Circulating miR-15b, Annexin A1, procalcitonin and interleukin-6 levels differentiate children with metabolically unhealthy obesity from those with metabolically healthy obesity: A case-control study

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Abstract. The present study assessed serum miR-15b, Annexin A1, procalcitonin, and interleukin-6 (IL-6) levels in children with metabolically healthy obesity (MHO) and metabolically unhealthy obesity (MUO) and compared them to these levels in a non-obese healthy control group. It also tested the ability of each of these parameters to early differentiate children with MUO from those with MHO. The present study included 620 children [434 males (70%) and 186 females (30%); aged 9-15 years] divided into the following groups: G1, healthy non-obese controls (n=200); G2, MHO (n=246); G3, MUO (n=174). Serum miR-15b, Annexin A1 procalcitonin, IL-6, and other metabolic parameters levels were measured, and clinical examinations were conducted for all of the children. After testing the normality of the variable, Kruskal-Wallis one-way-ANOVA, and Spearman correlation coefficients were used. The area under the receiver operating characteristic curve (AUC) was determined to test the variable's ability to differentiate MUO from MHO. miR-15b, procalcitonin, and IL-6 levels were significantly higher while Annexin A1 levels were significantly lower in G2 and G3 when compared to G1, and in G3 when compared to G2. These levels were positively correlated (Annexin A1 was negatively correlated) with body mass index (BMI) and waist circumference percentiles, and with serum levels of LDL-cholesterol, glucose, HbA1c, insulin, and C-reactive protein (CRP) and with the homeostasis model of insulin resistance (HOMA-IR). The AUC was 0.92, 0.84, 0.82, and 0.67 for miR-15b, Annexin A1, procalcitonin, and IL-6, respectively. In conclusion, determination of serum miR-15b, Annexin A1, and procalcitonin levels could differentiate children with MUO from those with MHO. This may help the early management of these cases and their accompanying complications.

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Abbreviations: MHO, metabolically healthy obesity; MUO, metabolically unhealthy obesity; BMI, body mass index; ANOVA, analysis of variance; RNA, ribonucleic acid; IDF, International Diabetes Federation; WC, waist circumference; ROC, receiver operating characteristic; AUC, area under the ROC curve; LDL-c, low-density lipoprotein cholesterol; HDL-c, high-density lipoprotein cholesterol; TAG, triacylglycerol; HbA1c%, glycosylated hemoglobin percentage; T2DM, type 2 diabetes mellitus; WHO, World Health Organization; HOMA-IR, homeostasis model of insulin resistance; TNF-α, tumor necrosis factor-α; IL-6, interleukin-6; CRP, C-reactive protein; RNF, ring finger protein; FPR2, formyl-peptide receptor-2; ELISA, enzyme-linked immunosorbent assay

Key words: miR-15b, Annexin A1, procalcitonin, interleukin-6, metabolically healthy obesity, metabolically unhealthy obesity

Introduction

The increasing prevalence of childhood obesity in the current century has made it an alarming worldwide health issue. Around the world, currently more than 43 million children are reported to be obese or overweight (1). The condition is recognizable mainly in developed and developing countries (1,2).

The mechanisms involved in the progression of childhood obesity and its complications are still undetermined which hinders the timely management of these conditions (3,4). Some obese individuals may have a significantly low risk to develop cardiometabolic complications [metabolically

healthy obesity (MHO)] when compared with others [metabolically unhealthy obesity (MUO)] (5,6). Blüher reported that cases with MHO have higher insulin sensitivity and less obesity-associated inflammation than those with MUO (6). Some other studies reported that MHO is not a strictly cardio-metabolic benign condition, and individuals with MHO still have an increased risk of developing type 2 diabetes mellitus (T2DM) and atherosclerosis than healthy lean individuals (6,7). In addition, obesity is considered an inflammatory condition that is associated with an increase in adipocyte-derived adipokines such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and C-reactive protein (CRP) (8). The control of these inflammations may slow down the progression of obesity-associated complications (8). To understand these inflammations and other consequences of obesity, many previous studies have been conducted on microRNAs and other inflammatory-related biomarkers such as Annexin A1, procalcitonin, and IL-6 (9,10).

MicroRNAs (miRNAs/miRs) are small non-coding ribonucleic acids that can modify the process of adipogenesis and its related complications (9). In this regard, miR-15b expression was reported in many previous studies to correlate with insulin resistance and inflammatory markers in obese individuals (10-12).

Annexin A1 (lipocortin-1, 37 kDa) is a protein that resolves inflammations by diminishing the production of pro-inflammatory cytokines in neutrophils (13). It can enhance the apoptosis of neutrophils and suppress the process of adipogenesis (13,14). Studies that have investigated the levels of Annexin A1 in obese and/or type 2 diabetic cases have revealed contradicting results (14-16).

Procalcitonin is secreted mainly by thyroid C cells and adipose tissues and is considered a marker for systemic inflammation (17). Obese individuals were reported to have high procalcitonin levels that were correlated with body mass index (BMI) and insulin resistance (17,18).

IL-6 controls diverse immune, inflammatory, and metabolic processes through a complex pathway that requires incorporation between a variety of cells and tissues. Its levels were reported by many studies to be higher in obese individuals than in non-obese controls (19-21).

Since the current differentiation of patients with MUO from those with MHO depends on the development of major clinical signs (22), which is considered late, the objectives of the present study were to measure serum miR-15b, Annexin A1, procalcitonin, and IL-6 levels in children with MHO and MUO and to compare them to those of a non-obese healthy control group. The study also tested the ability of each of these parameters to early differentiate children with MUO from those with MHO which may help the timely management of these cases and their accompanying complications.

Patients and methods

The present work was conducted in three primary health care centers in Unaizah, Qassim area, Saudi Arabia after being reviewed and approved by the Qassim University Medical Ethics Board (approval # mduc-2019-2-2-I-5441). All procedures performed were in accordance with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed written consents were obtained from all children's guardians.

Calculation of sample size. The formula $(n)=(r+1/r)\ (\sigma^2)\ (Z_\beta+Z\alpha/2)^2/(\text{difference})^2$ was used to calculate the sample size (r is the ratio of healthy non-obese children to obese children; σ is the estimated standard deviation; Z_β is the desired power (typically 0.84 for 80% power); and $Z\alpha/2$ is the desired statistical significance level (1.96). The difference was set at 5. Thus, a sample size of at least 171 participants in each arm was used.

