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Exercise, fitness and health in childhood obesity: a focus on transcriptomics, epigenomics, and proteomics

Ejercicio físico, condición física y salud en la obesidad infantil: un enfoque desde la transcriptómica, la epigenómica y la proteómica

PROGRAMA DE DOCTORADO EN BIOMEDICINA

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Exercise, fitness and health in childhood obesity: a focus on transcriptomics, epigenomics, and proteomics

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Lo que sabemos es una gota de agua;

lo que ignoramos es el océano (Isaac Newton)

A todos mis compañeros que me ayudan a mejorar cada día

A mis padres y hermanos,

gracias por todo vuestro amor y apoyo

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LIST OF ABBREVIATIONS

ANCOVA: Analysis of covariance

BFP: Body fat percentage

BMIQ: Beta-Mixture Quantile

BMI: Body mass index

Bp: Base pair

CpGs: Cytosine-phosphate-guanine site

CRF: Cardiorespiratory fitness

CVs: Coefficients of variation

CVD: Cardiovascular disease

DNA: Deoxyribonucleic acid

DXA: Dual-energy X-ray absorptiometry

EGF: Epidermal growth factor

eQTM: Expression quantitative trait methylation

FDR: False discovery rate

FMI: Fat mass index

GEO: Gene Expression Omnibus

GO: Gene ontology

HOMA-IR: Homeostasis model assessment for insulin resistance

HR: Heart rate

IL: Interleukin

IPA: Ingenuity Pathway Analysis

KEGG: Kyoto Encyclopedia of Genes and Genomes

LMI: Lean mass index

METs: Metabolic equivalents

MHO: Metabolic healthy overweight/obesity

miRNA: micro ribonucleic acid

mRNA: messenger ribonucleic acid

MUO: Metabolic healthy overweight/obesity

NF-kB: Nuclear factor kappa-light-chain-enhancer of activated B cells

OW/OB: Overweight/obesity

PBMCs: Peripheral blood mononuclear cells

RCT: Randomized controlled trial

RER: Respiratory exchange ratio

RNA: Ribonucleic acid

RPE: Rating of perceived exertion

SPSS: Statistical package for social sciences (IBM SPSS statistics, IBM corporation)

STRT: Single-cell tagged reverse transcription

TFBS: Transcription factor binding site

TNF-α: Tumor necrosis factor-α

TSS: Transcription start site

UTR: Untranslated region

VAT: Visceral adipose tissue

VEGFA: Vascular endothelial growth factor A

VO_2peak: Peak oxygen consumption

WC: Waist circumference

WGCNA: Weighted gene coexpression network analysis

ABSTRACT

The present Doctoral Thesis examines the relationships of metabolic health, fitness, and exercise with health performing high-throughput omics analyses in blood samples from children with overweight/obesity (OW/OB). Thus, the aims of this Doctoral Thesis were: 1) to examine the differences in the whole-blood transcriptome profiles (RNA-sequencing) of children with metabolic healthy OW/OB compared to metabolic unhealthy OW/OB; 2) to characterize whole-blood transcriptome profile differences between fit and unfit children with OW/OB; 3) to investigate the differences on plasma targeted proteomics between fit and unfit children with OW/OB; 4) To summarize the state-of-the-art of the effect of exercise on gene expression and epigenetic regulation in pediatric population; 5) to study the effects of a 20-week exercise intervention on whole-blood transcriptome profile in children with OW/OB; 6) to report the effects of a 20-week exercise intervention on whole-blood genome-wide DNA methylation profile in children with OW/OB. Six studies were performed within the ActiveBrains project (http://profith.ugr.es/activebrains?lang=en), which were organized into two sections. **Section I,** Metabolic health and cardiorespiratory fitness levels in children with overweight/obesity: a focus on transcriptomics and proteomics (**Studies I, II**, and **III**); and **section II**: Effects of exercise on transcriptome and epigenome in children with overweight/obesity (**Studies IV, V,** and **VI**).

The main findings from this thesis were: I) Thirty-two genes were differentially expressed in children with metabolic healthy OW/OB compared to metabolic unhealthy OW/OB, which were mainly related to metabolism, mitochondrial, and immune functions (**Study I**); II) A different transcriptome profile (256 genes) was identified in fit compared to unfit children with OW/OB, which enriched several molecular pathways related to the immune system and inflammation (**Study II**); III) 16 proteins involved in neurogenesis,

signal transduction, immune function, inflammatory response were differentially expressed in plasma of fit compared to unfit children with OW/OB (**Study III**); IV) A systematic review was performed, where we gathered the state-of-the-art of the effect of exercise on gene expression and epigenetic regulation, which gathered ten studies that used the candidate gene approach, while two studies performed high-throughput analyses. Most of the studies showed that exercise (acute and chronic effects) alter gene expression, and DNA methylation of candidate genes related to cardiovascular disease, asthma, and immune function (**Study IV**); V) a 20-week exercise intervention regulated gene pathways involved in immune processes in blood cells in children with OW/OB (**Study V**); VI) a 20-week exercise intervention demonstrated an impact on DNA methylation profiles of genes involved in metabolic and immune gene pathways. Also, changes of DNA methylation profiles were associated with changes on gene expression levels involved in immune pathways (**Study VI**). The effects exercise at transcriptomic and epigenomic levels generally did not persist after multiple corrections, so these findings should be considered preliminary and be confirmed in more powerful studies in the future.

RESUMEN

La presente Tesis Doctoral examina la relación de la salud metabólica, la condición física y el ejercicio con la salud mediante la realización de análisis ómicos de alto rendimiento en muestras de sangre de niños con sobrepeso/obesidad. Así, los objetivos de esta Tesis Doctoral fueron: 1) examinar las diferencias en los perfiles de transcriptoma en sangre (secuenciación de ARN) de niños con sobrepeso/obesidad metabólicamente sanos en comparación a niños metabólicamente no sanos; 2) caracterizar las diferencias en el perfil del transcriptoma de sangre completa entre niños con "alto" y "bajo" nivel de condición física cardiorespiratoria; 3) investigar las diferencias en la proteómica dirigida en plasma entre niños con sobrepeso/obesidad con "alto" y "bajo" nivel de condición física cardiorespiratoria; 4) resumir la evidencia existente respecto al efecto del ejercicio sobre la expresión génica y la regulación epigenética en población pediátrica; 5) estudiar los efectos de una intervención de ejercicio de 20 semanas en el perfil del transcriptoma de sangre completa en niños con sobrepeso/obesidad; 6) investigar los efectos de una intervención de ejercicio de 20 semanas en el perfil de metilación del ADN de todo el genoma en sangre completa de niños con sobrepeso/obesidad. Se realizaron seis estudios dentro del proyecto ActiveBrains, que se organizaron en dos secciones. **Sección I**, Salud metabólica y condición física cardiorrespiratoria en niños con sobrepeso/obesidad: un enfoque en transcriptómica y proteómica (**Estudios I, II** y **III**); y **sección II**: Efectos del ejercicio sobre el transcriptoma y el epigenoma en niños con sobrepeso/obesidad (**Estudios IV, V** y **VI**).

Los principales hallazgos de esta tesis fueron: I) Treinta y dos genes se expresaron diferencialmente en niños con sobrepeso/obesidad metabólicamente sanos en comparación a los niños con sobrepeso/obesidad metabólicos no saludables, estos genes

se relacionaron principalmente con el metabolismo, las funciones mitocondriales e inmunes (**Estudio I**); II) Se identificó un perfil de transcriptoma diferente (256 genes) en los niños con sobrepeso/obesidad con "alto" nivel de condición física en comparación a los niños con sobrepeso/obesidad y "bajo" nivel de condición física. Este transcriptoma enriqueció varias vías moleculares relacionadas con el sistema inmunológico y la inflamación (**Estudio II**); III) 16 proteínas implicadas en la neurogénesis, la transducción de señales, la función inmunitaria y la respuesta inflamatoria se expresaron de forma diferencial en el plasma de los niños con "alto" nivel de condición física en comparación con los niños con "bajo" nivel de condición física (**Estudio III**); IV) Se realizó una revisión sistemática, donde recopilamos el estado del arte del efecto del ejercicio en la expresión génica y la regulación epigenética, que reunió diez estudios que utilizaron el enfoque del gen candidato, mientras que solo dos estudios emplearon enfoques ómicos de última generación. La mayoría de los estudios mostraron que el ejercicio (efectos agudos y crónicos) altera la expresión génica y la metilación del ADN de genes candidatos relacionados con enfermedades cardiovasculares, asma y función inmunológica (**Estudio IV**); V) una intervención de ejercicio físico de 20 semanas de duración puede regular procesos inmunitarios en las células sanguíneas de niños y niñas con sobrepeso/obesidad (**Estudio V**); VI) una intervención de ejercicio de 20 semanas demostró un impacto en los perfiles de metilación del ADN de los genes involucrados en rutas metabólicas e inmunes. Además, los cambios en los perfiles de metilación del ADN se asociaron con cambios en los niveles de expresión génica implicados en vías inmunes (**Estudio VI**). Los efectos del ejercicio a nivel transcriptómico y epigenómico generalmente no persistieron después de ajustes por comparaciones múltiples, por lo que estos hallazgos deben considerarse preliminares y confirmarse en estudios con mayor potencia estadística en el futuro.

GENERAL INTRODUCTION

1. THE HUMAN GENOME AND "OMICS"

The human genome has three billion base pairs of deoxyribonucleic acid (DNA) and 20.000 estimated protein-coding genes (1-2% of the whole genome) [1]. Thus, completing human genome sequencing in 2001 meant the beginning of the post-genomic research area. As a result, several high-throughput omics techniques, such as genomics, epigenomics, transcriptomics, proteomics, and metabolomics emerged, thus allowing researchers analyze the information of thousands of molecules simultaneously. In this regard, genomics/epigenomics/transcriptomics indicate "*what may happen*", while proteomics/metabolomics, which are influenced by both the genome and environment, indicate "*what is happening/has indeed happened*" because it reflects the closest omics levels to the phenotype [2]. In the present doctoral thesis, we will focus on three different omics levels: epigenomics, transcriptomics, and proteomics in the context of fitness, exercise, and cardiometabolic risk in children with overweight/obesity (OW/OB).

Epigenetics refers to regulatory mechanisms of gene expression that are independent from changes in DNA sequence [3]. In this context, DNA methylation (i.e, the addition of methyl groups [CH3] to DNA) can be quantified in cytosine-guanine base pairs (CpG). Thus, increased DNA methylation at CpG sites located at promoter regions of genes reduces gene expression, and vice versa. However, this interpretation is challenging and much more complex [4]. Transcriptomics reflects the ribonucleic acid (RNA) profile in a cell. Indeed, some RNAs encode proteins (e.g., messenger RNA [mRNA]) and small and long non-coding RNAs that regulate the translation of mRNA to protein at the post-transcriptional level [1]. The coding transcriptome (i.e., mRNAs) is translated into proteins, while proteomics studies hundreds/thousands of proteins (in a given biological sample) that regulate cell functions [1]. The number of genes is lower than the number of proteins, highlighting the increase in complexity when we move from

genomics to proteomics [1]. This increase in the number of proteins *vs.* genes is partially explained by a process known as alternative splicing. Briefly, different combinations of exons (part of a DNA sequence that will be part of the mature RNA) allow to produce diverse mRNA strands. Alternative splicing lets a single gene to code for multiple proteins with different functions (90-95% of human genes undergo through alternative splicing) [1]. The integration of molecular data through multi-omics analyses combined with clinical data and systems biology analyses will allow researchers to discover potential novel biomarkers related to fitness, cardiometabolic risk, and exercise adaptation, thereby improving personalized interventions (**Figure 1**).

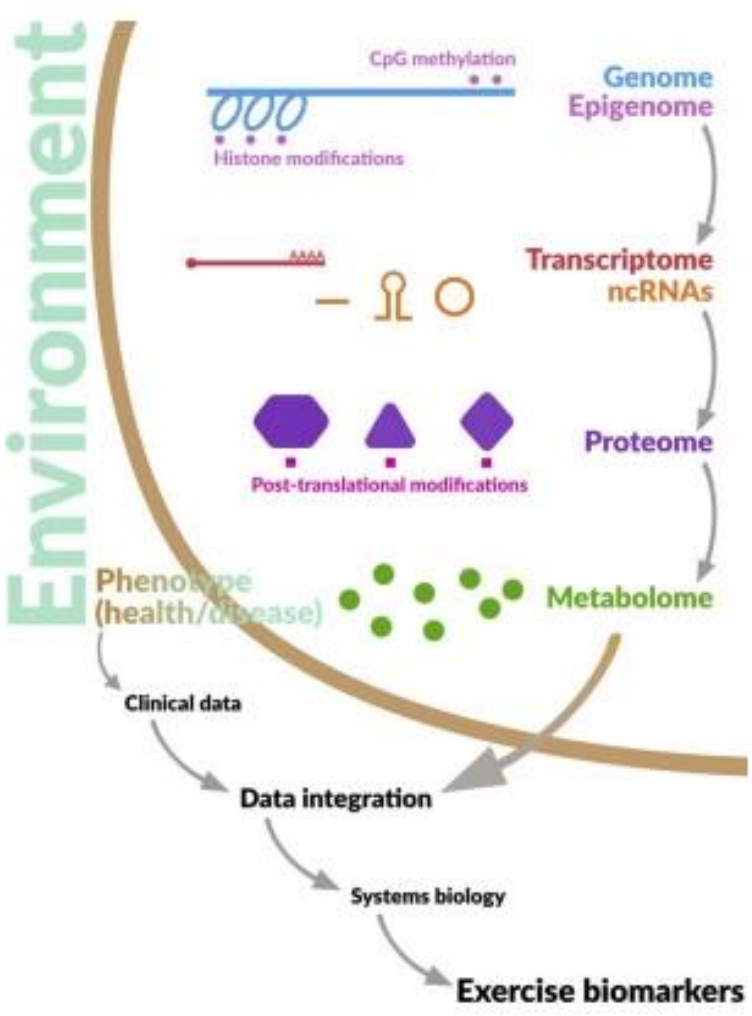

Figure 1. The integration of omics data (i.e., genomics, epigenomics, transcriptomics, proteomics, and metabolomics) and clinical data can be helpful to the discovery of novel biomarkers related to exercise adaptations improving the implementation of exercise as a

paersonalized medicine. Reprinted from Gomes CPC et al., [1], with permission from the publisher Elsevier.

2. OBESITY, METABOLIC HEALTH, AND TRANSCRIPTOMICS IN CHILDREN

Obesity is a growing public health concern in the world [5]. The prevalence of children with OW/OB has been increasing in the last decades, yet has stabilized in most of European countries over the last years [6,7]. Some estimations point out that more than 90 million children and adolescents will present OW or OB in 2025 [6,7]. Childhood obesity is associated with increased cardiovascular disease (CVD) risk factors (e.g., metabolic syndrome markers and circulating pro-inflammatory cytokines) and brain health impairments (i.e., cognitive and brain development) [8–10]. Importantly, it is well known that childhood obesity can predict the development of CVD during adulthood [11], which is the leading cause of mortality globally [12].

Excessive fat accumulation is related to low-grade systemic inflammation that is associated with type II diabetes and atherosclerosis [13]. Adipose tissue is not a "simple" storage site for excess energy. In contrast, adipose tissue is considered an active endocrine organ that regulates the homeostasis of multiple organs through the secretion of circulating adipokines [13]. Crucially, circulating immune cells can be infiltrated in adipose tissue, further contributing to the development of systemic chronic low-grade inflammation [13]. Several immune cells are increased in number and activity within the adipose tissue (e.g., macrophages, mast cells, neutrophils, T and B lymphocytes) while at the same time, other immune cells decreased in number and/or activity (e.g., eosinophils, subsets of T lymphocytes such as T helper 2 and T regulatory cells) [14]. This imbalance of immune cells infiltrated in the adipose tissue leads to insulin resistance and chronic

low-grade systemic inflammation, which is reflected by an increased production of pro-inflammatory cytokines by adipose tissue in obesity.

Interestingly, a subset of the OW/OB population has been considered metabolically "healthy" overweight/obese (MHO), presenting a better CVD risk profile in comparison to the metabolically "unhealthy" overweight/obese (MUO) [9,15]. One of the most frequently used definitions in the literature is to classify children as MHO if they have OW/OB according to the standard definition of body mass index (BMI) and do not meet any metabolic syndrome criteria. Besides, despite the excessive amount of weight/adipose tissue, the individuals presenting MHO phenotype display a lower inflammatory profile than MUO individuals [16].

The molecular mechanisms underlying the MHO phenotype in children are poorly understood. In November 2019, a panel of experts highlighted the lack of studies performing multi-omics analyses to better understand the molecular mechanisms underlying the obesity-related cardiometabolic alterations in children (**Figure 2**) [17]. In this line, several studies reported different transcriptome profiles in adipose tissue (subcutaneous and visceral), liver, and peripherical blood mononuclear cells (PBMCs) of adults with MHO compared to MUO [18–21]. In children, however, for obvious ethical and technical aspects, blood is accessible tissue for exploring the molecular mechanisms underlying cardiometabolic obesity-related alterations. Interestingly, one study reported that individual's whole-blood transcriptome predicted skeletal muscle expression levels for 81% of the genes and 75% of the genes in adipose tissue [22]. Furthermore, whole blood could indicate the low-grade systemic inflammation that is observed in obesity [23]. Thereby, the transcriptome profile in blood cells is considered as an interesting reservoir of CVD biomarkers [24]. Thus, whole-blood transcriptome profile differences between MHO *vs*. MUO can provide new insights into the molecular mechanisms

underlying the better CVD risk profile in this population that remain metabolic "healthy" despite the excessive body weight and adiposity. Therefore, in this doctoral thesis, one cross-sectional study reporting differences on whole-blood transcriptome profiles between MHO *vs*. MUO children was conducted (**Section I, Study I**).

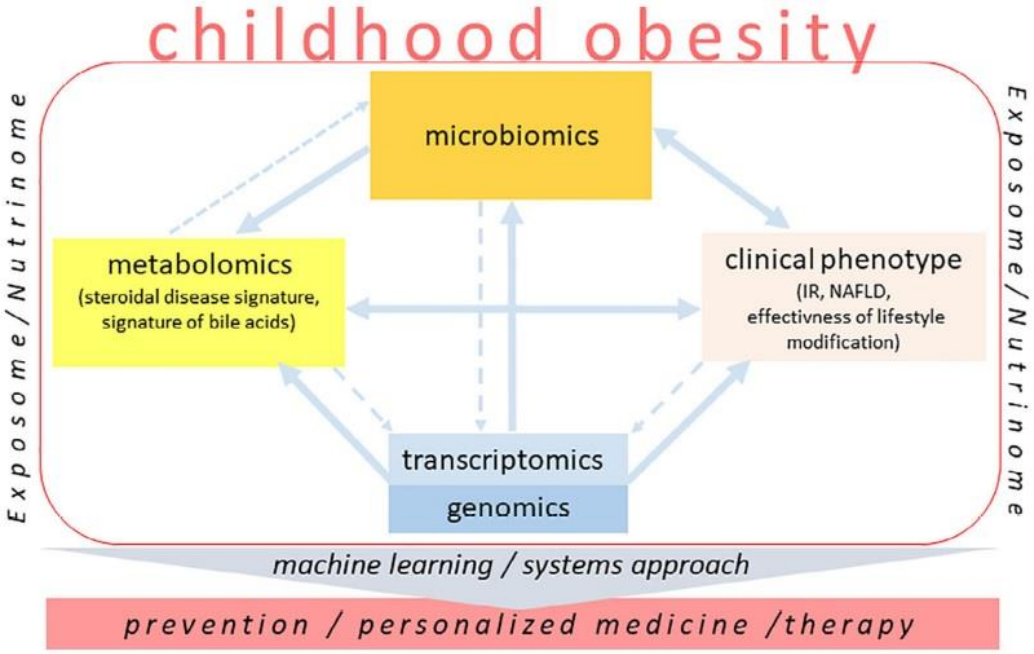

Figure 2. Machine learning and systems biology approach to reveal the interconnection between different omics levels and environmental factors, which might predispose to develop and prevent childhood obesity. Reprinted from Gawlik et al., 2021 [17], with permission from John Wiley and Sons. *IR: insulin resistance; NAFLD: Non-alcoholic fatty liver disease.*

3. CARDIORESPIRATORY FITNESS, TRANSCRIPTOMICS AND PROTEOMICS IN CHILDREN

As mentioned above, childhood obesity has a negative impact on CVD risk factors and brain health parameters in children [8–10]. CVD risk factors and brain health impairment share common pathophysiological mechanisms, which is known as "the heart-brain connection" [25]. Importantly, cardiorespiratory fitness (CRF) is related to adiposity/body composition, being CRF a powerful marker of health in youth populations [26]. Higher CRF levels are related to a better CVD risk factors profile during childhood

[27,28]. Likewise, higher CRF levels are associated with healthier brain parameters (e.g., volume at different regions related to better academic performance) in children with OW/OB [29,30]. Recently, a longitudinal study reported an inverse association between CRF and inflammatory markers in children, independently of body composition and lifestyle behaviours [31]. In this scenario, "good" or higher CRF levels can counteract the negative impact of obesity on CVD risk factors profile and brain health in pediatric populations, being this phenomena known as "the fat-but-fit paradigm" [32,33]. However, the molecular mechanisms underlying the positive impact of good CRF levels on children's health are still poorly understood.

One study showed that "fit" normal-weight children reported higher *PPARG* gene expression (a gene involved in lipid metabolism and inflammation) in leukocytes compared to "unfit" normal-weight children [34]. However, the molecular mechanisms related to higher CRF levels and consequently a better CVD risk profile using high-throughput technology (e.g., RNA-sequencing for transcriptome) in children with and without OW/OB are still lacking. In addition, specific plasma proteomics profiles have been associated with higher CRF levels in adults [35,36]. To the best of our knowledge, transcriptomics and proteomics analyses have not been considered in the context of different CRF levels in pediatric populations. In this regard, in this doctoral thesis, two cross-sectional studies addressed the relationship of "high" and "low" CRF levels with transcriptome/"proteome" profiles in children (**Section I, Studies II and III**).

4. EXERCISE, TRANSCRIPTOMICS AND EPIGENOMICS IN CHILDREN

Exercise interventions provide health benefits (e.g., improve CVD risk factors profile decreasing blood pressure, pro-inflammatory cytokines in circulation, etc) in children with OW/OB [37,38]. However, studies using high-throughput technologies to

explore the molecular mechanisms underlying the health benefits of an exercise intervention in children are scarce. Several studies have reported the effects of an exercise intervention on the transcriptome profile in blood cells of young and middle-aged adults [39–43]. Concerning pediatric populations, only two studies reported the effects of a single bout of exercise on transcriptome profile in blood cells of healthy boys and girls [44,45]. To date, the transcriptome profile's characterization in children's blood in response to an exercise intervention is non-existent.

Epigenetic mechanisms (e.g., DNA methylation) are influenced by environmental factors (e.g., exercise, diet) and can regulate the transcriptome profile independently of alterations of DNA sequence [3]. The link between DNA methylation patterns and gene expression changes is essential to track down the complete molecular mechanisms underlying exercise effects and to validate the findings in epigenetic studies. To the best of our knowledge, the existing evidence about the effects of exercise on transcriptome and DNA methylation profile in pediatric population has not yet been systematically reviewed. Therefore, within this doctoral thesis a systematic review on the genomic response to sedentary behavior and physical activity was conducted (**Section II, Study IV**). Previous researchers have reported the effects of an exercise intervention on genome-wide DNA methylation profile in blood cells of healthy adults and patients with cancer [42,43,46,47]. Differentially methylated genes by an exercise intervention were linked to specific enriched gene pathways involved in cardiovascular disease, immune function, and type 1 and 2 diabetes, among others [42,43,46,47]. In this regard, to the best of our knowledge, the effects of an exercise intervention on whole blood genome-wide DNA methylation profile and the integration with transcriptome data in pediatric population is warranted. In this doctoral, we reported the effects of a 20-week exercise intervention on whole-blood transcriptome and DNA methylation profile in children with OW/OB

(**Section II, Studies V and VI**). This knowledge altogether will contribute to promote the prescription of exercise as a personalized medicine in children.

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AIMS

AIMS

This doctoral thesis aims to increase the understanding of the molecular mechanisms (through omics analyses) related to metabolic health, fitness, and exercise in children with OW/OB. This general aim is addressed in six studies organized into two sections.

Section 1 – Metabolic healthy and cardiorespiratory fitness levels in children with OW/OB: A focus on transcriptomics and proteomics

Aim 1: To characterize transcriptome and proteomics profiles related to metabolic health and cardiorespiratory fitness levels in children with OW/OB.

- Specific aim 1.1: To characterize the whole-blood transcriptome profile of children with MHO compared to MUO.
- Specific aim 1.2: To characterize the whole-blood transcriptome profile of fit children with OW/OB compared to unfit children with OW/OB.
- Specific aim 1.3: To characterize the targeted proteomics profile involved in brain and cardiovascular health in fit children with OW/OB compared to unfit children with OW/OB.

Section 2 – Effects of exercise on transcriptome and epigenome in children with OW/OB

Aim 2: To examine the impact of a 20-week exercise intervention on the transcriptome and epigenome profiles in children with OW/OB

- Specific aim 2.1: To summarize the current knowledge of the effects of sedentary behavior and exercise on gene expression and epigenetic mechanisms in children and adolescents.

- Specific aim 2.2: To examine the effects of a 20-week exercise intervention on whole-blood transcriptome profile in children with OW/OB.
- Specific aim 2.3: To investigate the effects of a 20-week exercise intervention on whole blood genome-wide DNA methylation profile in children with OW/OB.

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GENERAL METHODS

The present doctoral thesis was performed under the umbrella of the ActiveBrains project [1]. This project is a randomized controlled trial investigating the effects of a 20-week exercise intervention on brain health outcomes and secondary variables such as bone health, cardiometabolic risk factors, and molecular outcomes among others, in a cohort of 109 children with OW/OB [1]. The ActiveBrains project was approved by the Committee for Research Involving Human Subjects at the University of Granada (Reference: 848, February 2014). Furthermore, all parents received information about the study and gave their consent following the Declaration of Helsinki guidelines. This doctoral thesis selected a subsample for transcriptomics and epigenomics analyses (N = 24 and 23, respectively). In contrast, a bigger subsample size was available for targeted proteomics analysis in plasma samples of 87 children with OW/OB. A methodological summary of the six studies included in this doctoral thesis is provided below.

REFERENCE

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Table 1. Methodological overview of the six studies included in this Doctoral Thesis

Study	Design	Project (reference)	Participants characteristics	Groups comparisons	Mian outcome	Statistical approach and analyses
Study I	Cross-sectional study	ActiveBrains project [1]	27 children with OW/OB (10.1 ± 1.3 years, 59% boys)	MHO *vs.* MUO	Transcriptome (RNA-seq)	- Mann–Whitney U and chi-squared test (sample characteristics) - Linar models in Limma (main outcome) - WGCNA (main outcome)
Study II	Cross-sectional study	ActiveBrains project [1]	27 children with OW/OB (10.1 ± 1.3 years, 59% boys)	Fit *vs.* Unfit	Transcriptome (RNA-seq)	- Student t test, chi-squared test, and ANCOVA (sample characteristics) - Linar models in Limma (main outcome)
Study III	Cross-sectional study	ActiveBrains project [1]	- Sample brain health proteomics 87 children with OW/OB (10.1 ± 1.1 years, 59% boys) - Subsample CVD proteomics 44 children with OW/OB (10.2 ± 1.1 years, 52% boys)	Fit *vs.* Unfit	Taregeted proteomics involved in brain and CVD (proximity extension assay technology)	- Student t test and chi-squared test (sample characteristics) - ANCOVA (main outcome)
Study IV	Systematic Review	NA	Children and adolescents	NA	- gene expression - epigenetics mechanisms (e.g., DNA methylation)	- NA
Study V	Randomized controlled trial	ActiveBrains project [1]	24 children with OW/OB (10.2 ± 1.3 years, 54% boys)	Exercise *vs.* control	Transcriptome (RNA-seq)	- Linear modelsin Limma (main outcome) - IPA gene networks (main outcome)
Study VI	Randomized controlled trial	ActiveBrains project [1]	23 children with OW/OB (10.1 ± 1.4 years, 44% boys)	Exercise *vs.* control	DNA methylation (Infinium EPIC array 850K Illumina)	- Linear models in Limma (main outcome) - eQTM analysis for the integration of DNA methylation and transcriptome data (secondary analysis)

Participants characteristics were presented as mean ± standard deviation otherwise stated, N (age and percentage of boys). ANCOVA: Analysis of covariance; CVD: Cardiovascular disease; eQTM: expression quantitative trait methylation; MHO: Metabolic healthy overweight/obesity; MUO: Metabolic unhealthy overweight/obesity; NA: Not applicable; IPA: Ingenuity pathway analysis; OW/OB: Overweight/obesity; WGCNA: Weighted gene coexpression network analysis.

RESULTS AND DISCUSSION

SECTION I. Metabolic health and cardiorespiratory fitness levels in children with overweight/obesity: A focus on transcriptomics and proteomics

STUDY I: Distinct whole-blood transcriptome profile of children with metabolic healthy overweight/obesity compared to metabolic unhealthy overweight/obesity

1. INTRODUCTION

Childhood obesity is strongly associated with increased cardiometabolic risk factors (e.g. dyslipidemia and hypertension) and type 2 diabetes [1,2]. However, there is a subset of children and adolescents with overweight/obesity (OW/OB) (6% to 22%, depending on the classification criteria [3,4], who present a normal/healthy metabolic profile that might reflect a better cardiovascular disease prognosis [3,5]. Metabolically "healthy" obesity (MHO) is defined as a subset of individuals with obesity (OB) according to the standard definition of body mass index (BMI) who lack metabolic syndrome symptoms. In contrast, MUO (i.e. metabolically unhealthy overweight/obesity) children and adolescents have 1 to 4 metabolic syndrome classification criteria [5,6]: fasting glucose (≥ 100.9 mg/dl in boys and girls), high serum triglycerides (≥ 127.4 mg/dl in boys and ≥ 141.6 mg/dl in girls), low high-density lipoprotein cholesterol (≤ 43.7 mg/dl boys and ≤ 48.3 mg/dl in girls), and systolic or diastolic blood pressure (Systolic ≥ 121 mmHg in boys and girls, diastolic ≥ 76 mmHg in boys and ≥ 80 mmHg in girls). In pediatric populations, MHO has mostly included children with both overweight and obesity (for simplicity the abbreviation MHO will be hereinafter used instead of MHO-O or others) to maximize sample size and power [7,8].

The underlying mechanisms for the normal/healthy metabolic profile of individuals with OW/OB are poorly understood [9]. Naukkarinen *et al*. reported lower expression of inflammatory genes and higher expression of mitochondrial genes in subcutaneous adipose tissue of young adults with MHO compared to MUO [10]. Das *et al*. also evaluated the transcriptome profile in the subcutaneous adipose tissue of middle-aged adults with MHO compared to MUO [11] and found differences in genes involved in cardiovascular disease-related processes and immune/inflammatory responses. However, a study by Gómez-Ambrosi *et al*. [12] analyzed candidate genes involved in

inflammation and tissue remodeling and detected no differences between adults with MHO and MUO in the liver and visceral adipose tissue. The inconsistent results in the different studies may be explained by different ways to classify individuals with MHO and MUO [5,13,14] and/or different tissues used to analyze transcriptome profiles (e.g. visceral adipose tissue, liver, subcutaneous adipose tissue, and peripheral blood mononuclear cells) [10–12,15].

Nevertheless, to the best of our knowledge, no previous studies have characterized the transcriptome profiles of children with MHO and MUO. Due to ethical considerations tissue biopsies are not permitted in pediatric populations, so that the main tissue to be studied in this population is whole blood, which can reflect the systemic inflammation in the body. Obesity is characterized by a low-grade systemic inflammation which is involved in the pathophysiology of type 2 diabetes mellitus and arteriosclerosis [16,17]. Studies have pointed to the tissue macrophages as a source which may contribute to increases in inflammation in adipose tissue, resulting in the dysregulation of the endocrine function inducing low-grade systemic inflammation in persons with obesity [18]. In this regard, one of the main factors that could contribute to an MHO phenotype is the presence of a low pro-inflammatory profile [19]. However, why some individuals would have a lower pro-inflammatory profile than others in the presence of an excessive weight/adiposity, is still poorly understood and the study of differences in gene expression levels between MHO *vs.* MUO can shed light on this.

The present study aimed to characterize the whole-blood transcriptome profile of children with MHO and MUO to promote a better understanding of why a subset of the population remains metabolically healthy despite having overweight/obesity.

2. METHODS

2.1 Participants

In total 27 children with OW and OB, ages 10.1 ± 1.3 years, 59% boys, from the ActiveBrains project (www.profith.ugr.es/activebrains, Clinical Trial: NCT02295072) were included in this analysis. Detailed design, methods, and inclusion/exclusion criteria have been described elsewhere [20]. The study was approved by the Committee for Research Involving Human Subjects at the University of Granada (Reference: 848, February 2014). All parents were informed about the study objective and written informed consent following the Declaration of Helsinki.

2.2 Body composition measurements and maturational status

Body weight and height were measured with an electronic scale and a stadiometer (Seca instruments, Germany, Ltd). BMI was calculated as kg/m^2 and participants were then classified as children with overweight or obesity according to the sex and age-specific World Obesity Federation cut-off points [21]. Waist Circumference (WC) was evaluated as using the International Society for the Advancement of Kinanthropometry (ISAK) procedures [22]. Body weight, height, and WC were collected twice consecutively by the same trained evaluator, and the average for each metric was used. Body fat percentage, fat-free mass and visceral adipose tissue were measured by dual energy X-ray absorptiometry (DXA, Discovery densitometer from Hologic). The positioning of the participants and the analyses of the results were undertaken following recommendations from the International Society of Clinical Densitometry [23]. All DXA analyses were performed by the same evaluator using the GE encore software (version 4.0.2).

The maturational status of the participants was reflected by peak height velocity (PHV) using age and height in validated algorithms [24]: in boys: −8.1 + (0.0070346 × (age × sitting height) and, in girls: −7.7 + (0.0042232 × (age × height). Maturity offset was calculated by subtracting the PHV age from the chronological age.

2.3 Definition of MHO and MUO in youth

Cardiometabolic risk factors used in our study to define the MHO and MUO (i.e. fasting glucose, serum triglycerides, and high-density lipoprotein cholesterol) were measured from fasting blood samples. Blood pressure (systolic and diastolic) was measured from the left arm with an automatic sphygmomanometer (Omron M6, the Netherlands) on two different days, and the minimum value was registered for the analyses.

Our participants were classified using the harmonization definition for MHO and MUO in youth using the Jolliffe and Janssen metabolic syndrome study [25], based on a comprehensive review of different criteria available (for more details see Ortega *et al*. [5,6]). In this study, age and sex specific cut-off points for each marker of metabolic syndrome were developed for adolescent populations from 12 to 18 years [25]. Cut-off points for boys and girls aged 12 [25] were used in our study, since it was the closest to the age range of our sample, i.e. 9 to 11 years. The strength of these cut-off points are equivalent to those proposed for adults by the International Diabetes Federation and Adults Treatment Panel and the adaptation by age- and sex-specific based on growth curves in youth [5]. The metabolic syndrome is defined as having fasting glucose (≥ 100.9 mg/dl in boys and girls), high serum triglycerides (≥ 127.4 mg/dl in boys and ≥ 141.6 mg/dl in girls), low high-density lipoprotein cholesterol (≤ 43.7 mg/dl boys and ≤ 48.3 mg/dl in girls), and systolic or diastolic blood pressure (Systolic ≥ 121 mmHg in boys

and girls, diastolic ≥ 76 mmHg in boys and ≥ 80 mmHg in girls). Children lacking all the metabolic syndrome criteria (excluding high WC) were classified as MHO. Children with one or more of the previous criteria were classified as MUO. Both, OW and OB were included in the analysis (n = 27; 6 OW and 21 OB) to maximize sample size and in line with previous studies in pediatric populations [7,8]. Homeostasis model assessment for insulin resistance (HOMA-IR) as an index of insulin resistance were provided.

2.4 Blood Sampling and Analysis

Participants arrived at the laboratory between 8-9 AM after an overnight fasting of at least 12 hours. Venous blood collected in tubes with EDTA was spun immediately at 1000×*g* for 10 min. Plasma was isolated and stored at -80ºC until analyses. In addition, 500 μL blood were collected into tubes containing 1.3 mL RNAlater solution (Ambion Inc; Austin, Texas, USA) for transcriptome analysis and was stored at -80ºC until further processing.

