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Low miR-222 expression in human visceral adipose tissue is associated with insulin resistance and PTEN and p53 mRNA levels

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Abstract

Introduction: The aim of this is study was to analyse the expression of miR-193b, miR-378, miR-Let7-d, and miR-222 in human visceral adipose tissue (VAT), as well as their association with obesity, insulin resistance (IR), and their role in the regulation of genes controlling adipose tissue homeostasis, including adipocytokines, the phosphatase and tension homologue (PTEN), and tumour protein 53 (p53).

Material and methods: VAT was obtained from normal-weight (NW), overweight, and obese (OW/OB) subjects with and without IR. Stem-loop RT-qPCR was used to evaluate miRNA expression levels. miRTarBase 4.0, miRWalk, and DIANA-TarBase v8 were used for prediction of validated target gene of the miRNA analysed. A qPCR was used to evaluate PTEN, p53, leptin (LEP), and adiponectin (ADIPOQ) mRNA.

Results: miR-222 was lower in IR subjects, and miR-222 and miR-378 negatively correlated with HOMA-IR. PTEN and p53 are miR-222 direct targets according to databases. mRNA expression of PTEN and p53 was lower in OW/OB subjects with and without IR, compared to NW group and its levels positively associated with miR-222. Additionally, p53 and PTEN are positively associated with serum leptin levels. On the other hand, miR-193b and miR-378 negatively correlated with serum leptin but not with mRNA levels. Moreover, miR-Let-7d negatively correlated with serum adiponectin but not with adiponectin mRNA levels.

Conclusions: Lower miR-222 levels are associated with IR, and PTEN and p53 expression; the implication of these genes in adipose tissue homeostasis needs more research. **(Endokrynol Pol 2022; 73 (5): 846–855)**

Key words: *microRNA (miRNA); visceral adipose tissue; obesity; insulin resistance; adipocytokines*

Introduction

Obesity leads to adipose tissue (AT) hypertrophy; when adipocyte buffer capacity is exceeded, free fatty acids accumulate in other organs such as liver and skeletal muscle, favouring insulin resistance (IR) development [1]. Adipocyte hypertrophy also promotes activation of macrophages resident in AT, which in turn release pro-inflammatory cytokines [2-4]. In addition, obesity causes dysregulation of the AT endocrine function, with an increase in leptin and a reduction in adiponectin expression; all these changes promote IR development [5–6].

Moreover, it has been demonstrated that genes like the phosphatase and tension homologue (PTEN) and tumour protein 53 (p53) are involved in adipocyte biology function [7–8]. PTEN and p53 act as tumour suppressor genes, additionally p53 positively regulates PTEN gene expression [8]. PTEN suppresses the PI3K-AKT-mTOR proliferative pathway, which also is involved in insulin signalling [9]. p53 is recognized for its roll in cancer development; however, recent evidence has shown the impact of this gene on adipocyte differentiation and function [7].

Epigenetics could explain part of the molecular mechanisms responsible for AT dysfunction. Among the epigenetic mechanisms are microRNA (miRNA), which are single-stranded RNA molecules ≈ 22 nucleotides in length, of endogenous expression, which post-transcriptionally regulate gene expression. miRNA-codifying genes represent 1% of the human ge-

nome, and it is considered that the expression of approximately 30% of all genes is regulated by miRNA [11–13]. miRNA bind by base-pairing to a mRNA 3′UTR region, and the miRNA-mRNA interaction leads to the inhibition of the mRNA translation or to its degradation [14–16]. A lower miR-193, miR-Let-7d, and miR-378 expression, and higher miR-222 expression in subcutaneous adipose tissue (SAT) have been reported in obese subjects compared to controls [16–18]. These differences in miRNA expression are related to alterations in adipocytokine expression and to IR development. miR-193b negatively regulates IL-6 and monocyte chemoattractant protein-1 (MCP-1) expression [17]. On the other hand, miR-193b overexpression in human SAT adipocytes increases adiponectin synthesis [19]. Moreover, miR-378 negatively regulates adiponectin expression [20]. Additionally, in vitro analyses have showed that a reduction in the expression of miR-Let-7d family could be related to an IR increase because it negatively regulates interleukin 6 (IL-6) and interleukin 1β (IL-1β) [2–23]. Finally, there is limited information about miRNA expression in human visceral adipose tissue (VAT) associated with body fat excess and IR. Thus, the aim of this is study was to analyse miR-193b, miR-378, miR-Let7-d, and miR-222 expression in human VAT and to assess their association with obesity, IR, and genes related to AT homeostasis control.

Material and methods

Subjects
A total of 29 unrelated Mexican-Mestizo participants were recruited in this cross-sectional study. Adult women and men (18–50 years old), programmed for elective surgery (cholecystectomy and/or Nissen funduplicature), were invited to participate in this study. All participants were recruited at the General Hospital of the State of Queretaro and were divided into the following 3 study groups: normal weight (NW; n = 10), overweight, and obese (OW/OB) without IR (n = 10) and OW/OB with IR (n = 9). Participants with type 2 diabetes (T2DM) and those who consumed vitamin supplements and/or minerals in the last 3 months were excluded. Also, people with a body mass index (BMI) ≥ 40 kg/m², who performed intense physical activity, who had a weight loss greater than 10% in the last 3 months, who were under treatment to lose weight, menopausal women, pregnant, breastfeeding, or under hormonal treatment were also excluded. The present study was conducted according to the Declaration of Helsinki Guidelines and was approved by the Bioethics Committee of the School of Natural Sciences at the Universidad Autonoma de Queretaro and by the Queretaro State Health Research Committee. All subjects received complete oral and written information about the study procedures, and voluntary written informed consent was obtained.

