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A polymorphism in the IL18RAP gene modifies miR-136 target and is involved in body mass regulation

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ORIGINAL ARTICLE

A polymorphism in the IL18RAP gene modifies miR-136 target and is involved in body mass regulation.

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ABSTRACT

Objectives: We investigated the association of IL-18RAP with body mass index (BMI) and obesity and verified the effect of a polymorphisms located in miR-136 (miR136) binding region in IL-18RAP,

Design: We analyzed two sample groups drawn from cross-sectional general population-based studies.

Setting: We studied two different Spanish regions with different sample characteristics: the VALCAR and the Hortega studies. Five single-nucleotide polymorphisms in the IL18RAP gene were analyzed by SNPlex.

Results: Of these polymorphisms, rs2293225, rs2272127, and rs7559479 were associated with obesity. The presence of the G allele in rs7559479 was associated with a higher risk of obesity (p = 0.01; odds ratio (OR) = 1.82 for the VALCAR group; p = 0.033; OR = 1.35 for Hortega population) and higher BMI values (p = 0.0045; p = 0.1 for VALCAR and Hortega, respectively). Furthermore, we found a significant association with obesity (p = 0.0024, OR = 1.44) and BMI values (p = 0.008) for the pooled population. We only found an association between rs2293225 and rs2272127 and obesity in the VALCAR population; the T allele in rs2293225 was associated with a lower risk of obesity (p = 0.036; OR = 0.60) and lower BMI values (p = 0.0038; OR = 1.41) and the G allele in rs2272127 was associated with a lower risk of obesity (p = 0.028; OR = 0.66) but was not associated with BMI.

Conclusions: Our results support the hypothesis that polymorphisms in the IL18RAP gene influence susceptibility to obesity. We demonstrated that the rs7559479 polymorphism A allele increases miR-136 binding, regulating IL18 system activity.

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Keywords: obesity, genetics, variations, cytokine, genetic polymorphism, microRNA.

Strengths and limitations of this study

- Our study, carried out in two Spanish populations from different regions with noticeably distinct characteristics, supports the hypothesis that IL18RAP gene polymorphisms may influence susceptibility to obesity.
- The number of individuals included in this study is limited, but the statistical power is sufficient for the reduced number of polymorphisms we analyzed.
- We did not expect to study total genetic variability in this gene because many other polymorphisms may modulate its activity which would require further study with a higher number of polymorphisms.
- The possible role of IL-18RAP SNPs in obesity phenotypes should be confirmed by other studies, including analyses of different populations, and with larger sample sizes.

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INTRODUCTION

Obesity is a state in which excess of energy is accumulated in body adipose tissue due to a chronic imbalance between energy intake and energy expenditure (1); individuals respond differently to this imbalance owing to 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, nonalcoholic fatty liver disease, colon cancer, and obstructive sleep apnea (3).

It has been suggested that interleukin-18 (IL-18) is an adipogenic cytokine (4) which is associated with excess adiposity and increased IL-18 concentrations. It is also hypothesized to have a pathophysiological role in obesity and metabolic syndromes by increasing adipogenesis. Obesity is a state of low-grade inflammation that is associated with an increase in cytokine production (5); IL-18 is a member of the IL-1 cytokine superfamily and is recognized as an important regulator of innate and acquired immune responses. Importantly, IL-18 is expressed at sites of chronic inflammation, in autoimmune diseases, in a variety of cancers, and in the context of numerous infectious diseases (6). IL-18 expression has been reported in macrophages, dendritic cells, Kupffer cells, keratinocytes, osteoblasts, adrenal cortex cells, intestinal epithelial cells, microglial cells, and synovial fibroblasts (7-14). This cytokine, acts by binding to a heterodimeric receptor (IL-18R) comprising a binding α chain, termed IL-18Rα or IL-18R1, and a signal-transducing β chain, termed IL-18Rβ or IL-18RAP (receptor accessory protein; 15-17). Both chains form a high-affinity complex that is required for inducing IL-18 signaling.

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Some polymorphisms can modulate a gene activity via different mechanisms such as modifying the binding or the action of specific miRNAs. In the past decade, the discovery of non-coding microRNAs which can post-transcriptionally regulate thousands of genes, has generated enormous research interest (18,19) and miRNA biomarkers have now been found in many chronic diseases such as cancer, cardiovascular disease, and type 2 diabetes (18, 20-22), and evidence of miRNA dysregulation has recently been reported in human obesity (23). In the future, miRNA biomarkers may be useful in the early diagnosis of chronic diseases and might also provide new therapeutic targets (24).

There are many studies in the literature on IL-18 and IL18R1, but very few specifically about IL-18RAP; therefore, we investigated the possible association between IL-18RAP, BMI, and obesity. To do this, we verified the effect of polymorphism rs7559479 located in the miR-136 binding site in mRNA of IL-18RAP and the mRNA levels of this gene.

MATERIALS AND METHODS

Population samples

We analyzed 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 VALCAR and HORTEGA samples were originally collected for the study of cardiovascular risk and cardiovascular disease development in general population. The Hortega study was approved by the Ethical Committee at the *Hospital Río Hortega*, and the VALCAR study and the genetic studies presented here were

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approved by the Ethical Committee at the *Hospital Clínico Universitario de Valencia*. All participants gave 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) along with anthropometric parameters were collected using standard procedures. The presence of obesity, hypertension (HTN), and type 2 diabetes (as defined using the World Health Organization criteria; http://www.who.int) was recorded and the BMI (kg/m^2) was calculated. Briefly, obesity was diagnosed when a BMI value was 30 kg/m^2 or higher, HTN was diagnosed when systolic and diastolic blood pressure values were higher than 140 and/or 90 mm Hg respectively, and diabetes was defined as a fasting plasma glucose level ≥7.0mmol/l (126mg/dl) or a 2 h plasma glucose level ≥ 11.1mmol/l (200mg/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 analyzed are shown in Table 1. Missing data for these parameters were, expressed as (subjects with missing data in VALCAR population, subjects with missing data in HORTEGA population): BMI (35/58), systolic blood pressure (53/10), Diastolic blood pressure (53/10), glucose (5/2)

The exclusion criteria were the same in both populations: the presence of serious concomitant diseases, disorders that could influence the collection of reliable information, or any mental or social condition which might complicate or prevent the subject's participation in the study.

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

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was quantified and diluted to a final concentration of 100 ng/μL. The IL-18RAP SNPs were selected for genotyping based on the conjunction of several parameters selected in the SYSNPs web tool (25): heterozygosity in a Caucasian population (higher than 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 IL-18RAP gene were selected (Table 2) and were genotyped using an oligonucleotide ligation assay (SNPlex; Applied Biosystems, Foster City, CA, USA) following the manufacturer's guidelines.

Statistical analysis

Samples with poor genotyping results (lower than 90%) were removed for all the SNPs. All quantitative values are expressed as the mean ± the standard deviation (*SD*) or as a percentage when describing qualitative variables. The ANOVA test was used to compare quantitative variables between groups and the chi-square test was used for categorical variables. Allele and genotype frequencies were calculated for each SNP using SPSS and SNPSTATS (http://bioinfo.iconcologia.net/index.php). The Hardy–Weinberg equilibrium (HWE) was tested using a chi-square distribution with one degree of freedom using SNPSTAT software. The HWE was maintained for all the polymorphisms analyzed.

The association 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. ANOVA was used to compare mean differences for continuous variables among genotypes. The association between obesity and each polymorphism was tested using logistic regression models, and the association

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

Cell lines and plasmids

As the rs7559479 polymorphism is located in the 3´-UTR of the IL18RAP gene, we thought that it might represent a microRNA target; we used microRNA.org to test this prediction and found that rs7559479 is a target for miR-136.

HuH-7 cells, an immortal epithelial-like tumorigenic cell line, were used for the miRNA assays. Cells were cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum, L-glutamine, and antibiotics, at 37° C in a 5% CO_2 environment. The pEZX-MT05 control reporter, reporters containing the IL18RAP 3´-UTR with either the most frequent rs7559479 A-allele polymorphism or the rs7559479 G-allele polymorphism, the pEZX-MR04 control reporter, and the miR-136 reporter were purchased from GeneCopoeia™.

Transfection and luciferase reporter assay

HuH-7 cells were seeded in 6-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 mediums and measured the activity of Gaussia luciferase (GLuc) and secreted alkaline phosphatase (SEAP) using a

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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 IL-18RAP gene polymorphisms and obesity traits was first identified in the VALCAR population; similar positive results were subsequently found in the Hortega study, and finally also when we analyzed both samples together. The main characteristics of the two samples studied are shown in Table 1. The VALCAR study included 468 individuals (24.80% obese; 47.22% men) and the Hortega study included 1502 subjects (23.70% obese; 49.47% men).

