

BMJ Open Are *IL18RAP* gene polymorphisms associated with body mass regulation? A cross-sectional study

Vanesa Martínez-Barquero,^{1,2} Griselda de Marco,² Sergio Martínez-Hervas,^{1,3,4} Victoria Adam-Felici,² Cristina Pérez-Soriano,² Verónica Gonzalez-Albert,² Gemma Rojo,^{3,5} Juan Francisco Ascaso,^{1,3,4} José Tomás Real,^{1,3,4} Ana Barbara Garcia-Garcia,^{2,3} Juan Carlos Martín-Escudero,⁶ Raquel Cortes,² Felipe Javier Chaves^{2,3}

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¹Department of Medicine, University of Valencia, Valencia, Spain

²Department of Genomic and Genetic Diagnosis Unit, Hospital Clínico Research Foundation (INCLIVA), Valencia, Spain

³CIBER of Diabetes and Associated Metabolic Diseases (CIBERDEM), Madrid, Spain

⁴Department of Endocrinology and Nutrition Service, Hospital Clínico Universitario de Valencia, Valencia, Spain

⁵Department of Endocrinology and Nutrition Service, Hospital Regional Universitario, Instituto de Biomedicina de Málaga (IBIMA), Málaga, Spain

⁶Department of Internal Medicine, Rio Hortega Hospital, University of Valladolid, Valladolid, Spain

Correspondence to

Dr Ana Barbara Garcia-Garcia; a.barbara.garcia@ext.uv.es

ABSTRACT

Objectives To investigate the association between *IL18RAP* and body mass index (BMI) and obesity and to verify the effect of a polymorphism in the microRNA136 (MIR136) *IL18RAP* binding region.

Design We analysed samples from two Spanish cross-sectional studies, VALCAR (Spanish Mediterranean coast) and Hortega (Spanish centre). These studies aimed at analysing cardiovascular risk and development of cardiovascular disease in the general population. Both populations correspond to regions with different characteristics.

Setting Five *IL18RAP* single nucleotide polymorphisms were selected using the SYSNPs web tool and analysed by oligonucleotide ligation assay (SNPlex). For the MIR136 functional study, cells were transfected with plasmids containing different rs7559479 polymorphism alleles and analysed by luciferase reporter assays.

Participants 1970 individuals (Caucasian, both genders): VALCAR (468) and Hortega (1502).

Results rs2293225, rs2272127 and rs7559479 showed the following associations: rs7559479 G allele correlated with a higher obesity risk ($P=0.01$; OR=1.82; 95% CI 1.15 to 2.87 for the VALCAR group; $P=0.033$; OR=1.35; 95% CI 1.03 to 1.79 for the Hortega population) and higher body mass index (BMI) values ($P=0.0045$; $P=0.1$ for VALCAR and Hortega, respectively); a significant association with obesity ($P=0.0024$, OR=1.44, 95% CI 1.14 to 1.82) and increased BMI values ($P=0.008$) was found when considering both populations together. rs2293225 T allele was associated with lower obesity risk ($P=0.036$; OR=0.60; 95% CI 0.35 to 0.96) and lower BMI values ($P=0.0038$; OR=1.41) while the rs2272127 G allele was associated with lower obesity risk ($P=0.028$; OR=0.66; 95% CI 0.44 to 0.97) only in the VALCAR population. A reporter assay showed that the presence of the A allele in rs7559479 was associated with increased MIR136 binding to *IL18RAP*.

Conclusions Our results suggest that polymorphisms in *IL18RAP* influence susceptibility to obesity. We demonstrated that the A allele in rs7559479 increases MIR136 binding, which regulates IL-18 system activity.

Strengths and limitations of this study

- Our study was carried out in two Spanish populations from different regions with noticeably distinct characteristics, although the results were similar for both.
- The number of individuals included in this study was limited, but its statistical power was sufficient for the reduced number of polymorphisms we analysed.
- We did not exhaustively study genetic variability in this gene because this gene region has too many variations, and would have required the study of more polymorphisms.
- Our results should be confirmed by other studies, which include analyses of different populations and with larger sample sizes.

