



Trabajo Original

Obesidad y síndrome metabólico

Relation of serum IL-32 levels and gene polymorphism rs45499297 with obesity in Mexican patients: a laboratory and *in silico* analysis

Relación de los niveles séricos de IL-32 y del polimorfismo rs45499297 con la obesidad en pacientes mexicanos: un análisis de laboratorio e in silico

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Abstract

Background: many genes have been involved in the development of obesity. Interleukin 32 (IL-32) is a proinflammatory cytokine; rs45499297 is a T/C promoter, single-nucleotide polymorphism of the *IL-32* gene.

Objectives: this study aimed to evaluate the rs45499297 polymorphism and its association with obesity. Another objective of this study was to carry out an *in silico* analysis.

Methods: this study was cross-sectional, and included 333 subjects classified by body mass index and fat percentage. The plasma glucose and lipid profile were measured. We measured serum IL-32 protein by ELISA and the rs45499297 polymorphism by PCR-RFLP. We used several databases to build the *IL-32* gene network and infer transcription factors that bind to this polymorphic site.

Results: subjects underweight and with low fat percentages had lower levels of IL-32. CT genotype and allele C were less frequent in the overweight/obesity group than in the normal-weight group. Interestingly, this result remained only in the male gender. We found that the transcription factors Hepatocyte Nuclear Factor and Specificity Protein 1 bind to this polymorphic site. In addition, we infer that *IL-32* is involved in metabolic pathways related to viral infections.

Conclusion: the TC genotype is associated with overweight/obesity. The decrease in levels of IL-32 observed in underweight and low fat percentage groups could be due to an impaired inflammatory profile. The *in silico* analysis showed that several transcriptional factors bind at this polymorphic site, and that the enrichment of the metabolic pathways is diverse.

Keywords:

Obesity. Interleukin 32. BMI. Fat percentage. Polymorphism. *In silico*.

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Resumen

Introducción: la interleucina 32 es una citocina proinflamatoria. El rs45499297 es un polimorfismo de nucleótido simple del gen de *IL-32*, situado en la región promotora y caracterizado por un cambio de T/C.

Objetivo: evaluar el polimorfismo rs45499297 y su asociación con la obesidad, y realizar un análisis *in silico*.

Métodos: el estudio fue transversal e incluyó 333 sujetos clasificados por índice de masa corporal y porcentaje de grasa. Se midieron la glucosa y el perfil lipídico, así como los niveles séricos de IL-32 mediante ELISA y el genotipo del polimorfismo rs45499297 mediante PCR-RFLP. Para el análisis *in silico* se utilizaron varias bases de datos para hacer la red de genes de *IL-32* e inferir factores de transcripción unidos al sitio polimórfico.

Resultados: los sujetos con bajo peso y bajo porcentaje de grasa tienen niveles más bajos de IL-32. El genotipo TC y el alelo C se encontraron con menos frecuencia en los sujetos con sobrepeso/obesidad que en los normopeso, resultado que permaneció solo en el género masculino. Se encontró que el factor nuclear de los hepatocitos y la proteína de especificidad 1 se unen a este sitio polimórfico. Se infiere que *IL-32* está involucrado en vías metabólicas relacionadas con las infecciones virales.

Conclusión: el genotipo TC está asociado al sobrepeso/la obesidad. La disminución de los niveles de IL-32 observada en los sujetos con bajo peso y bajo porcentaje de grasa podría ser por un perfil inflamatorio alterado. El análisis *in silico* mostró que varios factores de transcripción se unen al sitio polimórfico y que el enriquecimiento de las vías metabólicas es diverso.

Palabras clave:

Obesidad. Interleucina 32. IMC. Porcentaje de grasa. Polimorfismo. *In silico*.

INTRODUCTION

The incidence of obesity is rapidly increasing in the entire world. In 2016, the World Health Organization (WHO) estimated that more than 1.9 billion adults aged 18 and older were overweight (1). In Mexico, data showed that the prevalence of being overweight between 20 and 29 years old was 29.5 % for men and 25.3 % for women for obesity (2).

Obesity results from a disruption of energy balance that leads to weight gain and metabolic disturbances that cause tissue stress, inflammation, and endothelial dysfunction (3) involving various environmental and genetic factors (4). Gene-association studies have identified several genes that affect obesity-related traits (5) and have suggested that single nucleotide polymorphisms (SNP) account for around 30 % of BMI (body mass index) variance (6).

