Involvement of Circulating MicroRNAs in the Pathogenesis of Atherosclerosis in Young Patients With Obesity

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Received March 29, 2024
Accepted August 5, 2024

Summary

Obesity is considered an important factor contributing to the development of atherosclerosis. Inflammation plays a key role in endothelial dysfunction (ED), an initial stage of the atherosclerotic process. Several microRNAs (miRNAs) may play an important role in the inflammatory process, but there is a lack of information about their participation in the early stages of atherosclerosis development in patients with obesity. We aimed to assess the relations between plasma concentration of selected miRNAs, ED evaluated by reactive hyperemia index (RHI), inflammatory markers and other factors involved in the pathogenesis of atherosclerosis in adolescents and young adults with obesity. Participants (30 males, 30 females; aged 15-25 years) were divided into two groups: those with overweight/obesity (OW/O) (20 males, 20 females) and controls (C) (10 males, 10 females). The plasma concentrations of inflammatory markers, cytokines, adipocytokines, markers of lipid profile and glucose metabolism and selected miRNAs (miR-92, -126, -146a, -155) were analyzed. No significant differences in any of the miRNAs were found between the groups. MiR-146a correlated positively with RHI. Dividing the group by sex showed more significant associations between miRNA and analyzed parameters (IL-6, fasting glycemia) in men. Several observed correlations indicate a potential role of miRNAs in inflammation, the atherosclerotic process and glycemic control, primarily in male subjects with obesity. The relatively low number of observed associations between assessed parameters related to obesity and the pathogenesis of its complications could be attributed to the early stage of the atherosclerotic process in young subjects with obesity, where only subtle abnormalities are expectedly found.

Key words

Endothelial dysfunction • Atherosclerosis • Obesity • MicroRNA • Reactive hyperemia index

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Introduction

Obesity is a chronic, complex and multifactorial disease characterized by the excessive accumulation of body fat. A large body of evidence shows that complications of obesity, which previously occurred only in adulthood – e.g. dyslipidemia, atherosclerosis, arterial

hypertension, impaired glucose tolerance, diabetes mellitus type 2 – are increasingly found in adolescents and young adults with obesity [1].

Various mechanisms connecting obesity with cardiovascular diseases have been proposed, and most of them involve endothelial dysfunction (ED) – the initial stage and very early step in the pathogenesis and development of atherosclerosis. ED refers to the inability of the endothelium to control vascular homeostasis and essentially describes a loss in the balanced release of endothelial-derived relaxing and contracting factors [2]. In recent years, ED has received increasing attention as an early predictive marker of vascular disorders including atherosclerosis since its manifestation markedly precedes the appearance of significant clinical symptoms. Mechanical or inflammatory damage to the integrity of the endothelium is the main stimulus for the initiation of atherosclerotic plaque formation, while the inflammatory-fibroproliferative response occurs as a result of various types of endothelial damage [3].

Inflammation and oxidative stress are known to be key factors contributing to ED [4]. A state of chronic low-grade inflammation and the nonspecific activation of the immune system are characterized by high levels of inflammatory markers and fat tissue produced hormones – adipocytokines, including interleukin (IL)-6, IL-8, tumor necrosis factor (TNF)-α, resistin and leptin. They are secreted by a large variety of cells, mostly by the adipocytes, endothelial cells, and adipose tissue immune cells [5].

With childhood obesity on the rise, it is important to focus on the early stages of obesity-related complications, including atherosclerosis, and to introduce and test methods for assessing their progression. Recent research has shown that microRNAs (miRNAs) can be used as biomarkers in various clinical settings. MiRNAs are small non-coding RNAs expressed in a wide variety of organs and cells, that play important roles in many cellular processes, including differentiation, proliferation, apoptosis, and stress response [6,7]. In the immune system, miRNAs are emerging as key regulators of immune cell development, immune responses, autoimmunity, and inflammation, capable of affecting both pro- and anti-inflammatory mechanisms. Dysregulation of miRNAs affects the function of various tissues and organs, including endothelial function, potentially contributing to the development of atherosclerosis [8].

MiRNAs are extremely stable and can be reliably detected from plasma or serum. It points towards their potential application in clinical practice – their role as diagnostic biomarkers has emerged in recent years in many pathological states [9,10].

The aim of this study was to evaluate the levels of selected miRNAs in relation to the presence of obesity and early atherosclerotic changes in young patients with obesity. Accordingly, we focused on the interrelations between early changes related to atherosclerosis, measured by a noninvasive clinical-physiological method, and the levels of selected miRNAs in adolescents and young adults with overweight or obesity – i.e. during the early subclinical stage of obesity-related complications. Furthermore, we assumed that a more detailed characterization of the relations between miRNA levels, adipocytokines and subclinical chronic low-grade inflammation markers would improve the understanding of the role of miRNAs in the pathogenesis of obesity-associated cardiovascular complications. The originality of this study also lies in assessing the relationships between selected anthropometric parameters and miRNA concentrations, with separate analyses for male and female subjects. We suggest that assessing these relationships could be an important step toward better understanding the connection between excessive fat accumulation and its distribution and the future occurrence of cardiovascular complications related to obesity.

Materials and Methods

Participants and clinical measurements

In this study, 60 adolescents and young adults (30 females, 30 males) with ages ranging from 15 to 25 years were enrolled. Participants were recruited to ensure equal distribution of gender and to encompass a wide range of body mass index (BMI) categories from normal weight through overweight to the obesity range. Participants were divided into two groups based on their BMI and age according to the Cole´s chart [11]. The overweight/obese (OW/O) group consisted of sex and age matched subjects: 20 participants with overweight (10 females, 10 males) and 20 participants with obesity (10 females, 10 males) aged 17.7 [15.0-25.8] (median [interquartile range]) years. The control group consisted of 20 participants (10 females, 10 males) aged 17.9 [15.4-25.6] (median [interquartile range]) years. Detailed characteristics of the study groups are presented in Table 1.