INTRODUCTION

Obesity is a state in which excess energy accumulates in corporal adipose tissue due to a chronic imbalance between energy intake and energy expenditure¹; individuals respond differently to this imbalance because of their differing genetic backgrounds.² Over the past 20 years there has been an alarming increase in the number of overweight and obese individuals. This is especially worrying because obesity significantly increases the risk of chronic diseases, such as type 2 diabetes, cardiovascular disease, non-alcoholic fatty liver disease, colon cancer and obstructive sleep apnoea.³

Obesity is defined as a low-grade inflammatory state characterised by increased cytokine production.⁴ Interleukin-18 (IL-18) concentration increases in obesity, suggesting it is involved in obesity and metabolic syndrome by increasing adipogenesis.⁵ IL-18 is a member of the IL-1 cytokine superfamily. It regulates the immune response and is expressed in cells involved

in chronic inflammation, autoimmune diseases and several cancers and infectious diseases: macrophages, dendritic cells, Kupffer cells, keratinocytes, osteoblasts, adrenal cortex cells, intestinal epithelial cells, microglial cells and synovial fibroblasts.^{6–14} IL-18 signalling is induced by binding of this cytokine to a heterodimeric receptor called IL-18R. IL-18R is formed of two chains, IL-18R1 (with a binding role) and IL18RAP (receptor accessory protein, with a signal-transducing role). The interaction of both IL-18R chains is mandatory to induce IL-18 signalling.^{15–17}

Some polymorphisms can modulate gene activity via different mechanisms, such as modifying the binding or action of specific microRNAs (miRNAs). They are small non-coding RNAs which modulate expression of thousands of genes, and studies performed over the past decade suggested their involvement in disease regulation.^{18–19} Several miRNAs have been described as biomarkers for cancers, cardiovascular disease and type 2 diabetes.^{18–20–22} Furthermore, it has been suggested that a failure in miRNA regulation plays a part in obesity.²³ These facts support the use of miRNA as biomarkers for the early diagnosis of chronic diseases and as therapeutic targets.²⁴

Many studies have reported on IL-18 and IL18R1, but few specifically discussed IL18RAP; therefore, we investigated, for the first time, the possible association between IL18RAP, body mass index (BMI) and obesity. To do this, we verified the effect of polymorphism rs7559479 located in the MIR136 binding site in the mRNA of *IL18RAP* and the mRNA levels of this gene.

MATERIALS AND METHODS

Population samples

We analysed two general-population-based study samples (a total of 1970 people) from two different regions of Spain: the VALCAR study, with 468 subjects from the Valencian region and the Hortega study, with 1502 subjects from the province of Valladolid. Both the VALCAR and Hortega samples were originally collected in order to study cardiovascular risk and the development of cardiovascular disease in the general population. The Hortega sample comprised subjects from the healthcare authority area representing half the Valladolid province, while the VALCAR patients came from the health authority area covered by the Hospital Clínico Universitario de Valencia. The Hortega study was approved by the ethics committee at the Hospital Río Hortega and the VALCAR study, as well as the genetic studies presented here, were approved by the ethics committee at the Hospital Clínico Universitario de Valencia. All participants gave their signed informed consent. The research was carried out according to the code of ethics of the World Medical Association (Declaration of Helsinki).

Demographic data (age and gender) and anthropometric parameters were collected using standard

procedures. The presence of obesity, hypertension (HTN) and type 2 diabetes (as defined by the WHO criteria; <http://www.who.int>) was recorded and the BMI (kg/m^2) was calculated. Briefly, obesity was diagnosed when the BMI was $\geq 30 \text{ kg/m}^2$, HTN was diagnosed when systolic and diastolic blood pressure values were >140 and/or >90 mm Hg, respectively, and diabetes was defined as a fasting plasma glucose level $>7.0 \text{ mmol/L}$ (126 mg/dL) or a 2-hour plasma glucose level $\geq 11.1 \text{ mmol/L}$ (200 mg/dL). A previous diagnosis of type 2 diabetes or HTN and detection of the disease at the time of sample collection was also recorded. The general characteristics of the samples analysed are shown in [table 1](#).

The exclusion criteria were the same in both populations: the presence of serious physical conditions which prevented the subject from responding to the survey or donating the samples required, disorders that might influence the collection of reliable information, or any mental or social condition which might complicate or prevent the subject's participation in the study. The decision to exclude patients was made on an individual basis by the clinician.