2.4.1 Inflammatory markers

Interleukin-6 (IL-6), interleukin-1β (IL-1β), and tumor necrosis factor-α (TNF-α) cytokines were measured in plasma using multiple analyte profiling technology (MILLIPLEX® MAP Human High Sensitivity T Cell Magnetic Bead Panel, EMD Millipore Corporation, Missouri, USA) with a kit plex (HCYIL6-MAG Anti-Human IL-6 Beads set, HCYIL1B-MAG Anti-Human IL-1β Bead, and HCYTNFA-MAG Anti-Human TNFα Beads set) [26]. The intra- and inter-assay coefficients of variation (CVs) for IL-6 were ≤ 5% and ≤ 20%, respectively, and sensitivity was 0.11 pg/mL. For both, IL-1β and TNF-α the intra- and inter-assay CVs were ≤ 5% and ≤ 15%, respectively, with a sensitivity of 0.14 pg/mL for IL-1β, and of 0.16 pg/mL for TNF-α [26]. Vascular endothelial growth factor A (VEGF-A) and epidermal growth factor (EGF) were

quantified by multiple analyte profiling technology (MILLIPLEX® MAP Human Angiogenesis/Growth Factor Magnetic Bead Panel 1, EMD Millipore Corporation, Missouri, USA) using a kit plex (HVEGF-MAG Anti-Human VEGF-A Bead, and HAGEGF-MAG Anti-Human EGF Bead) [27]. The intra- and inter-assay CVs for VEGF-A were ≤ 3.5% and ≤ 10%, respectively, and sensitivity was 8.1 pg/mL. For EGF, the intra- and inter-assay CVs were ≤ 3.2% and ≤ 6.8%, respectively, with a sensitivity of 1 pg/mL [27].

2.4.2 RNA extraction and sequencing

Total RNA was isolated from the blood samples that contained RNA later using RiboPureTM-Blood Kit (Thermo Fisher Scientific; Waltham, Massachusetts, USA). Subsequently, GlobinLock molecular mechanism was applied to block the high globin mRNA content of erythrocytes, which is abundant in the blood and could hamper the whole blood RNA analyses [28]. Full transcriptome analysis was performed following the modified version of single-cell tagged reverse transcription (STRT) protocol as described in detail before [29], where 10 ng of high-quality input RNA was converted into cDNA and amplified to form an Illumina-compatible library. The STRTprep pipeline, available at https://github.com/shka/STRTprep/tree/v3dev, was used for processing raw sequencing reads, aligning to the hg19 genome, quantitating the expression levels. RNA-seq data is available at www.ncbi.nlm.nih.gov/geo under accession number GSE146869.

2.5 Statistical analyses

The differences in sample characteristics between children with MHO and MUO were tested using the non-parametric Mann-Whitney U and chi-squared test for continuous and categorical variables, respectively. Winsorizing the data was performed

to limit the influence of extreme values. Briefly, the winsorization method replaces extreme high/low values for the closest (highest/lowest) valid values [30]. The analyses were performed using SPSS version 21.0 (IBM Corporation, NY, USA), and the statistical significance was defined at the level of $p < 0.05$. Differential expression analysis between children with MHO and MUO was performed with Limma R/Bioconductor software package [31]. Prior to performing the analyses, quantile normalization was performed on gene expression data of RNA-sequencing. These analyses were adjusted by sex and maturational status. Statistically differentially regulated genes were defined by a FDR < 5% (Benjamini and Hochberg correction on multiple testing). These genes were characterized by functional enrichment analysis using g:Profiler [32]. Biological process and pathways with a FDR < 0.05 were considered significantly enriched.

Additionally, we performed a weighted gene coexpression network analysis (WGCNA) following the guidelines for these analyses [33–35] in order to explore possible gene networks. Two different networks of genes coexpressed based on terms of correlations were created, i.e. one network in children with MHO and another network of genes coexpressed in the group of children with MUO. The subsequent step was to define and characterize the non-preserved modules or subnetworks (i.e. clusters of genes named by colors) and their function in the network constructed in children with MHO respect to the network computed in children with MUO. The "modulePreservation" WGCNA function was used to compute a range of preservation across the two networks. Module preservation statistics were calculated to evaluate whether a given color module defined in one data set (reference network, in our study the reference network was computed in children with MHO) can also be found in another data set (test network, in our study the test network was computed in children with MUO). Module preservation among children

with MHO and MUO was reported as a composite Zsummary measurement of connectivity and density. Zsummary scores greater than 10 indicate high network preservation (i.e. a module color or group of genes is similarly clustered in children with MHO and MUO), scores between 2 and 10 are indicative of moderate network preservation, and Zsummary measures below 2 provide no evidence of preservation (i.e. a color module or group of genes is clustered in children with MHO but not clustered in MUO). Hub genes (i.e. highly connected intramodular genes, referring to bigger role within the network) were identified in color modules with low preservation based on the module membership value (MM). The MM has been calculated as the correlation between the module eigengene (i.e. first principal component) of a module and the expression profile of a gene. Genes whose MM was >0.8 were considered as hub genes in each module [36].

The Venn Diagram [37] was used to perform the overlapping between differentially expressed genes (i.e. Limma analyses) and the hub genes in color modules with low preservation (i.e. WGCNA analyses) in children with MHO compared to MUO. A molecular interaction network was created using GeneMania app in CYTOSCAPE platform [38], the most relevant genes in the molecular network were defined based on the highest value of centrality degree, which is defined as the number of interactions in which a node (i.e. gene) is involved.

In silico validation analysis was performed using two publicly available (PHENOPEDIA database) lists of obesity and metabolism genes. We performed the overlapping between the most important genes found in our study (i.e. genes overlapped between Limma and WGCNA analyses) and the two lists. These relevant genes in obesity and metabolism were identified following a semi-automatic workflow for the identification of the most relevant genes in a pathology [39]. For the interested reader

wishing to retrace our analyses, all files are available from the Open Science Framework (https://osf.io/xt8v4/).

Table 1. Participants' characteristics

Variables	Total sample n=27 (16 boys/11 girls)	MHO n=13 (10 boys/3 girls)	MUO n=14 (6 boys/8 girls)	P value
Age and Maturational status				
Age (years)	10.1 ± 1.3	10.2 ± 1.4	10.0 ± 1.2	0.76
PHV offset (years)	-2.2 ± 0.9	-2.3 ± 1.0	-2.0 ± 0.8	0.76
BMI group by Cole *et al.*				
Overweight	6 (22.2%)	4 (30.8%)	2 (14.3%)	0.30
Obesity	21 (77.8%)	9 (69.2%)	12 (85.7%)	
Body composition and anthropometry				
Weight (kg)	57.3 ± 10.3	56.9 ± 13.8	57.7 ± 6.0	0.79
Height (cm)	145.7 ± 9.1	146.4 ± 11.5	145.0 ± 6.4	0.62
Waist circumference (cm)	91.7 ± 7.3	90.0 ± 9.3	93.3 ± 4.4	0.40
BMI (kg/m^2)	26.8 ± 2.7	26.1 ± 3.2	27.4 ± 1.9	0.38
BF (%)	42.7 ± 4.7	41.1 ± 5.5	44.3 ± 3.3	0.09
DXA total VAT (g)	414.3 ± 85.9	395.1 ± 107.5	432.1 ± 58.2	0.23
DXA FFM (Kg)	32.1 ± 5.7	32.7 ± 7.7	31.5 ± 3.0	0.72
Cardiometabolic markers				
Fasting Glucose (mg/dl)	87.0 ± 5.2	88.1 ± 4.5	85.9 ± 5.8	0.40
Insulin (uU/mL)	13.4 ± 8.3	10.8 ± 4.2	15.8 ± 10.4	0.17
HOMA-IR	2.9 ± 1.8	2.3 ± 0.9	3.3 ± 2.3	0.21
Triglycerides (mg/dl)	109.0 ± 86.6	**71.6 ± 19.7**	**122.9 ± 65.4**	**0.02**
HDL-Cholesterol (mg/dl)	49.6 ± 9.9	**56.5 ± 9.2**	**42.0 ± 5.4**	**<0.001**
Blood pressure				
Systolic (mmHg)	101.9 ± 7.7	100.8 ± 7.6	102.9 ± 8.0	0.52
Diastolic (mmHg)	58.0 ± 9.3	56.9 ± 8.3	58.9 ± 10.4	0.79
Inflammatory markers*				
IL-1β (pg·mL-1)	1.8 ± 0.7	1.5 ± 0.7	2.0 ± 0.7	0.11
IL-6 (pg·mL-1)	2.3 ± 2.0	**1.5 ± 0.7**	**2.6 ± 1.2**	**0.02**
TNF-α (pg·mL-1)	3.9 ± 1.1	**3.3 ± 0.8**	**4.4 ± 1.2**	**0.02**
EGF (pg·mL-1)	1.8 ± 1.7	1.9 ± 2.1	1.6 ± 1.2	0.89
VEGF-A (pg·mL-1)	27.39 ± 13.0	25.2 ± 12.1	29.3 ± 13.9	0.63

Data presented as mean ± SD, and as number and frequency. MHO: metabolically healthy overweight/obesity, MUO: metabolically unhealthy overweight/obesity, BMI: Body mass-index, BF: Body fat, VAT: Visceral adipose tissue, FFM: Fat free mass, PHV: Peak height velocity, HOMA-IR: Homeostasis model assessment for insulin resistance, HDL: High density lipoprotein, IL: interleukin, TNF-α: tumor necrosis factor alpha, EGF:

epidermal growth factor, VEGF-A: vascular endothelial growth factor A. Bold numbers indicates $p < 0.05$

* IL-1β, IL-6, insulin and HOMA-IR (n = 25), TNF-α and VEGF (n = 26), EGF (n = 24).

3. RESULTS

Anthropometric characteristics and cardiometabolic and inflammatory markers of participants are presented in **Table 1.** Ten boys and three girls were classified as MHO and six boys and eight girls were classified as MUO. MUO had abnormal values of triglycerides and HDL-cholesterol ($p < 0.05$), and higher values of pro-inflammatory cytokines (e.g. TNF-α and IL-6) compared to MHO ($p < 0.05$).

Differential gene expression analyses, using Limma R/Bioconductor software package, showed 40 differentially expressed genes (19 up-regulated and 21 down-regulated, FDR < 0.05) in children with MHO compared to MUO **(Figure 1**). These 40 genes were mainly related to metabolism, mitochondrial and immune functions **(Supplemental Table S1 [online]**). Differentially expressed genes were enriched in pathways related to cardiovascular growth via hypertrophy (FDR < 0.05) (**Table 2**). Additionally, WGCNA analysis identified 23 genes coexpression modules with low preservation (Zsummary < 2) (i.e. genes clustered in children with MHO but not clustered in MUO), three with moderate preservation (2 > Zsummary < 10) and two with high preservation (Zsummary > 10) (i.e. genes similarly clustered in children with MHO and MUO) in children with MHO compared to MUO **(Figure 2**). The gene coexpression modules size ranged from 65 to 1217 genes (**Supplemental Table S2 [online]**). The hub genes (i.e. highly connected intramodular genes) identified in the 23 modules with low preservation ranged from 35 to 826 genes (**Supplemental Table S2 [online]**).

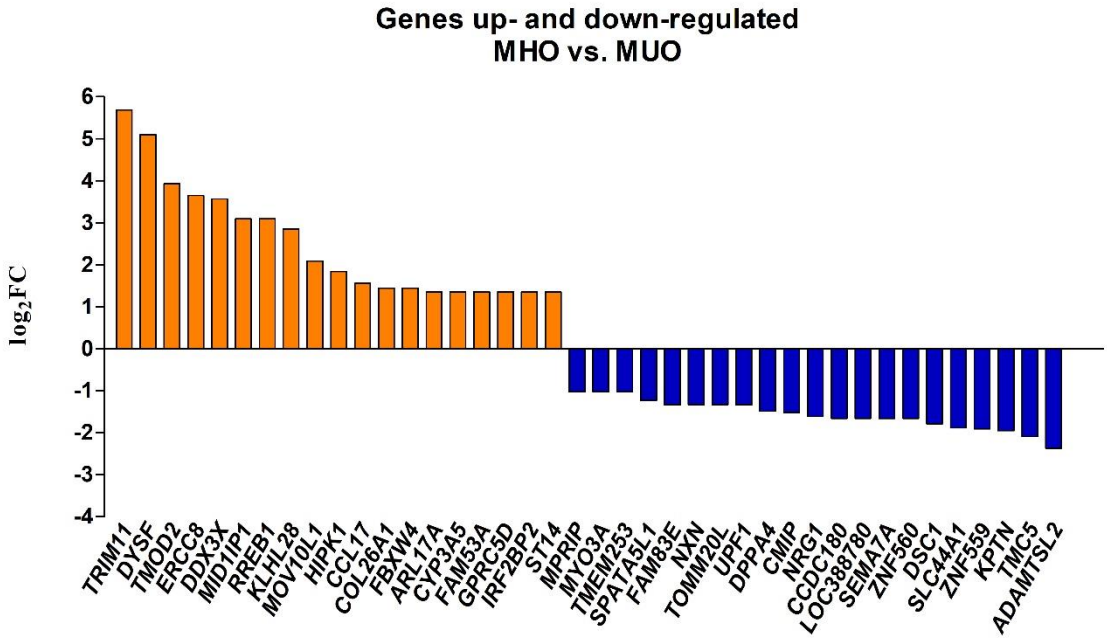

Figure 1. Fold change (FC) of genes significantly up- and down-regulated in children with metabolic healthy overweight/obesity (MHO) compared to metabolic unhealthy overweight/obesity (MUO) according to Limma analyses (False discovery rate, FDR < 0.05).

From 40 differentially expressed genes in the Limma analyses, 32 genes were found among the hub genes (i.e. highly connected intramodular genes) identified within WGCNA modules with low preservation clustered in the Tan and Midnight color. These genes were *ZNF559, MPRIP, MYO3A, ADAMTSL2, LOC388780, FAM83E, TOMM20L, DSC1, TMC5, ZNF560, CMIP, UPF1, SLC44A1, KPTN, NXN, SPATA5L1, TMEM253, NRG1, SEMA7A, CCDC180, FBXW4, FAM53A, CYP3A5, ARL17A, IRF2BP2, HIPK1, MOV10L1, COL26A1, GPRC5D, CCL17, RREB1* and *ST14*. From the molecular network composed by these 32 genes (**Figure 3**) we selected the top-5 based on the highest values in centrality degree (i.e. the number of interactions in which a gene is involved). These genes: *RREB1, FAM83E, SLC44A1, NRG1* and, *TMC5* are involved in type 2 diabetes, macrophage phospholipid metabolism, inflammation and cardiovascular growth through exercise **(Table 3 and Supplemental Table S3 [online]).**

Table 2. Functional Enrichment analyses of differentially expressed genes in children with metabolic healthy overweight/obesity phenotype compared to children with

metabolic unhealthy overweight/obesity phenotype using g:Profiler (30). Significance threshold (False discovery rate, FDR < 0.05)

Molecular function	**Genes involved**
Chemorepellent activity	*NRG1*↓, *SEMA7A*↓
Pathways	**Genes involved**
miR-222 in exercise-induced Cardiac Growth	*HIPK1*↑, *NRG1*↓
Cardiac hypertrophic response	
Cardiac progenitor differentiation	
MicroRNAs in cardiomyocyte hypertrophy	

In silico corroboration analysis using PHENOPEDIA database provided a list of 2,263 genes with the term "obesity" and 5324 genes with the term "metabolic diseases". Next, GeneMania app in CYTOSCAPE platform was applied for the identification of the most significant genes in both lists, creating 2 molecular interaction networks composed by 2,263 and 5,324 genes, respectively. When testing our common list of 32 genes (**Figure 3**), 3 genes were found in the *in silico* lists of top-genes involved in obesity and metabolism, based on the highest centrality degree (i.e. the number of interactions in which a gene is involved) threshold by their means (**Supplemental Table S4 and S5 [online]**): *RREB1*, *NRG1* and, *CYP3A5*. Genes *RREB1* and *NRG1* were identified in the top-5 genes based on the highest values in centrality degree (**Table 3 and Figure 3**).

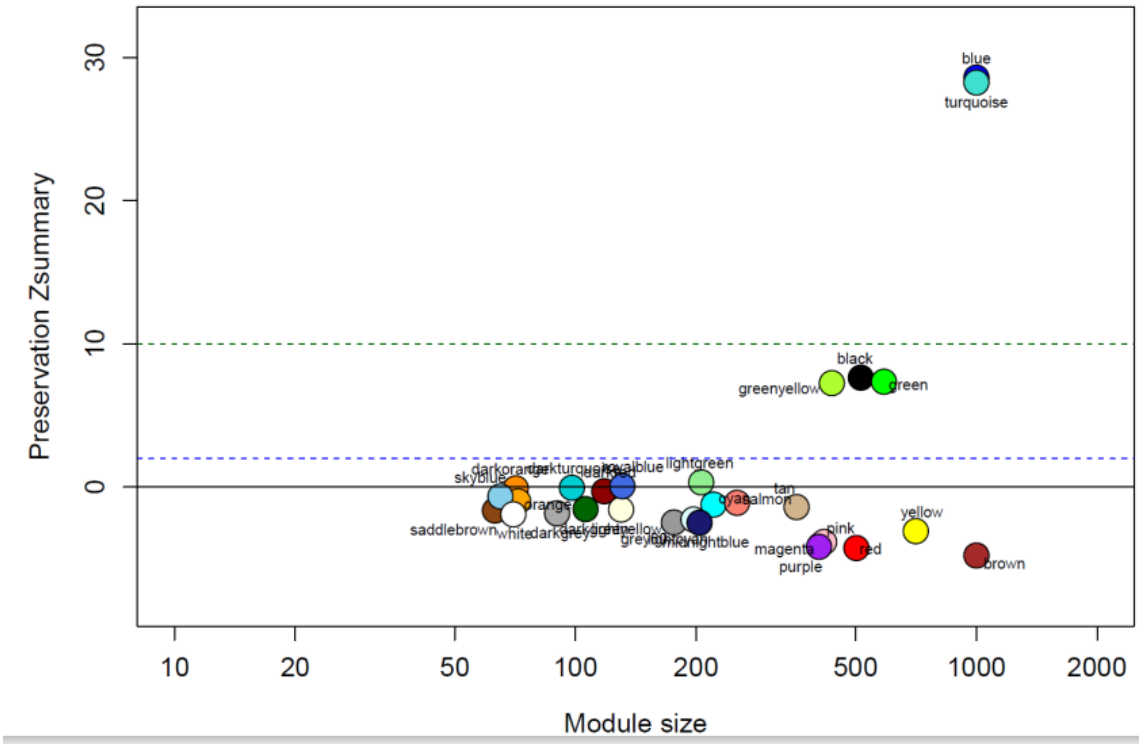

Figure 2. Gene coexpression modules preservation between children with metabolic healthy overweight/obesity (MHO) and children with metabolic unhealthy overweight/obesity (MUO) as compound Zsummary (y-axis). In total, 23 gene coexpression modules were low preserved (Zsummary < 2, y-axis) in children with MHO compared with MUO (i.e. genes clustered in children with MHO but not clustered in MUO).

4. DISCUSSION

We identified 32 differentially expressed genes in children with MHO compared to MUO, which were related to metabolism, mitochondrial and immune functions. These findings provide the first step towards the understanding the underlying mechanisms that differentiate MHO from MUO in young ages. Concerning the most up-regulated and down-regulated genes in our study (i.e. MHO *vs*. MUO) we found highest expression of *TRIM11* gene and lowest expression of the *ADAMTSL2* gene in children with MHO compared to MUO. TRIM11 is involved in the degradation of AIM2 inflammasome [40]. The AIM2 inflammasome cytosolic signaling complex lead to the maturation of pro-inflammatory cytokines [40]. Indeed, children with MHO showed *TRIM11* gene up-

regulation and lower levels of pro-inflammatory cytokines compared to MUO. In fact, the MHO phenotype has been characterized by a favorable inflammatory profile compared to MUO [9].

Body mass index and adiposity correlate positively with circulating levels of transforming growth factor beta (TGF-β1) protein [41]. This protein is involved in the regulation of inflammation, immune function, and glucose homeostasis, among other biological processes [41–43]. Interestingly, mutations in the *ADAMTSL2* gene may lead to a dysregulation of TGF- β signaling [44]. Furthermore, previous evidence has shown that the blockade of TGF- β signaling protects mice from obesity, diabetes, and hepatic steatosis [41]. Likewise, Snelling *et al*. reported up-regulation of *ADAMTSL2* gene in human damage cartilage compared with undamaged cartilage [45]. In our study, inflammatory markers TNF-α and IL-6 were significantly higher in MUO compared to MHO children. We hypothesize that a higher *ADAMTSL2* together with lower *TRIM11* gene expression levels in children with MUO could promote higher inflammatory profile in those children compared to children with MHO.

Telle-Hansen *et al*. reported higher peripheral blood mononuclear cells expression of some candidate genes involved in lipid metabolism (i.e. *UCP2, HSL, and PPARG*) in young and middle-aged adults with MHO compared to MUO [15]. Similarly, we found higher whole blood expression of some genes involved in fatty acids synthesis and cholesterol metabolism (e.g. *CYP3A5 and, IRF2BP*) in children with MHO compared to MUO. The increase in peripheral blood mononuclear *CYP3A4* and *CYP3A5* gene expression has been associated with a greater LDL-cholesterol reduction in adults with hypercholesterolemia after treatment with atorvastatin [46]. On the contrary to our knowledge, the relation of the higher expression of *CYP3A5* gene with HDL-cholesterol metabolism has not been reported. Furthermore, the IRF2BP2 protein is a novel regulator

of lipid metabolism and inflammation in macrophages [47]. This protein is necessary for the expression of the anti-inflammatory transcription factor KLF2 in macrophages, and deficiency in IRF2BP2 protein leads to an increase in lipid accumulation [47]. Likewise, IRF2BP2 protein deficiency has been associated with an increase in atherosclerosis and coronary artery disease in mice and humans, respectively [47]. Interesting, *IRF2BP2* gene was up-regulated in children with MHO compared to MUO and could contribute partially to a better cardiometabolic and inflammatory profile. Interestingly, Sánchez et al. identified some genes (e.g., *ZNF418, NPPA, POLR1C, ADRB3, P2RX2*) that might discriminate children aged 4.7-8 years with OW presenting high or low triglycerides levels [48]. These genes were not detected in our study; some differences between studies could partially explain the lack of gene overlapping. In this regard, different high throughput technologies (microarrays *vs.* RNA-seq) were used, different weight status (children with OW *vs.* children with OW/OB), and different age range of the participants (4.7-8 years *vs.* 9-11 years) among others.

On the other hand, Zhang *et al*. showed association between metabolic abnormalities (i.e. MUO and metabolic unhealthy non-obesity) and left ventricular hypertrophy (LVH) in young adults, while not in MHO group [49]. In our study, key genes involved in cardiovascular growth via hypertrophy that are regulated by physical exercise, i.e. *HIPK1* and *NRG1* genes [50,51], were found up- and down-regulated in children with MHO compared to MUO. Our results suggest a "balance" on the expression of genes and pathways involved in cardiac growth via hypertrophy between children with MHO and MUO. As we did not perform echocardiography measurements, it is not possible elucidate the clinical implications of these results in cardiovascular health via cardiac hypertrophy in children with weight disturbances.

Table 3. Top-5 genes (based on centrality degree, i.e. the number of interactions in which a gene is involved) from molecular network composed by 32 genes overlapped between differentially expression-Limma and hub genes from WGCNA analyses (MHO compared to MUO) and their possible connection to metabolism, immune function or obesity

Gene	Official Full Name	Centrality degree	Possible link to metabolism, immune function and obesity
RREB1	Ras responsive element binding protein 1	15	*RREB1* gene has been considered a novel candidate gene for type 2 diabetes
FAM83E	Family with sequence similarity 83 member A	11	May be involved in MAPK signalling and has been associated with diabetes-related traits in humans
SLC44A1	Solute carrier family 44 member 1	11	Involved in macrophage phospholipid metabolism and inflammation
NRG1	Neuregulin 1	8	Chronic NRG1 treatment improved glucose tolerance in diabetic mice. Involved in cardiac growth through exercise
TMC5	Transmembrane channel like 5	8	A variant of this gene from abdominal subcutaneous white adipose tissue was associated with obesity in adults.

Our constructed molecular network composed of 32 genes could highlight the most relevant genes in understanding the molecular basis underlying the MHO phenotype in children. *RREB1* and *NRG1* genes could be of special interest, as we found them among top five genes of our network of 32 genes and that were also confirmed as top genes in the in-silico analyses on two different publicly available datasets. *RREB1* gene (i.e. the gene with the highest value of centrality degree of our network of 32 genes), is considered a novel candidate gene for type 2 diabetes in humans [52]. Furthermore, genome-wide association studies identified loci in *RREB1* gene associated with fasting glucose and fat distribution [53,54]. Further, the *RREB1* gene might modulate different clinical phenotypes (e.g. hypertension, fat distribution, fasting glucose, non-diabetic end-stage

kidney disease) [52]. *RREB1* gene could serve as a promising candidate for prognosis of cardiometabolic health in pediatric population.

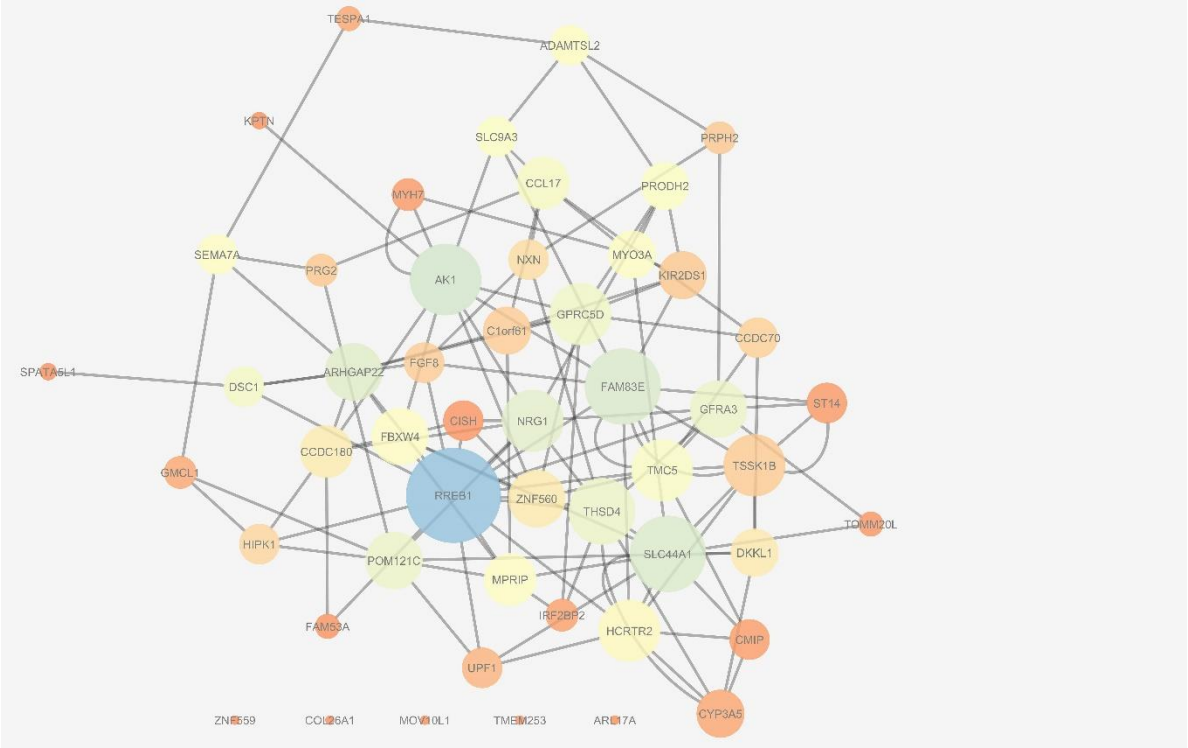

Figure 3. Molecular network representation composed by 32 genes overlapped using 2 bioinformatics approaches (i.e. differentially gene expression-Limma and hub genes from WGCNA analyses). The node size marks the level of centrality degree, i.e. the number of interactions in which a gene is involved

Several limitations in our study need to be highlighted. First, we analyzed whole-blood transcriptome profile. Different subsets of leukocytes have different roles in immune function and metabolism. However, the whole blood RNA-sequencing has been considered promising for the identification and tracking of biomarkers and useful as a diagnostic tool for rare diseases [55]. Secondly, our sample size was relatively small and most of children in the MHO group were boys (10 of 13), which could have influenced WGCNA analyses. Nevertheless, we adjusted the Limma analyses by sex (in addition to maturation i.e. PHV), and the selected genes overlapped with those from the WGCNA analyses, aiming to minimize the potential confounding effect of sex. Likewise, we cannot

assume a causal relationship due to our cross-sectional study design. Lastly, the cut-off points used in our study were specific for boys and girls aged 12, due to was the closest to the age range of 9 to 11 years. Despite these limitations, to the best of our knowledge, this is the first study that reports differences on whole-blood transcriptome profile in children with MHO and MUO. Further, blood samples for transcriptome analysis were obtained in a unified manner (fasting blood in first hour in the morning), we applied GlobinLock as a novel robust globin mRNA reduction tool to preserve RNA quality [28], and two different bioinformatics approaches together with *in silico* data mining were applied to corroborate the results.

5. CONCLUSION

The distinct gene expression pattern includes genes related to metabolism, mitochondrial and immune functions. The most relevant genes in understanding the molecular basis underlying the MHO phenotype in children could be: *RREB1*, *FAM83E*, *SLC44A1*, *NRG1*, *TMC5*, *CYP3A5*, *TRIM11* and *ADAMTSL2*. The results contribute to the development of hypothesis directed to a better understanding of why a subset of the population remains metabolically healthy despite having overweight/obesity. Future studies should confirm our findings in larger cohorts of children and in other populations.

SUPPLEMENTARY MATERIAL

The article contains supplementary information online at journal website (https://www.nature.com/articles/s41390-020-01276-7)

In addition, the supplementary files can be downloaded in this link:

https://osf.io/unpje/?view_only=d3eeb5a70be44b84a845efc96607c08a

Supplemental Table S1. Differentially expressed genes (FDR < 0.05) in children with MHO compared to non-MHO and their possible connection to metabolism, immune function and obesity

Supplemental Table S2. The gene coexpression modules size and the hub genes (i.e., highly connected intramodular genes) identified in the 23 modules with low preservation ranged from 35 to 826 genes.

Supplemental Table S3. Data generated from the molecular network representation composed by 32 genes overlapped using 2 bioinformatics approaches (i.e., differentially gene expression-Limma and hub genes from WGCNA analyses).

Supplemental Table S4. *In silico* lists of top genes involved in obesity based on the highest centrality degree (i.e., the number of interactions in which a gene is involved) threshold by their means

Supplemental Table S5. *In silico* lists of top genes involved in metabolism based on the highest centrality degree (i.e., the number of interactions in which a gene is involved) threshold by their means

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STUDY II: Cardiorespiratory fitness in children with overweight/obesity: Insights into the molecular mechanisms

1. INTRODUCTION

Childhood obesity is associated with increased cardiovascular disease (CVD) risk factors (e.g., high fasting glucose, triglycerides and inflammatory markers) that might promote the development of CVD during adulthood [1], the main cause of mortality worldwide. Cardiorespiratory fitness (CRF) is a powerful marker of health in youth [2], and is inversely associated with CVD risk factors [3,4]. Notably, youth with low CRF levels have a higher risk to develop CVD during adulthood [5,6]. In fact, there is extensive evidence to support the fat-but-fit paradigm which shows that CRF is able to counteract the adverse effects of obesity on CVD risk factors. [3,4]. Understanding the molecular mechanisms underlying the health benefits of fitness will promote the use of exercise as a form of medicine in a more precise and personalized way.

Previously, a single-gene analysis approach demonstrated higher expression of *PPARG* gene in leukocytes of normal-weight children with high CRF levels compared to normal-weight children with low CRF levels [7]. The *PPARG* gene is involved in lipid, glucose metabolism, and inflammatory response and is a proposed therapeutic target for CVD treatment [8]. Another cross-sectional study using microarray analysis detected higher expression of mitochondrial genes and lower expression of inflammatory genes in leukocytes of healthy young adults endurance athletes compared to healthy young adults non-athletes [9]. These are the first studies to explore the molecular mechanisms in fitness in young population, and clearly more research is warranted.

Due to ethical considerations in pediatric studies muscle and/or adipose tissue biopsies are limited and the blood is the primary tissue to study using the cutting edge omics platforms. Thus, recent technological advances let us to explore, for the first time, the molecular mechanisms related to fitness using high throughput technology such as RNA-seq in children with OW/OB. Importantly, whole-blood includes immune cells that

play an important role in the atherothrombotic process and might manifest the organism's systemic inflammatory state associated with obesity and CVD [10,11]. Furthermore, the transcriptome profile in blood cells has been informative to provide biomarkers for molecular diagnostics and management of CVD [12]. Altogether this indicates that the whole-blood transcriptome profile could provide accessible biomarkers related to CVD and CRF levels in children with OW/OB.

The present study aimed to characterize the whole-blood transcriptome profile (RNA-seq) of fit children with OW/OB compared to unfit children with OW/OB. Our findings will promote a better understanding of the fat-but-fit paradigm and how fitness can counteract some of the adverse effects of obesity on CVD risk factors.

2. MATERIALS AND METHODS

2.1 Study sample

The present cross-sectional study used data from the ActiveBrains project (Clinical Trial: NCT02295072). Twenty-seven children with OW/OB (10.14 ± 1.3 years, 59% boys) were included in the current study. The methodology of the project, as well as the inclusion/exclusion criteria, have been reported in detail elsewhere [13]. This study was approved by the Committee for Research Involving Human Subjects at the University of Granada (Reference: 848, February 2014). According to the Declaration of Helsinki, the study's information was provided to all parents/legal guardians, which gave written informed consent.

2.2. Maturational status and body composition

Peak height velocity (PHV) was calculated as an indicator of maturational status using age and height in validated algorithms for boys and girls [14]. Body weight and height were collected using an electronic scale and a stadiometer (Seca instruments, Germany, Ltd). Body mass index (kg/m^2) was calculated, and participants were accordingly classified as OW/OB following the World Obesity Federation body mass index standards, specific for sex and age [15]. Waist Circumference (WC) was reported as an indicator of central fat distribution. Body composition parameters were measured by dual-energy X-ray absorptiometry (DXA, Discovery densitometer from Hologic) following the recommendations from the International Society of Clinical Densitometry.

2.3 Cardiorespiratory fitness

Cardiorespiratory fitness (i.e., VO_2peak) was quantified using a gas analyzer (General Electric Corporation) while performing a maximal incremental treadmill test (HP-Cosmos ergometer). The incremental test adapted for children with weight disturbances consisted of walking as long as possible at a constant speed (4.8 Km/h). The slope started at 6% with grade increments of 1% every minute until volitional exhaustion. Oxygen consumption, HR (beats/min), and respiratory exchange ratio (RER) were continuously measured and recorded every 10 s, whilst the rating of perceived exertion (RPE) scale was reported at the end of each 1 min stage using children's OMNI scale ranging from 0 to 10. CRF (i.e., VO_2peak) was reported relative to body weight (ml/kg/min). We classified the participants as "fit" and "unfit" according to the health-related cut points for CRF, i.e. 42 and 35 ml/kg/min relative to body weight for boys and girls respectively, derived from a meta-analysis of studies relating CRF to CVD risk in children and adolescents [16].

2.4 Blood sampling and analysis

Blood sampling was performed in the morning (8-9 AM) after an overnight fasting. Venous blood was drawn and collected in EDTA tubes. For transcriptome analyses, 500 μL of whole-blood with 1.3 mL RNAlater (Ambion Inc; Austin, Texas, USA), was stored at -80ºC until further processing. In regard to inflammatory markers quantification, blood was centrifuged at 1000×g for 10 minutes, and isolated plasma was stored at -80ºC.

2.4.1 RNA extraction and sequencing

Briefly, blood samples that contained RNA later were processed to isolate total RNA using RiboPureTM-Blood Kit (Thermo Fisher Scientific; Waltham, Massachusetts, USA), and the abundant globin mRNA content of erythrocytes was blocked using the GlobinLock mechanism [17]. The modified version of the single-cell tagged reverse transcription (STRT) protocol was followed to perform the full transcriptome analysis as described before [18]. High-quality RNA (10 ng) was converted into cDNA and amplified to form an Illumina-compatible library. The processing of the raw sequencing reads, alignment to the hg19 genome, and the quantification of the expression levels were done using the STRTprep pipeline, available at https://github.com/shka/STRTprep/tree/v3dev. The RNA-seq data are available in the Gene Expression Omnibus repository (GEO), accession number GSE164873.