Participants. This case-control study included 620 consecutive children [434 males (70%) and 186 females (30%)] who visited the primary health care centers in Unaizah governorate, Qassim area, Saudi Arabia between January 2019 and October 2021. Their ages ranged between 9 and 15 years (mean age, 12.6±1.5). Medical histories were collected, and examinations were conducted for all children. The children's waist circumference (WC) percentile and body mass index (BMI) percentile were calculated, and a child was considered obese when his/her WC was ≥90th percentile and BMI was ≥95 percentile for age and sex (1). Children were considered to have MUO when their WC was ≥90th percentile and presented with one or more of the following findings (22): a) Serum triacylglycerol (TAG) ≥150 mg/dl; b) serum high-density cholesterol (HDL-c) (<40 mg/dl); c) blood pressure ≥90th percentile (age, sex, and height) or systolic blood pressure ≥130 mm Hg or diastolic blood pressure ≥85 mm Hg when the age was between 10 and 16 years; d) fasting blood glucose ≥100 mg/dl.

Exclusion criteria included children with T2DM, T1DM, genetic or endocrinal disorders, inflammatory, or any other general acute or chronic illness (3,4).

The eligible children were divided into a healthy non-obese control group [G1, n=200 (32.3%)] and obese group [n=420 (67.7%)]. According to the classification of international diabetes federation (IDF) (22); the obese group was further subdivided into 2 subgroups; G2 [children with MHO, n=246 (39.7%)] and G3 [children with MUO, n=174 (28.0%)].

Sampling and laboratory analysis. Blood (5 ml) was withdrawn from the antecubital vein of all of the children after overnight fasting. One milliliter was used to measure glycosylated hemoglobin percentage (HbA1c%) in the whole blood immunoturbidimetrically by an autoanalyzer (COBAS INTEGRA 400, Roche Diagnostic). The remaining portion was left to be clotted and then centrifuged for 10 min at 3,000 x g, and the sera were collected in aliquots and stored at -80˚C until assay. Fasting serum glucose was measured by glucose oxidase activity high-density assay kit (ab219924; Abcam). Serum total cholesterol (TC), high density lipoprotein-cholesterol (HDL-c), and triacylglycerol (TAG) were measured calorimetrically by corresponding kits (-Spectrum Diagnostics; cat. nos. 230003, 266002t and 314003 respectively). Low density lipoprotein-cholesterol (LDL-c) was calculated by the Friedewald equation (LDL-c equals $TC-(HDLc+\frac{TAG}{5})$. Insulin levels were measured using an insulin assay kit (cat. no. INS31-K01; Eagle Biosciences, Inc.), and serum CRP was assayed using the Human C-Peptide ELISA kit (cat. no. EZHCP-20K; Sigma-Adrich; Merck KGaA). The homeostasis model of insulin resistance (HOMA-IR) was calculated

as described by Matthews *et al* (23). The case was considered as being insulin-sensitive when HOMA-IR was >1.9, early insulin resistant when HOMA-IR was 1.9 to >2.9, and significant insulin resistance when HOMA-IR was ≥2.9 (23).

Serum Annexin A1 levels were measured using the Human Annexin A1 ELISA (cat. no. ELH-ANXA1-1; RayBiotech Life) with detection range 1.64-400 ng/ml, and coefficient of variation 4.0 and 5.3% for intraassay and interassay, respectively, according to the manufacturer's protocols. Serum levels of procalcitonin were assayed using the human procalcitonin ELISA kit (cat no. ab100630; Abcam) and coefficient of variation 5.0 and 6.0% for intraassay and interassay, respectively. Human IL-6 Quantikine HS ELISA Kit (R&D Systems, Inc.) was used to measure serum IL-6 levels with assay range 0.2-10 pg/ml and coefficient of variation 3.4 and 5.2% for intraassay and interassay, respectively.

RNA extraction. Serum miRNAs were extracted and purified by the miRNeasy kit (Ambion® miRNA Isolation Kit, cat. no. K157001; Thermo Fisher Scientific, Inc.). The serum was transferred into a spine cartridge with a collection tube, centrifuged for 1 min at 12,000 x g and then 700 μl of 96-100% ethanol was added and mixed well by vertexing. An amount 700 μl of the mixed sample was then transferred to a new spin cartridge and centrifuged again for 1 min at 12,000 x g. Wash buffer (500 μl) was used to wash the spin cartridge with centrifugation at 12,000 x g for 1 min (this step was repeated twice). Next, 50-100 μl of RNase-free water was added and the sample was incubated for 1 min before being centrifuged for 1 min at the maximum velocity. The spine cartridge was then removed and discarded, and the purified RNA was left in the recovery tube, stored at -80˚C until being utilized.

Reverse transcription. For this step, High-Capacity cDNA Reverse Transcription Kit was used (Applied Biosystems, cat. no. 4368814; Thermo Fisher Scientific, Inc.) according to the manufacturer protocol. Ten microliters of 2X reverse transcription master mix was pipetted into the reaction plate wells; 10 μl of RNA sample was added into each well and pipetted up and down to mix them. The plate was then sealed and centrifuged to spin down the contents and to get rid of the air bubbles. The plate was put on ice until being loaded to the thermal cycler (Applied Biosystems; Thermo Fisher Scientific, Inc.) under conditions that were optimized for the high-capacity cDNA RT kits (25˚C for 10 min, 37˚C for 120 min and 85˚C for 5 min).

RT-qPCR. RT-qPCR was conducted using TaqMan® MicroRNA RT Kit (Applied Biosystems; Thermo Fisher Scientific, Inc.). miR-24 was used as an internal control [stem-loop RT primer sequence: 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCTTCTG-3', forward primer: 5'-GCGGCGGTGGCTCAGTACAGC-3' for miR-24, and (universal) reverse primer: 5'-GTGCAGGGTCCGAGGT-3'] during the quantification using specific stem-loop primers for miR-15b (stem-loop RT primer sequence: 5'-GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACTGTAAACC-3'; forward primer: 5'-ATCCAGTGCGTGTCGTG-3', and reverse primer: 5'-TGCTTAGCAGCACATCATG-3') to normalize it as follows: ΔCt=ΔCtmiR-ΔCtmiR-24 (24). The analysis of the results was performed using Sequence Detection Software version 2.3 (Applied Biosystems; Thermo Fisher Scientific, Inc.). The difference in the miR-15b relative expression levels between samples was calculated using the $2^{-\Delta\Delta Cq}$ method (25).

Statistical analysis. The collected data were analyzed by SPSS software (v.26) (IBM Corp). The normality of the quantitative data was tested. Kruskal-Wallis one-way-ANOVA was used to compare data among the three studied groups and the Dunn's post hoc test was conducted for pairwise comparisons after Kruskal-Wallis ANOVA. The correlations between the different studied continuous variable levels were tested by Spearman's correlations coefficient. The receiver operating characteristic curve (ROC) was conducted to test the variable's ability to differentiate children with MUO from those with MHO. P-values ≤0.05 were considered to indicate a significant difference.