SNP selection and genotyping

Venous blood samples were collected in tubes containing EDTA in order to obtain genomic DNA using the Chemagic system (Chemagen, Baesweiler, Germany). DNA was quantified and diluted to a final concentration of $100 \text{ ng}/\mu\text{L}$. The *IL18RAP* single nuclear polymorphisms (SNPs) were selected for genotyping based on the conjunction of several parameters selected in the SYSNPs web tool²⁵: heterozygosity in a Caucasian population ($>10\%$ for the minor allele frequency), position and spacing along the gene and a possible functional effect (<http://www.ensembl.org/index.html>). Five SNPs in the *IL18RAP* gene were selected ([table 2](#)) and were genotyped using an oligonucleotide ligation assay (SNPlex; Applied Biosystems, Foster City, California, USA), according to the manufacturer's guidelines. The genomic information about the selected SNPs was obtained from Ensembl release 89 and the single nucleotide polymorphism database (dbSNP) build 149.

Statistical analysis

Before the study, we estimated the statistical power using the lower frequency included in the selection criteria of the selected polymorphisms (minor allele frequency=0.10) for the lower sample size (distributed in cases and controls) by the GAS Power Calculator (http://csg.sph.umich.edu/abecasis/gas_power_calculator/index.html). We considered a P value of 0.01 (based on multiple comparisons). The statistical power obtained for OR=2.5 was 91.9% for one stage study. Applying the same criteria for whole sample the statistical power was 0.975 for OR=1.5.

After genotyping, samples with poor genotyping results ($<90\%$) were removed for all the SNP analyses.

Table 1 General characteristics of the population

Characteristics	VALCAR	Hortega	VALCAR+Hortega
Number of subjects	468	1502	1970
Age (years)	46.4±14.9*	54.4±19.3	52.6±18.7
BMI† (kg/m ²)	28±4.7*	26.4±4.1	27.7±4.4
Waist circumference (cm)	92.6±12.8*	89.5±13.0	90.2±13.0
Obesity (n, %)	114 (24.4)*	356 (23.7)	470 (23.9)
Abdominal obesity (n, %)	151 (32.3)*	414 (27.6)	565 (28.7)
Diabetes mellitus (n, %)	55 (11.8)*	114 (7.6)	169 (8.6)
Glucose† (mg/dL)	99.7±24.4*	92.5±20.4	91.9±29.9
Hypertension (n, %)	129 (27.5)*	642 (42.7)	771 (39.1)
Systolic blood pressure† (mm Hg)	126.5±16.1*	130.7±21.6	129.5±20.5
Diastolic blood pressure† (mm Hg)	77.1±9.9*	79.1±10.6	78.2±10.4
Total cholesterol (mg/dL)	222.7±61.7*	201.3±38.1	207.6±46.2
HDL cholesterol (mg/dL)	56.4±15.1*	51.7±14.2	53.9±14.5

Values are mean±SD deviation. For qualitative variables, data are expressed as (n, %).

*P<0.0001.

†Number of missing data in VALCAR/Hortega populations: BMI 35/38, systolic blood pressure 53/10, diastolic blood pressure 53/10, glucose 5/2.

BMI, body mass index; HDL, high-density lipoprotein.

All quantitative values are expressed as the mean±SD or as a percentage for qualitative variables. Analysis of variance was used to compare quantitative variables between groups, and χ^2 tests were used for categorical variables. Allele and genotype frequencies were calculated for each SNP using SPSS Statistics package version 22.0 and SNPStats (<http://bioinfo.iconcologia.net/SNPstats>). The Hardy–Weinberg equilibrium (HWE) was tested using a χ^2 distribution with one degree of freedom using SNPStat software. HWE was maintained for all the polymorphisms analysed.

The associations between polymorphisms and anthropometric parameters, BMI as a quantitative trait and obesity as a qualitative trait were examined, first using a co-dominant inheritance model in SPSS and SNPStat and then using the remaining models if two genotypes had similar means. Analysis of variance was used to compare the mean differences for continuous variables among genotypes. The association between obesity and each polymorphism and, where appropriate, the haplotype,

was tested using logistic regression models. Linkage disequilibrium measurements were calculated using the R2 statistic. Odds ratios were used to evaluate the risk for the presence of obesity for each polymorphism, and age and gender were used as two potential confounding covariates.

MiRNA target prediction

Because the rs7559479 polymorphism is in the 3'-untranslated region (3'-UTR) of the *IL18RAP* gene, the microRNA.org tool (August 2010 release) was used to predict the microRNA target.

