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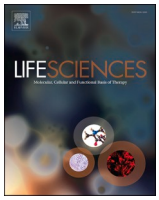

Circulating microRNA levels differ in the early stages of insulin resistance in prepubertal children with obesity

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ABSTRACT

Aims: The increasing prevalence of childhood obesity escalates the risk for related complications. Circulating microRNAs (miRNAs) have been suggested as good predictive markers of insulin resistance in those with obesity. The aim was to identify a circulating miRNA profile that reflects insulin resistance in prepubertal children with obesity.

Material and methods: Plasma miRNAs were measured in prepubertal children ($n = 63$, 5–9 years) using TaqMan Advanced miRNA Human Serum/Plasma plates and then were validated by RT-qPCR. Subjects were divided into normal weight ($n = 20$, NW) and overweight or obese ($n = 43$, OW/OB) groups according to their BMI z-scores. The OW/OB group was further subdivided into insulin sensitive or metabolically healthy obese ($n = 26$, MHO) and insulin resistant or metabolically unhealthy obese ($n = 17$, MUO) according to HOMA-IR.

Key findings: While no differences were observed in the fasting plasma glucose levels, serum insulin levels were significantly elevated in the OW/OB compared to the NW group. Of 188 screened miRNAs, eleven were differentially expressed between the NW and OW/OB groups. Validation confirmed increased circulating levels of miR-146a-5p and miR-18a-5p in the OW/OB group, which correlated with BMI z-score. Interestingly, miR-146a-5p was also correlated with HOMA-IR index. While only miR-18a-5p was upregulated in the OW/OB children, independently of their degree of insulin sensitivity, miR-146-5p, miR-423-3p and miR-152-3p were associated with insulin resistance.

Significance: The present study provides evidence of molecular alterations that occur early in life in prepubertal obesity. These alterations may potentially be crucial for targeted prevention or prompt precision therapeutic development and subsequent interventions.

1. Introduction

About 1.9 billion adults worldwide are suffering from being overweight or obese, which is a major public health concern [1]. Obesity is not merely a condition of enlarged adipose tissue depots, but is also accompanied by metabolic alterations that affect whole-body metabolism, especially dyslipidemia, insulin resistance, hypertension, and induce a persistent low-grade chronic inflammatory state [2]. Moreover, the high prevalence of obesity currently observed in adults is associated not only with unhealthy habits during adulthood, but also with metabolic alterations, that for many, may have started years earlier during childhood [3].

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According to the World Health Organization (WHO), in 2016 over 340 million children and adolescents (5–19 years old) were considered overweight (OW) or obese [1]. This escalates the risk for the development of several obesity-related complications early in life, especially early development of type 2 diabetes (T2DM) and cardiovascular disease, which are two major causes of death worldwide [1].

However, a substantial part of the population with obesity display a phenotype that can be described as metabolically "healthy" (MHO), in which subjects display a lower degree of metabolic complications despite the presence of clinically defined obesity [4–8]. However, it is still unclear whether MHO subjects remain metabolically healthy or if at some point, they become metabolically unhealthy (MUO). In the Bogalusa Heart study [9], MHO status persisted from childhood into adulthood with better cardiometabolic profiles compared to MUO subjects. They presented with lower levels of circulating cholesterol, triglycerides, and had normal blood pressure (BP) measurements, as well as reduced carotid intima-media thickness, a key marker of atherosclerosis. On the other hand, in the English Longitudinal Study of Ageing [10], a tendency to an increasingly deteriorated metabolic status in aged MHO people was observed. Therefore, identifying the underlying mechanisms that differentiate MHO from MUO individuals early in life will be crucial for preventing the development and escalation of obesity-related metabolic syndrome complications and for the development of targeted novel therapeutic approaches.

MicroRNAs (miRNAs) are small non-coding RNAs, comprised of approximately 21 nucleotides. These molecules act as post-transcriptional regulators of gene expression by targeting sequence-specific mRNAs and increasing their rate of degradation [11,12]. Interestingly, miRNAs can also be released into the circulation and can be detected in body fluids such as blood, plasma or serum, urine, and saliva [13]. Because miRNAs display specific characteristics that can be used as disease markers, including easy access via body fluids, high specificity and sensitivity of detection, and high analyte stability, they have attracted scientific interest [14]. In prepubertal children (4 to 6 years old) with obesity, altered levels of circulating miR-486-5p, miR-146b-5p and miR-15b-5p have been correlated with BMI and abdominal fat mass [15]. Therefore, these specific miRNAs have been suggested as predictors of insulin resistance and future metabolic complications [15]. However, further studies are needed to investigate reliable biomarkers that can distinguish between MHO and MUO phenotypes in prepubertal children and to identify predictors for the development of future metabolic disease prior to further deterioration. The present study was aimed to evaluate the impact of insulin resistance on the circulating miRNAs profile of prepubertal children with obesity.

2. Material and methods

2.1. Participants and clinical measurements

Our study population included 63 prepubertal children, 5–9 years of age, recruited after informed, written parental or legal guardian consent. The study was approved by the Institutional Review Board (IRB), permit number 206164, at the University of Arkansas for Medical Sciences. This clinical study was registered in ClinicalTrials.gov (NCT03323294), which includes information pertaining to the study population and design, as well as inclusion and exclusion criteria, as required by Arkansas Children Research Institute, where the studies were performed. The prepubertal Tanner stage I was reported by parent or legal guardian, and confirmed by the team physician, whenever needed. However, no sexual hormones were measured. Participants were characterized based on anthropometric measurements including sex, age (years), height (cm), weight (kg) and waist circumference (cm) as previously described [16] and shown in Table 1. In children, obesity is defined according to body mass index (BMI, calculated as kg/m^2) and adjusted for age and sex, as recommended by the Center for Disease Control and Prevention (www.cdc.gov/growthcharts) [17]. For this study, participants with BMI between the 85th and the 95th percentile scores were considered overweight (OW, $n = 18$) and participants with BMI at or higher than the 95th percentile score were considered obese (OB, $n = 15$). However, for the statistical analysis, all participants with BMI $\geq$ 85th percentile score were grouped to form the overweight/obese (OW/OB) group, Table 1. The clinical outcomes of systolic and diastolic blood pressure (BP) were also converted to percentiles to avoid age and sex biases and are presented as BP z-scores in Table 1.

Table 1
Anthropometric, clinical, and biochemical characteristics of the study participants.