Results

Comparison of the different studied variables in G1, G2, and G3. The three groups were age and sex-matched (Table I). There were significantly higher serum miR-15b, procalcitonin, and IL-6 levels while significant lower serum Annexin A1 levels in the children with MHO (G2) and MUO (G3) when compared with the healthy non-obese control group (G1); and children with MUO (G3) when compared with levels in the children with MHO (G2) (Table I). In addition, significantly higher values of BMI and WC percentiles and serum levels of TAG, glucose, HbA1c%, insulin, and CRP were found in children with MHO (G2) and with MUO (G3) when compared with these in the healthy non-obese control group (G1); and in children with MUO (G3) when compared with levels in the children with MHO (G2) (Table I). The percentages of cases with insulin resistance (early and significant) were higher in G3 than G2 and G1 and G2 than G1 (Table I). The systolic and diastolic pressure for all participants were less than 90th percentile (normotensive; data not shown).

Comparison of serum levels of miR-15b, Annexin A1, procalcitonin, and IL-6 in the whole study sample according to the degree of their insulin sensitivity. The participants in the present study were insulin sensitive [n=228 (36.8%)], early insulin resistant [n=290 (46.8%)], and significant insulin resistant [n=102 (16.5%)]. Significant high serum levels of miR-15b, procalcitonin, and IL-6 and low levels of Annexin A1 were found in children with early and significant insulin resistance compared to those who were insulin sensitive; and in children with significant insulin resistance compared to those with early insulin resistance (P<0.001 for all) (Fig. 1A-D).

Correlations of serum miR-15b, Annexin A1, procalcitonin, and IL-6 levels in the whole study sample. The serum levels of miR-15b, procalcitonin, and IL-6 were positively correlated (while the serum levels of Annexin A1 were negatively correlated) with BMI and WC percentiles and with serum levels of LDL-c, TAG, cholesterol, glucose, HbA1c%, insulin, and CRP and with HOMA-IR (Table II). The serum levels of miR-15b, procalcitonin, and IL-6 levels were negatively correlated with

Table I. Comparison of different variables among the healthy non-obese control (G1), children with MHO (G2) and children with MUO (G3).

Variables	Healthy non-obese control (G1; n=200)	Children with MHO (G2; n=246)	Children with MUO (G3; n=174)	P-values			
				G1 vs. G2 vs. G3	G1 vs. G2	G1 vs. G3	G2 vs. G3
Sex, n (%)[a]							
Male	139 (69.5%)	173 (70.3%)	122 (70.1%)	0.982	0.803	0.871	0.991
Female	61 (30.5%)	73 (29.7%)	52 (29.9%)				
Age, mean (years)	12.6±1.3	12.5±1.6	12.6±1.5	0.688	0.380	0.911	0.432
BMI percentile	77.5±7.1	95.9±0.9	97.1±1.5	<0.001[b]	<0.001[b]	<0.001[b]	<0.001[b]
WC percentile	79.2±5.4	93.5±2.1	95.2±2.1	<0.001[b]	<0.001[b]	<0.001[b]	<0.001[b]
HDL-c (mg/dl)	41.2±2.2	41.3±3.7	29.4±7.7	<0.001[b]	0.975	<0.001[b]	<0.001[b]
LDL-c (mg/dl)	99.1±20.4	105.3±27.0	110.4±19.1	<0.001[b]	0.002[b]	<0.001[b]	0.435
TAG (mg/dl)	107.8±19.6	112.7±21.3	142.4±37.1	<0.001[b]	<0.001[b]	<0.001[b]	<0.001[b]
Cholesterol (mg/dl)	164.4±21.2	172.4.4±30.7	177.2±21.1	<0.001[b]	<0.001[b]	<0.001[b]	0.361
Glucose (mg/dl)	86.5±11.5	90.1±18.2	109.9±11.7	<0.001[b]	0.006	<0.001[b]	<0.001[b]
HbA1c (%)	4.6±0.6	5.1±0.9	5.5±0.4	<0.001[b]	<0.001[b]	<0.001[b]	0.009[b]
Insulin (μg/ml)	7.7±1.4	9.0±0.7	10.5±0.8	<0.001[b]	<0.001[b]	<0.001[b]	<0.001[b]
CRP (ng/ml)	2.8±0.8	2.9±0.3	3.0±0.2	<0.001[b]	0.041[b]	<0.001[b]	0.014[b]
HOMA-IR	1.6±0.4	2.4±0.5	2.6±0.5	<0.001[b]	<0.001[b]	<0.001[b]	<0.001[b]
Degree of IS, n (%)[a]							
IS (HOMA-IR <1.9)	138 (69%)	84 (34.1%)	6 (3.4%)	<0.001[b]	<0.001[b]	<0.001[b]	<0.001[b]
Early IR (1.9< HOMA-IR <2.9)	62 (31%)	114 (46.4%)	114 (65.5%)				
Significant IR (HOMA-IR ≥2.9)	0 (0%)	48 (19.5%)	54 (31.1%)				
miR-15bR	3.5±0.6	4.0±0.5	4.4±0.5	<0.001[b]	0.012[b]	<0.001[b]	0.009[b]
Annexin A1 (ng/ml)	177.7±14.5	146.7±54.4	132.8±52.7	<0.001[b]	0.002[b]	<0.001[b]	0.016[b]
Procalcitonin (ng/ml)	0.033±0.019	0.051±0.013	0.065±0.011	<0.001[b]	<0.001[b]	<0.001[b]	0.018[b]
IL-6 (pg/ml)	1.9±0.4	2.3±0.8	2.6±1.0	<0.001[b]	0.037[b]	<0.001[b]	0.032[b]

Unless otherwise indicated, independent-samples Kruskal-Wallis test was used to compare the three groups. [a]Compared by Chi-square test; [b]Value shows a significant result. MHO, metabolic healthy obesity; MUO, metabolic unhealthy obesity; BMI, body mass index; WC, waist circumference; HDL-c, high density lipoprotein cholesterol; LDL-c, low density lipoprotein cholesterol; TAG, triacylglycerol; HbA1c, glycosylated hemoglobin; CRP, C-reactive protein; HOMA-IR, Homeostatic Model Assessment-Insulin Resistance; IR, insulin resistance; IS, insulin sensitive; miR-15bR, miR-15b relative expression; IL-6, interleukin 6.

serum levels of HDL-c, while Annexin A1 levels were positively correlated with serum levels of HDL-c (Table II).