Cell lines and plasmids

HuH-7 cells, an immortal epithelial-like tumorigenic cell line, were used for miRNA assays. Cells were cultured in Dulbecco's modified Eagle medium containing 10% fetal bovine serum, L-glutamine and antibiotics and were grown in a humidified environment at 37°C with 5% CO₂. The pEZEX-MT05 control reporter, reporters containing

Table 2 Characteristics of selected polymorphisms from the *IL18RAP* gene (Ensembl ID: ENSG00000115607)

SNP name	Chromosome position	Location	Reference	% Genotype	HWE	MAF	Allele reference vs minor
rs4851581	102418289	Upstream	NM_003853.3:c.-989A>G	95.2	0.196	0.053	A>G
rs2293224	102419319	Intron 1	NM_003853.3:c.-337-242T>C	96.8	0.522	0.466	C>T
rs2293225*	102419429	Intron 1	NM_003853.3:c.-337-132C>T	98.6	0.129	0.167	C>T
rs2272127	102423413	Intron 3	NM_003853.3:c.70+66C>G	99.9	0.149	0.195	C>G
rs7559479*	102452327	3'-UTR	NM_003853.3:c.*146G>A	99.7	0.42	0.277	A>G

*Tag-SNP by HapMap in Caucasian patients.

SNP name: dbSNP build 149. Reference: begins in the first nucleotide of exon 1, build 149 and Ensemble release 89.

HWE, Hardy–Weinberg equilibrium; MAF, minor allele frequency.

Table 3 Association between obesity and body mass index (BMI) and the rs7559479 polymorphism, adjusted by age and sex in both samples (values for BMI are the mean \pm SE; “% Non-obese” indicates the % of non-obese subjects carrying AA or AG-GG genotypes, “% Obese” indicates the % of obese subjects carrying AA or AG-GG genotypes; bold indicates significance)

Genotype	N	BMI	% Non-obese	% Obese	Obesity, OR (95% CI)
AA	1062	26.41 (0.13)	877 (59.6%)	185 (50.7%)	1.00
AG-GG	774	26.96 (0.16)	594 (40.4%)	180 (49.3%)	1.44 (1.14 to 1.82)
P value		0.008			0.0024

the *IL18RAP* 3'-UTR with either the most frequent rs7559479 A-allele polymorphism or the rs7559479 G-allele polymorphism, as well as the pEZX-MR04 control reporter and the MIR136 reporter, were purchased from GeneCopoeia.

Transfection and luciferase reporter assay

HuH-7 cells were seeded in six-well plates the day before transfection and were 70–90% confluent at the time of use. Transfections were carried out with a calcium phosphate Profection Mammalian Transfection System kit (Promega, Madison, USA), according to the manufacturer's instructions. Seventy-two hours after transfection, we collected the media and measured the Gaussia luciferase and secreted alkaline phosphatase activity using a Secrete-Pair Dual Luminescence assay kit (GeneCopoeia, Rockville, USA), according to the manufacturer's instructions. Luminescence was measured using a Perkin Elmer Wallac 1420 VICTOR2 multilabel counter.

RESULTS

Characteristics of the study population

The association between *IL18RAP* gene polymorphisms and obesity traits was first identified in the VALCAR population, and similar results were subsequently found in the Hortega study; when we analysed both samples together this association remained. The main characteristics of the two samples are shown in [table 1](#). The VALCAR study included 468 individuals (24.8% obese; 47.2% male) and the Hortega study included 1502 subjects (23.7% obese; 49.5% male).

Association between *IL18RAP* gene polymorphisms and obesity traits

All the polymorphisms analysed were in HWE. Of the five SNPs analysed in the *IL18RAP* gene, rs2293225, rs2272127 and rs7559479 were associated with obesity. We focused on rs7559479 because it is the one most strongly associated with obesity. The presence of the G allele was associated with a higher obesity risk ($P=0.0024$, $OR=1.44$) and BMI values ($P=0.008$) in the pooled population ([table 3](#)) and was significantly associated with obesity risk in each individual population. We found an association for rs2293225 and rs2272127 only in the VALCAR population: the T allele in rs2293225 was associated with a lower obesity risk ($P=0.036$; $OR=0.60$) and with lower BMI values ($P=0.0038$; $OR=1.41$) and the G allele in rs2272127 was associated with a lower obesity risk ($P=0.028$; $OR=0.66$) but not with BMI values. These associations are summarised in [table 4](#).

