Expression of candidate genes associated with obesity in peripheral white blood cells of Mexican children

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Abstract

Introduction: Obesity is a chronic, complex, and multifactorial disease, characterized by excess body fat. Diverse studies of the human genome have led to the identification of susceptibility genes that contribute to obesity. However, relatively few studies have addressed specifically the association between the level of expression of these genes and obesity.
Material and methods: We studied 160 healthy and obese unrelated Mexican children aged 6 to 14 years. We measured the transcriptional expression of 20 genes associated with obesity, in addition to the biochemical parameters, in peripheral white blood cells. The detection of mRNA levels was performed using the OpenArray Real-Time PCR System (Applied Biosystems).
Results: Obese children exhibited higher values of fasting glucose ($p = 0.034$), fasting insulin ($p = 0.004$), low-density lipoprotein ($p = 0.006$), triglycerides ($p < 0.001$), systolic blood pressure and diastolic blood pressure ($p < 0.001$), and lower values of high-density lipoprotein ($p < 0.001$) compared to lean children. Analysis of transcriptional expression data showed a difference for ADRB1 ($p = 0.0297$), ADIPOR1 ($p = 0.0317$), GHRL ($p = 0.0060$) and FTO ($p = 0.0348$) genes.
Conclusions: Our results suggest that changes in the expression level of the studied genes are involved in biological processes implicated in the development of childhood obesity. Our study contributes new perspectives for a better understanding of biological processes involved in obesity. The protocol was approved by the National Committee and Ethical Committee Board from the Mexican Social Security Institute (IMSS) (IMSS FIS/IMSS/PRIO/10/011).

Key words: single nucleotide polymorphisms, triglycerides, obesity, Mexican children, gene expression.

Introduction

Obesity is the result of a chronic, positive imbalance between energy intake and energy expenditure [1]. The adverse metabolic effects caused by obesity may result in increased risk for type 2 diabetes, many forms of cancer, fatty liver disease, hormonal disturbance, hypertension,

cardiovascular disease, and increased mortality, among others [2, 3]. On the other hand, childhood obesity increases the odds for earlier adolescence, gynecomastia in boys, and polycystic ovary syndrome, among other maladies; in addition, obese children and teenagers have an increased probability of remaining obese in adulthood [4]. Several genome-wide association studies (GWAS) [5–7] have shown the association of common genetic variants with body mass index (BMI); furthermore, a significant association of some single nucleotide polymorphisms (SNPs) in several genes with obesity has been reported [8]. However; studies have usually not addressed in full the mechanism which results in increased energy intake associated with gene variants in humans. Additionally, only a few studies have explored the effect of risk SNPs on gene expression [9, 10]. However, some studies have reported on the relationship between obesity and gene expression in both humans and animal models [11–13].

The analysis of gene expression on a genomic scale is potentially significant since it facilitates rapid progress in the identification of molecular pathways associated with several diseases [14]. The peripheral blood tissue is an excellent model for gene expression studies, as it is considered to be a tissue affected by the health condition of the host, and may reflect changes caused by high levels of glucose, insulin, and free fatty acids, among other factors [15, 16]. In this work, going beyond the typical genotyping study, we studied and reported the transcriptional level of expression of several candidate genes, which have been reported to contain polymorphic variants associated with obesity in Mexican children (Table I). Our results show the existence of differences in the transcriptional expression level of some candidate genes associated with obesity, which might explain their role in the development of this disease.

Material and methods

Study subjects

A total of 160 unrelated children, aged between 6 and 14 years, were randomly selected from two different Sport Unit facilities belonging to the public health system (IMSS) in Mexico City (Cuauhtémoc and Independencia Units) from July 2011 to July 2012. Children with different diseases and with pharmacological treatment in the last month before the study were excluded. Both parents and children signed the informed consent form in accordance with the Helsinki Declaration revised in 2000. The National Committee and Ethical Committee Board from the IMSS (IMSS FIS/IMSS/PRIO/10/011) approved the protocol.