2.4.2 Inflammatory markers

Pro-inflammatory cytokines IL-1β, TNF-α, and IL-6 were detected by multiple analyte profiling technologies (MILLIPLEX® MAP Human High Sensitivity T Cell Magnetic Bead Panel, EMD Millipore Corporation, Missouri, USA). For IL-1β and TNF-α the inter- and intra-assay coefficients of variation were ≤ 15% and ≤ 5%, respectively, and sensitivity of 0.14 pg/mL for IL-1β, and of 0.16 pg/mL for TNF-α. The inter- and

intra-assay coefficients of variation for IL-6 were ≤ 20% and ≤ 5%, respectively, with a sensitivity was 0.11 pg/mL.

2.5 Statistical analysis

The sample characteristics differences between fit and unfit children with OW/OB were tested using the t-student test and chi-squared test for continuous and categorical variables, respectively. ANCOVA analysis was performed to obtain adjusted mean differences between fit and unfit children with OW/OB after including sex and maturational status (i.e., PHV) as confounders. The analysis was performed using SPSS version 21.0 (IBM Corporation, NY, USA); statistical significance was defined at the level of $P < 0.05$.

Gene expression data was normalized using a quantile normalization. Subsequently, differential expression analysis between fit and unfit children with OW/OB was performed with Limma R/Bioconductor software package and were adjusted by sex and PHV (maturation), since these two factors are known to be highly influential at this period of life. Statistically significant differentially regulated genes were defined by a FDR < 5% (Benjamini and Hochberg correction on multiple testing). Scripts used to perform this analysis are available for readers: https://osf.io/neuys/. These genes were characterized by functional enrichment analysis using DAVID Bioinformatic resource. Pathways with an EASE score < 0.05 were considered significantly enriched. EASE score is a modified Fisher Exact P value in DAVID Bioinformatic resource used for functional enrichment analysis (EASE score P = 0 shows a perfect enrichment). In addition, in *silico* validation mining was performed with gene lists associated with different diseases publicly available in the PHENOPEDIA database. Briefly, PHENOPEDIA provides information about genetic associations studies in relation to different diseases, which is continuously updated from PubMed. Thus, differentially expressed genes in our study

were overlapped with lists of genes involved in different diseases, i.e., CVD, metabolic syndrome, hypertension, inflammation, and asthma.

3. RESULTS

Descriptive characteristics are presented in **Table 1**. In the fit group, 25% of children were boys and 75% girls, while in the unfit group 87% of children were boys and 13% girls. Fit children presented higher CRF (i.e., VO_2peak relative to body weight; unadjusted mean difference of 3 ml/kg/min, and a difference of 8.5 ml/kg/min in adjusted models) and lower values of pro-inflammatory cytokine IL-1β (unadjusted mean difference of -0.50 pg·mL-1, and a difference of -1.12 pg·mL-1 in adjusted models) compared to unfit children after adjusting for sex and PHV (adjusted P value < 0.05). Also, borderline differences were found for pro-inflammatory cytokine IL-6 after adjusting for sex and PHV (adjusted P value = 0.09; unadjusted mean difference of -0.46 pg·mL-1, and a difference of -1.06 pg·mL-1 in adjusted models).

Table 1. Characteristics of the participants

Variables	Total sample n=27 (16 boys/11 girls)	Fit n=12 (3 boys/9 girls)	Unfit n=15 (13 boys/2 girls)	Unadjusted P value	Adjusted P value*
Age and Maturational status					
Age (years)	10.1 ± 1.3	10.1 ± 1.2	10.2 ± 1.4	0.74	0.17
PHV offset (years)	-2.15 ± 0.94	-1.76 ± 0.80	-2.47 ± 0.96	0.50	N.A.
BMI group					
Overweight	6 (22.2%)	3 (25.0%)	3 (20.0%)	0.56	N.A.
Obesity	21 (77.8%)	9 (75.0%)	12 (80.0%)		
Body composition and anthropometry					
Weight (kg)	57.31 ± 10.30	58.07 ± 9.23	56.70 ± 11.37	0.74	0.49
Height (cm)	145.65 ± 9.06	147.18 ± 8.16	144.43 ± 9.83	0.44	0.30
Waist circumference (cm)	91.72 ± 7.26	91.50 ± 5.54	91.89 ± 8.58	0.89	0.09
BF (%)	42.73 ± 4.71	42.91 ± 5.37	42.58 ± 4.29	0.86	0.05
DXA FM (kg)	24.22 ± 5.71	24.50 ± 5.10	24.00 ± 6.31	0.83	0.16
DXA total VAT (g)	414.29 ± 85.91	424.48 ± 89.43	406.14 ± 85.22	0.59	0.72
DXA LM (Kg)	30.71 ± 5.43	31.12 ± 5.81	30.38 ± 5.30	0.73	0.51
Inflammatory markers					
IL-1β (pg·mL-1)	1.77 ± 0.72	1.49 ± 0.56	1.99 ± 0.77	0.08	**0.002**
IL-6 (pg·mL-1)	2.31 ± 1.98	1.79 ± 0.62	2.25 ± 1.33	0.26	0.09
TNF-α (pg·mL-1)	3.92 ± 1.13	3.91 ± 1.22	3.93 ± 1.11	0.95	0.37
Cardiorespiratory fitness					
VO2peak BW (ml/kg/min)	37.68 ± 4.44	39.39 ± 5.27	36.32 ± 3.21	0.07	< **0.001***

Data presented as non-adjusted means ± SDs, and as number and frequency. BMI: Body mass-index, BF: Body fat, FM: Fat mass, VAT: Visceral adipose tissue, LM: lean mass, PHV: Peak height velocity. BW: Body weight, LM: Lean mass, abs: absolute, N.A.: not applicable. Bold numbers indicates $P < 0.05$; * p values derived from ANCOVA models adjusted for sex and maturation (i.e., PHV). IL-1β and IL-6 (n = 25), TNF-α (n = 26)

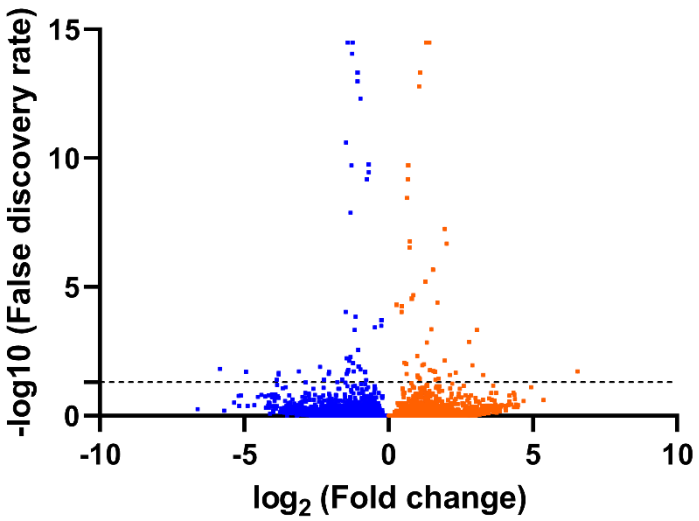

Figure 1. Volcano plot indicating the 256 differential expressed genes (up-regulated in yellow and down-regulated in blue) between fit and unfit children with overweight/obesity. The x-axis reflects the log_2 fold change (FC) value, while the y-axis corresponds to the false discovery rate (FDR). Statistically significance threshold (i.e., FDR < 0.05 corresponds to –log10 > 1.30 in the horizontal dashed line).

Two hundred and fifty six genes were differentially expressed (145 up-regulated and 111 down-regulated, log_2FC ranged from -5.83 to 6.55, FDR < 0.05) in fit children compared to unfit children after adjusting by sex and PHV (**Figure 1; Supplementary Table 1**). The differentially expressed genes were enriched in two pathways related to inflammation: dopaminergic synapse and GABAergic synapse (EASE score < 0.05) (**Table 2**). Genes identified in dopaminergic and GABAergic synapse pathways were linked to obesity (**Supplementary Table 2**). *In silico* validation data mining within the PHENOPEDIA database detected that 9 of the differentially expressed genes between fit and unfit children were involved in CVD, 11 genes in metabolic syndrome, 30 genes in hypertension, 25 genes inflammation, and 13 genes in asthma (**Table 3**). Further, 33 top-genes were selected based on the highest log_2FC (threshold ≥1.5) (**Figure 2**). Three of these 33 genes were enriched in the detected dopaminergic synapse, and GABAergic synapse pathways: *GNG10, CREB3L3, and PPP2R5E* (**Table 2**), while 3 of these 33

genes were detected in the *in silico* validation data mining using the PHENOPEDIA database: *IL2RA, GRB2, and MAL* (**Table 3**).

Table 2. Pathway enrichment analysis of differentially expressed genes in fit children compared to unfit children with overweight/obesity using DAVID bioinformatic resource (EASE score < 0.05).

Selected pathways (KEGG)	**Genes identified in each pathway**
Dopaminergic synapse	*GNAO1*↑ *GNAL*↑ *GNG10*↑ *GNG8*↓ *CREB3L3*↑ *PPP2R5E*↑
GABAergic synapse	*GNAO1*↑ *GNG10*↑ *GNG8*↓ *GABARAP*↑ *GABBR1*↓
Molecular function	**Genes involved**
Histone deacetylase binding	*DNMT3B*↓ *KLF4*↓ *CCND1*↑ *KAT2B*↑ *MEF2B*↑ *ZMYND15*↑
zinc ion transmembrane transporter activity	*SLC30A4*↓ *SLC30A8*↑ *SLC39A4*↓

KEGG: Kyoto Encyclopedia of Genes and Genomes.

4. DISCUSSION

Our study highlight different transcriptome profiles between fit and unfit children with OW/OB, where a number of molecular pathways related to immune system and inflammation are involved, such as dopaminergic and GABAergic synapse pathways.

Exercise and physical activity are the main environmental factors able to modify CRF, and therefore, fit and unfit groups might be in part indicative of more and less active children, respectively. Importantly, Fit and unfit groups presented a unadjusted mean difference of 3 ml/kg/min in VO2peak, and a difference of 8.5 ml/kg/min in adjusted models. A threshold of 1.75 ml/kg/min in VO2peak has been considered clinically relevant [19]. Thus, the transcriptome analyses between Fit and Unfit groups could be of interest to gain a better understanding of the molecular mechanisms related to CRF and health in children with OW/OB. In our study, differentially expressed genes between fit

and unfit children enriched dopaminergic and GABAergic synapse pathways; most of the genes were up-regulated in this pathways (*GNAO1, GNAL, GNG10, CREB3L3, PPP2R5E,* and *GABARAP*). In this context, exercise could increase levels of neurotransmitters, such as dopamine and amino acid γ-aminobutyric acid (GABA) in plasma and in different brain regions in humans [20–22]. Besides, neurological disorders have elucidated that dopamine might play an important role in controlling movement [23]. Interestingly, impairments in dopamine synthesis, release and receptor function (mainly in the nervous system cells) could be underlying the lack of physical activity in humans with obesity [23].

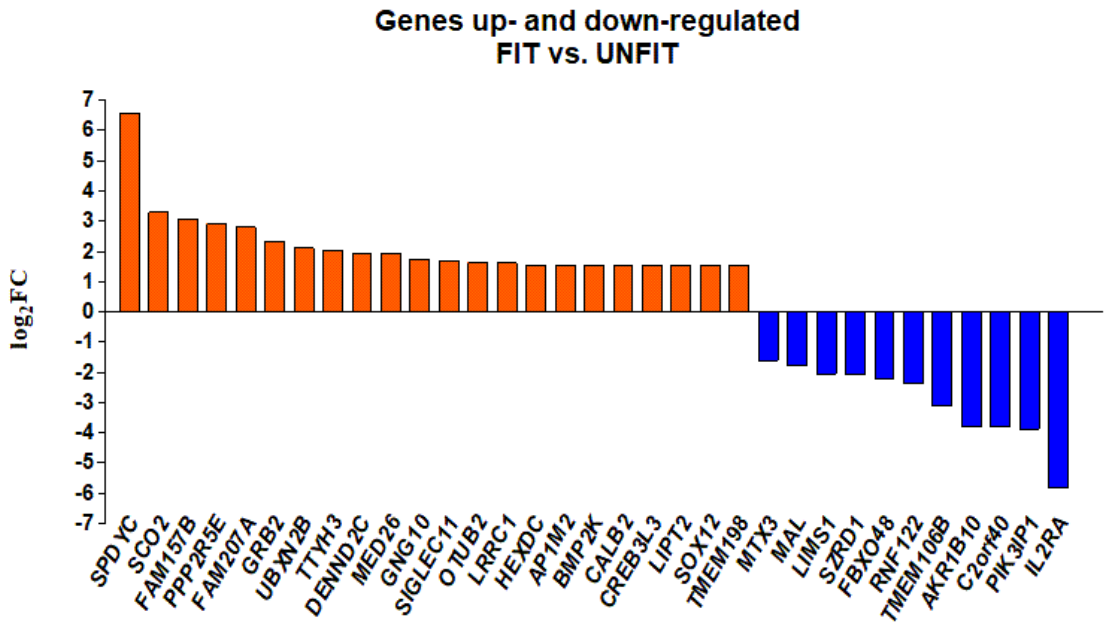

Figure 2. 33 top-genes significantly up- and down-regulated (fold change [FC]) ≥1.5) in fit children compared to unfit children with overweight/obesity according to Limma analysis (up-regulated in orange and down-regulated in blue). False discovery rate, FDR < 0.05

Importantly, dopaminergic and GABAergic receptors are expressed in different types of immune cells with different roles in the immune system [24,25]. Thus, dopaminergic pathways has been related to obesity-associated inflammation, although the specific molecular mechanisms in different type of immune cells need to be clarified [25].

Furthermore, GABA reduced the secretion of 47 cytokines (IL-1β included) in peripheral blood mononuclear cells (PBMC) and CD4+ T cells of type 1 diabetes patients [26]. Also, Reyes-García et al. reported that GABA decreased IL-6 production in peripheral macrophages of rodents [27], while Bhat et al. showed that increased GABAergic activity reduced autoimmune inflammation [28]. Therefore, dopaminergic and GABAergic pathways in immune cells have been related to inflammation such as an emergent research area [24,25]. Indeed, we did observed in our study that fit children presented a more favorable inflammatory profile than unfit children, i.e., lower values of circulating pro-inflammatory cytokines such as IL-1β and IL-6, which are involved in CVD. Importantly, obesity might impair the dopaminergic and GABAergic systems [23,29,30]. Thus, good CRF levels (modifiable by physical activity and exercise) could attenuate the negative impact of obesity on the dopaminergic and GABAergic systems (i.e., the *fat but fit* paradigm). These findings, however, should be interpreted with caution, as for example IL-6 can exert both pro- and anti-inflammatory effects [31]. High levels of circulating IL-6 (measured at resting conditions) could induce pro-inflammatory effects and are related to pediatric obesity, insulin resistance and lipid metabolism [32]. Otherwise, IL-6 has been considered a pleiotropic myokine with anti-inflammatory properties when is released by skeletal muscle in response to acute exercise [31]. In our study, IL-6 was considered a pro-inflammatory cytokine because was quantified at resting conditions in children with overweight/obesity. The interpretation of circulating IL-6 levels could be different in response to acute exercise.

It is well known that low CRF levels are associated with more CVD events [33], a higher risk of asthma incidents [34], and unfavorable cardiometabolic and inflammatory profiles [35]. In order to test the validity of our findings, i.e. of the differentially expressed genes in fit *vs.* unfit children with OW/OB, we performed an *in silico* validation data

mining using PHENOPEDIA database. Our findings showed that differentially expressed genes according to fitness groups were involved in CVD, metabolic syndrome, hypertension, inflammation, and asthma, matching therefore well with previous epidemiological evidence [33–35]. These results suggest that these differentially expressed genes could contribute partially to a better cardiovascular profile in those children with higher CRF levels. Further studies should analyze these genes' mechanistic role in developing CVD in the pediatric population with weight disturbances.

On single gene level, of specific interest are *SCO2* and *IL2RA* genes that showed the highest and lowest regulation in fit *vs.* unfit OB/OW children. The IL-2 receptor (IL-2R) comprises three subunits (IL-2Rα, IL-2Rβ, and IL-2γc). The IL-2Rα subunit encoded by *IL2RA* gene regulates T lymphocyte activation, playing an important role in the atherothrombotic process, although the precise mechanisms are unclear [36,37]. Interestingly, high concentrations of plasma soluble IL-2Rα have been positively associated with CVD risk factors and mortality in older adults [38]. Therefore, that the *IL2RA* gene was found down-regulated in the fit group in our study supports the notion that this could be one of the mechanisms why higher CRF linked to better cardiovascular health.

Table 3. *In silico* validation of differentially expressed genes in fit *vs*. unfit children with overweight/obesity using PHENOPEDIA database

PHENOPEDIA input term	Genes overlapped
Cardiovascular disease	*TNNI3*↓ *TNFRSF11A*↓ *APOC3*↑ *KAT2B*↑ *MEIS2*↑ *SPTA1*↑ *AHSG*↑ *SLC30A8*↑ *NPY*↑
Metabolic syndrome	*TNNI3*↓ *PTGES*↓ *IGF2R*↓ *TNFRSF11A*↓ *APOC3*↑ *CCND1*↑ *AHSG*↑ *SLC30A8*↑ *NPY*↑ *LRP8*↑ ***GRB2***↑
Hypertension	***IL2RA***↓ *MYBPH*↓ *TNNI3*↓ *DYNC1H1*↓ *NAT8*↓ *PTGES*↓ *IGF2R*↓ *TNFRSF11A*↓ *TOX2*↓ *ARVCF*↓ *ATP1B1*↓ *APOC3*↑ *CCND1*↑ *CXCL13*↑ *DDAH1*↑ *IL9*↑ *MLXIP*↑ *LTBP4*↑ *MFAP2*↑ *SPTA1*↑ *TNKS*↑ *NF1*↑ *SLC30A8*↑ *MYLK*↑ *NDST1*↑ *TXN*↑ *NPY*↑ *RFX7*↑ *LRP8*↑ ***GRB2***↑
Inflammation	***IL2RA***↓ ***MAL***↓ *SMAD7*↓ *DNMT3B*↓ *MYBPH*↓ *C3orf18*↓ *PTGES*↓ *IGF2R*↓ *GABBR1*↓ *TNFRSF11A*↓ *CDH6*↓ *KIF3B*↓ *APOC3*↑ *CCND1*↑ *CXCL13*↑ *DDAH1*↑ *GNAO1*↑ *IL9*↑ *RGMA*↑ *KAT2B*↑ *AHSG*↑ *SLC30A8*↑ *MYLK*↑ *TXN*↑ *NPY*↑
Asthma	*MYLK*↑ ***IL2RA***↓ *NPY*↑ *PTGES*↓ *TNFRSF11A*↓ *SLC22A15*↓ ***GRB2***↑ *IGSF11*↑ *TMEM79*↓ *CCND1*↑ *CXCL13*↑ *IL9*↑ *NRXN1*↑

Bold genes are contained in the list of 33 genes with the highest $\log_2$ fold changes (FC) (threshold $\log_2$FC 1.5; false discovery rate, FDR < 0.05).

Interestingly, the bioenergetic capacity of PBMC (i.e., higher maximal respiration of PBMC) was associated with lower circulating IL-6 in adults with OW/OB [39]. In this regard, SCO2 protein is fundamental for the assembly of cytochrome c oxidase, which is essential for cellular respiration and the aerobic ATP production in the mitochondria [40]. Markedly, an increase in age has been negatively associated with *SCO2* gene expression, while exercise training increased the *SCO2* gene expression levels in cardiac cells of old and young rodents [41]. Interestingly, mutations in *SCO2* gene have been associated with infantile cardioencephalomyopathy [42]. Furthermore, promoter hypermethylation and reduced *SCO2* gene expression were reported in cardiac cells of patients with congenital heart diseases [43]. We hypothesize that a lower *IL2RA* and higher *SCO2* gene expression levels in blood cells of fit children could promote a better cardiovascular profile in those children compared to unfit children.

Our study presents three main limitations. First, the cross-sectional study design does not allow us to assume causal relationships. Second, our sample size was relatively small and most of children in the fit group were girls (9 of 12) while in the unfit group were boys (13 of 15), which could have influenced the analysis. Nevertheless, the Limma analysis was controlled by sex, to attenuate the potential confounding role in this analysis. Third, whole-blood samples were used to perform whole transcriptome analysis. In this regard, it is known that different leukocyte populations have specific roles in the immune system and CVD. Nonetheless, the whole-blood RNA-seq reflects the general system's response to the stimulus and it has served as useful approach to identify "aberrant" gene expression patterns associated with different diseases.

Despite these limitations, some strengths in our study need to be acknowledged. To our knowledge, this is the first study to analyse the whole-blood transcriptome profiles using high throughput technology such as RNA-seq in fit children compared to unfit children with OW/OB. Besides, transcriptome analysis was performed using blood samples obtained in first hour in the morning at fasting conditions in a unified manner. Futhermore, GlobinLock molecular mechanism was applied as a novel robust method to block abundant globin mRNA in erythrocytes [17], which hinder the whole-blood transcriptome analysis.

In conclusion, differentially expressed genes between fit and unfit children with OW/OB are involved in dopaminergic and GABAergic synapse pathways. Further, in *silico* validation data mining using PHENOPEDIA database detected several differentially expressed genes related to CVD, metabolic syndrome, hypertension, inflammation, and asthma. The top candidate genes involved in link between CRF and CVD include *IL2RA*, *SCO2*, *GRB2*, *MAL*, *GNG10*, *CREB3L3*, and *PPP2R5E*. Our results promote a better understanding of how fitness might contribute to a favorable CVD risk factors profile in youth and potentially reduce CVD later in adulthood.

5. PERSPECTIVE

CRF is a powerful marker of health in children [2], which is modifiable by physical activity and exercise. For the first time, a distinct pattern of whole-blood transcriptome profile (RNA-seq) was identified in fit children with overweight-obesity (OW/OB) compared to unfit children with OW/OB. The identified whole-blood transcriptome profile in fit children with OW/OB might be related to inflammation and promote a better understanding of how fitness might contribute to reduce CVD later in

adulthood. Therefore, understanding the molecular mechanisms underlying the health benefits of CRF promote the use of exercise as a form of medicine in a more precise and personalized way in children with OW/OB.

SUPPLEMENTARY MATERIAL

Supplementary material may be found online in the Supporting information section (https://onlinelibrary.wiley.com/doi/10.1111/sms.14028).

In additin, the supplementary files can be downloaded in this link:

https://osf.io/unpje/?view_only=d3eeb5a70be44b84a845efc96607c08a

Supplementary Table 1. 256 differentially expressed genes between Fit and Unfit children with overweight/obesity (False dicovery rate, FDR < 0.05).

Supplementary Table 2. Differentially expressed genes in fit children compared to unfit children with overweight/obesity enriched in the domapinergic and GABAergic synapse pathways and possible link to obesity or inflammation.

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STUDY III: Cardiorespiratory fitness and targeted proteomics involved in brain and cardiovascular health in children with overweight/obesity

1. INTRODUCTION

Childhood obesity is related to increased cardiovascular disease (CVD) risk factors (e.g., fasting glucose, triglycerides, blood pressure, atherosclerosis, inflammation), and negatively affects brain health (i.e., cognitive and brain development) [1–3]. Both, CVD risk factors and brain health impairment share similar pathophysiology mechanisms, what has been called "the heart-brain connection" [4,5]. Closely related to adiposity and body composition are physical fitness components, and particularly, cardiorespiratory fitness (CRF). Consistent evidence support the notion that CRF is a powerful marker of health in youth [6]. Higher CRF levels are inversely associated with CVD risk factors during childhood (even early subclinical atherosclerosis) [7,8], and later in adulthood [9]. Besides, CRF is positively associated with brain volume at different regions related to better academic performance [10], and improved hippocampal connectivity and cognitive function [11–13] in children with overweight/obesity (OW/OB).

The fat-but-fit paradigm shows how CRF can reduce the detrimental impact of obesity on CVD risk factors, brain structure, cognitive function, and academic achievement in children [14–16]. However, less is known regarding the molecular mechanisms relating CRF to cardiovascular and brain health in children with OW/OB [17]. In this context, high-througput technological advances in the last decades provide the tools to explore the molecular mechanisms of CRF in children with OW/OB detecting thousands of molecules simultaneously [17]. We have recently performed the first whole-blood transcriptome profiling (RNA-sequencing) in fit and unfit children with OW/OB, where gene pathways related to CVD risk factors (i.e., mainly systemic inflammation) were detected [18]. In the current study, we applied targeted proteomic approach for search of cardiovascular and brain health biomarkes in different fitness levels in

childhood obesity by analyzing 184 proteins (92 brain health- and 92 CVD-related proteins) in plasma of fit compared to unfit children with OW/OB.

2. METHODS

2.1 Participants and study design

This cross-sectional study included eighty-seven children with OW/OB (10.14 ± 1.3 years, 59% boys) from the ActiveBrains project (Clinical Trial: NCT02295072) with available data of proteins included in the Olink Neurology panel and CRF. Due to budget restrictions, proteins included in the Cardiovascular panel II were measured in a subsample of 44 participants from the ActiveBrains project. This subsample was randomly selected using the SPSS software (version 21.0; Armonk, NY, USA). Subsequently, we verified that the subsample of 44 participants did not differ in key characteristics of this study, i.e., sex, age, maturation, or CRF, being therefore the subsample representive of the complete sample. The ActiveBrains project was approved by the Committee for Research Involving Human Subjects at the University of Granada (Reference: 848, February 2014). The inclusion/exclusion criteria and the methodology have been described in detail before [19]. Briefly, the inclusion criteria were: (i) children aged 8 – 11 years old with OW/OB using the World Obesity Federation cut-off points [20], (ii) not have neurological disorders that might have an impact on movement performance; (iii) the girls have not started menstruation; (iv) right-handed (differences in brain health between left- and right-handed may exist). All parents/legal guardians written informed consent following the Declaration of Helsinki and received the study's information.

2.2. Anthropometry and maturation

An electronic scale and a stadiometer (Seca Instruments, Germany, Ltd) were used to obtain weight, height, and body mass index (BMI) calculated as kg/m^2. Children were classified with OW/OB using the sex- and age-specific cut-off points (World Obesity Federation) [20]. Peak height velocity (PHV) reported the maturational status of children using validated algorithms for boys and girls [21]. PHV was calculated as follows for boys: −8.1 + (0.0070346 × (age x sitting height)) and girls: −7.7 + (0.0042232 × (age × height)) [21].

2.3. Cardiorespiratory fitness

CRF (i.e., VO_2peak) assessment has been described previously [18]. Briefly, an incremental treadmill test (HP-Cosmos ergometer) was performed using a gas analyzer (General Electric Corporation). The slope started at 6% with grade increments of 1% every minute until volitional exhaustion, while the speed was constant during the exercise test (i.e., 4.8 Km/h). CRF (i.e., VO_2peak) was reported relative to body weight (mL/kg/min). CRF levels higher than 42 ml/Kg/min (for boys) and 35 ml/Kg/min (for girls) relative to body weight were considered to classify children as "fit", while children with lower values were considered as "unfit". These health-related cut-off points for CRF relative to body weight were provided by a meta-analysis addressing the cardio-protective role of CRF in children [22].

2.4. Brain health- and CVD-related targeted proteomics

Blood collected in EDTA tubes (in the morning between 8-9 a.m. after 12h of fasting) was centrifuged at 1000×g for 10 min. Isolated plasma was stored at −80ºC. The 92 brain health-related and 92 CVD-related proteins were quantified in plasma (1 microliter) at the Olink laboratory in Uppsala using the PEA technology (Proseek Multiplex Neurology and Cardiovascular panel II 96 × 96 reagents kit [Olink®

Bioscience, Uppsala, Sweden]). The PEA technology has been described in detail (https://www.olink.com/). Briefly, antibody pairs labeled with DNA oligonucleotides bind to the target protein in plasma. Then, oligonucleotides in proximity hybridize and are extended by a DNA polymerase. Finally, the new DNA sequence (specific for each protein) is detected and quantified using a microfluidic qPCR. Normalized protein expression values (NPX values) are presented as arbitrary units in the log2 scale. NPX values are calculated from Ct values and are interpreted in the opposite direction (i.e., higher NPX values show higher protein concentrations, while higher Ct values indicate lower concentration). Intra- and inter-assay coefficients of variations, detection limits, and specific information for each protein are reported on the manufacturer's website (https://www.olink.com/).

2.5 Statistical analyses

SPSS version 21.0 (IBM Corporation, NY, USA) was used for statistical analyses, while a threshold of $p < 0.05$ was considered statistically significant. Student t-test (continuous variables) and chi-square (categorical variables) tests were used to study the differences on sample characteristics between fit and unfit children with OW/OB. ANCOVA was performed to obtain adjusted mean differences on 92 brain health-related and 92 CVD-related proteins in plasma between fit and unfit children with OW/OB after including sex, maturational status (i.e., PHV), and BMI as confounders. Analyses were adjusted for multiple comparisons using false discovery rate (FDR) based on the Benjamini-Hochberg method.

3. RESULTS

Characteristics of participants are shown in **Table 1**. In the fit group, 27% of participants were boys and 73% girls, while 79% were boys and 21% were girls in the unfit group. The fit group presented higher VO2peak relative to body weight, lower body weight, BMI, compared to the unfit group ($p < 0.05$). Descriptive information about the subsample of 44 children with data for CVD-related proteins is presented in **Supplementary Table 1**.

Table 1. Characteristics of the participants

Variables	Total sample n=87	Fit children n=34	Unfit children n=53	P value
Sex, age, and maturational status				
Boys, n (%)	51 (59%)	9 (27%)	42 (79%)	**<0.001**
Age (years)	10.08 ± 1.12	9.86 ± 1.14	10.22 ± 1.10	0.15
Years from PHV	-2.25 ± 0.97	-2.02 ± 0.91	-2.39 ± 0.98	0.08
BMI group by Cole *et al.*				
Overweight	31 (36%)	19 (56%)	12 (23%)	**0.003**
Obesity	56 (64%)	15 (44%)	41 (77%)	
Anthropometry				
Weight (kg)	55.34 ± 10.30	51.57 ± 10.08	57.77 ± 11.35	**0.01**
Height (cm)	144.01 ± 8.60	143.53 ± 9.63	144.31 ± 7.96	0.68
Waist circumference (cm)	89.50 ± 9.84	85.27 ± 8.83	92.22 ± 9.57	**0.001**
BMI (kg/m^2)	26.45 ± 3.55	24.81 ± 2.65	27.50 ± 3.67	**<0.001**
Cardiorespiratory fitness				
VO2peak relative to BW (ml/Kg/min)	37.56 ± 4.55	40.59 ± 4.26	35.61 ± 3.59	**<0.001**

Data presented as unadjusted mean ± SD, and as number and frequency. BMI: Body mass-index, BF: Body fat, FM: Fat mass, LM: lean mass, PHV: Peak height velocity. BW: Body weight, LM: Lean mass, abs: absolute, N.A.: not applicable. A. Bold numbers show $P < 0.05$; * reflects p values adjusted for sex and maturation (i.e., PHV) after performing ANCOVA analysis

Concerning 92 brain health-related proteins (N = 87; 34 Fit *vs.* 53 Unfit), 1 protein (MAPT) was below the limit of detection across all the plasma samples and was not included in statistical analyses. Ten proteins were differentially expressed between study groups. Specifically, 9 proteins were down-regulated (PLXNB3, sFRP3, CLEC1B, RSPO1, Gal8, GCP5, MDGA1, CTSC, LAT; adjusted mean differences ranged from -1.00 to -0.23 NPX values, $p < 0.05$) and 1 up-regulated (CLEC10A; adjusted mean differences 0.28 NPX values , $p<0.05$), in fit children compared to unfit children after adjusting by sex, maturation (i.e., PHV), and BMI (**Table 2; Figure 1, Panel A**). These proteins were involved in diverse diseases categories: neurological, cardiovascular, cancer, and inflammatory and biological processes: immune/inflammatory response, neurogenesis, signal transduction, and cellular metabolic process (**Table 3**). However, when applying the multiple testing correction, the expression level of these proteins did not reach significance level.

Regarding 92 CVD-related proteins (N = 44; 19 Fit *vs.* 25 Unfit), 6 proteins were differentially expressed. Specifically, 3 proteins were down-regulated (PRSS27, CXCL1, Gal9; adjusted mean differences ranged from -1.04 to -0.21 NPX values, $p<0.05$) and 3 up-regulated (GT, IL4RA, MERTK; adjusted mean differences ranged from 0.19 to 0.54 NPX values, $p < 0.05$) in fit children compared to unfit children after adjusting by sex, maturation (i.e., PHV), and BMI (**Table 2; Figure 1**, **Panel B**). These proteins were involved in diverse diseases categories: cardiovascular, pulmonary, and inflammatory, among others and biological processes: cell adhesion, immune response, and inflammaotry response, among others (**Table 3**). The protein expression levels, however, did not survive multiple hypothesis testing correction (FDR > 0.05). The non-significant results for 81 brain health- and 86 CVD-related proteins are presented in **Supplementary Tables 2 and 3** (all $p > 0.05$).

Table 2. Differentially expressed proteins, analysed with Olink Neurology Panel and Cardiovascular II panel between Fit and Unfit children with overweight/obesity.

Fit *vs.* Unfit - Neurologic panel (main study simple, N = 87)											
	Fit children (N = 34)			Unfit children (N = 53)			Difference between groups				
	Adjusted Mean	Lower limit 95% CI	Upper limit 95% CI	Adjusted Mean	Lower limit 95% CI	Upper limit 95% CI	Adjusted Mean	Lower limit 95% CI	Upper limit 95% CI	p-value	FDR
CLEC10A	5.84	5.70	5.99	5.56	5.44	5.69	0.28	0.07	0.50	0.01	0.17
CLEC1B	9.70	9.26	10.14	10.52	10.14	10.89	-0.82	-1.47	-0.17	0.02	0.17
CTSC	2.76	2.57	2.96	3.12	2.96	3.28	-0.35	-0.64	-0.07	0.01	0.17
gal8	5.62	5.26	5.97	6.21	5.91	6.51	-0.59	-1.11	-0.07	0.03	0.28
GCP5	5.21	4.97	5.45	5.67	5.47	5.87	-0.46	-0.82	-0.10	0.01	0.17
LAT	5.49	4.95	6.02	6.49	6.04	6.94	-1.00	-1.79	-0.22	0.01	0.17
MDGA1	4.85	4.59	5.10	5.23	5.01	5.45	-0.39	-0.76	-0.01	0.04	0.41
PLXNB3	4.12	3.84	4.41	4.66	4.42	4.90	-0.54	-0.96	-0.12	0.01	0.17
RSPO1	1.86	1.74	1.97	2.08	1.98	2.18	-0.23	-0.40	-0.06	0.01	0.17
sFRP3	3.05	2.75	3.36	3.67	3.41	3.92	-0.61	-1.06	-0.17	0.01	0.17
Fit *vs.* Unfit - Cardiovascular panel (randomly selected subsample, 50% of main study simple, N = 44)											
	Fit children (N = 19)			Unfit children (N = 25)			Difference between groups				
	Adjusted Mean	Lower limit 95% CI	Upper limit 95% CI	Adjusted Mean	Lower limit 95% CI	Upper limit 95% CI	Adjusted Mean	Lower limit 95% CI	Upper limit 95% CI	p-value	FDR
CXCL1	8.58	7.88	9.27	9.61	9.05	10.18	-1.04	-2.01	-0.07	0.04	0.67
Gal9	7.96	7.84	8.09	8.18	8.08	8.28	-0.21	-0.39	-0.04	0.02	0.49
GT	3.02	2.67	3.36	2.48	2.20	2.75	0.54	0.06	1.02	0.03	0.62
IL4RA	2.35	2.21	2.49	2.16	2.05	2.27	0.19	0.00	0.39	0.04	0.67
MERTK	6.28	6.12	6.44	5.99	5.87	6.12	0.29	0.07	0.51	0.01	0.49
PRSS27	8.79	8.52	9.06	9.25	9.03	9.47	-0.46	-0.83	-0.09	0.02	0.49

ANCOVA analyses adjusted by sex, maturational status (i.e., peak height velocity) and body mass index. Data are presented using NPX (Normalized Protein eXpression) values. A 1 NPX value difference means a doubling of protein concentration.

CI: Confidence intervals, CLEC10A: C-type lectin domain family 10 member A, CLEC1B: C-type lectin domain family 1 member B, CTSC: Dipeptidyl peptidase 1, CXCL1: C-X-C motif chemokine 1, FDR: False discovery rate, Gal-8: Galectin-8, Gal-9: Galectin-9, GCP5: Glypican-5, GT: Gastrotropin, IL-4RA: Interleukin-4 receptor subunit alpha, LAT: Linker for activation of T-cells family member 1, MERTK: Tyrosine-protein kinase Mer, MDGA1: MAM domain-containing glycosylphosphatidylinositol anchor protein 1, PLXNB3: Plexin-B3, PRSS27: Serine protease 27, RSPO1: R-spondin-1, sFRP3: Secreted frizzled-related protein 3

Table 3. Disease area and biological process (obtained from https://www.olink.com/) enriched in the 16 differentially expressed proteins (10 from the Neurology Panel and 6 from the Cardiovascular II panel) between Fit and Unfit children with overweight/obesity.