Association between IL-18RAP gene polymorphisms and obesity traits

All the polymorphisms analyzed were in HW equilibrium. Of the five polymorphisms analyzed in the IL-18RAP gene, rs2293225, rs2272127, and rs7559479 were associated with obesity. We focused on rs7559479 because it is the most significantly 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). In each population we have found significant association for obesity risk.

We only found an association for rs2293225 and rs2272127 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

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in rs2272127 was associated with a lower obesity risk (p = 0.028; OR = 0.66) but was not associated with BMI values.

Haplotype analysis

We performed haplotype analysis with the rs2272127, rs2293225 and rs7559479 variations, which all have a high linkage disequilibrium (D´ > 0.90). The haplotype CCG (number two in Table 4) was associated with a higher risk of obesity (p<0.0015) and with BMI (p<0.0088). This haplotype include the risk genotypes found in each variation and was present in 23% of the individuals in whole study population.

Validation of the interaction between miR-136 and the IL-18RAP 3´-UTR

Our analysis indicates that the rs4851581, rs2293224, rs2293225, and rs2272127 polymorphisms do not have a clear functional effect (http://www.ensembl.org/index.html), however, rs7559479 is located in the 3´-UTR of IL18RAP in the miR-136 miRNA-binding region (http://microrna.org). This variation can change the binding of this miRNA, and therefore the levels of IL-18RAP mRNA. Thus, it may also affect the downstream adipogenic effect of IL-18 and might be involved in BMI regulation.

In order to clarify whether the effect of the rs7559479 SNP on the miR-136 binding site in IL18RAP mRNA is functional, we performed an in vitro study in HuH7 cells. Five plasmids were used: the control reporter pEZX-MT05, a reporter containing the IL18RAP 3´-UTR with either the rs7559479 A-allele or G-allele polymorphism, the control pEZX-MR04 reporter, and the miR-136 reporter, as well as a plasmid that

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expresses SEAP 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 miR-136, and figure 1B shows that co-transfection of the reporters containing the IL18RAP 3´-UTR rs7559479 G-allele or A-allele polymorphisms with the miR-136 reporter gave a significantly higher signal (p value < 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 miR-136 binding and thus is likely to reduce IL18RAP protein production.

DISCUSSION

In this population-based study we examined five IL18RAP gene polymorphisms which are related to IL-18 adipogenic signaling pathway. The rs7559479 AG-GG genotypes were associated with a higher BMI and greater risk of obesity. It is particularly noteworthy that even though the Hortega and VALCAR populations had different characteristics the findings were replicated in each of them. Furthermore, when we studied the pooled population we found an even stronger association with BMI values and obesity. 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 IL18 system may be involved in BMI regulation and obesity.

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These relationships have not been found in previous genome-wide association (GWA) studies, but this may be the result of different environmental factors in the populations studied, for example, differences in nutrition. Mediterranean population might specifically differ from other populations, this may be especially true because it is usually underrepresented in GWA studies and many times no Spanish samples are included in these studies (26-28). Nevertheless, there is one study of obesity-related genes in Spanish populations, but of the 22 gene loci it analyzed, only FTO was clearly associated with BMI (29). Considering the high heritability of obesity, it is highly likely that new variants remain to be discovered, especially in the Spanish/ Mediterranean population where polymorphisms do not seem to have the same previously-described effects.

There are no previous studies that describe the association between increased weight or obesity and the rs7559479 SNP that we establish in this work. Nevertheless, obesity is strongly associated with inflammation (30), and it has been hypothesized that increased IL-18 concentrations have a pathophysiological role in obesity and metabolic syndromes (31).

The association that we describe is important because rs7559479 is located in the 3´UTR region of the IL18RAP gene which is a miRNA-136 target. We used a functional assay to demonstrate that the A allele, which is associated with a lower risk of obesity, reduce the mRNA levels of IL18RAP. MiR-136 has been widely studied and in particular it has been described to have roles in cancer, for example by modulating the sensitivity of glioma cells to different treatments (32, 33), in human non-small cell lung cancer cells (34), and in metastasis-associated traits in lung adenocarcinoma cells (35). Although miR-136 is not expressed in adipocytes (Ortega et al, 2010; Keller et al, 2011), is has been associated with obesity due to its role in adipogenic differentiation

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(Zaragosi et al, 2011) and its expression in hypothalamic neurons which are implicated in appetite and whole-body energy-balance control (Schneeberger et al., 2015).

The association between inflammatory cytokine genes and obesity is less studied than other candidate genes. In humans, circulating IL-18 levels positively correlate with BMI, adiposity, and metabolic syndrome disorders (40-44). 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 (45). In addition, adipocytes from obese humans secrete three-fold more IL-18 than those from lean donors (46) and the expression of IL-18 in subcutaneous adipose tissue is elevated in obesity and metabolic syndrome disorders (40, 43, 47, 48). Consistent with a metabolic-state signal, circulating IL-18 levels are increased by hyperglycemia or a high-fat meal (41, 49), and intermittent glucose exposure increases IL-18 secretion by adipocytes (50); conversely, weight loss and exercise decrease IL-18 levels (40, 43, 49). Some adipokines, whose levels increase with obesity (e.g. leptin), oppose the positive energy-balance via negative feedback (51, 52). Interestingly, and perhaps reminiscent of obesity-related leptin resistance, leukocytes from patients with obesity or type 2 diabetes are resistant to IL-18 (53). Moreover, polymorphisms of the IL-18 gene or its receptor have been associated with obesity (54) and metabolic syndrome disorders (55-57) in humans. Nevertheless, the total loss of IL-18 (58-60) has the opposite effect by other pathways (dysregulation of adipogenesis, appetite suppression and energy expenditure).

Our data indicate that a reduction in IL18RAP can reduce BMI and obesity; this could happen by decreasing IL18 proinflammatory signaling mediated by IL18RAP (61,62). In addition, the effect of these polymorphisms is important in cells during periods of miRNA-136 expression because of the potential consequences for cell differentiation,

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regulation, etc. Future studies should aim to clarify the pathways by which IL18RAP may mediate this reduction in BMI and obesity-risk.

As we have previously mentioned, IL-18 has been suggested as an adipogenic cytokine (4) which is associated with excess adiposity. IL18RAP enhances IL18 binding-activity by the IL18 receptor and plays a role in IL18 signaling. Therefore, although there are no previous studies that relate IL18RAP to obesity, our findings suggest that polymorphisms in this gene could modify susceptibility to obesity. Although the number of subjects included in this study is limited, statistical power is sufficient for the reduced number of polymorphisms we analyzed. The possible role of IL-18RAP SNPs in obesity phenotypes should be confirmed by other studies, including analyses of different populations, and with larger sample sizes.

Footnotes

Contributors: All authors participated in protocol and experiment design. VMB, GdM, VGA, CPS and VAF performed the experimental procedures. VMB and FJC analyzed data. SMH, GR, JFA, JTR, RC, JCME and ABGG revised results and contributed intellectually to data discussion and interpretation. Manuscript was written by VMB, FJC and ABGG and reviewed by all the authors.

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Disclaimer: neither funder played a role in the study design, data analysis or interpretation of the data.

Ethics approval: VALCAR and HORTEGA studies were approved by the Ethics Committee from the Hospital Clinico Universitario de Valencia and the Hospital Rio Hortega (Valladolid), respectively. All patients gave their written permission to participate in the study.

Competing interests: There are not competing interests to declare

Data sharing statement: No additional data are available.

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TABLES

Table 1 General characteristics of the population

	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/m2)	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 (24.8)*	356 (23.7)	470 (23.9)
Abdominal obesity (n, %)	151 (32.8)*	414 (27.6)	565 (28.8)
Diabetes mellitus (n, %)	55 (11.9)*	114 (7.6)	169 (8.6)
Glucose (mg/dL)	99.7 ± 24.4*	92.5 ± 20.4	91.9 ± 29.9
Hypertension (n, %)	129 (26.7)*	642 (42.7)	771 (39.3)
Systolic blood pressure (mmHg)	126.5 ± 16.1*	130.7 ± 21.6	129.5 ± 20.5
Diastolic blood pressure (mmHg)	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 ± standard deviation. For qualitative variables, data are expressed as (n, %).
* *p-value < 0.0001*

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Table 2. Characteristics of selected polymorphisms from the IL18RAP gene (Ensemble ID ENSG00000115607).

