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Research article

Epigenetic insights into pediatric obesity: microRNA profiling across puberty and insulin resistance-related stratification

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Abstract

Introduction: Pediatric obesity is a major global health challenge due to its increasing prevalence and elevated risk of early metabolic disease, yet the molecular mechanisms underlying adipose tissue dysfunction in childhood remain poorly understood.

Methods: In this cohort-based study, we profiled the expression of 10 obesity-associated microRNAs (miRNAs) and four target genes in subcutaneous adipose tissue from 29 children classified as normal-weight (NW) or overweight/obese (OW/OB). Molecular data were integrated with anthropometric, pubertal, and metabolic parameters, including the homeostatic model assessment for insulin resistance (HOMA-IR) as a fasting surrogate index of insulin resistance.

Results: OW/OB children exhibited a distinct miRNA and gene expression signature that accurately discriminated them from NW subjects. Physiological correlations between miRNAs and clinical parameters observed in NW were largely disrupted in OW/OB. In NW children, *miR-221* and *miR-26b* correlated with growth and lipid parameters, whereas in OW/OB children *miR-130a-3p*, *miR-34a* and *let-7d* associated with pubertal stage and HOMA-IR. Pubertal stratification revealed distinct molecular profiles in prepubertal OW/OB subjects, identifying puberty as a critical window for obesity-related molecular remodeling. Notably, miRNA alterations were detectable in OW/OB children in the lower HOMA-IR stratum, suggesting that adipose tissue molecular dysregulation precedes systemic metabolic impairment.

Conclusion: These findings identify a puberty- and HOMA-IR–associated miRNA signature in pediatric adipose tissue, highlighting early epigenetic remodeling in obesity. Because these signatures were identified in adipose tissue, future studies should determine whether comparable molecular profiles can also be detected in blood before any clinical application is considered. This molecular profile may represent a promising candidate for investigation in early risk stratification and targeted intervention in pediatric populations.

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Introduction

Over the past few decades, pediatric obesity has increased significantly worldwide, becoming one of the major global health challenges of the 21st century[1]. In Italy, according to the most recent data from the national surveillance system OKkio alla Salute, coordinated by the Italian National Institute of Health [2], 20.1% of children aged 8–9 years are overweight, and 9.2% have obesity. According to ISTAT data related to Goal 2 of the 2030 Agenda [3], the proportion of children and adolescents with overweight in 2021 was 33.3% among those aged 3–5 years and 27% among those aged 3–17 years, with a marked incidence in socio-economically disadvantaged groups.

Beyond its well-established clinical implications, pediatric obesity also represents a significant issue in terms of health equity [4]. Genetic, epigenetic, environmental, and behavioral factors interact to drive its onset and progression [5,6]. Current prevention and treatment strategies often fail to account for this interindividual variability, thereby limiting the effectiveness of targeted interventions [7]. Consequently, there is an urgent need for innovative biomarkers that can identify early molecular alterations and support precision-medicine approaches in pediatric populations.

Among the epigenetic regulators, microRNAs (miRNAs), small non-coding RNAs that modulate gene expression at the post-transcriptional level, have emerged as key mediators linking obesity to its metabolic and systemic complications. Recent studies have demonstrated that specific miRNAs regulate adipocyte differentiation, inflammatory responses, and insulin sensitivity, acting as critical nodes in the signaling networks connecting obesity, type 2 diabetes, and cardiovascular disease [8,9]. Furthermore, altered miRNA profiles have been associated with endothelial dysfunction, reproductive impairment, and cancer risk in obesity [10–12].

Despite growing evidence from adult studies, data on miRNA expression directly within pediatric adipose tissue remains limited. Most pediatric investigations have focused on circulating miRNAs, which may not fully capture tissue-specific regulatory mechanisms. Adipose tissue is a central metabolic and endocrine organ, especially during childhood and adolescence, when it undergoes dynamic remodeling in response to growth, hormonal changes, and energy balance [13,14].

Puberty represents a critical developmental window characterized by profound hormonal and metabolic changes that can interact with excess adiposity to accelerate metabolic dysfunction[15]. Insulin resistance, in particular, frequently emerges during pubertal

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transition and may be exacerbated by obesity, increasing the risk of long-term cardiometabolic complications [16].

To investigate the epigenetic mechanisms underlying pediatric adipose tissue dysfunction, we selected 10 key microRNAs and 4 representative target genes based on literature evidence and their involvement in the major biological pathways underlying obesity. Specifically, miR-130a, miR-26b, miR-335, and miR-125a are involved in adipogenesis, adipocyte differentiation, and tissue remodeling, whereas miR-29a, miR-22-5p, and miR-22-3p contribute to metabolic homeostasis [17–25]. In addition, miR-34a, miR-221, and let-7d have been associated with insulin resistance, chronic inflammation, and metabolic stress [26–35]. Accordingly, SNAIL1, IL-6, VEGFA, and IGF1 were selected as representative target genes and key regulators of extracellular matrix remodeling, inflammation, and growth signaling [36–38]. Together, these molecules represent interconnected pathways underlying adipose tissue dysfunction and obesity progression. The overarching goal is to identify potential early epigenetic markers of obesity-related complications in children, providing novel insights into the molecular mechanisms underlying metabolic dysregulation in early life.

Materials and Methods

Study population

For molecular analysis, periumbilical subcutaneous adipose tissue (scAT) samples were collected by surgical biopsy from 29 pediatric patients aged 1–18 years (7 F and 22 M, mean age 10.0±4.0 yrs) enrolled at Buzzi Children's Hospital (Milan, Italy).

Inclusion criteria were: boys and girls aged 1 to 18 years, at any Tanner stage (1 to 5), and with an informed consent form signed by a parent or legal guardian.

Exclusion criteria were: secondary obesity, ongoing pharmacological treatment, and the presence or history of chronic illnesses.

