Scandinavian Journal of Medicine & Science in Sports

WILEY

ORIGINAL ARTICLE OPEN ACCESS

An Integrated Neuromuscular Training Intervention Applied in Primary School Induces Epigenetic Modifications in Disease-Related Genes: A Genome-Wide DNA Methylation Study

Fidanka Vasileva[1,2] | Raquel Font-Lladó[2,3,4,5] | Víctor López-Ros[3,4] | Jordi Barretina[6] | Aleix Noguera-Castells[7,8,9] | Manel Esteller[7,9,10,11] | Abel López-Bermejo[1,12,13] | Anna Prats-Puig[2,14]

[1]Pediatric Endocrinology Research Group, Biomedical Research Institute of Girona, Girona, Spain | [2]University School of Health and Sport, University of Girona, Girona, Spain | [3]Faculty of Education and Psychology, University of Girona, Girona, Spain | [4]Research Group of Culture, Education and Human Development, Institute of Educational Research, University of Girona, Girona, Spain | [5]Chair of Sport and Physical Education – Centre of Olympic Studies, University of Girona, Girona, Spain | [6]Germans Trias i Pujol Research Institute, Barcelona, Spain | [7]Cancer Epigenetics Group, Josep Carreras Leukaemia Research Institute, Barcelona, Spain | [8]Department of Biosciences, Faculty of Science, Technology and Engineering, University of Vic-Central University of Catalonia, Barcelona, Spain | [9]Biomedical Research Centre in Cancer Network, Madrid, Spain | [10]Catalan Institution for Research and Advanced Studies, Barcelona, Spain | [11]Physiological Sciences Department, School of Medicine and Health Sciences, University of Barcelona, Barcelona, Spain | [12]Department of Medical Sciences, University of Girona, Girona, Spain | [13]Pediatric Endocrinology, Dr. Josep Trueta Hospital, Girona, Spain | [14]Research Group Health and Health Care, Nursing Department, University of Girona, Girona, Spain

Correspondence: Anna Prats-Puig (aprats@euses.cat)

Received: 25 October 2024 | **Revised:** 14 December 2024 | **Accepted:** 23 December 2024

Funding: This work was supported by European Union. Secretariat for Universities and Research of the Ministry of Business and Knowledge of the Government of Catalonia. European Social Fund. Ministerio de Ciencia e Innovación. "ERDF A way of making Europe".

Keywords: children | core genes | diseases | DNA methylation | integrated neuromuscular training | school

ABSTRACT

Physical exercise has been shown to induce epigenetic modifications with various health implications, directly affect DNA methylation (DNAm), as well as reverse the epigenetic age. Hence, we aimed to identify differential methylation changes and assess the epigenetic age in the saliva of 7–9-year-old school children following a 3-month integrated neuromuscular training (INT), as well as to explore if any of the methylation changes are in core genes. Core genes are defined as genes of high relevance and essential importance within the human genome. Forty children (17 boys and 23 girls) were recruited from schools in Girona, Spain, and allocated into control ($N = 20$) or INT ($N = 20$) group. The INT group performed a 3-month INT as a warm-up during the physical education (PE) classes, encompassing strength, coordination, dynamic stabilization, plyometrics, speed, and agility exercises, whereas the control group performed traditional warm-up activities, encompassing aerobic exercises that will prepare the cardiovascular system and increase the joint mobility for the upcoming effort during the class. Genome-wide DNAm analysis was performed with the Illumina 900 K microarray. Core genes were recognized based on the accomplishment of a rigorous and widely accepted 3-point criteria: participation in the enriched pathways, high connectivity (≥ 10), and target genes of key transcription factors. There were 1200 differentially methylated positions (DMPs) in the control group and 414 DMPs in the INT group (FDR < 0.05, $p < 0.05$, $A\beta < |0.1|$), suggesting a non-significant trend of epigenetic age acceleration in the control group (1.18 months, $p > 0.05$) and a non-significant 1-month decrease of the epigenetic age in the INT group ($p > 0.05$). The genes with DMPs in the control group showed low similarity between enriched pathways and low interconnectivity, encompassing

distinct pathways, mostly development and growth-related. Additionally, no core genes were identified in the control group. Interestingly, the genes with DMPs in the INT group showed high similarity between enriched pathways and high interconnectivity, encompassing related pathways involving signaling mechanisms, as well as hormone and protein metabolism pathways. Moreover, 17 DMPs in the children from the INT group were in core genes. The main findings of the present study are suggesting an integrated response to the training stimulus in 7–9-year-old school children that performed a 3-month INT, including epigenetic modifications in 17 genes considered as core genes.
Trial Registration: The study protocol was registered in the ISRCTN registry (ISRCTN16744821)

1 | Introduction

Integrated neuromuscular training (INT) is a specific exercise program with progressive load that improves fundamental motor skills, physical fitness, and overall health [1–5]. It encompasses a combination of efficient cognitive processing, correct movement patterns, and muscle force production, providing a solid context for expanding the motor learning experience and promoting health, especially when applied to the pediatric population [2].

Epigenetics is defined as the study of heritable changes in gene expression that are not caused by alterations in the DNA sequence [6]. These changes include histone modification, miRNA expression, and DNA methylation (DNAm) [6]. One of the most commonly studied epigenetic modifications with an extensive potential to impact health and disease is DNAm [7]. DNAm is defined as an addition of a methyl group on the 5th carbon position within the cytosine base of the DNA strand, directly affecting gene expression [7, 8]. It predominantly occurs at cytosines within CG dinucleotides ("CpG" sites) in mammalian genomes, and when close to gene promoter regions, it represses transcription, leading to gene silencing [7–9]. Gene silencing is particularly important for genes whose overexpression is related to the development and/or progression of certain diseases [10]. The assessment of DNAm across the entire genome is possible with the application of genome-wide approaches such as Illumina microarrays [11]. Genome-wide DNAm studies provide insights into DNAm levels simultaneously at millions of CpG sites within the genome [11].

Among the lifestyle habits that have been shown to directly affect DNAm is physical exercise [12]. Regular exercise training prevented age-related alterations in DNAm, as well as reversed aged skeletal muscle methylome profiles, that is, the epigenetic age toward patterns observed in younger profiles [13–17]. The epigenetic age is defined as an estimation of the biological age based on DNAm patterns across the genome [13, 15]. Reliable tools for assessing the epigenetic age are the epigenetic clocks [14]. While initially epigenetic clocks were developed for adult aging, nowadays pediatric epigenetic clocks are an important tool to monitor dynamic changes in DNAm that occur during childhood as a result of growth, development, and, more importantly, environmental influences [18].

Previous studies assessing DNAm after training interventions, including endurance and resistance training, reported both hypermethylation and hypomethylation changes in blood, sperm, adipose tissue, and skeletal muscle [19–30]. When hypermethylation occurs close to gene promoter regions, that is, near the transcription start site (TSS), transcription is repressed and the gene is silenced [31]. However, when hypomethylation occurs under the same conditions, transcription is activated and the gene is expressed [32]. In summary, genes that were differentially methylated in blood, sperm, adipose tissue, and skeletal muscle after physical exercise (including both endurance and resistance training) are mainly related to signaling mechanisms, inflammatory and immune pathways, glucose metabolism, oxidative and fatty acid metabolism, protein metabolism, cardiovascular and muscle physiology, contractile properties, hypertrophy, neurogenesis, and neuronal differentiation [20–30, 33, 34]. Moreover, training interventions have been shown to induce health-beneficial changes in the DNAm of disease-related genes [20, 23, 35, 36]. For instance, a 6-month endurance training modified the DNAm of genes related to obesity and type 2 diabetes mellitus in the adipose tissue of healthy adults [20]. A 3-month sprint training induced changes in the DNAm of genes related to neurodegenerative and cardio-metabolic diseases in the sperm of healthy young men [23]. Exercise interventions have also induced DNAm changes in tumor-related genes in prostate tumor tissue of rats, as well as in genes related to breast cancer in the blood of adult women [35, 36]. Aerobic exercise training reversed altered DNAm in the skeletal muscle of female breast cancer survivors [37]. Moreover, a recent systematic review that assessed DNAm changes after physical exercise in blood, sperm, adipose tissue, and skeletal muscle of healthy adults reported that training interventions (including endurance and resistance training) modified some core genes whose altered expression is disease-related [38].

Core genes are defined as genes of high relevance and essential importance within the human genome, playing a crucial role in the fundamental processes of a living organism [39]. Systematic methodologies for core gene screening, which are widely and commonly accepted in the bioinformatics field, are typically based on rigorous and stringent bioinformatics analyses, including enrichment analysis, gene–gene interaction analysis, and transcription factor target analysis [38, 40–43]. In this line, in order to recognize and consider a certain gene as a core gene, this gene must participate in the enriched pathways, must exhibit a high degree of connectivity ($\geq$ 10 connections), and must be a target gene of a key transcription factor, or in other words, this gene must accomplish these 3-point criteria.

Existing evidence assessing the effects of physical exercise on genome-wide DNAm has been focused on adults [20, 21, 23–26, 29, 30, 36, 44], older individulas [13, 27], and animal models [35, 45–47], while genome-wide DNAm studies in children are scarce. A plausible explanation for this gap could be the invasiveness of the sampling techniques, which so far

have included blood collection [24, 29, 36] or even biopsy sampling (e.g., skeletal muscle, adipose tissue, etc.) [20, 21, 25–27, 30]. However, in the last decades the use of saliva has become widely accepted in clinical settings [48, 49], opening new horizons for research, especially in pediatric populations, mainly because saliva collection is a practical and non-invasive procedure [48, 49]. Recent studies have shown that the genome-wide DNAm profile of saliva is more than 90% similar to blood [50, 51]. Indeed, there is less than a 20% difference in methylation patterns of more than 96% of the CpGs between saliva and blood in children [50].

Because previous evidence suggests that physical exercise affects DNAm in various human biospecimens (blood, sperm, adipose tissue, and skeletal muscle) in the adult population [12, 20, 21, 23–30], we hypothesized that a 3-month INT applied in schools will also induce DNAm changes detectable in cells present in the saliva of healthy children. Furthermore, we also hypothesized that the 3-month INT may modify some core genes in these children. This hypothesis is based on a recent systematic review where we showed that physical exercise interventions epigenetically modified 19 core genes in healthy adults [38].

Taking into consideration previous findings and the literature gap, we aimed to assess the effects of a 3-month INT on genome-wide DNAm in the saliva of healthy 7–9-year-old school children. More precisely, our objectives were: 1) to identify differential methylation changes and assess the epigenetic age in saliva of 7–9-year-old school children following a 3-month INT; and 2) to explore if any of the methylation changes are in core genes. An additional goal was to identify the genomic location of the methylation changes in those genes in relation to the TSS and the CpG site.

2 | Methods

2.1.1 | Sample Size Estimation

The GRANMO 7.12 program was used to identify the sample size for inclusion based on a previous study including genome-wide DNAm analysis [52]. Accepting an alpha risk of 0.05 and a beta risk of 0.2 in a two-sided test, the estimated sample size for the present study was 34 participants in total or 17 participants in each group.

2.1.2 | Population and Ethics

A total of 40 apparently healthy children (17 boys and 23 girls) were recruited from schools in Cassà de la Selva and Salt (Girona, Northeastern Spain). Due to ethical reasons, randomization of children within the same school and during the same physical education (PE) class was not possible; thus, the schools were randomly assigned to control or INT, with a total of 20 children being included in each school. However, following the completion of the study, the INT was also offered to the control school to ensure that all children who participated in the study were treated fairly and equally, as well as to prevent any perception of discrimination due to the randomization process. Inclusion criteria were: 1) no evidence of chronic or acute illness in the month preceding potential enrollment; and 2) age between 7 and 9 years. Exclusion criteria were: 1) major congenital abnormalities; 2) illness or chronic use of medication; 3) musculoskeletal or neurological disorder and/or a medication therapy that could alter postural stability and cardiorespiratory function; and 4) attending fewer than 80% of the PE classes. The research was approved by the Institutional Review Board of Dr. Josep Trueta Hospital, Girona, Spain (CEIm:2016.134), and the study protocol was registered in the ISRCTN registry (ISRCTN16744821). The study was conducted in accordance with the Declaration of Helsinki regulations, and signed informed consent was obtained from parents of all participating children. Anthropometric measurements and sample collection were conducted on the same day. Both schools participating in the study were located in the same province and country, ensuring homogeneity of the curricular content for the PE classes. Prior to the study, the PE teacher at the intervention school was trained to conduct the intervention sessions together with the researcher-expert in INT [3].