	NW	OW/OB	p-Value
N	20	43	
Sex: male/female	14/6	21/22	0.12
Age (years)	7 (6–8)	7 (6–9)	0.18
BMI z-score	0.08 ± 0.67	2.05 ± 0.62	**≤0.001**
Waist circumference (cm)	54.3 ± 4.2	69.3 ± 12.8	**≤0.001**
Systolic BP z-score	0.3 ± 1	1 ± 0.9	**0.001**
Diastolic BP z-score	0.2 ± 0.9	0.5 ± 9	0.17
Fasting plasma insulin (μU/mL)	3.49 (2.38–4.71)	7.27 (4.91–11.10)	**≤0.001**
HOMA-IR	0.79 (0.52–0.92)	1.58 (1.17–2.53)	**≤0.001**
Fasting plasma glucose (mmol/L)	4.92 ± 0.35	5.07 ± 0.65	0.69
HDL cholesterol (mmol/L)	1.48 (1.24–1.58)	1.25 (1.12–1.53)	0.06
LDL cholesterol (mmol/L)	2.15 ± 0.61	2.49 ± 0.75	0.09
Triglycerides (mmol/L)	0.52 (0.38–0.67)	0.66 (0.43–1.02)	0.09
Total cholesterol (mmol/L)	3.73 ± 0.69	3.96 ± 0.76	0.26
Leptin (μg/mL)	0.09 (0.05–0.10)	1.01 (0.26–1.61)	**≤0.001**
Adiponectin (μg/mL)	13.93 (11.23–17.63)	11.30 (7.79–16.92)	0.06
Leptin/Adiponectin	0.006 (0.003–0.008)	0.10 (0.016–0.18)	**≤0.001**
CRP (mg/L)	0.15 (0.15–0.26)	0.81 (0.34–2.81)	**≤0.001**
IL-6 (pg/mL)	13.72 (4.67–56.53)	9.96 (2.34–2.81)	0.13
IL-8 (pg/mL)	4.88 (4.01–16.12)	5.48 (3.17–11.21)	0.73
IL-1β (pg/mL)	0.90 (0.48–1.62)	0.62 (0.57–1.45)	0.70
TNF-α (pg/mL)	6.49 (5.41–8.13)	5.11 (2.71–6.84)	**0.02**
MCP-1 (pg/mL)	127.70 ± 36.69	119.70 ± 37.4	0.44

Table includes mean ± SD for parametric data and median (interquartile range) for non-parametric data. The data from the categorical variable "sex" were analyzed by χ^2 test (2 sided). Bold font indicates statistical significance; BMI, body mass index; BP, blood pressure; HDL, high density lipoprotein; LDL, low density lipoprotein; CRP, C-reactive protein; IL-6, interleukin-6; IL-8, interleukin-8; IL-1β, interleukin-1 beta; TNF-α, tumor necrosis factor alpha; MCP-1, monocyte chemoattractant protein-1.

2.2. Blood collection and biochemical analysis

After overnight fasting, morning blood samples were collected in EDTA tubes. After centrifugation, plasma samples were stored at −80 °C. The plasma insulin concentration (μU/mL) was evaluated using the Mesoscale Discovery platform (MSD Multi-Array Assay System, Gaithersburg, MD, USA), according to the manufacturer's protocol. The plasma glucose concentration (mmol/L) was evaluated using an YSI 2900 biochemistry analyzer (YSI Life Sciences Yellow Springs, OH, USA). Insulin sensitivity was calculated according to the homeostatic model assessment for insulin resistance (HOMA-IR) index using the formula HOMA-IR = Fasting plasma insulin (μIU/mL) * fasting plasma glucose (mmol/L)/22.5. Participants with HOMA-IR $\geq$ 1.95 were considered insulin resistant [18–20]. Therefore, 26 participants were insulin sensitive and defined as MHO (OW:OB ratio is 10 to 16 participants), while the remaining 17 participants with a HOMA-IR $\geq$ 1.95 were defined as insulin resistant and as MUO (OW:OB ratio is 2 to 10 participants), Table 2.

Plasma lipid profiles including, low density lipoprotein (LDL: mmol/L), high density lipoprotein (HDL: mmol/L), total cholesterol (mmol/L),

Table 2
The anthropometric, clinical, and biochemical characteristics of normal weight (NW) participants *versus* metabolically healthy obese (MHO), and metabolically healthy obese (MHO) *versus* metabolically unhealthy obese (MUO) participants.

	NW	MHO	p-Value*	MUO	p-Value**
N	20	26		17	
Sex: Male/Female	14/6	14/12	0.27	7/10	0.42
Age (years)	7 (6–8)	7 (6–8)	0.47	8 (7–9)	0.31
BMI z-score	0.8 ± 0.67	1.81 ± 0.56	**≤0.001**	2.43 ± 0.53	**≤0.001**
Waist circumference (cm)	54.4 ± 4.2	63.9 ± 10.6	**≤0.001**	77.7 ± 11.9	**≤0.001**
Systolic BP z-score	0.3 ± 1	0.9 ± 1	0.09	1.3 ± 0.6	0.06
Diastolic BP z-score	0.2 ± 0.9	0.5 ± 0.83	0.25	0.5 ± 1	0.76
Fasting plasma insulin (μU/mL)	3.65 ± 1.55	5.43 ± 1.77	**≤0.001**	18.16 ± 10.38	**≤0.001**
HOMA-IR	0.79 (0.52–0.92)	1.19 ± 0.37	**≤0.001**	4.37 ± 9.09	**≤0.001**
Fasting plasma glucose (mmol/L)	4.92 ± 0.35	4.98 ± 0.52	0.69	5.23 ± 0.81	0.22
HDL cholesterol (mmol/L)	1.47 ± 0.61	1.41 ± 0.30	0.51	1.16 ± 0.16	**0.001**
LDL cholesterol (mmol/L)	2.15 ± 0.61	2.32 ± 0.76	0.42	2.77 ± 0.65	0.06
Triglycerides (mmol/L)	0.52 (0.38–0.67)	0.53 (0.36–0.80)	0.79	0.78 (0.65–1.24)	**0.024**
Total cholesterol (mmol/L)	3.73 ± 0.69	3.87 ± 0.81	0.55	4.12 ± 0.65	0.30
Leptin (μg/mL)	0.09 (0.05–0.10)	0.59 (0.17–1.43)	**0.006**	1.49 (0.93–2.07)	**0.006**
Adiponectin (μg/mL)	13.93 (11.23–17.63)	14.13 (10.70–19.20)	0.97	7.71 (6.72–10.08)	**≤0.001**
Leptin/Adiponectin	0.006 (0.003–0.008)	0.05 (0.01–0.11)	**≤0.001**	0.18 (0.12–0.32)	**≤0.001**
CRP (mg/L)	0.15 (0.15–0.26)	0.76 (0.27–2.25)	**≤0.001**	1.94 (0.37–2.92)	0.46
IL-6 (pg/mL)	13.73 (5.32–49.58)	8.31 (2.41–44.55)	0.31	9.96 (1.92–22.92)	0.59
IL-8 (pg/mL)	4.88 (4.07–15.36)	5.55 (3.96–17.29)	0.85	4.51 (3.00–8.80)	0.45
IL-1β (pg/mL)	0.90 (0.51–1.51)	0.74 (0.57–0.74)	0.94	0.57 (0.57–1.06)	0.46
TNF-α (pg/mL)	6.49 (5.44–7.91)	6.12 (3.91–9.00)	0.37	4.36 (2.28–5.40)	**0.037**
MCP-1 (pg/mL)	127.78 ± 36.69	122.20 ± 38.48	0.63	115.86 ± 37.82	0.59