The serum miR-15b levels were positively correlated with serum levels of procalcitonin (0.544, P<0.001), and IL-6 (0.261, P<0.001) while negatively correlated with serum Annexin A1 levels (-0.265, P<0.001) (data not shown).

The serum Annexin A1 levels were negatively correlated with serum levels of procalcitonin (-0.477, P<0.001) and IL-6 (-0.331, P<0.001) (data not shown).

Serum procalcitonin levels were positively correlated with serum IL-6 levels (0.441, P<0.001) (data not shown).

Correlations of serum miR-15b, Annexin A1, procalcitonin, and IL-6 levels in the three studied groups

Serum miR-15b levels in the healthy control group (G1). Serum miR-15b levels in the healthy control group (G1) were positively correlated with BMI and WC percentiles and with serum levels of LDL-c, TAG, TC, glucose, and HbA1C% and were negatively correlated with HDL-c (Table III). Its correlations with serum levels of Annexin A1, procalcitonin, and IL-6 were non-significant (Fig. 2A-C).

Serum miR-15b levels in children with MHO (G2) and MUO (G3). Serum miR-15b levels in children with MHO (G2) and MUO (G3) were positively correlated with BMI and WC percentiles and serum levels of LDL-c, TAG, TC, glucose, HbA1c%, insulin, and CRP and with HOMA-IR while correlated negatively with HDL-c (Table III). In addition, serum miR-15b levels were positively correlated with serum levels of procalcitonin and IL-6 (Fig. 2B and C). In contrast, they were negatively correlated with serum Annexin A1 levels (Fig. 2A).

Serum Annexin A1 levels in the healthy control group (G1). Serum Annexin A1 levels in the healthy control group (G1) showed non-significant correlations with all studied metabolic and anthropometric parameters (Table III) and, with serum levels of procalcitonin and IL-6 (Fig. 3A and B).

Serum Annexin A1 levels in children with MHO (G2) and MUO (G3). Serum Annexin A1 levels in children with MHO (G2) and MUO (G3) were negatively correlated with BMI and WC percentiles and with serum levels of LDL-c, TC, glucose, HbA1c%, insulin, and CRP and with HOMA-IR (Table III) and with serum levels of procalcitonin and IL-6 (Fig. 3A and B). They were also negatively correlated with TAG in G3 (Table III). On the other hand, Annexin A1 levels were correlated positively with serum HDL-c levels in both groups (Table III).

Serum procalcitonin levels in the healthy control group (G1). Serum procalcitonin levels in the healthy control group (G1) were positively correlated with serum levels of LDL-c, TAG, TC, glucose, and HbA1c% and with HOMA-IR (Table III).

Serum procalcitonin levels in children with MHO (G2) and MUO (G3). Serum procalcitonin levels in children with MHO (G2) and MUO (G3) were positively correlated with BMI and WC percentiles and serum levels of LDL-c, TAG, TC, glucose, HbA1c%, insulin, and CRP and with HOMA-IR while negatively correlated with HDL-c (Table III). In addition, they were

Table II. Correlations of serum miR-15b, Annexin A1, procalcitonin and IL-6 levels with anthropometric and metabolic parameters in the whole study sample.

Variables	Correlation/ significance	BMI percentile	WC percentile	HDL-c (mg/dl)	LDL-c (mg/dl)	TAG (mg/dl)	Cholesterol (mg/dl)	Glucose (mg/dl)	HbA1c (%)	Insulin (μg/ml)	CRP (ng/ml)	HOMA-IR
miR-15bR	r_s	0.686	0.558	-0.653	0.345	0.450	0.335	0.516	0.486	0.484	0.287	0.540
	P-value	<0.001	<0.001[a]	<0.001[a]	<0.001[a]	<0.001[a]	<0.001[a]	<0.001[a]	<0.001[a]	<0.001[a]	<0.001[a]	<0.001[a]
Annexina1 (ng/ml)	r_s	-0.498	-0.575	0.311	-0.280	-0.306	-0.250	-0.381	-0.402	-0.473	-0.317	-0.475
	P-value	<0.001	<0.001[a]	<0.001[a]	<0.001[a]	<0.001[a]	<0.001[a]	<0.001[a]	<0.001[a]	<0.001[a]	<0.001[a]	<0.001[a]
Procalcitonin (ng/ml)	r_s	0.708	0.672	-0.638	0.368	0.516	0.360	0.608	0.521	0.659	0.385	0.666
	P-value	<0.001	<0.001[a]	<0.001[a]	<0.001[a]	<0.001[a]	<0.001[a]	<0.001[a]	<0.001[a]	<0.001[a]	<0.001[a]	<0.001[a]
IL-6 (pg/ml)	r_s	0.400	0.402	-0.304	0.280	0.273	0.235	0.384	0.339	0.337	0.308	0.405
	P-value	<0.001	<0.001[a]	<0.001[a]	<0.001[a]	<0.001[a]	<0.001[a]	<0.001[a]	<0.001[a]	<0.001[a]	<0.001[a]	<0.001[a]

[a]Value indicates a significant result. r_s, Spearman's correlation coefficient; BMI, body mass index; WC, waist circumference; HDL-c, high density lipoprotein cholesterol; LDL-c, low density lipoprotein cholesterol; TAG, triacylglycerol; HbA1c, glycosylated hemoglobin; CRP, C-reactive protein; HOMA-IR, Homeostatic Model Assessment-Insulin Resistance; miR-15bR, microRNA-15b relative expression; IL-6, interleukin 6.