Anthropometry and body composition
Height and weight were used to calculated BMI. Subjects were categorized according to the WHO cut-off points [22]. Body composition was determined with a bioimpedance scale (TANITA BC-558, USA).

Biochemistry measurements
A 12-h fasting blood sample was taken before surgery from each subject by venous puncture in the cubital vein. Automatized spectrophotometry (VITROS® 350 Chemistry System, Ortho Clinical Diagnostic) was used for the determination of glucose, total cholesterol, low-density lipoprotein (LDL) cholesterol (LDL-C), high-density lipoprotein (HDL) cholesterol (HDL-C), and triglycerides.

Adipocytokines and insulin determinations
Adiponectin, leptin, C-reactive protein (CRP), and tumour necrosis factor alpha (TNF-α) were determined with commercial ELISA kits from Life Technologies Corporation (Invitrogen, USA), and interleukin 6 (IL-6) was assessed using a Quantikine HS ELISA kit (R&D Systems, UK). Insulin was measured with a commercial ELISA kit for Human Insulin Animal Serum Free (MERCK Sigma-Aldrich, GER). All ELISA assays were performed according to the manufacturer's instructions and were read with a microplate photometer Multiskan Ascent 354 (Thermo Electron Corporation). IR was calculated by using the Homeostasis Model Assessment (HOMA-IR) index using the HOMA2 Calculator (v2.2.3) from the ©Diabetes Trials Unit, University of Oxford. A participant with a HOMA2-IR value ≥ 2.5 was considered to have IR [23].

RNA extraction
During surgery a VAT sample from the omentum was collected; biopsies were immediately washed with sterile saline solution and frozen with liquid nitrogen until laboratory arrival. All samples were stored at –80°C until further RNA extraction. For RNA isolation, ≈100 mg of tissue were homogenized with liquid nitrogen. RNA isolation was performed with TriZol reagent (Life Technologies Corporation, Invitrogen, USA), according to the manufacturer's instructions. RNA quantification was performed with a NanoDrop spectrophotometer (Thermo Scientific™ ONE, USA), and its integrity was verified by using 2% agarose gel electrophoresis.

Reverse transcription and qPCR
miRNA expression was performed according to Chen et al. [24]. Briefly, this procedure consists of 2 steps: the formation of a cDNA stem-loop by reverse transcription (RT) and the qPCR. The primers for miRNA analyses were designed according to the methodology reported by Czimmerer et al. [25]. Mature miRNA sequences were taken from the miRBase database with the following accession numbers; hsa-mir-193b (MIMAT0004767), hsa-miR-378 (MIMAT0014999), hsa-let-7d-5p (MIMAT0000065), and hsa-mir-222 (MIMAT0004569). Synthesis of cDNA stem-loop was performed using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA). The 20 μL RT reactions contained 200 ng of total RNA, 1 μL stem-loop primer (1 μM), 1 μL dNTPs (10 mM), 1 μL RevertAid M-MuLV RT, 4 μL 5X Reaction Buffer, and 1 μL RiboLock RNase Inhibitor. The template, dNTPs and stem-loop primers were mixed and heated to 65°C for 5 min, and chilled on ice for 5 min. Then the remaining reagents were added, and the complete reaction was incubated at 16°C for 30 min, followed by 60 cycles of 20°C/30 s, 42°C/30 s, and 50°C/1 s. The RevertAid M-MuLV RT was inactivated by heating the reactions to 85°C for 10 min and then cooling at 4°C. qPCR was performed using Maxima SYBR Green qPCR Master Mix (Thermo Scientific, USA) according to the manufacturer's protocol on a CFX96 Touch Real-Time PCR Detection System (BIO-RAD). The qPCR protocol for miRNA consisted of an incubation at 95°C for 3 min, followed by 40–45 cycles of 95°C/10 s, 53°C/10 s and 65°C/7 s. RNU6 was used to normalize the miRNA gene expression. qRT-PCR was performed for analysis of the miRNA targets including the PTEN and p53, and adipocytokines including ADIPOQ and LEP. β-actin was used to normalize the mRNA of target genes and adipocytokines. The qPCR protocol consisted of an incubation at 95°C for 3 min, followed by 40–45 cycles of 95°C/15 s, Tm/30 s, and 72°C/30 s; the primer sequences are provided in Table 1. Gene expression was calculated by using the ΔΔCt method. miRNA and mRNAs expressions were logarithmically transformed to normalize the data.