Table 1. Anthropometric, clinical and biochemical characteristics of adolescents and young adults.

	ENTIRE STUDY POPULATION			FEMALE			MALE		
	Mean (SD)/Median [IQR]			Mean (SD)/Median [IQR]			Mean (SD)/Median [IQR]		
	C (N=20)	**OW/O (N=40)**	**p**	**C (N=10)**	**OW/O (N=20)**	**p**	**C (N=10)**	**OW/O (N=20)**	**p**
A Anthropometry									
Height (cm)	175.1 [166.7-181.2]	169.3 [163.2-176.7]	**0.000**	164.7 (8.7)	165.5 (5.6)	0.779	181.2 [177.5-182.8]	175.6 [170.7-179.1]	**0.012**
Weight (kg)	66.9 (10.6)	85.2 (17.3)	**0.100**	58.7 [54.0-64.1]	80.4 [76.6-86.1]	**0.000**	73.2 (8.6)	88.5 (22.1)	**0.005**
BMI (kg/m^2)	22.2 [20.1-23.4]	29.3 [27.2-31.3]	**0.000**	21.9 [20.6-23.0]	29.5 [28.5-31.2]	**0.000**	22.4 (2.7)	29.2 (6.2)	**0.000**
Fat mass (%)	20.61 (9.05)	35.49 (8.00)	**0.000**	26.72 (8.47)	40.53 (3.95)	**0.000**	14.5 (4.28)	30.70 (7.97)	**0.000**
WHR (-)	0.78 (0.06)	0.83 (0.08)	**0.014**	0.75 (0.07)	0.79 (0.05)	0.156	0.81 (0.04)	0.87 (0.09)	**0.020**
VFA (cm^2)	45.25 [23.95-56.65]	99.90 [86.10-126.05]	**0.000**	43.80 [23.90-50.90]	96.80 [91.15-117.00]	**0.000**	45.28 (22.55)	112.58 (42.49)	**0.000**
WHtR (-)	0.45 [0.43-0.47]	0.57 [0.53-0.61]	**0.000**	0.45 [0.43-0.45]	0.58 [0.54-0.61]	**0.001**	0.45 [0.41-0.47]	0.55 [0.53-0.61]	**0.000**
B Parameter of endothelial function									
RHI	1.37 [1.22-1.69]	1.36 [1.24-1.56]	0.672	1.41 [1.22-1.72]	1.43 [1.23-1.57]	0.692	1.37 [1.11-1.65]	1.34 [1.25-1.55]	0.860
C Cytokines and adipocytokines									
Adiponectin (μg/ml)	72.76 [26.34-110.10]	103.43 [76.66-123.92]	0.057	75.21 [33.14-121.48]	98.88 [76.66-120.51]	0.356	68.18 (43.95)	96.46 (40.57)	0.109
Leptin (μg/ml)	2.04 [0.94-5.92]	12.29 [5.16-20.59]	**0.000**	5.92 [4.30-10.27]	17.89 [11.23-22.38]	**0.003**	0.94 [0.64-1.10]	9.23 [4.02-18.27]	**0.000**
IL-6 (pg/ml)	0.76 [0.55-1.07]	1.16 [0.84-1.79]	**0.006**	0.77 [0.45-0.95]	1.37 [1.04-1.84]	**0.005**	0.76 [0.60-1.29]	1.01 [0.77-1.71]	0.262
TNF-α (pg/ml)	5.28 [3.67-7.00]	6.41 [5.13-8.02]	0.103	4.35 [3.83-5.73]	6.01 [5.40-7.20]	**0.035**	6.15 (2.78)	6.97 (2.98)	0.466
D Lipid profile and glucose metabolism									
Total cholesterol (mmol/l)	3.86 (0.70)	4.14 (0.83)	0.210	4.21 (0.46)	4.17 (0.90)	0.875	3.554 (0.75)	4.116 (0.78)	0.089
HDL-cholesterol (mmol/l)	1.40 (0.28)	1.23 (0.26)	**0.043**	1.50 (0.25)	1.25 (0.25)	**0.039**	1.322 (0.30)	1.212 (0.28)	0.363
LDL-cholesterol (mmol/l)	2.10 (0.56)	2.36 (0.60)	0.140	2.32 [1.93-2.62]	2.28 [1.95-2.35]	0.718	1.92 (0.65)	2.41 (0.67)	0.087
Cortisol (mmol/l)	383.12 (103.71)	365.60 (151.55)	0.626	337.28 (86.87)	379.33 (180.20)	0.430	423.84 (104.60)	351.87 (120.04)	0.126
Glucose (mmol/l)	5.19 (0.42)	5.09 (0.48)	0.475	5.10 (0.40)	4.99 (0.42)	0.529	5.27 (0.45)	5.20 (0.53)	0.736
Insulin (mU/l)	5.90 [4.10-7.20]	11.30 [7.25-16.10]	**0.000**	5.15 (1.74)	12.90 (6.33)	**0.000**	6.30 (2.10)	12.20 (7.56)	**0.006**
HOMA-IR	1.34 [0.92-1.63]	2.57 [1.64-3.58]	**0.000**	1.18 (0.46)	2.86 (1.42)	**0.000**	1.49 [1.27-1.73]	2.41 [1.69-3.57]	**0.021**

All values were reported as mean (SD) or median [interquartile range]. Bold font in p-values column indicates statistical significance. Abbreviations: BMI: body mass index, WHR: waist to hip ratio, VFA: Visceral Fat Area; WHtR: waist to height ratio; RHI – reactive hyperemia index; IL-6: interleukin 6; TNF-α: tumor necrosis factor alpha; HDL-C: high-density lipoprotein cholesterol, LDL-C: low-density lipoprotein cholesterol, HOMA-IR: homeostatic model assessment for insulin resistance; C: control group (subjects with normal weight); OW/O: subjects with overweight/obesity. Groups were compared by Student´s t-test (for the data with normal distribution) and the Mann-Whitney U-test (non-normally distributed data).