2.1.3 | Intervention

A 3-month INT intervention was applied to children during PE classes by the researcher-expert in INT and the PE teacher [3]. The PE classes in both groups (control and INT) were conducted twice weekly (60 min each) and were structured into an introductory segment (20 min), a main segment (30–35 min), and a concluding segment (5–10 min). While the main and the concluding segments were the same across both groups, the introductory segment was different.

The main difference between the introductory segments in the control and the INT groups is the type of the exercises performed (Supplementary Figure S1). While the introductory segment in the control group consisted of aerobic activities, the children from the INT group performed the INT, which consisted of both aerobic and anaerobic activities. More precisely, the introductory segment for the control group consisted of traditional warm-up activities, that is, exercises designed to prepare the cardiovascular system and increase the joint mobility for the upcoming physical effort during the class (Supplementary Figure S1A) [53]. On the other hand, the INT group during the introductory segment of the PE classes engaged in INT sessions as warm-up activities for 3 months, that is, 24 sessions of progressively structured exercises focusing on strength, coordination, dynamic stabilization, plyometrics, speed, and agility, organized in circuits and games (Supplementary Figure S1B) [3]. Another important difference between the introductory segment in the control and the INT group is the diversity of movement patterns and motor experiences that is provided with the INT, as well as the progression in terms of complexity of movement patterns.

Following the 20-min warm-up during the introductory segment, the children from both groups proceeded to the main segment of the PE class. The main segment of the PE classes covered specific curricular content outlined in the national PE curricula, which was didactically delivered by the PE teacher: 1) aerobic activities (running, jumping a rope) and activities

that include solving motor tasks in environmental conditions (outdoor circuits and polygons, orienteering activities); 2) activities that will induce development of fundamental motor skills, motor abilities and motor competence (motor challenges that contain elements from individual sports: athletics, gymnastics, tennis); 3) activities that will induce development of interaction skills and team-work (cooperative motor challenges that contain elements from sport games: football, basketball, handball, volleyball); 4) traditional and contemporary dances; and 5) outdoor activities in the natural environment (hiking, cycling, rollerblading, skating).

Finally, the concluding segment of the PE classes in both groups covered a short period with less dynamic activities that allowed children to cool down gradually and also provided an opportunity for reflection of the acquired skills during the class.

2.1.4 | Anthropometric Measurements

Anthropometric measurements were performed at the schools in the morning hours (between 8.00 and 10.00 AM). Body mass was measured with a calibrated digital scale (Portable TANITA, 240MA, Amsterdam, Netherlands) while participants were barefoot and wearing light clothes. Height was measured with a wall-mounted stadiometer (SECA SE206, Hamburg, Germany). BMI was calculated as body mass in kg divided by the square of height in m. Age- and sex-adjusted standard deviation scores (SDS) for body mass, height, and BMI were calculated using regional normative data [54]. All measurements were performed twice, at baseline and after 3 months.

2.1.5 | Biological Samples Collection

Saliva samples were collected in a fasting state with Oragene Discover (OGR-500; DNA Genotek, USA) in the morning between 8.00 and 10.00 AM, and stored according to the manufacturer's protocol. Participants were instructed to discharge 1–2 mL of saliva into the collection funnel, which allowed the saliva to flow easily into the collection tube pre-filled with a stabilizing solution. Once participants filled the collection tube, the researcher in charge inverted each tube several times to mix the saliva with the stabilizing solution in the tube, ensuring the preservation of DNA integrity during transportation and storage. Note that children were not allowed to drink water before collection (per manufacturer's protocol). The same procedure was performed twice, at baseline and after 3 months.

2.1.6 | DNAm Assessment and Epigenetic Clock Analysis

First, genomic DNA was extracted from saliva using the prep-IT•L2P reagent (PT-L2P-5; DNA Genotek, USA) and 500 μL of Oragene samples, according to the manufacturer's instructions. The quantity of the extracted DNA was assessed with a Qubit 2.0 fluorometer (Invitrogen—Thermo Fisher Scientific, USA), and DNA sample integrity was checked by electrophoresis. Finally, DNA was bisulfite-converted using an EZ-96 DNAm kit (D5003; Zymo, USA) following the manufacturer's instructions.

Genome-wide DNAm analysis was performed at the Genomics Unit of the Josep Carreras Leukemia Research Institute (Barcelona, Spain) with the Illumina 900 K (EPIC v2) microarray [55]. The raw data files generated from the microarray were processed with the software Genome Studio 2011.1 developed by Illumina. This included quality assessment and normalization. Then, DNAm β values were obtained for each CpG probe. β values range from 0 to 1. A β value of 0 indicates that the corresponding CpG probe is 0% methylated, and a β value of 1 indicates that the corresponding CpG probe is 100% methylated. Following this, several quality and control steps were implemented to remove low-quality probes, masked probes, and sex-chromosome probes, with the aim of minimizing errors and ensuring the accuracy of the further DNAm analysis. Subsequently, β value imputation with the media was performed in probes that have failed (detection $p < 0.01$) in 10% of the samples.

Thereafter, the CpG probes underwent differential methylation analysis. The differential methylation analysis was performed by applying linear regression models for microarray data (limma package v3.48.3 in R studio) adjusting for age, sex, BMI, and technical batch effects (time of experiment). The FDR applied to consider a CpG probe as a differentially methylated position (DMP) was FDR < 0.05, and the significance level was set at $p < 0.05$. Note that the differential methylation analysis was performed comparing the post values versus pre values in each group in order to understand which pathways are being changed in the control group during the 3-month period and which pathways are being changed in the INT group during the 3-month INT. This approach allowed a more rigorous and less biased analysis considering both time points (pre and post) and both conditions (control and experimental).

Finally, the epigenetic clock analysis was performed with the Methylclock R package [56]. More precisely, DNAm age was estimated with the saliva-based epigenetic clock specifically designed for individuals aged 0–20 years [18]. The epigenetic age acceleration or the deviation between epigenetic age and chronological age was calculated based on the residuals obtained from regressing DNAm age against chronological age.

2.1.7 | Core Genes Analysis

After obtaining the differentially methylated probes following the 3-month period in both groups, we proceeded with core gene analysis. We applied this approach to comprehensively understand the methylation changes we observed, as well as to avoid overestimation of significant observations through applying a meticulous filter consisting of further bioinformatics analyses. Moreover, the core gene analysis allowed us to identify which of the DMPs after the 3-month period are in core genes, that is, genes of high relevance and essential importance within the human genome. The methodological approach used to recognize core genes consisted of the accomplishment of the following 3 criteria: 1) participation in the enriched pathways; 2) a high degree of connectivity (≥ 10 connections); and 3) target genes

of key transcription factors [38, 40–43]. The accomplishment of these 3 criteria was verified through enrichment analysis, gene–gene interaction analysis, and transcription factor target analysis.

2.2 | Enrichment Analysis

Enrichment analysis of DMPs after the 3-month period in the children from both groups was performed with the bioinformatics software Metascape [57]. To verify if the DMPs fulfill the first criterion, that is, participation in the enriched pathways, we first inputted the corresponding lists in the Metascape platform. All statistically enriched terms (which can be Gene Ontology and/or Kyoto Encyclopedia of Genes and Genomes terms, canonical pathways, hallmark gene sets, etc., based on the default choices of the bioinformatics software Metascape) were then identified. Subsequently, accumulative hypergeometric p-values and enrichment factors were calculated and used for filtering. Remaining significant terms were hierarchically clustered into a tree based on Kappa-statistical similarities among their gene memberships. A kappa score of 0.3 was applied as a threshold to cast the tree into term clusters. Finally, the representative terms from the full cluster were converted into a network layout. Terms with a similarity score greater than 0.3 are connected by an edge whose thickness represents the strength of the similarity score. Thicker edges indicate higher similarity between terms. The enriched term clusters are visualized with Cytoscape [58]. This analysis allowed us to comprehensively understand which pathways are being changed in the control group and which pathways are being changed in the INT group during the 3-month period. Also, it gives us information on the similarity between the pathways that are being changed.

2.3 | Gene–Gene Interaction Analysis

To verify the accomplishment of the second criterion, that is, a high degree of connectivity (≥ 10 connections), and obtain the gene–gene interactions, we used the bioinformatics software Metascape [57]. We first inputted the corresponding lists in the Metascape platform. Subsequently, based on known interactions between the genes, Metascape constructed the gene–gene interactions. Note that to construct gene–gene interactions, Metascape uses only physical interactions with a physical score higher than 0.132. The gene–gene interaction network was then visualized with the bioinformatics software Cytoscape [58]. The resultant network contains the subset of genes that form physical interactions with at least one other member in the list. In case the network contained between 3 and 500 genes, the Molecular Complex Detection algorithm has been applied to identify the most densely connected network components [59]. Finally, we exported the Cytoscape output in an Excel spreadsheet and identified the genes with ≥ 10 connections. This analysis allowed us to understand if the observed methylation changes are potentially a response to a single stimulus (e.g., a training intervention) or if they involve temporal variations. In case the interconnectivity is high, and the physical interaction scores are higher than 0.132, it is considered that these changes could be a response to a certain stimulus because the input genes act in a collaborative manner, aiming to provide an integrated response [57, 58, 60]. Otherwise, low interconnectivity and physical interaction scores lower than 0.132 are considered physiologically irrelevant [57].

2.4 | Transcription Factor Target Analysis

To identify if some of the potential candidate DMPs (DMPs that fulfilled the above-mentioned 2 criteria) are in target genes of key transcription factors, that is, fulfill the third criterion, we used the ENCODE dataset of transcription factor targets as a reference [61]. This analysis allowed us to identify which of the DMPs are in genes that are targets of important transcription factors. This is especially relevant because these transcription factors play a crucial role in regulating gene expression and are mainly related to signaling pathways, metabolic pathways, as well as immune response [62]. So, the input genes that are targets of key transcription factors are considered to have a crucial role in the fundamental biological processes of a living organism.

2.5 | Genomic Location of the Methylation Changes

The genomic location of methylation changes in the core genes was identified in relation to the TSS and the CpG site. In relation to the TSS, the methylation changes in core genes were annotated to the following locations: TSS1500 (1500 bases upstream of the TSS), TSS200 (200 bases upstream of the TSS), 5'UTR—5 prime, First Exon, Body, 3'UTR—3 prime, and Intergenic. Subsequently, 2 groups were created: 1) close to the TSS (TSS1500, TSS200, 5'UTR, and First Exon) and 2) distant from the TSS (Body, 3'UTR, and Intergenic). In relation to the CpG site, the methylation changes in core genes were annotated as follows: CpG island, N and S shores (< 2000 bases from the CpG island), N and S shelves (2000 to 4000 bases from the CpG island), and open sea (> 4000 bases from the CpG island).

3 | Results

Demographic characteristics of the studied children at baseline and after 3 months are presented in Table 1. It is worthy to note that the control and the INT group were comparable because there were no statistically significant differences in DNAm between them at baseline ($p > 0.05$). However, the number of DMPs (FDR < 0.05, $p < 0.05$) after the 3-month period in the control and the INT group are presented in Table 2. Details are presented in the Supporting Information (in Supplementary File 1), and all DNAm data (including raw data files) are publicly available in GEO (Accession: GSE279803). There were 1200 DMPs (of which 677 were hypomethylated and 523 were hypermethylated) after the 3-month period in the control group. On the other hand, we observed 414 DMPs (of which 263 were hypomethylated and 151 were hypermethylated) after the 3-month INT in the children that performed the INT (Table 2 and Supplementary File 1). Note that the observed methylation changes were not sufficient enough to exhibit biological relevance ($A\beta < |0.1|$; Supplementary File 1), as well as they did not remain when applying an FDR < 0.005 (Table 2).

TABLE 1 | Demographic characteristics and comparison between the control and the INT group at baseline and after 3 months.