Clinical evaluation

Participants were measured for weight with a digital scale (Seca, Hamburg, Germany) and height with a portable stadiometer (Seca 225, Hamburg, Germany). Body mass index (BMI) was calculated and classified according to the Centers for Disease Control and Prevention 2000 (CDC 2000) references [17]. CDC 2000 growth charts are based on 5 U.S. nationally representative surveys conducted between 1963 and 1994, in which Mexican-American children were included. According to those growth charts, for ages 2 to 20 years, eutrophic children were defined as body mass index (BMI)-for-age between the 10^{th} and 85^{th} percentile, *z*-score < +1, and obese children when BMI-for-age was higher than the 95^{th} percentile, *z*-score > +2. Waist circumference (WC) was measured at the midpoint between the lowest rib and the iliac crest after a normal exhalation with children in the standing position. Systolic and diastolic blood pressure was obtained using a mercurial sphygmomanometer (ALPK2, Tokyo, Japan). Two blood pressure readings were taken for each participant, on the right arm in a sitting position, resting 5 min between each measurement, and considering the level of blood pressure as the mean of the readings. Insulin resistance was defined as homeostasis model assessment of insulin resistance (HOMA-IR) ≥ 3.4, which is the 90^{th} percentile of HOMA-IR in the population of healthy Mexican children [18]. HOMA-IR = (fasting glucose [mg/dl]) (fasting insulin [µU/ml])/405.

Biochemical studies

For studies, blood samples were taken after 12 h overnight fasting, one into an EDTA tube, and another into a BD Vacutainer Rapid Serum Tube (RST). We measured glucose, total cholesterol, high-density lipoprotein (HDL) and low-density lipoprotein (LDL) cholesterol and triglycerides in mg/dl using an ILab 350 Clinical Chemistry System (Instrumentation Laboratory IL) for clinical laboratory evaluation. Insulin (µU/ml) was measured by chemiluminescence (IMMULITE).

Selection of candidate genes

We selected candidate genes containing reported single nucleotide polymorphisms which have been associated with obesity by the presence of a polymorphism (SNP) in different populations. For selection, we also considered the existence of studies for these genes in children and adolescents, their expression in peripheral blood, as well as their role in the metabolism and energy expenditure. The set of chosen genes is listed in Table I.

mRNA extraction and RT-PCR

An aliquot of 250 µl of fresh blood sample with EDTA was stabilized with TriPure Isolation reagent (Roche), immediately frozen in dry ice, and stored at –70°C. Total RNA was purified from this sample using the TriPure protocol; the procedure is an improvement of the single-step RNA isolation method [19]. All samples were treated with DNase I (Fermentas) to remove traces of genomic DNA. RNA purity and integrity were verified by 260/280 nm measurements using an EpochBiotek spectrophotometer and by electrophoretic fractionation in 1% agarose gels stained with ethidium bromide 0.01 mg (data not shown). Only samples with appropriate RNA integrity were processed. RNA was reverse-transcribed using 2 µg of RNA by RT-PCR and High Capacity cDNA Reverse Transcription Kits (Applied Biosystems) following the manufacturer's instructions. Random primers were employed to copy pre-mRNA and mRNA in a Maxygene thermocycler (Axygene).

Table I. Studied candidate genes associated with obesity

Gene	Name	GenBank	AE_ID	Phenotype	Ref.
ABCA1	ATP-binding cassette, sub-family A (ABC1), member 1	NM_00502.3	Hs00194045_m1	Obesity, BMI	35
ADIPOQ	Adiponectin, C1Q and collagen domain containing	NM_004797	Hs02564413_s1	Obesity, WHR	36
ADIPOR1	Adiponectin receptor 1	NM_015999	Hs01114951_m1	Obesity, BMI	37
ADIPOR2	Adiponectin receptor 2	NM_024551	Hs01047563_m1	Obesity, BMI	37
ADRB1	Adrenergic, β1-receptor	NM_000684	Hs00265096_s1	BMI, body fat	38
APOA4	Apolipoprotein A-IV	NM_000482.3	Hs00166636_m1	Obesity	39
ATRN	Attractin	NM_139321	Hs00390610_m1	Obesity, BMI	40
CALCR	Calcitonin receptor	NM_001742	Hs00156229_m1	BMI	41
FTO	Fat mass and obesity associated	NM_001080432.2	Hs01057143_m1	T2D, BMI	42
GHRL	Ghrelin/obestatin prepropeptide	NM_016362	Hs00175082_m1	Obesity, insulin resistance	43
INSIG2	Insulin induced gene 2	NM_016133.2	Hs00379223_m1	Obesity, BMI	44
INSR	Insulin receptor	NM_000208	Hs00961550_m1	Obesity, BMI	45
LEP	Leptin	NM_000230	Hs00174877_m1	Obesity, BMI	46
LEPR	Leptin receptor	NM_002303	Hs00174497_m1	Extreme obesity	47
MC3R	Melanocortin 3 receptor	NM_019888	Hs00252036_s1	Obesity, BMI	48
PPAR-α	Peroxisome proliferator-activated receptor α	NM_005036	Hs00947539_m1	Reduces obesity	49
PPAR-γ	Peroxisome proliferator-activated receptor γ	NM_015869	Hs01115513_m1	Obesity, body fat	50
UCP1	Uncoupling protein 1 (mitochondrial, proton carrier)	NM_021833	Hs00222453_m1	Obesity, BMI	50
PPAR-γ-C1-α	Peroxisome proliferator-activated receptor γ, coactivator 1α	NM_013261	Hs01016722_m1	Obesity, BMI	51
SORT1	Sortilin 1	NM_002959	Hs00907094_m1	Obesity, BMI	52