Haplotype analysis

We performed haplotype analysis for the rs2272127, rs2293225 and rs7559479 variations, which all have a high degree of linkage disequilibrium ($D'>0.90$). The CCG haplotype (number 2 in [table 5](#)) was associated with a higher risk of obesity ($P<0.0015$) and with BMI ($P<0.0088$). This haplotype includes the risk genotypes found in each variation and was present in 23% of the individuals in the whole study population.

Confirmation of the interaction between MIR136 and the *IL18RAP* 3'-UTR

Our analyses indicate that the rs4851581, rs2293224, rs2293225 and rs2272127 polymorphisms ([**Table 4** Summary table for the associations found in the *IL18RAP* gene \(bold indicates significance\)](http://www.</p>
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SNP name	Gene location	Associated phenotype	Populations where an association has been found	Associated allele
rs4851581	2:102418289	No associations	--	--
rs2293224	2:102419319	No associations	--	--
rs2293225	2:102419429	Lower obesity, lower BMI	VALCAR	T
rs2272127	2:102423413	Lower obesity	VALCAR	G
rs7559479	2:102452327	Higher obesity	VALCAR	G
		Higher obesity	Hortega	
		Higher obesity, higher BMI	VALCAR+Hortega	

BMI, body mass index

Table 5 Haplotype association analysis for rs2272127, rs2293225 and rs7559479 in the *IL18RAP* gene with the body mass index (BMI) and obesity risk adjusted for age and sex

No	Haplotype	Frequency		Freq.	OR (95% CI)	P value	BMI	
		Non-obese	Obese				Difference (95% CI)	P value
1	CCA	0.344	0.308	0.337	1.00	–	0.00	–
2	CCG	0.220	0.277	0.232	1.44 (1.15 to 1.80)	0.0015	0.48 (0.12 to 0.85)	0.0088
3	GCA	0.225	0.232	0.226	1.13 (0.90 to 1.43)	0.30	0.08 (–0.29 to 0.44)	0.68
4	CTA	0.206	0.175	0.199	0.95 (0.74 to 1.21)	0.68	–0.2 (–0.57 to 0.17)	0.29

SNPs used in the haplotype construction: rs2272127, rs2293225 and rs7559479.

Bold indicates significance.

Freq, frequency.

ensembl.org/index.html) do not have a clear functional effect; however, rs7559479 is located in the 3'-UTR of *IL18RAP* in the MIR136 miRNA-binding region (<http://microrna.org>). This variation can change the binding of this miRNA and therefore can affect the levels of *IL18RAP* mRNA. Thus, it may also influence the downstream adipogenic effect of IL-18 and might be involved in BMI regulation.

To clarify the functional effect of the rs7559479 SNP on the *IL18RAP* mRNA MIR136 binding site, we performed an in vitro study in HuH-7 cells. Five plasmids were used: the control reporter pEZX-MT05, a reporter containing the *IL18RAP* 3'-UTR with either the rs7559479 A- or G-allele polymorphism, the control pEZX-MR04 reporter and the MIR136 reporter, as well as a plasmid that expresses secreted alkaline phosphatase as a transfection control. The expression level was represented as the ratio between the activities of the two enzymes. Figure 1A shows similar luminescence in the reporters containing the *IL18RAP* 3'-UTR with the rs7559479 A-allele and G-allele polymorphisms in the absence of MIR136. Figure 1B shows that co-transfection of the reporters containing these *IL18RAP* 3'-UTR rs7559479 polymorphisms alongside the MIR136 reporter produced a significantly higher signal ($P<0.05$) for the G allele, meaning that miRNA binding was lower

for this SNP than for the A-allele polymorphism. Therefore, the A allele (which is associated with a lower risk of obesity) increases MIR136 binding and is thus likely to reduce *IL18RAP* protein production. Figure 2 shows the characteristics of the interaction between MIR136 and rs7559479 presented according to Piletič and Kunej.²⁶

DISCUSSION

In this population-based study we examined five *IL18RAP* gene polymorphisms related to the IL-18 adipogenic signalling pathway and found that the rs7559479 AG-GG genotypes were associated with higher BMIs and a greater risk of obesity. It is particularly noteworthy that even though the Hortega and VALCAR populations had different characteristics, the findings were replicated in both; furthermore, when we studied the pooled population, the strength of this association with BMI values and obesity became even stronger. As noted in table 4, a haplotype that is associated with a higher obesity risk and which is frequent in the population is congruent with the data obtained in individual polymorphism analyses (haplotype CCG for rs2272127, rs2293225 and rs7559479). These results reinforce the idea that the IL-18 cascade may be involved in BMI regulation and obesity.