Table includes mean ± SD for parametric data and median (interquartile range) for non-parametric data. The data from the categorical variable "sex" were analyzed by χ^2 test (2 sided). Bold font indicates statistical significance; BMI, body mass index; BP, blood pressure; HDL, high density lipoprotein; LDL, low density lipoprotein; CRP, C-reactive protein; IL-6, interleukin-6; IL-8, interleukin-8; IL-1β, interleukin-1 beta; TNF-α, tumor necrosis factor alpha; MCP-1, monocyte chemoattractant protein-1.
* NW *versus* MHO comparison.
** MHO *versus* MUO comparison.

triglyceride (mmol/L) and the inflammatory marker C-reactive protein (CRP) concentrations were evaluated using an RX Daytona Clinical Analyzer, according to the manufacturer's protocols (Randox Laboratories-IS, Kearneysville, WV, USA), as previously described [21,22].

Plasma concentrations of the cytokines interleukin (IL)-1β, IL-6, IL-8, tumor necrosis factor (TNF)-α, monocyte chemoattractant protein (MCP)-1, and the adipokine leptin, were measured using a Milliplex® Map Human Adipokine Panel (Millipore®, Burlington, MA, USA). Adiponectin levels were evaluated using an ELISA (Millipore®, Burlington, MA, USA). All procedures were performed in accordance with the manufacturers' instructions.

2.3. RNA isolation

Total RNA was extracted from 200 μL of plasma using the MagMax™ total RNA isolation kit (ThermoFisher, Waltham, MA, USA, A27828) on a KingFisher™ Flex Purification System (ThermoFisher, Waltham, MA, USA) according to the manufacturer's instructions.

2.4. cDNA synthesis and miRNA discovery analysis

cDNA synthesis was performed using the TaqMan Advanced miRNA cDNA Synthesis Kit (Thermofisher, Waltham, MA, USA, cat no A28007), containing reagents for polyA tailing, 5′ adaptor ligation, cDNA synthesis, followed by a miRNA-Amp pre-amplification reaction, which uses universal primers (Thermofisher, Waltham, MA. USA). A 2 μL RNA input was used for the initial polyA tailing step. Pre-amplified cDNA was diluted 1:10 in 0.1× Tris-EDTA (TE, Ambion A9260G, pH = 8) and TaqMan Advanced miRNA discovery plates with pre-stamped miRNAs assays, and added TaqMan FAST Advanced master mix (Thermofiser, Waltman, MA, USA, 4444557) were loaded onto a QuantStudio 6 Flex real-time PCR instrument (Thermofisher, Waltham, MA, USA). Thermal cycling consisted of 40 cycles of denaturation (15 s at 95 °C) and annealing/extension (60s at 60 °C), after an enzyme activation step at 95 °C for 20s. Although each plate contained assays for the spike-ins cel-miR-39-3p and ath-miR-159a, their amplifications did not live up to quality control estimates (high Ct levels, high variation between replicates and multiple absent values). Thus, data were normalized to the endogenous control mir-16-5p, also provided on the plate. Of note, miR-16-5p Ct levels were stable between the patient groups. For the initial miRNA discovery analysis 16 samples from the 63 study participants were used. The difference between the normalized NW ($n = 8$) samples, considered as the control group, and the OW/OB (n = 8) samples was calculated (ΔΔCt), followed by $2^{-\Delta\Delta Ct}$ to show fold change differences.

2.5. Validation and targeted analysis of selected miRNAs

After the discovery analysis, 11 miRNAs were selected and validated by RT-qPCR for all study participants. For target miRNA amplification, cDNA was prepared as described above. Diluted cDNA was mixed with the individual miRNA TaqMan assays and added to 2× TaqMan FAST Advanced master mix (Thermofisher, Waltham, MA, USA, cat no 4444557). Data were acquired and for each comparison the difference between the normalized groups was calculated $2^{-\Delta\Delta Ct}$.

2.6. Statistical analysis

Before statistical analysis, the study cohort was divided into two major groups according to the body mass index z-scores (BMI z-score), adjusted for sex and age, to avoid growth-related biases, calculated based on the BMI percentile [23]. Data were tested for normal distribution using the Shapiro-Wilk test and for variance homogeneity using Levenes' test. Comparisons between groups were made using an independent *t*-test for normally distributed variables and presented as mean ± standard deviation (SD), or by the Mann-Whitney non-parametric test for non-normally distributed variables and presented as median (interquartile range, Q1–Q3). The non-normally distributed variables, miRNAs levels and HOMA-IR, were log transformed before statistical analysis. For all the comparisons, the categorical variable "sex" was tested using a χ^2 test. Correlation between continuous variables was assessed using the Pearson or Spearman's correlation for normal or non-

normally distributed variables, respectively. The coefficient (rho, r) is shown for each correlation. Univariate linear regression models were made with adjustment for age and sex as co-variates. A receiver operating characteristic (ROC) curve was generated for each miRNA and the Area under the curve (AUC) with a respective 95% confidence interval was calculated to evaluate sensitivity and specificity. Predicted target genes for the individual miRNAs were identified using TargetScan (v.7.2, human, http://www.targetscan.org/vert_72/, accessed on 17 February 2022) [24], and pathway enrichment analysis was performed using the PANTHER (Protein ANalysis THrough Evolutionary Relationships) Classification System (v.15.0, http://pantherdb.org/, accessed on 17 February 2022), as well as the PANTHER pathways annotation set [25]. The enrichment analysis was visualized by RStudio (v. 1.3.1093, RStudio, PBC Boston, MA, USA, http://www.rstudio.com) using the ggplots2 (v.3.1.0, https://ggplot2.tidyverse.org/) package [24]. All the analyses were performed using IBM SPSS version 27 (SPSS Inc., Chicago, IL, USA) or RStudio (v.1.3.1093, RStudio, PBC Boston, MA, USA, http://www.rstudio.com). GraphPad Prism version 8 (GraphPad Inc., La Jolla, CA, USA) was used for graphical representation. A p-value of <0.05 was considered significant.