Chronic low-grade inflammation contributes to obesity-associated comorbidities, and adipose tissue is a major immunologically active organ contributing to this inflammation (8,9). Adipose tissue is metabolically active that can secrete a variety of adipokines and proinflammatory cytokines such TNF α and IL-1 β in addition to anti-inflammatory cytokines like IL-10 (7).

Interleukin 32 (IL-32) is a proinflammatory cytokine that performs essential immune regulation. It is expressed by human peripheral blood mononuclear cells upon activation, induced in human epithelial cells by IFN- γ , and in natural killer cells by IL-12 plus IL-18 (9). IL-32 participates in inflammatory disorders such as rheumatoid arthritis, ulcerative colitis, multiple sclerosis, and cancer (10). Despite this characterization, the specific role of this protein in obesity remains to be determined.

The gene encoding IL-32 protein is located on chromosome 16p13.3 and contains six exons. Several SNPs have been studied in various diseases (5,11-13). The polymorphism rs45499297 is located in the promoter region of the *IL-32* gene, characterized by a change from T to C. However, the association between this polymorphism and BMI, fat percentage, and lipid profile has not been reported. This study aimed to evaluate the role of IL-32 serum levels and the rs45499297 polymorphism in obese subjects. Another objective was to carry out an *in silico* analysis to

investigate the interaction of this gene with factors that regulate its expression and analyze the network of this gene.

SUBJECTS, MATERIALS AND METHODS

STUDY PARTICIPANTS

This study was cross-sectional and included 333 subjects – 245 females and 88 males – all between 18 and 69 years of age. The participants were classified into the following groups: 22 underweight (BMI < 18.5 kg/m²), 188 normal-weight (BMI = 18.5-24.9 kg/m²), and 122 overweight/obese (BMI > 25 kg/m²) subjects. Additionally, we also classified them by percentage of fat. This interpretation was made considering gender and age as follows: for men, low (F% < 8), standard (F% = 8-19.9) and high/obese (F% \geq 20); in women, low (F% < 21), standard (F% = 21-32.9), and high/obese (F% \geq 33) (14). These guidelines yielded 16 low-fat, 153 standard-fat, and 164 high-fat/obese subjects.

Subjects were recruited from September 2017 to August 2019. The demographic and clinical characteristics of the studied population were recorded in an appropriate questionnaire. The exclusion criteria were based on subjects taking anti-inflammatory medications, having a history of autoimmune diseases, chronic alcohol usage, pregnancy, or women in lactation. All study subjects signed an informed consent form. The local ethics committee approved the study (6/2017-2018).

BIOCHEMICAL ANALYSIS

Blood samples were taken after eight hours of fasting and obtained from the antecubital vein in test tubes without anticoagulants. The serum was obtained by centrifuging at 3500 rpm for 20 minutes. An Abbott Aero Set autoanalyzer with the original kit was used to measure plasma glucose, total cholesterol, triglycerides, and HDL levels. The Friedewald equation was used to calculate LDL levels.

MEASUREMENT OF IL-32 LEVELS

We quantified serum levels of IL-32 by ELISA DuoSet (R&D Systems, USA). Briefly, plates were coated by 100 μ L per well with Capture Antibody, then 100 μ L of standard or sample was added per well, and the procedure was performed according to the manufacturer's instructions. The absorption was determined at 450 nm.

GENETIC ANALYSIS

DNA extraction was carried out from peripheral blood leukocytes using the Purelink Genomic DNA mini kit, following manufacturer's instructions. IL-32 SNP rs45499297 (T/C) was genotyped by the restriction fragment length polymorphism-based method, as previously described (12). Forward 5'-GATTGCTGAGACCAGTGA-3' and reverse 5'-TCTCTGAGCCCAGGAATG-3' primers were used to obtain a fragment of 445 bp. Amplification was performed with PCR conditions in a gradient thermocycler. The thermal profile was as follows: an initial denaturation step at 95 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 62 °C for 30 s, and an extension at 72 °C for 45 s, with a final extension step at 72 °C for 5 min. Amplified products were visualized by 6 % acrylamide gel electrophoresis.

We treated amplified products with the enzyme *Bam*HI. The outputs obtained were as follows: the TT genotype presented two fragments, 306 bp and 139 bp. The TC genotype showed 445 bp, 306 bp, and 139 bp fragments. The CC genotype is a fragment of 445 bp. Amplified products were visualized by 6 % acrylamide gel electrophoresis and stained with silver nitrate.