SNP name	Chromosome position	Reference	% Genotype	HWE	MAF	Allele Reference vs minor
rs4851581	102401181	NM_003853.2: c.-989A>G	95.2	0.196	0.053	A>G
rs2293224	102402211	NM_003853.2: c.-337-242T>C	96.8	0.522	0.466	C>T
rs2293225¥	102402321	NM_003853.2: c.-337-132C>T	98.6	0.129	0.167	C>T
rs2272127	102406305	NM_003853.2: c.70+66C>G	99.9	0.149	0.195	C>G
rs7559479¥	102435219	NM_003853.2: c.*146G>A	99.7	0.420	0.277	A>G

¥:Tag-SNP by Hapmap in Caucasian patients.
SNP name: dbSNP126. Reference: Begins in the first nucleotide of exon 1, Build 126, Ensembl release 41.
HWE, Hardy–Weinberg equilibrium; MAF, minor allele frequency;

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Table 3. Association between obesity and BMI and rs7559479 adjusted by age and sex in both samples. (Values are mean ± standard error. 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 (1.14-1.82)
P-value		**0.008**			**0.0024**

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Table 4. Haplotype association analysis of rs2272127, rs2293225 and rs7559479 of IL18RAP gene with BMI and obesity risk adjusted by age and sex.

		Frequency					BMI	
No	Haplotype	Non-obese	Obese	Freq.	OR (95% CI)	P-value	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 - 1.80)	**0.0015**	0.48 (0.12 - 0.85)	**0.0088**
3	GCA	0.225	0.232	0.226	1.13 (0.90 - 1.43)	0.30	0.08 (-0.29 - 0.44)	0.68
4	CTA	0.206	0.175	0.199	0.95 (0.74 - 1.21)	0.68	-0.2 (-0.57 - 0.17)	0.29

SNPs used in the haplotype construction: rs2272127, rs2293225 and rs7559479.Freq: frequency. Bold indicates significance

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FIGURE TITLES AND LEGENDS.

Figure 1: Luciferase assays for rs7559479 (target for miR-136) using different reporters and combinations. Reporters used were: rs7559479 polymorphism A allele (1), rs7559479 polymorphism G allele (2), control reporter pEZX-MT05 (3), miR-136 (4), and control reporter pEZX-MR04 (5) . Data are represented as the mean ± SE. Reporters 4, 5 and cells are negative controls.

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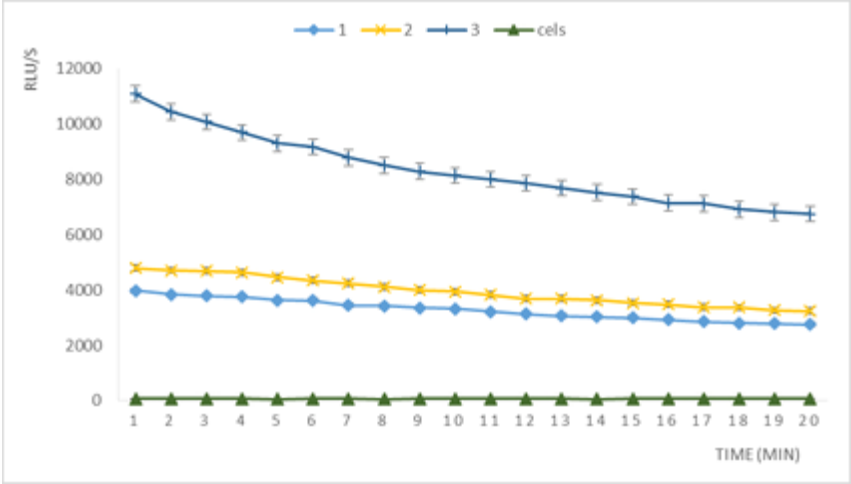
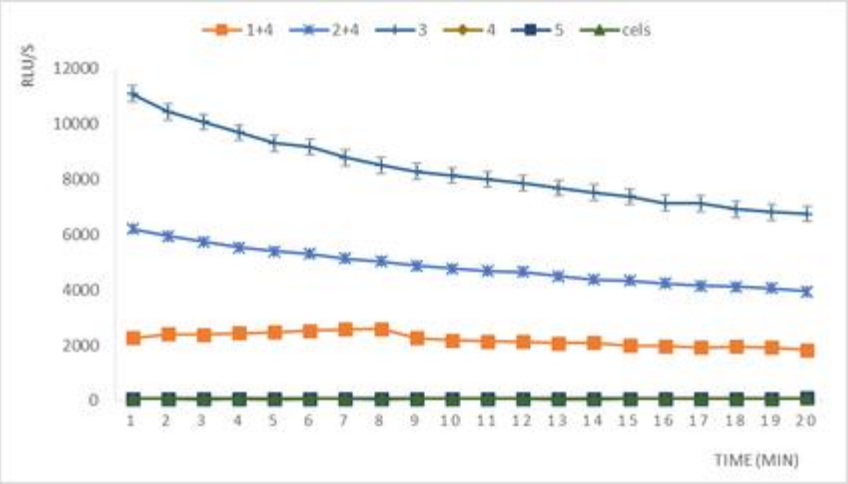
Figure 1

1A



1B



STROBE 2007 (v4) Statement—Checklist of items that should be included in reports of *cross-sectional studies*

Section/Topic	Item #	Recommendation	Reported on page #
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	abstract
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	2
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	4-5
Objectives	3	State specific objectives, including any prespecified hypotheses	5
Methods			
Study design	4	Present key elements of study design early in the paper	5-6
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	5-6
Participants	6	(*a*) Give the eligibility criteria, and the sources and methods of selection of participants	6
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	6
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	6
Bias	9	Describe any efforts to address potential sources of bias	
Study size	10	Explain how the study size was arrived at	5
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	6-7
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	7
		(*b*) Describe any methods used to examine subgroups and interactions	7
		(*c*) Explain how missing data were addressed	7
		(*d*) If applicable, describe analytical methods taking account of sampling strategy	
		(*e*) Describe any sensitivity analyses	
Results			

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Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	6
		(b) Give reasons for non-participation at each stage	
		(c) Consider use of a flow diagram	
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	5-6
		(b) Indicate number of participants with missing data for each variable of interest	6-7
Outcome data	15*	Report numbers of outcome events or summary measures	
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	9,26
		(*b*) Report category boundaries when continuous variables were categorized	6
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	
Discussion			
Key results	18	Summarise key results with reference to study objectives	11
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	13-14
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	13-14
Generalisability	21	Discuss the generalisability (external validity) of the study results	13-14
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	15

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.

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Are IL18RAP gene polymorphisms associated to body mass regulation? A cross sectional study

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Primary Subject Heading:	Cardiovascular medicine
Secondary Subject Heading:	Diabetes and endocrinology, Cardiovascular medicine, Genetics and genomics
Keywords:	obesity, variations, cytokine, genetic polymorphism, microRNA, Genetics < TROPICAL MEDICINE

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ORIGINAL ARTICLE

Are *IL18RAP* gene polymorphisms associated to body mass regulation? A cross sectional study

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lower risk of obesity (p = 0.028; OR = 0.66) only in the VALCAR population. On the other hand, reporter assay showed that MIR136 binding to was increased with the rs7559479 A allele

Conclusions: Our results suggest that polymorphisms in influence susceptibility to obesity. We demonstrated that the rs7559479 A allele increases MIR136 binding, regulating IL18 system activity.

Keywords: obesity, genetics, variations, cytokine, genetic polymorphism, microRNA.

Strengths and limitations of this study

- Our study has been carried out in two Spanish populations from different regions with noticeably distinct characteristics, being results similar in both of them.
- The number of individuals included in this study is limited, but the statistical power is sufficient for the reduced number of polymorphisms we analyzed.
- We did not study total genetic variability in this gene because there are too many variations in gene region, which would require further study with a higher number of polymorphisms.
- Our results should be confirmed by other studies, including analyses of different populations, and with larger sample sizes.

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INTRODUCTION

Obesity is a state in which excess of energy is accumulated in body adipose tissue due to a chronic imbalance between energy intake and energy expenditure (1); individuals respond differently to this imbalance owing to 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, nonalcoholic fatty liver disease, colon cancer, and obstructive sleep apnea (3).

It has been suggested that interleukin-18 (IL-18) is an adipogenic cytokine (4) which is associated with excess adiposity and increased IL-18 concentrations. It is also hypothesized to have a pathophysiological role in obesity and metabolic syndromes by increasing adipogenesis. Obesity is a state of low-grade inflammation that is associated with an increase in cytokine production (5); IL-18 is a member of the IL-1 cytokine superfamily and is recognized as an important regulator of innate and acquired immune responses. Importantly, IL-18 is expressed at sites of chronic inflammation, in autoimmune diseases, in a variety of cancers, and in the context of numerous infectious diseases (6). IL-18 expression has been reported in macrophages, dendritic cells, Kupffer cells, keratinocytes, osteoblasts, adrenal cortex cells, intestinal epithelial cells, microglial cells, and synovial fibroblasts (7-14). This cytokine, acts by binding to a heterodimeric receptor (IL-18R) comprising a binding α chain, termed IL-18Rα or IL-18R1, and a signal-transducing β chain, termed IL-18Rβ or IL-18RAP (receptor

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accessory protein; 15-17). Both chains form a high-affinity complex that is required for inducing IL-18 signaling.