Fit *vs.* Unfit - Neurologic panel (N = 87; Fit = 34 and Unfit = 53)		
	Disease Area	Biological process
CLEC10A	Cardiovascular, neurological, infectious, cancer	Immune response
CLEC1B	Cardiovascular, skeletal, renal, metabolic, digestive, cancer	Cell differentiation, signal transduction
CTSC	Cardiovascular, metabolic, cancer, cutaneous	Cell death, cellular metabolic process, immune response, proteolysis
Gal-8	Neurological, inflammatory, cancer	Other GO terms
GCP5	Cardiovascular, renal, neurological, metabolic, digestive, cancer	Cellular metabolic process
LAT	Renal, neurological, inflammatory, infectious, cancer	Cell adhesion, cellular metabolic process, immune response, MAPK cascade, signal transduction
MDGA1	Neurological	Cell differentiation, neurogenesis
PLXNB3	Neurological, cancer	Axon development, axon guidance, cell adhesion, cell differentiation, neurogenesis, signal transduction
RSPO1	Digestive, cancer, cutaneous	Cellular metabolic process, signal transduction
sFRP3	Skeletal, cancer	Cell death, cell differentiation, cell growth, signal transduction
Fit *vs.* Unfit - Cardiovascular panel (N = 44; Fit = 19 and Unfit = 25)		
	Disease Area	Biological process
CXCL1	Cardiovascular, Pulmonary, inflammatory, hepatic, digestive, skeletal	Immune response, inflammatory response

Gal-9	Skeletal, neurological, Hemic and Lymphatic, cancer	Cell adhesion, immune response, inflammatory response, MAPK cascade
GT	Metabolic, digestive, cancer	Catabolic process
IL-4RA	Cardiovascular, Pulmonary, neurological, inflammatory, infectious, cutaneous, cancer	Cell adhesion, immune response, inflammatory response
MERTK	Cardiovascular, Pulmonary, neurological, inflammatory, digestive	Cell adhesion, coagulation, platelet activation, wound healing
PRSS27	Cardiovascular, cancer	Other GO terms

CLEC10A: C-type lectin domain family 10 member A, CLEC1B: C-type lectin domain family 1 member B, CTSC: Dipeptidyl peptidase 1, CXCL1: C-X-C motif chemokine 1, Gal-8: Galectin-8, Gal-9: Galectin-9, GCP5: Glypican-5, GO: Gene ontology, GT: Gastrotropin, IL-4RA: Interleukin-4 receptor subunit alpha, LAT: Linker for activation of T-cells family member 1, MERTK: Tyrosine-protein kinase Mer, MDGA1: MAM domain-containing glycosylphosphatidylinositol anchor protein 1, PLXNB3: Plexin-B3, PRSS27: Serine protease 27, RSPO1: R-spondin-1, sFRP3: Secreted frizzled-related protein 3.

4. DISCUSSION

This study indicates that a number of cardiovascular and brain health biomarkers might be differentially expressed in plasma of fit compared to unfit children with OW/OB. Specifically, from neurology panel, 9 biomarkers were down-regulated (PLXNB3, sFRP3, CLEC1B, RSPO1, Gal8, GCP5, MDGA1, CTSC, LAT) and 1 up-regulated (CLEC10A); and from cardiovascular panel, 3 were down-regulated (PRSS27, CXCL1, Gal9) and 3 up-regulated (GT, IL4RA, MERTK). These proteins were involved in several diseases categories (e.g., neurological, cardiovascular, inflammatory, pulmonary) and biological process (e.g., neurogenesis, signal transduction, immune function, inflammatory response). Thus, these findings provide novel plasma biomarkers related to CRF levels in children with OW/OB. However, statistical analyses did not survive multiple correction, and therefore these findings should be interpreted as preliminary and should be confirmed in studies involving larger sample size.

Interestingly, our fit and unfit groups showed a mean difference of 4.98 mL/kg/min in VO_2peak relative to body weight. In this regard, differences of 1.75 mL/kg/min of VO_2peak relative to body weight have been considered relevant from a clinical point of view [23]. Thus, the different patterns of protein expression between fit and unfit children may help to understand better the molecular mechanisms relating CRF to health in children with OW/OB. Several proteins (i.e., differentially expressed in fit compared to unfit children) are discussed in the context of existing knowledge linking these protein biomarkers to human diseases, animal experiments, or cell cultures. We will discuss proteins presenting higher mean differences between fit and unfit groups, and interesting proteins with possible implications for brain and cardiovascular health. The biological process and disease categories of 16 differentially expressed proteins between fit and unfit groups were presented in Table 3.

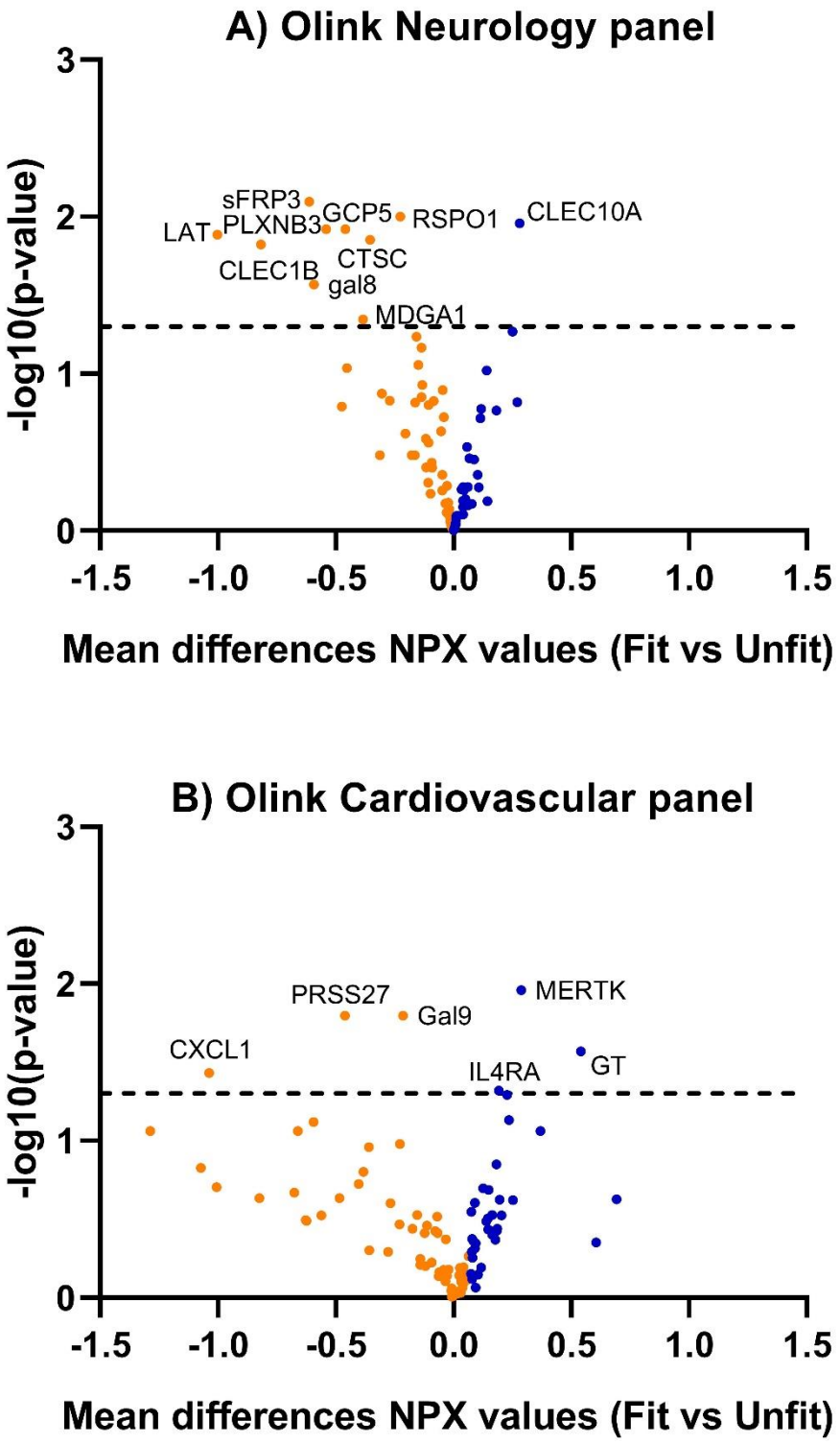

Figure 1. Volcano plot shows the 16 differential expressed proteins (10 Neurology panel [A] and 6 Cardiovascular II panel [B]) between fit and unfit children with overweight/obesity controlling for sex, maturational status (i.e., peak height velocity), and body mass index. Up-regulated proteins are highlighted in orange, while down-regulated proteins are in blue. The x-axis reflects the NPX values mean differences (i.e.,

A 1 NPX value difference means a doubling of protein concentration), while the y-axis indicates statistical significance $p < 0.05$, which is $-\log10 > 1.30$ in the horizontal dashed line.

LAT and CXCL1 proteins (from Neurology and Cardiovascular II panels) showed the highest mean differences between fit and unfit groups (downregulated in plasma of fit compared to unfit children with OW/OB). LAT (Linker for activation of T-cells family member 1) is part of the T-cell receptor complex (TCR). It works as an integrator node of several signaling pathways regulating T cell activation [24]. In this context, obesity is characterized by chronic "over-activation" of the immune system, which is reflected by altered T cell activity and infiltration in adipose tissue contributing to systemic low-grade chronic inflammation [25]. Thus, we infer that lower levels of LAT in plasma of fit compared to unfit children with OW/OB could be indicative of a lower chronic "over-activation" of the immune system. In the brain health context, an experiment in the zebrafish model showed that LAT could impact early neurogenesis [26]. LAT suppression was associated with increasing brain cells number and size. Conversely, LAT overexpression was associated with decreased cell proliferation in the brain and microcephaly in zebrafish [26].

CXCL1 (C-X-C Motif Chemokine Ligand 1) is involved in the immune-inflammatory response (i.e., contribute to attracting immune cells into injury sites) [27,28]. Angiotensin II infusion (to induce vasoconstriction and increase blood pressure) was associated with increased CXCL1 expression (the most expressed chemokine compared to 11 different chemokines), as well as increased infiltration of macrophages and neutrophils in the heart of rodents [27]. Besides, increased blood pressure induced by angiotensin II infusion contributed to left ventricular hypertrophy associated with heart failure [27]. Likewise, in the clinical scenario, hypertensive humans with heart failure showed higher concentrations of CXCL1 in serum compared to normotensive controls

[27]. Therefore, the neutralization of CXCL1 has been proposed as a therapeutic target for CVD treatment [27,28]. Interestingly, regular physical activity, which can increase CRF levels, decreased CXCL1 levels in serum of rodents [29,30], while anti-inflammatory diet interventions reduced CXCL1 in serum of patients with rheumatoid arthritis [31]. In our study, the LAT and CXCL1 proteins were downregulated in the fit group compared to the unfit group. This supports the notion that these proteins could indicate a better brain and cardiovascular health status in fit compared to unfit children with OW/OB.

CLEC1B and sFRP3 were down-regulated proteins in fit children, showing higher mean differences between the study groups. CLEC1B is a type II transmembrane receptor involved in platelet activation in hemostasis and thrombosis [32]. Increased CLEC1B levels in plasma were related to a higher risk of coronary artery disease [33,34]. In addition, a study of proteomics biomarkers discovery in CVD showed that CLEC1B was one of the most important predictors of subclinical atherosclerosis in humans [35]. Besides, activation of immune cells and platelet might contribute to produce an adverse thromboinflammatory response that might destroy the blood-brain barrier and cause neuronal damage [32,36]. In this regard, traumatic brain injury is related to inflammation and dysfunctional coagulation influenced by altered platelet activation [32,37]. Interestingly, traumatic brain injury patients reported high levels of CLEC1B in plasma, which were associated with a higher risk of mortality [38]. Likewise, higher levels of CLEC1B were observed in plasma of adults with Alzheimer's disease compared to adults with mild cognitive impairment [39]. To note, secreted frizzled-related proteins (sFRPs) regulate the Wnt signaling pathway involved in hypertrophy cardiac growth and remodeling [40]. In this regard, higher plasma sFRP3 has been related to a higher risk of all-cause and cardiovascular mortality in middle-aged adults with heart failure [41]. In

the neurological context, sFRP3 reduction contributes to faster new neuron development influencing neurogenesis in the hippocampus of mice [42]. Interestingly, neuronal activation induced by voluntary running reduced sFRP3 expression in the hippocampus of mice (specifically in the dentate gyrus) [42]. Thus, downregulation of CLEC1B and sFRP3 proteins in fit compared to unfit children indicates these biomarkers' promising role in linking CRF to brain and cardiovascular health in children with OW/OB.

Galectins is a family of proteins with multiple roles in the organism (neuroinflammation, Tregs differentiation, antimicrobial immunity, wound healing, etc) involved in CVD and brain health [43,44]. Interestingly, in our study, two different galectins, Gal9 and Gal8, were downregulated in fit compared to unfit children with OW/OB. Patients with large artery atherosclerotic stroke [45] and type 2 diabetes with coronary artery disease [46] showed higher Gal9 in serum compared to controls. In the neurological context, Gal9 is highly expressed in microglia and astrocytes [44], while higher Gal9 concentrations in cerebrospinal fluid were positively associated with cognitive decline in adults with human immunodeficiency virus (HIV) [47]. Besides, serum Gal9 levels were higher in patients with Alzheimer's disease and mild cognitive impairment compared to controls [48], and were negatively associated with global cognitive function [48]. On the other hand, Gal8 can activate human platelets playing a role in thrombosis/inflammation [49], while this protein was upregulated in tumoral endothelial cells [50]. In contrast, Gal8 may play a neuroprotective role in the brain [44,51]. In cell culture, Gal8 contributes to the survival of hippocampal neurons under stress conditions found in neurocognitive diseases (e.g., oxidative stress, glutamate-induced excitotoxicity, nutrient deficits) [51]. Besides, Gal8 knock-out rodents reported higher apoptosis in the hippocampus compared to wild-type [51]. Nevertheless, more research is needed to understand the health implications and molecular mechanisms

underlying the lower levels of Gal9 and Gal8 in plasma of fit compared to unfit children with OW/OB.

Several limitations should be considered. First, causality cannot be assumed due to the cross-sectional study design. Second, proteins included in the Cardiovascular II panel were quantified in a subsample of 44 participants. Third, our sample size was relatively small, and most of the children in the unfit group were boys (8 of 53) with OB (41 of 53) while most of the children in the fit group were girls (25 of 34) with OW (although better balanced, 19 of 34), which could have influenced the analysis. Nevertheless, the ANCOVA analysis was controlled by sex, maturation (PHV), and BMI to attenuate the potential confounding role in this analysis. On the other hand, several strengths should be highlighted. First, blood samples were extracted, and plasma was isolated in the first hour in the morning (at fasting conditions) following a unified protocol. Second, CRF was objectively quantified with a gas analyzer using adapted treadmill protocol for children with OW/OB. Third, our study is the first to use a wide proteomics approach to link cardiovascular and brain health biomerkers with CRF levels in children with OW/OB.

5. CONCLUSION

The differential protein expression detected in fit compared to unfit children with OW/OB contribute to identify novel brain health- and CVD-related biomarkers (i.e., PLXNB3, sFRP3, CLEC1B, RSPO1, Gal8, CLEC10A, GCP5, MDGA1, CTSC, LAT, IL4RA, PRSS27, CXCL1, Gal9, MERTK, and GT) associated with CRF levels in children with OW/OB. However, these findings should be interpreted with caution due to the cross-sectional study design and the relatively small sample size, with significant differences not persisting after multiple correction. Cross-sectional studies using larger

sample sizes and randomized controlled trials are needed to test the impact of exercise interventions on CRF and these brain health- and CVD-related proteins.

SUPPLEMENTARY MATERIAL

The supplementary files can be downloaded in this link:

https://osf.io/unpje/?view_only=d3eeb5a70be44b84a845efc96607c08a

Supplementary Table 1. Descriptive information about the subsample of 44 children with data for cardiovascular disease-related proteins

Supplementary Table 2. The list of 81 proteins from the Neurology panel, non-differentially expressed between Fit and Unfit children with overweight/obesity.

Supplementary Table 3. The list of 86 proteins from the Cardiovascular II panel, non-differentially expressed between Fit and Unfit children with overweight/obesity.

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SECTION II. Effects of exercise on transcriptome and epigenome in children with overweight/obesity

STUDY IV: Genomic Response to Sedentary Behavior and Physical Activity in Children and Adolescents: A Systematic Review

1. INTRODUCTION

The relationship of sedentary behavior and physical activity with different health-related outcomes (cardiometabolic risk factors, brain health, among others) in children and adolescents is well known [1–5]. However, little is known about the molecular mechanisms underlying the effects of sedentary behavior and physical activity (acute or chronic effects) on health in children and adolescents. Sedentary behavior is considered any behavior that implies energy expenditure ≤1.5 metabolic equivalents (METs) while sitting, reclining or laying [6–8]. Physical activity is defined as any body movement that requires energy expenditure higher than in resting conditions [6–8]. Observational studies (cross-sectional [data collected a single moment in time] or longitudinal [data is collected at different time points]) record data about participants without manipulating the environment or exposure (e.g., physical activity). Experimental studies include both the acute effects of physical activity (i.e., the molecular response to a single bout/session of physical activity) or the chronic effects of physical activity (i.e., the molecular response to long-term physical activity interventions lasting weeks/months).

At the single-gene approach, many of the studies have reported how physical activity modulates the association between candidate gene sequence variants (e.g. single nucleotide polymorphisms) and cardiometabolic risk factors (e.g. blood pressure, body fat, among others) in pediatric population [9–12]. Epigenetic modifications (i.e. DNA methylation, histone acetylation and microRNAs [miRNAs]) which are known to be modulated by lifestyle factors such as sedentary behavior and/or physical activity [13,14], should be further studied to understand their effect on gene expression. Interestingly, non-coding RNAs such as microRNAs (miRNAs) are considered one of the novel molecular biomarkers in the physical activity-mediated interactions, which might modulate protein and metabolite expression at the post-transcriptional level by binding to coding

messenger RNAs (mRNAs) [15,16]. Therefore, transcription (i.e. gene expression) and translation (i.e. protein expression) are influenced by epigenetic modifications, which might have an impact on phenotype and physiological functions [13].

Technological advances in molecular biology, such as high through-put omics platforms allow exact and simultaneous examinations of thousands of genes, proteins and metabolites at the genome-wide level [17]. These molecular mechanisms are still poorly understood in the pediatric population, and comprehensive overview on the topic is lacking. This systematic review aimed to provide a summary of the current literature on the effects of sedentary behavior and physical activity (acute and chronic effects) on gene expression and epigenetic mechanisms in children and adolescents.

2. METHODS

For this systematic review, we used the Preferred Reporting Items for Systematic Review and Meta-Analysis (PRISMA) 2020 guidelines. [18]. The review protocol was registered in the International Prospective Register of Systematic Reviews (PROSPERO) with the reference number: CRD42021235431.

2.1 Search strategy and eligibility criteria

A systematic search was conducted in PubMed, Web of Science, and Scopus databases up to 07 May 2021. Detailed search strategy is available in **Table S1**. Search terms were selected based on the exercise and molecular biology concepts of interest. **Table 1** lists the definitions of the main molecular biology-related terms used in this systematic review, for those researchers, exercise physiologists, or clinicians less familiarized with molecular biology-related terminology. The inclusion criteria were: (1) children and/or adolescents aged ≤18years; (2) observational articles (cross-sectional or

longitudinal) that study the relationship of sedentary behavior and/or physical activity with gene expression and epigenetics modifications (both candidate gene and high-throughput approaches); (3) articles that report the acute and/or chronic effects of physical activity on gene expression and/or epigenetics modifications (both candidate gene and high-throughput approaches). The exclusion criteria were defined as follows: 1) studies that reported the acute and/or chronic effects of physical activity combined with other lifestyle interventions such as nutrition interventions, probiotic or prebiotic supplementation or caloric restriction, so that the independent effect of sedentary behavior or physical activity could not be extracted; (2) articles written in any language other than English or Spanish; (3) letters to the editor, editorials, meeting abstracts, study protocols, or reviews.

2.2 Study selection and data extraction

The relevant articles were identified by two researchers (AP-F and IP-P) screening by the title and abstract using the Covidence tool (https://www.covidence.org/). Then, full-text articles were reviewed by the same researchers to determine final eligibility. Conflictive articles were solved through common consensus. The data extraction, performed by one researcher (IP-P) and double checked by one independent researcher (AP-F), included the following information: (1) study design; (2) sample characteristics (i.e., size, gender, age, and ethnicity/race); (3) characteristics of the exposure (i.e., sedentary behavior or physical activity); (4) tissue; (5) dependent outcome (i.e., gene expression or epigenetics); (6) main findings.

Table 1. Definition of the main molecular biology-related terms used in this systematic review

Term	Definition
mRNA	Messenger RNA (mRNA) carries the genetic information from nucleus to ribosomes necessary to synthesize proteins. Gene expression analysis is based on analysing mRNA molecules.
Epigenetics	Epigenetic modifications (i.e., DNA methylation, histone acetylation, miRNA expression) that act on DNA structure. These mechanisms can activate or repress transcription (i.e., gene expression).
CpG site	DNA region prone to methylaton where a cytosine nucleotide is followed by a guanine nucleotide linked by a phosphate group.
DNA methylation	One of the most studied epigenetic modifications that consists in adding a methyl group to C nucleotide in DNA.
Histone acetylation	Epigenetic modification that involves the addition of an acetyl group to the histone proteins.
Microarray	Microarray is a technology that detects the expression levels of thousands of genes at the same time. Briefly, thousands of genetic sequences are located on a chip, and based on the complementary sequences of the transcripts in a biological sample the hybridization takes place and produces light that can be measured to detect gene expression level.
miRNA	Non-coding micro RNA (miRNA) molecule that is small in length, 18-24 pair of bases. These small RNA molecules are able to regulate gene expression.
omics	Refers to analyses of large amount of data, representing an entire set of molecules such as proteins (i.e., proteomics), metabolites (i.e., metabolomics), DNA sequence variants (i.e., genomics), mRNA expression (i.e., transcriptomics), or DNA methylation profile (i.e., epigenomics) within the sample.
RNA-seq	RNA sequencing technique to quantity the gene expression profile (i.e., transcriptome) in a biological sample.
qPCR	Polymerase chain reaction (PCR) technique widely used in molecular biology to obtain millions to billions of copies of a specific DNA sample. This technique is able to quantify gene expression levels.
Transcriptome	Analysis of transcripts (typically mRNA molecules) in order to assess the gene expression levels. Both microarray and RNA-seq approaches are used. The difference between these methods is that in the array a set of possible genes is defined, while RNA-seq allows detection of known and unknown genes.

RNA, Ribonucleic acid; mRNAs, messenger ribonucleic acids; miRNA, micro-RNA DNA, Desoxirribonucleic acid; CpG, Cytosine-phospate-Guanine; qPCR, quantitative polymerase chain reaction; RNA-seq, RNA sequencing.

2.3 Risk of Bias Assessment

Risk of bias for each eligible article was performed by two researchers (AP-F and IP-P) using the Joanna Briggs Institute Critical Appraisal Tool for Systematic Reviews [19]. The different checklists included in this tool are specific for each study design (e.g., cross-sectional studies, non-randomized controlled trials). The items in each checklist had 4 possible answers: "yes" (criterion met), "no" (criterion not met), "unclear" or "not applicable". Particularly, the checklists used were those for cross-sectional studies and non-randomized controlled trials, which include eight and nine items, respectively. For acute physical activity studies we used a modified version of the Downs and Black checklist [20]. This checklist contains 17 questions and was previously adapted for the risk of bias assessment of articles that reported the acute effects of physical activity on bone biomarkers [21]. The quality score per item (%) was calculated by dividing the number of studies that met the quality criteria in one specific item (e.g., answer as yes in item number 1) by the total number of studies (e.g., 5 cross-sectional studies). The lower is the score in each item (expressed in %) the lower is the quality of that item and therefore the higher is the bias in that item. As an example, a 40 % score in the item number 1 and a 100% in the number 2 is indicating a lower quality and higher bias in item 1 compared to item number 2.

3. RESULTS

PRISMA checklist 2020 shows the appropriateness of the methods performed in our systematic review (**Tables S2 and S3**). **Figure 1** illustrates the PRISMA 2020 flow diagram for the selection process of the studies: a total of 1336 articles were included from the three databases, and after removing the duplicates and non-eligible studies, 12

articles remained eligible for this review. All the relevant information extracted from each article is presented in **Table 2**. In addition, a graphical summary of the mains results is presented in **Figure 2.** Specific genes and related pathways found in the studies are interpreted and discussed in the context of existing knowledge in the Discussion section.

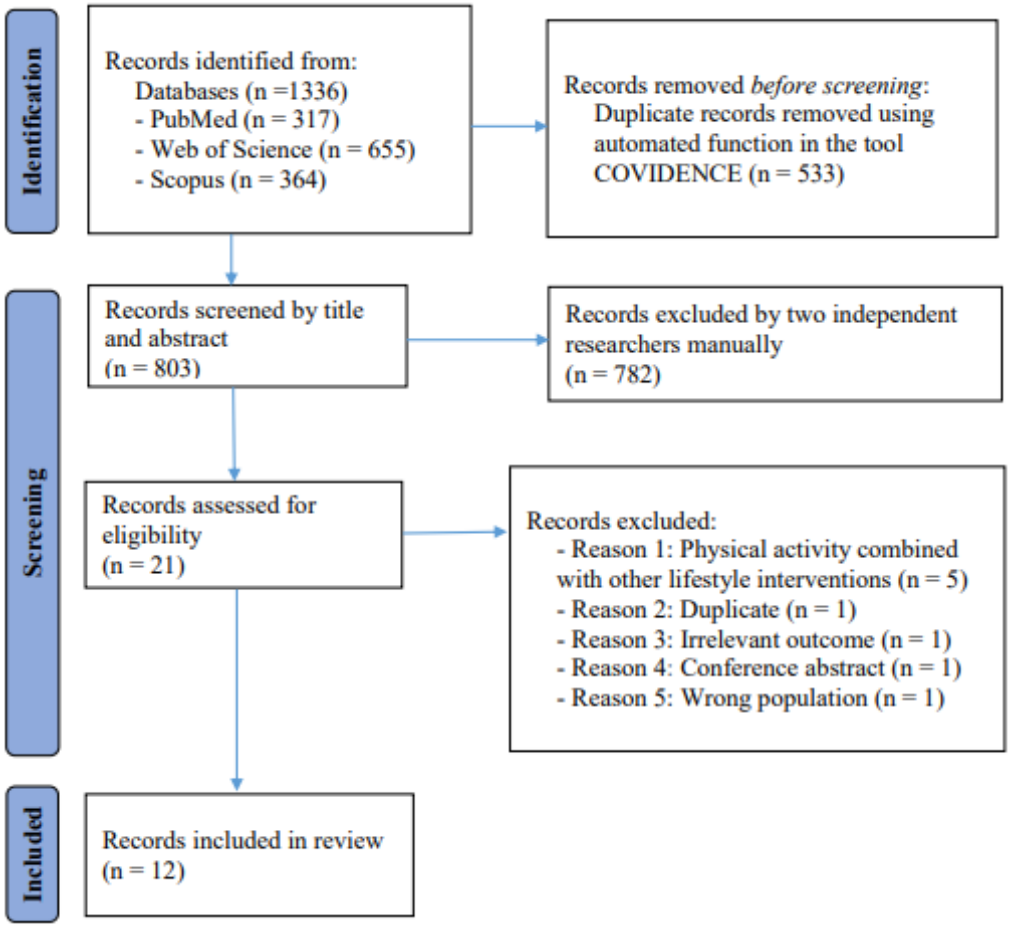

Figure 1. Study selection process based on the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) 2020 flow diagram

3.1 Sedentary behavior and physical activity: cross-sectional evidence

Five cross-sectional studies out of the twelve articles presented in **Table 2** reported the associations of sedentary behavior or physical activity with DNA methylation or gene expression using candidate gene analyses [22–26]. Three studies reported significant associations [23–25], while one study showed significant associations that disappeared after multiple hypothesis testing corrections [22]. Also, one study did

not report significant associations between physical activity and gene expression of the 2 candidate genes (interleukin-2 and brain-derived neurotrophic factor) that were tested [26]. Sedentary behavior and physical activity were reported by questionnaires in three studies, with an assessment time ranging from 6 months [22] to 2 years [25,26]. Two studies measured physical activity and/or sedentary behavior objectively using accelerometers on the nondominant wrist for 6 days [23,24]. Gene expression and DNA methylation levels were obtained from white blood cells in three studies [22,24,26] and saliva in two studies [23,25]. All observational studies/gene expression analyses were performed at cross-sectional level, while longitudinal studies/analyses were not performed.

3.2 Acute effects of physical activity: experimental studies

Five out of the twelve articles presented in **Table 2** reported significant effects of acute bout of physical activity on gene expression [27–31]. Among the five studies, three reported the effects of acute bout of physical activity using candidate gene analyses (i.e., mRNA or miRNA expression) [27,28,31], while two studies performed high-throughput transcriptomics analyses using microarrays [29,30]. Four studies used circulating peripheral blood mononuclear cells (PBMCs) to quantify gene expression [28–31], while one study used capillary blood samples from the earlobe [27].

3.3 Chronic effects of physical activity: experimental studies

Two out of the twelve articles presented in **Table 2** reported significant effects of chronic physical activity on gene expression [31,32], while one study did not detect any effect of chronic physical activity on DNA methylation [33] using candidate gene analyses. The duration of the physical activity interventions was 12-week [32,33] and 8-week [31]. In one study, the intensity of physical activity was unclear [32], while in two

studies the intensity was well-defined using specific % of maximal heart rate (HRmax) during physical activity and breaks [31,33]. The three studies used blood samples to quantify gene expression or DNA methylation [31–33].

3.4 Risk of bias assessment

Among the 5 cross-sectional studies the items 1 and 3 obtained the lowest bias per item score. These items reflect that the sample inclusion criteria were not clearly defined, and the exposure (i.e., sedentary behavior and physical activity) was not measured in a valid a reliable way (**Table S4**). Concerning the 5 studies that reported the effects of acute physical activity, item 16 obtained the lowest bias per item score. This reflects that most acute physical activity studies did not consider unusual activity or nutritional factors the day before performing the physical activity test (**Table S5**). Regarding the chronic effects of physical activity, item 4 obtained the lowest bias per item score that reflects the absence of a control group not exposed to the interest intervention (i.e., long-term physical activity intervention) (**Table S6**).

Table 2. Summary of study characteristics of articles included in this review

Sedentary behavior and physical activity: cross-sectional evidence						
Ref.	**Study design**	**Target population (Sample size [N]); Sex (boys %); Age (SD or range in yrs); Ethnicity/Race (%)**	**Characteristics of the exposure (SB, PA) or PA intervention**	**Tissue**	**Dependent outcome**	**Main findings**
Wu *et al.* 2015 [22]	Cross-sectional	Group 1: Children with obesity (N=59); Boys+Girls (45.8%); 13.8±3.0y; Chinese (100 %) Group 2: Normal-weight children (N=39); Boys+Girls (61.5%); 10.3±1.1y; Chinese (100 %)	SB and PA across 6 months (questionnaire completed by parents or guardians)	Leukocytes	DNA methylation at *FAIM2* promoter (Sequenom MassARRAY platform)	Differentially methylation levels at *FAIM2* promoter between obese and normal-weight children according to SB and PA levels. Results were not significant after multiple hypothesis testing corrections
Lovinsky-Desir *et al.* 2017 [23]	Cross-sectional	Group 1: Active children (N=77); Boys+Girls (45%); 12.2y (9.2y–14.0y); Hispanic (60%), African American (40%) Group 2: Non-active children (N=58); Boys+Girls (55%); 12.7y (10.5y–14.0y); Hispanic (72%), African American (28%)	PA across 6 days (accelerometer on the nondominant wrist)	Buccal swabs (squamous epithelial cells)	DNA methylation at *FOXP3* promoter (pyrosequencing) and gene expression	Active children had lower *FOXP3* promoter methylation compared to Non-active children exposed to high air pollutant black carbon concentrations. No significant association was reported between *FOXP3* promoter methylation and gene expression
Vriens *et al.* 2018 [25]	Cross-sectional	Children with normal-weight 70%, overweight 12.5%, and underweight 17.5% (N=80); Boys+Girls (46.3%); 10.44±0.97y; Caucasian (91.3%)	SB and PA across ~2 years (out-of-school sport activities and screen time use questionnaires filled out by the parents)	Extracellular fraction of saliva	Expression levels of miRNA-222 and miRNA-146a (qPCR)	SB, represented by screen time use, was positively associated with miRNA-222 and miRNA-146a levels. PA was not significantly associated with either miRNA-222 or miRNA-146a

Wu *et al.* 2020 [24]	Cross-sectional	Adolescents (N=369); Boys+Girls (47.2%); 14.22±1.99y for boys/13.95±2.04y for girls; Mexican (100 %)	SB and PA across 7 days (accelerometer on the nondominant wrist)	Leukocytes	DNA methylation at *PPARA*, *H19*, *LINE-1* and *HSD11B2* (pyrosequencing)	Substituting 30-min of vigorous PA for 30-min of SB daily was associated with higher methylation at *HSD11B2* promoter in boys
Gopalan *et al.* 2020 [a] [26]	Cross-sectional	Group 1: Exercisers (N=20); Boys+Girls with HIV infection (75%); 10.5y; Indian (100 %) Group 2: Non-exercisers (N=20); Boys+Girls with HIV infection (44.4%); 12.5y; Indian (100 %)	Children who practiced 20 to 45 min/day, 4 times per week from year 0 to year 2 were categorized as "exercisers" (physical activity questionnaire suited for Indian children)	PBMC	*IL-2* and *BDNF* gene expression (qPCR)	The gene expression of *IL-2* and *BDNF* was not significantly different between exercisers and non-exercisers groups
Acute effects of physical activity: experimental studies						
Radom-Aizik *et al.* 2009 [29]	Within-subjects experiment	Group 1: Early-pubertal boys (N=10); Boys; 10.5±0.4y; NR Group 2: Late-pubertal boys (N=10); Boys; 17.4±0.4y; NR	Cycle ergometer test, 10x2min bouts, the work rate was individualized for each boy (~90 % of HR_{peak}) with 1-min rest intervals	PBMC	Microarray gene expression (Affymetrix U133+2 arrays)	A single bout of PA induced changes in PBMC gene expression in both groups, particularly 1246 genes (517 up, 729 down) in late-pubertal boys and 109 (79 up, 30 down) in early pubertal boys. 13 gene pathways involved in immune function and type I diabetes, were altered by acute PA in both early- and late-pubertal boys
Radom-Aizik *et al.* 2009 [30]	Within-subjects experiment	Group 1: Early-pubertal girls (N=10); Girls; 10.0±0.3y; NR Group 2: Late-pubertal girls (N=10); Girls; 6.1±0.4y; NR	Cycle ergometer test, 10x2min bouts, the work rate was individualized for each girl (~90 % of HR_{peak}) with 1-min rest intervals	PBMC	Microarray gene Expression (Affymetrix U133+2 arrays)	A single bout of PA induced changes in PBMC gene expression in both groups, particularly, 877 genes (611 up, 266 down) in late-pubertal girls and 1,320 (829 up, 491 down) in early-pubertal girls. 5

						gene pathways related to inflammation, stress, and apoptosis, were altered by acute PA in both early- and late-pubertal girls
Kochanska-Dziurowicz *et al.* 2013 [28]	Within-subjects experiment	Youth ice hockey players (N=19); Boys; 17.1±0.5y; Polish (100 %)	Cycle ergometer test until voluntary exhaustion (starting with 1.0 W•kg^{-1} load and increasing the intensity by 0.5 W•kg^{-1} each 3 min)	PBMC	*ADRB2* and *ACTB* gene expression (qPCR)	*ADRB2* and *ACTB* (internal control) gene expression increased in 74% of players after the PA test
Kilian *et al.* 2016 [27]	Cross-over experiment	Competitive young cyclists (N=12); Boys; 14.4±0.8y; NR	Session 1: HIIT, 4x4min at 90-95% PPO with 3-min active recovery intervals at 45% PPO Session 2: HVT, 90 min at 60% PPO	Capillary blood samples	Expression levels of miRNA-16, miRNA-21, miRNA-126 and VEGF mRNA (qPCR)	HVT significantly increased miRNA-16 and miRNA-126 during and after the PA test, whereas HIIT showed no significant influence on the miRNAs. VEGF gene expression significantly increased during and after HIIT and HVT
Lu *et al.* 2017 [b] [31]	Within-subjects experiment	Group 1: Asthmatics adolescents (N=12); Boys+Girls (33.3%); 15.7y (14.0y-17.0y); White (50%), Asian (42%), more than one ethnicity (1%) Group 2: Healthy adolescents (N=14); Boys+Girls (57.1%); 15.0y (14.0y-17.0y); White (71%), Asian (21%), more than one ethnicity (7%)	Acute effects of PA: Cycle ergometer test, 10x2min at ~75 % of VO_{2peak} with 1-min rest intervals Chronic effects of PA: 8-weeks, 3 days/week (1 h-session)	PBMC	*GR (NR3C1)*, *GRβ*, *HSP70*, *TGFβ1*, and *TGFβ2* gene expression (qPCR)	No effect on PBMC gene expression of *NR3C1*, *GR β*, *TGFβ1*, and *TGFβ2* in both healthy and asthmatic adolescents. In addition, *HSP70* gene expression was increased after acute PA while was decreased after chronic PA intervention
Chronic effects of physical activity: experimental studies						

Woo *et al.* 2011 [c] [32]	Non-randomized controlled trial	Group 1: Children with overweight (N=20); Boys; 11.30±1.17y; Korean (100 %) Group 2: Normal-weight children (N=19); Boys; 11.32±1.06y; Korean (100 %)	12-weeks PA intervention. The characteristics of the PA intervention were unclear (i.e., intensity, frequency, among others)	PBMC	*SOD* and *GPX* gene expression (qPCR)	*SOD* and *GPX* gene expression was up-regulated after 12-weeks of PA in both groups. In addition, *SOD* and *GPX* gene expression was up-regulated after 24-weeks of PA in children with overweight
Blüher *et al.* 2019 [33]	Non-randomized controlled trial	Adolescents with overweight/obesity (N=28); Boys+Girls (46.5%); 15.5±1.4y; NR	HIIT, 6-months, 2 sessions/week, 60 min/session at 80–95% HR_{max} with active breaks at 50–60% of HR_{max}	Blood samples	DNA methylation at *RALBP1* (pyrosequencing)	No significant changes in levels of methylation at *RALBP1* were observed after 6-months of PA intervention in children with overweight/obesity

[a] The study design was retrospective cohort study. However, gene expression analysis was performed only at year 2 (cross-sectional) between "exercisers" and "non-exercisers"

[b] This study reported acute and chronic effects of physical activity in gene expression.