Demographic characteristics at baseline	Control group ($N=20$)	INT group ($N=20$)	p
Age (years)	7.71 ± 0.38	7.48 ± 0.27	> 0.05
Sex (m/f)	10/10	7/13	> 0.05
Body mass (kg)	27.01 ± 2.99	26.63 ± 5.42	> 0.05
Body mass SDS	−0.13 (−0.43–0.13)	−0.37 (−0.73–0.26)	> 0.05
Height (cm)	129.78 ± 5.19	127.06 ± 5.73	> 0.05
Height SDS	0.35 (−0.12–0.78)	0.40 (−0.56–0.80)	> 0.05
BMI (kg/m^2)	16.10 ± 1.46	16.37 ± 2.20	> 0.05
BMI SDS	−0.43 (−0.80–0.04)	−0.55 (−0.82–0.04)	> 0.05
Demographic characteristics after 3 months	**Control group ($N=20$)**	**INT group ($N=20$)**	p
Age (years)	7.95 ± 0.39	7.73 ± 0.25	> 0.05
Sex (m/f)	10/10	7/13	> 0.05
Body mass (kg)	27.61 ± 3.53	26.36 ± 4.36	> 0.05
Body mass SDS	0.05 (−0.42–0.21)	−0.42 (−0.56–0.17)	> 0.05
Height (cm)	131.09 ± 5.45	128.15 ± 5.63	> 0.05
Height SDS	0.50 (0.01–1.07)	0.68 (−0.17–1.24)	> 0.05
BMI (kg/m^2)	16.05 ± 1.64	15.97 ± 1.78	> 0.05
BMI SDS	−0.41 (−0.87–0.11)	−0.60 (−0.87–0.16)	> 0.05

Note: Data for Gaussian variables are presented as mean ± standard deviation. Data for non-Gaussian variables are presented as median and interquartile range. The p-value for Gaussian variables is from the t-test. The p-value for non-Gaussian variables is from the Mann–Whitney U test. The p-value for categorical variables is from the chi-squared test. The significance level is set at $p < 0.05$. BMI: body mass index; INT: integrated neuromuscular training; SDS: standard deviation score.

TABLE 2 | Differential methylation analysis (post versus pre) in the control and the INT groups.

Differential methylation analysis (post versus pre)	Control group ($N=20$)	p
Hypomethylated positions (N)	677	**< 0.05**
Hypermethylated positions (N)	523	**< 0.05**
Total DMPs (N)	1200	**< 0.05**
Differential methylation analysis (post versus pre)	**INT group ($N=20$)**	p
Hypomethylated positions (N)	263	**< 0.05**
Hypermethylated positions (N)	151	**< 0.05**
Total DMPs (N)	414	**< 0.05**

Note: The differential methylation analysis was performed with linear regression models for microarray data (limma package v3.48.3 in R studio) adjusting for age, sex, BMI, and technical batch effects (time of experiment). The FDR applied to consider a CpG probe as a DMP was FDR < 0.05 and the significance level was set at p < 0.05 (marked in bold). Note that the DMPs did not remain when applying an FDR < 0.005. DMP: differentially methylated position; INT: integrated neuromuscular training.

The epigenetic clock analysis (Figure 1) indicates an interesting, even though statistically non-significant, trend. We observed a non-significant epigenetic age acceleration of 1.18 months in the control group after the 3-month period, whereas we observed a non-significant decrease of 1 month in the epigenetic age of the children from the INT group after the 3-month INT ($p > 0.05$).

The enrichment analysis of DMPs (post versus pre) in the control and the INT group is presented in Figure 2. The DMPs after the 3-month period in the children from the control group were mainly related to children's development, cell cycle, hematopoiesis, and growth (Figure 2A). The DMPs in the children that performed the 3-month INT were related to disease pathways (including cancer pathways), signaling mechanisms, hormone metabolism, protein metabolism, phosphorylation, and stress response (Figure 2B). Interestingly, the similarity between the enriched term clusters in the INT group is higher as compared to the control group (Figure 2). This indicates a more focused and targeted response encompassing similar and related pathways in the children from the INT group. On the other hand, in the children from the control group, we observed distinct and less related pathways.

Furthermore, a gene–gene interaction network from the genes with a DMP in the INT group is presented in Figure 3. Note that a gene–gene interaction network in the control group was not constructed because Metascape uses only the subset of genes that form physical interactions with at least one other member in the list, exhibiting a physical score higher than 0.132. More precisely, if the gene–gene interactions do not meet this threshold, the interaction network is considered irrelevant. These results suggest that the genes with a DMP in the control group interact less with each other and/or the physical interaction scores are lower as compared to the INT group. Conversely, the genes with a DMP in the INT group were highly interconnected, exhibiting interactions with higher physical scores as compared to the control group. The higher interconnectivity and the stronger interactions between the genes with a DMP in the INT group may potentially indicate that these genes act in a collaborative manner rather than in isolation, probably being part of common and/or cooperative biological processes, resulting in an integrated, more synchronized, and coordinated response in the children from the INT group.

The results from the core gene screening procedure are presented in Supplementary Table S1. Based on this table, the first criterion, that is, participation in the enriched pathways, was fulfilled by 1038 genes with a DMP in the control group and 410 genes with a DMP in the INT group. Interestingly, none of the 1038 potential candidate genes in the control group fulfilled the second criterion, that is, high degree of connectivity (≥ 10). On

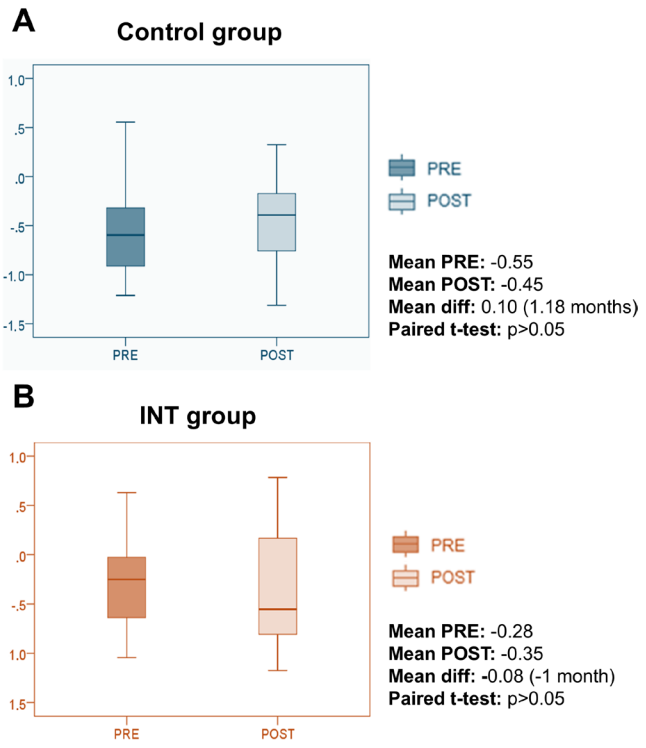

FIGURE 1 | Box plots representing the epigenetic age of the children at baseline and after the 3-month period. The DNAm age was estimated with the saliva-based epigenetic clock specifically designed for individuals aged 0–20 years, and the analysis was performed with the Methylclock R package. The deviation between epigenetic age and chronological age was calculated based on the residuals obtained from regressing DNAm age against chronological age. (A) Box plots representing the epigenetic age of the children from the control group at baseline and after 3 months. The box plots are showing a non-significant trend of epigenetic age acceleration in the children from the control group. (B) Box plots representing the epigenetic age of the children from the INT group at baseline and after the 3-month INT. The box plots show a non-significant 1-month decrease in the epigenetic age of the children from the INT group. DNAm: DNA methylation; INT: Integrated neuromuscular training; Mean diff: Mean difference.

the other hand, 17 genes out of 410 potential candidate genes fulfilled the second criterion in the INT group. Finally, all candidate genes identified in the INT group fulfilled the third criterion, that is, target gene of key transcription factors.

The number of connections for each candidate gene, as well as the corresponding transcription factor of which the gene is a target, are presented in Supplementary Table S2. The candidate genes' connections vary from 10 to 32, and the transcription factors of which candidate genes are targets are mainly NF-κB, ARID3A, BHLHE40, and ATF2.

Finally, we did not identify any core genes in the control group because potential candidates did not fulfill the second criterion, that is, a high degree of interconnectivity (Supplementary Table S1). Interestingly, we identified 17 core genes with a DMP (11 hypomethylated and 6 hypermethylated positions) after the 3-month INT in 7–9-year-old school children ($\log FC = |0.61|$ to $\log FC = |1.56|$, $\Delta\ (\%) = \leq 1\%$, $p < 0.05$; Table 3). Even though the observed methylation changes were not sufficient enough to exhibit biological relevance and did not remain when applying an FDR < 0.005, it is worth considering that the DMPs after the 3-month INT were in core genes, that is, genes considered to have high relevance within the human genome. In brief, all these genes—*BIRC3, CLTC, CSNK2B, CTSL, DCTN2, DDB1, FASN, FBXW11, FGFR1, GNAI2, HSPA2, MTOR, NDC80, PRPF19, PRPF8, RPL13A*, and *SUPT16H*—were related to inflammation, fat metabolism, protein metabolism, ATP binding, and growth (details are presented in Table 3). Moreover, their altered expression appears to be related to metabolic *(FASN, FGFR1, GNAI2)*, cardiovascular *(CTSL, GNAI2, PRPF19*, and *RPL13A)*, neurodegenerative diseases *(CLTC, CSNK2B, CTSL, DCTN2, FBXW11, PRPF8*, and *SUPT16H)*, musculoskeletal disorders *(DCTN2, DDB1*, and *FGFR1)*, cancer *(BIRC3, CLTC, CTSL, FASN, FGFR1, GNAI2, HSPA2, MTOR*, and *NDC80)*, and various syndromes: Noonan syndrome, Poirier-Bienvenu syndrome, respiratory syndrome, Perry syndrome, White-Kernohan syndrome, and Muenke syndrome (*CLTC, CSNK2B, DCTN2, DDB1*, and *FGFR1*; details are presented in Table 3).

The genomic location of methylation changes in the core genes in relation to the TSS and the CpG site is presented in Figure 4. Remarkably, 88% of the methylation changes were close to the TSS, while only 12% were distant from the TSS (Figure 4A). According to the location in CpG sites, 59% were in CpG islands, 41% were in shores, whereas no methylation changes were observed in shelves and open sea sites (Figure 4B).

4 | Discussion

There were 1200 DMPs in the children from the control group after the 3-month period, and 414 DMPs in the children from the INT group after the 3-month INT. Interestingly, we observed a statistically non-significant trend of epigenetic age acceleration (1.18 months) in the control group, whereas we observed a non-significant decrease of 1 month in the epigenetic age of the children from the INT group. Moreover, the enrichment and gene–gene interaction analyses revealed distinct and less related pathways with lower interconnectivity among the genes with a DMP in the control group, in contrast to the higher similarity of enriched pathways and higher interconnectivity among the genes with a DMP in the INT group. These results may indicate a chaotic response in the children from the control group, encompassing mainly development and growth-related pathways, versus the integrated and coordinated response to the training stimulus observed in the children from the INT group, encompassing signaling pathways, as well as hormone and protein metabolism pathways. Even though the observed methylation changes were not sufficient enough to exhibit biological relevance in both groups, and the DMPs did not remain when applying an FDR < 0.005, it is worth considering that some of the epigenetic modifications in the children that performed the 3-month INT in the school were in genes considered as core genes. Moreover, 88% of the methylation changes in the core genes were close to the TSS (near to the promoter regions), and 59% were in CpG islands.