Some polymorphisms can modulate a gene activity via different mechanisms such as modifying the binding or the action of specific microRNAs (miRNAs). In the past decade, the discovery of non-coding miRNAs which can post-transcriptionally regulate thousands of genes, has generated enormous research interest (18,19) and miRNA biomarkers have now been found in many chronic diseases such as cancer, cardiovascular disease, and type 2 diabetes (18, 20-22), and evidence of miRNA dysregulation has recently been reported in human obesity (23). In the future, miRNA biomarkers may be useful in the early diagnosis of chronic diseases and might also provide new therapeutic targets (24).

There are many studies in the literature on IL-18 and IL18R1, but very few specifically about IL-18RAP; therefore, we investigated for the first time the possible association between IL-18RAP, BMI, and obesity. To do this, we verified the effect of polymorphism rs7559479 located in the MIR136 binding site in mRNA of
and the mRNA levels of this gene.

MATERIALS AND METHODS

Population samples

We analyzed 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 (recruited between 2007 and 2010), and the HORTEGA study, with 1502 subjects from the province of Valladolid (collected from 2004 to 2005). Both

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VALCAR and HORTEGA samples were originally collected for the study of cardiovascular risk and cardiovascular disease development in general population. HORTEGA sample was composed by subjects from health areas representing half the Valladolid province, while the VALCAR patients came from the health area of Hospital Clinico Universitario of Valencia. The Hortega study was approved by the Ethical Committee at the , and the VALCAR study and the genetic studies presented here were approved by the Ethical Committee at the . All participants gave 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) along with anthropometric parameters were collected using standard procedures. The presence of obesity, hypertension (HTN), and type 2 diabetes (as defined using the World Health Organization criteria; http://www.who.int) was recorded and the BMI (kg/m^2) was calculated. Briefly, obesity was diagnosed when a BMI value was 30 kg/m^2 or higher, HTN was diagnosed when systolic and diastolic blood pressure values were higher than 140 and/or 90 mm Hg respectively, and diabetes was defined as a fasting plasma glucose level $\geq$7.0mmol/l (126mg/dl) or a 2 h plasma glucose level $\geq$ 11.1mmol/l (200mg/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 analyzed are shown in Table 1. Missing data for these parameters were, expressed as (subjects with missing data in VALCAR population, subjects with missing data in HORTEGA population): BMI (35/58), systolic blood pressure (53/10), Diastolic blood pressure (53/10), glucose (5/2)

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The exclusion criteria were the same in both populations: the presence of serious physical conditions which prevented from taking the survey or sample donation, disorders that could 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 rule out a patient was taken by a 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 ng/µL. The SNPs were selected for genotyping based on the conjunction of several parameters selected in the SYSNPs web tool (25): heterozygosity in a Caucasian population (higher than 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 gene were selected (Table 2) and were genotyped using an oligonucleotide ligation assay (SNPlex; Applied Biosystems, Foster City, CA, USA) following the manufacturer's guidelines. Ensemble 89 and dbSNP149 releases were used for genomic information regarding the selected SNPs

Statistical analysis

Samples with poor genotyping results (lower than 90%) were removed for all the SNPs. All quantitative values are expressed as the mean ± the standard deviation ( ) or as a percentage when describing qualitative variables. The ANOVA test was used to compare quantitative variables between groups and the chi-square test was used for categorical variables. Allele and genotype frequencies were calculated for each SNP using SPSS Statistics package 22.0 and SNPSTATS

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(http://bioinfo.iconcologia.net/SNPstats). The Hardy–Weinberg equilibrium (HWE) was tested using a chi-square distribution with one degree of freedom using SNPSTAT software. The HWE was maintained for all the polymorphisms analyzed.

The association 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. ANOVA was used to compare mean differences for continuous variables among genotypes. The association between obesity and each polymorphism was tested using logistic regression models, and the association between obesity and each polymorphism and haplotype was tested using logistic regression models. Linkage disequilibrium measurements were calculated using the R2 statistic. The odds ratios (OR) was used to evaluate the risk for the presence of obesity for each polymorphism, and age and gender were used as two potential confounding covariates.

Cell lines and plasmids

As the rs7559479 polymorphism is located in the 3´-UTR of the gene, microRNA target prediction was performed using microRNA.org tool, release August, 2010

HuH-7 cells, an immortal epithelial-like tumorigenic cell line, were used for miRNA assays. Cells were cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum, L-glutamine, and antibiotics, at 37° C in a 5% CO_2 environment. The pEZX-MT05 control reporter, reporters containing the 3´-UTR with either the most frequent rs7559479 A-allele polymorphism or the rs7559479

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G-allele polymorphism, the pEZX-MR04 control reporter, and the MIR136 reporter were purchased from GeneCopoeia™.

Transfection and luciferase reporter assay

HuH-7 cells were seeded in 6-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 mediums and measured the activity of Gaussia luciferase (GLuc) and secreted alkaline phosphatase (SEAP) 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 gene polymorphisms and obesity traits was first identified in the VALCAR population; similar positive results were subsequently found in the Hortega study, and finally also when we analyzed both samples together. The main characteristics of the two samples studied are shown in Table 1. The VALCAR study included 468 individuals (24.80% obese; 47.22% men) and the Hortega study included 1502 subjects (23.70% obese; 49.47% men).

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Association between *IL18RAP* gene polymorphisms and obesity traits

All the polymorphisms analyzed were in HW equilibrium. Of the five SNPs analyzed in the gene, rs2293225, rs2272127, and rs7559479 were associated with obesity. We focused on rs7559479 because it is the most significantly associated with obesity. The presence of the G allele was associated with a higher obesity risk (= 0.0024, OR = 1.44) and BMI values (= 0.008) in the pooled population (Table 3). In each population we have found significant association for obesity risk.

We only found an association for rs2293225 and rs2272127 in the VALCAR population: the T allele in rs2293225 was associated with a lower obesity risk (= 0.036; OR = 0.60) and with lower BMI values (= 0.0038; OR = 1.41) and the G allele in rs2272127 was associated with a lower obesity risk (= 0.028; OR = 0.66) but was not associated with BMI values.

Table 4 summarizes the found associations

Haplotype analysis

We performed haplotype analysis with the rs2272127, rs2293225 and rs7559479 variations, which all have a high linkage disequilibrium (D´ > 0.90). The haplotype CCG (number two in Table 5) was associated with a higher risk of obesity ($p<0.0015$) and with BMI ($p<0.0088$). This haplotype include the risk genotypes found in each variation and was present in 23% of the individuals in whole study population.

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Validation of the interaction between MIR136 and the *IL18RAP* 3´-UTR

Our analysis indicates that the rs4851581, rs2293224, rs2293225, and rs2272127 polymorphisms do not have a clear functional effect (http://www.ensembl.org/index.html), however, rs7559479 is located in the 3´-UTR of in the MIR136 miRNA-binding region (http://microrna.org). This variation can change the binding of this miRNA, and therefore the levels of mRNA. Thus, it may also affect the downstream adipogenic effect of IL-18 and might be involved in BMI regulation.

In order to clarify whether the effect of the rs7559479 SNP on the MIR136 binding site in mRNA is functional, we performed an in vitro study in HuH7 cells. Five plasmids were used: the control reporter pEZX-MT05, a reporter containing the 3´-UTR with either the rs7559479 A-allele or G-allele polymorphism, the control pEZX-MR04 reporter, and the MIR136 reporter, as well as a plasmid that expresses SEAP 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 3´-UTR with the rs7559479 A-allele and G-allele polymorphisms in the absence of MIR136, and figure 1B shows that co-transfection of the reporters containing the 3´-UTR rs7559479 G-allele or A-allele polymorphisms with the MIR136 reporter gave a significantly higher signal (value < 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 thus is likely to reduce IL18RAP protein production. Figure 2 shows the characteristics of the interaction between MIR136 and rs7559479 (26)

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DISCUSSION

In this population-based study we examined five gene polymorphisms which are related to IL-18 adipogenic signaling pathway. The rs7559479 AG-GG genotypes were associated with a higher BMI and greater risk of obesity. It is particularly noteworthy that even though the Hortega and VALCAR populations had different characteristics the findings were replicated in each of them. Furthermore, when we studied the pooled population we found an even stronger association with BMI values and obesity. 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 IL18 system may be involved in BMI regulation and obesity.

These relationships have not been found in previous genome-wide association (GWA) studies, but this may be the result of different environmental factors in the populations studied, for example, differences in nutrition. Mediterranean population might specifically differ from other populations, this may be especially true because it is usually underrepresented in GWA studies and many times no Spanish samples are included in these studies (27-29). Nevertheless, there is one study of obesity-related genes in Spanish populations, but of the 22 gene loci it analyzed, only FTO was clearly associated with BMI (30). Considering the high heritability of obesity, it is highly likely that new variants remain to be discovered, especially in the Spanish/ Mediterranean population where polymorphisms do not seem to have the same previously-described effects.