[c] To assess the detraining effect in *SOD* and *GPX* gene expression, children with overweight were divided (at the end of 12-weeks of physical activity program) into an overweight training group (i.e., in addition, performed 12-weeks of physical activity) and an overweight detraining group (i.e., in addition, performed 12-weeks of detraining).

Abbreviations: Boys (B); Girls (G); Glucocorticoid receptor (GR); High intensity interval training (HIIT); High volume session (HVT); Kilogram (Kg); Kilometer (Km); Maximal heart rate (HR_{max}); Micro-RNA (miRNA); Minutes (min); Not reported (NR); Peak heart rate (HR_{peak}); Peak oxygen consumption (VO_2peak); Peak power output (PPO); Peripheral blood mononuclear cells (PBMC); Physical Activity (PA); quantitative polymerase chain reaction (qPCR); Sedentary behavior (SB); Wattios (W); Years (Y)

4. DISCUSSION

The main findings and gaps identified by this systematic review in children and adolescents were: (1) there is very limited information of the molecular mechanisms of sedentary behavior and/or physical activity on gene expression and its regulation in pediatric population; (2) most of the studies showed that sedentary behavior and physical activity (acute and chronic effects) alter gene and MicroRNA expression, and DNA methylation of candidate genes related to obesity, asthma, immune function, and cardiovascular disease; (3) the studies are hardly comparable due to different candidate genes selected, characteristics of the exposure, health and training status of the participants, and study designs; (4) only two studies performed high-throughput transcriptomics analyses and detected thousands of genes differentially altered by acute bout of physical activity in boys and girls at different pubertal stages [29,30]. Studies using high-throughput techniques (i.e. sequencing) and longitudinal study approach and/or randomized controlled trials on bigger cohorts are lacking in children and adolescents.

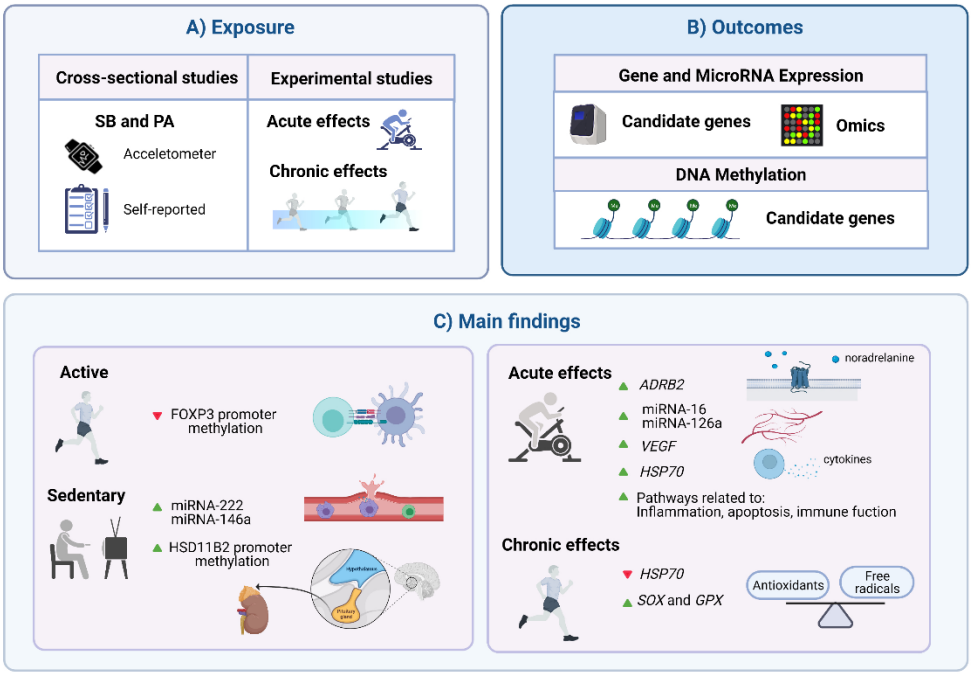

Figure 2. Summary of the main candidate genes and gene pathways related to sedentary behavior (SB) and physical activity (PA) (i.e., acute and chronic effects) in the pediatric population. A) Exposure: SB and PA (acute and chronic effects). B) Outcomes: gene expression and epigenetics (candidate genes and high-throughput transcriptomics analyses). C) Main findings: relevant genes identified in our systematic review. Green arrows reflect up-regulation and red arrows down-regulation. This figure was created with BioRender.com.

4.1 Sedentary behavior and physical activity: cross-sectional evidence

The candidate genes selected by the five cross-sectional studies detected in our review, were related to obesity (*FAIM2*) [34], cardiac hypertrophy, angiogenesis and inflammation (miRNA-222 and miRNA-146a) [25,35,36], signalling molecule in the immune system (*IL-2*) [26], brain health (*BDNF*) [26], T regulatory cells differentiation and function (*FOXP3*) [37], and stress/cortisol metabolism (*HSD11B2*) [38]. Wu et al. reported differential methylation levels at several CpG sites at the *FAIM2* promoter region between obese and normal-weight children according to sedentary behavior and physical activity levels assessed by questionnaires (physical activity threshold 150min/week) [22].

FAIM2 is involved in apoptosis and neurogenesis and is also influenced by food restriction in rodents [39,40]. Some studies reported that polymorphisms near *FAIM2* as well as promoter methylation levels might be associated with obesity [41–43]. Thus, *FAIM2* promoter methylation levels could be influenced by sedentary behavior and physical activity affecting health status in children with obesity.

Vriens et al. showed positive associations of sedentary behavior (represented by screen time) with body mass index, salivary miRNA-222 and miRNA-146a expression [25], while on the contrary, circulating plasma levels of miRNA-146a were up-regulated after acute physical activity in young endurance athletes [44]. It is known that acute physical activity is associated with a transitory immunological/stress response, which in the long-term could be beneficial to the organism [45–47]. In this context, miRNA-146a plays an essential role in the inflammatory signaling in different type of cells and might reflect the inflammatory state after prolonged aerobic physical activity [44]. Thus, the increase of miRNA-146a after a single bout of physical activity might reflect the transitory stress/acute inflammatory response. However, high salivary miRNA-146a levels at resting conditions (i.e., not a transitory response to acute physical activity) could be interpreted as a biomarker of chronic inflammation, which might be related to higher body mass index, cardiovascular, and metabolic diseases. Importantly, several differences among studies must be considered for infering biological implications (e.g., different tissues analyzed [saliva, plasma], populations [adolescents, young adults], and trained status [sedentary, endurance athletes, recreational or professional athletes]).

On the other hand, Gopalan et al. did not report any differences on genes involved in the regulation of the immune and neurophysiological function (*IL-2* and *BDNF*) between "exercisers" (i.e., 24-weeks, 4 sessions/week 20-45 min + 15-30 min yoga) and "non-exercisers" children with HIV infection [26]. To note, the duration of running and

yoga was reported but the intensity of running (e.g., % of HRpeak or perceived exertion) was lacking. Another study by Lovinski-Desirt et al. showed that active children (at least 60 min of moderate-to-vigorous physical activity daily objectively measured by accelerometry) had lower methylation levels at *FOXP3* compared to non-active children (not met at least 60 min of moderate-to-vigorous physical activity daily), among those with higher air pollutant black carbon exposure [23]. FOXP3 controls the differentiation and function of T regulatory cells, where increased *FOXP3* promoter methylation negatively associated with FOXP3 expression and linked to higher air pollution exposure [48]. Furthermore, lung function outcomes (e.g., the ratio between forced expiratory volume in 1 s [FEV1]/forced vital capacity [FVC]) were negatively associated with *FOXP3* promoter methylation. These results suggest that urban children may obtain immunological/cardiorespiratory protection by an active lifestyle.

Wu et al. reported that substituting 30-min of vigorous physical activity for 30-min of sedentary behavior daily was associated with higher methylation levels at *HSD11B2* promoter in boys [24]. *HSD11B* genes catalyze the interconversion of cortisol and corticosterone [38], and thereby vigorous acute physical activity might be associated with an increased transitory stress/immunological response [45–47]. Thus, *HSD11B* could be involved in stress/immunological response to acute physical activity or related to vigorous physical activity levels. In summary, very few single genes have been analysed and clearly bigger studies using the whole genome analysis approaches together with longitudinal studies are warranted.

4.2 Acute effects of physical activity: experimental studies

In our systematic search, three candidate-gene studies reported the acute effect of physical activity on gene expression. Kochanska-Dziurowicz et al. showed that an acute

bout of physical activity on a cycle ergometer until voluntary exhaustion increased *ADRB2* gene expression in the whole-blood assessment of adolescent ice hockey players [28]. The beta-2 adrenergic receptor (ADRB2) is the main target of catecholamines such as noradrenaline involved in the stress response (e.g., acute physical activity). Thus, *ADRB2* gene up-regulation in the whole blood after a single bout of intense physical activity could be related to the stress response. Interestingly, the ADRB2 gene was downregulated in lymphocytes of children with asthma compared to healthy controls [49]. *ADRB2* up-regulation after an intense acute bout of physical activity may induce partially immunological protection in children with asthma, which should be tested in future studies.

Killian et al. used a cross-over design to test the impact of two different types of acute physical activity (e.g., High volume session [HVT] *vs.* High intensity interval training [HIIT]) on circulating miRNAs (miRNA-16, miRNA-21, and miRNA-126) and *VEGF* gene expression in healthy boys as competitive cyclists [27]. These genes are highly expressed in the endothelium cells and are involved in angiogenic processes such as the formation of new capillaries [27,50,51]. MiRNA-16, miRNA-21, and miRNA-126 were up-regulated during and after HVT, while no changes were detected with the HIIT (4x4min at 90-95% of peak power output with 3-min active recovery intervals at 45% of peak power output), suggesting that physical activity volume (duration) of physical activity might play a role in regulating vascular circulating miRNA expression in children. On the other hand, Radom-Aizik et al. reported contrary results where miRNA-16 and miRNA-126 were down-regulated in neutrophils of healthy young adults after interval acute physical activity bouts (10 × 2 min at 76% VO2peak) [52]. Different factors could explain partially the opposite findings between the two abovementioned studies (e.g., miRNA isolated from capillary blood from the earlobe *vs.* miRNA in circulating

neutrophils, children *vs* young adults, continuous *vs.* interval physical activity bouts). Future studies should analyze the impact of acute physical activity with different characteristics (e.g., volume, intensity, continuous or intermittent) on miRNA expression in children with different diseases and, if possible, performing high-throughput analyses (e.g., miRNA-sequencing).

Another study by Lu et al. reported up-regulation of *HSP70* after a single bout of physical activity (10x2min at ~75 % of VO2peak with 1-min rest intervals), while the expression of candidate genes involved in the glucocorticoid receptor pathway (*GR*, *GRβ*, *TGFβ1*, and *TGFβ2*) in PBMCs of asthmatics and healthy adolescents did not change [31]. HSP70 protein can reduce the expression of pro-inflammatory cytokines such as TNF-α or IL-1β [53,54], while physical activty can increase HSP70 levels in lymphocytes [54,55]. Besides, the coordinated action of HSP70 and HSP90 can regulate glucocorticoid receptor function [56]. It is possible that other genes related to glucocorticoid function are also regulated by acute physical activity. This is a "limitation" of the single gene approach that could be overcome by using high-throughput analyses. Further research need to contrast or confirm whether the abovementioned genes could be altered in children with different clinical conditions such as obesity and implementing physical activity protocols with different characteristics.

In regards to high-throughput analyses, two studies reported the acute effects of physical activity (cycle ergometer test, 10x2min bouts, ~90 % of HRpeak with 1-min rest intervals) on gene expression profile in PBMCs of healthy boys and girls at different pubertal stages using microarrays analysis [29,30]. The expression of 1246 genes were altered following the acute physical activity bout in late-pubertal boys [29], while the expression level of 109 genes was found to be altered in early-pubertal boys [29]. 13 gene pathways related to immune function and type I diabetes, among others were enriched

[29]. Contrary to boys, the difference in the number of genes their expression was altered following the same acute bout of physical activity was much smaller; 877 genes in late-pubertal girls [30] and 1320 genes in early-pubertal girls [30]. 622 genes overlapped between the groups. These genes enriched gene pathways involved in inflammation, stress, and apoptosis [30]. These pioneering studies highlight the need to account for sex and pubertal stage when interpreting genomic data in response to acute bout of physical activity [29,30], and the need to apply high-throughput approach to better understand the molecular mechanisms involved in the response to physical activity.

In conclusion, more studies are needed to address the acute effects of physical activity on gene expression profile and its regulation in healthy children and children with clinical conditions (e.g., obesity, leukemia, anemia, cystic fibrosis) using high-throughput sequencing technologies (e.g., RNA-seq).

4.3 Chronic effects of physical activity: experimental studies

Only a few studies have investigated the chronic effects of physical activity on gene expression. Woo et al. reported the up-regulation of *SOD* and *GPX* in PBMCs of children with obesity and normal-weight after 12-weeks of physical activity intervention [32]. These genes encode antioxidant enzymes [32], suggesting that long-term physical activity intervention (i.e., chronic effects) might improve the oxidative stress profile of these children. However, the characteristics of the physical activity intervention were unclear (i.e., intensity, frequency, type, among others) [32]. Another study by Lu et al. reported that 8-week physical activity down-regulated the expression of *HSP70* in asthmatics and healthy adolescents [31], which can regulate glucocorticoid receptor function [56]. Interestingly, acute physical activity altered the expression of *HSP70* in the

opposite direction (i.e., up-regulated) indicating that acute and chronic physical activity may have a different impact on *HSP70* expression level.

Lastly, a well-defined physical activity intervention based on HIIT (6-months, 2 sessions/week, 60 min/session at 80–95% HRmax with active breaks at 50–60% of HRmax) showed no changes in methylation levels at *RALBP1* in whole blood samples of adolescents with overweight/obesity. RALBP1 is involved in the pathogenesis of metabolic syndrome and obesity-associated inflammation [57,58]. Increased methylation at the *RALBP1* promoter region has been linked to decreased *RALBP1* gene expression in adipose tissue of healthy men after 6-months of physical activity intervention (one session of 1 hour spinning and two sessions of 1 hour aerobics per week, no more details were provided) [59]. Heterogeneity among studies hampers comparisons and could partially explain the contradictory findings (e.g., different tissues, characteristics of physical activity interventions, age range of participants, and weight status).

It is evident that further studies should assess the chronic effects of physical activity attending to its different characteristics (i.e., type, intensity, volume, continuous *vs.* interval, etc.) on genes and molecular pathways in healthy children and children with different clinical conditions and training status. Besides, longitudinal randomized controlled trials (i.e., children randomly assigned into a physical activity or control group) are needed to characterize the molecular response to long-term physical activity interventions (i.e., chronic effects) in children and adolescents.

5. FUTURE DIRECTIONS

Our systematic review highlights the small body of knowledge available on the genomic response to sedentary behavior and physical activity (i.e., acute and chronic

effects) in children and adolescents. Most of the studies focused on candidate gene analyses, and only two studies performed high-throughput analyses to assess the acute effects of physical activity on gene expression profile in children. It is clear from the studies performed in adults and animals that sedentary behavior/physical activity alter the expression of thousands of gene in an orchestrated manner [13,17,60,61]. In order to move forward the knowledge of molecular effects of physical activity in children, studies applying the high-throughput omics techniques such as RNA/DNA sequencing together with epigenome analyses (genome-wide DNA methylation, acetylation, miRNA-seq) are warranted.

The main reason why research in the pediatric population is lagging behind other age groups is the limitation of obtaining study material for technical and ethical reasons. Interestingly, a recent study assessing 32 tissues in human concluded that the analysis of the whole blood gene expression predicts well the tissue-specific transcriptome profile (60% of the genes across 32 tissues), and especially accurate in the skeletal muscle (81% of the genes) [62]. This whole-blood transcriptome profile was a good predictor of different diseases such as type 2 diabetes or hypertension [62]. These findings are promising and have the potential to enhance the field by increasing the number and the quality of studies exploring sedentary behavior/physical activity effects on genomic response in the pediatric population.

Recently, Contrepois et al. investigated the effects of acute physical activity at molecular level performing multi-omics analyses in middle-aged adults [61]. In the future, multi-omics analysis should be implemented to understand the link between changes on gene expression and epigenetics modifications at genome-wide level in response to physical activity (acute and chronic effects) in children and adolescents. **Figure 3** illustrates the complexity and the potential of an integrated omics perspective.

In this regard, the Molecular Transducers of Physical Activity Consortium (MoTrPAC) funded by National Institute of Health (NIH) Common Fund will address these gaps in 320 healthy children by characterizing the molecular response to physical activity (acute and chronic effects) performing multi-omics analyses (i.e., genomics, transcriptomics, epigenomics, metabolomics, proteomics) [60]. The body of knowledge derived from MoTrPAC would be helpful to understand how physical activity is beneficial for health and how it can potentially be prescribed as a personalized medicine in children and adolescents. Further initiatives in this direction are needed to understand and unravel the complex effects of physical activity on health.

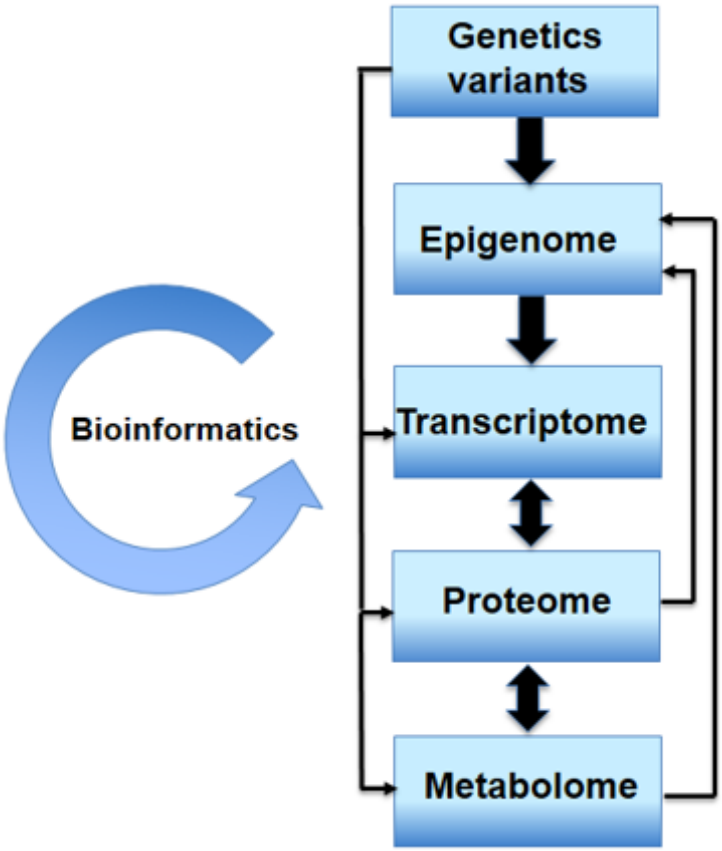

Figure 3. The complex integration of 'omics' data (i.e., multi-omics analysis) might contribute to a better understanding of the molecular mechanisms underlying the health-related benefits of physical activity in children and adolescents. The human genome is essentially invariant and comprises more than 25000 genes, which encode ~100000–

200000 transcripts and 1 million proteins, and a smaller number of metabolites (2500-3000) make up the human metabolome [63]. The epigenome, which can be influenced by physical activity in adults [13], shows a low/moderate temporal variance and influences both transcriptome and proteome. The transcriptome can be affected by a single bout of physical activity [29,30] in children and presents a high temporal variance and is translated into the proteome, influencing the metabolome in a tissue-specific manner. Figure modified from Altmäe et al., 2014 [64] with permission of the Publisher.

6. CONCLUSIONS

This review highlights how scarce is our knowledge on the molecular effects of sedentary behavior and physical activity on pediatric population. The few studies in the field suggest that sedentary behavior and physical activity are associated with miRNA expression and DNA methylation, where the most relevant candidate genes in the cross-sectional studies were: *FOXP3*, *HSD11B2*, miRNA-222, and miRNA-146a, and the acute chronic effects of physical activity regulated the expression of *ADRB2, miRNA-16, miRNA-126, HSP70, SOX,* and *GPX* genes. Transcriptomic analyses identified gene pathways involved in inflammation, apoptosis or diabetes among others, that are influenced by acute physical activity in boys and girls. In conclusion, very few genes and genetic regions have been studied, and the field is yawing for studies on bigger cohorts, longitudinal studies and randomized controlled trials together with high-throughput multi-omics analyses.

SUPPLEMENTARY MATERIAL

The supplementary files can be downloaded in this link:

https://osf.io/unpje/?view_only=d3eeb5a70be44b84a845efc96607c08a

Supplementary Table 1. Search terms used in PubMed, Web of Science, and Scopus databases.

Supplementary Table 2. PRISMA main checklist 2020.

Supplementary Table 3. PRISMA abstract checklist 2020.

Supplementary Table 4. Risk of bias assessment of included cross-sectional studies.

Supplementary Table 5. Risk of bias assessment of acute physical activity studies (i.e., acute effects).

Supplementary Table 6. Risk of bias assessment of included long-term physical activity interventions studies (i.e., chronic effects).

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STUDY V: Effects of exercise on whole-blood transcriptome profile in children with overweight/obesity

1. INTRODUCTION

Childhood overweight/obesity (OW/OB) is related to higher future morbidity and mortality [1,2], and exercise has the potential to attenuated this adverse consequences by improving a wide range of health-related markers (e.g., fitness, body composition, cardiometabolic risk markers, among others) [3–5]. However, the understanding of the molecular processes underlying the health benefits of regular exercise in pediatric population are limited.

Probably, the ethical and technical difficulty in obtaining biopsies from skeletal muscle and adipose tissue in children has limited the investigation on the effects of exercise at molecular level, compared to research in adult population. An alternative target tissue for studying the effects of exercise on molecular processes is the whole-blood, which is less invasive compared to biopsies and therefore more suitable for pediatric research. In fact, a recent study predicted tissue-specific gene expression from the whole-blood transcriptome across 32 tissues and found that individual's whole-blood transcriptome predicted skeletal muscle expression levels for 81% of the genes and 75% of the genes in adipose tissue [6]. Hence, a significant part of the muscular and adipose tissue transcripts are reflected in the whole-blood transcriptome, and additionally whole blood could indicate the low-grade systemic inflammation that is observed in obesity [7], and could be influenced by exercise [8,9].

A few studies have investigated the effects of exercise interventions on gene expression profiles in blood cells of young and middle-aged adults [10–14]. A 8-week resistance exercise intervention changed the expression of growth factor genes (*GHRH* and *FGF1*) in leukocytes of healthy young men, and a 4-week exercise intervention based on sprints altered the expression of genes involved in cardiovascular health in leukocytes

of healthy young men [10]. Likewise, an endurance exercise intervention of longer duration (e.g., 18-week) regulated the expression of genes involved in immune function and development in peripheral blood mononuclear cells (PBMCs) of healthy young men [14]. In the middle-aged group, a 24-week exercise intervention up-regulated the expression of genes involved in inflammation and immune response (e.g., *IL-2*, *IL-4*, *IL-8*) in men [12]. While a 12-week exercise intervention showed that several genes detected in oxidative phosphorilation gene pathway were up-regulated by exercise in whole-blood of middle-aged women [13]. To the best of our knowledge, the effects of an exercise intervention (i.e., the effects of chronic exercise) on whole-blood transcriptome profile in children and adolescents are so far unstudied.

In pediatric population, a short-term/acute exercise effects on PBMCs transcriptome profile in healthy boys and girls has been shown [15,16], where genes involved in pediatric inflammatory diseases likes' asthma, apoptosis and, cytotoxic killing factors were regulated by acute exercise, suggesting that acute exercise could initiate a "danger" response in PBMCs that might be of benefit to the organism [15,16]. These are the first studies performed in children to unravel the effects of acute exercise on molecular events, and clearly more studies in different pediatric target groups and testing chronic exercise effects are warranted.

We set out to investigate the effects of a 20-week exercise intervention on whole-blood transcriptome profile in pre-pubertal children with OW/OB in order to understand the beneficial effects of exercise on molecular processes in childhood obesity. As there is a growing evidence of the sex differences on transcriptome landscapes [17–19], we analysed boys and girls separately.

2. METHODS

2.1 Study design and participants

One hundred and nine children with OW/OB were included in the ActiveBrains randomized controlled trial (ClinicalTrials.gov [identifier: NCT02295072]) [20]. Out of 109 children in the randomized controlled trial, 24 children (10.21 ± 1.33 years, 46% girls) qualified to our study (considering also the >66% participation/adherence to the training intervention, samples obtained in all time-points, and the RNA quality for transcriptome analysis). Ten children were allocated into the exercise group (5 boys and 5 girls) and 14 children into the control group (8 boys and 6 girls). Children included in the current study were pre-pubertal (8-11 years) with OW/OB according to the sex- and age-specific international body mass index (BMI) standards (World Obesity Federation) [21]. The ActiveBrains project was approved by the Committee for Research Involving Human Subjects at the University of Granada (Reference: 848, February 2014). All parents received information about the study and gave their consent following the Declaration of Helsinki guidelines. The assessments were performed from October 2014 to February 2016. Detailed design and methods of this randomized controlled trial have been described elsewhere [20]. Briefly, the randomization was done after the pre-intervention data collection. Likewise, the person who performed the computer random generation and exercise trainers were not involved in the pre-intervention data collection.

2.2 Exercise training intervention

The 20-week exercise training intervention has been described in detail [20]. In short, the exercise training intervention based on multi-games is in line with the international physical activity guidelines [22,23]. The minimum frequency of exercise was three sessions per week, and the desired requency was five sessions. Each session

lasted for 90 min. The general structure of the sessions was: 1) warm-up (5-10 min) consisting of 1-2 physical games; 2) aerobic part (60-min) at moderate-to-vigorous intensities based on 4-5 multi-games (emphasis above 80% of heart rate (HR) max) and 3) resistance training (20-min) comprising of strength exercises involving large-muscle-groups in sets of 10-12 repetitions using bodyweight, theraband and/or fitballs; and 4) cool-down (5-10 min) based on relaxation and stretching exercises.

The intensity of the exercise intervention was controlled individually in each session using heart rate monitors (Polar RS300X, Polar Electro Oy Inc, Kempele, Finland). The maximum heart rate achieved by each child in a maximal incremental treadmill test was used to individually program the heart rate monitors. The intensity was checked each week to ensure an accurate training stimulus along the exercise intervention. The heart rate data were assessed for the aerobic and resistance training part independently due to the different physiological demand of the cardiovascular system in these parts of the sessions. The children allocated in the control group were advised to continue with their usual life and information of healthier lifestyle regarding physical activity and nutrition were provided.

2.3 Body composition measurements and maturational status

Body composition measurements were performed before and after the exercise intervention period. Body weight and height were measured with an electronic scale and a stadiometer (Seca instruments, Germany, Ltd) and BMI was calculated as kg/m^2. Waist circumference was evaluated as an indicator of central fat using the International Society for the Advancement of Kinanthropometry (ISAK) procedures [24]. Fat mass index (FMI), body fat percentage (BFP) and lean mass index (LMI) were measured by dual energy X-ray absorptiometry (DXA, Discovery densitometer from Hologic), and were

calculated as the ratio between the fat mass and fat-free mass (kg) with the squared height (m^2) and the percentage (%) of adipose tissue relative to body weight, respectively. Also, visceral adipose tissue (VAT) was measured. The maturational status of the participants was reflected by the peak height velocity (PHV) [25]. This variable was derived from height and seated height. Maturity offset was calculated by subtracting the PHV age from the chronological age.

2.4 Cardiorespiratory fitness

Cardiorespiratory fitness (i.e., VO_2peak) was assessed before and after the 20-week exercise intervention period in the laboratory using a gas analyzer (General Electric Corporation) while performing a maximal incremental treadmill test (HP-Cosmos ergometer) adapted for low-fit children [26]. Briefly, the incremental test consisted of walking on a treadmill at a constant speed (4.8 Km/h) starting at a 6% slope with grade increments of 1% every minute until volitional exhaustion [26]. Oxygen consumption (ml/min), HR (beats/min) and respiratory exchange ratio (RER) were continuously recorded every 10 s, whilst the rating of perceived exertion (RPE) scale was reported at the end of each 1 min stage using children's OMNI scale ranging from 0 to 10 [27]. Absolute peak oxygen consumption (VO_2peak absolute; ml/min) and oxygen consumption relative to body weight (VO_2peak; ml/kg/min) were provided as indicators of cardiorespiratory fitness.

2.5 Blood sampling and analysis

Peripheral blood sample was collected at 2 time-points, before and after the exercise intervention period. Participants arrived at the laboratory between 8:00-9:00 AM after an overnight fasting of at least 12 hours. Venous blood was collected into EDTA tube, which was subsequently centrifuged at 1000g for 10 min, and the plasma was

isolated and stored at -80ºC until biochemical analyses. For RNA-seq analysis, 500 ul whole-blood was collected into the tube that contained 1.3ml RNA later solution (Ambion Inc; Austin, Texas, USA) and was stored at -80ºC until further processing.

2.5.1 Pro-inflammatory markers

Interleukin-6 (IL-6), interleukin-1β (IL-1β), and tumor necrosis factor-α (TNF-α) cytokines were quantified by multiple analyte profiling technology (MILLIPLEX® MAP Human High Sensitivity T Cell Magnetic Bead Panel, EMD Millipore Corporation, Missouri, USA) using a kit plex (HCYIL6-MAG Anti-Human IL-6 Beads set, HCYIL1B-MAG Anti-Human IL-1β Bead, and HCYTNFA-MAG Anti-Human TNFα Beads set) [28]. Vascular endothelial growth factor A (VEGFA) was measured using multiple analyte profiling technology (MILLIPLEX® MAP Growth Factor Magnetic Bead Panel 1, EMD Millipore Corporation, Missouri, USA) [28]. The intra- and inter-assay coefficients of variation for IL-6 were ≤ 5% and ≤ 20%, and sensitivity was 0.11 pg/mL. For IL-1β and TNF-α the intra- and inter-assay coefficients of variation were ≤ 5% and ≤ 15%, respectively, with a respective sensitivity of 0.14 pg/mL and 0.16 pg/mL.

2.5.2 RNA extraction and sequencing

Total RNA was isolated from the whole-blood samples using RiboPureTM-Blood Kit (Thermo Fisher Scientific; Waltham, Massachusetts, USA), followed by treatment with GlobinLock system (GlobinLock, GL; Tartu, Estonia) in order to block the high globin mRNA content of erythrocytes, which is abundant in the blood and could hamper the whole-blood RNA analyses [29]. Total transcriptome analysis was performed following the single-cell tagged reverse transcription (STRT2) RNA-seq protocol as described in detail before [30]. Briefly, 10 ng of high-quality input RNA was converted into cDNA and amplified to form an Illumina-compatible library. The STRTprep pipeline

v.3.0.0, available at https://github.com/shka/STRTprep/tree/v3dev, was used for processing the raw sequencing reads, aligning to the hg19 genome and quantitating the expression levels. RNA-seq data is publicly available at www.ncbi.nlm.nih.gov/geo under accession number GSE193771.

2.6 Data analyses

2.6.1 Descriptives and physiological effects of exercise training

Descriptive data are presented as mean and standard deviation (mean ± SD), or frequency and percentage for categorical variables. The effects of exercise intervention were performed on body composition variables, cardiorespiratory fitness, and inflammatory markers in boys and girls separately. The analyses were performed using SPSS version 21.0 (IBM Corporation, NY, USA), and the statistical significance was defined at the level of $p < 0.05$. Analysis of covariance (ANCOVA) was performed using post-exercise data as dependent variables, group (i.e. exercise vs. control) as a fixed factor, and baseline data as covariates. The z-scores for each variable at post-exercise (i.e. (post-exercise individual value – baseline value) / baseline SD) were calculated as an effect size indicator of the changes caused by the exercise, being considered a value around 0.2 a small effect size, 0.5 medium effect size and, 0.8 a large effect size [34].

2.6.2 Gene expression profiles

Quantile normalization was performed for RNA-seq data. Subsequently, the differential gene expression analysis between the exercise and the control groups (i.e., interaction: group [exercise; control] x time [pre-intervention; post-intervention]) was performed using the Limma R/Bioconductor software package [31], in boys and girls separately. For further interest, our scripts files of the Limma analyses are available in the Open Science Framework (https://osf.io/5a9nh/). The differentially expressed genes were

further characterized by enrichment analysis using the LIMMA function topKEGG in R software environment ($p < 0.05$). All analyses were controlled for Benjamini-Hochberg DFR multiple testing correction (false discovery rate [FDR] < 0.05). Further, the gene networks were computed with QIAGEN's Ingenuity® Pathway Analysis (IPA®, QIAGEN Redwood City, www.qiagen.com/ingenuity), using as input the list of differentially expressed genes between the exercise and control groups (boys and girls separately). Networks were generated based on the connectivity among genes and top-IPA networks, with the cut-point of 35 molecules (default settings) and the highest IPA score per network.

2.7 *In silico* data mining and validation

We performed in *silico* data mining and validation of the transcriptome data in two steps. First, PHENOPEDIA database was used to perform *in silico* data mining with the aim to find common genes/pathways with obesity and its associated diseases. PHENOPEDIA provides lists of genes involved in different diseases (based on genetic associations studies), which are continuously updated from PubMed [32]. Genes regulated by exercise intervention (i.e., exercise vs. control) in our study were explored with lists of genes involved in obesity and other diseases and risk factors associated with obesity (i.e., CVD, inflammation, and metabolic syndrome). Next, the *in silico* validation was performed using a up-to-date database https://extrameta.org/, which is derived from a extensive meta-analysis database using 43 publicly available transcriptome data from human skeletal muscle and blood in response to acute and exercise interventions [33]. Our validation was focused on genes that were identified in: 1) KEGG gene pathways (FDR < 0.05) (i.e., pathways analyses using the LIMMA function topKEGG in R); 2) Top-IPA networks; and 3) were detected in at least one of the PHENOPEDIA disease terms.

3. RESULTS

3.1 Sample characteristics and physiological effects of training

Descriptive characteristics of participants at baseline are summarised in **Table 1.** The study sample had an average chronological age of 10.76 ± 1.34 and biological maturation age of -2.17 ± 0.97 years for boys, while for girls the chronological age was 9.57 ± 1.35, and biological maturation age -1.88 ± 0.87 years.