This study was performed in accordance with the Declaration of Helsinki. This human study was approved by Lombardia Milano Area 1 (MI-Area 1) Ethics Committee (approval: 12/2016; amendment 07/2020). Surgical biopsies were not performed solely for research purposes. Subcutaneous adipose tissue (approximately 0,5-1 cm^3) was collected opportunistically during routine surgery for independent clinical indications, including abdominal, abdominal-wall, inguinoscrotal, and ovarian procedures. Only tissue exposed or removed as part of the planned operation and otherwise destined for disposal was collected. Sampling required no additional incision, invasive maneuver, modification, or prolongation

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of the procedure and posed no additional risk to the child. Written informed consent was obtained from the parents or legal guardians of all participants.

Anthropometric measurements and biochemical profile

Each participant underwent anthropometric and biochemical assessments according to our previously published protocol [39]. Trained personnel recorded height, weight, waist circumference, and pubertal stage using predefined procedures.

Anthropometric assessments were performed with participants barefoot and wearing only underwear. Body weight was measured using a calibrated scale with a precision of ±100 g, with the participant standing still in the center of the platform. Height was measured using a wall-mounted stadiometer accurate to ±1 mm, with the participant standing upright, heels together, arms relaxed at the sides, head positioned in the Frankfurt horizontal plane, and heels, buttocks, shoulders, and occiput aligned as appropriate.

Waist circumference was measured using a non-elastic flexible tape positioned horizontally midway between the lower margin of the last palpable rib and the top of the iliac crest. Measurements were taken with the participant standing upright, feet slightly apart, abdomen relaxed, arms at the sides, and after a normal expiration. Care was taken to ensure that the tape was snug but did not compress the skin and that it remained horizontal around the body.

Body mass index was calculated as weight divided by height squared (kg/m²) and converted into BMI z-scores according to WHO reference standards [40].

Based on BMI z-score values, participants were classified as having normal-weight (NW, $-2 \leq$ BMI z-score < 1; n=13) or overweight/obesity (OW/OB, BMI z-score ≥ 1; n=16).

Pubertal development was evaluated during the clinical examination using Tanner staging [41,42], and participants were classified as stage 1 (prepubertal), stages 2–3 (early puberty), or stages 4–5 (late to post-pubertal).

Venous blood samples were collected after an overnight fast between 8:30 and 9:00 a.m. Plasma glucose, insulin, triglycerides (TG), total cholesterol, and high-density lipoprotein (HDL)- and low-density lipoprotein (LDL)-cholesterol were measured using standardized clinical laboratory assays.

The homeostatic model assessment for insulin resistance (HOMA-IR) was calculated as a fasting surrogate index of insulin resistance using the formula (fasting insulin [mU/L] × fasting glucose [mmol/L])/22.5 [43]. For analytical purposes only, participants were stratified according to a HOMA-IR value exceeding +2 standard deviations, corresponding to values above the 97th percentile according to sex- and pubertal stage–specific reference values,

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hereafter abbreviated as HOMA-IR>2 [44]. This threshold was used solely to define HOMA-IR strata for group comparisons and was not used as a diagnostic criterion for insulin resistance.

Gene expression analysis by Real-Time Quantitative PCR (RT-qPCR)

Immediately after collection, tissue specimens were processed for RNA extraction or stored at −80°C until use. Total RNA was isolated from adipose tissue biopsies using TRIzol™ reagent (Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. RNA concentration and purity were determined using a Micro-volume spectrometer (LifeReal, Zhejiang, China).

For miRNA quantification, 100 ng of total RNA was reverse transcribed using the Mir-X™ miRNA First-Strand Synthesis Kit (TaKaRa, Diatech, Italy). Quantitative PCR was performed using SYBR™ Green Fast qPCR Mix (Fisher Molecular Biology, Rome, Italy) on a CFX Connect Real-Time PCR Detection System (Bio-Rad, USA) with specific primers (Table S3).

Relative expression levels of selected miRNAs were calculated using the $2^{-}\Delta Ct$ method [45] using U6 small nuclear RNA as an internal control.

For gene expression analysis, 200 ng of total RNA was reverse transcribed using SuperScript IV Reverse Transcriptase (Invitrogen, Thermo Fisher Scientific). The obtained cDNA was amplified using the CFX Connect Real-Time PCR Detection System using SYBR™ Green Fast qPCR Mix, with gene-specific primers (Table S4). Relative expression levels were calculated using the 2^-ΔΔCt method [46] normalized on the expression of 14S gene.

Linear Discriminant Analysis for patient class identification

To explore the global differences between patients with normal weight and overweight/obesity, we performed a Linear Discriminant Analysis (LDA) using miRNA/mRNA expression profiles. Before LDA computation, missing values were imputed, and all variables scaled. Then, LDA was used to derive a single discriminant axis separating the two groups, onto which the group distributions were projected.

Statistical Data Analysis

Statistical analyses were performed using GraphPad Prism (version 10.0). Group differences were assessed using the Mann–Whitney U test for non-parametric data, unpaired Student's t test for parametric comparisons between two groups, and one-way ANOVA for parametric

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data involving multiple groups. Associations between molecular and clinical variables were evaluated using Spearman's rank correlation analysis. All statistical tests were two-tailed, and a p-value<0.05 was considered statistically significant.

Results

Clinical and biochemical parameters

Table 1 summarizes the clinical and biochemical characteristics of the study participants. The mean age of the total sample was 10±4.0 years, with a slightly younger mean age in the NW group compared to the OW/OB group. The distribution of pubertal stages was similar across participants. As expected, body weight, height, BMI, and waist circumference were higher in the OW/OB group. The OW/OB group also showed higher fasting glucose and insulin levels total and LDL-cholesterol and triglycerides, together with lower HDL-cholesterol values, compared to the NW group.