STATISTICAL ANALYSIS

The characteristics of subjects were described using simple frequency, percentages, mean and standard deviation. After applying the corresponding normality tests, the association between study variables was performed using the chi-square test, Kruskal-Wallis test, or ANOVA. A $p < 0.05$ was considered statistically significant.

IN SILICO ANALYSIS

The investigation of the factors that regulate transcription of the *IL-32* gene due to the effect of the rs45499297 polymorphism was carried out using the HaploReg (15), PROMO (16), rVar-Base (17), and AliBaba (18) software.

To analyze the *IL-32* gene network we used the COXPRESdb database (19) to obtain the genes coexpressed with *IL-32*. We used the first 50 genes and then constructed these genes' protein interaction network using STRING (20). KEGG pathway enrichment analyses of these coexpressed genes were carried out in the Database DAVID (21) and WebGestalt (22).

RESULTS

The demographic and biochemical characteristics of the study population classified according to their BMI and body fat percentage are shown in table I. As expected, the overweight/obesity group presented elevated glucose, total cholesterol, triglycerides, LDL-C, and VLDL-C levels. Overweight/obese subjects also presented lower HDL-C levels ($p < 0.001$). When separating subjects by body fat percentage, the high-fat/obesity group presented higher glucose, total cholesterol, triglycerides, LDL-C, and VLDL-C levels.

Table I. Demographic and biochemical characterization of the study population classified by BMI and fat percentage

BMI			
Variables	Underweight	Normal weight	Overweight/obesity
Gender (male/female)	6/16	41/147	41/81
Age (years)	20.7 \pm 4.45	21.6 \pm 5.27	26.7 \pm 11.20* [†]
Glucose (mg/dL)	85.24 \pm 12.74	85.30 \pm 17.79	92.24 \pm 19.37 [†]
Total cholesterol (mg/dL)	139.78 \pm 39.39	156.14 \pm 38.43	172.58 \pm 54.15* [†]
Triglycerides (mg/dL)	72.03 \pm 45.63	89.83 \pm 46.35*	115.59 \pm 63.83* [†]
HDL-C (mg/dL)	70.08 \pm 50.40	57.56 \pm 25.26	44.04 \pm 17.21* [†]
LDL-C (mg/dL)	58.47 \pm 53.12	84.44 \pm 45.85	107.71 \pm 55.29* [†]
VLDL-C (mg/dL)	14.40 \pm 9.12	17.96 \pm 9.27*	23.11 \pm 12.76* [†]
Fat percentage			
Variables	Low	Standard	High/obesity
Gender (male/female)	3/13	29/124	56/108
Age (years)	21.3 \pm 5.14	21.1 \pm 5.37	25.8 \pm 10.04 [†]
Glucose (mg/dL)	79.90 \pm 13.80	84.14 \pm 14.22	92.04 \pm 21.03* [†]
Total cholesterol (mg/dL)	136.79 \pm 22.84	156.87 \pm 45.54	167.29 \pm 46.74* [†]
Triglycerides (mg/dL)	59.27 \pm 18.18	89.56 \pm 47.66*	109.83 \pm 60.58* [†]
HDL-C (mg/dL)	64.62 \pm 37.30	57.19 \pm 28.30	48.50 \pm 21.91 [†]
LDL-C (mg/dL)	55.54 \pm 37.92	83.67 \pm 52.93*	101.80 \pm 49.36* [†]
VLDL-C (mg/dL)	11.85 \pm 3.63	17.91 \pm 9.53*	21.96 \pm 12.11* [†]

* $p < 0.05$ compared with the underweight group or low-fat group; [†] $p < 0.05$ compared with the normal-weight group or standard group.

Figure 1 shows the analysis of serum IL-32 levels. Underweight subjects had lower levels of IL-32 when compared to normal-weight subjects ($p = 0.035$). Besides, IL-32 was lower in subjects with low fat than in those with a standard fat percentage ($p = 0.025$) and high/obesity groups ($p = 0.026$).