Table 1. Descriptive characteristics of the study sample

Variables	Total sample (n=24, 46% girls)	Boys (n=13, exercise 5/control 8)	Girls (n=11, exercise 5/control 6)
Age and Maturational status			
Age (years)	10.21 ± 1.33	10.76 ± 1.34	9.57 ± 1.05
PHV offset (years)	-2.04 ± 0.92	-2.17 ± 0.97	-1.88 ± 0.87
Body composition			
Weight (kg)	58.37 ± 9.99	59.73 ± 11.82	56.76 ± 7.53
Height (cm)	146.53 ± 9.03	146.04 ± 10.34	143.56 ± 6.43
Waist circumference (cm)	92.46 ± 6.82	92.10 ± 7.95	92.88 ± 5.55
BMI (kg/m^2)	27.01 ± 2.51	26.61 ± 2.48	27.49 ± 2.57
FMI (kg/m^2)	11.52 ± 2.11	10.82 ± 1.88	12.36 ± 2.15
BF (%)	43.15 ± 4.69	41.14 ± 4.12	45.52 ± 4.33
Visceral adipose tissue (g)	426.03 ± 83.67	422.91 ± 92.81	429.71 ± 75.75
LMI (kg/m^2)	14.35 ± 1.03	14.67 ± 1.07	13.98 ± 0.90
Cardiorespiratory fitness			
VO2peak absolute (ml/min)	2194.83 ± 485.54	2336.62 ± 578.54	2027.27 ± 289.06
VO2peak relative (ml/kg/min)	37.56 ± 4.63	39.07 ± 5.48	35.77 ± 2.62
Inflammatory markers			
IL-1β (pg·mL-1)*	1.80 ± 0.75	1.68 ± 0.63	1.93 ± 0.89
IL-6 (pg·mL-1)*	2.11 ± 1.10	2.03 ± 1.23	2.22 ± 0.95
TNF-α (pg·mL-1)*	4.02 ± 1.17	3.87 ± 1.24	4.21 ± 1.11
VEGFA (pg·mL-1)*	28.08 ± 13.97	32.04 ± 14.65	23.32 ± 12.11

Data presented as mean ± SD, and as number and frequency. BMI: Body mass-index, BF: Body fat, FMI: Fat mass index, IL: interleukin, PHV: Peak height velocity, TNF-α: tumor necrosis factor alpha, VEGFA: vascular endothelial growth factor A.

* Sample size baseline: IL-1β (boys 12; girls 10); IL-6 (girls 9); TNF-α (girls 10); VEGFA (boys 12; girls 10 girls)

The main effects of the exercise intervention on body composition, cardiorespiratory fitness, and inflammatory markers are reported in the complete sample from the ActiveBrains project (manuscript in preparation). In this subsample (N = 24) with transcriptome data in all time-points, in boys, the exercise intervention reduced waist circumference and improved significantly cardiorespiratory fitness (medium and large effect sizes: -0.41 SDs to 1.29, all $p \leq 0.041$) (**Supplementary File 1**. Among girls, body fat percentage and inflammatory marker VEGFA decreased after exercise intervention (large effect sizes: -0.87 SDs, $p = 0.080$ and -1.44 SDs, $p = 0.056$) (**Supplementary File 2).** Time of 80%HR max during exercise sessions was significantly higher in boys than in girls (**Supplementary File 3**), supported by the significant improvement of VO_2 values in boys, which could add to the gender-specific differences.

3.2 Gene expression profiles

One hundred and sixty-one genes were differentially expressed (71 up-regulated and 90 down-regulated; **Figure 1**) in boys allocated in the exercise group compared to boys in the control group (log_2FC ranged from -1.93 to 2.36; $P < 0.05$) (**Supplementary File 4**). Among girls, 121 genes were differentially expressed (51 up-regulated and 70 down-regulated; **Figure 2**) in the exercise group compared to the control group (log_2FC ranged from -2.35 to 1.65; $P < 0.05$) (**Supplementary File 4)**. Among the two gene lists, only 8 genes overlapped between the sexes: *RAB7A*, *TXNL4B*, *CKS1B*, *TPP1*, *CCN1*, *CALR*, *EMD*, and *LASP1*. After applying multiple hypothesis testing, none of the genes (161 genes in boys and 121 genes in girls) were significantly differentially expressed between exercise and control groups (all FDR > 0.05) (**Supplementary File 4**). The KEGG pathway analysis of the genes regulated by exercise in boys was significantly enriched in five pathways, such as antigen processing and presentation, and infections (yersinia, salmonella, human immunodeficiency virus, pathogenic Escherichia coli), and

GO (Gene ontology) terms T cell receptor complex and endoplasmic reticulum chaperone complex (FDR < 0.05) (**Table 2**). In addition, immune gene pathways such as Toll-like receptor, NOD-like receptor, nuclear factor-κB (NF-κB) were regulated by exercise in boys (P < 0.05). However, the abovementioned pathways were not significant after multiple correction testing (FDR > 0.05) (**Supplementary File 5**). In girls, the genes regulated by exercise were enriched in Fc epsilon RI signaling pathway and GO terms including endoplasmic reticulum-Golgi intermediate compartment membrane, platelet alpha granule membrane, endoplasmic reticulum-Golgi intermediate compartment, melanosome, pigment granule, and U12-type spliceosomal complex (FDR < 0.05) (**Table 2**). Besides, gene pathways involved in inflammation such as Fc gamma R-mediated phagocytosis, VEGF signaling, and platelet activation were regulated by the exercise intervention in girls (P < 0.05), nevertheless they were not significant after multiple correction testing (FDR > 0.05) (**Supplementary File 5**). Several pathways involved in immune processes, including antigen processing and presentation, B cell receptor signaling, phagosome, lysosome, and leukocyte transendothelial migration were common pathways regulated by exercise in both obese/overweight boys and girls (p < 0.05), however did not survive the multiple correction testing (FDR > 0.05) (**Supplementary File 5**).

Table 2. Enrichment analyses of differentially expressed genes by a 20-week exercise intervention (i.e., exercise vs control) in boys and girls (statisticaly significant pathways FDR < 0.05)

KEGG pathways and GO terms	Genes identified in each pathway
Boys, 5 KEGG gene pathways and 2 GO terms	
Antigen processing and presentation (KEGG)	***CD4*** (↑), ***HLA-C*** (↑), *NFYB* (↑), ***CD8B*** (↓), ***CALR*** (↓)
Yersinia infection (KEGG)	*ACTG1* (↑), ***CD4*** (↑), ***CD8B*** (↓), *FOS* (↑), ***MYD88*** (↑), ***NFKBIA*** (↓), ***PTK2B*** (↑), *SKAP2* (↑), *WASF2* (↓)
Salmonella infection (KEGG)	*ACTG1* (↑), *CYFIP2* (↑), *DYNC1LI1* (↑), *FOS* (↑), *GSDMD* (↓), ***MYD88*** (↑), *MYL9* (↑), ***NFKBIA*** (↓), *RAB7A* (↑), ***TNFRSF1A*** (↑)
Human immunodeficiency virus 1 infection (KEGG)	***CALR*** (↓), ***CD4*** (↑), *FOS* (↑), *GNG2* (↑), ***HLA-C*** (↑), ***MYD88*** (↑), ***NFKBIA*** (↓), ***PTK2B*** (↑), ***TNFRSF1A*** (↑)
Pathogenic Escherichia coli infection (KEGG)	*ACTG1* (↑), *CYFIP2* (↑), *FOS* (↑), ***MYD88*** (↑), ***NFKBIA*** (↓), *SLC9A3R1* (↓), ***TNFRSF1A*** (↑), *WASF2* (↓)
T cell receptor complex (GO)	***CD4*** (↑), *SKAP1* (↓), ***CD8B*** (↓), ***CD6*** (↓)
endoplasmic reticulum chaperone complex (GO)	*PPIB* (↓), *P4HB* (↓), *MZB1* (↓)
Girls, 1 KEGG gene pathway and 6 GO terms	
Fc epsilon RI signaling pathway (KEGG)	***RAC1*** (↑), ***FYN*** (↓), ***INPP5D*** (↓), *ALOX5* (↓), ***PIK3CD*** (↓)
Endoplasmic reticulum-Golgi intermediate compartment membrane (GO)	*TMED1* (↑), *RAB2A* (↑), ***CALR*** (↓), *TAPBP* (↓), *RAB1B* (↓)
Platelet alpha granule membrane (GO)	***ITGA2B*** (↑), ***SPARC*** (↓), *APLP2* (↓)
Endoplasmic reticulum-Golgi intermediate compartment (GO)	*TMED1* (↑), *MYDGF* (↑), *RAB2A* (↑), ***CALR*** (↓), *TAPBP* (↓), *RAB1B* (↓)
Melanosome (GO)	***RAC1*** (↑), *RAB2A* (↑), *RAB7A* (↑), *RAB5C* (↓), ***ANXA6*** (↓), *TPP1* (↓)
Pigment granule (GO)	***RAC1*** (↑), *RAB2A* (↑), *RAB7A* (↑), *RAB5C* (↓), ***ANXA6*** (↓), *TPP1* (↓)
U12-type spliceosomal complex (GO)	*SNRNP35* (↑), *SF3B4* (↓), *SF3B2* (↓)

(↑): gene up-regulated (exercise vs. control), (↓): gene down-regulated (exercise vs. control). Bold indicate genes overlapped between genes identified in gene pathways regulated by exercise and Top-IPA gene networks. GO: Gene ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes.

Top-IPA gene network in boys (IPA score 41) highlighted the molecular functions such as cell death and survival, cellular development, and inflammatory response influenced by exercise intervention in boys (**Figure 3**), where genes *CD4, HLA-C, TNFRSF1A, MYD88, PTK2B, CD8B, CD6, CALR, NFKBIA* are of special interest as they also belong to the KEGG pathways significantly influenced by exercise (**Table 2**). In girls, the top-IPA gene network (IPA score 51) highlighted the involvement of cell-mediated immune response, cellular function and maintenance, and hematological system development and function stimulated by exercise intervention (**Figure 4)**, where genes *RAC1, ITGA2B, FYN, INPP5D, PIK3CD, CALR, TAPBP, SPARC, ANXA6* are of interest as they were also detected in the KEGG pathway analyses influenced by exercise (**Table 2**).

3.2.1 *In silico* data mining and validation

In *silico* data mining of the differentially expressed genes in boys after exercise intervention using the PHENOPEDIA database detected 21 of the genes to be involved in obesity, 9 genes in CVD, 31 genes in inflammation, and 10 genes in metabolic syndrome (**Supplementary File 6**). Interestingly, genes *CD4, CD6, HLA-C, TNFRSF1A, MYD88, PTK2B, CALR, NFKBIA* were identified in our gene pathways (**Table 2**), Top-IPA network (**Figure 3**), and in PHENOPEDIA disease terms (**Supplementary File 6**). Five out of these 8 genes, *CD6, HLA-C, TNFRSF1A, MYD88, and NFKBIA* validated in the large meta-analysis database of exercise effects identified as significantly (although reported high I^2) regulated genes by exercise *(*https://extrameta.org/).

In girls, 11 of the genes regulated by exercise were related to obesity, 4 genes involved in CVD, 21 genes involved in inflammation, and 10 in metabolic syndrome (**Supplementary File 6**). Eight genes, *RAC1, ITGA2B, FYN, INPP5D, PIK3CD, CALR,*

SPARC, ANXA6 were identified in our gene pathways (**Table 2**), Top-IPA network (**Figure 4**), and in PHENOPEDIA disease terms (**Supplementary File 6**). Six out of these top 8 genes, *RAC1, ANXA6, FYN, INPP5D, PIK3CD* and *SPARC* validated in the comprehensive meta-analysis database of exercise effects as significantly regulated genes by exercise *(*https://extrameta.org/).

4. DISCUSSION

This study shows that a 20-week exercise intervention based on multi-games may impact on whole-blood transcriptome profile (RNA-sequencing) in pre-pubertal children with OW/OB. Differential gene expression analyses detected 161 and 121 genes regulated by exercise in boys and girls, nevertheless, the effects was pronounced on molecular pathway level rather than on a single gene level. A number of molecular pathways involved in immune responses were influenced by the exercise intervention, and differences on molecular responses between boys and girls were detected.

Although few genes were in common that were influenced by exercise in both boys and girls, a number of gene pathways involved in immune processes were detected, including antigen processing and presentation, phagosome, lysosome, leukocyte transendothelial migration and B cell receptor signaling pathway. The knowledge of exercise effects on molecular level is limited, especially on pediatric population, but the stimulating effect of physical exercise on immune responses is one of the few molecular processes commonly detected in previous studies [8,35,36]. Regardless of the few shared molecular processes between both sexes in our current study, the whole-blood transcriptome profiles in pre-pubertal OB/OW children were rather different in boys and girls. We believe that in addition to the sex differences, the lack of overlap in molecular responses could be

explained, at least in part, by the findings that we observed in our exercise intervention, in which boys spent more time exercising at higher intensities (i.e. over the 80% of the maximal heart rate) than girls with different effect of training on body composition and cardiorespiratory fitness.

4.1 Exercise intervention and blood transcriptome in boys

We detected the exercise intervention to influence antigen processing and presentation and T cell receptor complex gene pathways in boys. Antigen-presenting cells (i.e., monocytes, macrophages, dendritic cells) lead to the recognition of foreign proteins (antigen) by T lymphocytes (particularly T-cell receptors on T-helper cells) [37]. T-helper cells release cytokines to activate other immune cells (e.g., can stimulate antibody production by B lymphocytes) [37]. Interestingly, the abovementioned gene pathways were up-regulated after a single bout of exercise in transcriptome analysis of PBMCs of healthy boys [15] and middle-aged adults [38]. In addition, exercise regulated other gene pathways related to different infections (e.g., salmonella, yersinia) that involve diverse immune processes. Further, several genes regulated by exercise that were detected in infection pathways and *in silico* validation (e.g., *NFKBIA*, *MYD88*) were detected in well-characterized immune gene pathways such as Toll-like receptor, NOD-like receptor, nuclear factor-κB (NF-κB). Toll-like and NOD-like receptors are important for identifying pathogen-associated molecular patterns by antigen-presenting cells [39], while the transcription factor NF-κB is a master regulator of inflammation inducing pro-inflammatory cytokine production (e.g., IL-6, TNF-α, IL-1β) [40]. In fact, Toll-like receptors can induce the production of the pro-inflammatory cytokine IL-1β through the activation of NF-κB [39].

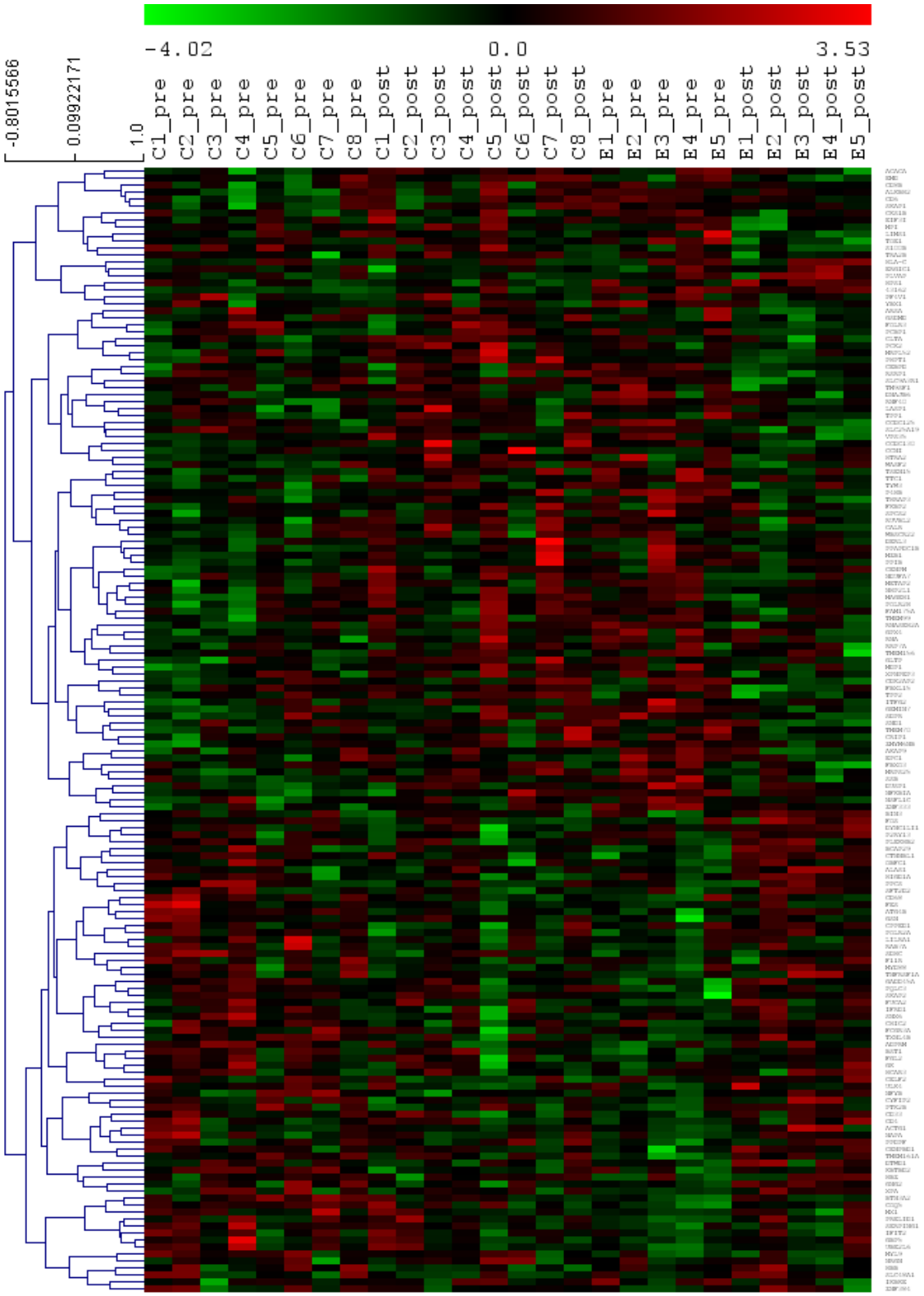

Figure 1. Heatmap of differentially expressed genes between exercise and control groups at different time-points (pre- and after intervention) in boys (161 genes, unadjusted p-value < 0.05). Red indicates up-regulated genes and green down-regulated genes. Columns represent each subject in the specific group (i.e., exercise or control) and the time-point (i.e., pre- or post-intervention).

Interestingly, *NFKBIA* was identified as a hub gene in the "Top" IPA network in boys (also detected within *in silico* validation). This gene encodes the protein NF-κB Inhibitor Alpha (IκBα) [40]. In this regard, up-regulation of *NFKBIA* could be expected by a long-term exercise intervention leading to the inhibition of NF-κB, contributing partially to decreased levels of pro-inflammatory cytokines in circulation. However, the current study reported changes in the opposite direction (e.g., exercise intervention reduced *NFKBIA* gene expression). Intriguingly, a previous study reported a positive association of body mass index and insulin resistance with IκBα protein and gene expression levels (i.e., *NFKBIA*) in PBMCs of adults with type II diabetes compared to healthy controls [41]. IκBα may be regulated by NF-kB activity (higher in type II patients compared to healthy controls), when NF-kB activity is increased will increase the expression of IκBα quickly to reduce the activity of NF-kB [41]. Another study showed that *NFKBIA* gene expression was significantly higher in whole-blood of non-survivors melioidosis patients than survivors [42]. Thus, the reduction of *NFKBIA* gene expression levels could partially indicate a lower pro-inflammatory profile after exercise intervention in boys. However, in our limited sample size we did not detect any differences in pro-inflammatory cytokines IL-6, TNF-α, and IL-1β. On the other hand, CRF increased and waist circumference was reduced after exercise intervention, improving CVD risk profile in boys, independently of changes on pro-inflammatory cytokines. Similarly, a 12-week exercise intervention improved CRF levels, while exercise did not affect cardiometabolic blood markers and total body fat in children with obesity [3].

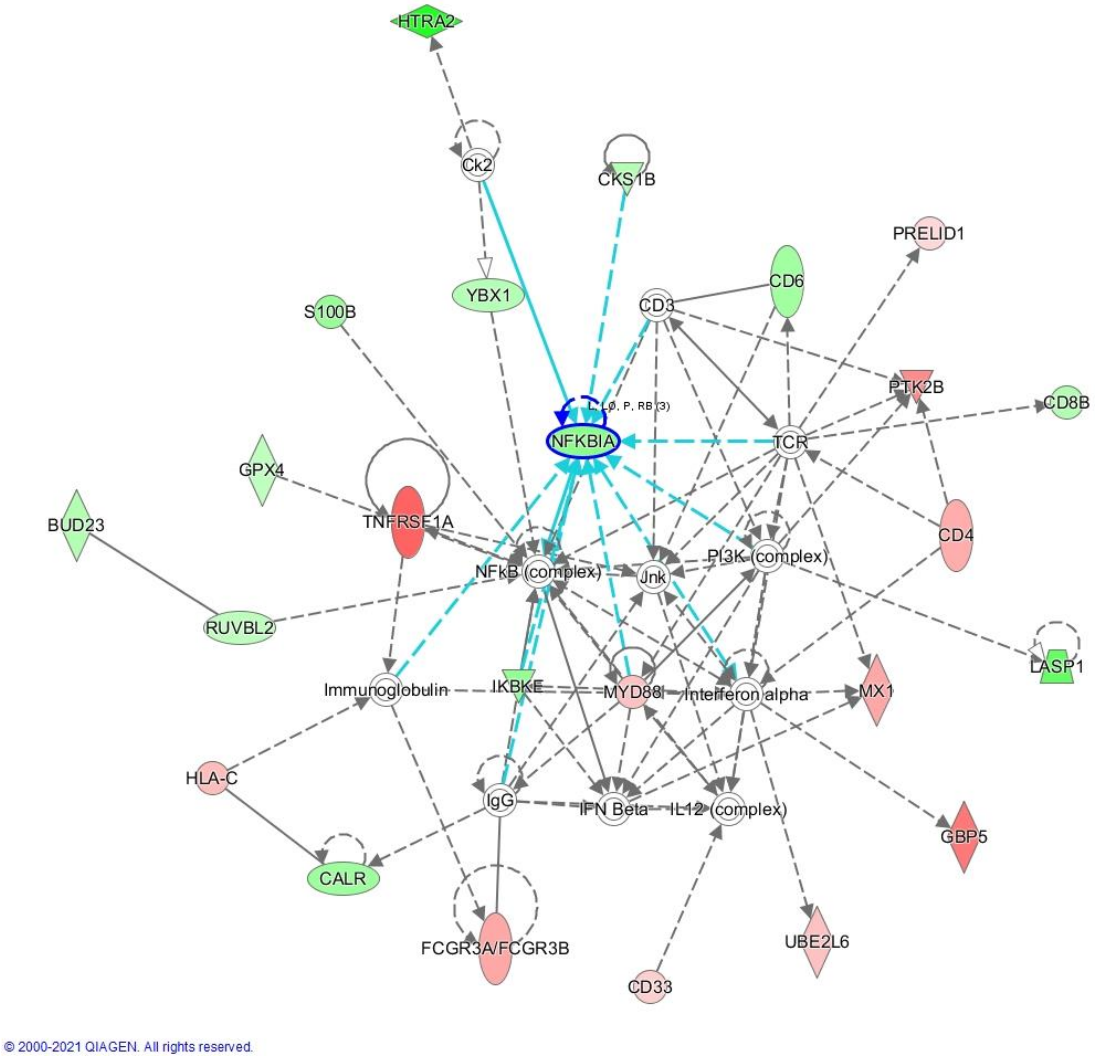

Figure 3. Top-IPA gene network in boys, enriched with IPA disease terms and functions such as cell death and survival, cellular development, and inflammatory response. The red color indicates up-regulated genes and the green color down-regulated genes in the exercise group compared to control group. Molecule shapes and relationship lines are available in: IPA®, QIAGEN Redwood City, www.qiagen.com/ingenuity. Transcription regulator: ⬭, transmembrane receptor: ⬯, enzyme: ◈, kinase: ▽, transporter: ⏢, other: ◯ . Dashed lines and solid lines reflect indirect and direct relationships between molecules. Circles lines shows that the molecule interacts with itself (e.g.,

autophosphorilation). Blue lines indicate the hub gene (i.e., *NFKBIA*) with more interactions inside the gene network. For more details about molecule shapes and relationship lines, please see: https://qiagen.secure.force.com/KnowledgeBase/articles/Basic_Technical_Q_A/Legend

4.2 Exercise intervention and molecular responses in girls

The whole-blood transcriptome analysis in girls demonstrated that the exercise intervention regulated Fc epsilon RI signaling pathway (considered a pro-inflammatory pathway [43]), where most genes were down-regulated after exercise intervention in this pathway). Fc receptors modulate humoral and innate immunity being important to prevent chronic inflammation and auto-immune diseases [43,44]. Interestingly, acute exercise down-regulated the expression of miR-96 in neutrophils of healthy adults [45]. To note, miR-96 targets genes in the Fc epsilon RI signaling pathway [45]. Otherwise, platelet activation is involved in the atherothrombotic process and long-term exercise interventions can reduce platelet activation in adults with hypertension [46]. Also, platelet activation releases growth factors into the circulation, such as VEGFA, which has been considered a pro-inflammatory marker in rheumatoid and pediatric obesity-associated inflammation [47–49]. VEGFA is an angiogenic factor that may regulate adipose tissue expansion by forming new blood vessels in adipose tissue [50,51]. Some studies reported positive associations between circulating VEGFA levels and adiposity/body weight [51,52]. In this regard, VEGFA levels decreased in circulation after weight loss induced by bariatric surgery or multicomponent interventions (i.e., exercise, behavioral and nutritional counseling) [52,53]. Interestingly, in our study, an exercise intervention regulated the platelet alpha granule membrane gene pathway, while circulating VEGFA

levels and body fat percentage decreased after exercise intervention in girls with OW/OB. In addition, other gene pathways (e.g., Fc gamma R-mediated phagocytosis, VEGF signaling, and platelet activation) related to inflammation and platelet function may be regulated by the exercise intervention in girls with OW/OB.

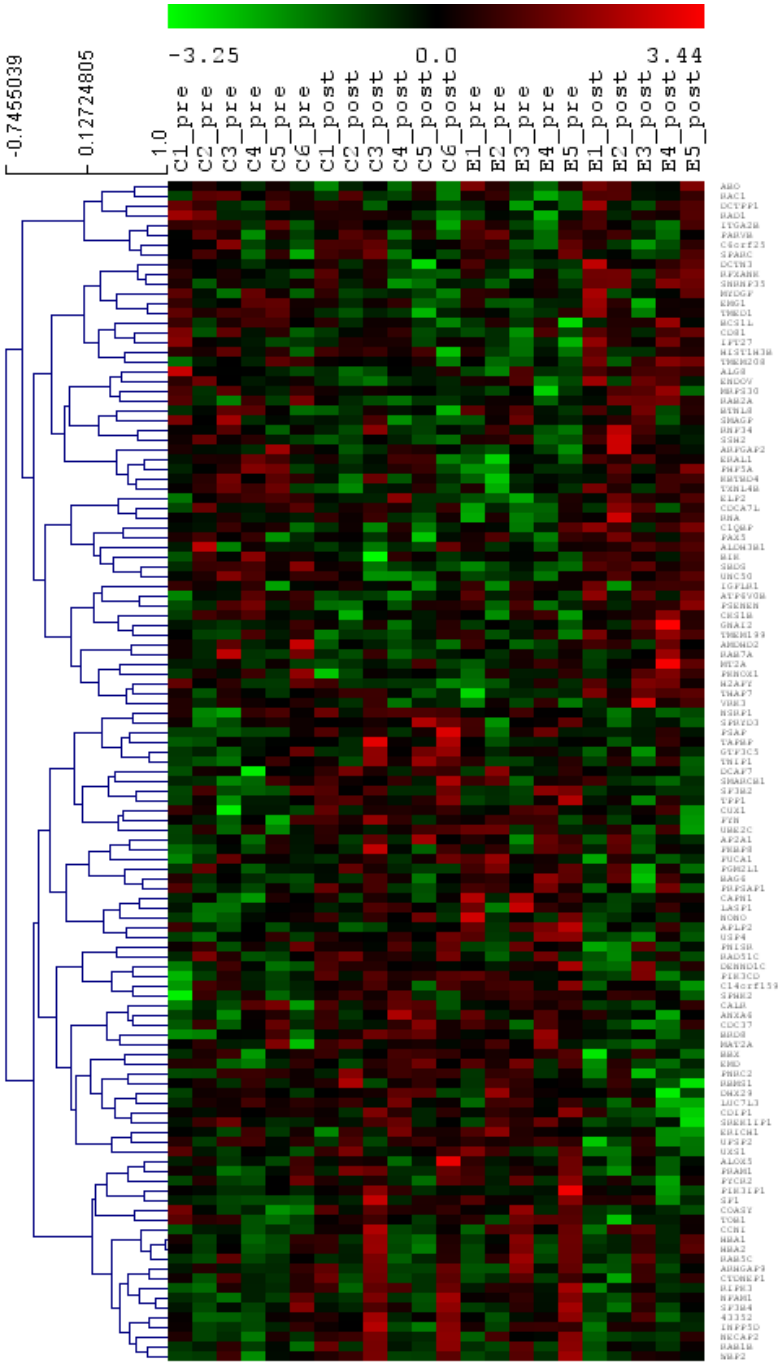

Figure 2. Heatmap of differentially expressed genes between exercise and control groups at different time-points (pre- and after intervention) in girls (121 genes, unadjusted p-value < 0.05). Red indicates up-regulated genes and green down-regulated genes.

Columns represent each subject in the specific group (i.e., exercise or control) and the time-point (i.e., pre- or post-intervention).

RAC1 (up-regulated by exercise in the current study) was a hub gene in the "Top" IPA network and identified in *in silico* validation in girls. RAC1 is a member of the Rac family of guanosine triphosphate phosphohydrolases (GTPases) that might contribute to inflammation and cardiovascular disease [54]. Also, the inhibition of RAC1 in macrophages from rodents could reduce the risk of atherosclerosis [55]. In our study, the exercise intervention increased *RAC1* gene expression levels in the whole-blood of girls with OW/OB (in the opposite direction of the expected results based on previous literature [55]). However, we cannot assume that increased *RAC1* gene expression after exercise intervention is directly related to increased RAC1 protein expression levels in whole blood (post-transcriptional mechanisms will regulate protein levels). In addition, post-translation modifications play a key role in RAC1 protein activation by regulating the protein localization in cell [54]. Besides, the exercise intervention did not affect plasma indicators of systemic inflammation (IL-6, TNF-α, IL-1β). At the same time, VEGFA (interpreted as a pro-inflammatory marker) decreased after exercise intervention in girls with OW/OB. Thus, we cannot interpret the increase of *RAC1* gene expression in whole blood after exercise intervention as an indicator of a higher pro-inflammatory status after exercise in girls.

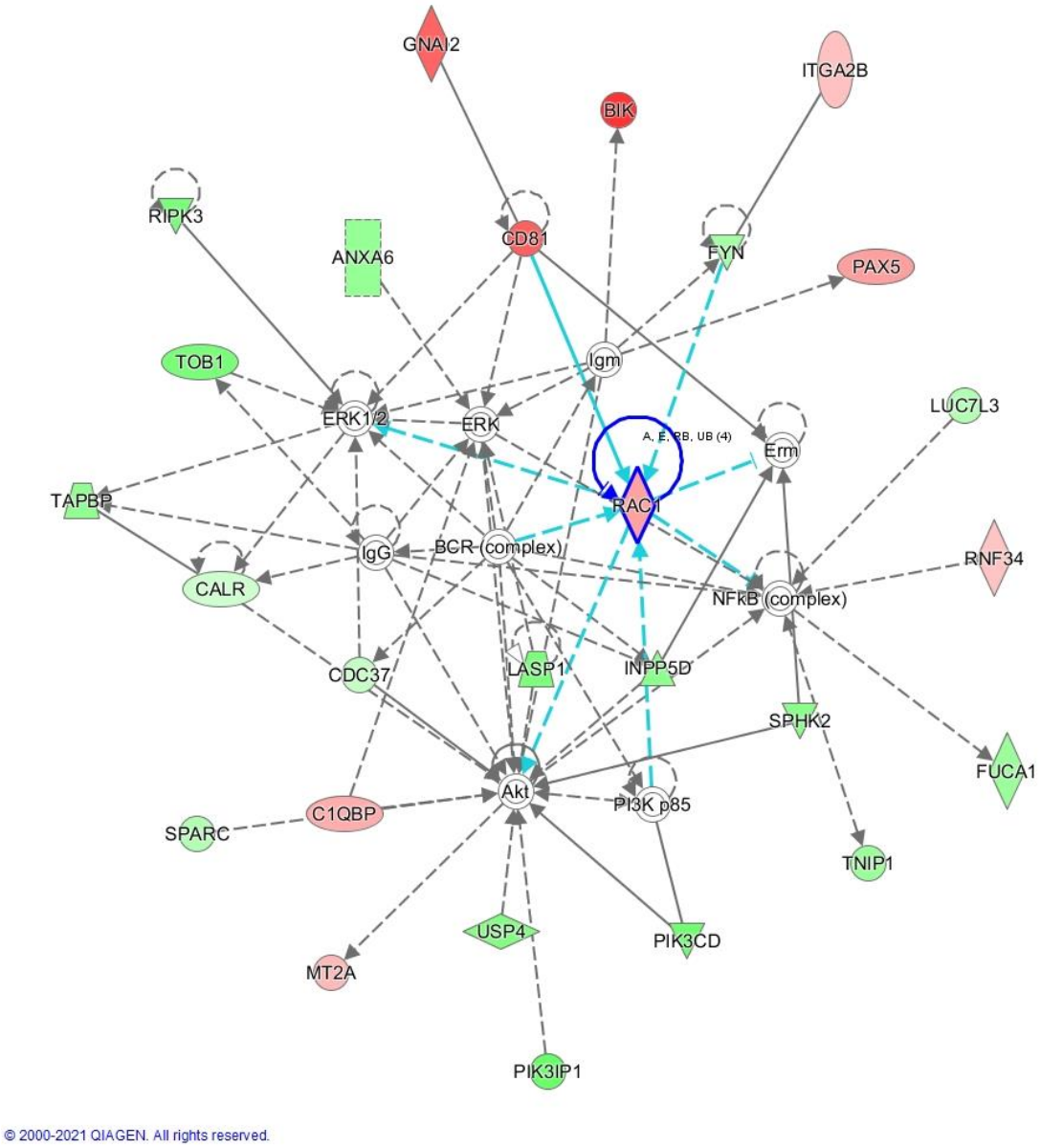

Figure 4. Top-IPA gene network in girls enriched with IPA disease terms and functions such as cell-mediated immune response, cellular function and maintenance, and hematological system development and function. The red color indicates up-regulated genes and the green colo down regulated genes in the exercise group compared to control group. Molecule shapes and relationship lines are available in: IPA®, QIAGEN Redwood City, www.qiagen.com/ingenuity. Transcription regulator:⬭,transmembrane receptor:, ⬯ enzyme:⧫, kinase:▽, peptidase:◁▷phosphatase: △, transporter:⏢

ion channel: ⬚ , other: ◯ Dashed lines and solid lines reflects indirect and direct relationships between molecules. Circles lines shows that the molecule interacts with itself (e.g., dimerization). Blue lines indicate the hub gene (i.e., *RAC1*) with more interactions inside the gene network. Please see: https://qiagen.secure.force.com/KnowledgeBase/articles/Basic_Technical_Q_A/Legend for more details about molecule shapes and relationship

4.3 Limitations

Although providing novel information, several limitations need to be acknowledged. Whole-blood gene expression profile was analysed, while blood is a complex tissue comprised of different cell populations (e.g., T cells, B cells, NK killers, neutrophils, among others) with different gene expression profiles [56]. Likewise, the relatively low sample size limited our statistical power to detect statistically significant individual gene expression differences between groups. Despite these limitations, our study is the first randomized controlled trial to report the effects of a 20-week exercise intervention on whole-blood transcriptome profile in boys and girls with OW/OB. Likewise, a RNA-sequencing method was used to perform whole-transcriptome untargeted analysis. Additionally, the high globin mRNA of erythrocytes was reduced by a locking assay enabling better detection of less abundant transcripts in the blood [29].

5. CONCLUSIONS

We hereby present novel findings on whole-blood transcriptome in pediatric population, increasing the understanding into the beneficial effects of regular physical activity on molecular processes. We were able to identify a number of immune processes to be influenced by the exercise intervention in OB/OW children, with some overlap in gene pathways in boys and girls. The general trend of transcriptome differences between

boys and girls could partly be explained, in addition to the sex differences, by the distinct intensity in the exercise intervention in boys and girls. We believe that our preliminary findings will be helpful input to advance our understanding of the molecular basis of the health benefits of exercise in children with OW/OB.