All subjects were instructed not to use substances influencing autonomic nervous system activity or the cardiovascular system (alcohol, caffeine, energetic beverages) and were asked to refrain from smoking for 12 h before the examination. All measurements were performed in a quiet thermo-neutral environment (22-25 °C) in the morning hours (8-11 AM).

We excluded subjects with any current or previous infectious disease (within three weeks prior to the examination date), cardiovascular disease including hypertension (diagnosed using 24-h ambulatory blood pressure monitoring after examination), diabetes mellitus, psychiatric disorders, hypothyroidism and those with a higher BMI due to an increased amount of skeletal muscle, as determined by body composition analysis. All female subjects were examined in the proliferative phase (6-13th day) of their menstrual cycle.

Procedures and Measurements

Clinical and anthropometric data

A detailed medical history was obtained from each participant, and an experienced physician checked their current health status to identify subjects meeting any of the exclusion criteria.

Anthropometric measures were taken immediately after arrival in fasting condition using an InBody J10 device (InBody, South Korea). This body composition analyzer uses the direct segmental multi-frequency bioimpedance analysis method (DSM-BIA) and provides a detailed analysis of body composition including fat mass, skeletal muscle mass, percentage of fat mass, visceral fat area, etc. This method was validated against dual X-ray absorptiometry and is recommended as an acceptable surrogate method for the estimation of body composition in research studies [12]. The circumferences of the waist and hip were measured using a measuring tape. WHR (waist-to-hip ratio) and the WHtR (waist-to-height ratio) were calculated.

Blood sampling and biochemical parameter analyses

Venous blood samples from an antecubital vein were collected in ethylenediamine tetraacetic acid tubes (EDTA). The samples were centrifuged for 30 min (1500× g, 15 min) at room temperature, and plasma samples were immediately stored at -80 °C. Plasma concentrations of the cytokines IL-6, TNF-α, and selected adipocytokine (leptin) were measured using the Evidence Investigator^{TM} Metabolic Syndrome Array I *(Randox Laboratories, Crumlin, UK).* It is routinely used for the simultaneous quantitative detection of multiple related metabolic immunoassays (in parallel) from a single sample. Adiponectin levels were determined using the Human Adiponectin ELISA Kit (KHP0041, Invitrogen). Clinical chemistry tests for plasma concentrations of total cholesterol, high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C) and fasting glycemia were determined as part of routine laboratory tests in a central laboratory (University Hospital in Martin). Plasma concentrations of cortisol and insulin were determined using a chemiluminescent enzyme immunoassay (cortisol: Access Cortisol Kit; Beckman Coulter Cat# 33600, Inc., Tokyo, Japan; insulin: Access Insulin Kit, Beckman Coulter Cat# 33410, Inc., Tokyo, Japan). The homeostatic model assessment for insulin resistance index (HOMA index) was calculated as insulin levels (μU/ml) multiplied by glucose level (mmol/l)/22.5 [13]. All procedures were performed in accordance to the manufacturer´s instructions.

RHI measurement

Endothelial function was assessed by Reactive Hyperemia Peripheral Arterial Tonometry (RH-PAT) using the Endo-PAT 2000 device (Itamar Medical, Israel). Using this method, flow-induced vasodilation was evaluated following a provocation of reactive hyperemia induced by rapid release of brachial artery occlusion lasting for 5 min. The RHI was defined as the ratio of the post-deflation pulse amplitude to the baseline (pre-ejection) pulse amplitude [14]. Subjects were in a supine position for at least 5 min prior to the examination and were asked to minimize movements and refrain from speaking during measurement.

Analysis of individual miRNAs

Based on previous studies in adults, we have selected miRNAs with an assumed significant association with ED and atherosclerosis, and with the production of inflammatory cytokines and adipocytokines. A miRNA panel consisting of 5 miRNAs (miR-92a [15], miR-126 [16], miR-146a [17], miR-155 [18], miR-181b [19]) was quantified in plasma samples using RT-qPCR. Our final selection of miRNAs (miR-92a, miR-126, miR-146a and miR-155) for statistical analysis was achieved by implementing a stepwise exclusion based on several quality control parameters.

We picked the eight putative reference target

miRNAs (miR-16, miR-17, miR-103, miR-106a, miR-140, miR-191, miR-423, cel-miR-39) and reassess their potential contributions as normalizers by geNorm mathematical method. The analyses were tested on 12 samples in duplicates.

The geNorm algorithm is the preferred method to select (a combination of) reference genes for relative quantification. This algorithm uses the geometric mean to determine the most stable housekeeping genes. The optimal number of reference targets in this experimental situation is 3 (geNorm V<0.15 when comparing a normalization factor based on the 3 or 4 most stable targets). This method calculates a normalization factor based on multiple reference miRNA for more accurate and reliable normalization of miRNA expression data. It is based on the principle that the expression ratio of any two reference miRNAs (using standard deviation of log-transformed reference miRNA ratios) should be identical in all samples. Stability measures will be returned which are the average pairwise variation between a reference miRNA and all other reference miRNAs using stepwise exclusion of the worst scoring miRNAs. The predefined miRNAs with the best geNorm-calculated stability measures are listed if their value is less than 1.5 [20].