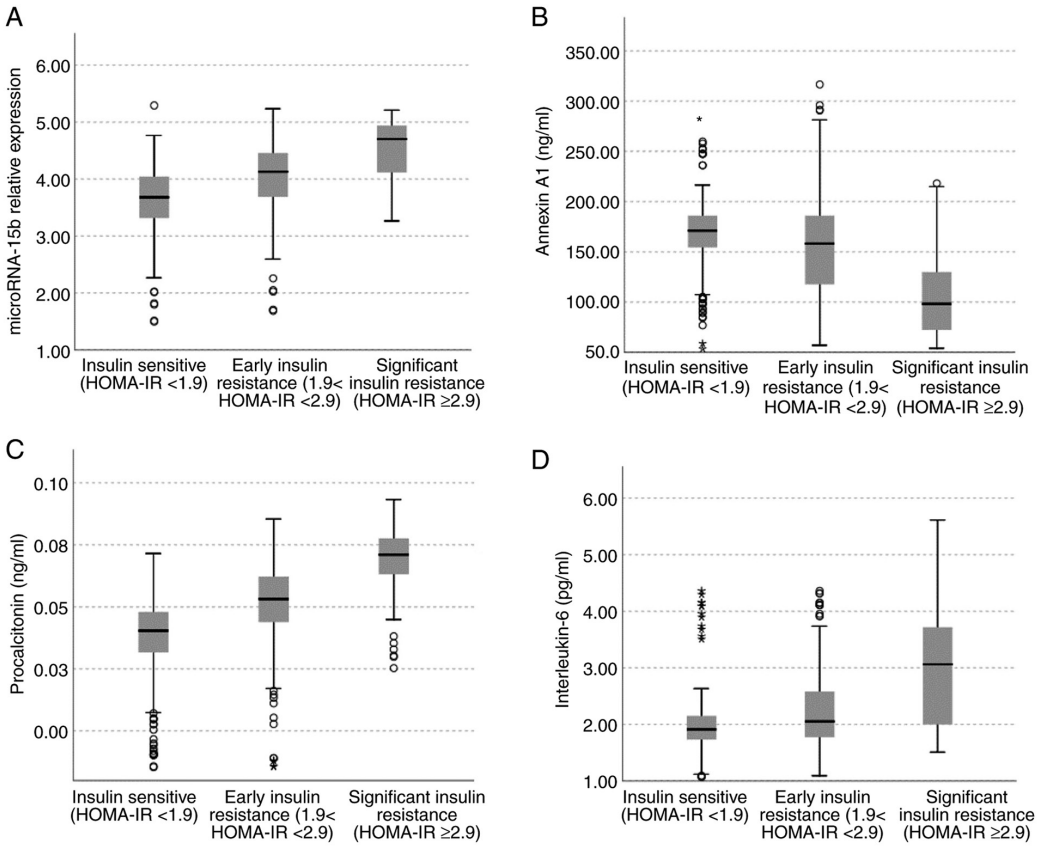

Figure 1. Comparison of (A) microRNA-15b (relative expression), (B) serum Annexin A1, (C) procalcitonin and (D) interleukin-6 levels in the whole sample according to the degree of insulin-resistance by independent-samples Kruskal-Wallis test. The P-value was found to be significant for all at ≤0.001). (*) indicates the outliers. HOMA-IR, homeostasis model of insulin resistance.

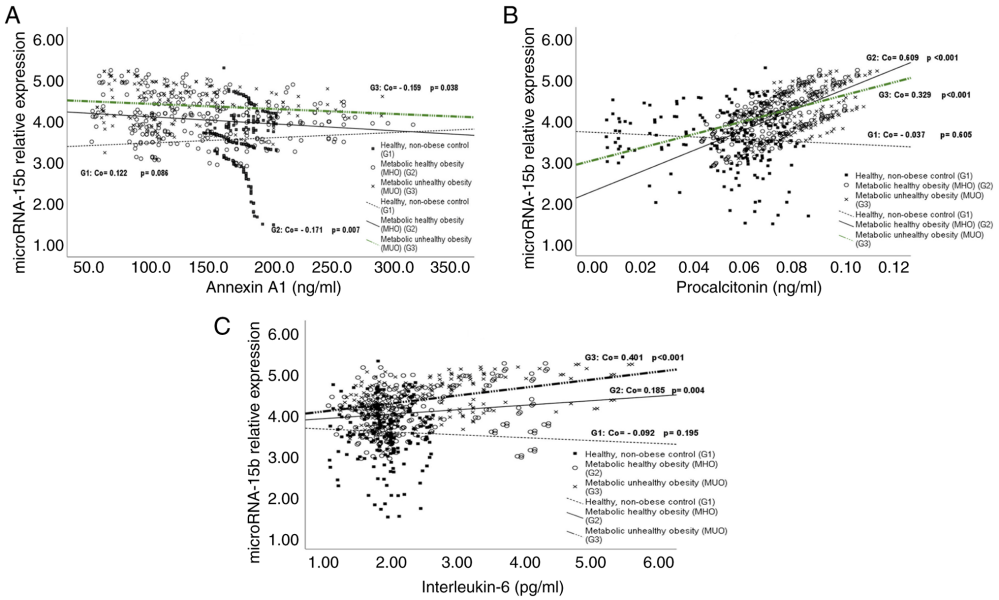

Figure 2. Correlations of circulating microRNA-15b (relative expression) with (A) serum Annexin A1, (B) serum procalcitonin, and (C) serum interleukin-6 levels in G1 (healthy, non-obese children), G2 [children with metabolic healthy obesity (MHO)] and G3 [children with metabolic unhealthy obesity (MUO)]. P-values (p) as well as correlation coefficients (Co) are shown.

positively correlated with serum levels of IL-6 (G2; 0.432, P<0.001and G3; 0.862, P<0.001). On the other hand, serum procalcitonin levels were negatively correlated with serum HDL-c levels (Table III).

Serum levels of IL-6 in the healthy control group (G1). Serum levels of IL-6 in the healthy control group (G1) were positively correlated with serum levels of TAG and HbA1C% (Table III).

Table III. Correlations of serum miR-15b, Annexin A1, procalcitonin and IL-6 levels with anthropometric and metabolic parameters in healthy non-obese control (G1), children with MHO (G2) and children with MUO (G3).