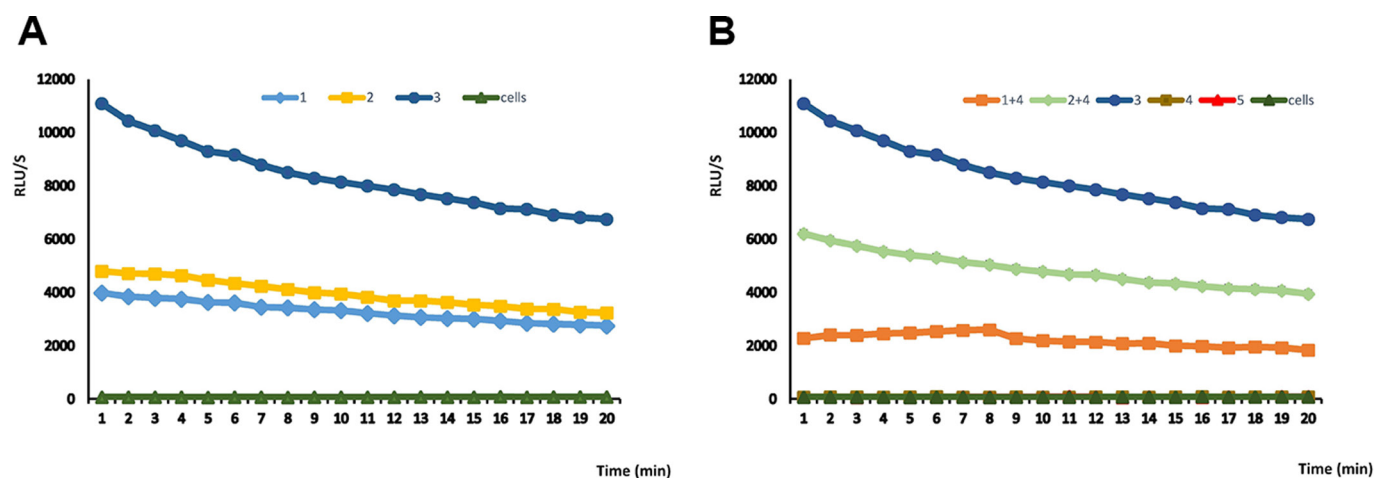


Figure 1 Luciferase assays for rs7559479 (a MIR136 target) using different reporters and combinations. Reporters used were: rs7559479 polymorphism A allele (1), rs7559479 polymorphism G allele (2), control reporter pEZX-MT05(3), MIR136 (4) and control reporter pEZX-MR04 (5). Data are represented as the mean±SE. Reporters 4, 5 and cells are negative controls.

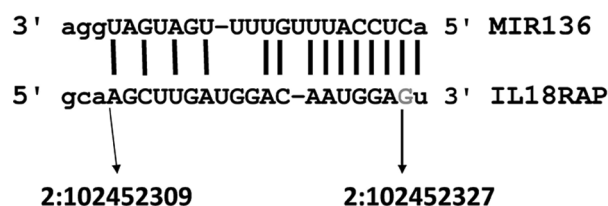


Figure 2 Characteristics of the interaction between MIR136 and *IL18RAP*. MicroRNA targets predicted using the microRNA.org tool are shown; the base in grey corresponds to the position of the rs7559479 polymorphism.

These relationships have not been found in previous genome-wide association studies, but this may be the result of different environmental factors, such as nutrition, in the populations studied. Mediterranean populations might specifically differ from other populations, especially because they are usually under-represented in genome-wide association studies, which often do not include any Spanish samples.^{27–29} Nevertheless, one study analysed 22 obesity-related gene loci in Spanish populations and found that only the *FTO* gene was clearly associated with BMI.³⁰ Considering the high heritability of obesity, it is very likely that new variants remain to be discovered, especially in the Spanish/Mediterranean population, where polymorphisms do not seem to have the same effects as described elsewhere in the literature.