Based on geNorm analysis, three miRNAs (miR-17, miR-106a, cel-miR-39) were identified as the most stable combination for further normalization. The average arithmetic mean of these three miRNAs for the patient group was 24.84 and for the control group 24.82 – the difference of 0.02 between groups indicates low variability in expression across samples. All Ct values of the normalizing miRNAs are provided in the Supplementary material (Suppl. Table S1).

MiRNAs were quantified using miRNA quantitative reverse transcription PCR (RT-qPCR). Plasma samples were thawed on ice and centrifuged for 10 min (4 °C, 16000× g). Preceding qPCR, total RNA was isolated using the Mirvana Paris Kit (ThermoFisher) enriched for miRNAs as described previously [21]. A synthetic cel-miR-39 was added to the sample before RNA extraction as spike-in control. TaqMan miRNA primers (ThermoFisher) were used for reverse transcription and preamplification. The multiplex qPCR was done in a recently calibrated CFX96 thermal cycler (BioRad, California, USA) as described previously [21].

The tested samples spanned multiple plates. As PCR efficiency can vary between PCR runs, it is appropriate to normalize the data across different PCR plates and runs. In this case, one representative sample from the control group was chosen and used as an interplate calibrator to eliminate plate-to plate variation and improve the accuracy of the results. Normalization of the raw cycle threshold (Ct) values by this calibrator enhances the reproducibility of the results. All reactions and analyses were performed in duplicate. The coefficient of variation accepted for intra-assay replicates was set at 4 %.

The output of the RT-qPCR provided a Ct value for each miRNA examined, which indicates the cycle number at which the fluorescence signal exceeds the detection threshold. Ct values between 19 and 29 indicate a high presence of the target miRNA. Medium amounts are expressed by Ct values between 30 and 35, while values above 35 indicate a minimal amount of miRNA in the examined sample. We eliminated Ct values that were undetermined or >35 from the analysis, to minimize statistical confounding by high quantification cycle values.

We converted all Ct values to relative quantities, comparing the relative change in the amount of the selected miRNA in the examined sample with the endogenous controls and with the control sample. Finally, normalized Ct values were used to calculate relative quantities of miRNAs by $2^{-\Delta\Delta Ct}$ method that is a widely used approach for relative quantification in RT-qPCR experiments [22].

Ethical approval

The study was approved by the Ethics Committee of the Jessenius Faculty of Medicine, Comenius University (EK 16/2022) and the Antwerp University Hospital (UZA). The study protocol is in accordance with the ethical guidelines of the Declaration of Helsinki of 1975.

Statistical analysis

The normality of the data distribution was assessed using the Shapiro-Wilk test. To analyze the differences between OW/O and C groups, Student´s *t*-test was used for data with a normal distribution, and the Mann-Whitney *U*-test was used for data that were not normally distributed. The associations between parameters were analyzed using Pearson´s (variables with normal distribution) or Spearman´s (variables with non-normal distribution) correlation coefficients. Variables are presented as mean ± SD or median [interquartile range]. Statistical analysis was performed using the software SYSTAT 13 (Systat Software Inc., USA). A p-value <0.05 was considered statistically significant.

Results

Anthropometric, clinical, and biochemical characteristics of the 60 participants included in our study, along with a subgroup analysis by sex, are presented in Table 1. For the whole cohort, anthropometric parameters, including body weight, BMI, percentage of fat mass, WHR, VFA and WHtR were significantly higher in the OW/O group compared to C group, as expected (Table 1A). In contrast, height was significantly lower in the OW/O group compared to the C group. When the data were examined separately for female and male subjects, WHR was significantly higher and height was significantly lower in OW/O compared to controls in the male subgroup only. All other anthropometric measures were significantly higher in OW/O group in both males and females (Table 1A).

RHI showed no significant differences between OW/O and C. Subanalysis by sex confirmed an absence of significant differences in this parameter between groups (Table 1B).

Biochemical parameters (cytokines and adipocytokines) that could contribute to the proinflammatory status in OW/O patients are presented in Table 1C. Leptin and IL-6 were significantly increased in OW/O group compared to C (Table 1C) in the entire cohort. When the data were analyzed for female and male subjects separately, the level of leptin was significantly higher in OW/O group compared to C group in both females and males, but IL-6 and TNF-α were significantly increased in OW/O females only (Table 1C).

The parameters of lipid profile and glucose metabolism are presented in Table 1D. For the entire cohort, we observed that the patients with OW/O had higher insulin levels and HOMA-IR, and lower HDL cholesterol levels compared to C. In the subgroups, a lower level of HDL cholesterol was found in females only.

The circulating levels of miR-92a, -126, -146a and -155 were compared between the C (N=20) and OW/O (N=40) groups. The results showed no significant differences in any of the target miRNAs between groups (Fig. 1). The means (SD) or medians [interquartile ranges] of miRNAs between the C and OW/O groups, as well as between the C, OW and O groups for the entire cohort are depicted in the Supplementary Materials (Suppl. Table S2A, B).

Correlation analysis

Table 2 and Table 3 summarize the correlations of each circulating miRNA with the anthropometric parameters, measure of endothelial function (RHI), adipocytokine and cytokine concentrations and parameters of lipid profile and glucose metabolism in the entire study cohort and in subgroups divided by sex.