BMI – body mass index, WHR – waist-to-hip ratio, T2D – type 2 diabetes, GenBank – accession number, AE_ID – assay expression ID, Applied Biosystems; Ref. – reference.

Measurement of mRNA expression

Detection of mRNAs was performed by TaqMan gene expression assay in an OpenArray Real-Time PCR System (Applied Biosystems), following the manufacturer's instructions using 100 ng/µl of cDNA. We tested β-actin (NM_001101.3, gene expression assay Hs99999903_01 Applied Biosystems); HPRT (NM_000194.2, gene expression assay Hs03929098_m1 Applied Biosystems), and TLR9 (NM_017442.3, gene expression assay Hs00370913_s1 Applied Biosystems) as housekeeping genes. The validation of an internal reference was made for this study under our experimental conditions [20]. The normalized level of expression of a particular gene is the amount of measured expression of that gene in relation to the measured expression of the reference gene. We used the comparative $2^{-\Delta CT}$ method to calculate the expression level for each group, because the data come from different individual samples, and there is no way to justify which obese sample is compared with which lean sample, so the $2^{-\Delta\Delta CT}$ method cannot be used [21]. A melting curve analysis was also performed to confirm the amplification of a single amplicon for each gene analyzed.

Quantitative PCR

Random sampling of 40 individuals was performed to validate by qPCR the OpenArray Real-Time PCR results for FTO, GHRL and TLR9 genes, where a significant difference in gene expression was found (data not shown). For this, we used Assay Expression ID primers and probes from Applied Biosystems (Table I). The PCR reactions were set up in 384-well plates, using the above primer and probe sets, 30 ng of cDNA, and TaqMan Gene Expression Master Mix (Applied Biosystems PN 4304437), in a total volume of 5 µl per reaction. qPCR was performed using the 7900HT Fast Real-Time PCR System (Applied Biosystems). Triplicate amplification reactions were performed using the following conditions: 50°C for 2 min, 95°C for 10 min and 40 cycles of 95°C for 10 s and 60°C for 1 min. In the negative control, cDNA was replaced with sterile water. Crossing of threshold (C_t) values obtained for the target gene were normalized against TLR9 C_t values. Relative quantification of PCR products was determined using the $2^{-\Delta CT}$ method [21].

Statistical analysis

Anthropometric and biochemical measurements were expressed as mean ± SEM (standard error of the mean), and gene expression measurements were expressed as mean ± SEM. Data analysis was performed using the Student *t*-test or Mann-Whitney *U* test according to the data. *P*-values ≤ 0.05 were considered to be significant. Statistical operations were performed using the SPSS (version 14.0) and GraphPad Prism software (version 5.0).

Results

Subject characteristics

The general characteristics of all 160 participants are summarized in Table II. Subjects were grouped as healthy children when BMI-for-age was between the 10th and 85th percentile, and obese children when BMI-for-age was higher than the 95th percentile. We obtained 81 lean children and 79 obese children, of whom 64.20% were lean girls and 44.30% were obese girls. According to the statistical analysis, comparing healthy and obese children, the mean values of the variables do not show significant differences by gender ($p = 0.012$); however, we observed a significant difference in height ($p < 0.001$) according to the Student's *t*-test result, and waist circumference ($p < 0.001$) according to the Mann-Whitney *U* test result in agreement with their phenotype (Table II). The study of metabolic quantitative traits included BMI, fasting plasma glucose, fasting insulin, cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL), triglycerides, systolic blood pressure (SBP) and diastolic blood pressure (DBP). In our sample, obese children exhibited higher values of fasting glucose ($p = 0.034$), fasting insulin ($p = 0.004$), LDL ($p = 0.006$), and triglycerides ($p < 0.001$), and a lower HDL value ($p < 0.001$), compared to lean children. Unexpectedly, unlike lean children, obese children had higher values of SBP and DBP ($p < 0.001$).