Table 1. *Sequences and information of primers*

Gene	Primer sequence	Length [bp]	Annealing temperature [Tm°C]
RNU6	**F** 5'-CTCGCTTCGGCAGCACA-3'	96	60
	R 5'-AACGCTTCACGAATTTGCGT-3'		
PTEN	**F** 5'-AAGCTGGAAAGGGACGAACT-3'	145	60
	R 5'-CGCCTCTGACTGGGAATAGT-3'		
TP53	**F** 5'-TCCTCAGCATCTTATCCGAG-3'	85	62
	R 5'-CACCACCACACTATGTCGA-3'		
ADIPOQ	**F** 5'-GGCCGTGATGGCAGAGAT-3'	110	60
	R 5'-CCTTCAGCCCCGGGTACT-3'		
LEPTIN	**F** 5'-CCAACGACCTGGAGAACCTCCGGGATC-3'	176	57
	R 5'-GTCCTGCAGAGACCCCTGCAGCCTGCT-3'		
β-ACTIN	**F** 5'-AAGGAGAAGCTGTGCTACGTC-3'	256	60
	R 5'-CTGTGTTGGCGTACAGGTCT-3		

RNU6 — RNA, U6 Small Nuclear 1; *PTEN* — phosphatase and tensin homolog; *TP53* — tumour protein 53; *ADIPOQ* — adiponectin

Prediction of miRNA target genes

The miRTarBase 4.0 (https://mirtarbase.cuhk.edu.cn/~miRTarBase/miRTarBase_2022/php/index.php), miRWalk 2.0 (http://mirwalk.umm.uni-heidelberg.de/) and DIANA-TarBase v8 (https://carolina.imis.athena-innovation.gr/diana_tools/web/index.php?r=tarbasev8%2Findex) were used for prediction of validated target genes. Target genes were selected if they were validated in at least 2 of the databases.

Statistical analyses

Normality of the distribution of all variables was assessed by the D'Agostino-Pearson omnibus test in the entire cohort and each of the study groups. Variables with normal distribution were analysed with one-way ANOVA (two-tailed) with a Bonferroni multiple comparisons test or Student's t-test (two-tailed), depending on whether we compared 3 or 2 groups; data were expressed as mean ± standard deviation (SD). Variables with non-Gaussian distribution were analysed with Kruskal-Wallis test (two-tailed) with a Dunn's post-hoc test; data were expressed as medians (interquartile range 25–75). Two-tailed bivariate and partial Pearson's correlations were used to analyse the association between miRNA with adipocytokine variables and p53 and PTEN expression. Body fat percentage, age, and sex were used as covariates for partial correlations. All data were analysed using SPSS version 23 (IBM® SPSS®) and GraphPad Prism 6 (GraphPad Software). Statistical significance was established with a p value < 0.05.

Results

Metabolic characterization of the studied groups

Significant differences were found in anthropometric and composition variables between study groups (Tab. 2). Also, significant differences were found in HOMA-IR, adiponectin, CRP, IL-6, and leptin between the study groups (Tab. 2).

miR-222 expression is lower in IR subjects

No differences were found in miR-193b (p = 0.287), miR-Let-7d (p = 0.873), miR-378 (p = 0.076), and miR-222

(p = 0.053) expression in VAT between the study groups (Fig. 1A–G). However, when miRNA expression was analysed considering only the presence of IR, there was a trend towards reduction in miR-378 expression (p = 0.054), and miR-222 was found to be lower in the IR subjects (p = 0.019) (Fig. 1H); no differences were found in miR-193b and miR-Let-7d expression when considering only IR.

PTEN and p53 mRNA expression is lower in the VAT of overweight and obese subjects with IR and is associated with miR-222 expression

According to the validated target prediction databases, miRTarBase 4.0 and miRWalk 2.0, PTEN, and p53 are direct targets of miR-222. PTEN expression in VAT was decreased in the OW/OB with IR group compared to the OW/OB without IR (p = 0.002) and NW (p < 0.001) groups (Fig. 2A). Moreover, p53 expression in VAT was decreased in the OW/OB with IR group compared to the OW/OB without IR (p < 0.009) and NW (p < 0.002) groups (Fig. 2B). A negative correlation was found between HOMA-IR with PTEN and p53 expression levels (r = –0.567, p = 0.001; r = –0.415, p = 0.035, respectively), the association between HOMA-IR and PTEN expression levels remained significant after adjustment with covariates (r = –0.456, p = 0.022); however, the association between HOMA-IR and p53 expression levels was no longer significant after covariate adjustment (r = –0.074, p = 0.744). Moreover, correlations were performed to analyse the association between miR-222 with PTEN and p53 expression. miR-222 was positively correlated with PTEN expression (r = 0.584, p < 0.001) and with p53 (r = 0.504, p = 0.008) (Fig. 2C–D). Correlations between PTEN

Table 2. General and metabolic characteristics of the participants.