No significant correlations were found between anthropometric parameters and the targeted miRNAs in the entire group of participants. BMI only weakly negatively correlated with miR-92a and miR-146a in the female subgroup. Importantly, a positive correlation of RHI with miR-146a was found when analyzing the entire group of participants (Table 2, Fig. 2).

From the assessed adipocytokines and cytokines, plasma adiponectin concentration negatively correlated with miR-92a, miR-126 and miR-146a. These correlations were significant in both sexes except for miR-92a in males. The traditional marker of inflammation, IL-6, was negatively correlated with miR-146a in the entire cohort of participants. When dividing the group by sex, the negative association between IL-6 and miR-146a was confirmed only in the male subgroup. Additionally, in men, significant correlations were observed between IL-6 and miR-126 and miR-155.

From the lipid profile and glucose metabolism parameters, only glucose level was found to be associated with miRNAs; miR-92a, miR-126 and miR-146a were negatively correlated with glucose in the entire cohort. Analysis by sex revealed that these associations were present in men only.

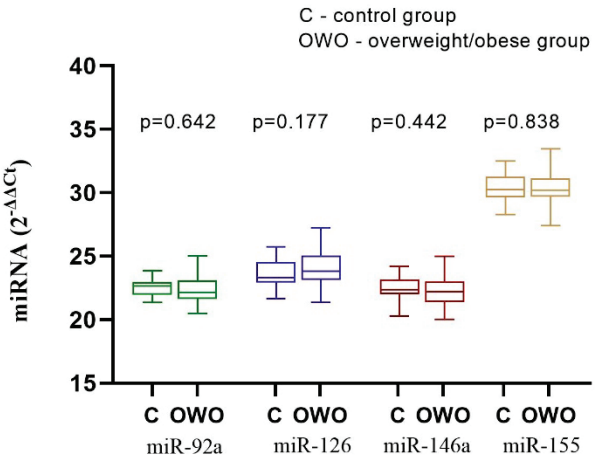

Fig. 1. Circulating miRNA (miR-92a, -126, -146a, -155) levels in adolescents and young adults with normal weight (C group) and in participants with overweight/obesity (OW/O). Groups were compared using Student´s *t*-test (for the data with normal distribution) and Mann-Whitney U-test (for data that were not normally distributed).

Table 2. Correlations between miRNAs levels and anthropometric, clinical and biochemical characteristics in the entire study population.

	miR-92a		miR-126		miR-146a		miR-155	
	R[a]	p	R[a]	p	R[a]	p	R[a]	p
Anthropometry								
Height (cm)	0.021	0.873	-0.025	0.850	0.016	0.903	-0.011	0.934
Weight (kg)	-0.077	0.556	-0.037	0.779	-0.116	0.377	0.026	0.844
BMI (kg/m^2)	-0.110	0.404	-0.051	0.699	-0.179	0.171	-0.007	0.958
Fat mass (%)	0.112	0.397	0.211	0.109	-0.017	0.899	0.148	0.263
WHR (-)	-0.142	0.287	0.051	0.704	0.098	0.466	-0.007	0.958
VFA (cm^2)	-0.087	0.514	0.051	0.704	-0.100	0.452	0.066	0.619
WHtR (-)	-0.213	0.108	0.007	0.958	-0.147	0.271	0.034	0.800
Endothelial function								
RHI	0.192	0.142	0.204	0.118	**0.277**	**0.032**	0.006	0.964
Cytokines and adipocytokines								
Adiponectin (µg/ml)	**-0.263**	**0.044**	**-0.381**	**0.003**	**-0.442**	**0.000**	-0.082	0.537
Leptin (µg/ml)	0.055	0.676	0.069	0.600	-0.090	0.494	0.092	0.484
IL-6 (pg/ml)	-0.200	0.125	-0.212	0.104	**-0.344**	**0.007**	-0.118	0.369
TNF-α (pg/ml)	-0.046	0.727	-0.074	0.574	-0.188	0.150	0.017	0.897
Lipid profile and glucose metabolism								
Total cholesterol (mmol/l)	-0.109	0.437	-0.246	0.076	-0.286	0.038	-0.147	0.294
HDL-cholesterol (mmol/l)	0.127	0.365	-0.073	0.603	0.023	0.870	-0.169	0.200
LDL-cholesterol (mmol/l)	-0.130	0.355	-0.228	0.101	-0.182	0.191	-0.134	0.339
Glucose (mmol/l)	**-0.321**	**0.019**	**-0.426**	**0.001**	**-0.340**	**0.013**	-0.184	0.187
Cortisol (mmol/l)	-0.216	0.120	-0.219	0.115	-0.248	0.073	-0.090	0.522
Insulin (mU/l)	-0.104	0.459	0.024	0.865	-0.132	0.346	0.094	0.503
HOMA-IR	-0.149	0.287	-0.051	0.717	-0.200	0.151	0.056	0.690

Abbreviations: BMI: body mass index, WHR: waist to hip ratio, VFA: Visceral Fat Area; WHtR: waist to hight ratio; RHI – reactive hyperemia index; IL-6: interleukin 6; TNF-α: tumor necrosis factor alpha; HDL-C: high-density lipoprotein cholesterol, LDL-C: low-density lipoprotein cholesterol, HOMA-IR: homeostatic model assessment for insulin resistance; miR: micro-RNA. [a] Correlation coefficient R values are Spearman´s or Pearson´s correlation coefficients, *** p<0.001; ** p<0.01; * p<0.05.