Expression level of candidate genes for obesity reveals differences in obese children

To analyze whether the gene expression in white blood cells obtained from obese children differs from that obtained from lean children, we used the $2^{-\Delta CT}$ method for each group as individual data points (as mentioned in Material and methods). A Mann-Whitney *U* test was performed on the data to determine whether the difference was statistically significant. The results showed a difference in expression levels of genes, which was increased for ADIPOR1 ($p = 0.0317$), GHRL ($p = 0.0060$), and FTO ($p = 0.0348$), and decreased for ADRB1 ($p = 0.0297$) in obese children (Figure 1). In the case of genes such as ABCA1, ADIPOQ, ADIPOR2, ATRN, INSIG2, INSR, LEP, LEPR, PPARα, PPARγ, and SORT1, no difference was observed in the level of expression by the same method. On the other hand, we confirmed the undetectable expression of APOA4, CALCR, MC3R, PPARγ-C1-α and UCP1 genes by our method, in white blood cells,

Table II. Clinical characteristics of lean and obese children

Characteristics	Lean children	Obese children	*P*-value
	BMI < 85 pc *N* = 81	BMI ≥ 95 pc *N* = 79	
Female (%)	52 (64.20)	35 (44.30)	0.012*
Age [years]	8.41 ±1.60	9.19 ±1.73	0.004£
Anthropometric data:			
Weight [kg]	32.69 ±10.41	48.65 ±14.28	< 0.001£
Height [m]	1.33 ±0.12	1.42 ±0.13	< 0.001¥
BMI [kg/m^2]	18.07 ±3.16	23.53 ±4.21	< 0.001£
WC [cm]	61.96 ±1.05	77.34 ±1.41	< 0.001£
Metabolic factors:			
Fasting glucose [mmol/l]	4.44 ±0.06	4.62 ±0.06	0.034¥
Fasting insulin [µU/ml]	3.03 ±0.33	7.83 ±1.05	0.004£
Cholesterol [mmol/l]	3.78 ±0.08	4.05 ±0.11	0.225£
HDL [mmol/l]	1.38 ±0.04	1.19 ±0.03	< 0.001¥
LDL [mmol/l]	2.41 ±0.06	2.69 ±0.08	0.006£
Triglycerides [mmol/l]	0.83 ±0.03	1.42 ±0.09	< 0.001£
SBP [mm Hg]	94.69 ±8.42	100.21 ±9.30	< 0.001£
DBP [mm Hg]	63.24 ±7.77	67.81 ±7.36	< 0.001£

*Data are mean ± SEM: Standard error of the mean; P-value according to *χ^2 test for categorical variables, £Mann-Whitney U test for continuous variables with non-normal distribution, ¥Student's t test for continuous variables with normal distribution, BMI – body mass index, HDL – high-density lipoprotein, LDL – low-density lipoprotein, SBP – systolic blood pressure, DBP – diastolic blood pressure, pc – percentile, WC – waist circumference.*

in accordance with the GeneCards Human Gene Database. OpenArray results for the FTO and GHRL genes were confirmed by qPCR using the 7900HT Fast Real-Time PCR System (data not shown).

Discussion

In this study, we measured the transcriptional expression of 20 genes associated with obesity, in peripheral white blood cells of well-characterized healthy and obese Mexican children aged 6 to 14 years. Detection of mRNA levels was performed using the OpenArray Real-Time PCR System (Applied Biosystems). We found that obese Mexican children have higher levels of fasting glucose, triglycerides, LDL, SBP and DBP than lean children from the same sample; moreover, obese children have lower levels of HDL. All these traits are associated with adiposity, and similar data have been reported in various studies related to obesity in children [22–24]. Our biochemical results reinforce the observation that obese children have an important metabolic imbalance due to obesity. The assessment of biochemical data for overweight and obesity in children is not well standardized in Mexico, because the country does not have a standard information chart for children.