Groups	Variables	Correlation/ significance	BMI percentile	WC percentile	HDL-c (mg/dl)	LDL-c (mg/dl)	TAG (mg/dl)	Cholesterol (mg/dl)	Glucose (mg/dl)	HbA1c (%)	Insulin (μg/ml)	CRP (ng/ml)	HOMA-IR
Healthy, non-obese control (G1, n=100)	miR-15bR	r_s	0.296	0.319	-0.179	0.201	0.177	0.207	0.297	0.183	-0.073	-0.048	0.069
		P-value	<0.001[a]	<0.001[a]	0.011[a]	0.004[a]	0.012[a]	0.003[a]	<0.001[a]	0.009[a]	0.307	0.501	0.331
	Annexin A1	r_s	0.026	0.000	-0.014	0.022	-0.085	0.004	-0.032	-0.052	-0.004	-0.002	-0.033
	(ng/ml)	P-value	0.719	0.995	0.845	0.757	0.231	0.955	0.656	0.461	0.951	0.980	0.638
	Procalcitonin	r_s	0.090	0.103	-0.238	0.210	0.203	0.200	0.273	0.216	0.067	0.030	0.140
	(ng/ml)	P-value	0.205	0.146	0.001[a]	0.003[a]	0.004[a]	0.004[a]	<0.001[a]	0.002[a]	0.346	0.670	0.048[a]
	IL-6 (pg/ml)	r_s	<0.001	0.021	0.027	0.122	0.188	0.168	0.032	0.171	-0.097	-0.050	-0.002
		P-value	0.995	0.767	0.704	0.085	0.008	0.017	0.652	0.015	0.173	0.486	0.974
Metabolic healthy obesity (MHO) (G2, n=41)	miR-15bR	r_s	0.994	0.387	-0.985	0.446	0.282	0.431	0.458	0.433	0.369	0.382	0.469
		P-value	<0.001[a]	<0.001[a]	<0.001[a]	<0.001[a]	<0.001[a]	<0.001[a]	<0.001[a]	<0.001[a]	<0.001[a]	<0.001[a]	<0.001[a]
	Annexin A1	r_s	-0.156	-0.479	0.167	-0.293	-0.117	-0.277	-0.272	-0.279	-0.293	-0.286	-0.291
	(ng/ml)	P-value	0.014[a]	<0.001[a]	0.009[a]	<0.001[a]	0.066	<0.001[a]	<0.001[a]	<0.001[a]	<0.001[a]	<0.001[a]	<0.001[a]
	Procalcitonin	r_s	0.591	0.431	-0.607	0.461	0.230	0.439	0.466	0.417	0.430	0.471	0.479
	(ng/ml)	P-value	<0.001[a]	<0.001[a]	<0.001[a]	<0.001[a]	<0.001[a]	<0.001[a]	<0.001[a]	<0.001[a]	<0.001[a]	<0.001[a]	<0.001[a]
	IL-6 (pg/ml)	r_s	0.191	0.280	-0.184	0.303	0.279	0.056	0.270	0.245	0.283	0.259	0.299
		P-value	0.003[a]	<0.001[a]	<0.001[a]	<0.001[a]	<0.001[a]	0.380	<0.001[a]	<0.001[a]	<0.001[a]	<0.001[a]	<0.001[a]
Metabolic unhealthy obesity (MUO) (G3, n=29)	miR-15bR	r_s	0.487	0.452	-0.427	0.269	0.265	0.089	0.429	0.426	0.363	0.403	0.414
		P-value	<0.001[a]	<0.001[a]	<0.001[a]	<0.001[a]	<0.001[a]	0.245	<0.001[a]	<0.001[a]	<0.001[a]	<0.001[a]	<0.001[a]
	Annexin A1	r_s	-0.532	-0.547	0.391	-0.393	-0.174	-0.227	-0.486	-0.526	-0.564	-0.530	-0.550
	(ng/ml)	P-value	<0.001[a]	<0.001[a]	<0.001[a]	<0.001[a]	0.022[a]	0.003[a]	<0.001[a]	<0.001[a]	<0.001[a]	<0.001[a]	<0.001[a]
	Procalcitonin	r_s	0.598	0.544	-0.498	0.332	0.324	0.452	0.558	0.520	0.443	0.569	0.527
	(ng/ml)	P-value	<0.001[a]	<0.001[a]	<0.001[a]	<0.001[a]	<0.001[a]	<0.001[a]	<0.001[a]	<0.001[a]	<0.001[a]	<0.001[a]	<0.001[a]
	IL-6 (pg/ml)	r_s	0.761	0.640	-0.654	0.386	0.324	0.131	0.616	0.585	0.571	0.689	0.646
		P-value	<0.001[a]	<0.001[a]	<0.001[a]	<0.001[a]	<0.001[a]	0.084	<0.001[a]	<0.001[a]	<0.001[a]	<0.001[a]	<0.001[a]

[a]Indicates a statistically significant result. r_s, Spearman's correlation coefficient; MHO, metabolic healthy obesity; MUO, metabolic unhealthy obesity; BMI, body mass index; WC, waist circumference; HDL-c, high density lipoprotein cholesterol; LDL-c, low density lipoprotein cholesterol; TAG, triacylglycerol; HbA1c, glycosylated hemoglobin; CRP, C-reactive protein; HOMA-IR, Homeostatic Model Assessment-Insulin Resistance; miR-15bR, microRNA-15b relative expression; IL-6, interleukin 6.

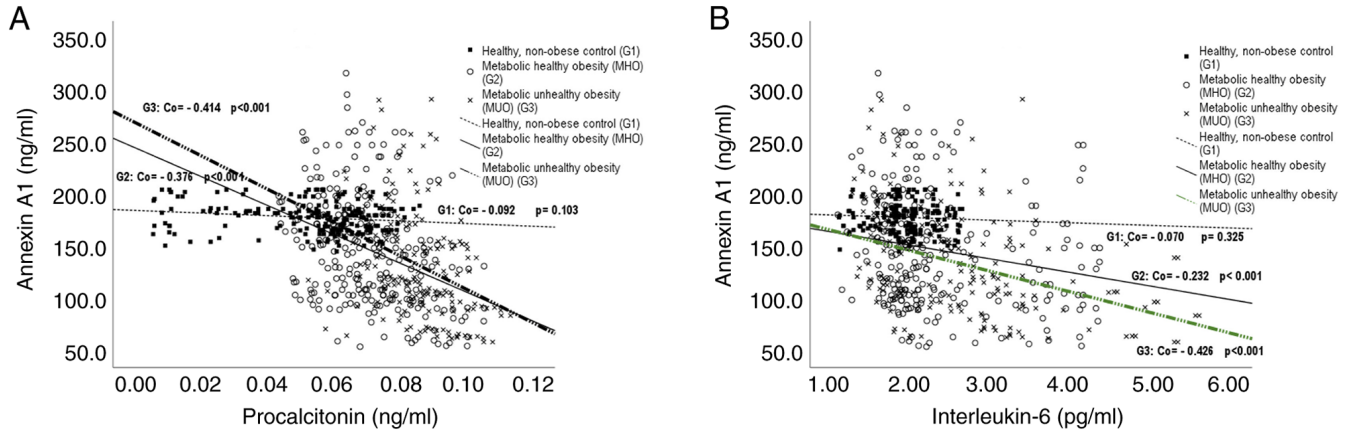

Figure 3. Correlations of Annexin A1 serum levels with (A) serum procalcitonin levels, and (B) serum interleukin-6 levels in G1 (healthy, non-obese children), G2 [children with metabolic healthy obesity (MHO)] and G3 [children with metabolic unhealthy obesity (MUO)]. P-values (p) as well as correlation coefficients (Co) are shown.