Variable	NW (n = 10)	OW/OB without IR (n = 10)	OW/OB with IR (n = 9)	Total (n = 29)	p[1]
Age [years]	31.2 ± 8.6	31.8 ± 8.6	33.1 ± 11.3	32.0 ± 9.2	0.908
Gender [female/male]	9/1	8/2	6/3	23/6	
BMI [kg/m²]	22.4 ± 2.2[a]	31.2 ± 4.6[b]	33.1 ± 3.1[b]	28.8 ± 5.0	< 0.001
Waist [cm]*	77.7 (74.5–82.7)[a]	100.1 (87.8–107.3)[b]	101.8 (92.0–106.7)[b]	93.6 (79.6–103.6)	0.018
Hip [cm]*	90.5 (89.4–94.3)[a]	113.0 (101.6–114.9)[b]	109.9 (103.6–113.9)[b]	106.2 (93.7–114.1)	0.018
WHR	0.8 ± 0.0	0.8 ± 0.0	0.9 ± 0.0	0.8 ± 0.0	0.087
Body fat (%)	30.9 ± 3.3[a]	36.4 ± 8.0[ab]	40.6 ± 6.4[bc]	36.0 ± 7.2	0.011
Glucose [mg/dL]*	83.7 (77.0–86.5)	86.5 (79.7–103.2)	92.0 (84.0–101.1)	86.0 (80.5–98.5)	0.162
Insulin [uU/mL]	13.0 ± 2.7[a]	13.5 ± 2.4[a]	26.7 ± 5.6[b]	17.4 ± 7.3	0.006
HOMA2-IR	1.6 ± 0.3[a]	1.7 ± 0.3[a]	3.3 ± 0.7[b]	2.2 ± 0.9	< 0.001
Cholesterol [mmol/L]*	159.0 (125.5–176.0)	192.5 (125.7–213.2)	162.0 (133.0–202.0)	162.0 (130.5–197.5)	0.481
Triglycerides [mmol/L]*	126.0 (89.7–156.7)	112.5 (72.2–167.0)	174.0 (127.5–193.5)	134.0 (87.5–179.5)	0.154
HDL [mmol/L]*	43.5 (34.5–50.7)	37.5 (34.0–41.7)	39.0 (32.0–46.5)	39.0 (32.0–46.5)	0.680
LDL [mmol/L]	86.6 ± 22.0	103.1 ± 41.1	93.1 ± 35.3	94.3 ± 33.2	0.501
CRP [mg/L]*	0.4 (0.1–0.9)[a]	2.2 (1.1–8.6)[b]	2.0 (1.6–5.5)[b]	1.5(0.6–3.7)	0.002
Leptin [pg/mL]	161.5 ± 90.9[a]	315.3 ± 163.1[ab]	520.2 ± 332.3[bc]	325.8 ± 254.1	0.004
TNF-α [pg/mL]	11.5 ± 6.7	10.3 ± 5.1	9.7 ± 5.5	10.6 ± 5.7	0.202
IL-6 [pg/mL]	1.6 ± 0.6[a]	4.1 ± 3.1[bc]	2.9 ± 0.8[ab]	2.8 ± 2.0	0.036
Adiponectin [ng/mL]	2.2 ± 1.1[a]	1.4 ± 0.6[ab]	1.1 ± 0.4[bc]	1.6 ± 0.9	0.025

Data are expressed as mean ± standard deviation (SD) for parametric variables and median (percentile 25–75) for non-parametric variables. Variables with a Gaussian distribution were analysed using one-way ANOVA (two-tailed) with a Bonferroni correction for multiple comparisons. *Non-parametric variables were analysed using a Kruskal-Wallis test (two-tailed) with a Dunn's post-hoc test. Significance level, p < 0.051. NW — normal weight; OW/OB — overweight and obesity; IR — insulin resistance; WHR — waist-hip ratio; BMI — body mass index; HOMA2-IR — Homeostatic Model Assessment Index 2 — Insulin Resistance; HDL — high-density lipoprotein; LDL — low-density lipoprotein; CRP — C-reactive protein; TNF-α — tumour necrosis factor alpha; IL-6 — interleukin 6

and p53 with miR-222 remained significant after adjusting with sex, age, and body fat percentage (r = 0.735, p < 0.001; r = 0.546, p = 0.009; respectively). Finally, PTEN and p53 were positively correlated (Fig. 3E), and the association remained significant after adjusting with covariates (r = 0.601, p = 0.003).

p53 and PTEN are negatively associated with serum leptin levels

Correlations between leptin mRNA and serum leptin with mRNA PTEN and p53 expression levels, were performed. A negative correlation between PTEN mRNA expression and serum leptin was observed (Fig. 3A), and the association remained significant after adjusting with covariates (r = –0.681, p ≤ 0.001; data not shown). No association was found between PTEN and leptin mRNA levels (Fig. 3C). A negative correlation between p53 mRNA expression and serum leptin was also observed (Fig. 3B); however, the association was not significant after adjusting with covariates (r = –0.332, p = 0.131; data not shown). A positive association was found between p53 and leptin mRNA levels (Fig. 3D), and the association remained significant

after adjusting with covariates (r = 0.427, p = 0.048; data not shown).