ADRB1 expression levels were higher in normal weight children than in obese ones. This result is consistent with a study in mice suggesting that its expression is important to prevent induced obesity caused a high fat diet [25]; to our knowledge, this is the first description of ADRB1 mRNA expression in human peripheral white blood cells. This report gives clues which might help to prevent obesity in children by increasing the expression of ADRB1. This also means that the mechanism of ADRB1 action might be stimulated by an agonist that could provoke a lipolysis increase in white adipocytes and thermogenesis in brown adipocytes, mainly by cAMP (cyclic adenosine monophosphate) production, activation of hormone-sensitive lipase and protein kinase A, among other pathways. Consequently, the adrenergic beta receptors affect lipid metabolism [26].

On the other hand, we found that ADIPOR1 is overexpressed in peripheral white cells of obese children in our sample. This receptor regulates several physiological aspects, including lipid metabolism [27]. A study in human adipose tissue showed reduced ADIPOR1 gene expression in obese adult subjects; the same study concluded that weight loss increased gene expression significantly. Additionally, the study demonstrated that weight loss induced improved insulin sensitivity, which may be mediated by up-regulation of adiponectin [28]. Moreover, it has also been reported that higher levels of receptor expression are associated with insulin resistance [29], which could be a compensatory mechanism to mitigate the effects of decreased adiponectin levels.