3. Results

3.1. Characteristics of the study population

A group of 63 prepubertal children, 5 to 9 years of age, was recruited and stratified according to BMI z-scores. No differences were identified in sex and age distribution between normal weight (NW) and overweight or obese (OW/OB) groups. The OW/OB group showed higher BMI z-scores ($p \leq 0.001$) and on average, 10 cm larger waist circumference ($p \leq 0.001$) when compared with the NW participants. A higher ($p \leq 0.001$) systolic BP z-score was also observed (Table 1). The fasting plasma insulin ($p \leq 0.001$) was increased by 50 % in the OW/OB group compared with the NW group. Interestingly, although the fasting glucose concentration was similar in the two groups ($p = 0.70$), the OW/OB group displayed a significantly higher HOMA-IR index ($p \leq 0.001$). Moreover, both leptin ($p \leq 0.001$) and CRP ($p \leq 0.001$) concentrations were elevated >5 times in the OW/OB group compared to the NW group. No differences were observed regarding cholesterols or triglycerides levels between groups (Table 1). In addition, despite no differences in inflammation markers IL-6, IL-8, IL-1β, and MCP-1, the concentration of TNF-α was lower ($p = 0.02$) in the OW/OB *versus* the NW group.

3.2. MicroRNA discovery analysis in children with normal weight versus overweight or obese

Samples from 16 of the 63 participants ($n = 8$ NW and n = 8 OW/OB) were used to carry out miRNA discovery and to identify unique miRNA circulating signatures in prepubertal children with obesity. A similar anthropometric and biochemical output was observed in the miRNA discovery cohort when the NW and the OW/OB group were compared, Supplementary Table 1. Briefly, the OW/OB group showed higher BMI z-score ($p \leq 0.001$), and increased waist circumference ($p \leq 0.001$) and systolic BP z-scores ($p = 0.005$), when compared to the NW group, as presented in Supplementary Table 1. Additionally, the OW/OB group also presented increased fasting plasma insulin ($p \leq 0.001$), HOMA-IR index ($p = 0.001$), leptin ($p \leq 0.001$), CRP ($p = 0.009$) and LDL ($p = 0.026$), while HDL ($p = 0.004$) and adiponectin ($p = 0.001$) levels were decreased in comparison to the NW group (Supplementary Table 1). All 188-circulating miRNAs were detected in both groups. In accordance with the literature and the fold-change differences in levels between the two groups, eleven miRNAs were selected. Supplementary Table 2 summarizes the information regarding the selected miRNAs in the context of metabolic dysfunction, in particular childhood obesity and insulin resistance [3,15,26–38].

3.3. Circulating miRNAs related to prepubertal obesity

The circulating levels of the selected miRNA candidates: let-7d, miR-15b-5p, miR-18a-5p, miR-130b-3p, miR-142-5p, miR-146-5p, miR-152-3p, miR-423-3p, miR-425-3p, miR-532-3p and miR-766-3p were validated in individual samples by RT-qPCR in the NW ($n = 20$) and OW/OB ($n = 43$) groups. Two miRNAs were differentially expressed between the two groups. Participants with obesity showed a 2.3-fold upregulated expression of miR-18a-5p ($p = 0.017$) and a 5-fold upregulation of miR-146a-5p ($p = 0.03$) (Fig. 1).

3.4. Obesity and insulin resistance related miRNAs in prepubertal children

To evaluate the direct association of obesity on miRNA expression, the characteristics of the insulin sensitive groups (MHO and NW) were compared. On the other hand, the influence of insulin resistance was also investigated by comparing the MHO and MUO groups, (Table 2).

No differences were observed in sex and age distribution between the NW and MHO or MHO and MUO groups. As expected, the BMI z-score ($p \leq 0.001$) and waist circumference ($p \leq 0.001$) were higher in the MHO participants *versus* NW (Table 2). Moreover, even though participants from both MHO and MUO groups were considered OW/OB, the BMI z-score was 25 % higher ($p \leq 0.001$) in the MUO group and on average, the MUO group had a 14 cm larger waist circumference ($p \leq 0.001$) when compared to the MHO group (Table 2).

No significant differences were observed in BP z-scores or fasting plasma glucose levels between groups.

Interestingly, even though the MHO participants were considered insulin sensitive, their plasma insulin concentrations were on average 2μU/mL higher as compared to the NW group ($p \leq 0.001$, Table 2). Consequently, the HOMA-IR index was also significantly increased ($p \leq 0.001$) in the MHO group. On the other hand, the MUO study participants presented significantly elevated fasting plasma insulin levels ($p \leq 0.001$), and consequently higher HOMA-IR index ($p \leq 0.001$) when compared with the MHO participants (Table 2).

Interestingly, despite no significant differences observed in the lipid profile from the participants in the NW and MHO groups, the HDL ($p = 0.001$) concentrations were significantly lower in MUO *versus* MHO (Table 2), whereas triglycerides ($p = 0.024$) levels were elevated in the MUO as compared to the MHO (Table 2). Moreover, adiponectin concentrations were decreased by half ($p \leq 0.001$) in the MUO *versus* MHO, whereas triglycerides ($p = 0.024$) and leptin ($p = 0.006$) concentrations were elevated in the MUO as compared to the MHO. In addition, both leptin ($p = 0.006$) and CRP ($p \leq 0.001$) levels were 5-fold higher in the MHO group when compared to the NW group (Table 2). However, when the inflammatory markers were measured in both groups with obesity,

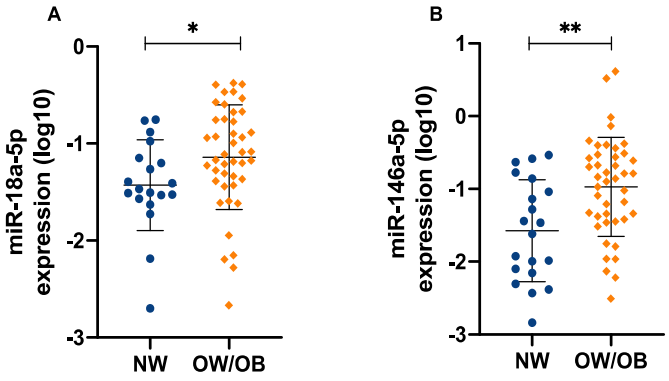

Fig. 1. Altered circulating miRNA levels in prepubertal children with obesity. Expression levels of **A)** MiR-18a-5p and **B)** miR146a-5p expression. The p-values were determined using an independent t-test after logarithmic (log10) transformation. Data are presented as mean ± SD. *$p \leq 0.05$, **$p \leq 0.001$.

only the TNF-α levels were decreased ($p = 0.037$) in the MUO when compared to the MHO group (Table 2).