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There are no previous studies that describe the association between increased weight or obesity and the rs7559479 SNP that we establish in this work. Nevertheless, obesity is strongly associated with inflammation (31), and it has been hypothesized that increased IL-18 concentrations have a pathophysiological role in obesity and metabolic syndromes (32).

The association that we describe is important because rs7559479 is located in the 3´UTR region of the gene which is a MIR136 target. We used a functional assay to demonstrate that the A allele, which is associated with a lower risk of obesity, reduce the mRNA levels of . MIR136 has been widely studied and in particular it has been described to have roles in cancer, for example by modulating the sensitivity of glioma cells to different treatments (33, 34), in human non-small cell lung cancer cells (35), and in metastasis-associated traits in lung adenocarcinoma cells (36). Although MIR136 is not expressed in adipocytes (37,38), is has been associated with obesity due to its role in adipogenic differentiation (39) and its expression in hypothalamic neurons which are implicated in appetite and whole-body energy-balance control (40). To our knowledge, this is the first time the target pair MIR136:IL78RAP is validated.

The association between inflammatory cytokine genes and obesity is less studied than 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 three-fold more IL-18 than those from lean donors (47) and the expression of IL-18 in subcutaneous adipose tissue is elevated in obesity and metabolic syndrome disorders

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(41, 44, 48, 49). Consistent with a metabolic-state signal, circulating IL-18 levels are increased by hyperglycemia or a high-fat meal (42, 50), and intermittent glucose exposure increases IL-18 secretion by adipocytes (51); conversely, weight loss and exercise decrease IL-18 levels (41, 44, 50). Some adipokines, whose levels increase with obesity (e.g. leptin), oppose the positive energy-balance via negative feedback (52, 53). Interestingly, and perhaps reminiscent of obesity-related leptin resistance, leukocytes 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 obesity (55) and metabolic syndrome disorders (56-58) in humans. Nevertheless, the total loss of IL-18 (59-61) has the opposite effect by other pathways (dysregulation of adipogenesis, appetite suppression and energy expenditure).

Our data indicate that a reduction in IL-18RAP can reduce BMI and obesity; this could happen by decreasing IL18 proinflammatory signaling mediated by IL-18RAP (62,63). In addition, the effect of these polymorphisms is important in cells during periods of MIR136 expression because of the potential consequences for cell differentiation, regulation, etc. Future studies should aim to clarify the pathways by which IL18RAP may mediate this reduction in BMI and obesity-risk.

As we have previously mentioned, IL-18 has been suggested as an adipogenic cytokine (4) which is associated with excess adiposity. IL-18RAP enhances IL-18 binding-activity by the IL-18 receptor and plays a role in IL-18 signaling. Therefore, although there are no previous studies that relate to obesity, our findings suggest that polymorphisms in this gene could modify susceptibility to obesity. Although the number of subjects included in this study is limited, statistical power is sufficient for the reduced number of polymorphisms we analyzed. The possible role of SNPs

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in obesity phenotypes should be confirmed by other studies, including analyses of different populations, and with larger sample sizes.

Footnotes

Contributors: All authors participated in protocol and experiment design. VMB, GdM, VGA, CPS and VAF performed the experimental procedures. VMB and FJC analyzed data. SMH, GR, JFA, JTR, RC, JCME and ABGG revised results and contributed intellectually to data discussion and interpretation. Manuscript was written by VMB, FJC and ABGG and reviewed by all the authors.

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Ethics approval: VALCAR and HORTEGA studies were approved by the Ethics Committee from the Hospital Clinico Universitario de Valencia and the Hospital Rio Hortega (Valladolid), respectively. All patients gave their written permission to participate in the study.

Competing interests: There are not competing interests to declare

Data sharing statement: No additional data are available.

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TABLES

Table 1 General characteristics of the population

	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/m2)	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 (24.8)*	356 (23.7)	470 (23.9)
Abdominal obesity (n, %)	151 (32.8)*	414 (27.6)	565 (28.8)
Diabetes mellitus (n, %)	55 (11.9)*	114 (7.6)	169 (8.6)
Glucose (mg/dL)	99.7 ± 24.4*	92.5 ± 20.4	91.9 ± 29.9
Hypertension (n, %)	129 (26.7)*	642 (42.7)	771 (39.3)
Systolic blood pressure (mmHg)	126.5 ± 16.1*	130.7 ± 21.6	129.5 ± 20.5
Diastolic blood pressure (mmHg)	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 ± standard deviation. For qualitative variables, data are expressed as (n, %).

* *p-value < 0.0001*

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Table 2. Characteristics of selected polymorphisms from the *IL18RAP* gene (Ensemble 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: dbSNP149. Reference: Begins in the first nucleotide of exon 1, Build 149, Ensembl release 89.

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

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Table 3. Association between obesity and BMI and rs7559479 adjusted by age and sex in both samples. (Values are mean ± standard error. 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 (1.14-1.82)
P-value		**0.008**			**0.0024**

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Table 4. Summary table for the found associations in *IL18RAP* gene.

SNP name	Gene Location	Associated phenotype	populations where the 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 higher obesity higher obesity, higher BMI	VALCAR HORTEGA VALCAR + HORTEGA	G

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Table 5. Haplotype association analysis of rs2272127, rs2293225 and rs7559479 of IL18RAP gene with BMI and obesity risk adjusted by age and sex.

		Frequency					BMI	
No	**Haplotype**	**Non-obese**	**Obese**	**Freq.**	**OR (95% CI)**	**P-value**	**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 - 1.80)	**0.0015**	0.48 (0.12 - 0.85)	**0.0088**
3	GCA	0.225	0.232	0.226	1.13 (0.90 - 1.43)	0.30	0.08 (-0.29 - 0.44)	0.68
4	CTA	0.206	0.175	0.199	0.95 (0.74 - 1.21)	0.68	-0.2 (-0.57 - 0.17)	0.29

SNPs used in the haplotype construction: rs2272127, rs2293225 and rs7559479.Freq: frequency. Bold indicates significance

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FIGURE TITLES AND LEGENDS.

Figure 1: Luciferase assays for rs7559479 (target for miR-136) 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 icroRNA target prediction performed using microRNA.org tool is shown. The base in green corresponds to rs7559479 position.

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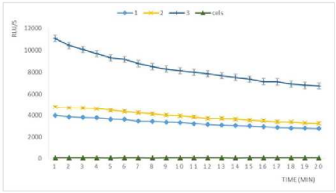
Figure 1

1A



1B

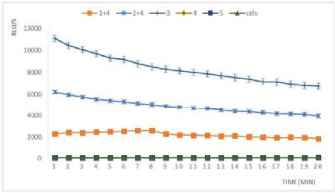


Figure 1: Luciferase assays for rs7559479 (target for miR-136) using different reporters and combinations. Reporters used were: rs7559479 polymorphism A allele (1), rs7559479 polymorphism G allele (2), control reporter pEZX-MT05 (3), MIR136 (4), and control reporter pEZX-MR04 (5) . Data are represented as the mean ± SE. Reporters 4, 5 and cells are negative controls.

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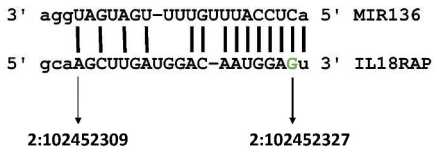
Figure 2.



miRNA gene name	MIR136
miRNA ENTREZ ID	406927
Target gene name	*IL18RAP*
Target Gene ENTREZ ID	8807
TRANSCRIPT ID	IL18RAP-201 (ENST000002642606)
Species Name (EntrezID)	Homo Sapiens (9606)
Method for experimental validation	luciferase reporter assay
Cell line	HuH7cells
Sequence variants within MIR136	rs7559479
Associated phenotype	obesity

Figure 2: Characteristics of the interaction between MIR136 and IL18RAP. MicroRNA target prediction performed using microRNA.org tool is shown. The base in green corresponds to rs7559479 position.