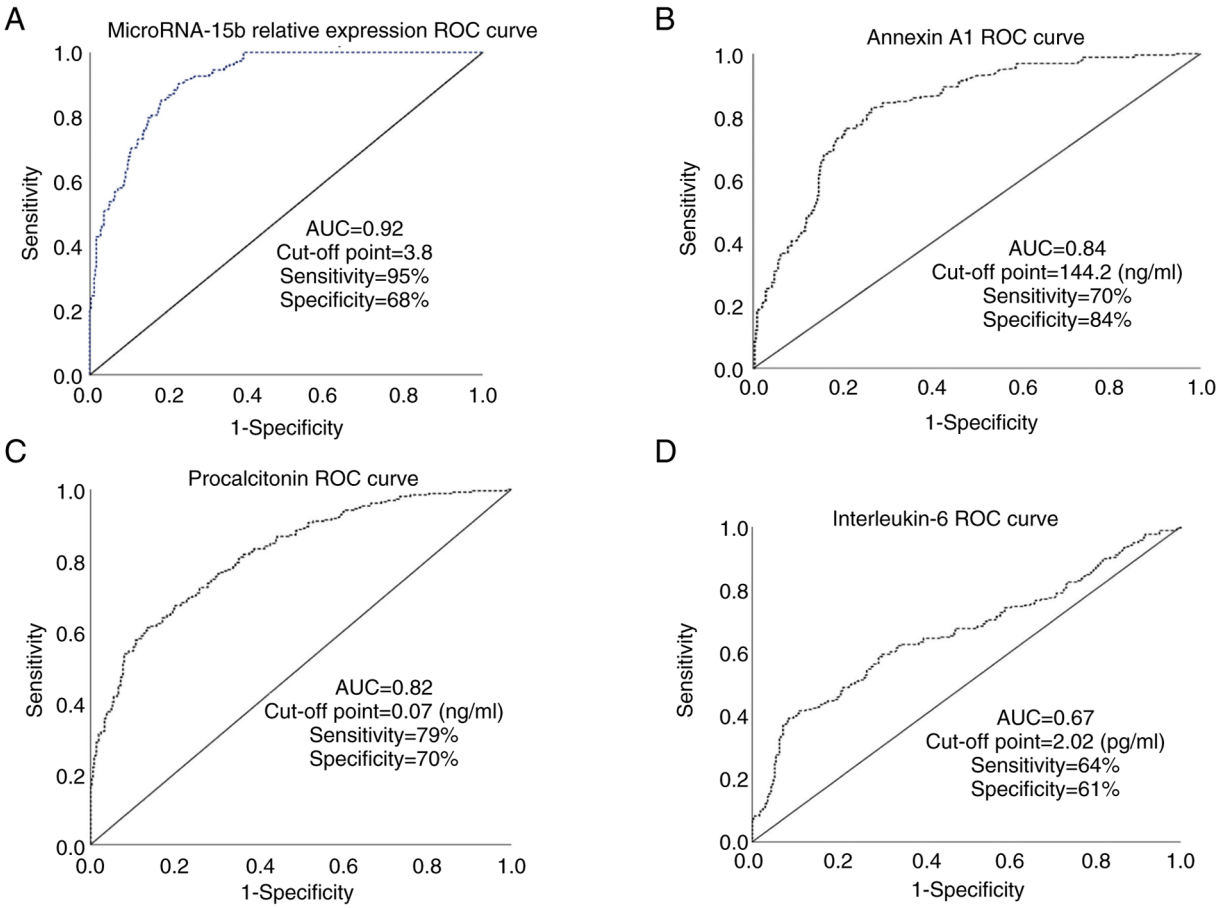

Figure 4. The receiver operating characteristic (ROC) shows the efficacies of (A) circulating microRNA-15b (relative expression), (B) serum levels of Annexin A1, (C) procalcitonin and (D) interleukin-6 in differentiating children with metabolic healthy obesity (MHO) from those with metabolic unhealthy obesity (MUO). AUC, area under the ROC curve.

Serum levels of IL-6 in children with MHO (G2) and MUO (G3). Serum levels of IL-6 in children with MHO (G2) and MUO (G3) were positively correlated with BMI and WC percentiles and serum levels of LDL-c, TAG, glucose, HbA1c%, insulin, and CRP and with HOMA-IR while serum levels of IL-6 in children with MHO (G2) and MUO (G3) were negatively correlated with serum HDL-c levels (Table III).

ROC curve results. The areas under the ROC curve (AUC) for the ability of serum levels of miR-15b, Annexin A1, procalcitonin, and IL-6 to differentiate children with MUO from those with MHO were (0.92, 0.84, 0.82, and 0.67, respectively) (Fig. 4A-D). While AUCs for other studied parameters were 0.27 (BMI percentile), 0.28 (waist circumference percentile), 0.11 (serum cholesterol), 0.48 (LDL-c), 0.15 (TAG), 0.58

(serum cholesterol), 019 (serum glucose), 0.50 (serum insulin), and 0.42 (serum CRP) (data not shown).

Discussion

The increased serum levels of miR-15b, procalcitonin, and interleukin (IL)-6 and the decreased levels of Annexin A1, that were found in the present study, emphasize the status of low inflammation that accompanies obesity (8). The accumulated fat in adipocytes activates immune cells in adipose tissues to initiate an immune-inflammatory process that results in unfavorable metabolic consequences (8,12).

Several existing obesity management protocols (diagnosis and treatment) are based on a body mass index (BMI) ≥30 kg/m^2 (26). Yet, research has revealed the incapability of BMI to correctly predict obesity-associated complications (27). According to the IDF classification, some obese individuals have a high risk to develop cardiometabolic complications and are called as presenting with metabolically unhealthy obesity (MUO), while those with lower risk present with what is termed metabolically healthy obesity (MHO) (22). The criteria to differentiate between both types were previously discussed in the Patients and methods section (5,6). Some research has reported that MHO is a strictly cardiometabolic benign condition (28) while other studies did not agree (6). The present study aimed to find new biomarkers that can differentiate between these two types to allow timely management.

MicroRNAs play important roles in the development and functions of the immune system and their abnormal expression levels have been reported in obesity and its accompanying complications (4,12). They fine-tune and regulate the production of many inflammatory cytokines by regulating expression of their genes (10). Of these microRNAs, the overexpression of miR-15b was found to enhance the inflammatory process, induced by certain viruses, by targeting the ring finger protein 125 (RNF-125). This leads to the induction of retinoic acid-inducible gene I and excess production of pro-inflammatory cytokines (29). In addition, miR-15b binds to the 3'untranslated ends of the insulin receptor substrate 1 mRNAs to repress them. This results in decreasing glucose uptake by cells and ultimately, insulin resistance (11).