The present findings allow us to accept the first hypothesis, according to which we expected to find DNAm changes in the saliva of healthy children after a 3-month INT. A previous study

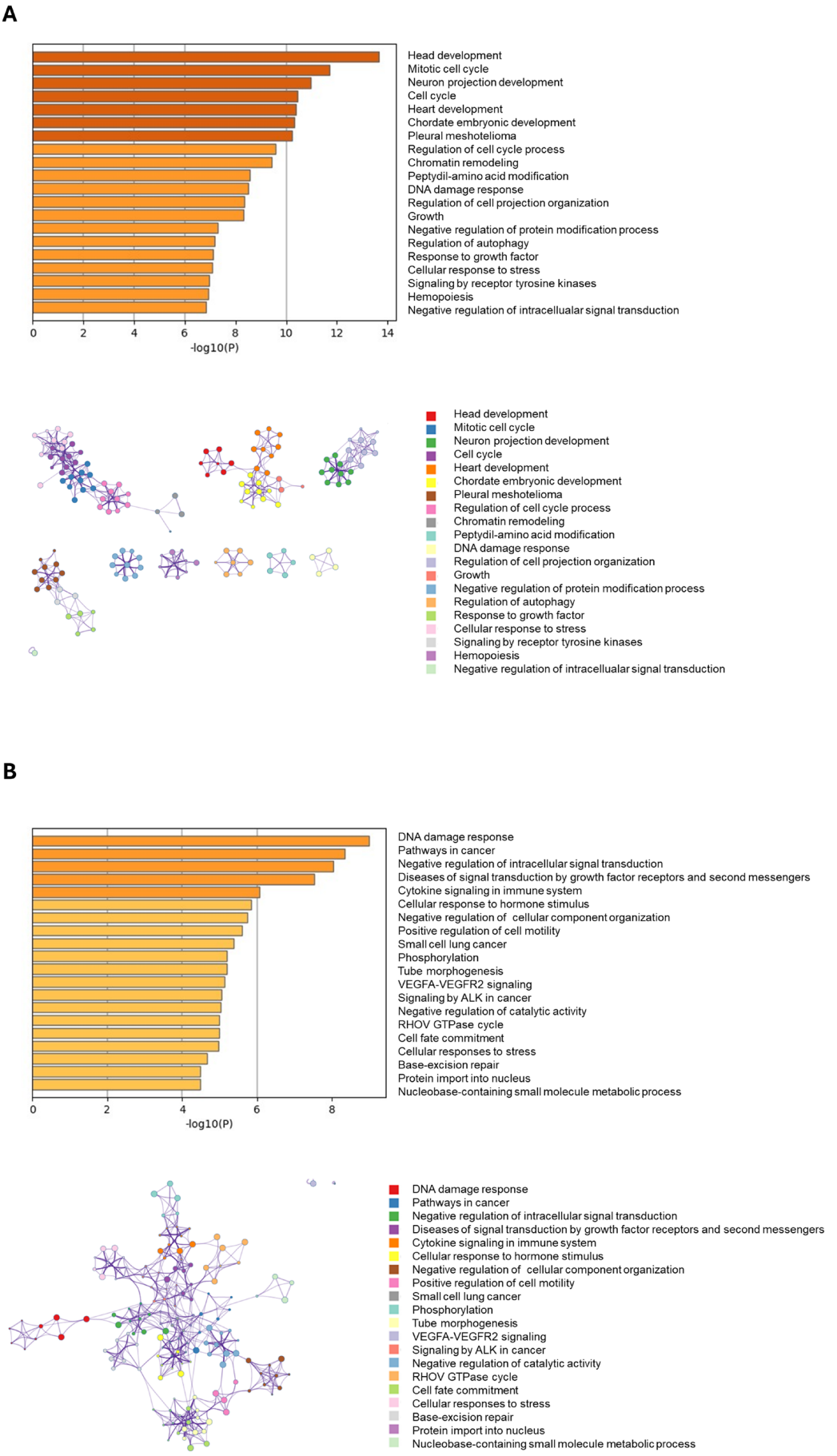

FIGURE 2 | Legend on next page.

FIGURE 2 | Enrichment analysis of DMPs. All statistically enriched terms (which can be Gene Ontology and/or Kyoto Encyclopedia of Genes and Genomes terms, canonical pathways, hallmark gene sets, etc., based on the default choices of the bioinformatics software Metascape) were first identified. Then, accumulative hypergeometric p-values and enrichment factors were calculated and used for filtering. Remaining significant terms were hierarchically clustered into a tree based on Kappa-statistical similarities among their gene memberships. A kappa score of 0.3 was applied as a threshold to cast the tree into term clusters. Finally, the representative terms from the full cluster were converted into a network layout that was visualized with the bioinformatics software Cytoscape. The network layout represents sets of genes with their respective biological functions. More precisely, the circle nodes represent the enriched term (set of genes), and the size of the circle node is proportional to the number of genes that fall under that term. The color of the circle node represents the identity of the cluster (i.e., biological function), and the circle nodes with the same color represent sets of genes with the same biological function. The edges between the circle nodes represent the similarity score between the sets of genes, while thicker edges indicate higher similarity. (A) Enrichment analysis of DMPs in the children from the control group. There are multiple distinct pathways and low similarity (thin connector edges) between enriched term clusters in the children from the control group. This is indicating a chaotic response encompassing distinct and less related pathways. (B) Enrichment analysis of DMPs in the children from the INT group. There are multiple connector edges (most of them thick) forming a unique network indicating a high similarity between the enriched term clusters in the children from the INT group. This similarity between pathways is suggestive of a targeted response to a certain stimulus, such as in this case the INT. DMPs: Differentially methylated positions; INT: Integrated neuromuscular training.

that employed an exercise intervention with the same frequency (twice per week) and the same duration (3 months) as the INT applied in the present study reported similar DNAm changes in the sperm of healthy young men [23]. More precisely, the authors of the previous work reported DNAm changes ranging from 0.7% to 3% in genes related to cardio-metabolic diseases, Alzheimer's disease, Tourette's syndrome, and obsessive compulsive disorder [23]. In addition, slightly higher DNAm changes (< 5%) were observed in genes related to development, morphogenesis, schizophrenia, Parkinson's disease, leukemia, and cancer [23]. On the other hand, exercise intervention with a longer duration (6 months) induced higher DNAm changes (< 11%) in the adipose tissue of healthy adults [20]. Furthermore, it has been reported that lifelong physical activity induces considerably more pronounced changes in DNAm in human skeletal muscle [27]. Indeed, the difference between DNAm levels of genes involved in metabolism, myogenesis, and oxidative stress resistance between active and inactive individuals was at least 30% [27]. Physical activity has also been accepted as an effective strategy to prevent age-related epigenetic changes, while regular exercise training has been shown to partially reverse aging-induced alterations in the skeletal muscle methylome [13], therefore leading to remarkable shifts of the epigenetic clock toward a younger profile [16, 17]. In this line, in the present study we observed an interesting, but statistically non-significant trend of a 1-month decrease in the salivary epigenetic age of the children from the INT group. On the other hand, the children from the control group showed non-significant epigenetic age acceleration (1.18 months). These statistically non-significant results may be indicative of the onset of adverse epigenetic programming in the children from the control group, which appears diminished in the children from the INT group, suggesting a potential, still non-significant reversing or protective effect of the 3-month INT. Given that studies assessing the epigenetic age in saliva of children after a training intervention are scarce, we encourage further research in the pediatric population because previous studies assessing the epigenetic age in skeletal muscle of adults, older individuals, and even female breast cancer survivors reported remarkable reversing effects induced by physical exercise [13, 16, 17, 37]. Taking into consideration the present and previous findings, we believe that interventions with longer duration may induce higher changes in DNAm as compared to interventions with shorter duration, while practicing lifelong physical activity may induce even greater impact on DNAm and potentially reverse the epigenetic clock toward a younger profile [17, 20, 23, 27]. Therefore, we suggest fostering physical activity from pediatric age and implementing exercise interventions with longer duration.

The genes with a DMP in the control group showed low similarity between the enriched pathways, weak interactions, and low interconnectivity, indicating a chaotic response encompassing distinct pathways (mostly development and growth-related). On the other hand, the genes with a DMP in the children from the INT group showed high similarity between the enriched pathways, strong interactions, and high interconnectivity, suggesting an integrated and coordinated response to the training stimulus. More precisely, the high interconnectivity and the dense interaction network observed in the INT group indicate that these genes act in a collaborative manner rather than in isolation, probably being part of common and/or cooperative biological processes [57, 58, 60]. In addition, the enrichment analysis in the children from the INT group revealed pathways such as signaling mechanisms, stress response, protein, and hormone metabolism. Even though studies assessing the genome-wide DNAm changes in saliva following an INT in children are missing, previous studies assessing exercise-induced methylation changes in various biospecimens (e.g., skeletal muscle, adipose tissue, sperm, and blood) in adults [20, 21, 23–26, 29, 30, 36, 44, 63], older individuals [13, 27], and animal models [35, 45–47], have reported similar findings. In human skeletal muscle, endurance exercise and high-intensity interval training induced DNAm changes in genes related to MAPK signaling, insulin pathways, and oxidative and fatty acid metabolism [21, 22, 64, 65], whereas single resistance exercise bout and resistance training induced DNAm changes in genes related to signaling pathways, focal adhesion, axon guidance, actin structure, remodeling, and hypertrophy-related pathways [25, 63]. Interestingly, it has been suggested that exercise-induced DNAm changes are tissue- and type-specific, and that the enriched pathways depend on the type of the exercise stimulus [25, 30, 38]. For instance, the enriched pathways of genes differentially methylated after high-load, short-duration exercise, in comparison to low-load, continuous exercise, were diverse [21, 25, 63–65]. In this line, in skeletal muscle of young adults, a single resistance exercise bout and resistance training (including detraining and retraining periods)

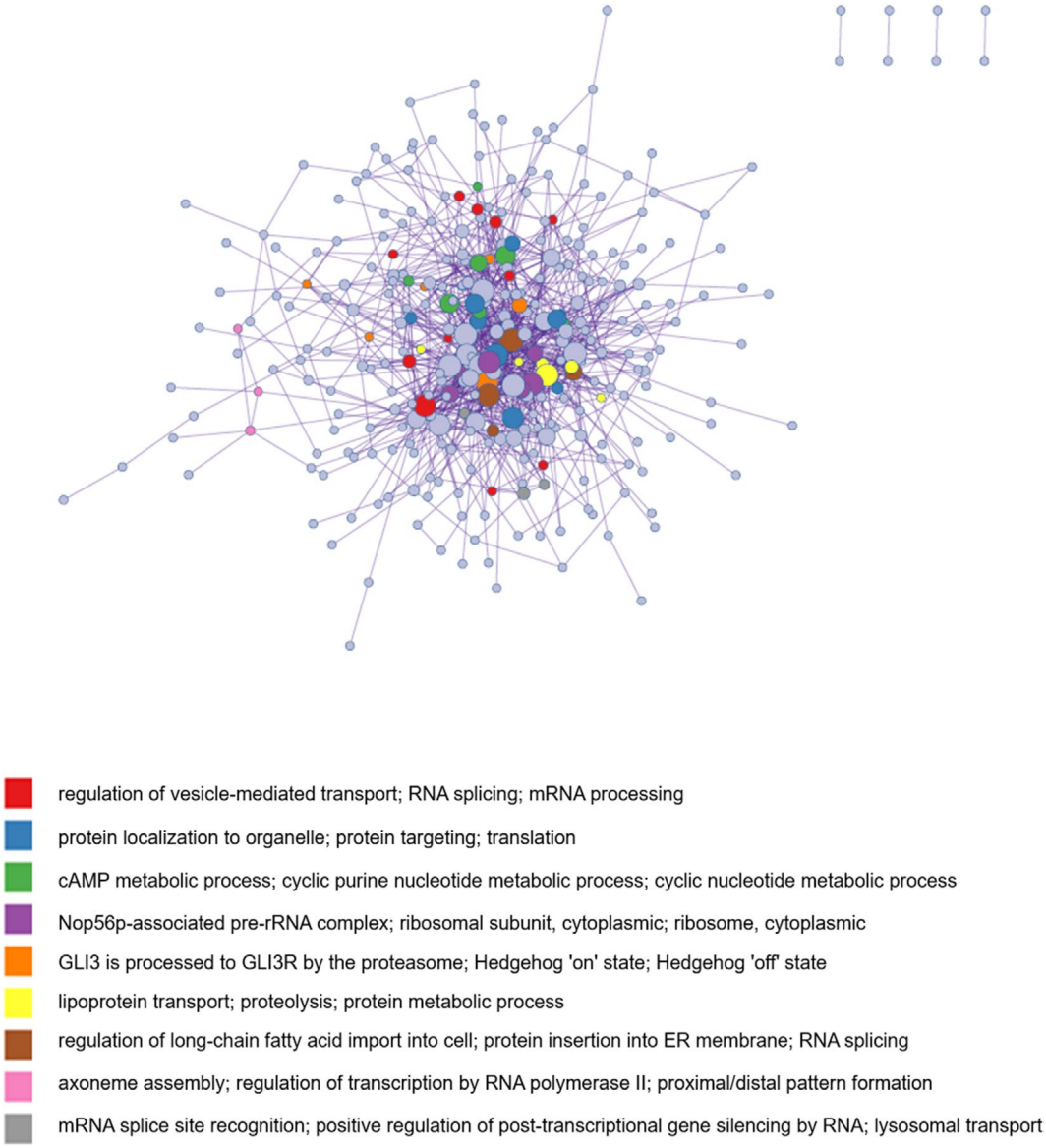

FIGURE 3 | Gene–gene interaction network in the children from the INT group. The gene–gene interaction network represents the interactions between the genes. The "circle nodes" represent sets of genes with diverse biological functions that belong to densely connected neighborhoods, and the connector edges between the circle nodes represent the interactions with a physical score higher than 0.132. The gene–gene interactions were constructed based on known interactions among the genes in the list, with the bioinformatics software Metascape, and the network was visualized with the bioinformatics software Cytoscape. Each color represents the biological function of the set of genes in the densely connected neighborhoods. To annotate the biological functions, Metascape retains the top three best p-values for the enriched terms in the densely connected neighborhoods. The genes with a DMP in the INT group exhibit strong interactions and high interconnectivity, suggesting an integrated and coordinated response to the training stimulus. This high interconnectivity indicates that these genes act in a collaborative manner rather than in isolation, probably being part of common and/or cooperative biological processes. On the other hand, note that the interaction network in the control group was not constructed. The genes with a DMP in the control group exhibit low interconnectivity and interactions with a physical score lower than 0.132, not meeting the threshold for constructing an interaction network established by Metascape (physical score higher than 0.132). cAMP: Cyclic adenosine monophosphate; DMP: Differentially methylated position; ER: Endoplasmic reticulum; INT: Integrated neuromuscular training.