No previous studies have described the association between increased weight or obesity and the rs7559479 SNP that we have established in this work. However, obesity is strongly associated with inflammation³¹ and increased IL-18 concentrations are thought to play a pathophysiological role in obesity and metabolic syndromes.³²

The association that we describe is important because rs7559479 is in the 3'-UTR region of the *IL18RAP* gene, which is an MIR136 target. We used a functional assay to demonstrate that the A allele, which is associated with a lower risk of obesity, reduces the mRNA levels of *IL18RAP*. MIR136 has been widely studied and, in particular, is implicated in several cancers—for example, by modulating the sensitivity of glioma cells to different treatments,^{33 34} in human non-small cell lung cancer cells³⁵ and in metastasis-associated traits in lung adenocarcinoma cells.³⁶ Although MIR136 is not expressed in adipocytes,^{37 38} it has been associated with obesity owing to its role in adipogenic differentiation³⁹ and its expression in hypothalamic neurons, which are involved in appetite and whole-body energy-balance control.⁴⁰ Moreover, to our knowledge, this is the first time the MIR136:IL18RAP target pair has been validated.

The association between inflammatory cytokine genes and obesity has been less studied than for other candidate genes. In humans, circulating IL-18 levels positively

correlate with BMI, adiposity and metabolic syndrome disorders.^{41–45} This finding is consistent with increased adipokine release from adipose tissue in obesity and accordingly, monocyte/macrophage-lineage cells which are resident in fat tissue are major sources of circulating IL-18.⁴⁶ In addition, adipocytes from obese humans secrete threefold more IL-18 than those from lean donors⁴⁷ and IL-18 expression in subcutaneous adipose tissue is elevated in obesity and metabolic syndrome disorders.^{41 44 48 49} Consistent with a metabolic-state signal, circulating IL-18 levels are increased by hyperglycaemia or a high-fat meal,^{42 50} and intermittent glucose exposure increases IL-18 secretion by adipocytes⁵¹; conversely, weight loss and exercise reduce IL-18 levels.^{41 44 50} Some adipokines, whose levels increase with obesity (eg, leptin), oppose the positive energy-balance via negative feedback.^{52 53} Interestingly and perhaps reminiscent of obesity-related leptin resistance, leucocytes from patients with obesity or type 2 diabetes are resistant to IL-18.⁵⁴ Moreover, polymorphisms of the IL-18 gene or its receptor have been associated with obesity⁵⁵ and metabolic syndrome disorders^{56–58} in humans. Nevertheless, total loss of IL-18^{59–61} has the opposite effect (dysregulation of adipogenesis, appetite suppression and energy expenditure) via alternative pathways.

Our data indicate that a reduction in IL18RAP can reduce BMI and obesity; this may occur by an IL18RAP-mediated decrease in IL-18 proinflammatory signalling.^{62 63} In addition, the effect of these polymorphisms is important in cells during periods of MIR136 expression—for example, because of their potential consequences for cell differentiation and regulation. Therefore, future studies should aim to clarify the pathways by which IL18RAP may mediate this reduction in BMI and obesity risk. As previously mentioned, it has been suggested that IL-18 is an adipogenic cytokine associated with excess adiposity.⁴ Moreover, IL18RAP enhances IL-18 binding activity via the IL-18 receptor and plays a role in IL-18 signalling. Therefore, although no previous studies relate *IL18RAP* to obesity, our findings suggest that polymorphisms in this gene might modify individual susceptibility to obesity. Although the number of subjects

included in this study is limited, its statistical power is sufficient for the reduced number of polymorphisms we analysed. However, the possible role of *IL18RAP* SNPs in obesity phenotypes should be confirmed by other studies, including analyses of different populations and with larger sample sizes.

Contributors All authors participated in protocol and experiment design. VM-B, GdM, VG-A, CP-S and VA-F performed the experimental procedures. VM-B and FJC analysed the data. SM-H, GR, JFA, JTR, RC, JCM-E and ABG-G revised the results and contributed intellectually to discussion and interpretation of the data. The manuscript was written by VM-B, FJC and ABG-G and reviewed by all the authors.

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Competing interests None declared.

Ethics approval The VALCAR and Horteiga studies were approved by the ethics committees at the Hospital Clínico Universitario de Valencia and the Hospital Rio Horteiga (Valladolid), respectively. All patients gave their written permission to participate in the study.

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