Lack of association between *LEP* rs2167270 (19 G>A) polymorphism and disease susceptibility and cardiovascular disease in patients with rheumatoid arthritis

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Abstract

Objective

To assess the potential association between LEP rs2167270 (19 G>A) gene polymorphism and disease susceptibility and cardiovascular disease (CV) in patients with rheumatoid arthritis (RA).

Methods

773 patients fulfilling the 1987 American College of Rheumatology classification criteria for RA, seen at the rheumatology outpatient clinics of Hospital Xeral-Calde, Lugo, and Hospital San Carlos, Madrid, Spain, and 957 matched controls, were studied. Patients were genotyped for the LEP rs2167270 (19G>A) polymorphism, located within the 5´UTR, using predesigned TaqMan single nucleotide polymorphism genotyping assay. Also, HLA-DRB1 genotyping was performed using molecular based methods. Subclinical atherosclerosis was assessed in a subgroup of patients with no history of CV events by brachial artery reactivity to determine flow-mediated endothelium-dependent and endothelium-independent vasodilatation (n=133) and by B-mode ultrasonography of the carotid artery intima-media thickness (n=113).

Results

No statistically significant differences in the genotype or allele frequencies of the LEP rs2167270 gene polymorphism between patients with RA and controls were seen. Likewise, LEP rs2167270 polymorphism did not influence the development of CV events. Also, no significant differences in LEP rs2167270 genotype or allele distribution were seen when results of surrogate markers of subclinical atherosclerosis were assessed.

Conclusion

LEP rs2167270 polymorphism does not seem to be a genetic risk factor for disease susceptibility or clinically evident CV disease and subclinical atherosclerosis in patients with RA.

Key words

Rheumatoid arthritis, atherosclerosis, cardiovascular disease, genetics,

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Introduction

A growing body of evidence indicates that rheumatoid arthritis (RA), a chronic autoimmune disease, is associated with accelerated atherosclerosis and increased incidence of cardiovascular (CV) events (1, 2). Chronic systemic inflammation (3, 4) and classic CV risk factors (5, 6) have a major role in the atherosclerosis process observed in patients with RA. Moreover, recent studies have emphasized the importance of genetic factors in the development of endothelial dysfunction that represents a key early step in atherogenesis (7-9), and the progression of atherosclerosis disease in RA (3, 9-11).

Leptin is an adipocytokine implicated in the regulation of body weight by inhibiting food intake and stimulating energy expenditure (12). This is also a potent modulator of immune response. This adipokine modulates both innate and adaptive immunity (13).

Leptin regulates and participates both in immune homeostasis and inflammatory mechanisms acting as a modulator of T-cell activity. Leptin is also a proinflammatory adipocyte-derived factor that operates in the cytokine network by linking immune and inflammatory processes to the neuroendocrine system (12, 14). Due to this, leptin seems to play a key role in some autoimmune inflammatory diseases such as type 1 diabetes, bowel inflammation and RA (12, 14). Moreover, leptin exerts many potential atherogenic effects and high leptin concentrations predict incident CV disease in non-RA subjects (15).

Polymorphisms in the leptin gene (*LEP*) and leptin receptor gene have been studied in conjunction with leptin levels and obesity (16, 17). With respect to this, *LEP* rs2167270 (19 G>A) polymorphism in the 5' flanking region, predicted the degree of obesity in severe obese women (16). Li *et al*. reported that the *LEP* rs2167270 (19 G>A) variant was associated with obesity and body mass index in severely obese compared with non-obese Caucasian women (16). Also, Hager *et al*. reported that this variant in the 5´ non-coding region of the *LEP* gene was associated with leptin levels in morbidly obese individuals suggesting that this polymorphism may play a role in leptin regulation (17). More recently, Hart Sailors *et al*. showed that women carrying the leptin A- allele had lower body mass index and lower plasma leptin levels than those not carrying this allele (18). Therefore, allelic variants of this polymorphism may influence the regulation of the *LEP* gene and thereby influence body weight. These results may have some implications in the development and protection against the process leading to the development of the metabolic syndrome and the accelerated atherogenesis of patients with RA. With respect to this, we have recently confirmed that in RA patients with severe and active disease, serum leptin levels positively correlated with body mass index (19).

Taking together all these observations and since *LEP* rs2167270 (19 G>A) variant has been found to exhibit functional importance and it is in linkage disequilibrium with other common *LEP* markers such as the rs7799039, in the present study we assessed for first time whether the *LEP* rs2167270 (19 G>A) polymorphism was associated with susceptibility to RA. In a second step we aimed to determine the potential influence of this gene polymorphism in the development of CV events as well as its implication in the presence of subclinical atherosclerosis of patients with RA.

These analyses, therefore, were designed to serve as an initial evaluation of the potential importance of *LEP* gene in RA and the CV risk associated with this chronic inflammatory rheumatic disease.