Identification of obesity-related miRNAs

To identify miRNAs potentially involved in pediatric obesity, we started from a literature-based selection of 10 miRNAs previously implicated in adult obesity: *miR-22-3p, miR-22-5p, miR-26b, miR-29a, miR-34a, miR-125a-3p, miR-130a-3p, miR-221, miR-335*, and *let-7d.* Their reported roles in obesity-related molecular pathways are summarized in Table 2**.** Quantitative RT-qPCR analysis revealed distinct expression patterns between pediatric NW and OW/OB samples. Several miRNAs, including *miR-29a, miR-221, miR-26b, and miR-22-3p*, showed a trend toward higher expression in obese children, although these differences did not reach statistical significance. *miR-335* and *miR-125a-3p* levels appeared largely unchanged in OW/OB children compared with NW controls, indicating stable expression in early-life adipose tissue. Importantly, *miR-22-5p* was the only miRNA significantly upregulated in obese children ($p<0.05$) (Fig.1A).

To further explore potential molecular targets, the expression levels of *SNAIL1*, *IL-6*, *VEGFA*, and *IGF1* genes were evaluated. *SNAIL1* and *IGF1* expression levels showed an increasing trend in the OW/OB group. A significant upregulation of the inflammatory mRNAs encoding *IL-6* and *VEGFA* was observed in OW/OB samples compared to controls ($p<0.05$) (Fig.1B).

Even though almost no single miRNA or mRNA reached statistical significance, the total data showed a moderate separation between the two groups when analyzed by Linear Discriminant Analysis (LDA). To support this observation, Fig. 1C shows the distributions of

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the two groups projected along the LDA discriminant axis. A statistically significant difference between the two distributions was confirmed by the Mann–Whitney U test ($p<0.001$).

Associations between adipose tissue miRNA, gene expression and anthropometric parameters

Correlation analyses between adipose tissue miRNA/gene expression and clinical parameters revealed distinct patterns in NW compared to OW/OB children. In NW samples (Supplementary Table 1), correlation analysis revealed significant associations between specific miRNAs and anthropometric or clinical parameters (Fig. 2). In lean subjects, *miR-221* showed an inverse correlation with age *(r=-0.77, p=0.015),* height *(r=-0.78, p=0.014),* and HDL *(r=-0,71, p=0,05)* while *miR-26b* correlated positively with total cholesterol *(r=0.67, p=0.018).* Moreover, *SNAIL1* negatively correlated with triglycerides (*r=-0,65, p=0,04*).

Conversely, in adipose tissue samples from OW/OB children (Supplementary Table 2), correlation analyses revealed a strong inverse association between the Tanner stage and miRNA *miR-130a-3p (r=−0.62, p=0.011),* and *miR-34a (r=−0.60, p=0.01).* Additionally, HOMA-IR factor exhibited a negative correlation with *miR-130a-3p (r=−0.59, p=0.04)* and *let-7d (r=−0.56, p=0.05).* In this group, *IL-6* expression levels were negatively correlated with total cholesterol *(r=-0,69, p=0,03)*, while *SNAIL1* positively correlated with it *(r= 0,52, p= 0,04).*

Notably, the correlations observed in NW children were not present in the OB group, suggesting a potential disruption of physiological regulatory mechanisms associated with obesity.

Differences in miRNA expression levels in children's adipose tissue according to prepubertal and pubertal classification

Patients were stratified according to pubertal stage based on Tanner classification into two groups: prepuberty (Tanner stage 1) and early/late puberty (Tanner stages 2–5) (Figure 3). In the whole cohort, comparison between these two groups revealed differences in the expression of several miRNAs, with a distinct expression profile observed between prepubertal and pubertal subjects. Indeed, *miR-29a*, *miR-130a-3p*, *miR-34a*, *miR-26b*, *miR-22-5p*, and *let-7d* were significantly upregulated in OW/OB prepubertal subjects, compared with both OW/OB pubertal subjects ($p<0.05$) and with NW prepubertal subjects ($p<0.05$).

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Additionally, the expression levels of *miR-26b* and *let-7d* resulted also higher in prepubertal OW/OB subjects than in pubertal NW subjects (p<0.05), whereas no significant differences were observed for *miR-22-3p*, *miR-221*, *miR-335* and *miR-125a*. In contrast, in NW subjects no significant differences in miRNA expression were found between prepubertal and pubertal groups. Importantly, gene expression analysis of *VEGFA*, *SNAIL1*, *IGF1*, and *IL-6* stratified by pubertal stage did not reveal significant differences (Supplementary Fig. 1), suggesting that pubertal modulation of these markers may be less pronounced or occur downstream of miRNA regulatory changes.

Differences in miRNA and gene expression levels in children's adipose tissue according to HOMA-IR strata

When subjects were stratified for analytical purposes according to HOMA-IR strata (≤2 versus >2), significant differences in the expression of several miRNAs were observed between the two groups (Fig. 3B). In particular, *miR-29a-5p*, *miR-34a*, *miR-26b*, *miR-22-5p, and Let-7d* showed a significantly lower expression in NW subjects compared to OW/OB with HOMA-IR≤2. Moreover, within OW/OB population, *miR-29a*, *miR-34a*, *miR26b*, *miR-22-5p* and *Let-7d* were all significantly (p<0.05) downregulated in subjects with HOMA-IR>2. Finally, *miR-26b* and *let-7d* were both increased (p<0.05 and <0.005, respectively) in OW/OB with HOMA-IR≤2 in respect to NW with HOMA-IR>2. Overall, these findings indicate that a specific subset of miRNAs is downregulated in subjects with higher HOMA-IR values, suggesting a potential association between miRNA expression profiles and metabolic status.

Furthermore, we observed that gene expression analysis of *SNAIL1*, *VEGFA*, *IL-6*, and *IGF1* did not reveal statistically significant differences between NW and OW/OB subjects when stratified according to HOMA-IR strata (Supplementary Fig. 2). Nevertheless, *IL-6* expression showed a consistent trend toward higher levels in OW/OB subjects compared with NW controls, regardless of whether HOMA-IR values were below or above the cut-off.