miRNA expression is associated with adipocytokines

No association was found between miR-193b with LEP and ADIPOQ mRNA expression levels (Tab. 3). Moreover, no significant association was found between miR-193b and serum leptin levels (r = –0.281, p = 0.141; data not shown). However, when partial correlations were performed adjusting for covariates, a significant negative association was found (Tab. 3). A negative correlation was found between miR-378 and serum insulin (r = –0.400, p = 0.039) and HOMA2-IR levels (r = –0.393, p = 0.043; data not shown), which remained significant after adjusting with covariates (Tab. 3). No association was found between miR-378 and LEP and ADIPOQ mRNA expression levels (Tab. 3). With unadjusted analysis, no correlation was found between miR-378 and serum leptin levels (r = –0.343, p = 0.08; data not shown), but when analyses were adjusted with covariates, miR-378 was negatively correlated with serum leptin (Tab. 3). miR-222 was negatively
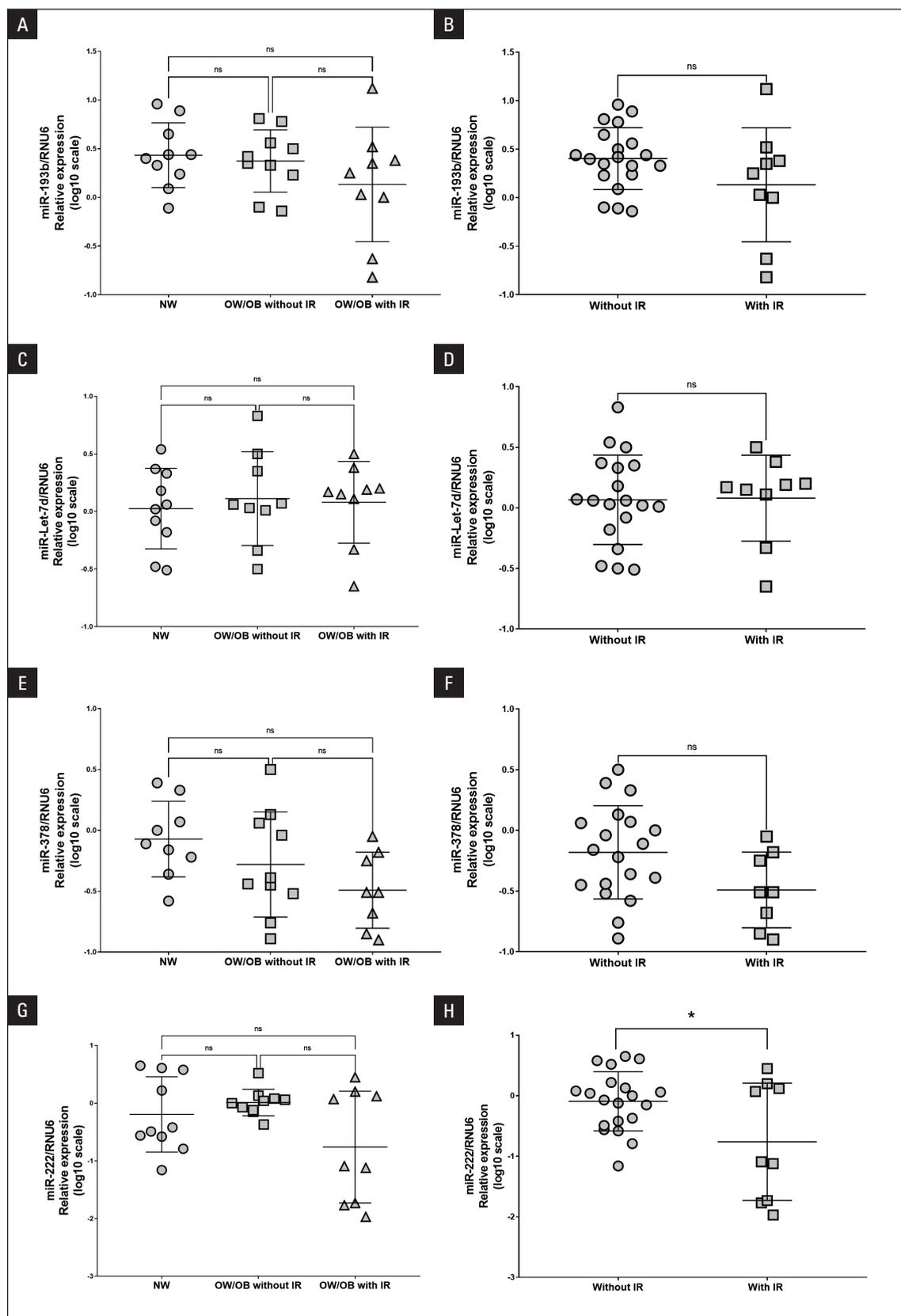
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Figure 1. *Comparison of miRNA expression in human visceral adipose tissue (VAT) in participants with normal weight (NW) and overweight and obesity (OW/OB) without and with insulin resistance (IR).* **A, B.** *miR-193b.* **C, D.** *miR-Let-7d;* **E, F.** *miR-378;* **G, H.