Table 3. Correlations between miRNAs levels and anthropometric, clinical and biochemical characteristics analyzed separately for female (F) and male (M) subgroups.

	miR-92a				miR-126				miR-146a				miR-155			
	F		M		F		M		F		M		F		M	
	R^a	p	R^a	p	R^a	p	R^a	p	R^a	p	R^a	p	R^a	p	R^a	p
Anthropometry																
Height (cm)	0.297	0.111	0.160	0.398	0.092	0.629	0.118	0.535	-0.003	0.988	0.213	0.258	0.224	0.234	0.048	0.801
Weight (kg)	-0.196	0.300	0.084	0.657	0.022	0.907	-0.058	0.761	-0.319	0.086	-0.013	0.945	0.179	0.344	0.009	0.961
BMI (kg/m^2)	**-0.370**	**0.044***	0.066	0.729	-0.034	0.859	-0.050	0.793	**-0.363**	**0.049***	-0.063	0.743	-0.015	0.937	0.022	0.908
Fat mass (%)	-0.320	0.091	0.144	0.449	0.124	0.522	0.159	0.401	-0.252	0.187	0.062	0.746	0.130	0.501	0.125	0.510
WHR (-)	0.072	0.710	-0.106	0.586	0.202	0.293	0.070	0.718	0.062	0.749	0.129	0.505	0.078	0.688	0.030	0.879
VFA (cm^2)	-0.208	0.280	0.028	0.883	0.156	0.420	0.002	0.992	-0.226	0.239	-0.028	0.883	0.210	0.274	-0.002	0.992
WHtR (-)	-0.357	0.057	-0.112	0.563	0.039	0.840	-0.034	0.861	-0.292	0.124	-0.008	0.967	0.072	0.711	0.024	0.902
Endothelial function																
RHI	0.195	0.302	0.257	0.170	0.262	0.162	0.173	0.361	0.310	0.095	0.261	0.164	-0.046	0.809	0.087	0.648
Cytokines and adipocytokines																
Adiponectin (μg/ml)	**-0.414**	**0.023***	-0.264	0.166	**-0.386**	**0.035***	**-0.434**	**0.019***	**-0.459**	**0.011***	**-0.496**	**0.006****	-0.231	0.219	-0.028	0.885
Leptin (μg/ml)	-0.055	0.773	-0.060	0.753	0.129	0.497	0.014	0.941	-0.122	0.521	-0.057	0.765	0.124	0.514	0.018	0.925
IL-6 (pg/ml)	-0.173	0.361	-0.288	0.123	0.059	0.757	**-0.455**	**0.012***	-0.202	0.284	**-0.433**	**0.017***	0.169	0.372	**-0.403**	**0.027***
TNF-α (pg/ml)	0.127	0.504	-0.094	0.620	0.217	0.249	-0.314	0.091	-0.039	0.838	-0.289	0.121	0.171	0.366	-0.164	0.388
Lipid profile and glucose metabolism																
Total cholesterol (mmol/l)	-0.081	0.696	-0.224	0.261	-0.282	0.163	-0.303	0.124	-0.349	0.080	-0.259	0.192	-0.283	0.161	-0.095	0.639
HDL-cholesterol (mmol/l)	0.054	0.793	0.210	0.293	-0.193	0.345	0.050	0.804	-0.029	0.889	-0.004	0.986	-0.234	0.250	-0.001	0.995
LDL-cholesterol (mmol/l)	0.170	0.406	-0.139	0.489	-0.143	0.486	-0.203	0.310	-0.173	0.398	-0.044	0.827	-0.317	0.115	-0.002	0.992
Glucose (mmol/l)	-0.156	0.447	**-0.435**	**0.023***	-0.280	0.166	**-0.553**	**0.003****	-0.178	0.384	**-0.426**	**0.027***	-0.165	0.421	-0.159	0.427
Cortisol (mmol/l)	-0.121	0.558	-0.339	0.084	-0.195	0.341	-0.368	0.059	-0.243	0.232	-0.271	0.172	0.199	0.330	-0.318	0.106
Insulin (mU/l)	-0.148	0.471	-0.153	0.446	0.133	0.517	-0.073	0.717	-0.182	0.374	-0.040	0.843	0.058	0.778	0.097	0.630
HOMA-IR	-0.160	0.435	-0.184	0.358	0.088	0.669	-0.143	0.477	-0.206	0.313	-0.106	0.599	0.047	0.820	0.083	0.681

Abbreviations: BMI: body mass index, WHR: waist to hip ratio, VFA: Visceral Fat Area; WHtR: waist to height ratio; RHI – reactive hyperemia index; IL-6: interleukin 6; TNF-α: tumor necrosis factor alpha; HDL-C: high-density lipoprotein cholesterol, LDL-C: low-density lipoprotein cholesterol, HOMA-IR: homeostatic model assessment for insulin resistance; miR: micro-RNA; F: female; M: male. [a] Correlation coefficient R values are Spearman´s or Pearson´s correlation coefficients, *** p<0.001; ** p<0.01; * p<0.05.

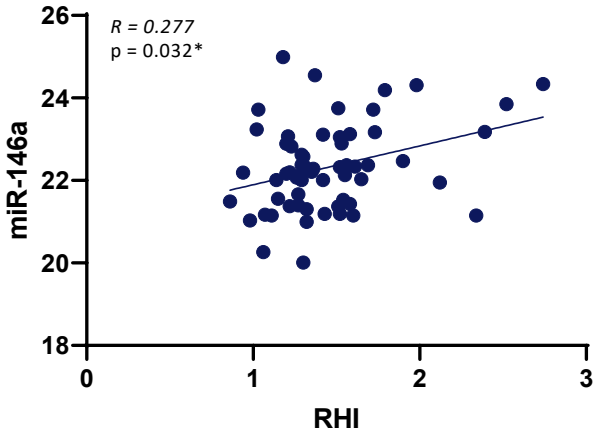

Fig. 2. Scatterplot of miR-146a level vs. RHI index. RHI: reactive hyperemia index; miR: microRNA. Spearman´s correlation analysis result is presented, * indicates a statistically significant correlation.