SUPPLEMENTARY MATERIAL

The supplementary files can be downloaded in this link:

https://osf.io/unpje/?view_only=d3eeb5a70be44b84a845efc96607c08a

Supplementary File 1. Effects of a 20-week exercise intervention (i.e., exercise vs control) on raw and z-score post-intervention body composition, cardiorespiratory fitness, and inflammatory markers in boys. Significant differences ($p < 0.05$) are highlighted in bold.

Supplementary File 2. Effects of a 20-week exercise intervention (i.e., exercise vs control) on raw and z-score post-intervention body composition, cardiorespiratory fitness, and inflammatory markers in girls. Marginal p-values ($p < 0.10$) are highlighted in bold.

Supplementary File 3. Time exercising (aerobic part of the sessions) during the 20-week exercise intervention at different intensities in boys and girls. Significant differences ($p < 0.05$) are highlighted in bold.

Supplementary File 4. Differentially expressed genes in boys and girls (exercise vs. control; $p < 0.05$). Red genes were up-regulated while green genes were down-regulated after the exercise intervention

Supplementary File 5. The KEGG pathways and GO terms of the genes regulated by exercise in boys and girls. Blue pathways (FDR < 0.05) and orange pathways ($p < 0.05$).

Bold reflects immune pathways of interest while asterisk indicates common pathways in boys and girls.

Supplementary File 6. In *silico* data mining of the differentially expressed genes in boys after exercise intervention. Bold genes were the most important genes identified in data mining (i.e., identified in gene pathways regulated by exercise, IPA networks, PHENOPEDIA and https://extrameta.org/).

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STUDY VI: Effects of exercise intervention on whole-blood genome-wide DNA methylation profile in children with overweight/obesity

1. INTRODUCTION

Childhood obesity is associated with the development of type 2 diabetes, cardiovascular disease (CVD), and premature death later in life [1–3]. Obesity is a multifactorial disease characterized by low-grade systemic inflammation [4,5], which can be prevented and even reversed with regular exercise [6]. Indeed, exercise interventions (i.e., chronic effects of exercise) have shown many health benefits, e.g., an improvement in cardiometabolic risk factors (metabolic syndrome markers, cardiorespiratory fitness [CRF], among others), body composition, and brain health indicators in children with overweight/obesity (OW/OB) [7–9]. However, the molecular mechanisms underlying these health-related benefits are poorly understood in pediatric population.

The "epigenome" refers to different gene regulatory mechanisms (e.g., DNA methylation, histone acetylation) that may be influenced by environmental factors (e.g., regular exercise) and can regulate gene expression without altering DNA sequence [10,11]. DNA methylation, specifically methylation of CpG sites in DNA is the most studied epigenetic modification of DNA [10]. DNA methylation profiles in blood cells have been associated with body mass index/obesity from birth to adulthood [12–14] and other diseases (e.g., cancer, type 2 diabetes) [10]. Furthermore, DNA methylation is the epigenetic mechanism most investigated in response to exercise interventions in adults [11,15], where, the majority of studies have focused on the effects of exercise interventions on DNA methylation profile in skeletal muscle [16,17], adipose tissue [18], or leukocytes from peripherial blood in adults [19,20]. Nevertheless, to our knowledge, effects of an exercise intervention on whole-blood genome-wide DNA methylation, i.e. methylome profile in children is still lacking.

DNA methylation patterns may alter the gene expression levels of nearby loci (which is known as cis-expression quantitative trait methylation (cis-eQTM)) but also at long distances from their occurrence (known as trans-eQTM). The study of which genes have their expression altered by DNA methylation pattern is essential to trace the complete molecular mechanisms underlying exercise effects as well as to validate findings in an epigenetic study. Recent technological advances and bioinformatics tools allow to analyze and integrate changes on DNA methylation with changes on gene expression patterns of thousands of genes at genome-wide level (i.e., multi-omics analyses) in response to exercise [21].

Due to ethical concerns of obtaining tissue biopsies in children, whole-blood is the primary choice tissue to study the molecular response to exercise interventions in pediatric population [21]. Indeed, blood is an interesting target tissue to investigate the effects of exercise on DNA methylation profile compared to other tissues (e.g., adipose tissue) in children with OW/OB for several reasons: i) infiltration of blood cells (e.g., macrophages) in adipose tissue may contribute to low-grade systemic inflammation in obesity [22,23]; while whole-blood may reflect the systemic inflammatory profile that can be reverted by regular exercise in humans with obesity [6]; ii) whole-blood genome-wide DNA methylation profile related to diet has been associated with CVD risk factors and all-cause of mortality [24]; and iii) there is previous evidence of the effect of exercise on the blood DNA methylation profiles in healthy adults [19,20] and patients with cancer [25,26].

In the present study we aimed: 1) to investigate the effects of a 20-week exercise intervention on whole-blood methylome and 2) to examine the association between changes induced by exercise intervention on whole-blood methylome and whole-blood transcriptome in children with OW/OB. This multi-omics approach would provide a

deeper understanding of the molecular mechanisms induced by exercise in children with OW/OB.

2. METHODS

2.1 Design and participants

In total, 109 children with OW/OB participated in the ActiveBrains randomized controlled trial trial (ClinicalTrials.gov [identifier: NCT02295072]) [27], from where a subsample for DNA methylation and transcriptome analyses was selected. Valid and complete DNA methylation data before and after the exercise intervention was obtained for 24 children. One child with DNA methylation data was excluded from the analyses for not attending a minimum of 2/3 of the training sessions, leading to the final sample of 23 children (aged 10.05 ± 1.39 years, 56% girls) included for the primary analyses in the present study. For the integration of DNA methylome and transcriptome data, in total 18 children provided valid data. The ActiveBrains project was approved by the Human Research Ethics Committee of the University of Granada. Details, inclusion and exclusion criteria, and, random allocation of the participants into exercise or control groups have been described elsewhere [27]. Briefly, all the participants included in this study were children with OW/OB according to the age- and sex-specific World Obesity Federation cut-off points [28] and aged 8-11 years old. The participants were randomly allocated into an exercise group (i.e., performed a 20-week exercise intervention) or a control group (i.e., continued with their usual lifestyle for 20 weeks). Body mass index (kg/m^2) was calculated using body weight and height assessed with an electronic scale and a stadiometer (Seca instruments, Germany, Ltd). Then, children's were classified as OW/OB according to the World Obesity Federation cut-off points, specific for sex and

age [28]. Peak height velocity was computed as an indicator of maturational status [29]. Both groups received at the beginning of the study a pamphlet with guidelines about physical activity and healthy eating. Due to ethical considerations, the exercise intervention was provided to the children allocated in the control group once the project was completed. The SPSS software was used (version 21.0; Armonk, NY, USA) to perform the randomization of the participants.

Three methodological aspects to reduce risk of bias were: (i) the randomization was performed after the baseline (i.e., pre-intervention) data collection; (ii) the person who performed the computer random generation was not involved in the baseline data collection; (iii) exercise trainers were not involved in the baseline data collection; and (iv) DNA and RNA analyses were performed in external labs by technical staff not involved in the randomized controlled trial (RCT), ensuring blinding in the main outcomes of this study.

2.2 Exercise intervention

The exercise intervention is in line with the international physical activity guidelines [30,31] and had a duration of 20-week. The participants were encouraged to attend to the program 5 days/week (1 session/day of 90 minutes) being the minimum attendance recommended 3 sessions/week. Sessions of the exercise intervention were divided into four parts: (i) Warm-up (5-10 minutes) consisting of 1-2 physical games; (ii) aerobic part (60-min) based on 4-5 multi-games at moderate-to-vigorous intensities (i.e., emphasis above 80% of maximal heart rate), (iv) resistance training part (20-min) consisting in strength exercises involving large-muscle-groups in sets of 10-12 repetitions using bodyweight, theraband and/or fitballs; and (v) Cool-down (5-10 minutes) based on relaxation and stretching exercises.

The intensity of the exercise intervention was controlled individually in each session using heart rate monitors (Polar RS300X, Polar Electro Oy Inc, Kempele, Finland). The maximum heart rate achieved by each child in a maximal incremental treadmill test (see cardiorespiratory fitness assessment section) was used to individually program the heart rate monitors. The intensity was checked each week to ensure an accurate training stimulus along the 20-week exercise intervention. The children allocated in the control group were advised to continue with their usual lifestyle (physical activity and nutrition recommendations for healthy lifestyle were provided).

2.3 DNA extraction and methylome analysis

Genomic DNA was extracted from whole-blood using the Qiamp DNA Investigator Kit for coagulated samples and the Qiamp DNA Mini & Blood Mini Kit for non-coagulated samples (QIAgen Systems, Inc., Valencia, CA, USA). The DNA Clean and Concentrator kit from Zymo Research (Zymo Research, Irvine, CA, USA) was used to purify the samples. The EZ-96 DNA Methylation Kit (Zymo Research Corporation, Irvine, CA) was used to treat DNA samples (≥ 500 ng) with bisulfite. DNA methylation was assessed using the Infinium Methylation EPIC array (Illumina, San Diego, CA, USA).

2.4 RNA extraction and sequencing (RNA-seq)

Whole-blood gene expression analyses have been previously described [32,33]. Briefly, the RiboPureTM-Blood Kit (Thermo Fisher Scientific; Waltham, MA, USA) was used to isolate total RNA from the whole-blood samples. Next, abundant globin mRNA of erythrocytes was blocked using GlobinLock molecular mechanism [34]. The modified version of single-cell tagged reverse transcription (STRT2) protocol was used to prepare Illumina-compatible library using 10 ng of high-quality RNA as input that was converted

to cDNA [35]. aw sequencing reads, aligning to the hg19 genome, and quantify the expression levels we used the STRTprep pipeline, available at https://github.com/shka/STRTprep/tree/v3dev. Transcriptome data is available at GEO database, www.ncbi.nlm.nih.gov/geo under accession number GSE193771.

2.5 Statistical analyses

2.5.1 Effect of exercise on whole-blood methylome

Descriptive data are presented as mean and standard deviation (mean ± SD), or frequency and percentage for clinical variables. The effects of exercise intervention on whole-blood genome-wide methylome and association with changes on transcriptome profile (RNA-seq) were performed in R software version 4.0.3. The Beta-Mixture Quantile (BMIQ) intra-array normalization was used to reduce the variation within and between samples considering all the participants and time points. DNA Methylation levels at CpG sites were reported using beta values (β-values = M/(M + U)) or M-values (M value = log2(M/U)). U and M refers to the Unmethylated and Methylated signals respectively. M-values were selected for statistical analyses because they presented lower heteroscedasticity than β-values. However, beta values (i.e., % of methylation at each CpG site) were used for exploratory purpose in visualization plots. The effects of exercise intervention on methylation levels of CpG sites were evaluated through a multi-level experiment, treating the patient as a random effect, and the experimental group and time as a combined fixed factor. The inter-subject correlation was the input for the linear model fit. The between groups contrast over the linear model was then applied using a moderated t-test. These analyses were implemented in the R environment using linear models from the *LIMMA* R package and considering the M-values of DNA methylation for each CpG as the outcome or dependent variable. We considered a raw $p < 0.001$ as the threshold for

statistical significance. Raw p-values were corrected using the false discovery rate (FDR) according to the Benjamin- Hochberg procedure for multiple comparisons.

The *illuminahumanmethylationepicanno.ilm10b4.hg19* and *missmethyl* R packages were used to perform functional annotation of CpG sites and regions. We reported 2 categories in terms of genomic regulatory elements: 1) annotation to gene region (i.e., CpGs identified in 1st exon, 3' untranslated regions [UTRs], 5' UTR, body, exonbnd, Transciprtion start site [TSS] 1500, TSS200, and intergenic regions) and 2) distance to a CpG island (i.e., island, N_shelf, N_shore, Opensea, S_shelf, and S_shore). Pathway analysis was conducted using as input genes that showed differentially methylated CpGs sites after exercise in boys and girls respectively. The *gometh* function from the R package *MANIFEST* was applied to obtain enrichment of CpG-annotated genes in KEGG terms [36], considering the number of CpG sites per gene on the EPIC array. Multiple hypothesis testing correction was performed (Benjamini-Hochberg FDR).

2.5.2 Integration of methylome and transcriptome data: Expression quantitative trait methylation (eQTM) analysis

The expression quantitative trait methylation (eQTM) analysis was performed to reveal whether the methylation changes (up- or down-regulated) of a specific CpG site may regulate the expression of a target gene transcript. The transcriptome analyses are described in detail in [37]. The *MatrixEQTL* R package was used to perform linear models between changes on methylation at CpG sites (post – pre) as predictors (i.e., from the list of CpG sites differentially methylated by exercise intervention) and changes on gene expression (post – pre) as the outcome (using all the genes quantified in our RNA-seq analyses). A quantile normalization was performed on transcriptome data and genes with many zeros were removed. If the distance between the CpG site and the gene transcript

was higher than 10.000 bp was considered trans-eQTM, while cis-eQTM refers to a CpG located in or around 10.000 bp of a gene transcript. Beta regression coefficients were reported as indicators of the eQTM effects size. All the p-values obtained in trans-eQTM were corrected by multiple hypothesis testing correction (Benjamini-Hochberg FDR). Finally, we studied the overlapping between the list of gene transcripts identified as statistical significant in cis- and trans-eQTMs (i.e., gene transcripts associated with changes on methylation at CpG sites) with the list of gene transcripts regulated by the same exercise intervention in boys and girls with OW/OB according to our previous transcriptomic study [37].

2.6 In silico data validation of genes

An *in silico* data mining and validation approach was performed to identify the top genes related to molecular effects of exercise using the PHENOPEDIA database [38] and database extrameta.org. First, the list of gene transcripts overlapped between eQTM analysis in this study and the list of genes transcript regulated by exercise in our previous study [37], were overlapped with lists of genes related to obesity, metabolic syndrome, inflammation, and/or CVD provided by PHENOPEDIA database (based on genetic associations studies continuously updated in PubMed). Next, gene transcripts identified in: 1) eQTM analysis; 2) regulated by the same exercise intervention in our previous study [37]; and 3) found in PHENOPEDIA disease terms were investigated in the resource https://extrameta.org/ [39]. Briefly, https://extrameta.org/ was created in a extensive meta-analysis using public data of transcriptome profiles (in total 43 datasets) in response to acute and chronic exercise in blood and skeletal muscle of humans [39] in order to cofirm the involvement of these genes in molecular responses to exercise.

3. RESULTS

Table 1. Descriptive characteristics of the study sample

Variables	Total sample (n=23, 13 girls/10 boys)	Boys (n=10, 4 exercise/6 control)	Girls (n=13, 6 exercise/7 control)
Age and Maturational status			
Age (years)	10.05 ± 1.39	10.90 ± 1.33	9.39 ± 1.06
Peak height velocity offset (years)	-2.08 ± 0.92	-2.10 ± 0.93	-2.06 ± 0.95
Body composition			
Weight (kg)	55.95 ± 10.75	60.37 ± 11.27	52.54 ± 9.37
Height (cm)	144.86 ± 9.99	149.02 ± 10.36	141.66 ± 8.77
Waist circumference (cm)	90.69 ± 7.97	93.12 ± 6.93	88.82 ± 8.48
Body mass index (kg/m^2)	26.44 ± 2.85	26.96 ± 2.26	26.04 ± 3.27

Data presented as mean ± SD, and as number and frequency.

3.1 Effect of exercise on whole-blood methylome

Descriptive characteristics of participants are presented in **Table 1**. The main results of this study are summarized in **Figure 1**. The whole-blood genome-wide DNA methylation data is available at under GEO accession number GSE193730. 485 CpG sites were regulated by the exercise intervention in boys (306 increased and 179 decreased methylation levels after exercise; log_2FC ranged from -5.83to 4.87; $p < 0.001$) (**Supplementary File 1**). In girls, 386 CpG sites, 108 increased and 278 decreased methylation after exercise (log_2FC ranged from -4.27 to5.61; $p < 0.001$) (**Supplementary File 1**). Regarding genomic location in boys, 2.89% of CpG sites were identified in 1st Exon, 4.74% in 3'UTR, 7.22% in 5'UTR, 41.03% in gene body, 1.03% exon boundaries, 11.75% in TSS1500, 8.66% in TSS200, and 22.68% in intergenic regions (**Supplementary Figure 1**). 69 CpG sites were located in enhancer regions (ENCODE consortium) and 84 CpG sites were located in transcription factor binding sites (TFBS), while 14 CpG sites were identified in both enhancer regions and TFBS (**Supplementary File 1)**. In girls, 1.04% of CpG sites were identified in 1st Exon, 2.85% in 3'UTR, 9.84%

in 5'UTR, 40.41% in gene body, 0.78% exon boundaries, 15.03% in TSS1500, 7.77% in TSS200, and 22.28% in intergenic regions (**Supplementary Figure 2)**. 57 CpG sites were located in enhancer regions (ENCODE consortium) and 60 CpG sites were located in TFBS, while 9 CpG sites were identified in both enhancer regions and TFBS (**Supplementary File 1).** The location of CpG sites concerning island is presented in **Supplementary Figures 3 and 4** for boys and girls respectively.

Table 2. Enrichment analyses of genes that showed alteration of CpGs sites after 20-week exercise intervention in boys and girls (P < 0.05). Bold font indicates that exercise intervention impacted DNA methylation in CpG sites located in these genes, which were associated with changes on gene expression by the same exercise intervention in a previous study [37].

KEGG Pathways regulated by exercise intervention	Genes identified in each pathway
Boys, 17 pathways input 485 CpGs	
NOD-like receptor signaling	*IKBKG, MAPK8, **MAPK10**, RNF31, TRPM7, **ITPR2**, NEK7, **RHOA**, **JAK1***
Inflammatory mediator regulation of TRP channels	*HTR2C, **ITPR2**, PPP1CB, TRPA1, ASIC2, MAPK8, **MAPK10**, TRPM8*
Adipocytokine signaling pathway	*MAPK8, **MAPK10**, IKBKG, ACSL1, LEPR, PRKAG2*
Foxo signaling pathway	*PRKAG2, MAPK8, **MAPK10**, FOXO1, FOXO3, **CDK2**, BCL6*
Type II diabetes mellitus	*MAPK8, **MAPK10**, PKM, CACNA1C*
Fatty acid degradation	*ACOX3, ACSL1, ADH6*
Insulin resistance	***RPS6KA2**, PPP1CB, MAPK8, **MAPK10**, PRKAG2, FOXO1*
Neurotrophin signaling pathway*	***RPS6KA2**, FOXO3, ABL1, **RHOA**, MAPK8, **MAPK10**, TP53*
Pentose phosphate pathway	*G6PD, RBKS, PGM1, RGN*
Long-term potentiation	*PPP1CB, GRIN2B, CACNA1C, **RPS6KA2, ITPR2***
Cell cycle	*ABL1, **HDAC1**, TFDP1, **CDK2**, STAG1, TP53, PRKDC*
Mitophagy - animal	*MAPK8, **MAPK10**, TP53, FOXO3, TBC1D15*
Shigellosis	***RHOA**, FOXO1, FOXO3, **ITPR2**, TP53, IKBKG, MAPK8, **MAPK10**, RNF31, U2AF1, PIK3R4*
Viral carcinogenesis	***JAK1**, **REL**, TP53, **CDK2**, H2BW1, GTF2E2, **HDAC1**, HDAC4**, HDAC7**, HDAC8, PKM, IKBKG, **RHOA***
Alcoholism*	*GRIN2B, **HDAC1**, HDAC4, **HDAC7**, HDAC8, H2AZ2, H2BW1, PPP1CB*
Huntington disease	*HTT, DNAH3, MAPK8, **MAPK10**, GRIN2B, SLC1A3, **HDAC1**, TP53, COX4I1, ATP5MC2, PSMA8, PIK3R4*
Pancreatic cancer	*IKBKG, MAPK8, **MAPK10**, **JAK1**, TP53, RAD51*
Girls, 17 pathways input 386 CpGs	
Lysine degradation	*SETD1B, ASH1L, SMYD3, EHMT2, PRDM2*
Cortisol synthesis and secretion	***PRKACB**, PBX1, **CREB5**, **NCEH1**, SCARB1*
Fat digestion and absorption	*PLA2G2E, SCARB1, NPC1L1*
IL-17 signaling pathway	*MAPK14, TRAF5, MUC5B, IL17B*
Neurotrophin signaling pathway*	*NTRK2, **RAPGEF1**, MAPK14, PLCG2, **CAMK4**, PTPN11*
Longevity regulating pathway	***PRKACB**, **CREB5**, EHMT2, ADIPOR1, **CAMK4***

RNA transport	*XPOT, CLNS1A, TPR, NUP35,* ***NUP214****, SUMO1,* ***AAAS****, EIF3J, EIF3D*
Inositol phosphate metabolism	***MTMR14****, PIP5K1C, PIP5K1A, PLCE1, PLCG2*
Vasopressin-regulated water reabsorption	***PRKACB, CREB5****, DCTN2*
Ubiquitin mediated proteolysis	***UBE2D1****, UBE2F, UBE2G1, UBE2G2, RCHY1, PIAS3, SKP2*
Prion disease	*STIP1,* ***PRKACB****, NDUFV2, NDUFA9, SDHC, COX8C, PSMD8, MAPK14,* ***CREB5****, TUBA8*
Parkinson disease	***PRKACB****, UBE2G2, UBE2G1, PSMD8, NDUFV2, NDUFA9, SDHC, COX8C, TUBA8*
Ferroptosis	*LPCAT3, PCBP1, ATG7*
Epithelial cell signaling in Helicobacter pylori infection	*PTPN11, PLCG2, MAPK14, ATP6V1H*
Amyotrophic lateral sclerosis	*NDUFV2, NDUFA9, SDHC, COX8C, PSMD8, TPR, NUP35, NUP214, MAPK14, TUBA8, DCTN2, NRG1*
Alcoholism*	*H2BC18, ADORA2B,* ***PRKACB****,* ***CREB5****, NTRK2,* ***CAMK4***
Progesterone-mediated oocyte maturation	*MAPK14,* ***PRKACB****, CCNA2, PKMYT1, MAD1L1*

KEGG: Kyoto Encyclopedia of Genes and Genomes. * Common pathways regulated by exercise intervention in boys and girls.

In boys, exercise regulated gene pathways related to metabolism and immune function such as type 2 diabetes, insulin resistance, fatty acid degradation, NOD-like receptor signaling, adipocytokine signaling pathway, among others ($p < 0.05$) (**Table 2**). In girls, gene pathways were enriched for metabolic and immune signaling pathways including lysine degradation, cortisol synthesis and secretion, fat digestion and absorption, IL-17 signaling pathway among others ($p < 0.05$) (**Table 2**). Neurotrophin signaling pathway was a common pathway regulated by exercise intervention in boys and girls ($p < 0.05$) (**Table 2**). The abovementioned analyses did not remain significant after false discovery rate correction (FDR > 0.05).

3.2 Integration of methylome and transcriptome data: Expression quantitative trait methylation (eQTM) analysis

In boys, methylation changes on 206 CpG sites (from a list of 485 CpG sites) were associated with expression changes of 192 transcripts from RNA-seq (the total number of associations were: 66 cis-eQTM and 274 trans-eQTM) (beta coefficients ranged from -2.14 to 2.69; $p < 0.05$ and $p < 0.001$ for cis- and trans-eQTM respectively) (**Supplementary File 2**). From the abovementioned associations, 51 gene transcripts (15 identified in cis-eQTM) were overlapped between eQTM analyses (i.e., 192 gene transcripts) and our list of gene transcripts regulated by the same exercise intervention (i.e., 161 gene transcripts) in our previous study [37] (**Supplementary File 2**). Sixty-one CpG sites (30% of the total CpG sites identified in significant eQTM associations [206 CpG sites]) were located in enhancer regions or TFBS. From these 61 CpG sites, 23 (3 cis- and 20-trans eQTM) were associated with 20 gene transcripts regulated by the exercise intervention in our previous study (i.e., from the abovementioned 51 gene transcripts) (**Supplementary File 2**). All these analyses did not survive false discovery rate correction (FDR > 0.05).

In girls, methylation changes on 140 CpG sites (from a list of 387 CpG sites) sites were associated with expression changes of 138 transcripts from RNA-seq (from total 38 cis-eQTM and 150 trans-eQTM) (beta coefficients ranged from -2.36 to 2.23; $p < 0.05$ and $p < 0.001$ for cis- and trans-eQTM respectively) (**Supplementary File 3**). From the abovementioned associations, 30 gene transcripts (5 identified in cis-eQTM) overlapped between eQTM analyses (i.e., 138 gene transcripts) and our list of genes regulated by the same exercise intervention (i.e., 121 gene transcripts) in our previous study [37] (**Supplementary File 3**). Thirty-three CpG sites ((21% of the total CpG sites identified in significant eQTM associations [140 CpG sites])) were located in enhancer regions or TFBS. From these 33 CpG sites (all trans-eQTM), 8 associated with 8 gene transcripts regulated by the exercise intervention in our previous study (i.e., from the abovementioned 30 gene transcripts) (**Supplementary File 3**). All these analyses did not survive false discovery rate correction (FDR > 0.05).

3.3. *In silico validation*

In boys, an *in silico* data mining approach using the PHENOPEDIA database detected that 10 out of the 51 genes transcripts overlapped between our current eQTM lists and findings from our previous study [37] were identified in different disease terms related to obesity (**Supplementary File 4**). These genes included *NFKBIA (*obesity, inflammation, and CVD terms in PHENOPEDIA*), PCK2* (obesity and metabolic syndrome), *XPA* (obesity and inflammation), *DNAJB6* (obesity), *CALR* (inflammation), *MX1* (inflammation), *TYW3* (inflammation), *CD6* (inflammation), *FES* (inflammation), *GADD45A* (inflammation). From these 10 top genes, 6 validated as significantly regulated genes by exercise (according to https://extrameta.org/): *NFKBIA*, *XPA*, *MX1*, *CD6*, *FES*, and *GADD45A* (**Table 3**)

Table 3. *In silico* data mining approach using the PHENOPEDIA (diseases linked) and Meta-analysis database extrameta (genes regulated by exercise) [39]. (↑): gene up-regulated (exercise vs. control), (↓): gene down-regulated (exercise vs. control). NS: No significant/reported

Gene	General function	PHENOPEDIA	extrameta (genes regulated by exercise) [39]				Gene expression after exercise in our previous study [37]
			Muscle		Blood		
			Acute	Chronic	Acute	Chronic	
NFKBIA	Inhibit transcription factor Nuclear factor kappa B (NF-κB)	Obesity, inflammation and cardiovascular disease	↓	NS	↓	NS	↓
XPA	Role in nucleotide excision repair	Obesity and inflammation	↓	↓	NS	NS	↑
MX1	Cellular antiviral response	Inflammation	NS	↑	NS	NS	↑
CD6	T cell activation	Inflammation	↓	NS	NS	NS	↓
FES	hematopoiesis and cytokine receptor signaling	Inflammation	↑	↑	↑	NS	↑
GADD45A	Activates the p38/JNKpathway	Inflammation	↑	NS	NS	NS	↑
RAC1	Control of cell growth, cytoskeletal reorganization, and the activation of protein kinases	Metabolic syndrome and inflammation	NS	↓	↑	NS	↑
BAG6	Control of apoptosis and response to DNA damage	Inflammation and cardiovascular disease	↓	↓	NS	NS	↓
BRD8	Involved in thyroid hormone-dependent activation	Metabolic syndrome	NS	↓	↑	NS	↓
FUCA1	Degradation of fucose-containing glycoproteins and glycolipids	Metabolic syndrome	NS	↓	NS	NS	↓

In girls, 8 out of the 30 gene transcripts overlapped our eQTM lists and findings from our previous study [37] were detected in PHENOPEDIA disease terms related to obesity (**Supplementary File 4**). These genes were: *RAC1* (metabolic syndrome and inflammation), *CD81* (obesity and inflammation), *BAG6* (inflammation and cardiovascular disease), *PAX5* (obesity), *CCNI* (metabolic syndrome), *BRD8* (metabolic syndrome), *ERAL1* (metabolic syndrome), *FUCA1 (metabolic syndrome)*. From these 8 top genes, 4 validated as significantly regulated genes by exercise (https://extrameta.org/) (**Table 3**): *RAC1*, *BAG6*, *BRD8*, and *FUCA1* (**Table 3**).

4. DISCUSSION

The findings of this study support that: 1) a 20-week exercise intervention may have an impact on the whole-blood DNA methylation profile of genes involved in metabolic and immune gene pathways in children with OW/OB; 2) changes on DNA methylation induced by exercise are to some extent associated with exercise-induced changes in gene expression related to immune function, obesity, metabolic syndrome, and inflammation; 3) boys and girls seem to have different molecular response to exercise invetvention stimulus. Given the relatively small sample size, our findings should be considered exploratory and preliminary.

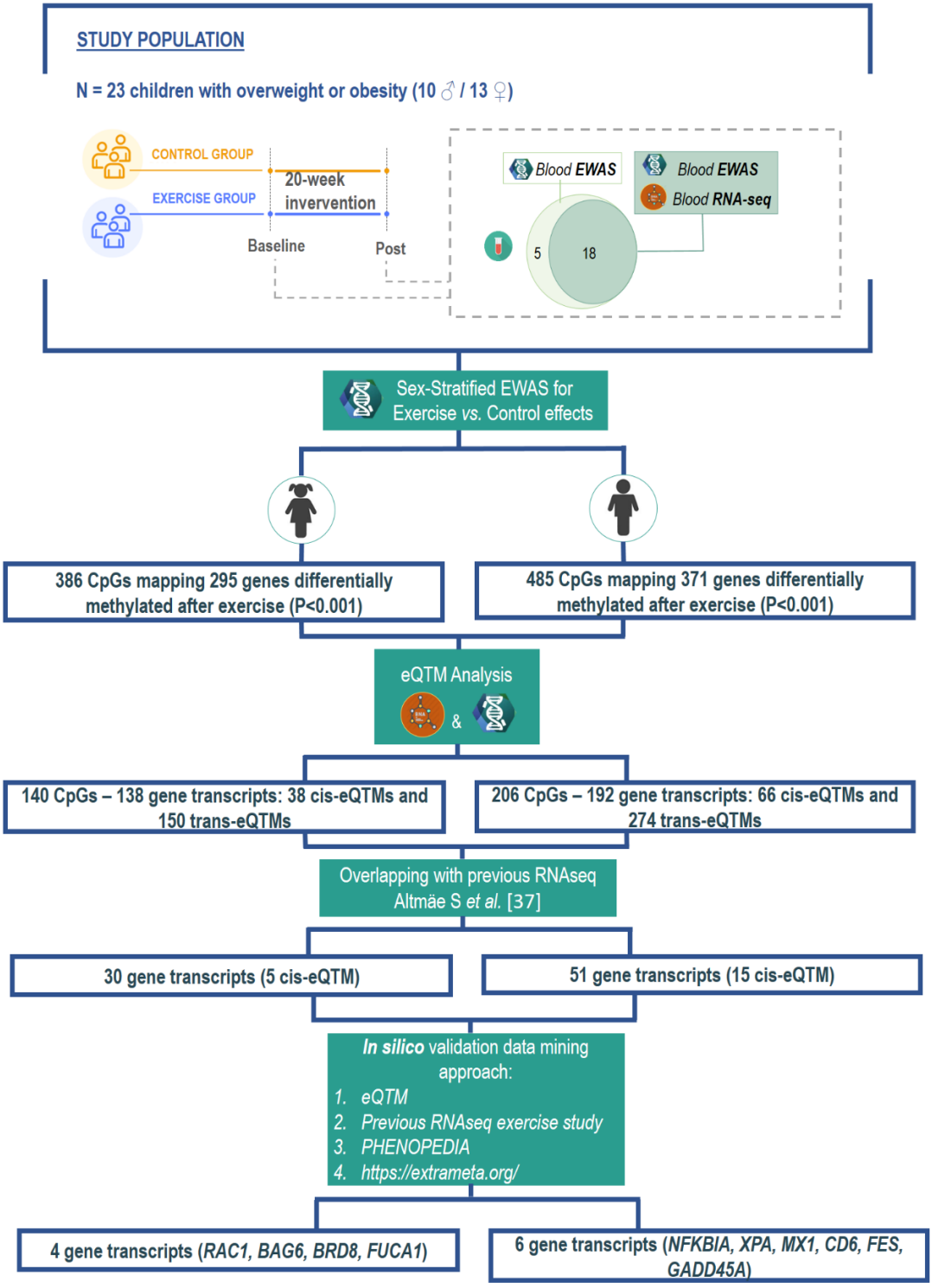

Figure 1. Summary of the study design and main findings. EWAS: Epigenome-wide association study; eQTM: Expression quantitative trait methylation; cis-eQTM: DNA methylation patterns that may alter the gene expression levels of nearby loci; trans-eQTM: DNA methylation patterns that may alter the gene expression levels at long distances from their occurrence.

The metabolic programming of immune cells is related to different diseases such as obesity and type 2 diabetes [40]. However, the evidence of the exercise and immunometabolism relationship is still limited [40]. According to our findings, exercise may regulate methylation at different CpG sites in genes related to molecular pathways such as type 2 diabetes, insulin resistance, fatty acid degradation, and pentose phosphate. In a previous study on young healthy men, a 4-week exercise intervention altered the methylation at CpG sites that enriched several molecular pathways involved in cardiovascular health in leukocytes [20]. One of these pathways was the MAPK signaling pathway related to several diseases such as cancer, inflammatory disease, obesity, and type 2 diabetes [41]. Interestingly, in the current study, *MAPK8* and *MAPK10* genes from the MAPK signaling pathway mapped to the type 2 diabetes and insulin resistance gene pathways regulated by exercise in boys. These genes were also detected in gene pathways involved in immune function, such as NOD-like receptor signaling and adipocytokine signaling pathway, which are involved in the pathogenesis of obesity and type 2 diabetes [42,43]. Also, a 8-week resistance training in healthy young men regulated methylation at CpG sites located in genes that enriched type 1 and type 2 diabetes gene pathways in leukocytes [19].

In boys, changes on methylation at CpG sites located in *TEX41* and *NPHP3* genes associated with the changes in *NFKBIA* gene expression. Furthermore, *NFKBIA* is one of the top genes which involvement in exercise-induced response [37] was confirmed in our *in silico* validation. Interestingly, an epigenome-wide association (EWAS) study using whole-blood from adults reported that CpG sites within *TEX41* and *RPS6KA2* (gene identified in insulin resistance pathway in our study and CpG site regulated by exercise in 5'UTR associated with TFBS) were differentially methylated between obese and lean adults [13]. *NFKBIA* gene encodes a protein (IκBα) that regulates the activity of nuclear

factor-kappa-B (a master regulator of the body's immune responses and inflammatory reactions) [44,45] and was down-regulated by the exercise intervention in boys with OW/OB [37]. A previous study showed that insulin resistance was positively associated with *NFKBIA* gene expression in peripheral blood mononuclear cells of patients with type 2 diabetes compared to controls [46]. Interestingly, one study reported that a 5-months interval walking training increased methylation at promoter regions of nuclear factor-kappa-B genes (*NFKB1* and *NFKB2*) in whole-blood in older women [47]. In sum, our study results highlight the molecular events (transcriptomics and epigenomics levels) in response to exercise involved in metabolism and inflammation in the whole-blood of boys.

Among girls, we identified exercise-induced DNA methylation changes in genes related to metabolic pathways such as aminoacid degradation (lysine), stress hormones (cortisol synthesis and secretion), as well as immune function (IL-17 signaling pathway). L-lysine is an aminoacid that can decrease pro-inflammatory responses [48] (e.g., lysine dietary restrictions were associated with up-regulation of pro-inflammatory genes [49]). Cortisol is a well-characterized glucocorticoid that increases in circulation to satisfy energy requirements during exercise (i.e., induce lipolysis, fatty acid oxidation, glycolysis depending of on duration and intensity of exercise) [50] and is beneficial for cognition [51]. However, chronic stress can increase cortisol levels with a negative impact on cognition (i.e., the exercise-glucocorticoid paradox) [51]. Otherwise, lymphocytes of sedentary children presented a higher IL-17 production compared to lymphocytes of active children [52]. Likewise, a 12-week exercise intervention decreased levels of IL-17 in serum of postmenopausal breast cancer survivors [53]. Interestingly, regular practice of resistance training has been associated with increased global methylation at the promoter of *IL-17A* gene in lymphocytes, suggesting anti-inflammatory effects of

resistance training in older women [54]. In concordance with literature, CpG sites located in promoter regions of genes identified in the IL-17 signaling pathway (e.g., *MAPK14*, *TRAF5)* increased methylation levels after a 20-week exercise intervention in girls in the current study.