Discussion

In this study, we provide evidence that pediatric obesity is associated with an early and selective disruption of adipose tissue miRNA regulatory networks, preceding or accompanying metabolic and developmental alterations. Pediatric obesity is a complex and multifactorial condition that represents a growing global health challenge [47]. There is a pressing need to identify innovative and reliable biomarkers capable of detecting early

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molecular alterations associated with obesity. Epigenetic regulation may contribute to the characterization of obesity-related molecular changes and may support the development of future precision-medicine strategies, particularly in pediatric populations, where early intervention could influence long-term health outcomes [48]. In this context, miRNAs have attracted increasing attention as potential regulators of metabolic processes. Therefore, we analyzed selected miRNAs previously implicated in adult obesity to explore whether comparable obesity-related molecular patterns may also be observed in children and adolescents. This exploratory approach was intended to generate preliminary evidence and does not imply a direct causal role of these miRNAs in pediatric obesity.

Growing evidence indicates that miRNAs play a central role in linking obesity to its associated metabolic and systemic complications, thereby contributing to disease onset, progression, and interindividual variability in clinical manifestations [49,50]. Thus, we focused our attention on 10 miRNAs previously implicated in adult obesity: *miR-22-3p, miR-22-5p, miR-26b, miR-29a, miR-34a, miR-125a-3p, miR-130a-3p, miR-221, miR-335*, and *let-7d.* We analyzed their expression in tissue isolated from pediatric obese subjects. While different studies have examined circulating miRNAs, particularly in plasma or serum, as non-invasive biomarkers of metabolic dysregulation in adults, data directly derived from adipose tissue remain scarce [51]. Analyzing miRNAs directly in adipose tissue offers a more precise view of local molecular mechanisms driving adipogenesis, inflammation, and metabolic regulation, revealing alterations that may not be detectable in circulation. Our results highlight a selective upregulation of *miR-22-5p* in OW/OB children compared with NW controls. This result is in line with previous literature in adult obesity [52]. The significant increase in *miR-22-5p* expression observed in OB children points to a possible role in adipocyte remodeling and inflammatory activation. Coherently, we observed a significant upregulation of *IL-6* and *VEGFA* expression in adipose tissue from OW/OB children. The concomitant increase in *miR-22-5p* and inflammatory markers supports the hypothesis that miRNA dysregulation contributes to the initiation of low-grade inflammation within growing adipose tissue. Moreover, given that *miR-22-5p* is known to modulate key metabolic regulators such as *PPARα*, *SIRT1*, and *FGF21*, its upregulation may promote lipid accumulation and oxidation, mitochondrial function, and energy balance [8,9,53]. *miR-22-5p* has GLP1 as a potential target (Targetscan): its upregulation could lead to a decrease of glucagon molecule, leaving its GLP1 receptor free from binding. This condition could sustain obesity, regulate glucose levels, and influence appetite and satiety, confirming a potential use of agonistic of GLP1 therapeutic drugs also in children [54]. In addition to single-marker

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analyses, multivariate integration of adipose tissue miRNA and gene expression data revealed that NW and OW/OB experimental groups could be significantly distinguished using LDA analyses. When miRNA and gene expression levels were plotted, OW/OB and NW subjects clustered into distinct classes. The limited overlap between the two groups was confined to the tails of the distributions, reflecting interindividual physiological variability. Importantly, this separation was achieved independently of anthropometric or clinical parameters, suggesting that obesity is associated with a molecular fingerprint at the adipose tissue level. These findings support the concept that pediatric obesity is characterized by an early and structured reprogramming of epigenetic and transcriptional networks, which can be captured through integrated molecular profiling and may precede overt clinical manifestations.

Then, we proceeded with the integration of all collected data, investigating the potential reciprocal correlation between molecular targets and clinical parameters. We observed that *miR-221* in NW groups were inversely correlated with growth-related parameters (age, height), two closely related parameters reflecting physiological development, as well as with HDL levels. These results may reflect a role for *miR-221* in modulating lipid homeostasis and tissue remodeling during physiological growth. The inverse correlation with growth-related parameters in children suggests a potential involvement in developmental regulation, possibly through effects on vascular remodeling or bone metabolism [55,56]. Furthermore, circulating *miR-221/222* signatures have already been associated with HDL-associated metabolic phenotypes, characterized by cardiovascular low-risk [57]. Similarly, the positive correlation between *miR-26b* expression and total cholesterol in NW children aligns with prior evidence implicating *miR-26b* in physiological lipid metabolic pathways. Instead of reflecting growth or physiological mechanisms, miRNA expression in OW/OB children became more closely associated with pathological parameters. The correlations described in NW were largely absent or altered in OW/OB children, indicating a disruption of normal miRNA–phenotype relationships in the context of pediatric obesity. Specifically, we observed that *miR-130a-3p* and *miR-34a* were inversely correlated with Tanner stage, a clinical index that reflects the degree of pubertal maturation, while *miR-130a-3p* and *let-7d* were negatively associated with HOMA-IR, indicating early alterations in insulin sensitivity in OW/OB children. In addition, inflammatory and transcriptional markers, including *IL-6* and *SNAIL1*, exhibited positive correlations with total cholesterol, suggesting that lipid overload may directly promote inflammatory activation and adipose tissue remodeling. Our findings align with epidemiological evidence linking obesity to altered pubertal timing. In a Chinese cohort, girls with obesity exhibited earlier puberty compared with normal-weight or

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overweight peers, likely mediated by increased aromatase activity in adipose tissue and higher estrogen production [58,59]. A recent systematic review confirmed that childhood obesity is associated with a significantly higher risk of early puberty in girls, with similar trends observed in boys, and comparable results have been reported in Mexican and Danish populations [60–63].

Such alterations are consistent with the concept of "epigenetic drift", whereby persistent metabolic challenges lead to cumulative deregulation of non-coding RNA networks, impairing tissue resilience [64]. This mechanism may help explain why obesity in early life predisposes individuals to long-term complications such as IR, cardiovascular disease, and infertility [10].