Discussion

In this study, we evaluated the relationships among the plasma concentration of selected miRNAs, endothelial function assessed by a clinical-physiological method, and other factors involved in the pathogenesis of atherosclerosis in adolescents and young adults with overweight and obesity – i.e. during the early subclinical stages of obesity-related complications.

The main findings of our study include:

- no significant differences in plasma levels of the selected miRNAs between C and OW/O group,
- a significant correlation between RHI and plasma level of miR-146a in the entire study population,
- stronger associations between miRNAs and several assessed parameters (adipocytokines, cytokines and fasting glycemia) in men compared to women.

The present study found no difference in RHI between the OW/O and C groups, indicating similar endothelial function in both groups. However, previous studies in humans have shown that obesity is independently associated with ED. Studies applying RH-PAT method in adults with obesity demonstrated impaired endothelial function expressed by decreased RHI values [23], followed by a significant increase in RHI after weight loss [24]. This is in agreement with previous studies that have used the flow mediated dilation method (FMD), based on the measurement of postocclusion brachial artery diameter using ultrasound imaging to assess endothelial function in adults with obesity [25]. FMD and RH-PAT are both similar and effective noninvasive methods for evaluating vascular endothelial function. Reduced values of FMD indicate ED associated with reduced NO bioavailability. We found several studies comparing FMD values in children and adolescents or adults with overweight/obesity. Generally, FMD is decreased in children and adolescents with obesity, mostly in patients with visceral obesity. FMD improves after obesity treatment [26,27]. However, the studies measuring RHI in children and adolescents have shown inconsistent results. Although several studies demonstrated significantly lower RHI values in adolescents with obesity and an inverse relation of RHI with percentage of fat mass [28,29], other studies did not confirm these associations [30,31]. In accordance with previous studies employing RHI, we did not find significant differences in RHI values in the OW/O group and the normal-weight group. This may indicate a well-preserved endothelial function in young patients with obesity. Alternatively, this result could suggest that the RH-PAT method may have insufficient sensitivity to detect impaired endothelial function in young patients with obesity.

Interestingly, we demonstrated a novel finding: a positive association of circulating miR-146a with RHI as a marker of endothelial function in adolescents and young adults. This indicates that higher levels of miR-146a were associated with increased RHI values in young subjects. Because miR-146a has been found to be associated with inflammation [17] and oxidative stress [32], and considering the important role of these processes in the initiation and progression of atherosclerosis, we suggest that the positive correlation of miR-146a with RHI could be attributed to these associations.

To the best of our knowledge, there is only one previous study [16] that focused on the association between plasma levels of a selected miRNA (miR-126) and RHI. In this study, the authors observed a concomitant increase of RHI and miR-126 level as a result of weight loss in adolescents with obesity. Furthermore, an experimental study in mice demonstrated a proatherogenic effect of miR-92a, preferentially expressed in endothelial cells promoting inflammation and ED [15]. Regarding miR-155, its role in the atherosclerotic process and inflammation has been demonstrated in humans [18]. However, in our study, we did not find evidence of an association between miR-126, miR-92 or miR-155 and RHI as a marker of endothelial function.

A growing body of research indicates that

circulating miRNAs are closely associated with the presence and severity of obesity in adults, adolescents, and children [7,33]. In the present study, there were no significant differences between groups in the plasma concentration of miR-92a, -126, -146a and -155. However, in previous studies, differences in these miRNA levels related to the presence of obesity and their association with pathophysiological processes, including inflammation and altered adipocytokines expression, as summarized below.

MiR-92a plays important roles in various biological processes, including cell proliferation, differentiation, migration, invasion and apoptosis [34]. It has been demonstrated to be associated with the development of cardiovascular diseases through the regulation of inflammation [15] and was proposed as a promising biomarker of metabolic status (glucose control) in obesity as reported by Cereijo *et al.* [35]. This miRNA was upregulated in adults with obesity compared to healthy lean individuals [36]. On the contrary, down-regulation of miR-92a in morbidly obese patients is associated with the presence of metabolic syndrome [37], suggesting that miR-92a can play a role in obesity and its related metabolic disorders. Our results showed no differences in concentration of miR-92a between the OW/O and control groups, indicating unchanged miRNA levels in young patients with obesity without comorbidities.

MiR-126 participates in inflammation, and contributes to the development and progression of atherosclerosis. Antiinflammatory and antiatherogenic effects of miR-126 were demonstrated in a murine model of accelerated atherosclerosis [38] and in elderly humans [39]. These findings are in agreement with an *in vitro* study on endothelial cells and fibroblasts [40,41]. Interestingly, the level of miR-126 was found to be increased in children with obesity [42] but decreased in middle-aged adults with obesity [19], suggesting age-related differences in its expression associated with obesity. Our results did not reveal any difference in miR-126 level between the OW/O and control groups. In line with previous studies, the role of miR-126 in the inflammatory response might explain the negative correlation between miR-126 and IL-6 observed in the present study.