primarily induced hypomethylation changes in hypertrophy-related genes [21, 25, 30, 63, 66]. In human skeletal muscle again, acute incremental cycling exercise on a cycle ergometer induced dose-dependent hypomethylation in promoters of exercise-responsive genes such as *PGC-1α*, *PDK4*, and *PPAR-δ* [67], whereas high-intensity interval training induced hypomethylation changes in genes related to lactate transport and calcium signaling (*SLC16A3*, *INPP5a*, and *CAPN2*) [68]. In adipose tissue of healthy adults, endurance training induced hypomethylation of genes involved in lipolysis regulation and adipogenesis, as well as hypermethylation of obesity and type 2 diabetes mellitus genes [20, 28]. Furthermore, in sperm of healthy adults, sprint training induced hypomethylation of genes related to insulin receptor signaling pathways and cardiac muscle contraction [23]. In the blood of healthy adults, sprint training induced hypomethylation of genes related to angiogenesis, blood vessel cell migration, and cardiovascular function, as well as hypermethylation of cardiovascular disease-related genes [29]. In plasma

TABLE 3 | DMPs in core genes after a 3-month INT in 7–9-year-old school children.

CpG probe	Gene name	Gene Aliases	Pre (%)	Post (%)	Direction of methylation changes (*post* versus *pre*)	logFC	*p*	Molecular function	Disease
cg10659575	*BIRC3*	*AIP1, API2, CIAP2, HAIP1, HIAP1, IAP-1, MALT2, MIHC, RNF49, c-IAP2*	8.02	8.14	Hypermethylated	−0.95	**< 0.05**	Inflammatory signaling and immunity	Pancreatic cancer, renal cancer, gastric cancer, prostate cancer, colorectal cancer, lung cancer, leukemia
cg21745184	*CLTC*	*CHC, CHC17, CLH-17L2, Hc, MRD56, CLTC*	4.68	4.53	Hypomethylated	−1.01	**< 0.05**	Protein binding	Mental retardation, renal carcinoma, Noonan syndrome
cg24277128	*CSNK2B*	*CK2B, CK2N, CSK2B, Ckb1, Ckb2, G5A, POBINDS*	11.59	11.53	Hypomethylated	−0.97	**< 0.05**	Protein binding and signaling	Poirier-Bienvenu neurodevelopmental syndrome, autosomal dominant non-syndromic intellectual disability
cg10289843	*CTSL*	*CATL1, MEP, CTSL*	6.04	5.87	Hypomethylated	−0.90	**< 0.05**	Protein, fibronectin and collagen binding	Respiratory syndrome, ischemia, Alzheimer disease, lung cancer, ovarian cancer
cg11087358	*DCTN2*	*DCTN50, DYNAMITIN, HEL-S-77, RBP50*	8.45	8.83	Hypermethylated	−0.83	**< 0.05**	Protein binding	Neuropathy, Perry syndrome
cg15202813	*DDB1*	*DDBA, UV-DDB1, WHIKERS, XAP1, XPCE, XPE, XPE-BF*	7.55	7.56	Hypermethylated	−0.85	**< 0.05**	Protein and nucleic acid binding	Macular dystrophy, White-Kernohan syndrome
cg11958594	*FASN*	*FAS, OA-519, SDR27X1*	11.05	10.82	Hypomethylated	−0.61	**< 0.05**	Fatty acid activity, catalytic activity	Glucose intolerance, diabetes mellitus, fatty liver, prostate cancer, endometrial cancer, breast cancer
cg25116329	*FBXW11*	*BTRC2, BTRCP2, FBW1B, FBXW1B, Fbw11, Hos, NEDJED*	4.82	4.55	Hypomethylated	−1.23	**< 0.05**	Protein binding	Neuropathy, intellectual disability
cg20913106	*FGFR1*	*BFGFR, CD331, CEK, ECCL, FGFBR, FGFR-1, FLG, FLT-2, FLT2, HBGFR, HH2, HRTFDS, KAL2, N-SAM, OGD, bFGF-R-1*	5.60	6.29	Hypermethylated	−1.29	**< 0.05**	Fibroblast growth factor receptor activity	Hypogonadism, Muenke syndrome, lung cancer

(Continues)

TABLE 3 | (Continued)

CpG probe	Gene name	Gene Aliases	Pre (%)	Post (%)	Direction of methylation changes (*post* versus *pre*)	logFC	*p*	Molecular function	Disease
cg12996903	*GNAI2*	*GIPB, HG1C, H_LUCA15.1, H_LUCA16.1, GNAI2*	5.39	5.16	Hypomethylated	−1.56	**< 0.05**	Protein binding	Ventricular tachycardia, hyperaldosteronism, pituitary adenoma, hypoglycemia
cg13782781	*HSPA2*	*HSP70-2, HSP70-3*	4.49	3.91	Hypomethylated	−1.53	**< 0.05**	ATP binding, ATP hydrolysis activity	Infertility, gastric cancer
cg08862778	*MTOR*	*FRAP, FRAP1, FRAP2, RAFT1, RAPT1, SKS*	6.01	6.73	Hypermethylated	−1.38	**< 0.05**	Nucleotide binding	Melanoma, cervical cancer, renal cancer, breast cancer
cg14868726	*NDC80*	*HEC, HEC1, HsHec1, KNTC2, TID3, hsNDC80*	8.32	8.15	Hypomethylated	−0.78	**< 0.05**	Protein binding	Endometrial carcinoma, uterine corpus cancer
cg19552494	*PRPF19*	*NMP200, PRP19, PSO4, SNEV, UBOX4, hPSO4*	6.76	6.55	Hypomethylated	−0.90	**< 0.05**	Protein binding	Neutropenia
cg22159483	*PRPF8*	*HPRP8, PRP8, PRPC8, RP13, SNRNP220*	9.51	9.22	Hypomethylated	−0.95	**< 0.05**	Protein binding	Neurodevelopmental disorder
cg02048674	*RPL13A*	*L13A, TSTA1, uL13*	8.08	8.26	Hypermethylated	−1.17	**< 0.05**	Cellular response to stress and stimuli	Anemia, spermatogenic failure
cg12445792	*SUPT16H*	*CDC68, FACTP140, NEDDFAC, SPT16, SPT16/CDC68*	8.57	7.55	Hypomethylated	−0.79	**< 0.05**	Protein binding	Neurodevelopmental disorder, autism spectrum disorder

Core genes were identified only in the INT group after the accomplishment of the following 3 criteria: 1) participation in the enriched pathways; 2) high degree of connectivity (≥ 10 connections); and 3) target genes of key transcription factors. Note that none of the candidate positions in the control group fulfilled the 3-point criteria for recognizing core genes. The core gene analysis was performed on DMPs (post versus pre) in both groups. Significance level was set at $p < 0.05$ (marked in bold). Gene aliases were extracted from the National Library of Medicine. Molecular function and disease were extracted from the Gene Cards database. DMPs: differentially methylated positions; INT: integrated neuromuscular training; logFC: log fold change (post versus pre); %: percentage.

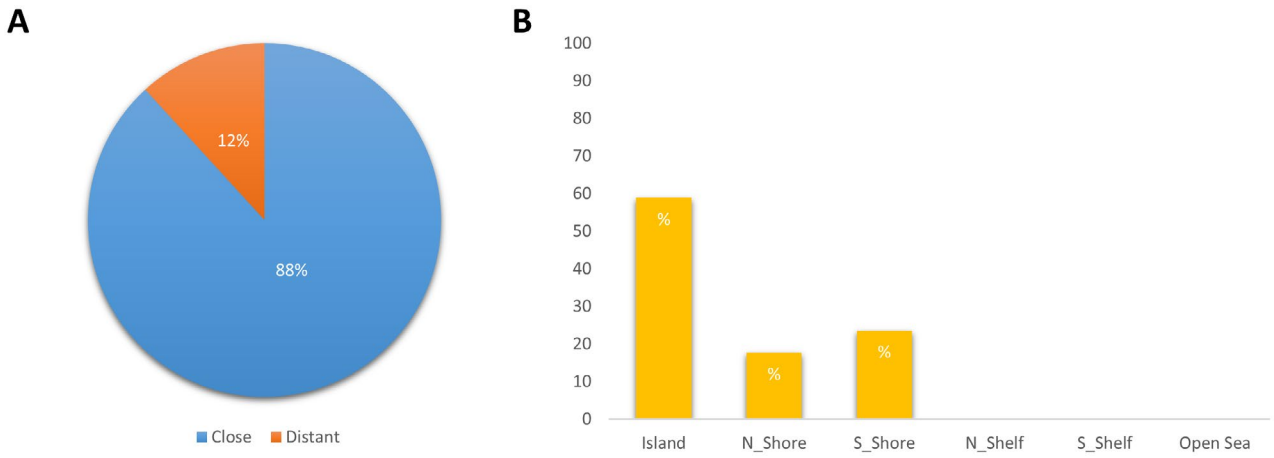

FIGURE 4 | Genomic location of methylation changes in the core genes. (A) In relation to the transcription start site (TSS): 1) close to the TSS (TSS1500, TSS200, 5'UTR, and First Exon) and 2) distant from the TSS (Body, 3'UTR, and Intergenic). (B) In relation to the CpG site: CpG island, N and S shores (< 2000 bases from the CpG island), N and S shelves (2000 to 4000 bases from the CpG island), and open sea (> 4000 bases from the CpG island).

of hypertensive rats, treadmill running induced hypermethylation of genes related to arterial function [19], while in blood, trotting exercise in horses induced hypermethylation of genes associated with cell division, signaling, adhesion, and transport [69]. Supporting previous scientific evidence, the present study also showed some differences, that is, diverse enriched pathways between groups, given that the INT is a short training intervention with a mixed type exercise modality, combining aerobic and anaerobic activities, whereas the traditional warm-up protocol in the control group included only aerobic activities.