To evaluate whether obesity or insulin resistance influence selected circulating miRNA levels, both insulin sensitive groups as well as the MHO and MUO children were compared.

Circulating levels of miR-152-3p ($p = 0.015$) and miR-423-3p ($p = 0.040$) were significantly higher in the MUO participants when compared with the MHO group (Fig. 2). Interestingly, the miR-146a-5p levels were upregulated (p = 0.040) in the MUO *versus* MHO and were also upregulated by 3-fold ($p = 0.036$) in MHO *versus* NW group. Thus, miR-146a-5p is the only miRNA differing between the two insulin sensitive groups (Fig. 2).

3.5. Correlations between miRNAs, anthropometric, clinical, and biochemical characteristics

BMI z-score and HOMA-IR were highly correlated ($r = 0.75$, $p \leq 0.001$, Table 3). Additionally, both BMI z-score and HOMA-IR were positively correlated with waist circumference ($r = 0.82$, $p \leq 0.001$ and $r = 0.73$, $p \leq 0.001$) as well as with several biochemical variables including fasting plasma insulin ($r = 0.76$, $p \leq 0.001$ and $r = 0.98$, $p \leq 0.001$), LDL cholesterol ($r = 0.34$, $p = 0.007$ and $r = 0.27$, $p = 0.032$), triglycerides ($r = 0.35$, $p = 0.006$ and $r = 0.47$, $p \leq 0.001$) and leptin levels ($r = 0.83$, $p \leq 0.001$ and $r = 0.75$, $p \leq 0.001$). In addition, both BMI z-score and HOMA-IR were negatively associated with HDL levels ($r = -0.46$, $p \leq 0.001$ and $r = -0.37$, $p = 0.003$), adiponectin levels ($r = -0.44$, $p \leq 0.001$ and $r = -0.57$ $p \leq 0.001$), as well as with the inflammatory marker TNF-α ($r = -0.32$, $p = 0.012$ and $r = -0.34$, $p = 0.007$) (Table 3).

Both miR-18a-5p and miR-146-5p were positively correlated with BMI z-score ($r = 0.32$, $p = 0.010$ and $r = 0.30$, $p = 0.018$) as well as with the inflammatory marker CRP ($r = 0.41$, $p = 0.001$ and $r = 0.27$, $p = 0.032$) (Table 3). MiR-146a-5p was also positively associated with both fasting plasma insulin levels ($r = 0.27$, $p = 0.035$) and consequently HOMA-IR ($r = 0.26$, $p = 0.038$) as well as LDL cholesterol ($r = 0.31$, $p = 0.015$) (Table 3). Moreover, a negative correlation was observed between TNF-α and expression levels miR-146a-5p ($r = -0.28$, $p = 0.029$), miR-152-3p ($r = -0.34$, $p = 0.006$) and miR-423-3p ($r = -0.31$, $p = 0.015$). In addition, miR-423-3p expression levels were positively associated with LDL cholesterol levels ($r = 0.28$, $p = 0.029$) (Table 3).

The association between BMI z-score and insulin resistance as well as each of the miRNAs of interest was evaluated using a univariate linear model (Supplementary Table 3). When the model included three specific parameters namely, insulin resistance, age, and sex, none of the three miRNAs were significantly associated with BMI z-score (Model A, Supplementary Table 3). However, when removing the non-significant covariates age and sex in a stepwise fashion, both HOMA-IR and miR-18a-5p were associated with BMI z-score (Model B, Supplementary Table 3). Thus, when controlling for the association with HOMA-IR, the results showed that miR-146a-5p, miR-152-3p and miR-423-3p were no longer significantly associated with BMI z-score, which suggests that HOMA-IR may be a primary association with these miRNAs.

3.6. miRNA alterations—an underlying feature of early life obesity

To evaluate whether the studied miRNAs could be used to differentiate states of obesity, IR or IS, at an early age, miRNAs found to be different in the various comparisons were used to perform ROC analysis (Supplementary Table 4). miR-146a-5p demonstrated individual discriminatory capacity between the NW and OW/OB group with the