MiR-146a is an inflammation associated miRNA inhibiting the expression of the inflammatory cytokines (IL-6, IL-1β, TNF-α) [17] suggesting its antiinflammatory effect. The association of obesity with miR-146a levels is inconsistent, as studies have reported both increased and decreased levels [43,44] in adults. Similarly, variable results were found in children and adolescents [45]. Furthermore, miR-146a upregulation is associated with cellular aging and oxidative stress [32], suggesting that the increased level of this miRNA in obese patients could be a response to, rather than a cause of inflammation. Although we did not find significant differences in plasma levels of miR-146a between patients with OW/O and controls, we observed a significant negative correlation between plasma levels of miR-146a and IL-6. This is in agreement with the concept of miR-146a as an antiinflammatory factor.

MiR-155 is implicated in the regulation of various physiological processes including inflammation and adipogenesis. The altered expression of miR-155 has been associated with several inflammation-related pathological states, including cancer, atherosclerosis, various cardiovascular diseases and obesity [18,46]. MiR-155 function was mostly assessed in adipocytes. The up-regulation of miR-155 was demonstrated in adipose tissue in obesity and its level was positively correlated with TNF-α and BMI, suggesting a role of miR-155 as a proinflammatory factor [18]. The results indicate that TNFα together with ox-LDL stimulate the transcription of miR-155 [47]. The relation between plasma level of miR-155 and the obesity status is dependent on age. While in older adults lower levels of miR-155 were reported, in younger adults increased miR-155 plasma levels were found [48,49]. Although, we found no significant correlation between miR-155 and TNF-α, the present study showed a significant negative association between miR-155 and inflammatory cytokine (IL-6), but only in men. Contrary to our findings, a study in adults with obesity did not show an association between plasma levels of miR-155 with cytokines (IL-6, IL-8) [50]. However, in this study no sex specific analysis of this relationship was performed.

Considering the comparable number of subjects in our study in the context of other studies in this field, we suggest that the lack of significant differences does not indicate that this study is underpowered. In general, it could be concluded that the investigated age group (young subjects with obesity and overweight without comorbidities often present in adults with obesity) did not exhibit detectable differences in the levels of the assessed miRNAs. It could be expected that the changes observed in adults with obesity occur only after several years of obesity and the development of its complications.

Considering the differences in fat distribution

and the influence of sex hormones, it is possible that associations between target miRNAs and evaluated parameters may differ by sex. Recent clinical data have shown significant sex differences in plasma levels of inflammatory markers in prepubertal children [51]. Accordingly, we divided the study sample by sex and performed a separate analysis of associations between miRNAs and selected anthropometric markers, adipocytokines, cytokines and markers of lipid profile. Sex differences have been associated with the development of obesity and associated comorbidities, especially in adults [52,53]. However, evidence on sex differences in miRNA expression in relation to cardiometabolic risk factors in children is lacking. We found more significant correlations in male subjects, suggesting that sex differences in the pathogenesis of overweight or obesity-related complications are already present in adolescence. This supports the concept that to optimize future clinical application, the cardiometabolic risk assessment in young patients with overweight/ obesity should integrate multiple markers, with different weighing for women and men.

Sex-related differences were also observed in the relationship between miRNA levels and indices related to glucose metabolism: three out of four microRNAs assessed in our study (miR-92a, miR-126 and miRNA-146a) were negatively correlated with fasting glycemia in male subjects only. It is well known that miRNAs play an important role in the regulation of glucose metabolism [54]. The positive association between serum levels of miR-92 and impaired glucose control, characterized by hyperinsulinemia, glycated hemoglobin and increased insulin resistance, were found in patients with obesity [34]. In contrast, a decreased plasma level of miR-126 was observed in patients with diabetes mellitus type 2 [55]. Previous studies have suggested potentially bidirectional interactions between miRNAs and blood glucose levels. While high glucose concentrations had no influence on the levels of pro-inflammatory miRNAs (miR-92a and -155), the levels of anti-inflammatory miRNAs (miR-126 and miR-146a) were significantly reduced in response to high glucose concentrations [42,56]. From our observational study, it is not possible to explain the mechanisms behind the observed associations. Nevertheless, future studies should investigate the effects of various miRNAs on blood glucose metabolism in more detail, considering potential sex-related differences.

Study limitations

Firstly, the sample size in our study was relatively small to generalize the results to the general population. Therefore, we cannot exclude the possibility of weaker associations and Type II statistical errors (false negative conclusions from statistical analysis). For future studies, it is recommended that the observed differences and associations should be confirmed in a larger study sample.

Secondly, we identified sex differences in the associations between plasma concentrations of miRNAs and other parameters. Numerous studies have confirmed the role of sex hormones in the immune response and significant sex differences in inflammatory markers also in prepubertal children. However, we did not investigate the association of sex hormones with the observed sex differences, to confirm their role in this relationship.

Lastly we did not determine plasma C-reactive protein level, often used as a marker reflecting the degree of inflammation. It would also be appropriate to analyze other markers of the inflammatory process in plasma samples from participants with overweight/obesity, although they were not available in our study. All these limitations should be considered when interpreting the results obtained in our study.

Conclusions

Although we did not find a significant effect of obesity on the levels of four miRNAs related to inflammation and endothelial function in young subjects, we did observe an association between miR-146a and endothelial function quantified by RHI. Several observed correlations suggest a potential role of miRNAs in inflammation, atherosclerotic process and glycemic control in males. The lack of significant associations among adipocytokines, cytokines, miRNAs, and other markers related to obesity and its complications could be attributed to the early stage of the atherosclerotic process present in adolescents and young adults with obesity. We suggest that a better understanding of the pathophysiological processes underlying the development of atherosclerosis could identify markers that enable the identification of patients at increased risk of more rapid atherosclerosis progression.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

The study was supported by grants VEGA 1/0283/21.

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