No core genes were identified in the control group following the implementation of the core gene analysis on the genes with a DMP after the 3-month period. Remarkably, aligning with the second hypothesis, according to which we expected to find DNAm changes in some core genes in children that performed the 3-month INT, we identified 17 core genes with a DMP, which were targets of key transcription factors that are regulating inflammation, immune response, cell differentiation, and tumor mechanisms. For instance, the transcription factor NF-κB plays a crucial role in regulating inflammation, immune response, cell proliferation, differentiation, and cell survival [62]. Indeed, targeting the NF-κB pathway has been proposed as a potential therapy for various chronic diseases [70, 71]. The transcription factor AIRD3A has diverse regulatory roles in immune response, hematopoiesis, DNA damage repair, and tumor and cancer suppression mechanisms [72, 73]. Furthermore, BHLHE40 is a transcription factor regulating various biological processes such as cell differentiation, inflammation modulation, immune response, and anti-tumor mechanisms [74, 75]. ATF2 is a transcription factor that has a role in stress response, DNA damage repair, immune response, and regulation of cell growth [76]. In addition, we consider that it is important to highlight that the DMPs in the children who performed the 3-month INT were in core genes related to inflammation, fat metabolism, protein metabolism, ATP binding, and growth pathways, involved in the regulation of metabolic, cardiovascular, neurodegenerative diseases, musculoskeletal disorders, cancer, and various syndromes [77]. Indeed, it is widely known that a potential cause for the development or progression of a certain disease can be the altered expression of genes associated with that disease, mediated by abnormal DNAm [38]. However, previous research has indicated that physical exercise may partially reverse aberrant methylation patterns [78]. In this line, 88% of the methylation changes in the core genes in the present study were close to the TSS (near a promoter region), and 59% were in CpG islands, therefore potentially regulating gene expression by inducing transcriptional silencing or activation [7–9, 79, 80]. Nevertheless, we must note that in this study, we did not assess the transcriptome or the proteome; thus, we are uncertain to which extent the observed methylation changes may be reflected in gene expression as previously described or on a protein concentration level [7–9, 79, 80]. However, previous studies integrating the methylome and the transcriptome in human skeletal muscle found similar alterations at both the DNAm and gene expression levels [21, 25, 30, 63]. For instance, after a single resistance exercise bout, as well as after resistance training, cancer-related genes were affected at both the methylome and transcriptome levels [30]. Furthermore, in middle-aged adults with morbid obesity and type 2 diabetes mellitus, differential DNAm and gene expression changes were found in response to endurance training in genes related to functional metabolic and microvascular plasticity, which are crucial for diabetes rehabilitation [21]. A single resistance exercise bout in untrained men induced differential methylation changes and altered gene expression in genes related to MAPK signaling, axon guidance, focal adhesion, and actin cytoskeleton [25]. Differential methylation changes and altered gene expression were also found in hypertrophy-related genes after resistance training intervention [25]. In addition, genes significantly altered at the mRNA level (upregulated or downregulated) 6 h following resistance exercise in trained college-aged men showed significantly inversed DNAm patterns across one or more CpG sites 3 h following exercise [63]. Indeed, a bioinformatics overlay analysis of DNAm and mRNA expression data showed that affected genes were related to signaling mechanisms (MAPK and PI3K-Akt signaling) and focal adhesion [63]. With regard to methylome and proteome integration studies, in mice skeletal muscle, changes at the methylome level were associated with changes at the proteome level in proteins related to mitochondrial translation after a progressive

weighted wheel running exercise [47]. Moreover, several transcription factors, including MYC, were proposed as regulators of the exercise-induced methylome-proteome landscape [47]. In skeletal muscle of trained men, a methylome-proteome integrative analysis showed a bidirectional relationship between methylome and proteome changes after 3 months of high-intensity interval training, mainly in genes and proteins related to mitochondrial and metabolic pathways [81]. Considering the previous and current findings, and also given that numerous studies have proposed physical exercise interventions for health promotion and disease prevention, or in addition to the traditional therapy for a more effective treatment for various diseases [82, 83], we believe that the present study should encourage future research integrating the salivary methylome, transcriptome, and proteome after an INT in children. These studies can reinforce and expand the present findings, and further explore the potential implications of an INT for health promotion and disease prevention in children.

4.1 | Limitations and Future Research

This is the first study assessing the effects of physical exercise, specifically INT, on the genome-wide DNAm in saliva from 7 to 9-year-old children, and the present findings should be interpreted considering the following limitations:

First, we have to note that skeletal muscle is the most affected tissue during exercise, not saliva [38, 84]. This means that one would potentially expect more prominent DNAm changes in skeletal muscle, thus considering it as a more suitable tissue for DNAm assessment in the context of exercise-induced effects. However, since the assessment of genome-wide DNAm in skeletal muscle requires biopsy, this kind of DNAm analysis in children is not possible due to ethical reasons. Although DNAm is tissue-specific [38], it is worthwhile to note that recent studies showed similar methylation profiles between saliva, blood, and various tissues, highlighting the utility of saliva as a non-invasive alternative for DNAm assessment [51, 85–87]. Indeed, salivary DNA has been shown to originate mainly from the polymorphonucleated leukocytes, thus apparently offering a homogeneous source of DNA for the methylation analysis [51]. However, the cellular composition of saliva is predominantly made up of polymorphonucleated leukocytes and epithelial cells, thus the extent to which DNA coming from these different cell types may impact the DNAm results should be further explored. Additionally, we also believe that future studies in adult populations should compare the genome-wide DNAm in skeletal muscle and saliva induced by the same exercise intervention and reinforce the idea of the potential utility of saliva in the assessment of exercise-induced DNAm changes.

Another limitation of the present study is the lack of transcriptome and proteome data. We think that it is really important to investigate if the observed methylation changes are subsequently reflected in gene expression, as well as on a protein concentration level. Thus, we suggest further research trying to integrate the methylome, the transcriptome, and even the proteome, aiming to obtain a more comprehensive understanding on the effects induced by INT interventions.

Furthermore, the absence of a control group that does not have structured exercise sessions should also be considered as a study limitation. Given that we targeted school children in the present study, the inclusion of such a control group was not possible because all children in schools have regular PE classes as part of the national curricula. However, we believe that it is necessary to design a further study involving pre-school children and include a control group with no structured exercise to overcome the current limitation.

The lack of continuous heart rate monitoring during the intervention or the absence of GPS measures can also be considered as a limitation of the present study. Thus, we propose future studies to overcome this limitation by introducing monitoring devices that will collect data during the training sessions in order to provide a better understanding on the effects of the INT.

Finally, the major limitations of this study are the short duration of the INT intervention and the sample size, consequently affecting the study's statistical power. Even though we tried to apply rigorous and stringent statistical and bioinformatics analyses (including a core genes analysis approach based on enrichment analysis, gene–gene interaction analysis, and transcription factor target analysis) that may help in overcoming the limitation related to the study's sample size, we still believe that future studies involving higher sample sizes should be designed to assure higher statistical power and further generalization of the study's findings. We also suggest future studies to implement INT interventions with longer duration in order to examine if more eminent changes in DNAm may be obtained after longer training interventions.

4.2 | Perspectives

Besides the previously mentioned limitations, the present findings observed in the saliva of 7–9-year-old children are in line with previous scientific reports from blood, sperm, adipose tissue, and skeletal muscle in adults, older individuals, and animal models, thus we believe that they should serve as a starting point for future research. The present study may encourage further studies on the methylome in saliva, especially in the pediatric population, where skeletal muscle tissue is not available and genome-wide DNAm studies are scarce. We believe that genome-wide DNAm studies in saliva after exercise training in children are important to bridge the current gap in scientific literature.

Also, it is worth considering that these findings seem to offer a plausible explanation for some of the epigenetic mechanisms that may underlie the health benefits induced by INT in children; thus, further research integrating the methylome, transcriptome, and proteome will be crucial to explore if these methylation changes are subsequently reflected in gene expression, as well as on a protein concentration level.

5 | Conclusion

Differential methylation changes after the 3-month period were observed in both the control and the INT groups, with a non-significant trend of epigenetic age acceleration in the control

group and a non-significant 1-month decrease in the epigenetic age of the children from the INT group. Interestingly, the 3-month INT applied twice weekly during PE classes at schools induced an integrated and coordinated response to the training stimulus in children, encompassing methylation changes in similar and related pathways, exhibiting strong interactions and high interconnectivity. On the other hand, the response in the children from the control group was more chaotic, encompassing distinct and less related pathways (most of them development and growth-related), exhibiting weak interactions and low interconnectivity. Remarkably, 17 out of 414 DMPs after the 3-month INT were in core genes that were target genes of key transcription factors. All these core genes were involved in disease regulation and related to inflammation, fat metabolism, protein metabolism, ATP binding, and growth. Eighty-eight percent of the methylation changes in the core genes were close to the TSS (near a promoter region), and 59% were in CpG islands.

Author Contributions

F.V. conceived and designed the study, performed the statistical analyses, analyzed data, interpreted the results, and wrote the first draft of the manuscript. R.F.-L. conceived and designed the study, supervised the intervention, collected data, analyzed data, interpreted the results, edited, and critically reviewed the manuscript. V.L.-R. interpreted the results, edited, and critically reviewed the manuscript. J.B. edited and critically reviewed the manuscript. A.N.-C. performed the statistical analysis, interpreted the results, edited, and critically reviewed the manuscript. M.E. interpreted the results, edited, and critically reviewed the manuscript. A.L.-B. interpreted the results, edited, and critically reviewed the manuscript. A.P.-P. conceived and designed the study, collected data, analyzed data, performed the statistical analyses, interpreted the results, edited, and critically reviewed the manuscript. All authors approved the final version submitted for publication.

Acknowledgments

The authors are grateful to all children who took part in the study and their parents, as well as to all researchers who helped with data collection and made their valuable contribution to this research.

Ethics Statement

The research was approved by the Institutional Review Board of Dr. Josep Trueta Hospital, Girona, Spain (CEIm:2016.134).

Consent

A signed informed consent was obtained from the parents of all participating children.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

DNAm data (including raw data files) are publicly available in GEO (Accession: GSE279803).

References

1. W. Zhao, C. Wang, Y. Bi, and L. Chen, "Effect of Integrative Neuromuscular Training for Injury Prevention and Sports Performance of Female Badminton Players," *BioMed Research International* 2021 (2021): 1–12, https://doi.org/10.1155/2021/5555853.

2. A. D. Faigenbaum, A. Farrell, M. Fabiano, et al., "Effects of Integrative Neuromuscular Training on Fitness Performance in Children," *Pediatric Exercise Science* 23, no. 4 (2011): 573–584, https://doi.org/10.1123/PES.23.4.573.

3. R. Font-Lladó, V. López-Ros, A. M. Montalvo, G. Sinclair, A. Prats-Puig, and A. Fort-Vanmeerhaeghe, "A Pedagogical Approach to Integrative Neuromuscular Training to Improve Motor Competence in Children: A Randomized Controlled Trail," *Journal of Strength and Conditioning Research* 34, no. 11 (2020): 3078–3085, https://doi.org/10.1519/JSC.0000000000003772.

4. B. Sañudo, J. Sánchez-Hernández, M. Bernardo-Filho, E. Abdi, R. Taiar, and J. Núñez, "Integrative Neuromuscular Training in Young Athletes, Injury Prevention, and Performance Optimization: A Systematic Review," *Applied Sciences* 9, no. 18 (2019): 3839, https://doi.org/10.3390/APP9183839.

5. M. J. Duncan, E. L. J. Eyre, and S. W. Oxford, "The Effects of 10-Week Integrated Neuromuscular Training on Fundamental Movement Skills and Physical Self-Efficacy in 6–7-Year-Old Children," *Journal of Strength and Conditioning Research* 32, no. 12 (2018): 3348–3356, https://doi.org/10.1519/JSC.0000000000001859.

6. E. R. Gibney and C. M. Nolan, "Epigenetics and Gene Expression," *Heredity (Edinb)* 105, no. 1 (2010): 4–13, https://doi.org/10.1038/hdy.2010.54.

7. S. Aurich, L. Müller, P. Kovacs, and M. Keller, "Implication of DNA Methylation During Lifestyle Mediated Weight Loss," *Front Endocrinol (Lausanne)* 14 (2023): 1181002, https://doi.org/10.3389/FENDO.2023.1181002/BIBTEX.

8. P. A. Jones, "Functions of DNA Methylation: Islands, Start Sites, Gene Bodies and Beyond," *Nature Reviews. Genetics* 13, no. 7 (2012): 484–492, https://doi.org/10.1038/NRG3230.

9. F. Brenet, M. Moh, P. Funk, et al., "DNA Methylation of the First Exon Is Tightly Linked to Transcriptional Silencing," *PLoS One* 6, no. 1 (2011): e14524, https://doi.org/10.1371/JOURNAL.PONE.0014524.

10. S. D. Li, S. Chono, and L. Huang, "Efficient Oncogene Silencing and Metastasis Inhibition via Systemic Delivery of siRNA," *Molecular Therapy* 16, no. 5 (2008): 942–946, https://doi.org/10.1038/MT.2008.51.