** *miR-222. Data are expressed as mean ± standard deviation (SD). Comparison analysis between 3 study groups were performed with one-way ANOVA (two-tailed) with a Bonferroni correction for multiple comparisons. Comparison analysis between 2 study groups were performed with Student's t-test. Significance level, *p < 0.05*
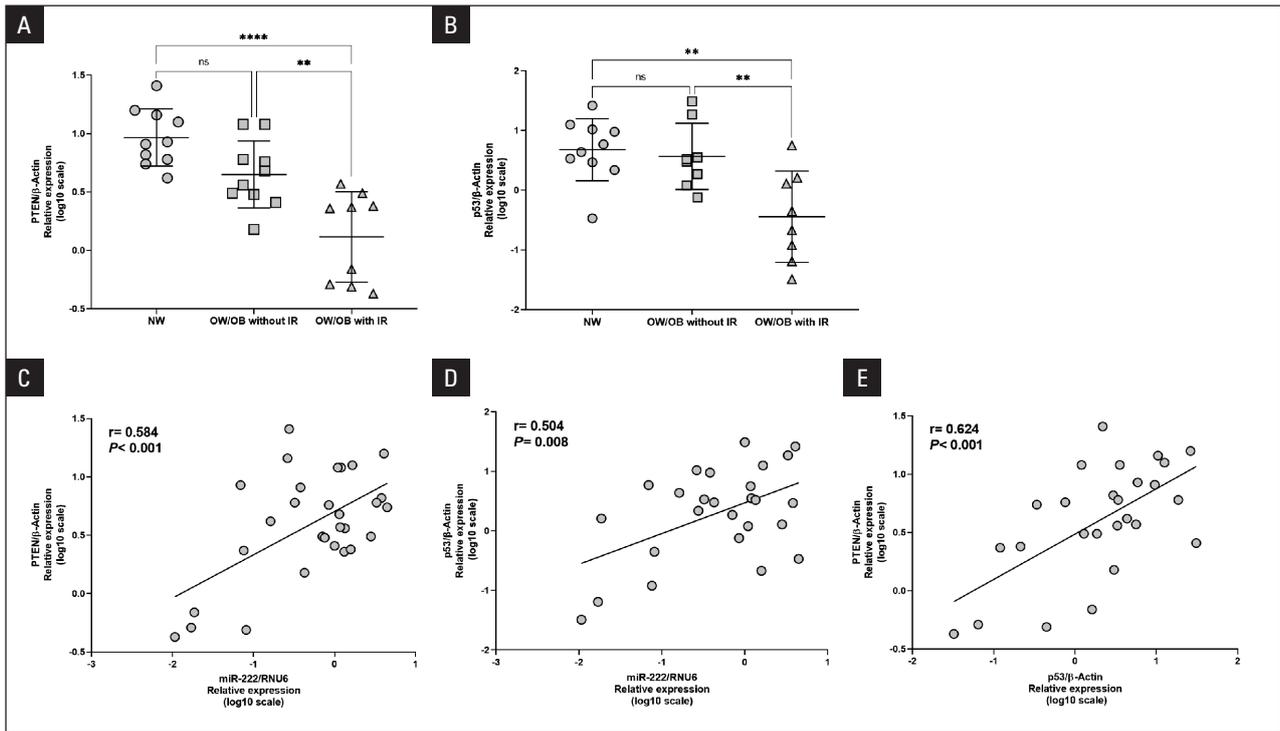
Figure 2. *mRNA phosphatase and tension homologue (PTEN) and tumour protein 53 (p53) expressions in human visceral adipose tissue (VAT) and their relationship with miR-222 expression levels. Comparison of mRNA PTEN (A) and p53 (B) expression in human VAT in participants with normal weight (NW) and overweight and obesity (OW/OB) without and with insulin resistance (IR). Data are expressed as mean ± SD. One-way ANOVA (two-tailed) with a Bonferroni correction for multiple comparisons was performed. Pearson correlations between mRNA PTEN (C) and p53 (D) with miR-222 expression levels. Pearson correlation between mRNA PTEN and p53 expression levels (E). Significance level, **p < 0.01; ***p < 0.001*
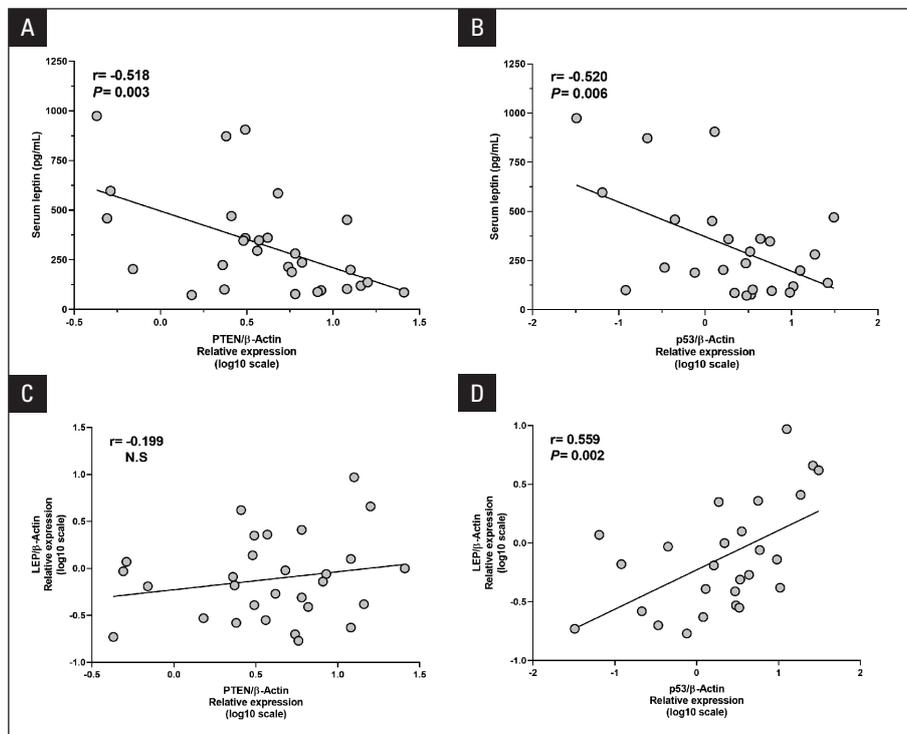
Figure 3. *Correlations between leptin mRNA and serum leptin with mRNA phosphatase and tension homologue (PTEN) and tumour protein 53 (p53) expression levels. Pearson correlations between serum leptin with mRNA PTEN (A) and p53 (B) expression levels. Pearson correlation between leptin mRNA with and PTEN (C) and p53 (D) expression levels*