From the eQTM analysis, we identified that changes on methylation in girls at one CpG site located in the 5'UTR of the *NCEH1* gene (the protein encoded by this gene regulate the efflux of cholesterol from macrophage foam cells [55]) and intergenic region (located in TFBS) were associated with changes in *RAC1* gene expression levels. Further, *RAC1* is the top gene which involvement in exercise-incuded stimulus was confirmed in our *in silico* validation. RAC1 is a key protein to induce GLUT4 translocation and glucose uptake in response to muscle contraction in skeletal muscle [56,57]. Furthermore, RAC1 has been related to systemic inflammation and the risk of atherosclerosis in rodents [58]. Interestingly, our 20-week exercise intervention increased *RAC1* gene expression levels in whole-blood of girls with OW/OB [37]. This finding could be in the opposite direction of expected in whole-blood (based on previous findings in macrophages [58]). Surprisingly, a previous study detected that high intensity interval training decreased *RAC1* gene expression while RAC1 protein expression increased in skeletal muscle of mice [59]. This finding highlights the lack of overlap between gene and protein expression [60]. In this regard, we cannot assume that increased *RAC1* gene expression after exercise intervention is directly related to increased RAC1 protein expression in whole-blood. Furthermore, the heterogeneity of whole-blood (i.e., T cells, B cells, monocytes, NK killers, etc) hamper the comparisons with other studies analysing specific cells populations [58].

In general, molecular effects of exercise intervention in children with OB/OW on epigenome level were different between boys and girls, with small overlap. In addition to

sex differences on DNA methylation profiles [61,62], the different exercise stimuli could partially explain the lack of overlap on gene and gene pathways between boys and girls (i.e., boys spent more time at higher intensities than girls [37]). The neurotrophin signaling pathway was identified as a common gene pathway influenced by exercise in boys and girls with OW/OB. Interestingly, the neurotrophin receptor signaling may play an important role in understanding the positive effect of exercise on cognitive functions and brain health [63].

Our study provides the first sight into the epigenetic together with transcriptome response to exercise intervention in children, nevertheless several limitations need to be acknowledged. Given the relatively small sample size and power of this study, our findings should be considered exploratory and preliminary. Indeed, our study results did not remain significant after false discovery rate correction. Also, differential leukocyte count or subset were not detected at specific levels (i.e., CD4 T cells, CD8 T cells, monocytes, etc) and may have an impact on whole-blood DNA methylation analyses [64]. Despite these limitations, our preliminary results provide important steps in the characterization of the genome-wide molecular responses to chronic exercise in pediatric population.

5. CONCLUSIONS

Our study findings indicate that 20-week exercise intervention could regulate the whole-blood DNA methylation profile associated with metabolism and immune function in boys and girls with OW/OB. The changes on DNA methylation were related to changes on gene expression levels involved in obesity, cardiometabolic risk factors, and inflammation. Top genes *NFKBIA*, *XPA*, *MX1*, *CD6*, *FES*, *GADD45A*, *RAC1*, *BAG6*, *BRD8*, and *FUCA1* regulated by exercise could serve as molecular targets in future

studies. More powered randomized controlled trials conducted in children would confirm, contrast or expand our study findings.

SUPPLEMENTARY MATERIAL

The supplementary files can be downloaded in this link:

https://osf.io/unpje/?view_only=d3eeb5a70be44b84a845efc96607c08a

Supplementary Figure 1. Genomic location of CpG sites differentially methylated by exercise in boys.

Supplementary Figure 2. Genomic location of CpG sites differentially methylated by exercise in girls.

Supplementary Figure 3. Island location of CpG sites differentially methylated by exercise in boys.

Supplementary Figure 4. Island location of CpG sites differentially methylated by exercise in girls.

Supplementary File 1. CpG sites differentially regulated by exercise in boys and girls.

Supplementary File 2. eQTM results in boys.

Supplementary File 3. eQTM results in girls.

Supplementary File 4. Genes regulated by exercise and detected within in silico data mining in PHENOPEDIA in boys and girls.

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GENERAL DISCUSSION

INTEGRATED SUMMARY OF THE MAIN FINDINGS

In the pediatric population, the molecular mechanisms underlying the MHO phenotype, the health benefits of being "fit" (based on CRF levels) and doing exercise are warranted. This doctoral thesis provides some novel and preliminary findings using high-throughput techniques at transcriptome, epigenome, and proteome levels in children with OW/OB. First, we investigated differences on transcriptome profiles (RNA-seq) between children with MHO and MUO phenotypes (**Study I**). Second, we studied transcriptome (using RNA-seq) and targeted proteome (applying Olink proximity extension assay technology) profiles in fit children compared to unfit children with OW/OB (**Studies II** and **III**). Third, we provided an overview and future directions on the effects of exercise on gene expression and epigenetic regulatory mechanisms in pediatric populations (**Study IV**). Lastly, we characterized transcriptome and epigenome (methylome) responses after a 20-week exercise intervention in boys and girls with OW/OB (**Studies V** and **VI**).

In general, we identified several genes, gene pathways, and proteins that were differentially regulated by exercise. In **section I** of this doctoral thesis, we found 32 genes involved in lipid metabolism and inflammation (e.g., *RREB1*, *FAM83E*, *SLC44A1*, *NRG1*, *TMC5*, *CYP3A5*, *TRIM11*, *ADAMTSL2*) differentially expressed in children with MHO compared to MUO (**Study I**). *RREB1* was one of the hub genes reported in our study; in this regard, a study published later using Infinium methylation EPIC showed specific methylation patterns at CpG sites related to overweight, which were mapped in target regions of RREB1 transcription factor [1]. Concerning CRF in children (**Study II** and **III**), we highlighted 256 genes detected in inflammation-related gene pathways, such as dopaminergic and GABAergic. Also, these genes were identified in CVD, inflammation, hypertension, and asthma PHENOPEDIA disease terms (**Study II**). In

addition, we provided 16 proteins (PLXNB3, sFRP3, CLEC1B, RSPO1, Gal8, CLEC10A, GCP5, MDGA1, CTSC, LAT, IL4RA, PRSS27, CXCL1, Gal9, MERTK, and GT) in circulation related to higher CRF levels in children with OW/OB (**Study III**). These proteins were involved in atherosclerosis, inflammatory response, neurogenesis, and/or cognitive decline, among others, although most of the previous evidence is based on adults or investigations in animal models. Longitudinal studies are still lacking to track these protein biomarkers and CRF changes in children and adolescents. The genes encoding the abovementioned proteins were not detected in transcriptomics analyses in **study II**. In this context, some issues could partially explain the lack of overlap: 1) changes in mRNA do not always imply changes in protein level [2], 2) we had different sample sizes in both studies (**studies II** and **III**), and 3) transcriptomics analyses were performed in the heterogeneous mix of whole blood cells while targeted proteomics was done in plasma. Likewise, the study of the different DNA methylation patterns at the genome-wide level between fit and unfit children with OW/OB and the integration with transcriptome data is still lacking.

Section II of this doctoral thesis highlight limited information on the effects of sedentary behavior and exercise on gene expression and its regulation in the pediatric population (**Study IV**). Most of the studies demonstrate that sedentary behavior and exercise alter mRNA and miRNA expression and DNA methylation of genes involved in obesity, immune function, angiogenesis, asthma, and cardiovascular disease (*FOXP3, HSD11B2, miRNA-222, miRNA-146a, ADRB2, miRNA-16, miRNA-126, HSP70, SOX*, and *GPX* genes). The only two transcriptomic studies reported how sex and pubertal stage should be considered relevant confounding factors that can influence the molecular response to exercise in pediatric populations. Concerning the **studies V** and **VI**, in general, several molecular pathways involved in metabolic (e.g., type 2 diabetes, insulin

resistance, fatty acid degradation, lysine degradation, cortisol synthesis, and secretion) and immune processes (e.g., antigen processing and presentation, T cell receptor complex, B cell receptor signaling, leukocyte transendothelial migration, IL-17 signaling pathway) were regulated by a 20-week of exercise program in boys and girls with OW/OB. At the same time, the lack of gene and gene pathways overlapping between boys and girls could be partially explained by the different exercise stimuli in both sexes. Our preliminary results are a first step in the characterization the molecular response to exercise interventions in children. Randomized controlled trials and high-throughput omics analyses on bigger cohorts of children are needed to advance further the field and clarify the molecular maps underlying the health benefits of exercise.

GENERAL LIMITATIONS

There are some limitations that should be highlighted:

- Transcriptome profile (**Studies I, II, V,** and **VI**) was performed in whole blood, which presents a high heterogeneity of immune cells. Thus, these cell subsets populations (e.g., monocytes, Natural killers, T lymphocytes, etc.) have different roles in immune function. Therefore, it is difficult to highlight specific conclusions or insights into immune mechanisms.
- Findings relating CRF levels and metabolic health to transcriptomics and proteomics profiles (**Studies I, II**, and **III**) were derived from cross-sectional study designs. Thus, causality cannot be stated in the aforementioned studies.
- For cross-sectional **studies I, II**, and **III**, we presented sex imbalance between groups of children after applying clinical-related cut-off points for metabolic parameters and CRF. Nevertheless, sex was included as a covariate in linear models to reduce its potential confounding effect.

- The sample size was relatively small in the six studies included in this doctoral thesis. Omics analyses should be performed in bigger cohorts of children.
- In **studies V** and **VI**, the exercise stimulus was not identical in boys and girls, hampering the gene pathways overlapping in response to exercise in both sexes.

FUTURE DIRECTIONS

There are some points to take into consideration for future research in pediatric exercise biology:

- Future studies should use flow and mass cytometry to study multi-omics response to exercise in specific immune cells from boys and girls with and without different clinical conditions. This approach has been performed so far to study the acute transcriptome response to exercise in Natural killers, neutrophils, and monocytes using flow cytometry in young adults [3–5].
- Metabolomics and lipidomics (a subsection of metabolomics) can provide an integrative view of metabolism and the prognosis/prediction of metabolic disease [6]. In this regard, exercise mode (aerobic and strength) had different and specific impact in the plasma metabolome of healthy young adults [7]. To our knowledge, the impact of exercise on metabolomics is still lacking in healthy children and those with diseases such as obesity, type 2 diabetes, among others.
- Kreb cycle intermediates are regulated during exercise in skeletal muscle [8,9]. Also, Kreb cycle intermediates such as succiante, fumarate, α-ketoglutarate can regulate epigenetic regulatory mechanisms of gene expression (e.g., DNA and histone methylation) [10]. Thus, the interconnection between metabolomics, epigenomics, and transcriptomics in the context of exercise is warranted in children.

- The metabolic and gene expression response to exercise may differ in pubertal stages. For example, early pubertal boys (8-12 years old) reported lower increases in lactate than late pubertal boys (15-18 years old) after performing the same single bout of exercise [11]. Thus, lower levels of muscle lactate dehydrogenase [12] and/or a lower dependence on glycolytic contribution to obtain energy during exercise in younger children [13] could partially explain these observations. Lactate can regulate metabolism and cytokine production in immune cells such as T cells, dendritic cells, monocytes [14–16]. Interestingly, only 109 genes were regulated by acute exercise in PBMCs of early pubertal boys, while 1246 genes were altered in late pubertal boys by the same bout of exercise [11]. The impact of the pubertal stage and different exercise intensities on the multi-omics response to exercise is unknown in children.
- It has been recently published the molecular choreography (transcriptomics, proteomics, metabolomics, lipidomics) in response to acute exercise in blood samples collected at different time points (baseline, 2 min, 15 min, 30 min, and 60 min after exercise) from 36 adults [17]. In pediatric population, the Molecular Transducers of Physical Activity Consortium (MoTrPAC) funded by National Institute of Health (NIH) Common Fund will characterize the molecular response to exercise (acute and chronic effects) at molecular level performing multi-omics analyses (i.e., genomics, transcriptomics, epigenomics, metabolomics, proteomics) in a cohort of 320 healthy children [18]. Multi-center and multi-interdisciplinary approaches like MoTrPAC should be applied in future projects in the field of omics and exercise in children with different diseases such as obesity, type 2 diabetes, leukemia, among others.

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CONCLUSIONS

GENERAL CONCLUSION

This doctoral thesis provides new insights into the understanding of exercise effects on children's health at molecular level via applying high-throughput omics analyses. Altogether these findings provide step towards the use of exercise as a form of precise and personalized medicine in children with OW/OB.

SPECIFIC CONCLUSIONS

Section 1: Metabolic health and cardiorespiratory fitness levels in children with OW/OB: A focus on transcriptomics and proteomics

- Children with MHO phenotype present 32 differentially expressed genes in whole-blood compared to MUO. These genes are involved in lipid metabolism and inflammation (e.g., *REB1, FAM83E, SLC44A1, NRG1, TMC5, CYP3A5, TRIM11*, and *ADAMTSL2*).
- Fit children with OW/OB show a distinct whole-blood transcriptome profile pattern compared to unfit children with OW/OB. The transcriptome profile enriches gene pathways such as Dopaminergic and GABAergic synapse, which are involved in inflammation. Genes identified in the abovementioned pathways were: *GNAO1, GNAL, GNG10, CREB3L3, PPP2R5E*, and *GABARAP*.
- Sixteen proteins (PLXNB3, sFRP3, CLEC1B, RSPO1, Gal8, CLEC10A, GCP5, MDGA1, CTSC, LAT, IL4RA, PRSS27, CXCL1, Gal9, MERTK, and GT) are differentially expressed in plasma of fit children compared to unfit children with OW/OB. These proteins enrich biological processes such as neurogenesis, inflammatory response and platelet activation.

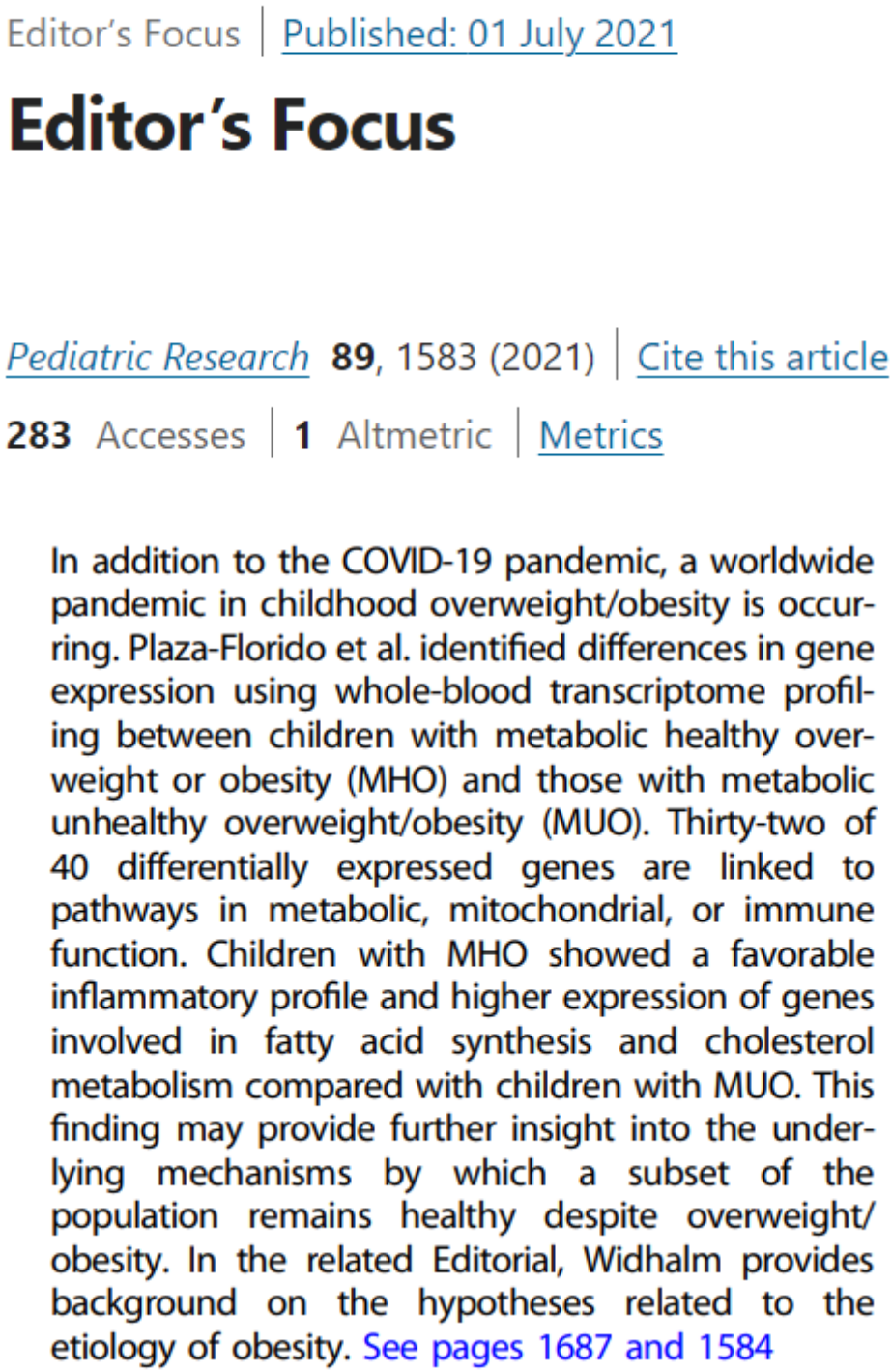
Section 2: Effects of exercise on transcriptome and epigenome in children with OW/OB

- Our systematic review highlights the lack of exercise studies about gene expression and epigenetics regulatory mechanisms in pediatric populations. Exercise impacted genes (*FOXP3, HSD11B2, miRNA-222, miRNA-146a, ADRB2, miRNA-16, miRNA-126, HSP70, SOX, GPX*) involved in diseases such as asthma, inflammation, angiogenesis, among others. The transcriptomic response to acute exercise can be modulated by sex and pubertal stage in pediatric populations. Randomized controlled trials performing multi-omic analyses are warranted.
- 20-week exercise intervention may regulate immune gene pathways in boys and girls with OW/OB. The lack of individual gene and gene pathways overlapping between boys and girls could be partially explained, in addition to sex differences, by the different exercise stimuli (more time spent at higher intensities in boys than girls). Also, the relatively low sample size and statistical power should be considered.
- 20-week exercise intervention regulated methylation at CpG sites located in genes enriched in metabolic and immune gene pathways. Changes on CpG sites regulated by exercise were associated with gene transcripts involved in inflammation and cardiometabolic risk. Boys and girls demonstrated differen methylome profiles, which could partially be explained, in addition to the sex differences, by different time spent exercising at higher intensities. Our study findings should be considered preliminary, because of limited statistical power, and studies using larger sample sizes are needed.

ANNEXES

ANNEX I-editorials related to study I included in this doctoral thesis

Study 1 included in this doctoral thesis was published in Pediatric Research (IF = 3.8; Rank PEDIATRICS 18/176; Q1). In addition, this paper was selected for the editor's choice (https://www.nature.com/articles/s41390-021-01597-1) in Pediatric Research (in total six manuscripts selected) and the editorial titled "Genetic Background of obesity" written by the professor Kurt Widhalm (https://www.nature.com/articles/s41390-021-01378-w).



Editor's Focus | Published: 01 July 2021

Editor's Focus

Pediatric Research **89**, 1583 (2021) | Cite this article

283 Accesses | **1** Altmetric | Metrics

In addition to the COVID-19 pandemic, a worldwide pandemic in childhood overweight/obesity is occurring. Plaza-Florido et al. identified differences in gene expression using whole-blood transcriptome profiling between children with metabolic healthy overweight or obesity (MHO) and those with metabolic unhealthy overweight/obesity (MUO). Thirty-two of 40 differentially expressed genes are linked to pathways in metabolic, mitochondrial, or immune function. Children with MHO showed a favorable inflammatory profile and higher expression of genes involved in fatty acid synthesis and cholesterol metabolism compared with children with MUO. This finding may provide further insight into the underlying mechanisms by which a subset of the population remains healthy despite overweight/obesity. In the related Editorial, Widhalm provides background on the hypotheses related to the etiology of obesity. See pages 1687 and 1584

www.nature.com/pr

EDITORIAL

Genetic background of obesity

Pediatric Research (2021) 89:1584–1585; https://doi.org/10.1038/s41390-021-01378-w

The paper by Plaza-Florido studies for the first time in two groups of obese children, one the metabolic healthy and the other the metabolic unhealthy subjects from the whole-blood transcriptome; surprisingly, they were able to show that the two groups revealed a distinct pattern of gene expression, which was linked to metabolism, mitochondrial, and immune function. They were able to identify 32 genes that were differentially expressed in children with healthy compared to unhealthy obesity. They found the most upregulated gene is *TRIMI1* and the most downregulated expression of the *ADAMTSL2* gene. The first is involved in the degradation of AIM2 inflammasome, which leads to the maturation of proinflammatory cytokines. In fact, the healthy obese children had a favorable inflammatory profile compared with the unhealthy obese children.

Furthermore, the authors were able to show that inflammatory markers tumor necrosis factor-α and interleukin-6 were significantly higher in children with unhealthy compared to those with healthy obesity. In addition, this study has shown that genes that are involved in the fatty acid synthesis and cholesterol metabolism (*CYP3A5* and *IRF2BP*) were higher expressed in whole blood.

These findings are without any doubt a very important mosaic stone in the understanding of the possible metabolic effects of obesity; it adds also some explanation why some children with the typical signs of the metabolic syndrome develop degenerative diseases and some not.

ANNEX II-Short curriculum vitae

SHORT CURRICULUM VITAE

ABEL ADRIÁN PLAZA FLORIDO

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Education

- **2012-2016** Bachelor degree in PHYSICAL ACTIVITY AND SPORTS SCIENCES (Grade: 9.13/10), Faculty of Sport Sciences, University of Granada, Spain.
- **2016-2017** Master degree in RESEARCH ON PHYSICAL ACTIVITY AND SPORT (Grade: 9.41/10), Faculty of Sport Sciences, University of Granada, Spain
- **2017 – 2022** PhD Student in BIOMEDICINE, University of Granada, Spain.

Grants/Fellowships

Abel Plaza-Florido has applied for nearly all scholarships and grants provided by Spanish Education and Research system, succeeding in the most of them. We indicate below the most relevant and research-related:

- **2015-2016 (8 months).** Research Fellowship: Training grant (National Level) for the students of the last year of bachelor studies. Spanish Ministry of Education. University of Granada, Spain.
- **2017 (5 months)** Technical research assistant. Junta de Andalucia.
- **2017 (october)- to 2022 (April)**. Research Fellowship: **FPU research fellow,** fellowship for formation of university faculty from the Spanish Ministry of Education. University of Granada, Spain.

Personal statement

My overall goal in my research career is to understand the molecular and physiological mechanisms underlying the health benefits of exercise in children and adolescents with different diseases (e.g. obesity and cancer among others). I'm interested in the combination of exercise immunology, physiology and multi-omics analyses (i.e. genomics, transcriptomics, epigenomics, metabolomics and proteomics) to discover the mechanisms that can link exercise with health benefits in pediatric population.

LIST OF PEER-REVIEWED MANUSCRIPTS PUBLISHED AS FIRST OR CORRESPONDING AUTHOR

[1] **Plaza-Florido A,** Altmäe S, Esteban FJ, Cadenas-Sanchez C, Aguilera CM, Einarsdottir E, Katayama S, Krjutškov K, Kere J, Zaldivar F, Radom-Aizik S, Ortega FB. Distinct whole-blood transcriptome profile of children with metabolic healthy overweight/obesity compared to metabolic unhealthy overweight/obesity. **Pediatr Res. 2020** Nov 23;. PubMed PMID: 33230195.

- *(IF = 3.8; Rank PEDIATRICS 18/176; **Q1**)*

[2] **Plaza-Florido A**, Altmäe S, Esteban FJ, Löf M, Radom-Aizik S, Ortega FB. Cardiorespiratory fitness in children with overweight/obesity: Insights into the molecular mechanisms. **Scand J Med Sci Sports. 2021** Aug 1;. doi: 10.1111/sms.14028. [Epub ahead of print] PubMed PMID: 34333829.

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* These authors have made an equal contribution.

- *(IF = 4.2; Rank MEDICINE, GENERAL & INTERNAL 39/169; **Q1**)*

[7] **Plaza-Florido A**, Alcantara JMA, Migueles JH, Amaro-Gahete FJ, Acosta FM, Mora-Gonzalez J, Sacha J, Ortega FB. Inter- and intra-researcher reproducibility of heart rate variability parameters in three human cohorts. **Sci Rep. 2020** Jul 9;10(1):11399. doi: 10.1038/s41598-020-68197-7.

- *(IF = 4.4; Rank MULTIDISCIPLINARY SCIENCES 17/73; **Q1**)*

[8] **Plaza-Florido A**, Alcantara JMA, Amaro-Gahete FJ, Sacha J, Ortega FB. Cardiovascular Risk Factors and Heart Rate Variability: Impact of the Level of the Threshold-Based Artefact Correction Used to Process the Heart Rate Variability Signal. **J Med Syst. 2020** Nov 25;45(1):2. PubMed PMID: 33237459.

- *(IF = 4.5; Rank HEALTH CARE SCIENCES & SERVICES 28/108; **Q1**)*

[9] **Plaza-Florido A**, Amaro-Gahete FJ, Acosta FM, Sacha J, Alcantara JMA. Heart rate rather than heart rate variability is better associated with cardiorespiratory fitness in adults. **Eur J Sport Sci. 2021** Feb 16;:1-38. PubMed PMID: 33591861.

- *(IF = 4.1; Rank SPORT SCIENCES 21/88; **Q1**)*

[10] **Plaza-Florido A**, Sacha J, Alcantara JMA. Short-term heart rate variability in resting conditions: methodological considerations. **Kardiol Pol. 2021** Jul 9. PubMed PMID: 34227676.

- *(IF = 3.1; Rank CARDIAC & CARDIOVASCULAR SYSTEMS 70/142; **Q2**)*

[11] **Plaza-Florido A.**, Migueles, J. H., Piepoli, A., Molina-Garcia, P., Rodriguez-Ayllon, M., Cadenas-Sanchez, C.,...& Ortega, F. B. Blood Flow-Restricted Training in Older Adults: A Narrative Review. **Journal of Science in Sport and Exercise. 2019**, 1-13.

- *(IF Not applicable, journal released in 2020)*

LIST OF PEER-REVIEWED MANUSCRIPTS PUBLISHED AS CO- AUTHOR

[1] Molina-Garcia P, H Migueles J, Cadenas-Sanchez C, Esteban-Cornejo I, Mora-Gonzalez J, Rodriguez-Ayllon M, **Plaza-Florido A**, Molina-Molina A, Garcia-Delgado G, D'Hondt E, Vanrenterghem J, Ortega FB. Fatness and fitness in relation to functional movement quality in overweight and obese children. **J Sports Sci. 2019** Apr;37(8):878-885.

- *(IF = 2.6; Rank SPORT SCIENCES 27/85; **Q2**)*

[2] Molina-Garcia P, Migueles JH, Cadenas-Sanchez C, Esteban-Cornejo I, Mora-Gonzalez J, Rodriguez-Ayllon M, **Plaza-Florido A**, Vanrenterghem J, Ortega FB. A systematic review on biomechanical characteristics of walking in children and adolescents with overweight/obesity: Possible implications for the development of musculoskeletal disorders. **Obes Rev. 2019** Jul;20(7):1033-1044.

- *(IF = 7.3; Rank ENDOCRINOLOGY & METABOLISM 12/143; **Q1**)*

[3] Gil-Cosano JJ, Gracia-Marco L, Ubago-Guisado E, Labayen I, Adelantado-Renau M, Cadenas-Sanchez C, Mora-Gonzalez J, **Plaza-Florido A**, Aguilera CM, Gómez-Vida J, Maldonado J, Jürimäe J, Ortega FB. Inflammatory markers and bone mass in children with overweight/obesity: the role of muscular fitness. **Pediatr Res. 2019** Sep 7

- *(IF = 2.7; Rank PEDIATRICS 25/128; **Q1**)*

[4] Molina-Garcia P, Miranda-Aparicio D, Molina-Molina A, **Plaza-Florido A**, Migueles JH, Mora-Gonzalez J, Cadenas-Sanchez C, Esteban-Cornejo I, Rodriguez-Ayllon M, Solis-Urra P, Vanrenterghem J, Ortega FB. Effects of Exercise on Plantar Pressure during Walking in Children with Overweight/Obesity. **Med Sci Sports Exerc. 2019** Sep 12

- *(IF = 5.4; Rank SPORT SCIENCES 8/88; **Q1**)*

[5] Migueles JH, Cadenas-Sanchez C, Rowlands AV, Henriksson P, Shiroma EJ, Acosta FM, Rodriguez-Ayllon M, Esteban-Cornejo I, **Plaza-Florido A**, Gil-Cosano JJ, Ekelund U, van Hees VT, Ortega FB. Comparability of accelerometer signal aggregation metrics across placements and dominant wrist cut points for the assessment of physical activity in adults. **Sci Rep. 2019** Dec 3;9(1):18235.

- *(IF = 3.9; Rank MULTIDISCIPLINARY SCIENCES 17/71; **Q1**)*

[6] Molina-Garcia P, **Plaza-Florido A**, Mora-Gonzalez J, Torres-Lopez LV, Vanrenterghem J, Ortega FB. Role of physical fitness and functional movement in the body posture of children with overweight/obesity. **Gait Posture. 2020** Apr 11;80:331-338. doi: 10.1016/j.gaitpost.2020.04.001.

- *(IF = 2.8; Rank SPORT SCIENCES 41/88; **Q2**)*

[7] Molina-Garcia P, Mora-Gonzalez J, Migueles JH, Rodriguez-Ayllon M, Esteban-Cornejo I, Cadenas-Sanchez C, **Plaza-Florido A**, Gil-Cosano JJ, Pelaez-Perez MA, Garcia-Delgado G, Vanrenterghem J, Ortega FB. Effects of Exercise on Body Posture, Functional Movement, and Physical Fitness in Children With Overweight/Obesity. **J Strength Cond Res. 2020** May 25;.

- *(IF = 3.8; Rank SPORT SCIENCES 25/88; **Q2**)*

[8] Torres-Lopez LV, Cadenas-Sanchez C, Migueles JH, Adelantado-Renau M, **Plaza-Florido A**, Solis-Urra P, Molina-Garcia P, Ortega FB. Associations of Sedentary Behaviour, Physical Activity, Cardiorespiratory Fitness and Body Composition with Risk of Sleep-Related Breathing Disorders in Children with Overweight/Obesity: A Cross-Sectional Study. **J Clin Med. 2020** May 20;9(5). doi: 10.3390/jcm9051544.

- *(IF = 4.2; Rank MEDICINE, GENERAL & INTERNAL 39/169; **Q1**)*

[9] Rodriguez-Ayllon M, Esteban-Cornejo I, Verdejo-Román J, Muetzel RL, Mora-Gonzalez J, Cadenas-Sanchez C, **Plaza-Florido A**, Molina-Garcia P, Kramer AF, Catena A, Ortega FB. Physical fitness and white matter microstructure in children with overweight or obesity: the ActiveBrains project. **Sci Rep. 2020** Jul 27;10(1):12469. doi: 10.1038/s41598-020-67996-2.

- *(IF = 4.4; Rank MULTIDISCIPLINARY SCIENCES 17/73; **Q1**)*

[10] Mora-Gonzalez J, Esteban-Cornejo I, Migueles JH, Rodriguez-Ayllon M, Molina-Garcia P, Cadenas-Sanchez C, Solis-Urra P, **Plaza-Florido A**, Kramer AF, Erickson KI, Hillman CH, Catena A, Ortega FB. Physical fitness and brain source localization during a working memory task in children with overweight/obesity: The ActiveBrains project. **Dev Sci. 2020** Oct 9;:e13048. PubMed PMID: 33037758.

- *(IF = 5.1; Rank PSYCHOLOGY, EXPERIMENTAL 7/90; **Q1**)*

BOOK CHAPTERS

[1] **Title**: *Advanced concepts of training with loads*. **Chapter number 11**. *Exercise Training in pathologies and special populations. Old people*. ISBN: 978-84-1374-237-3

REVIEWER ACTIVITY (JOURNALS)

[1] **Scandinavian Journal of Medicine & Science in Sports** (*Rank SPORT SCIENCES 18/88;* ***Q1)***

[2] **Scientific Reports** (*Rank MULTIDISCIPLINARY SCIENCES 17/73;* ***Q1)***

[3] **Cardiology in the Young** (*Rank CARDIAC & CARDIOVASCULAR SYSTEMS 139/141;* ***Q4)***

RESEARCH PROJECTS

Abel Plaza-Florido has participated in 5 projects:

1. Project: **ActiveBrains:** Effects of an exercise-based randomized controlled trial on cognitive performance, brain structure and brain function in overweight/obese preadolescent children.
   Funding: Funding: 120.000 €
   Source: Spanish Ministry of Economy and competitiveness.
   Principal investigator: Francisco B. Ortega Porcel
   Duration: from 2014 to 2016.
   Role: follow-up data collection, data analysis and writing manuscripts.

2. Project: **MUBI**: effects of an exercise program on movement biomechanics in children with overweight/obesity.
   Funding Source: The present study is part of the ActiveBrains project, which was funded by the Spanish Ministry of Economy and Competitiveness and the "Fondo Europeo de Desarrollo Regional (FEDER).
   Principal investigator: Francisco B. Ortega Porcel
   Duration: from 2016 to 2017
   Role: data collection and participation on manuscripts.

3. Project: **Smarter Move**: Exercise in the prevention and treatment of obesity and insulin resistance: Smart analysis-smart interventions.
   Principal Investigator: Ortega FB and Jonatan Ruiz
   Funding: 100.000 €
   Source: Spanish Ministry of Economy and Competitiveness and the Fondo Europeo de Desarrollo Regional (FEDER)
   Role of the Applicant: data collection and participation on manuscripts.

4. Project: **GENOBEX** - Genes, Obesity And Exercise.
   Principal Investigator: Ortega FB
   Funding: 33.400 €

Source: Programa Operativo Fondos FEDER-Junta de Andalucía. 2020-2022
Role of the Applicant: data collection, data analysis and participation on manuscripts

5. Project: **MECABRAIN** - Peripheral mechanisms inducing neurogenesis, hippocampal function and mental health in children: The role of exercise. Minister of Economy and Competitiveness – Proyecto EXPLORA (Call 2017, Ref: DEP2017-91544-EXP). 2018-2020. 48.400 EUR. y cofinanciado por la Fundación Alicia Koplowitz con 50.000€.
   Role of the Applicant: data analysis and writing manuscripts.

RESEARCH STAYS

- **2019.** Pediatric Exercise and Genomics Research Center (PERC). **University of California, Irvine**. School of Medicine. Department of Pediatrics.
  Country: **United States**. Duration: **3 months** (1 June- 28 August 2019)

- **2021**. **Karolinska Institutet**. Department of Physiology and Pharmacology.
  Country: **Sweden**. Duration: **3 months** (2 March- 30 May 2021)

UNIVERSITY TEACHING

- Subject**: Fundamentals of sports II: Basketball** (60 hours of teaching, 6 credits ECTS).
  Degree/Bachelor: **Physical Activity and Sport Sciences,** University of Granada (Spain). Academic course: **2018/2019**

- Subject: **Physical Activity and Health** (60 hours of teaching, 6 credits ECTS).
  Degree/Bachelor: **Physical Activity and Sport Sciences,** University of Granada (Spain). Academic course: **2019/2020**

- Subject: **Functional Anatomy of the Locomotor System** (60 hours of teaching, 6 credits ECTS).
  Degree/Bachelor: **Physical Activity and Sport Sciences,** University of Granada (Spain). Academic course: **2020/2021**

OTHER MERITS

- 20 **communications** or **posters** both in **national** and **international** conferences.
- **2015.** Book award to the best 150 best academic performances from the University of Granada (2014 – 2015)
- **2016.** Co-founder of the website "Ciencia del entrenamiento. Salud y rendimiento". The objective of this website is to disseminate the results of several investigations in the field of exercise and health. http://cienciadelentrenamiento.com/
- **2016-2017**. Award to the best Master Thesis Work in the Master Degree in

Research on Physical Activity and Sport. University of Granada.

- **2017. Lecturer** on **Postgraduate** studies on Physical prevention and rehabilitation. Course: Blood flow restriction exercise (2 credits, equivalent to 20-hours) University of Jaen, Faculty o Health Sciences.
- (Academic years **2016- 2017** and **2017-2018**) Invited lecturer (2-hours) to the subject "Fitness: últimas tendencias deportivas" in the degree on Physical Activity and Sports Sciences.
- **2017**. Seminar about heart rate variability analysis (6-hours), Virgen de las Nieves Hospital, Granada.
- **2021**. **Invited Lecturer** (1-hour) on the topic "Heart rate variability, fitness, and health" in the Sports medicine course that is held for medical students in their 6th semester of the medical program at **Karolinska Institutet, Sweden.**

ACKNOWLEDGEMENTS