To further investigate the role and impact of puberty on the OW/OB molecular signature, we stratified our cohort according to the pubertal stage. Importantly, prepubertal OW/OB children exhibited a significant upregulation of several miRNAs compared with both NW subjects and OW/OB children in the pubertal stage. These findings suggest that early hormonal changes during prepuberty may act as a critical "window of vulnerability," amplifying obesity-associated molecular alterations in adipose tissue. The observed miRNA upregulation in prepubertal children with OW/OB may disrupt the normal coordination of lipid metabolism, adipogenesis, and insulin sensitivity, predisposing these children to early metabolic dysregulation. In contrast, the attenuation of miRNA upregulation in pubertal OW/OB children may reflect partial adaptation or exhaustion of regulatory networks in response to sustained metabolic stress.

Moreover, we investigated the association between HOMA-IR strata and adipose tissue molecular profiles. Notably, two NW subjects exhibited a HOMA-IR>2, suggesting that higher HOMA-IR values may also occur independently of excess weight. However, in these individuals, miRNA expression levels were comparable to those observed in NW subjects with HOMA-IR≤2. In contrast, among children with obesity, significant differences in the expression of specific miRNAs were observed depending on the HOMA-IR stratum, highlighting the interaction between adiposity and metabolic stress in modulating miRNA networks. Interestingly, obese children with HOMA-IR≤2 exhibited upregulation of several key miRNAs, suggesting that early molecular adaptations occur at the adipose tissue level before overt metabolic dysfunction manifests. In contrast, in OW/OB children with HOMA-IR>2, the expression of these miRNAs returned to levels comparable to those observed in NW subjects, despite the presence of their higher HOMA-IR values. This pattern may indicate that the initial miRNA-mediated regulatory response represents an early, tissue-

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specific attempt to maintain metabolic homeostasis, which is subsequently lost in the higher HOMA-IR stratum. It is conceivable that in children with more pronounced metabolic impairment, the signaling pathways previously active within adipose tissue may no longer be detectable at the local level, as dysregulation shifts toward systemic circulation, reflecting a transition from tissue-specific molecular adaptation to a broader, organism-level metabolic disturbance. These findings underscore that critical molecular changes associated with obesity and higher HOMA-IR values may occur very early, highlighting the potential of miRNAs as early biomarkers of metabolic risk.

This study has some limitations. The relatively small sample size, mainly due to the difficulty of obtaining pediatric adipose tissue samples outside clinically indicated surgical procedures, limited the possibility of subgroup analyses. Consequently, participants with overweight and obese were combined into a single OW/OB group, which may have reduced the ability to detect differences specifically related to obesity severity. Moreover, NW children were slightly younger than OW/OB participants; although pubertal stage was considered, residual age- and puberty-related effects may have influenced the results. Finally, the limited number of participants, particularly females, precluded sex-stratified analyses. Since sex-related differences may affect adipose tissue remodeling, pubertal development, and metabolic risk, this represents an additional limitation for risk assessment. In addition, the molecular signatures were identified directly in adipose tissue, the biologically relevant compartment for local metabolic and inflammatory regulation; however, adipose tissue is not readily accessible for routine clinical assessment. Future studies should therefore investigate whether comparable signatures are detectable in circulating blood and determine their concordance with adipose tissue profiles. Overall, these findings should be considered exploratory and require confirmation in larger, sex-balanced cohorts allowing stratification by weight category and pubertal stage.

In conclusion, our study reveals a distinct adipose tissue miRNA signature in children and adolescents with obesity, shaped not only by excess adiposity but also by pubertal stage and HOMA-IR strata. These findings define a tissue-specific “molecular fingerprint” of early metabolic risk and suggest that puberty may represent a critical window during which adipose tissue dysfunction is amplified. In this context, miRNAs may act as integrative molecular links connecting obesity, developmental stage, and emerging metabolic complications such as insulin resistance. From a translational perspective, these results may guide future studies aimed at identifying candidate biomarkers for early risk stratification, particularly in children and adolescents with overweight/obesity at higher metabolic risk,

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including those with higher HOMA-IR values, central adiposity, adverse lipid profiles, rapid weight gain during puberty, or a family history of metabolic diseases. However, because the present signatures were identified in adipose tissue, their potential clinical relevance will depend on whether analogous and reproducible profiles can be detected in accessible biological samples, particularly blood. These findings should be considered exploratory and require validation in larger, sex-balanced cohorts and direct comparison of adipose tissue and circulating signatures before any clinical application can be proposed.

Statements

Study approval statement

The study protocol was reviewed and approved by the Institutional Ethics Committee (MI area 1–12/2016/2020) and carried out in accordance with the principles of the Declaration of Helsinki (1975, revised 2008).

Consent to participate statement

Informed written consent was obtained from the parents and/or legal representatives of the patients after they had received information about the study.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Author Contributions

Data curation, Formal analysis: C.G., A.L, C.C., B.G.G. G.R.B, V.C.; Clinical sample collection: G.P., G.Z., U.M.P., V.C.; Writing – original draft: C.G., A.L. C.C., B.G.G, G.R.B., V.C.; Conceptualization, Project administration, Supervision: C.G., G.R.B, V.C.

Data Availability Statement: All data generated or analyzed during this study are included in this article and its supplementary material files. Further enquiries can be directed to the corresponding author.

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Figure 1. Variations of miRNA and gene expression levels analyzed in children with NW compared to children with OW/OB

(A) RT-qPCR relative expression levels of: *miR-29a*, *miR-130a*, *miR-221*, *miR-34a*, *miR-26b*, *miR-22-5p*, *miR-22-3p*, *let-7d*, *miR-335* and *miR-125a* in obese compared to lean children's adipose tissue. **(B)** RT-qPCR fold change expression levels of: *SNAIL1*, *IL-6*, *VEGFA*, and *IGF1* in obese compared to lean children's adipose tissue. Data are presented as mean ± SEM; (NW n=13; OW/OB n=16); Mann–Whitney U test for non-parametric data was performed ($p<0.05$, *), unpaired t test for gene data was performed ($p<0.05$, *).

(C) Linear discriminant analysis (LDA) based on miRNA and mRNA expression levels. The one-dimensional LDA projection shows a clear separation between the two groups (NW vs OW/OB), as illustrated by the frequency histograms. Differences between distributions are statistically significant, as assessed by the Mann–Whitney U test ($p<0.001$).