Table 3. *Relationship between miRNA expression in human visceral adipose tissue (VAT) and metabolic biomarkers*

Variable	miR-193b/RNU6 (Log10)	miR-Let-7d/RNU6 (Log10)	miR-378/RNU6 (Log10)	miR-222/RNU6 (Log10)
HOMA-IR	−0.338	−0.187	−0.433*	−0.416*
LEP/β-actin [Log 10]	−0.083	−0.076	−0.129	0.148
LEP [pg/mL]	−0.544*	−0.245	−0.575*	−0.366
ADIPOQ [ng/mL]	−0.011	−0.404*	0.001	−0.068
ADIPOQ/β-actin [Log 10]	0.017	0.036	0.119	−0.261
CRP [mg/L]	0.135	0.201	0.338	0.061
TNF-α [pg/mL]	−0.164	−0.327	−0.053	0.139
IL-6 [pg/mL]	−0.066	−0.084	−0.051	0.096

Data are expressed as partial Pearson correlation (two-tailed) coefficients adjusted for sex, age, and body fat percentage. Significance level, *$p < 0.05$. HOMA2-IR — Homeostatic Model Assessment Index — Insulin Resistance; LEP — leptin; ADIPOQ — adiponectin; CRP — C-reactive protein; TNF-α — tumour necrosis factor alpha; IL-6 — interleukin 6

correlated with serum insulin (r = −0.385, p = 0.039) and HOMA2-IR (r = −0.386, p = 0.038; data not shown), which remained significant after adjusting with covariates (Tab. 3). No association was found between miR-222 and LEP and ADIPOQ mRNA and serum levels (Tab. 3). A negative correlation between miR-Let-7d and serum adiponectin was found (r = −0.426, p = 0.024; data not show); the correlation remained significant after adjusting for covariates (Tab. 3). However, there was no correlation between miR-Let-7 and ADIPOQ mRNA levels (Tab. 3). Finally, no association was found between analysed miRNA and CRP, IL-6, and TNF-α serum levels.

Discussion

Studies that have used microarray analyses have found differences in miRNA expression in human AT associated with obesity; however, miRNA differently expressed in obese subjects compared to controls has not shown consistent results [18–19, 27]. In these studies, subjects were selected under different criteria; for example, the BMI cut-off value for control groups is different and metabolic conditions such as IR were not always considered. Moreover most of these studies were performed in SAT, this is an important consideration because SAT and VAT have different metabolic characteristics and have a specific miRNA expression profile [26]. Data about changes in miRNA expression in VAT associated with obesity are limited.

In the present study no differences were found in miR-193b expression in OW/OB with and without IR. Arner et al. found a lower miR-193b expression in the SAT of obese subjects compared to subjects with a BMI < 30 kg/m² [17]. Although, the adipocytokines analysed in the present study are not recognized as direct targets of miR-193b according to databases, studies suggest that miR-193b could be involved in adiponectin regulation. It has been observed that miR-193b

overexpression in human adipocytes from SAT results in increased adiponectin secretion [19]. In this study, no association was found between miR-193b expression and serum adiponectin levels. Adiponectin is expressed 33% more in SAT than VAT in lean subjects [4]. Considering this, in the present study miR-193b expression analysis was performed in VAT and not in SAT; this could explain the results contrasting with other studies. In addition, according to our results, leptin levels were negatively associated with miR-193b expression, but only after adjusting for age, sex, and body fat percentage. More studies are needed to explore the association between miR-193b and leptin because information is limited.

In this study, there were no differences in miR-Let-7d expression between subjects with different metabolic characteristics. Jones et al. showed no differences in miR-Let-7d expression in VAT and SAT between obese and lean mice [27]. Arner et al. found a decrease miR-Let-7d expression in subjects with BMI > 30 kg/m² in SAT [17]. In this study, serum adiponectin levels were negatively associated with miR-Let-7d, but no association was found between miR-Let-7d and adiponectin mRNA in VAT. Regulation of miR-Let-7d in adiponectin production could act thorough an indirect mechanism. According to the miRTarBase 4.0 and miRWalk 2.0, adiponectin receptor 2 (ADIPOR2) is a direct target of miR-Let-7d. ADIPOR2 mediates adiponectin actions in liver [28] and is expressed in human VAT and SAT [29]. According to Bauche et al., adiponectin could auto-regulate its own synthesis through negative feedback, in which ADIPOR2 mRNA is upregulated in both inguinal and gonadal AT from adiponectin-knockout mice [30]. The relationship between miR-Let-7d and ADIPOR2 expression was not analysed in this study. Thus, more research is needed to evaluate the association between miR-Let-7d expression and adiponectin and the possible role of ADI-

POR2. Also, it has been reported that the miR-Let-7d family is involved in the modulation of inflammatory responses. Brennan et al. showed that transfection of miR-Let-7d in human carotid plaque tissue resulted in reduced of expression TNF-α, IL-1β, interferon γ, and vascular cell adhesion molecule-1. [31]. We did not find any association between miR-Let-7d and the expression of proinflammatory cytokines (CRP, TNF-α, and IL-6).

