immune relevant miRNAs important in virus response systems. *Functional Foods in Health and Disease*, 2022 Aug.

Karlic H, Krammer U, Haslberger A. Nutritional supplements for athletes and personalization; a short review. *Functional Food Science*, 2022 Oct.

Tomeva E, Krammer UDB, Switzeny O, Haslberger AG, Hippe B. Sex-specific miRNA differences and DNA methylation in liquid biopsies from subjects with solid tumors and healthy controls. Submitted *Epigenomes*, 2022 Oct.

Book chapter:

Krammer UDB and Hippe B. Epigenetische Marker als Grundlage salutogener Leistungsoptimierung. Submitted *Springer Verlag*, 2022 Mar.

8.2.2. Oral and poster presentations

VEÖ-Tagung 2019: Lebenswelt Darm – das Mikrobiom
Guided tour through the gut model (2019, Apr 10)

Targeting Metabesity – Extending Healthspan
Poster title: “The influence of exercise training on health and epigenetic regulation, especially on selected non-coding RNAs” (2020, Oct 12-15)

Epigenomics of Common Diseases
Poster title: “The effects of a 12-week sport intervention on health and epigenetic mechanisms, especially on selected non-coding RNAs” (2020, Nov 18-20)

Keystone eSymposia – Non-Coding RNAs: Biology and Applications
Poster title and sci talk: “Epigenetic markers, especially non-coding RNAs and their interaction between diet and exercise: a 12-week sports intervention” (2021, May 11-14)

8.2.3. Lectures and talks

Wiener Volkshochschule (VHS): Sport and Epigenetics – How physical activity changes cells (2020 May 7 and Oct 13)

Austrian Cycling Association (ÖRV): Epigenetic markers to assess athletic performance and health in athletes (2020 May 28)

Sigmund Freud Privat University (SFU): Personalized Health Management – Introduction to genetics and epigenetics (2020 Aug 10)

Wiener Volkshochschule (VHS): Sport and Epigenetics – Belly dancing perfect for men? (2021, Oct 5)