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Table 1. STREGA reporting recommendations, extended from STROBE Statement

Item	Item number	STROBE Guideline	Extension for Genetic Association Studies (STREGA)	Page # in Manuscript (or N/A if not applicable)
Title and Abstract	1	(a) Indicate the study's design with a commonly used term in the title or the abstract.		1-2
		(b) Provide in the abstract an informative and balanced summary of what was done and what was found.		2
Introduction				
Background rationale	2	Explain the scientific background and rationale for the investigation being reported.		4-5
Objectives	3	State specific objectives, including any pre-specified hypotheses.	***State if the study is the first report of a genetic association, a replication effort, or both.***	5
Methods				
Study design	4	Present key elements of study design early in the paper.		5-6

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Item	Item number	STROBE Guideline	Extension for Genetic Association Studies (STREGA)	Page # in Manuscript (or N/A if not applicable)
Setting	5	Describe the setting, locations and relevant dates, including periods of recruitment, exposure, follow-up, and data collection.		5-6
Participants	6	(a) **Cohort study –** Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up. **Case-control study –** Give the eligibility criteria, and the sources and methods of case ascertainment and control selection. Give the rationale for the choice of cases and controls. **Cross-sectional study –** Give the eligibility criteria, and the sources and methods of selection of participants.	***Give information on the criteria and methods for selection of subsets of participants from a larger study, when relevant***.	6
		(b) **Cohort study –** For matched studies, give matching criteria and number of exposed and unexposed. **Case-control study –** For matched studies, give matching criteria and the number of controls per case.		na
Variables	7	*(a)* Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable.	***(b) Clearly define genetic exposures (genetic variants) using a widely-used nomenclature system. Identify variables likely to be associated with population***	6-7

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Item	Item number	STROBE Guideline	Extension for Genetic Association Studies (STREGA)	Page # in Manuscript (or N/A if not applicable)
			stratification (confounding by ethnic origin).	
Data sources measurement	8*	*(a)* For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group.	***(b) Describe laboratory methods, including source and storage of DNA, genotyping methods and platforms (including the allele calling algorithm used, and its version), error rates and call rates. State the laboratory/centre where genotyping was done. Describe comparability of laboratory methods if there is more than one group. Specify whether genotypes were assigned using all of the data from the study simultaneously or in smaller batches.***	6-7
Bias	9	*(a)* Describe any efforts to address potential sources of bias.	***(b) For quantitative outcome variables, specify if any investigation of potential bias resulting***	NA

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Item	Item number	STROBE Guideline	Extension for Genetic Association Studies (STREGA)	Page # in Manuscript (or N/A if not applicable)
			from pharmacotherapy was undertaken. If relevant, describe the nature and magnitude of the potential bias, and explain what approach was used to deal with this.	
Study size	10	Explain how the study size was arrived at.		5
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen, and why.	***If applicable, describe how effects of treatment were dealt with.***	6-7
Statistical methods	12	(a) Describe all statistical methods, including those used to control for confounding.	***State software version used and options (or settings) chosen.***	7
		(b) Describe any methods used to examine subgroups and interactions.		7
		(c) Explain how missing data were addressed.		7

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Item	Item number	STROBE Guideline	Extension for Genetic Association Studies (STREGA)	Page # in Manuscript (or N/A if not applicable)
		(d) **Cohort study –** If applicable, explain how loss to follow-up was addressed. **Case-control study –** If applicable, explain how matching of cases and controls was addressed. **Cross-sectional study –** If applicable, describe analytical methods taking account of sampling strategy.		NA
		(e) Describe any sensitivity analyses.		
			(f) State whether Hardy-Weinberg equilibrium was considered and, if so, how.	7
			(g) Describe any methods used for inferring genotypes or haplotypes.	5-7
			(h) Describe any methods used to assess or address population stratification.	7-8
			(i) Describe any methods used to address multiple	7-8

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Item	Item number	STROBE Guideline	Extension for Genetic Association Studies (STREGA)	Page # in Manuscript (or N/A if not applicable)
			comparisons or to control risk of false positive findings.	
			(j) Describe any methods used to address and correct for relatedness among subjects	NA
Results				
Participants	13*	(a) Report the numbers of individuals at each stage of the study – e.g., numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed.	***Report numbers of individuals in whom genotyping was attempted and numbers of individuals in whom genotyping was successful.***	6-7
		(b) Give reasons for non-participation at each stage.		
		(c) Consider use of a flow diagram.		
Descriptive data	14*	(a) Give characteristics of study participants (e.g., demographic, clinical, social) and information on exposures and potential confounders.	***Consider giving information by genotype.***	5-6

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Item	Item number	STROBE Guideline	Extension for Genetic Association Studies (STREGA)	Page # in Manuscript (or N/A if not applicable)
		(b) Indicate the number of participants with missing data for each variable of interest.		6-7
		(c) **Cohort study –** Summarize follow-up time, e.g. average and total amount.		
Outcome data	15 *	**Cohort study-**Report numbers of outcome events or summary measures over time.	***Report outcomes (phenotypes) for each genotype category over time***	
		Case-control study – Report numbers in each exposure category, or summary measures of exposure.	***Report numbers in each genotype category***	
		Cross-sectional study – Report numbers of outcome events or summary measures.	***Report outcomes (phenotypes) for each genotype category***	9-10,27
Main results	16	(a) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (e.g., 95% confidence intervals). Make clear which confounders were adjusted for and why they were included.		9,26

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Item	Item number	STROBE Guideline	Extension for Genetic Association Studies (STREGA)	Page # in Manuscript (or N/A if not applicable)
		(b) Report category boundaries when continuous variables were categorized.		6
		(c) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period.		
			(d) Report results of any adjustments for multiple comparisons.	
Other analyses	17	(a) Report other analyses done – e.g., analyses of subgroups and interactions, and sensitivity analyses.		
			(b) If numerous genetic exposures (genetic variants) were examined, summarize results from all analyses undertaken.	26
			(c) If detailed results are available elsewhere, state how they can be accessed.	NA
Discussion				
Key results	18	Summarize key results with reference to study objectives.		11,26

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Item	Item number	STROBE Guideline	Extension for Genetic Association Studies (STREGA)	Page # in Manuscript (or N/A if not applicable)
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias.		13-14
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence.		13-14
Generalizability	21	Discuss the generalizability (external validity) of the study results.		13-14
Other Information				
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based.		15

STREGA = STrengthening the REporting of Genetic Association studies; STROBE = STtrengthening the Reporting of Observational Studies in Epidemiology.

* Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

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BMJ Open

Are IL18RAP gene polymorphisms associated with body mass regulation? A cross sectional study

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Primary Subject Heading:	Cardiovascular medicine
Secondary Subject Heading:	Diabetes and endocrinology, Cardiovascular medicine, Genetics and genomics
Keywords:	obesity, variations, cytokine, genetic polymorphism, microRNA, Genetics < TROPICAL MEDICINE

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ORIGINAL ARTICLE

Are IL18RAP gene polymorphisms associated with body mass regulation? A cross sectional study

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ABSTRACT

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

Design: We analyzed samples from 2 Spanish cross-sectional studies, VALCAR (Spanish Mediterranean coast) and Hortega (Spanish center). General goal for these studies was the analysis of cardiovascular risk and cardiovascular disease development in general population. Both populations correspond to regions with different characteristics.

Setting: Five *IL18RAP* SNPs were selected using the SYSNPs web tool and analyzed by oligonucleotide ligation assay (SNPlex). For the MIR136 functional study, cells were transfected with plasmids containing different rs7559479 polymorphism alleles and analyzed 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; odds ratio (OR) = 1.82; 95%CI=1.15-2.87 for VALCAR group; p = 0.033; OR = 1.35; 95%CI=1.03-1.79 for Hortega population) and higher 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=1.14-1.82) and increased BMI values (p = 0.008) was found when considering both populations together. Rs2293225 T allele was associated with lower obesity risk

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(p = 0.036; OR = 0.60; 95%CI=0.35-0.96) 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=0.44-0.97) only in the VALCAR population. 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 IL18 system activity.

Keywords: obesity, genetics, variations, cytokine, genetic polymorphism, microRNA

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 of them.
- The number of individuals included in this study was limited, but its statistical power was sufficient for the reduced number of polymorphisms we analyzed.
- We did not exhaustively study genetic variability in this gene because this gene region has too many variations, which 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.

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INTRODUCTION

Obesity is a state in which excess of energy is accumulated in corporal adipose tissue due to a chronic imbalance between energy intake and energy expenditure (1); 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, nonalcoholic fatty liver disease, colon cancer, and obstructive sleep apnea (3).

It has been suggested that interleukin-18 (IL-18) may be an adipogenic cytokine (4) associated with excess adiposity and increased IL-18 concentrations. It is also hypothesized to play a pathophysiological role in obesity and metabolic syndromes by increasing adipogenesis. Obesity is a state of low-grade inflammation that is associated with an increase in cytokine production (5); IL-18 is a member of the IL-1 cytokine superfamily and is recognized as an important regulator of innate and acquired immune responses. Importantly, IL-18 is expressed at sites of chronic inflammation, in autoimmune diseases, in a variety of cancers, and in the context of numerous infectious diseases (6). IL-18 expression has been reported in macrophages, dendritic cells, Kupffer cells, keratinocytes, osteoblasts, adrenal cortex cells, intestinal epithelial cells, microglial cells, and synovial fibroblasts (7-14). This cytokine, acts by binding to a heterodimeric receptor (IL-18R) comprising a binding α chain, termed IL-18Rα (or IL-18R1), and a signal-transducing β chain, termed IL-18Rβ (or IL-18RAP; receptor accessory protein; (15-17); both chains form a high-affinity complex that is required for inducing IL-18 signaling.