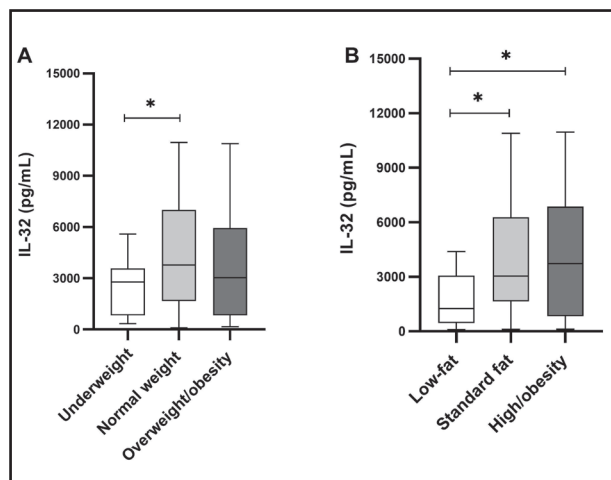


Figure 1. Analysis of serum IL-32 levels. A. Analysis according to BMI. The underweight group presented lower levels of IL-32 than the normal weight group. B. Analysis according to percentage of fat. The low-fat group presented lower levels of IL-32 than the standard-fat group and a high percentage of the high-fat/obesity group.

Later, we genotyped the *IL-32* rs45499297 polymorphism. As shown in table II, the CC genotype is not associated with obesity, according to BMI. However, the TC genotype showed a statistical difference between the three study groups ($p = 0.009$); specifically, this difference was between the normal-weight and overweight/obesity groups ($p = 0.003$). We obtained similar results for allele C ($p = 0.048$); and, like in the genotype analysis, the difference was between the normal-weight and overweight/obesity groups ($p = 0.024$). The rs45499297 polymorphism analysis showed no significant differences between the different fat percentage groups for both genotypes and alleles (Table II). Also, the rs45499297 polymorphism did not affect biochemical parameters or IL-32 protein levels (data not shown).

We also analyzed the association between obesity and gender. Men have a higher frequency of obesity by percentage of fat ($p = 0.007$) and a trend towards higher BMI ($p = 0.067$) than women (data not shown). However, when we included genotype in this analysis (we omitted the CC genotype for statistical reasons), we found a statistical difference between the three groups ($p = 0.026$). Interestingly, it was observed that the TC genotype is less frequent in the male overweight/obesity group ($p = 0.016$). This suggests that the TC genotype may have a protective effect against obesity in young men. Data are shown in table II.

For the *in silico* analysis, we examined the potential functional effect of this polymorphism in various databases. As seen in table III, we found several transcription factors that overlap in the position of the rs45499297 polymorphism. It is noteworthy that two

Table II. Association of the IL-32 polymorphism by BMI and fat percentage and by gender

	BMI				Fat percentage			
	Underweight % (n = 22)	Normal weight % (n = 189)	Overweight/obesity % (n = 122)	p-value	Low % (n = 16)	Standard % (n = 153)	High/obesity % (n = 164)	p-value
Genotype/Allele								
TT	86.4 (19)	70.4 (133)	84.5 (103)	Ref.	75.0 (12)	75.2 (115)	76.6 (128)	Ref.
TC	13.6 (3)	29.1 (55)	13.9 (17)*	0.009	18.7 (3)	24.8 (38)	22.5 (34)	0.790
CC	0	0.5 (1)	1.6 (2)	0.809	6.3 (1)	0	0.9 (2)	0.510
T	93.2 (41)	84.9 (321)	91.4 (223)	Ref.	93.7 (30)	87.6 (268)	88.4 (290)	Ref.
C	6.8 (3)	15.1 (57)	8.6 (21)*	0.048	6.3 (2)	12.4 (38)	11.6 (38)	0.756
Male								
TT	80.0 (5)	68.3 (28)	92.3 (36)	0.026	66.7 (2)	72.4 (21)	85.2 (46)	0.316
TC	20.0 (1)	31.7 (13)	7.7 (3)*		33.3 (1)	27.6 (8)	14.8 (8)	
Female								
TT	87.5 (14)	71.4 (105)	82.7 (67)	0.087	83.3 (10)	75.8 (94)	75.9 (82)	0.839
TC	12.5 (2)	28.6 (42)	17.3 (14)		16.7 (2)	24.2 (30)	24.1 (26)	

* $p < 0.05$ compared with the normal-weight group.