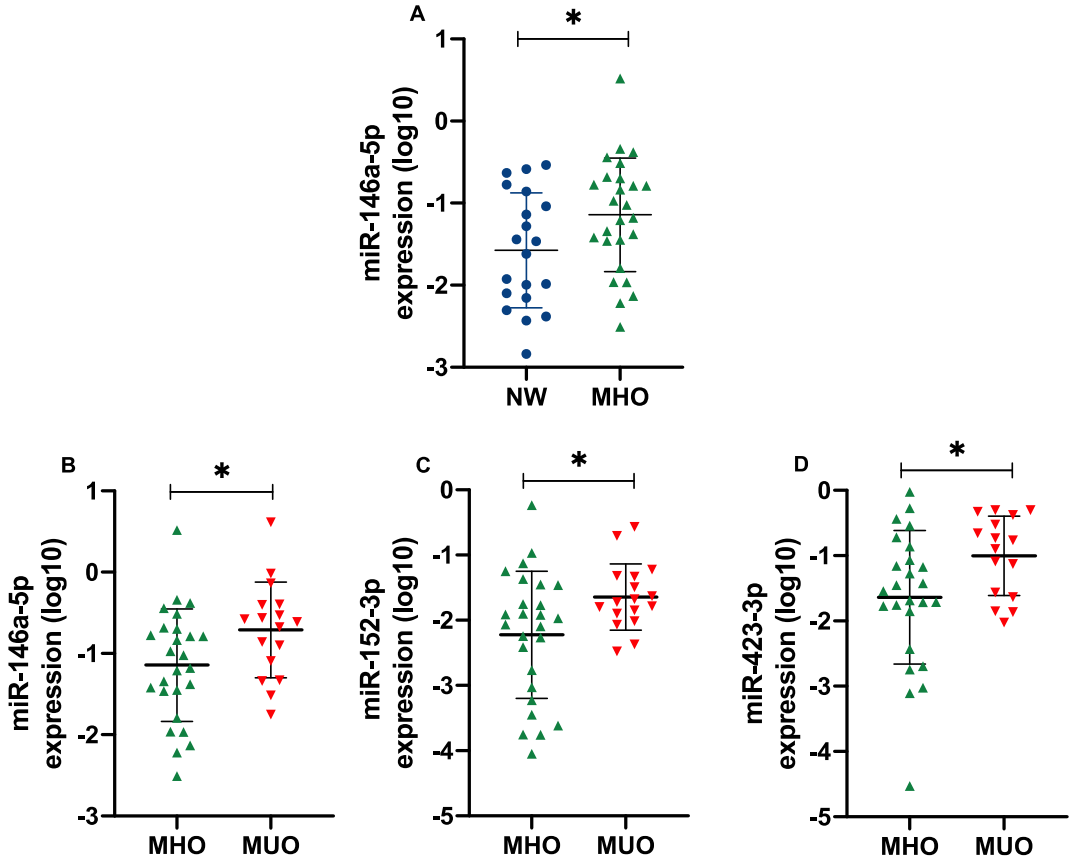

Fig. 2. The expression of specific circulating miRNAs in prepubertal children with insulin sensitivity with/without obesity. **A)** MiR-146a-5p, and in prepubertal children with obesity with/without insulin resistance, **B)** MiR-146a-5p, **C)** miR-152-3p and **D)** miR-423-3p. The *p*-values were determined using an independent *t*-test after logarithmic (log10) transformation. Data are presented as mean ± SD. *$p \leq 0.05$, **$p \leq 0.001$.

Table 3
Correlation between miRNAs, anthropometric, clinical, and biochemical characteristics.

	BMI z-score	HOMA-IR	miR-18a-5p	miR-146a-5p	miR-152-3p	miR-423-3p
BMI z-score	–	**0.75*****	**0.32***	**0.30***	0.11	0.17
Waist Circumference (cm)	**0.82*****	**0.73*****	0.17	0.18	−0.04	0.04
Systolic BP z-score	**0.45*****	**0.46*****	0.11	0.15	0.01	0.08
Diastolic BP z-score	0.22	0.16	−0.06	0.02	−0.06	0.05
Fasting plasma insulin (μU/mL)	**0.76*****	**0.98*****	0.18	**0.27***	0.14	0.16
HOMA-IR	**0.75*****	–	0.14	**0.26***	0.15	0.18
Fasting plasma glucose (mmol/L)	0.11	0.23	−0.17	−0.07	0.08	0.15
HDL cholesterol (mmol/L)	**−0.46*****	**−0.37****	−0.10	−0.25	−0.12	−0.24
LDL cholesterol (mmol/L)	**0.34****	**0.27***	0.10	0.31*	0.24	**0.28***
Triglycerides (mmol/L)	**0.35****	**0.47*****	0.09	0.18	0.11	0.15
Total cholesterol (mmol/L)	0.19	0.17	0.03	0.22	0.14	0.15
Leptin (μg/mL)	**0.83*****	**0.75*****	0.25	0.25	0.04	0.09
Adiponectin (μg/mL)	**−0.45*****	**−0.57*****	−0.09	**0.26***	0.01	−0.10
CRP (mg/L)	**0.68*****	**0.50*****	**0.41****	0.27	0.09	0.17
IL-6 (pg/mL)	−0.27*	−0.21	0.01	−0.13	−0.14	−0.15
IL-8 (pg/mL)	−0.12	−0.17	0.25	−0.06	−0.06	−0.07
IL-1β (pg/mL)	−0.12	−0.09	0.12	−0.08	−0.01	−0.11
TNF-α (pg/mL)	**−0.32***	**−0.34****	0.01	**−0.28***	**−0.34****	**−0.31***
MCP-1 (pg/mL)	0.14	−0.02	0.13	−0.28	−0.17	−0.23
miR-18a-5p	**0.32***	0.14	–	**0.59*****	**0.49****	**0.55*****
miR-146a-5p	**0.30***	**0.26***	**0.59*****	–	**0.79*****	**0.94*****

The Spearman correlation coefficient was evaluated. Bold font indicates statistical significance. BMI, body mass index; BP, diastolic blood pressure; HDL, high density lipoprotein; LDL, Low density lipoproteins; CRP, C-reactive protein; IL-6, interleukin-6; IL-8, interleukin-8; IL-1β, interleukin-1 beta; TNF-α, tumor necrosis factor alpha; MCP-1, monocyte chemoattractant protein-1.
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

highest AUC of 0.731 (sensitivity of 62.8 % and specificity of 65.0 %, $p = 0.003$). miR-18a-5p presented the second highest AUC of 0.690 (sensitivity of 74.4 % and specificity of 63.2 %, $p = 0.02$). Moreover, when both miRNAs were combined, the AUC was increased to 0.756 (sensitivity of 76.4 % and specificity of 68.4 %, $p = 0.001$). However, when the discriminatory capacity was evaluated regarding insulin resistance in the obese participants, only modest AUC with no significance were observed (Supplementary Table 4).

3.7. Predicted targets and pathway analysis

Since miRNAs are well known posttranscriptional regulators, it is of importance to evaluate whether the predicted target genes and their related pathways might be related with IR development and/or obesity. A total of 1319 transcripts were identified as targets of miR-152-3p, miR-18a-5p, miR-146-5p and miR-423-5p, 802 transcripts were targeted by miR-152-3p, followed by miR-18a-5p and miR-146a-5p with 321 and 283, respectively, and only 17 transcripts were targeted by miR-423-5p (Fig. 3A). From these, a total of 99 transcripts are shared by at least two or more miRNAs. However, there is no overlap when the transcripts of all four miRNAs were compared (Fig. 3A). Using the set of predicted mRNA targets, we performed a pathway enrichment analysis. The highest enrichment of 12.23-fold ($p \leq 0.001$) was observed for 'Regulation of PTEN (phosphatase and tensin homologue) mRNA translation', where PTEN is an important insulin regulator [39]. A positive enrichment was also found for the transcriptional activation of mitochondrial biogenesis pathways (enrichment fold of 3.26, $p \leq 0.001$) [40], signaling by the endothelial growth factor receptor (EGFR) (enrichment fold of 4.35, $p \leq 0.001$), and the Phosphatidylinositol 5 phosphatase (PI5P), protein phosphatase 2A (PP2A) and immediate early response 3 (IER3) (enrichment fold of 3.36, $p \leq 0.001$) both direct mediators of the PI3K/AKT signaling pathway, important in the regulation of insulin action [41] (Fig. 3B). Moreover, several lipid metabolism related pathways appear to be coordinately targeted by the miRNAs differentially regulated in pediatric obesity (Fig. 3B). The predicted genes of more than two miRNAs and their related pathways, with specific importance on IR development and/or obesity are listed in the Supplementary Table 5.