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Some polymorphisms can modulate gene activity via different mechanisms such as modifying the binding or action of specific microRNAs (miRNAs). The discovery of non-coding miRNAs which can post-transcriptionally regulate thousands of genes has generated enormous research interest over the past decade (18,19) and miRNA biomarkers have now been found in many chronic diseases such as cancer, cardiovascular disease, and type 2 diabetes (18, 20-22). Furthermore, evidence of miRNA dysregulation has recently been reported in human obesity (23). In the future, miRNA biomarkers may be useful in the early diagnosis of chronic diseases and might also provide new therapeutic targets (24).

There are many studies in the literature on IL-18 and IL18R1, but very few specifically about IL-18RAP; therefore, we investigated for the first time the possible association between IL-18RAP, BMI, and obesity. To do this, we verified the effect of polymorphism rs7559479 located in the MIR136 binding site in mRNA of *IL18RAP* and the mRNA levels of this gene.

MATERIALS AND METHODS

Population samples

We analyzed 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

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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 the 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) along with anthropometric parameters were collected using standard procedures. The presence of obesity, hypertension (HTN), and type 2 diabetes (as defined using the World Health Organization criteria; http://www.who.int) was recorded and the BMI (kg/m^2) was calculated. Briefly, obesity was diagnosed when a BMI value was 30 kg/m^2 or higher, HTN was diagnosed when systolic and diastolic blood pressure values were higher than 140 and/or 90 mm Hg respectively, and diabetes was defined as a fasting plasma glucose level $\geq$7.0 mmol/l (126 mg/dl) or a 2 h plasma glucose level $\geq$ 11.1 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 analyzed are shown in Table 1. Missing data for these parameters were, expressed as (subjects with missing data in VALCAR population / subjects with missing data in Hortega population): BMI (35/58), systolic blood pressure (53/10), Diastolic blood pressure (53/10), glucose (5/2).

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 could influence the collection of reliable information, or any mental or social condition which might complicate or prevent the

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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 ng/µL. The *IL18RAP* SNPs were selected for genotyping based on the conjunction of several parameters selected in the SYSNPs web tool (25): heterozygosity in a Caucasian population (higher than 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, CA, USA) following the manufacturer's guidelines. The genomic information about the selected SNPs was obtained from Ensemble release 89 and the Single Nucleotide Polymorphism database (dbSNP) build 149.

Statistical analysis

Previously to the study, we estimated the statistical power using the lower frequency included in the selection criteria of the selected polymorphisms (MAF=0.10) for the lower sample size (distributed in cases and controls) by 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 2.5 OR was 91,9% for one stage study. Applying the same criteria for whole sample the statistical power was 0.975 for 1.5 OR.

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After genotyping, samples with poor genotyping results (lower than 90%) were removed for all the SNP analyses. All quantitative values are expressed as the mean ± the standard deviation (*SD*) or as a percentage for qualitative variables. ANOVA tests were used to compare quantitative variables between groups and chi-square 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 chi-square distribution with one degree of freedom using SNPSTAT software. The HWE was maintained for all the polymorphisms analyzed.

The association 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. ANOVA 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. The odds ratios (OR) was 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´-UTR of the *IL18RAP* gene, the microRNA.org tool (August 2010 release) was used to predict the microRNA target.

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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 (DMEM) containing 10% fetal bovine serum, L-glutamine, and antibiotics, and were grown in a humidified environment at 37°C with 5% CO_2. The pEZX-MT05 control reporter, reporters containing 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 6-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 (GLuc) and secreted alkaline phosphatase (SEAP) 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

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the Hortega study; when we analyzed both samples together this association remained. The main characteristics of the two samples are shown in Table 1. The VALCAR study included 468 individuals (24.80% obese; 47.22% male) and the Hortega study included 1502 subjects (23.70% obese; 49.47% male).

Association between *IL18RAP* gene polymorphisms and obesity traits

All the polymorphisms analyzed were in HWE. Of the five SNPs analyzed 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 only found an association for rs2293225 and rs2272127 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 summarized 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 two in Table 5) was associated with a higher risk of obesity ($p<0.0015$) and with BMI ($p<0.0088$). This haplotype include the risk genotypes found in each variation and was present in 23% of the individuals in whole study population.

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Confirmation of the interaction between MIR136 and the *IL18RAP* 3´UTR

Our analyses indicate that the rs4851581, rs2293224, rs2293225, and rs2272127 polymorphisms (http://www.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 HuH7 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 SEAP 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 colleages (26)

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DISCUSSION

In this population-based study we examined five *IL18RAP* gene polymorphisms related to the IL-18 adipogenic signaling 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 IL18 cascade may be involved in BMI regulation and obesity.

These relationships have not been found in previous genome-wide association (GWA) studies, but this may be the result of different environmental factors, such as differences in nutrition, in the populations studied. Mediterranean populations might specifically differ from other populations, especially because they are usually underrepresented in GWA studies, which often do not include any Spanish samples (27-29). Nevertheless, one study analyzed 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.

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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 inflammation (31) 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 a 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 cells (35), and in metastasis-associated traits in lung adenocarcinoma cells (36). Although MIR136 is not expressed in adipocytes (37,38), is has been associated with obesity due to its role in adipogenic differentiation (39) 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:IL78RAP target pair has been validated.

The association between inflammatory cytokine genes and obesity is less studied than that 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 three-fold more IL-18 than those from lean donors (47) 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 hyperglycemia or a high-fat meal (42, 50), and intermittent glucose

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exposure increases IL-18 secretion by adipocytes (51); conversely, weight loss and exercise decrease IL-18 levels (41, 44, 50). Some adipokines, whose levels increase with obesity (e.g. leptin), oppose the positive energy-balance via negative feedback (52, 53). Interestingly, and perhaps reminiscent of obesity-related leptin resistance, leukocytes 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 obesity (55) 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 IL-18RAP can reduce BMI and obesity; this may occur by a IL-18RAP-mediated decrease in IL18 proinflammatory signaling (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, IL-18RAP enhances IL-18 binding activity via the IL-18 receptor and plays a role in IL-18 signaling. Therefore, although there are no previous studies that relate *IL18RAP* to obesity, our findings suggest that polymorphisms in this gene could 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 analyzed. 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.

Footnotes

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Contributors: All authors participated in protocol and experiment design. VMB, GdM, VGA, CPS, and VAF performed the experimental procedures. VMB and FJC analyzed the data; SMH, GR, JFA, JTR, RC, JCME, and ABGG revised the results and contributed intellectually to the discussion and interpretation of the data. This manuscript was written by VMB, FJC, and ABGG and its final version was approved by all the authors.

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Competing interests: The authors have no competing interests to declare.

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TABLES

Table 1 General characteristics of the population

	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 (24.8)*	356 (23.7)	470 (23.9)
Abdominal obesity (n, %)	151 (32.8)*	414 (27.6)	565 (28.8)
Diabetes mellitus (n, %)	55 (11.9)*	114 (7.6)	169 (8.6)
Glucose (mg/dL)	99.7 ± 24.4*	92.5 ± 20.4	91.9 ± 29.9
Hypertension (n, %)	129 (26.7)*	642 (42.7)	771 (39.3)
Systolic blood pressure (mmHg)	126.5 ± 16.1*	130.7 ± 21.6	129.5 ± 20.5
Diastolic blood pressure (mmHg)	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 ± standard deviation. For qualitative variables, data are expressed as (n, %).
* *p-value < 0.0001*

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Table 2. Characteristics of selected polymorphisms from the *IL18RAP* gene (Ensemble 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 Ensembl release 89.

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

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Table 3. Association between obesity and BMI and the rs7559479 polymorphism, adjusted by age and sex in both samples (values are the mean ± standard error; bold indicates significance). CI, confidence interval.

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 (1.14-1.82)
P-value		**0.008**			**0.0024**

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Table 4. Summary table for the associations found in *IL18RAP* gene.

SNP name	Gene Location	Associated phenotype	populations where the 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 higher obesity higher obesity, higher BMI	VALCAR Hortega VALCAR + Hortega	G

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Table 5. Haplotype association analysis for rs2272127, rs2293225, and rs7559479 in the IL18RAP gene with the BMI and obesity risk adjusted for age and sex.