Table III. Transcription factors and motifs overlapping the rs45499297 *IL-32* polymorphism according to different databases

SNP	Alleles	Location	HaploReg	PROMO	rVarBase	AliBaba
rs45499297	T/C	chr16:3115272-3115273	ATF3_disc2, ERalpha-a_disc2, Glis2, HNF4_known1, LRH1, Nkx2_9, Pax-5_disc1, RXR::LXR, RXRA_known1	HNF-3alpha, RXR-alpha	JUND, HNF4G, TCF12, POU2F2, FOS, FOSL2, MAX, HNF4A, STAT3, STAT1, HEY1, MAFK, ATF2, BCL3, HSF1, SP1, HDAC2, BCL3	SP1, NF-1

of them were inferred in three databases — Hepatocyte Nuclear Factor (HNF), with its variants. In addition, the transcriptional factor Specificity Protein 1 (SP1) was inferred in two databases. Therefore, this polymorphism appears to be a binding site for different transcription factors.

Based on the fact that IL-32 levels were decreased in the underweight group, we carried out the analysis of the IL-32 network (Fig. 2). We found that the 50 genes co-expressed with *IL-32* were interconnected with 327 interactions. The top 5 of the KEGG metabolic pathways are shown in figure 2A, along with the number of genes involved in each metabolic pathway. Figure 2B shows the network of genes co-expressed with *IL-32*. It should be noted that *IL-32* does not participate in any of the enriched routes of the top 5.

(11,23,24). We found significantly decreased levels of IL-32 in subjects who were underweight and low-fat when compared to normal-weight and standard-fat subjects. Fadaei et al. also performed IL-32 measurements and found higher levels in patients with type 2 diabetes *mellitus* versus controls, which supports the results from this study; in the diabetes group, they also found a positive correlation between BMI and IL-32 (25).

IL-32 production is predominantly induced by IL-1 β , TNF α , IL-2, or INF- γ in monocytes and epithelial cells (23, 26). Interestingly, malnutrition presents alterations of IL-1 β , TNF α , IL-2, and IFN- γ (27-30). Therefore, these cytokines may be regulating the production of IL-32 protein levels in underweight subjects. Rytter et al. found several levels of cytokines in children with malnutrition (27). Th1-cytokines IL-1, IL-2, IL-12, and INF- γ were lower in malnourished than in well-nourished children, while Th2-cytokines IL-10 and IL-14 were higher in those who were malnourished compared to those well-nourished. The authors conclude that malnourished children have an anti-inflammatory

DISCUSSION

IL-32 is a proinflammatory cytokine that is important for immune regulation and implicated in inflammatory disorders