11. W. S. Yong, F. M. Hsu, and P. Y. Chen, "Profiling Genome-Wide DNA Methylation," *Epigenetics & Chromatin* 9, no. 1 (2016): 1–16, https://doi.org/10.1186/S13072-016-0075-3.

12. W. M. Brown, "Exercise-Associated DNA Methylation Change in Skeletal Muscle and the Importance of Imprinted Genes: A Bioinformatics Meta-Analysis," *British Journal of Sports Medicine* 49, no. 24 (2015): 1567–1578, https://doi.org/10.1136/BJSPORTS-2014-094073.

13. S. Blocquiaux, M. Ramaekers, T. R. Van, et al., "Recurrent Training Rejuvenates and Enhances Transcriptome and Methylome Responses in Young and Older Human Muscle," *JCSM Rapid Communications* 5, no. 1 (2022): 10–32, https://doi.org/10.1002/RCO2.52.

14. S. Voisin, N. R. Harvey, L. M. Haupt, et al., "An Epigenetic Clock for Human Skeletal Muscle," *Journal of Cachexia, Sarcopenia and Muscle* 11, no. 4 (2020): 887–898, https://doi.org/10.1002/JCSM.12556.

15. S. Voisin, M. Jacques, S. Landen, et al., "Meta-Analysis of Genome-Wide DNA Methylation and Integrative Omics of Age in Human Skeletal Muscle," *Journal of Cachexia, Sarcopenia and Muscle* 12, no. 4 (2021): 1064–1078, https://doi.org/10.1002/JCSM.12741.

16. S. Voisin, K. Seale, M. Jacques, et al., "Exercise Is Associated With Younger Methylome and Transcriptome Profiles in Human Skeletal Muscle," *Aging Cell* 23, no. 1 (2024): e13859, https://doi.org/10.1111/ACEL.13859.

17. D. C. Turner, P. P. Gorski, M. F. Maasar, et al., "DNA Methylation Across the Genome in Aged Human Skeletal Muscle Tissue and

Muscle-Derived Cells: The Role of HOX Genes and Physical Activity," *Scientific Reports* 10, no. 1 (2020): 1–19, https://doi.org/10.1038/s41598-020-72730-z.

18. L. M. McEwen, K. J. O'Donnell, M. G. McGill, et al., "The PedBE Clock Accurately Estimates DNA Methylation Age in Pediatric Buccal Cells," *Proceedings of the National Academy of Sciences of the United States of America* 117, no. 38 (2020): 23329–23335, https://doi.org/10.1073/PNAS.1820843116.

19. Y. Chen, S. Li, Z. Xu, Y. Zhang, H. Zhang, and L. Shi, "Aerobic Training-Mediated DNA Hypermethylation of Agtr1a and Mas1 Genes Ameliorate Mesenteric Arterial Function in Spontaneously Hypertensive Rats," *Molecular Biology Reports* 48, no. 12 (2021): 8033–8044, https://doi.org/10.1007/S11033-021-06929-2.

20. T. Rönn, P. Volkov, C. Davegårdh, et al., "A Six Months Exercise Intervention Influences the Genome-Wide DNA Methylation Pattern in Human Adipose Tissue," *PLoS Genetics* 9, no. 6 (2013): e1003572, https://doi.org/10.1371/JOURNAL.PGEN.1003572.

21. D. S. Rowlands, R. A. Page, W. R. Sukala, et al., "Multi-Omic Integrated Networks Connect DNA Methylation and miRNA With Skeletal Muscle Plasticity to Chronic Exercise in Type 2 Diabetic Obesity," *Physiological Genomics* 46, no. 20 (2014): 747–765, https://doi.org/10.1152/PHYSIOLGENOMICS.00024.2014.

22. M. D. Nitert, T. Dayeh, P. Volkov, et al., "Impact of an Exercise Intervention on DNA Methylation in Skeletal Muscle From First-Degree Relatives of Patients With Type 2 Diabetes," *Diabetes* 61, no. 12 (2012): 3322–3332, https://doi.org/10.2337/DB11-1653.

23. J. Denham, B. J. O'Brien, J. T. Harvey, and F. J. Charchar, "Genome-Wide Sperm DNA Methylation Changes After 3 Months of Exercise Training in Humans," *Epigenomics* 7, no. 5 (2015): 717–731, https://doi.org/10.2217/EPI.15.29.

24. J. Denham, F. Z. Marques, E. L. Bruns, B. J. O'Brien, and F. J. Charchar, "Epigenetic Changes in Leukocytes After 8 Weeks of Resistance Exercise Training," *European Journal of Applied Physiology* 116, no. 6 (2016): 1245–1253, https://doi.org/10.1007/S00421-016-3382-2.

25. R. A. Seaborne, J. Strauss, M. Cocks, et al., "Human Skeletal Muscle Possesses an Epigenetic Memory of Hypertrophy," *Scientific Reports* 8, no. 1 (2018): 1898, https://doi.org/10.1038/S41598-018-20287-3.

26. M. E. Lindholm, F. Marabita, D. Gomez-Cabrero, et al., "An Integrative Analysis Reveals Coordinated Reprogramming of the Epigenome and the Transcriptome in Human Skeletal Muscle After Training," *Epigenetics* 9, no. 12 (2014): 1557–1569, https://doi.org/10.4161/15592294.2014.982445.

27. M. R. Sailani, J. F. Halling, H. D. Møller, et al., "Lifelong Physical Activity Is Associated With Promoter Hypomethylation of Genes Involved in Metabolism, Myogenesis, Contractile Properties and Oxidative Stress Resistance in Aged Human Skeletal Muscle," *Scientific Reports* 9, no. 1 (2019): 3272, https://doi.org/10.1038/S41598-018-37895-8.

28. O. Fabre, L. R. Ingerslev, C. Garde, I. Donkin, D. Simar, and R. Barrès, "Exercise Training Alters the Genomic Response to Acute Exercise in Human Adipose Tissue," *Epigenomics* 10, no. 8 (2018): 1033–1050, https://doi.org/10.2217/EPI-2018-0039.

29. J. Denham, B. J. O'Brien, F. Z. Marques, and F. J. Charchar, "Changes in the Leukocyte Methylome and Its Effect on Cardiovascular-Related Genes After Exercise," *Journal of Applied Physiology* 118, no. 4 (2015): 475–488, https://doi.org/10.1152/JAPPLPHYSIOL.00878.2014.

30. D. C. Turner, R. A. Seaborne, and A. P. Sharples, "Comparative Transcriptome and Methylome Analysis in Human Skeletal Muscle Anabolism, Hypertrophy and Epigenetic Memory," *Scientific Reports* 9, no. 1 (2019): 1–12, https://doi.org/10.1038/s41598-019-40787-0.

31. M. Ehrlich, "DNA Hypermethylation in Disease: Mechanisms and Clinical Relevance," *Epigenetics* 14, no. 12 (2019): 1141–1163, https://doi.org/10.1080/15592294.2019.1638701.

32. H. Maehara, T. Kokaji, A. Hatano, et al., "DNA Hypomethylation Characterizes Genes Encoding Tissue-Dominant Functional Proteins in Liver and Skeletal Muscle," *Scientific Reports* 13, no. 1 (2023): 1–19, https://doi.org/10.1038/s41598-023-46393-5.

33. F. He, J. Li, Z. Liu, C. C. Chuang, W. Yang, and L. Zuo, "Redox Mechanism of Reactive Oxygen Species in Exercise," *Frontiers in Physiology* 7 (2016): 486, https://doi.org/10.3389/FPHYS.2016.00486.

34. R. Furrer, B. Heim, S. Schmid, et al., "Molecular Control of Endurance Training Adaptation in Male Mouse Skeletal Muscle," *Nature Metabolism* 5, no. 11 (2023): 2020–2035, https://doi.org/10.1038/s42255-023-00891-y.

35. S. Dufresne, J. Guéritat, C. P. Wong, et al., "Exercise Training as a Modulator of Epigenetic Events in Prostate Tumors," *Prostate Cancer and Prostatic Diseases* 25, no. 1 (2021): 119–122, https://doi.org/10.1038/s41391-021-00380-x.

36. A. S. Gillman, T. Helmuth, C. E. Koljack, K. E. Hutchison, W. M. Kohrt, and A. D. Bryan, "The Effects of Exercise Duration and Intensity on Breast Cancer-Related DNA Methylation: A Randomized Controlled Trial," *Cancers (Basel)* 13, no. 16 (2021): 4128, https://doi.org/10.3390/CANCERS13164128.

37. P. P. Gorski, T. Raastad, M. Ullrich, et al., "Aerobic Exercise Training Resets the Human Skeletal Muscle Methylome 10 Years After Breast Cancer Treatment and Survival," *FASEB Journal* 37, no. 1 (2023): e22720, https://doi.org/10.1096/FJ.202201510RR.

38. F. Vasileva, R. Hristovski, R. Font-Lladó, et al., "Physical Exercise-Induced DNA Methylation in Disease-Related Genes in Healthy Adults-A Systematic Review With Bioinformatic Analysis," *Journal of Strength and Conditioning Research* 38, no. 2 (2024): 384–393, https://doi.org/10.1519/JSC.0000000000004686.

39. N. Segata and C. Huttenhower, "Toward an Efficient Method of Identifying Core Genes for Evolutionary and Functional Microbial Phylogenies," *PLoS One* 6, no. 9 (2011): e24704, https://doi.org/10.1371/JOURNAL.PONE.0024704.

40. B. Yang, M. Zhang, and T. Luo, "Identification of Potential Core Genes Associated With the Progression of Stomach Adenocarcinoma Using Bioinformatic Analysis," *Frontiers in Genetics* 11 (2020): 1282, https://doi.org/10.3389/FGENE.2020.517362/BIBTEX.

41. P. Kumar, A. K. Singh, K. N. Tiwari, et al., "Identification and Validation of Core Genes as Promising Diagnostic Signature in Hepatocellular Carcinoma Based on Integrated Bioinformatics Approach," *Scientific Reports* 12, no. 1 (2022): 1–10, https://doi.org/10.1038/s41598-022-22059-6.

42. A. Ratnakumar, N. Weinhold, J. C. Mar, and N. Riaz, "Protein-Protein Interactions Uncover Candidate 'Core Genes' Within Omnigenic Disease Networks," *PLoS Genetics* 16, no. 7 (2020): e1008903, https://doi.org/10.1371/JOURNAL.PGEN.1008903.

43. S. Liu, Y. Zhao, R. Duan, Y. Wu, X. Chen, and N. Li, "Identification of Core Genes Associated With Type 2 Diabetes Mellitus and Gastric Cancer by Bioinformatics Analysis," *Annals of Translational Medicine* 10, no. 5 (2022): 247–247, https://doi.org/10.21037/ATM-21-3635.

44. L. R. Ingerslev, I. Donkin, O. Fabre, et al., "Endurance Training Remodels Sperm-Borne Small RNA Expression and Methylation at Neurological Gene Hotspots," *Clinical Epigenetics* 10, no. 1 (2018): 1–11, https://doi.org/10.1186/S13148-018-0446-7/FIGURES/4.

45. F. Von Walden, M. Rea, C. B. Mobley, et al., "The Myonuclear DNA Methylome in Response to an Acute Hypertrophic Stimulus," *Epigenetics* 15, no. 11 (2020): 1151–1162, https://doi.org/10.1080/15592294.2020.1755581.

46. Y. Wen, C. M. Dungan, C. B. Mobley, T. Valentino, F. Von Walden, and K. A. Murach, "Nucleus Type-Specific DNA Methylomics Reveals Epigenetic "Memory" of Prior Adaptation in Skeletal Muscle," *Funct (Oxford, England)* 2, no. 5 (2021): zqab038, https://doi.org/10.1093/FUNCTION/ZQAB038.

47. T. L. Chambers, A. Dimet-Wiley, A. R. Keeble, et al., "Methylome-Proteome Integration After Late-Life Voluntary Exercise Training Reveals Regulation and Target Information for Improved Skeletal Muscle Health," *Journal of Physiology* (2024), https://doi.org/10.1113/JP286681. Online ahead of print.

48. N. J. Bonne and D. T. W. Wong, "Salivary Biomarker Development Using Genomic, Proteomic and Metabolomic Approaches," *Genome Medicine* 4, no. 10 (2012): 1–12, https://doi.org/10.1186/GM383/FIGURES/1.