4. Discussion

The increasing prevalence of childhood obesity, as a consequence of poor and unhealthy life-style habits, is of high importance, since it predisposes for the development of chronic obesity-related disorders [1]. An increase in body fat mass has been commonly related with a metabolic unhealthy phenotype characterized by an insulin resistant state and metabolic syndrome [42]. However, not all individuals with obesity display the same degree of metabolic dysfunction [5,43,44]. The MHO phenotype has previously been used to describe patients with obesity but reduced visceral fat and lower risk for developing obesity-related co-morbidities [45]. Altered miRNA profiles have been extensively described as predictors for the development of several chronic diseases including obesity related complications such as lipodystrophy, T2DM and cardiovascular disease [46,47]. Moreover, circulating miRNAs as potential markers of cellular dysfunction has been gaining scientific interest in recent years [12,14]. The present study importantly indicates the influence of insulin resistance on the signature of circulating miRNAs in prepubertal children with obesity.

From an initial discovery experiment (8 NW *versus* 8 OW/OB), eleven miRNAs were identified with increased expression in the OW/OB group: let-7d, miR-15b-5p, miR-18a-5p, miR-130b-3p, miR-142-5p, miR-146-5p, miR-152-3p, miR-423-3p, miR-425-3p, miR-523-3p and miR-766-3p. All these miRNAs have been reported to correlate with altered metabolic phenotypes across the life span, as extensively described in Supplementary Table 2. After validation using the whole set of samples, increased circulating levels of miR-18a-5p and miR-146a-5p were observed in children with obesity when compared to the NW group. In agreement, previous studies have identified increased circulating levels of miR-146a-5p in the plasma of children and adolescents (8 to 18 years old) [29] and in the serum of pre-school children from 4 to 6 years old [15] with obesity. Interestingly, both increased [34] or decreased [35] circulating levels of miR-18a have been observed in adult T2DM patients. To our knowledge, this study is the first to report increased miR-18a levels in a pediatric group of children with obesity.

Interestingly, no differences were observed in fasting glucose levels between the NW and the OW/OB groups, while increased fasting insulin levels in the OW/OB group were indicative of insulin resistance, as

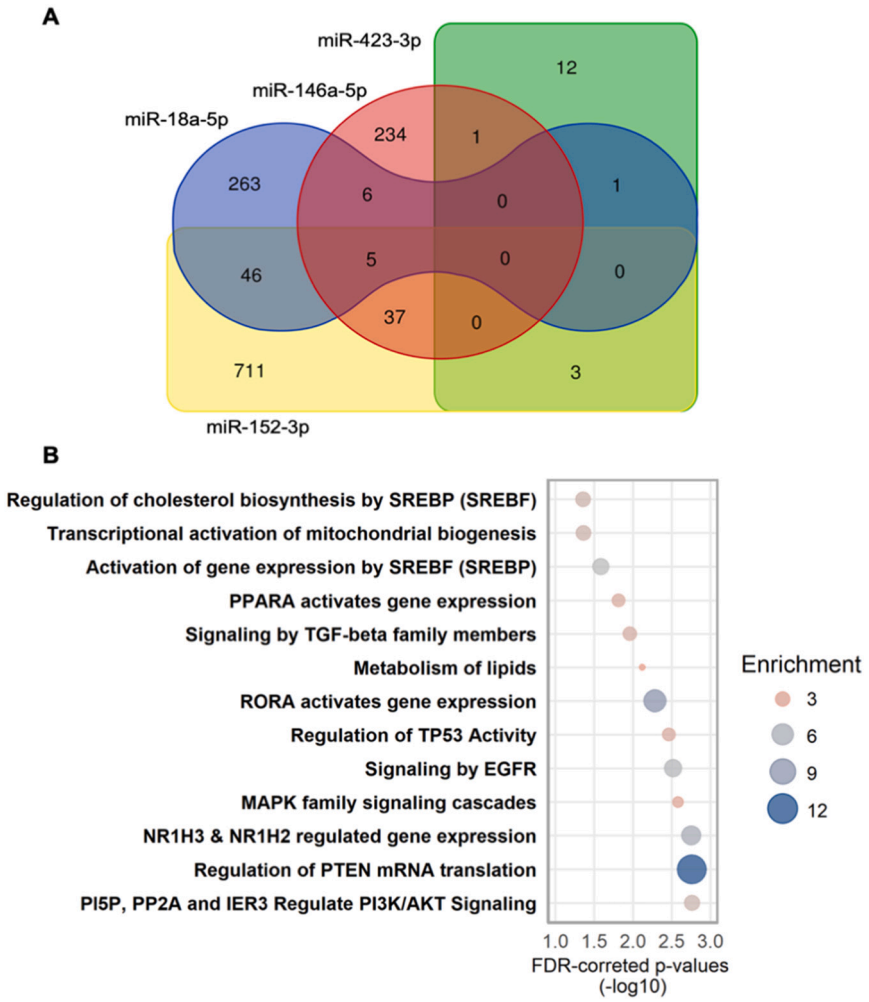

Fig. 3. Predicted target genes and related pathways. **A)** Venn diagram representing the number of shared target genes of the differentially expressed miRNAs, and **B)** enrichment analysis of the pathways regulated by the differentially expressed miRNAs.

previously observed in a similar prepubertal cohort [48].

In accordance with previous studies [26–29], the CRP and leptin levels were increased in the OW/OB group and were positively correlated with BMI z-score. In addition, an increase in systolic BP z-score, one of the key factors of cardiovascular risk, was also observed in the OW/OB group, when compared with the NW participants. However, all cholesterol levels were similar between NW and the OW/OB groups. In fact, previous studies have found that similar anthropometric and biochemical characteristics in pre-school children are already linked to inflammation and adipocyte dysfunction, which remains through adulthood [49].

When participants with obesity were stratified by their HOMA-IR index (HOMA-IR $\geq$ 1.95), both waist circumference and BMI z-score were correlated with HOMA-IR and both were increased in the MUO when compared to the MHO group.