Figure 2. Correlation analysis of clinical parameters, microRNA and genes expression

Spearman's rank correlation analysis was applied to assess associations between clinical variables and miRNA expression levels in normal-weight **(A)** and overweight/obese (OW/OB) children **(B)**, respectively. Correlation coefficients (ρ) are represented as a heatmap, with color intensity indicating the strength and direction of the correlations. Statistical significance of the Spearman correlation is indicated by asterisks ($p<0.05$, *; $p<0.01$, **; $p<0.001$, ***).

Figure 3. Classification of miRNA expression levels by puberty stages and HOMA-IR strata

MiRNA relative expression in NW compared to OW/OB children's adipose tissue classified by: **(A)** prepuberty (Tanner's score=1; NW n=8 and OW/OB n=5) or puberty (Tanner's score=2-5, NW n=5 and OW/OB n=11). **(B)** HOMA-IR strata, where a value > 2 denotes values exceeding +2 standard deviations from sex- and pubertal stage–specific pediatric reference values (HOMA-IR<2: NW n=11 and OW/OB n=3; HOMA-IR>2: NW n=2 and OW/OB n=10). Data are presented as mean ± SEM; One-way ANOVA test was performed ($p<0.05$, *; $p<0.005$, **, $p<0.0005$, ***).

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Table 1. Clinical and biochemical characteristics of the participants

	Total	**NW (n=13)**	**OW/OB (n=16)**
Age (years)	10.0±4.0	8.35±4.15	11.0±3.9
Sex (F/M)	7/22	4/9	3/13
Weight (kg)	45.8±20.7	29.9±15.9	58.7±13.6
Height (cm)	139.5±21.9	129.2±23.7	147.9±15.9
BMI (kg/m^2)	22.0±5.9	16.5±3.4	26.5±3.0
BMI z-score	1.2±1.2	0.23±0.74	2.2±0.5
Waist circumference (cm)	64.8±13.0	57.2±10.5	78.5±6.8
Pubertal stage			
Prepubertal (Tanner 1)	13	8	5
Pubertal (Tanner 2-5)	16	5	11
Fasting blood glucose (mg/dl)	87.9±11.5	84.7±11.5	90.4±10.9
Fasting Insulin	14.3±19.7	4.40±3.47	23.4±23.6
Total cholesterol	151.8±37.0	140.8±35.5	160.7±35.7
HDL-Cholesterol	43.1±13.2	48.8±16.3	38.6±7.3
LDL-Cholesterol	90.2±35.7	80.0±32.7	98.3±35.9
Triglycerides (mg/dl)	96.4±63.4	66.0±38.5	118.6±67.7
HOMA-IR	2.1±1.8	1.0±0.9	3.3±1.9

NW: normal-weight; OW/OB: overweight/obesity; HDL: high-density lipoprotein; LDL: low-density lipoprotein; HOMA-IR: homeostatic model assessment for insulin resistance.

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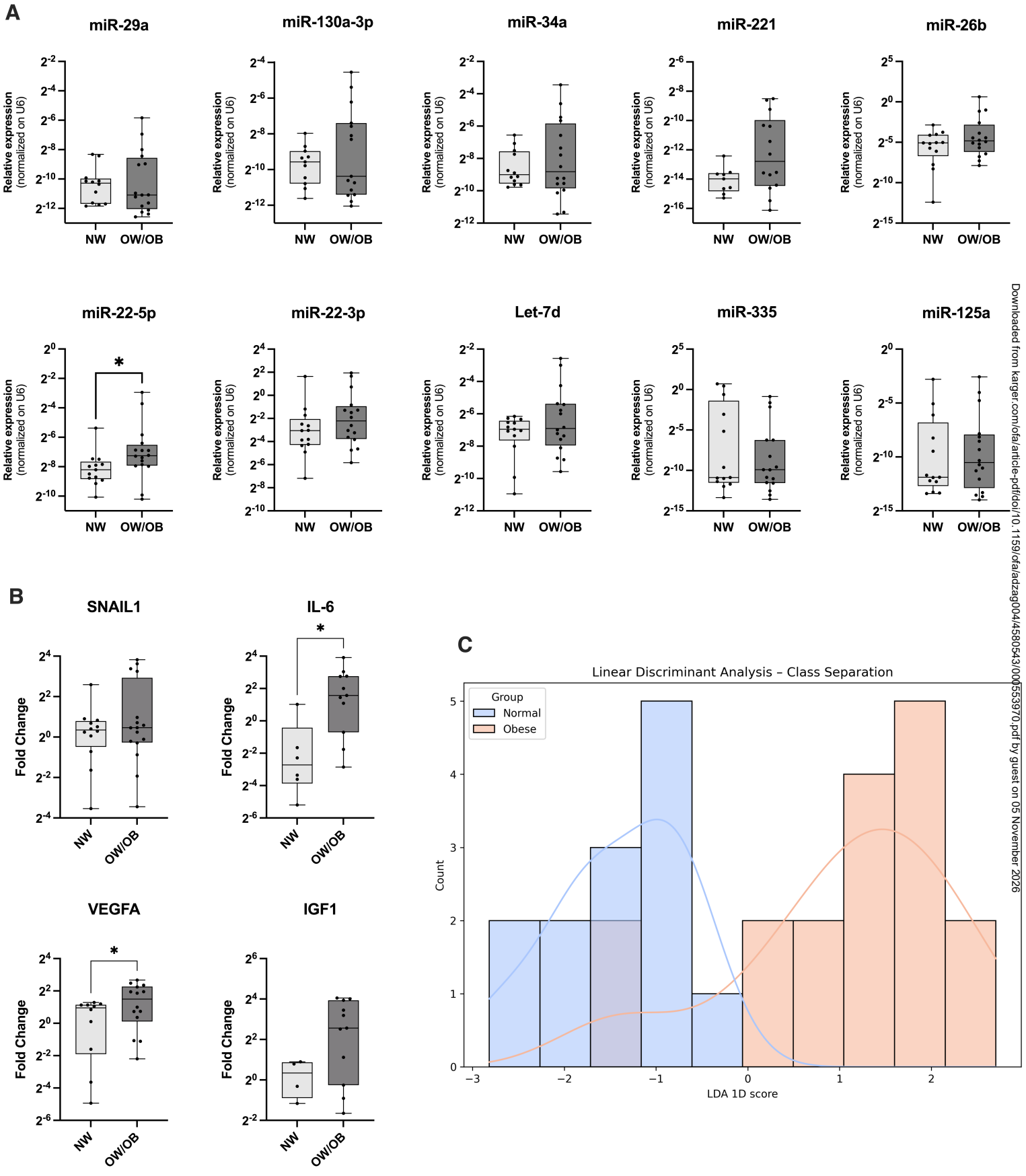
Table 2. Functional overview of the investigated microRNAs and their target genes in adipose tissue. The network shows predicted microRNA–gene interactions. Red nodes indicate microRNAs and blue nodes target genes. *IGF1*, insulin-like growth factor 1; *IL6*, interleukin 6; *SNAI1*, snail family transcriptional repressor 1; *VEGFA*, vascular endothelial growth factor A.