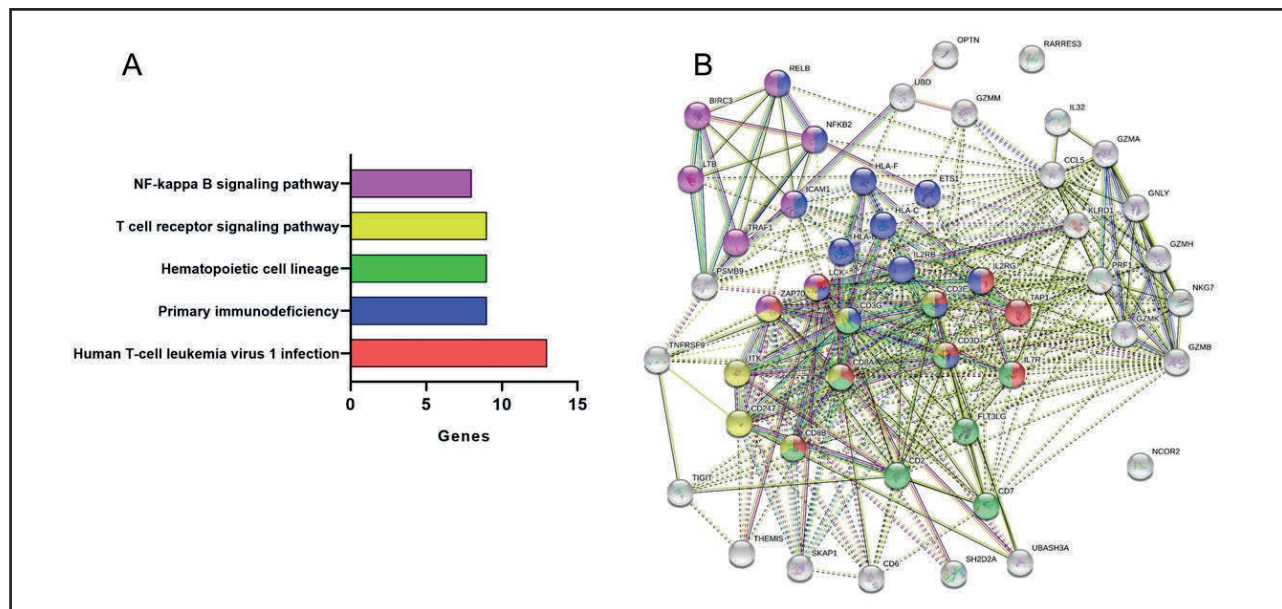


Figure 2. Enriched metabolic pathways and *IL-32* interaction network. A. Top 5 of the enriched metabolic pathways and the number of genes that participate in each of them. B. *IL-32* network — the genes of the enriched metabolic pathways match the color of the genes involved.

profile. Therefore, other altered cytokines in malnutrition may be affecting serum IL-32 levels in our study group.

We analyzed the rs45499297 polymorphism and its relationship with obesity for the first time in a Mexican population. We found that allele C and genotype TC of the IL-32 polymorphism are less frequent in the overweight/obesity group. Because so far there are no reports of this polymorphism in obesity or metabolic diseases, we cannot compare our results. However, Morsaljahani et al. analyzed this polymorphism in people from Iran with multiple sclerosis. They found that allele C was more frequent in patients than controls (11), so these results could indicate that this polymorphism is associated with another inflammatory disease.

We found that this polymorphism did not modify the biochemical parameters or serum levels of IL-32. There are also no reports of the rs45499297 polymorphism compared to biochemical parameters. However, in a study reported by Damen et al., which analyzed *IL-32* promoter rs4786370 in patients with rheumatoid arthritis, the lipid profile was affected (12); this may be because IL-32 is an essential regulator of cholesterol transporters ABCA1 and ABCG1 (31). Therefore, other polymorphisms of IL-32 may be capable of affecting biochemical parameters, although it is advisable to look for new factors associated with the phenotype of obesity, as in this case the rs45499297 polymorphism.

The high/obesity group was comprised of more men than women. Other studies have reported results similar to ours, where young men have a higher percentage of obesity than women (32,33). However, our results appear to show that the TC genotype acts as a protective factor in men. This result could be due to the different hormonal profiles existing between both genders, as it is well known that people with obesity have high levels of leptin (34,35). Leptin is present in higher amounts in females than in males (36,37). Therefore, the TC genotype may confer protection against being overweight and obese in men more than women due to hormonal reasons.

Since it is known that transcription factors regulate gene expression by binding to the promoter region of the gene, it is presumed that the polymorphisms present in these regions affect gene expression. Hepatocyte Nuclear Factor 4 alpha (HNF4 α), or its variants, and the transcriptional factor Specificity Protein 1 (SP1) were inferred in different databases. Previous studies reported that the *IL-32* gene has a binding site for these transcriptional factors (39,40). However, we have inferred that the binding site overlaps with the rs45499297 polymorphism.

Our network analysis also showed the interconnectivity between signaling pathways such as human T-cell leukemia virus 1 infection (HTLV1), primary immunodeficiency, hematopoietic cell lineage, T-cell receptor signaling pathway, and NF-kappa B signaling pathway. Malnutrition has been associated with HIV and HTLV1 (39,40), so it is inferred that genes co-expressed with *IL-32* are involved with these metabolic pathways, in addition to the fact that this cytokine is modified in malnourished states. This information is essential to understand disease mechanisms, especially in diseases with a high degree of complexity, such as obesity, in which several metabolic pathways are involved in the pathogenesis of the disease.

One limitation of the current study is the mean age of the participants, as the findings cannot be generalized to middle-aged or mature adults. So more research is needed with a greater range of ages. In addition, the results of the *in silico* analysis must be corroborated by experimental work.

CONCLUSION

Until now, there is little information on IL-32 and obesity. This study found an association of the rs45499297 polymorphism with overweight/obesity. However, there was no association of the polymorphism with lipid profile and serum IL-32 protein levels. The level of IL-32 was lower in underweight and low-fat subjects. On the other hand, the *in silico* analysis showed that this polymorphism is found in the binding site of several transcriptional factors. In addition to that, the enrichment of the metabolic pathways is diverse.

The results presented in this work could stimulate future research to clarify the pathophysiology of obesity, which has become a global problem that increases over time. Moreover, it is possible to better understand this pathology from a nutritional perspective and the genetic and immunology points of view.

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