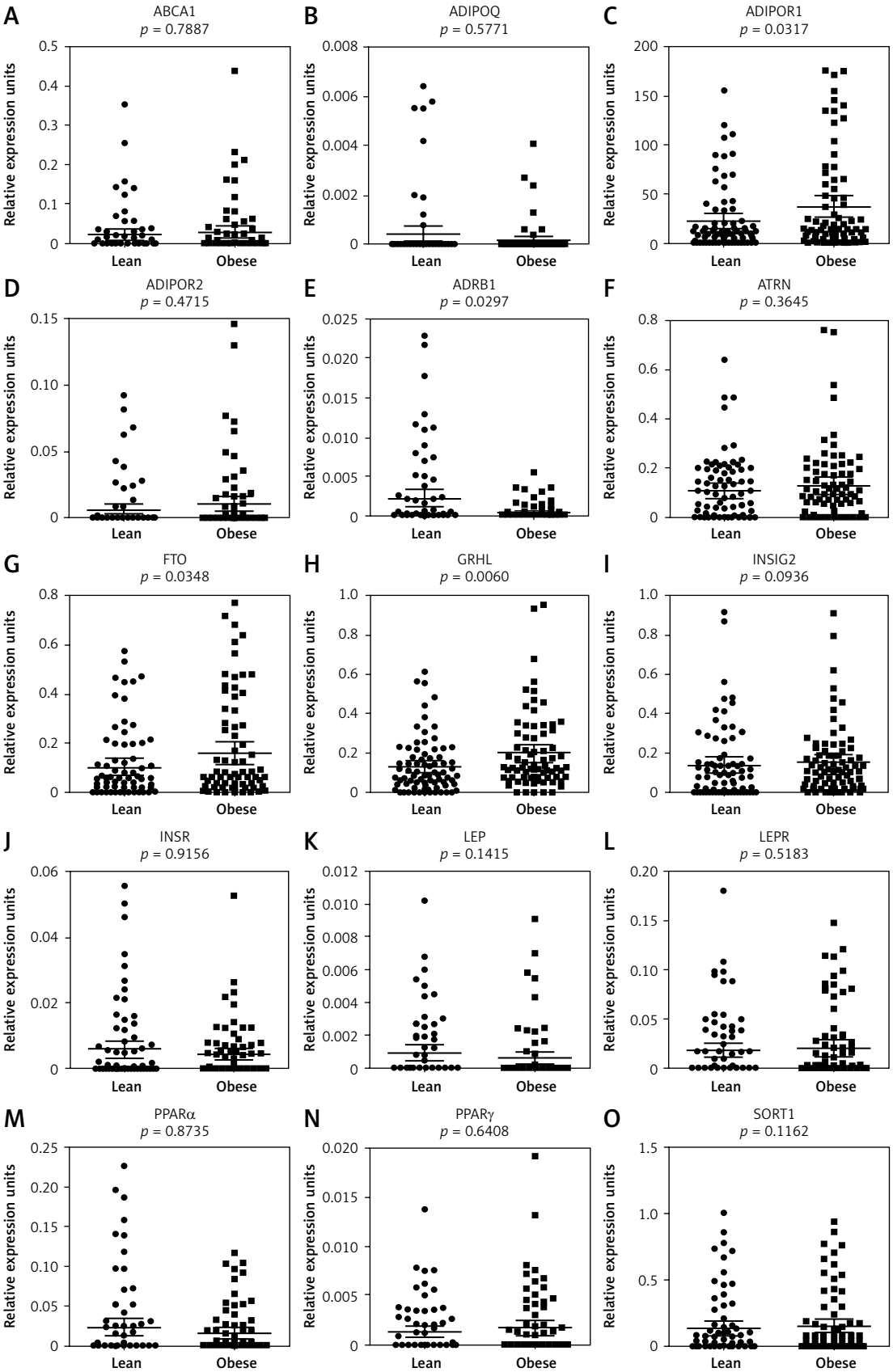

Figure 1. OpenArray quantitation of mRNA levels in peripheral white blood cells from lean and obese children. Data are expressed as relative expression units (arbitrary units) using TLR9 as a housekeeping gene. Bars represent standard error of the mean (SEM). *P*-value < 0.05 was considered statistically significant using the Mann-Whitney *U* test. Filled black circles, lean children; filled black squares, obese children. **A** – ABCA1, **B** – ADIPOQ, **C** – ADIPOR1, **D** – ADIPOR2, **E** – ADRB1, **F** – ATRN, **G** – FTO, **H** – GRHL, **I** – INSIG2, **J** – INSR, **K** – LEP, **L** – LEPR, **M** – PPARα, **N** – PPARγ, **O** – SORT1

It is remarkable that the levels of FTO expression were elevated in obese children. This result confirms what has been observed in other studies using murine and adult human models, where FTO is associated with the development of obesity. In a study conducted with mestizo Mexican adults, it was observed that the expression of FTO is greater in subcutaneous adipose tissue of obese individuals compared with healthy controls [30]. In another study, it was observed that mRNA FTO was associated with the body mass index (BMI) in adult persons of European origin, suggesting that expression may be regulated by the accumulation of fat in the body; specifically the visceral expression of FTO may contribute to the development of obesity [31]. In addition, using a murine model, it was found that an increase in FTO expression due to the inclusion of extra copies of the gene caused obesity. The mice that carried an extra copy of FTO exhibited an increase in body weight; it was also observed that FTO is directly related to food intake and mice metabolism, since an increase in the expression of FTO leads to an increase in the intake of food and body fat [32].

On the other hand, GHRL is also highly expressed in obese children. This gene has been associated with obesity because it produces an increase in adipose tissue and has a diabetogenic effect in the liver and pancreas. It has been observed that plasma ghrelin levels correlate inversely with BMI and food intake patterns; in general, its levels are reduced in obese individuals [33, 34].

In conclusion, in this study, we analyzed the level of gene expression of 20 candidate genes associated with obesity in peripheral white blood cells of Mexican children with and without obesity. We found a difference in the expression of ADRB1, ADIPOR1, FTO and GHRL. This suggested that these genes are involved in biological processes implicated in the development of childhood obesity.

We found that Mexican obese children have biochemical parameters comparable to adults. We found high levels of glucose, triglycerides, HDL, LDL and blood pressure in obese children of this study, suggesting the onset of metabolic imbalance that can lead to the development of various diseases mentioned above. This finding demonstrates the importance of enhancing medical actions to address this situation in children to minimize the risk of diseases associated with obesity. Obese children are at an increased risk of developing metabolic complications and cardiovascular disease risk factors.

We conclude that peripheral blood samples are a suitable tissue for gene expression studies and a promising tool in the field of transcriptomics. It is important to have biological samples in which we can easily analyze the expression levels of several genes and we might detect metabolic pathways to prevent and treat health problems with anticipation.

It is important to perform a comprehensive analysis of all parameters obtained from each individual and the expression of genes involved in various signaling pathways related to obesity in children. Hence, our study contributes new perspectives for a better understanding of biological processes that might be involved in obesity.

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Conflict of interest

The authors declare no conflict of interest.

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