		Frequency					BMI	
No	**Haplotype**	**Non-obese**	**Obese**	**Freq.**	**OR (95% CI)**	***P*-value**	**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 - 1.80)	**0.0015**	0.48 (0.12 - 0.85)	**0.0088**
3	GCA	0.225	0.232	0.226	1.13 (0.90 - 1.43)	0.30	0.08 (-0.29 - 0.44)	0.68
4	CTA	0.206	0.175	0.199	0.95 (0.74 - 1.21)	0.68	-0.2 (-0.57 - 0.17)	0.29

SNPs used in the haplotype construction: rs2272127, rs2293225, and rs7559479. Freq: frequency. Bold indicates significance.

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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 green corresponds to the position of the rs7559479 polymorphism.

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Figure 1

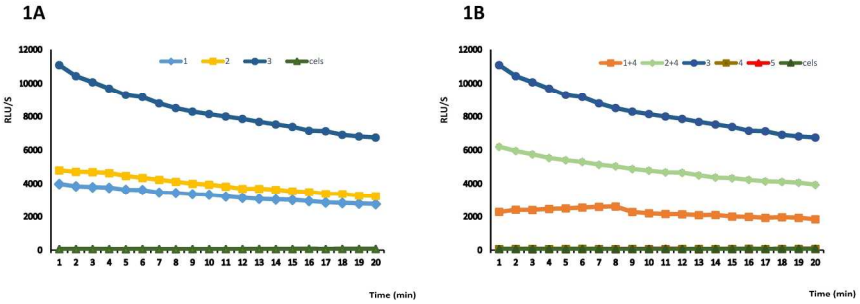

Figure 1: Luciferase assays for rs7559479 (a MIR136 target) using different reporters and combinations. Reporters used were: rs7559479 polymorphism A allele (1), rs7559479 polymorphism G allele (2), control reporter pEZX-MT05 (3), MIR136 (4), and control reporter pEZX-MR04 (5) . Data are represented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

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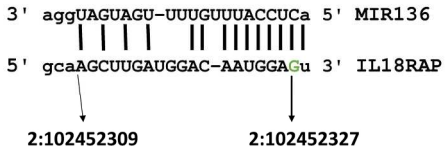
Figure 2.



miRNA gene name	MIR136
miRNA ENTREZ ID	406927
Target gene name	*IL18RAP*
Target Gene ENTREZ ID	8807
TRANSCRIPT ID	IL18RAP-201 (ENST000002642606)
Species Name (EntrezID)	Homo Sapiens (9606)
Method for experimental validation	luciferase reporter assay
Cell line	HuH7cells
Sequence variants within MIR136	rs7559479
Associated phenotype	obesity

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

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Table 1. STREGA reporting recommendations, extended from STROBE Statement

Item	Item number	STROBE Guideline	Extension for Genetic Association Studies (STREGA)	Page # in Manuscript (or N/A if not applicable)
Title and Abstract	1	(a) Indicate the study's design with a commonly used term in the title or the abstract.		1-2
		(b) Provide in the abstract an informative and balanced summary of what was done and what was found.		2
Introduction				
Background rationale	2	Explain the scientific background and rationale for the investigation being reported.		4-5
Objectives	3	State specific objectives, including any pre-specified hypotheses.	***State if the study is the first report of a genetic association, a replication effort, or both.***	5
Methods				
Study design	4	Present key elements of study design early in the paper.		5-6

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Item	Item number	STROBE Guideline	Extension for Genetic Association Studies (STREGA)	Page # in Manuscript (or N/A if not applicable)
Setting	5	Describe the setting, locations and relevant dates, including periods of recruitment, exposure, follow-up, and data collection.		5-6
Participants	6	(a) **Cohort study –** Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up. **Case-control study –** Give the eligibility criteria, and the sources and methods of case ascertainment and control selection. Give the rationale for the choice of cases and controls. **Cross-sectional study –** Give the eligibility criteria, and the sources and methods of selection of participants.	***Give information on the criteria and methods for selection of subsets of participants from a larger study, when relevant***.	6
		(b) **Cohort study –** For matched studies, give matching criteria and number of exposed and unexposed. **Case-control study –** For matched studies, give matching criteria and the number of controls per case.		na
Variables	7	*(a)* Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable.	***(b) Clearly define genetic exposures (genetic variants) using a widely-used nomenclature system. Identify variables likely to be associated with population***	6-7

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Item	Item number	STROBE Guideline	Extension for Genetic Association Studies (STREGA)	Page # in Manuscript (or N/A if not applicable)
			stratification (confounding by ethnic origin).	
Data sources measurement	8*	*(a)* For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group.	***(b) Describe laboratory methods, including source and storage of DNA, genotyping methods and platforms (including the allele calling algorithm used, and its version), error rates and call rates. State the laboratory/centre where genotyping was done. Describe comparability of laboratory methods if there is more than one group. Specify whether genotypes were assigned using all of the data from the study simultaneously or in smaller batches.***	6-7
Bias	9	*(a)* Describe any efforts to address potential sources of bias.	***(b) For quantitative outcome variables, specify if any investigation of potential bias resulting***	NA

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Item	Item number	STROBE Guideline	Extension for Genetic Association Studies (STREGA)	Page # in Manuscript (or N/A if not applicable)
			from pharmacotherapy was undertaken. If relevant, describe the nature and magnitude of the potential bias, and explain what approach was used to deal with this.	
Study size	10	Explain how the study size was arrived at.		5
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen, and why.	***If applicable, describe how effects of treatment were dealt with.***	6-7
Statistical methods	12	(a) Describe all statistical methods, including those used to control for confounding.	***State software version used and options (or settings) chosen.***	7
		(b) Describe any methods used to examine subgroups and interactions.		7
		(c) Explain how missing data were addressed.		7

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Item	Item number	STROBE Guideline	Extension for Genetic Association Studies (STREGA)	Page # in Manuscript (or N/A if not applicable)
		(d) **Cohort study –** If applicable, explain how loss to follow-up was addressed. **Case-control study –** If applicable, explain how matching of cases and controls was addressed. **Cross-sectional study –** If applicable, describe analytical methods taking account of sampling strategy.		NA
		(e) Describe any sensitivity analyses.		
			(f) State whether Hardy-Weinberg equilibrium was considered and, if so, how.	7
			(g) Describe any methods used for inferring genotypes or haplotypes.	5-7
			(h) Describe any methods used to assess or address population stratification.	7-8
			(i) Describe any methods used to address multiple	7-8

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			comparisons or to control risk of false positive findings.	
			(j) Describe any methods used to address and correct for relatedness among subjects	NA
Results				
Participants	13*	(a) Report the numbers of individuals at each stage of the study – e.g., numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed.	***Report numbers of individuals in whom genotyping was attempted and numbers of individuals in whom genotyping was successful.***	6-7
		(b) Give reasons for non-participation at each stage.		
		(c) Consider use of a flow diagram.		
Descriptive data	14*	(a) Give characteristics of study participants (e.g., demographic, clinical, social) and information on exposures and potential confounders.	***Consider giving information by genotype.***	5-6

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Item	Item number	STROBE Guideline	Extension for Genetic Association Studies (STREGA)	Page # in Manuscript (or N/A if not applicable)
		(b) Indicate the number of participants with missing data for each variable of interest.		6-7
		(c) **Cohort study –** Summarize follow-up time, e.g. average and total amount.		
Outcome data	15 *	**Cohort study-**Report numbers of outcome events or summary measures over time.	***Report outcomes (phenotypes) for each genotype category over time***	
		Case-control study – Report numbers in each exposure category, or summary measures of exposure.	***Report numbers in each genotype category***	
		Cross-sectional study – Report numbers of outcome events or summary measures.	***Report outcomes (phenotypes) for each genotype category***	9-10,27
Main results	16	(a) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (e.g., 95% confidence intervals). Make clear which confounders were adjusted for and why they were included.		9,26

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Item	Item number	STROBE Guideline	Extension for Genetic Association Studies (STREGA)	Page # in Manuscript (or N/A if not applicable)
		(b) Report category boundaries when continuous variables were categorized.		6
		(c) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period.		
			(d) Report results of any adjustments for multiple comparisons.	
Other analyses	17	(a) Report other analyses done – e.g., analyses of subgroups and interactions, and sensitivity analyses.		
			(b) If numerous genetic exposures (genetic variants) were examined, summarize results from all analyses undertaken.	26
			(c) If detailed results are available elsewhere, state how they can be accessed.	NA
Discussion				
Key results	18	Summarize key results with reference to study objectives.		11,26

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Item	Item number	STROBE Guideline	Extension for Genetic Association Studies (STREGA)	Page # in Manuscript (or N/A if not applicable)
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias.		13-14
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence.		13-14
Generalizability	21	Discuss the generalizability (external validity) of the study results.		13-14
Other Information				
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based.		15

STREGA = STrengthening the REporting of Genetic Association studies; STROBE = STtrengthening the Reporting of Observational Studies in Epidemiology.

* Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

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