References	Investigated microRNA	Target genes	Biological role in Adipose Tissue	Molecular Mechanism
Song et al.[19] Gharanei et al. [18]	***hsa-miR-26b***	*IGF1, IL6, SNAI1, VEGFA*	*Adipogenesis; Inflammation*	Regulates early adipocyte differentiation and lipid droplet growth. Its dysregulation shifts the adipokine profile, indirectly accelerating macrophage infiltration and triggering pro-inflammatory secretion.
Zhu et al. [29] Ju et al. [20]	***hsa-miR-335***	*IGF1, IL6, SNAI1*	*Adipogenesis; Tissue Hypoxia*	Control Adipose tissue expansion and lipid overload. It coordinates mesodermal lineage commitment, driven angiogenesis during rapid tissue growth.
Yiew at al. [21] Sung et al. [22]	***hsa-miR-130a-3p***	*IGF1, VEGFA*	*Lipid Homeostasis; Vascularization*	Coordinates the adipogenesis-angiogenesis axis. Its alters mature adipocyte differentiation and local tissue vascularization, modulating downstream hypoxic stress signaling pathways
Lino et al. [23]	***hsa-miR-22-3p -5p***	*SNAI, VEGFA*	*Metabolic Homeostasis; Nutrient Sensing*	Modulates key metabolic checkpoints. A loss of this metabolic control induces mitochondrial stress, driving chronic inflammation.
Jo et al. [24] He et al. [25]	***hsa-miR-29a***	*IGF1, IL6, SNAI1*	*Insulin Signaling; Fibrosis*	Alter glucose uptake and local somatic growth. It participates in fibrotic loops that modulate extracellular matrix deposition.
Cornejo et al. [30] Pan et al. [31] Park et al. [32]	***hsa-miR-34a***	*IGF1, IL6, SNAI1, VEGFA*	*Adipocyte Senescence; Inflammation*	Impair healthy tissue vascularization. It promotes cellular senescence and a pro-inflammatory state, driving insulin resistance.
Ortega et al. [33] Li et al. [34]	***hsa-miR-221***	*IGF1, IL6, SNAI1, VEGFA*	*Tissue Stress, Inflammation*	Trigger inflammator state in adipose tissue, lipid overload, tracking hypoxia-induced network remodeling.
Zhu et al. [35] Lightbody et al. [26]	***hsa-let-7d***	*IGF1, IL6, SNAI1, VEGFA*	*Tissue Remodeling, EMT-like Transitions*	Regulates cell fate and background proliferative capacity. It controls the activation of epithelial-to-mesenchymal-like transitions within the stromal vascular fraction, modulating fibrotic progression.
Chiu-Li Yeh et al [17]	***hsa-miR-125a-3p***	*VEGFA*	*Growth Axis, Pubertal Development*	Regulate systemic endocrine signals and proliferation pathways. It accelerates tissue remodeling.