49. E. M. O'Sullivan, P. Dowling, D. Swandulla, and K. Ohlendieck, "Proteomic Identification of Saliva Proteins as Noninvasive Diagnostic Biomarkers," *Methods in Molecular Biology* 2596 (2023): 147–167, https://doi.org/10.1007/978-1-0716-2831-7_12.

50. S. A. S. Langie, M. Moisse, K. Declerck, et al., "Salivary DNA Methylation Profiling: Aspects to Consider for Biomarker Identification," *Basic & Clinical Pharmacology & Toxicology* 121 (2017): 93–101, https://doi.org/10.1111/BCPT.12721.

51. M. E. Wren, E. A. Shirtcliff, and S. S. Drury, "Not all Biofluids Are Created Equal: Chewing Over Salivary Diagnostics and the Epigenome," *Clinical Therapeutics* 37, no. 3 (2015): 529–539, https://doi.org/10.1016/J.CLINTHERA.2015.02.022.

52. D. Fradin, P. Y. Boëlle, M. P. Belot, et al., "Genome-Wide Methylation Analysis Identifies Specific Epigenetic Marks in Severely Obese Children," *Scientific Reports* 7, no. 1 (2017): 1–8, https://doi.org/10.1038/srep46311.

53. R. Fernandez-Agullo and R. Merino-Marban, "Effect of Warm-up on Fitness Performance of Schoolchildren. A Systematic Review," *Polish Journal of Sport and Tourism* 29, no. 1 (2022): 3–8, https://doi.org/10.2478/PJST-2022-0001.

54. A. Carrascosa-Lezcano, J. Fernandez-Garcia, A. Ferandez-Longas, J. Lopez-Siguero, and E. Sanchez-Gonzales, *Cross-Sectional Study of Growth and Development*, 1st ed. (Pfizer: Barcelona, Spain, 2008).

55. A. Noguera-Castells, C. A. García-Prieto, D. Álvarez-Errico, and M. Esteller, "Validation of the New EPIC DNA Methylation Microarray (900K EPIC v2) for High-Throughput Profiling of the Human DNA Methylome," *Epigenetics* 18, no. 1 (2023): 2185742, https://doi.org/10.1080/15592294.2023.2185742.

56. D. Pelegi-Siso, P. De Prado, J. Ronkainen, M. Bustamante, and J. R. Gonzalez, "Methylclock: A Bioconductor Package to Estimate DNA Methylation Age," *Bioinformatics* 37, no. 12 (2021): 1759–1760, https://doi.org/10.1093/BIOINFORMATICS/BTAA825.

57. Y. Zhou, B. Zhou, L. Pache, et al., "Metascape Provides a Biologist-Oriented Resource for the Analysis of Systems-Level Datasets," *Nature Communications* 10, no. 1 (2019): 1523, https://doi.org/10.1038/S41467-019-09234-6.

58. P. Shannon, A. Markiel, O. Ozier, et al., "Cytoscape: A Software Environment for Integrated Models of Biomolecular Interaction Networks," *Genome Research* 13, no. 11 (2003): 2498–2504, https://doi.org/10.1101/GR.1239303.

59. G. D. Bader and C. W. V. Hogue, "An Automated Method for Finding Molecular Complexes in Large Protein Interaction Networks," *BMC Bioinformatics* 4, no. 1 (2003): 1–27, https://doi.org/10.1186/1471-2105-4-2/FIGURES/12.

60. G. Bebek, "Identifying Gene Interaction Networks," in *Statistical Human Genetics. Methods in Molecular Biology. Vol 850*, eds. R. Elston, J. Satagopan, and S. Sun (New Jersey, NJ: Humana Press, 2012), 483–494, https://doi.org/10.1007/978-1-61779-555-8_26.

61. R. M. Myers, J. Stamatoyannopoulos, M. Snyder, et al., "A User's Guide to the Encyclopedia of DNA Elements (ENCODE)," *PLoS Biology* 9, no. 4 (2011): e1001046, https://doi.org/10.1371/JOURNAL.PBIO.1001046.

62. A. Oeckinghaus and S. Ghosh, "The NF-κB Family of Transcription Factors and Its Regulation," *Cold Spring Harbor Perspectives in Biology* 1, no. 4 (2009): a000034, https://doi.org/10.1101/CSHPERSPECT.A000034.

63. C. L. Sexton, J. S. Godwin, M. C. McIntosh, et al., "Skeletal Muscle DNA Methylation and mRNA Responses to a Bout of Higher Versus Lower Load Resistance Exercise in Previously Trained Men," *Cells* 12, no. 2 (2023): 263, https://doi.org/10.3390/CELLS12020263.

64. M. F. Maasar, D. C. Turner, P. P. Gorski, et al., "The Comparative Methylome and Transcriptome After Change of Direction Compared to Straight Line Running Exercise in Human Skeletal Muscle," *Frontiers in Physiology* 12 (2021): 619447, https://doi.org/10.3389/FPHYS.2021.619447/BIBTEX.

65. P. P. Gorski, D. C. Turner, J. Iraki, J. P. Morton, A. P. Sharples, and J. L. Areta, "Human Skeletal Muscle Methylome After Low-Carbohydrate Energy-Balanced Exercise," *American Journal of Physiology. Endocrinology and Metabolism* 324, no. 5 (2023): E437–E448, https://doi.org/10.1152/AJPENDO.00029.2023.

66. S. Edman, R. G. Jones, P. R. Jannig, et al., "The 24-Hour Molecular Landscape After Exercise in Humans Reveals MYC Is Sufficient for Muscle Growth," *EMBO Reports* 25, no. 12 (2024): 5810–5837, https://doi.org/10.1038/S44319-024-00299-Z.

67. R. Barrès, J. Yan, B. Egan, et al., "Acute Exercise Remodels Promoter Methylation in Human Skeletal Muscle," *Cell Metabolism* 15, no. 3 (2012): 405–411, https://doi.org/10.1016/j.cmet.2012.01.001.

68. A. M. Pilotto, D. C. Turner, R. Mazzolari, et al., "Human Skeletal Muscle Possesses an Epigenetic Memory of High Intensity Interval Training," *American Journal of Physiology. Cell Physiology* 328, no. 1 (2024): C258–C272, https://doi.org/10.1152/AJPCELL.00423.2024. Online ahead of print.

69. J.-A. Gim, C. Pyo Hong, D.-S. Kim, et al., "Genome-Wide Analysis of DNA Methylation Before-and After Exercise in the Thoroughbred Horse With MeDIP-Seq," *Molecules and Cells* 38, no. 3 (2015): 210–220, https://doi.org/10.14348/molcells.2015.2138.

70. H. Yu, L. Lin, Z. Zhang, H. Zhang, and H. Hu, "Targeting NF-κB Pathway for the Therapy of Diseases: Mechanism and Clinical Study," *Signal Transduction and Targeted Therapy* 5, no. 1 (2020): 1–23, https://doi.org/10.1038/s41392-020-00312-6.

71. A. B. Kunnumakkara, B. Shabnam, S. Girisa, et al., "Inflammation, NF-κB, and Chronic Diseases: How Are They Linked?," *Critical Reviews in Immunology* 40, no. 1 (2020): 1–39, https://doi.org/10.1615/CRITREVIMMUNOL.2020033210.

72. M. L. Ratliff, M. Mishra, M. B. Frank, J. M. Guthridge, and C. F. Webb, "The Transcription Factor ARID3a is Important for in Vitro Differentiation of Human Hematopoietic Progenitors," *Journal of Immunology* 196, no. 2 (2016): 614–623, https://doi.org/10.4049/JIMMUNOL.1500355.

73. O. Alejo-Valle, K. Weigert, R. Bhayadia, et al., "The Megakaryocytic Transcription Factor ARID3A Suppresses Leukemia Pathogenesis," *Blood* 139, no. 5 (2022): 651–665, https://doi.org/10.1182/BLOOD.2021012231.

74. M. E. Cook, N. N. Jarjour, C. C. Lin, and B. T. Edelson, "Transcription Factor Bhlhe40 in Immunity and Autoimmunity," *Trends in Immunology* 41, no. 11 (2020): 1023–1036, https://doi.org/10.1016/J.IT.2020.09.002/ASSET/0617170C-C2AC-4889-A34C-B25A0E5F44C8/MAIN.ASSETS/GR3.SML.

75. C. Li, B. Zhu, Y. M. Son, et al., "The Transcription Factor Bhlhe40 Programs Mitochondrial Regulation of Resident CD8+ T Cell Fitness and Functionality," *Immunity* 51, no. 3 (2019): 491–507.e7.

76. A. Bhoumik, S. Takahashi, W. Breitweiser, Y. Shiloh, N. Jones, and Z. Ronai, "ATM-Dependent Phosphorylation of ATF2 Is Required for the DNA Damage Response," *Molecular Cell* 18, no. 5 (2005): 577–587, https://doi.org/10.1016/J.MOLCEL.2005.04.015.

77. Gene Cards, "The Human Gene Database," last modified December 23, 2024, https://www.genecards.org/.

78. B. K. Pedersen and B. Saltin, "Exercise as Medicine - Evidence for Prescribing Exercise as Therapy in 26 Different Chronic Diseases," *Scandinavian Journal of Medicine & Science in Sports* 25 (2015): 1–72, https://doi.org/10.1111/SMS.12581.

79. W. J. Lim, K. H. Kim, J. Y. Kim, S. Jeong, and N. Kim, "Identification of DNA-Methylated CpG Islands Associated With Gene Silencing in the Adult Body Tissues of the Ogye Chicken Using RNA-Seq and Reduced Representation Bisulfite Sequencing," *Frontiers in Genetics* 10, no. APR (2019): 346, https://doi.org/10.3389/FGENE.2019.00346.

80. A. Prats-Puig, S. García-Retortillo, M. Puig-Parnau, et al., "DNA Methylation Reorganization of Skeletal Muscle-Specific Genes in Response to Gestational Obesity," *Frontiers in Physiology* 11 (2020): 549509, https://doi.org/10.3389/FPHYS.2020.00938/BIBTEX.

81. M. Jacques, S. Landen, J. A. Romero, et al., "Methylome and Proteome Integration in Human Skeletal Muscle Uncover Group and Individual Responses to High-Intensity Interval Training," *FASEB Journal* 37, no. 10 (2023): e23184, https://doi.org/10.1096/FJ.202300840RR.

82. L. Montesi, S. Moscatiello, M. Malavolti, R. Marzocchi, and G. Marchesini, "Physical Activity for the Prevention and Treatment of Metabolic Disorders," *Internal and Emergency Medicine* 8, no. 8 (2013): 655–666, https://doi.org/10.1007/S11739-013-0953-7.

83. E. I. Rodríguez-Grande, O. C. Vargas-Pinilla, M. R. Torres-Narvaez, and N. Rodríguez-Malagón, "Neuromuscular Exercise in Children With Down Syndrome: A Systematic Review," *Scientific Reports* 12, no. 1 (2022): 1–12, https://doi.org/10.1038/s41598-022-19086-8.

84. L. S. Pescatello, R. Arena, D. Riebe, and P. D. Thomsen, *ACSM's Guidelines for Exercise Testing and Prescription*, 9th ed. (Philadelphia: Wolters Kluwer Health/Lippincott Williams & Wilkins, 2014).

85. A. K. Smith, V. Kilaru, T. Klengel, et al., "DNA Extracted From Saliva for Methylation Studies of Psychiatric Traits: Evidence Tissue Specificity and Relatedness to Brain," *American Journal of Medical Genetics* 168B, no. 1 (2015): 36–44, https://doi.org/10.1002/AJMG.B.32278.

86. N. L. Hearn, A. S. Coleman, V. Ho, C. L. Chiu, and J. M. Lind, "Comparing DNA Methylation Profiles in Saliva and Intestinal Mucosa," *BMC Genomics* 20, no. 1 (2019): 163, https://doi.org/10.1186/S12864-019-5553-0.

87. Ó. Rapado-González, C. Martínez-Reglero, Á. Salgado-Barreira, et al., "Salivary Dna Methylation as an Epigenetic Biomarker for Head and Neck Cancer. Part i: A Diagnostic Accuracy Meta-Analysis," *Journal of Personalized Medicine* 11, no. 6 (2021): 568, https://doi.org/10.3390/JPM11060568/S1.

Supporting Information

Additional supporting information can be found online in the Supporting Information section.