Increased levels of miR-146a-5p, miR-152-3p and miR-423-3p were observed in the MUO compared to the MHO group. Interestingly, while BMI z-score was correlated with both miR-18a-5p and miR-146a-5p, HOMA-IR was only correlated with the plasma levels of miR-146a-5p. However, after adjustment for the covariates age and sex, only miR-18a-5p was correlated with BMI z-scores, independently of the degree of insulin resistance. In T2DM patients, increased levels of circulating miR-18a are related with impaired glucose uptake and glucose metabolism in peripheral tissues [34]. Yet, previous studies have already identified that miR-18a overexpression enhances insulin sensitivity in 3 T3-L1 adipocytes by PKB activation [35]. In addition, the present study is the first to identify altered miR-18a-5p circulating levels in children with obesity, specifically in prepubertal obesity.

Both *in vitro* and *in vivo* studies have shown that miR-146a-5p is regulated by inflammation, cellular aging, and oxidative stress [15,50,51]. In addition, miR-146a-5p directly modulates the insulin cascade by targeting the insulin receptor and the insulin receptor substrate (IRS)-1 [52] and *via* downregulation of natriuretic peptide receptor 3 (NPR3) in adipocytes [53]. In agreement with our study, increased circulating levels of miR-146a-5p have previously been observed in the serum of pre-school children with obesity and insulin resistance [15]. Interestingly, increased cellular expression of miR-146a might improve insulin sensitivity, due to the downregulation of pro-inflammatory genes, including IL-6 and IL-1β [54]. However, in the present group of children, no associations were observed between the circulating levels of the described inflammatory markers and miR-146a-5p levels [15,50,52,53].

Previous studies have identified elevated miR-152-3p levels in the serum of adults with obesity and T2DM patients with obesity [55,56]. Interestingly, miR-152-3p is the only miRNA that can regulate most of the predicted targets. MiR-152-3p has been described important in modulating lipid metabolism due to its capacity to regulate protein kinase B/glycogen synthase kinase (PKB/GSK) pathway and glycogen synthesis in hepatocytes through PTEN modulation [55]. Previous studies have correlated increased levels of miR-152-3p with impaired cardiac mitochondrial metabolism and inflammation in animal models [40]. Moreover, in both pancreatic islets from hyperglycemic patients

and in the T2DM Goto-Kakizaki rat model, increased levels of miR-152 were involved in the progression of pancreatic β cell dysfunction with further consequences on the regulation of intracellular ATP levels [56]. Furthermore, circulating levels of miR-423-3p may be dependent on age and the degree of obesity. While higher plasma levels of this miRNA were observed in both prepubertal children [27] and pubertal (age mean = 12 years old) children with obesity [38], its downregulation was described in OW and obese adolescents (12 to 18 years old) [26] and in adults with morbid obesity [28] or with T2DM [31]. Moreover, previous studies have indicated increased circulating miR-423-3p levels as a risk factor for insulin resistance development [57]. As demonstrated in *in vitro* studies, miR-423-3p is a direct regulator of hepatic insulin resistance and glucose metabolism in obesity since miR-423-3p can directly regulate the insulin cascade *via* AKT/PKB inhibition, shown in 3 T3-L1 adipocytes [57] and in hepatocytes [58].

Like other studies, HOMA-IR was correlated with the secretory function of the adipocyte, particularly adipokine secretion [59]. Therefore, the increased circulating leptin and the decreased adiponectin levels observed in the MUO group compared to the MHO, may already reflect a critical adipocyte secretory dysfunction present in the MUO prepubertal children, as young as 5 years of age. Moreover, HOMA-IR was also correlated with a less favorable lipid profile, transcribed by higher circulating levels of triglycerides, while the HDL levels were decreased. In fact, this phenotype observed in MUO children seems conserved across the lifespan [15,60,61].

The influence of obesity in insulin sensitive prepubertal children was evaluated by comparing the participants from the NW group with the MHO. Despite normal fasting plasma glucose and lipid levels, the MHO group displayed increased plasma insulin, leptin and CRP levels, indicating early metabolic dysfunction [62]. Moreover, miR-146a-5p was increased in the MHO participants. Importantly, miR-146a-5p displayed higher plasma levels independently of which groups were being compared. These data suggest the direct involvement of miR-146a-5p in IR development and obesity related pathways.

The current data set did not allow the identification of miRNAs as predictive biomarkers for IR in our cohort of prepubertal children with obesity. However, twenty transcripts of importance for the regulation of key metabolic pathways were identified as predicted target genes from at least two or more of the identified miRNAs (Fig. 3B) [63]. Therefore, pathways including regulation of PTEN mRNA translation, PI3K/AKT signaling, and regulation of lipid metabolism can be directly influenced by the levels of the differentially expressed miRNAs [15,50,55,57,58].

A strength of the current study is that age and sex did not differ between the MHO and MUO group, contrary of what is observed in other studies [64,65] Besides, all participants were considered prepubertal, either reported by their parents or guardians, or when in doubt the team physician would assist to make the final distinction.

Because our study is cross-sectional, we cannot determine if the observed alterations in the levels of miRNA are a cause or a response to early metabolic alterations observed during obesity development in these prepubertal participants. Besides, new indexes, including the insulinogenic index and the disposition index have been shown effective in pre-diabetes diagnosis and even in relation to comorbidity development prevention in young adults (10–18 years of age) [66]. However, since no interventions were performed in our cohort, which were between 5 and 9 years of age, the HOMA-IR index is appropriate [18–20]. Additionally, since only circulating miRNAs were evaluated, the origin of the observed miRNAs is unknown. Moreover, larger studies need to be performed to validate these findings.

5. Conclusion

In the present study, we observed that the miR-18a-5p, miR-146a-5p, miR-152-3p and miR-423-3p are associated with obesity and insulin resistance in prepubertal children. However, more studies are needed not only to identify the underlying mechanism related with the differential miRNA profiles at this early stage of life, but also to determine their value as possible predictors for future metabolic dysfunction.

Declaration of competing interest

The authors declare no conflict of interest, financial or otherwise.

Data availability

Data will be made available on request.

Acknowledgments

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Author contributions

EC conceived the idea, designed the research, and achieved funding for the research. GG and LD recruited study participants; PPG, SR, EB, and EC conducted experiments and acquired data. EC, DS, AS and LTD analyzed and interpreted data. DS prepared figures and wrote the manuscript. All edited and revised the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2022.121246.

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