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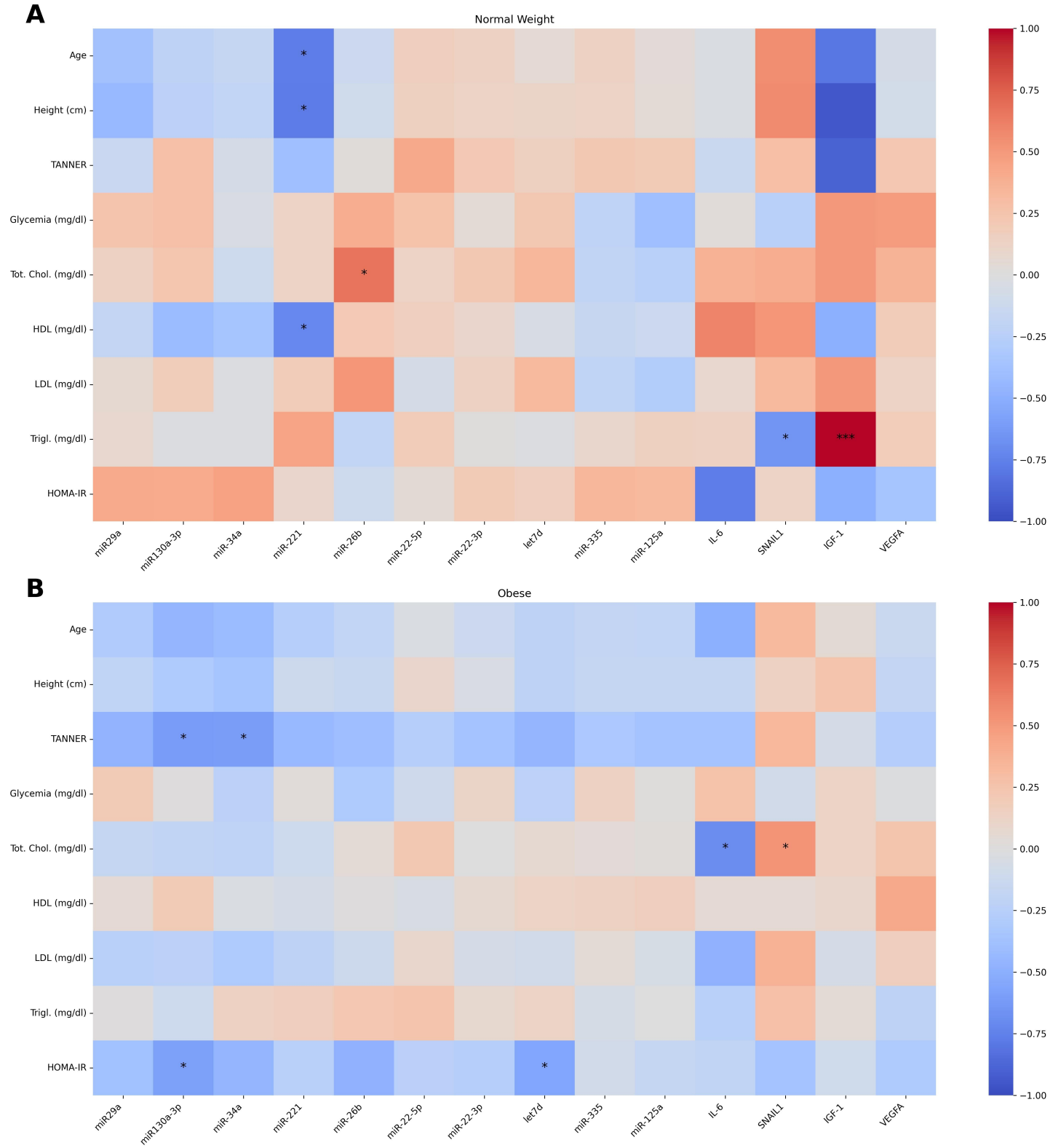
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Figure 1



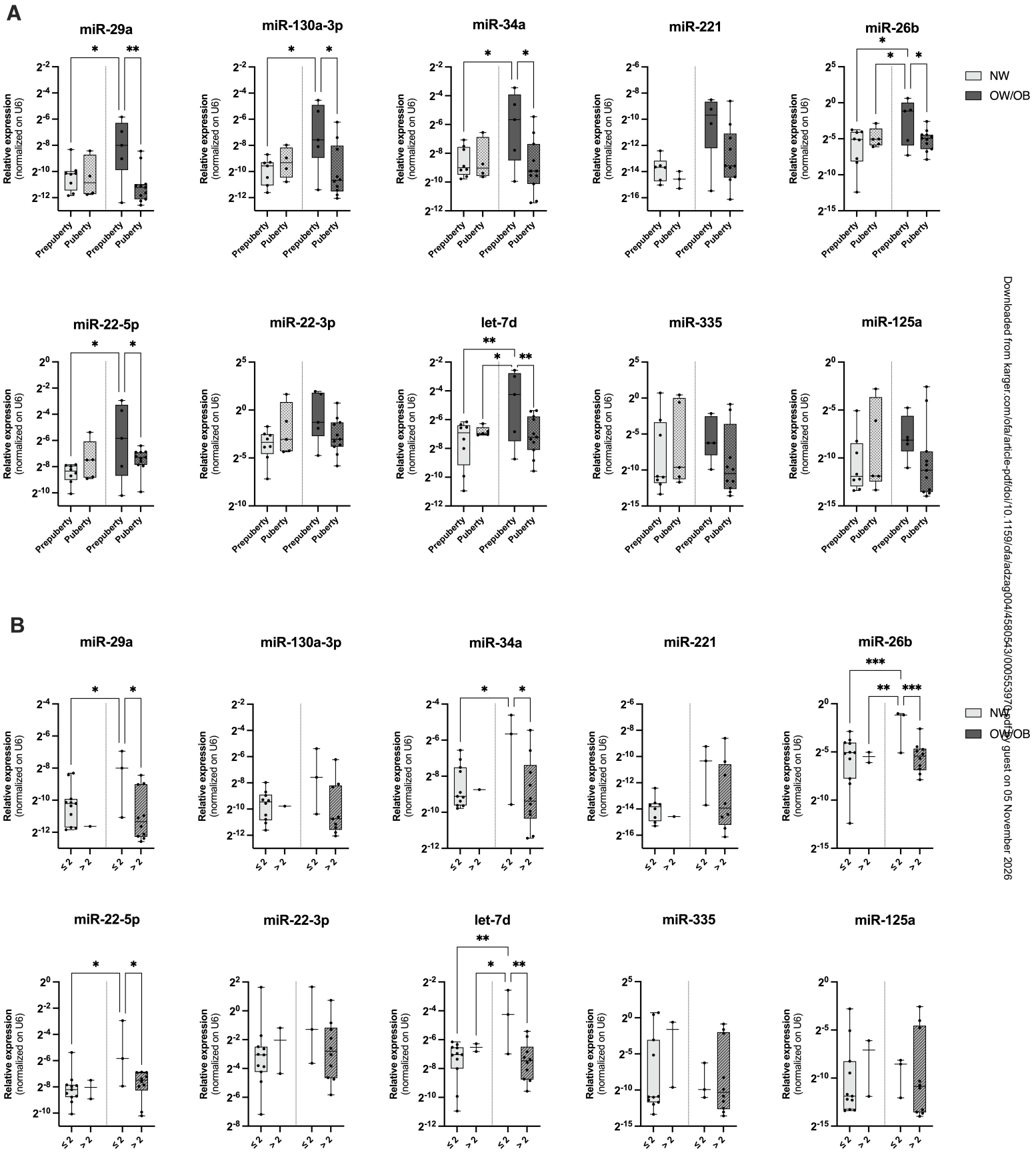
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Figure 2



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Figure 3



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