The findings of the present study are in accordance with the results reported by many previous studies. Yang *et al* (11) revealed an increase in serum miR-15b levels in mice fed a high-fat diet (obese) and these levels were associated with insulin resistance in hepatocytes. Cui *et al* (3) reported high circulating miR-15b in obese children and adults with T2DM. They revealed that miR-15b and miR-146b could suppress insulin secretion in response to high glucose levels and concluded that circulating miR-15b levels could predict the future risk of developing T2DM in obese children (3). Mohany *et al* (4) found an increase in serum miR-15b levels in obese children especially those with T2DM. They also revealed significant positive correlations between these levels and BMI percentile, serum levels of glucose, and HbA1c% (4).

Annexin A1 is an anti-inflammatory protein that acts through formyl-peptide-receptor-2 (FPR-2) in many cellular functions. It diminishes the production of pro-inflammatory cytokines by neutrophils and enhances the apoptosis of neutrophils, inhibits phospholipase A2 and consequently dampens the eicosanoid biosynthesis, and suppresses the process of adipogenesis (13,14,30).

The biosynthesis of Annexin A1 is regulated by glucocorticoids, the finding that was emphasized by the decreased anti-inflammatory response to glucocorticoids in Annexin A1-deficient mice (16).

The results of the present study agree with the findings of Kosicka *et al* (16) who found a reduction in plasma Annexin A1 levels in obese individuals and these levels were correlated negatively with BMI, total body fat, plasma levels of CRP, and leptin. They concluded that the decrease in Annexin A1 (anti-inflammatory protein) that occurred concurrently with the increase in body fat is strongly associated with obesity-related inflammation and cardiometabolic complications (16). In addition, the findings of the present study agree with Sajid *et al* (30) who concluded that Annexin A1 could decrease the accumulation of fat in adipocytes and suppress the process of adipogenesis. They also suggested a therapeutic role for Annexin A1/FPR2 to eliminate obesity accompanying inflammation (30).

By contrast, Aguilera *et al* (15) found overexpression of the Annexin A1 gene in the adipose tissues of obese children. Pietrani *et al* (14) detected a non-significant difference in the serum Annexin A1 levels between patients with T2DM and non-diabetic controls. When they classified the participants according to their BMI, the authors found higher serum levels of Annexin A1 in obese patients with T2DM than in normal-weight healthy individuals (14). These increased serum levels of Annexin A1 were assumed as an attempt by the body to counteract the systemic inflammatory process in obese subjects with/without T2DM. Unfortunately, these high serum levels of Annexin A1 were not effective to dampen the inflammatory cascades due to their cleavage in the adipose tissues (14).

Procalcitonin is produced by numerous tissues all over the body including the adipose tissue. The exact role of procalcitonin is still undetermined and is under investigation (31). Its secretion is increased in response to bacterial (but not viral) infections and other inflammatory stimuli and has been considered as a marker for systemic inflammations (32). Serum levels of procalcitonin have been used as a supportive test for diagnosis of sepsis; levels ≥ 0.1 ng/ml suggest bacterial infection that indicates antibacterial therapy and levels ≥0.5 ng/ml suggest severe sepsis (31,33).

The findings of the present study are in accordance with the results of Linscheid *et al* (34) and Van Gaal *et al* (35) who reported that the secretion of procalcitonin from the adipose tissue is induced by activation of the resident macrophages and is proportionate to the amount of accumulated fats. In addition, Abbasi *et al* (17) and Ghanem and Khalid (36) found an association between serum levels of procalcitonin and obesity and insulin resistance, and these levels were found to be correlated with HOMA-IR and serum levels of CRP. Moreover, El Kassas *et al* (18) revealed significantly high serum procalcitonin levels in obese children compared to non-obese controls (P<0.001), and these levels were positively correlated with BMI, HOMA-IR, and serum levels of insulin, cholesterol, TAG, and CRP. In contrast, Boursier *et al* (37) found non-significant correlations between plasma calcitonin levels and insulin resistance.

Regarding IL-6, the results of the present study are in accordance with Khaodhiar *et al* (19) who found significantly

increased levels of serum IL-6 in obese and morbidly obese individuals compared to normal-weight individuals, and these levels were positively correlated with BMI. They concluded that adipose tissue-derived IL-6 was proportionate to the degree of fat accumulations, especially in the abdominal region (19). Moreover, Ellulu *et al* (8) concluded that obesity is associated with increased serum levels of inflammatory markers such as TNF-α, IL-6, and CRP. Moreover, El-Mikkawy *et al* (21) reported high levels of serum IL-6 in obese and overweight individuals compared to normal-weight individuals ($P<0.001$), and these levels were found to be positively correlated with BMI and negatively with HDL-cholesterol. They concluded that levels of serum IL-6 were good indicators of obesity-associated low-grade inflammation (21).

Despite finding high serum levels of IL-6 in obese compared to non-obese subjects in the study conducted by Takumansang *et al* (20), they found no association between these levels and insulin resistance which is contrary to the findings of the present study. In addition, contrary to the present study, Neeland *et al* (27) reported a normal hormonal, inflammation, and immune profile in children with MHO.

To the best of our knowledge, the present study was the first to test the ability of serum levels of miR-15b, Annexin A1, procalcitonin, and IL-6 to differentiate children with MUO from those with MHO. In this regard, the present work revealed that serum levels of miR-15b (relative expression), Annexin A1 and procalcitonin are good biomarkers in this regard (when compared to the classically used parameters) with cut-off points=3.8, 144 ng/ml, 0.07 ng/ml and AUCs=0.92, 0.84, and 0.82, respectively.

One potential limitation of the present study was that the participants in the study groups were not sexually maturation matched.

In conclusion, high serum levels of miR-15b, procalcitonin, and IL-6, and low levels of Annexin A1 were found in obese children especially those with MUO. Measuring these levels could differentiate children with MUO from those with MHO which could help the early management of these cases and their accompanying complications. Future studies on a large-sized population are recommended to emphasize these findings.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

KMM was responsible for the conception and design of the study, the acquisition of the data, analysis of the data and interpretation of the data. This author also drafted the manuscript and substantially revised it. OAR was responsible for the conception and design of the study. This author also drafted the manuscript and substantially revised it. OAW was responsible for the conception of the study and substantially revised the manuscript. MA was responsible for the conception of the study and substantially revised the manuscript. AAN was responsible for the conception and design of the work, the acquisition of the data and substantially revised the manuscript. KMM, OAR and OAW confirm the authenticity of all the raw data. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work (including data) are appropriately investigated and resolved.

Ethics approval and consent to participate

The present work was conducted in three primary health care centers in Unaizah, Qassim area, Saudi Arabia after being reviewed and approved by the Qassim University Medical Ethics Board (approval no. mduc-2019-2-2-I-5441). All procedures performed were in accordance with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed written consents were obtained from all children's guardians.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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