Although we did not observe differences in miR-378 expression between the studied groups, miR-378 was inversely associated with insulin levels and HOMA2-IR. More research is needed regarding the miR-378 association with obesity and IR. Arner et al. found a decrease in miR-378 expression in SAT associated with adiposity [17]. In addition, it has been reported that miR-378 is overexpressed during adipocyte differentiation in SAT [16]. The capacity of SAT hyperplasia is related with metabolic features. A limited capacity of SAT hyperplasia promotes accumulation of fatty acids in other organs, thus favouring IR [32]. Moreover, an increase in the proportion of small visceral adipocytes in subjects with T2DM has been reported [33]; thus, impaired maturation of small cells of VAT could lead to accumulation of fatty acids promoting IR. As in SAT, miR-378 could also be involved in VAT hyperplasia, thus favouring IR development.

In this study, no differences in miR-222 levels between NW, and OW/OB with and without IR groups were found, but when subjects were classified according to the presence of IR, miR-222 was lower in subjects with IR compared to subjects without IR. Overexpression of miR-222 in SAT in obese and non-obese subjects with T2DM was reported [17, 34]. These differences may be explained by the fact that SAT and VAT have a differential miRNA expression profile; in this study miR-222 expression was analysed in VAT [26]. Moreover, miR-222 overexpression in obese subjects' serum has also been reported [36, 37]. AT is known to be a source of serum exosomal miRNA, capable of regulating gene expression in other organs; nevertheless, serum exosomal miRNA does not always correlate with miRNA expression in the source organ [37]; therefore, these findings cannot be compared with our results.

According to bioinformatics data, PTEN and p53 are miR-222 target genes. In this study PTEN and p53 expressions were lower in the OW/OB with IR group than in the NW and OW/OB without IR groups. A positive association was found between PTEN and p53 expression with miR-222. The most recognized miRNA mechanism of action is the recognition of the 3'UTR region of the mRNA, which leads to inhibition in mRNA translation or to its degradation [14–16]. Therefore, a negative association is expected

between miRNA and its target. Moreover, positive correlations between miRNA and its target genes have been reported in previous studies [38]. A positive association between miRNA and its target could be explained considering that miRNA has several mRNA targets that could be involved in the regulation of its expression and one mRNA can be regulated by several miRNA [39].

A strong positive association between PTEN and p53 was found; it has been reported that p53 positively regulates PTEN expression [8]. More studies are needed to understand the role of PTEN in AT and IR. Kadkhoda et al. found no differences in PTEN expression in VAT and SAT between normal weight and obese subjects [40]. Moreover, *in vivo* studies showed that AiPKO mice fed with a high-fat diet (HFD) for 6 weeks showed a higher insulin sensitivity than the control group, despite weight gain. After chronic exposure (5 months) to HFD, AiPKO mice still had lower glucose and insulin levels than the control group; they also showed larger SAT depots without change in adipocyte size, and no differences were found in VAT [41]. In contrast, specific PTEN deletion in inguinal mice fat resulted in increased VAT depots and adipocyte size, with an increase in serum leptin, without changes in serum glucose and adiponectin levels, compared to the control group [6]its roles in adipose homeostasis of adult animals are not yet fully understood. Here, we sought to determine the role of PTEN in whole-body adipose homeostasis. Methods: We genetically manipulated PTEN in specific fat depots through recombinant adeno-associated viral vector (rAAV). This result agrees with our findings, in which serum leptin levels were inversely associated with PTEN expression.

p53 is key in the regulation of adipogenesis; overexpression of p53 downregulates genes involved in pre-adipocyte differentiation [7]. The role of p53 in obesity and IR development remain a matter of discussion. Ortega et al. found an increase in p53 expression in the VAT of obese subjects and obese mice. The authors also showed that p53 expression is negatively correlated with IR [42]. Moreover, Huang et al. found a reduction in phosphorylated p53 in obese rats; according to these authors, p53 also inhibits Akt phosphorylation, which is needed for insulin signalling and GLUT4 translocation [1], [43]. According to our results, a negative correlation between p53 and serum leptin was found, but p53 is positively correlated to leptin mRNA expression levels. This result can be explained by the fact that mRNA abundance is not always related to an increase in protein levels, because cells are not in a permanent "steady state" [44].

One of the limitations of this study is that it is a cross-sectional study; therefore, causality cannot be

established. Another limitation is the relatively low sample; this was due to the complexity of collecting an omentum sample.

Conclusion

In summary, in the present study miR-222 was consistently related to IR. miR-222 expression was lower in human VAT in IR subjects. In addition, miR-222 was positively associated with mRNA PTEN and p53 expression. Finally, PTEN and p53 expression was lower in OW/OB with IR, and its levels were negatively associated with serum leptin.

Ethical approval and consent to participate
The present study was approved by the Bioethics Committee of the School of Natural Sciences at the Autonomous University of Queretaro and by the Queretaro State Health Research Committee.

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