Material and methods

Patients and controls

Between March 1996 and September 2008, 804 consecutive patients who fulfilled the 1987 American College of Rheumatology classification criteria for RA (20) were recruited from the rheumatology outpatient clinics of Hospital Xeral-Calde, Lugo and Hospital Clínico San Carlos, Madrid, Spain. Patients and 957 controls, matched by age, sex and ethnicity, from the same regions, were assessed for differences in the *LEP* rs2167270 (19 G>A) gene polymorphism. A DNA sample was

extracted from these patients at the time of recruitment.

Study protocol

Between December 2009 and January 2010 patient's clinical records were examined until patient's death, loss of follow-up or December 1st, 2009. Socio-demographical and clinical data regarding clinical manifestations, classic CV risk factors and history of CV events were registered. Clinical definitions for CV events and classic CV risk factors were established as previously described (3, 21). In this regard, patients were considered to have diabetes mellitus if before disease diagnosis they had been diagnosed as having diabetes mellitus by their family physicians or if 2 fasting plasma glucose levels on different days at the time of disease diagnosis or over the extended follow-up were >125 mg/dl (3). Smoking habit was considered to be present in those patients who smoked at the time of disease diagnosis, during the follow-up or who had smoked within the 10 years before the onset of RA symptoms or the disease diagnosis.

A CV event was considered to be present if the patient had ischaemic heart disease, heart failure, cerebrovascular accident or peripheral arteriopathy.

The definition of ischaemic heart disease (IHD) included acute coronary syndromes with or without persistent ST-segment elevation and chronic coronary heart disease. IHD was diagnosed if any of the following criteria were satisfied: a recorded diagnosis of ischaemic cardiopathy, on account of some acute coronary syndrome (acute myocardial infarction or unstable angina), the presence of pathological Q waves in the electrocardiogram, and coronary images showing >50% stenosis of at least one coronary vessel (3). Data regarding the clinical presentation of heart failure were also collected from all patients, based on the Framingham criteria (21). A patient was considered to have a cerebrovascular accident when he/she had a stroke and/or transient ischaemic attacks (TIAs). Strokes were classified according to their clinical features and they were confirmed by computed tomography and/or magnetic resonance imaging. TIAs were diagnosed if the symptoms were self-limited in less than 24 hours, without residual neurological damage (3). Peripheral arterial disease was considered to be present if it was confirmed by Doppler and arteriography (22).

Information on the main demographic characteristics, CV risk factors and CV events of patients in whom genotyping success was achieved (96%) is shown in Table I. One hundred and eighteen (17.91%) of these 773 patients with RA experienced clinically evident CV events.

To determine the potential association between the *LEP* rs2167270 polymorphism and the presence of subclinical atherosclerosis, between March 2007 and September 2009 a random subgroup of patients from the Lugo cohort with no previous history of CV events was selected. Presence of endothelial dysfunction was assessed by a brachial artery reactivity study in 133 patients. Flow-mediated endothelium-dependent vasodilatation-FMD (post-ischaemia) and endothelium-independent-NTG (post-nitroglycerin) vasodilatation were measured by brachial ultrasonography as previously reported (7, 23). A value of FMD less than 7% was considered pathologic, indicating the presence of endothelial dysfunction (23). Intra-observer variability for FMD and NTG was 1.3% and 1.9%, respectively, based on the repeat of the brachial ultrasonography in 32 healthy controls. Assessment of endothelial function of patients undergoing anti-TNF therapy was performed 24-48 hours before drug administration. Also, carotid ultrasonography studies were performed in 113 patients to determine the carotid artery intima-media thickness (IMT). It was assessed in the right common carotid artery as previously reported (24, 25). Based on a second carotid ultrasonography performed to 20 RA patients and 20 healthy controls within a week after the first assessment the correlation coefficient for carotid IMT was 0.98.

The subject's written consent was obtained according to the declaration of Helsinki, and the design of the work was approved by the Ethics Committee of Galicia (Spain). The Ethics Committee of the Hospital Clinico San Carlos (Madrid) also approved the study.

Genotyping

– LEP genotyping

DNA from patients was obtained from peripheral blood, using standard methods. Subjects were genotyped to determine *LEP* rs2167270 polymorphism status, located within the 5´UTR, using TaqMan Assays-on-Demand from Applied Biosystems following the manufacturer's protocol and analysed using the ABI 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA).

– Shared epitope determination

Several *HLA-DRB1* alleles (*HLA-DRB1**0401, *0404, *0405, *0408, *0101, *0102, *1001, *1402) are associated with susceptibility to RA. These alleles encode a conserved amino acid sequence (QKRAA, QRRAA, or RRRAA), called the shared epitope, at position 70-74 in the third hypervariable region of the HLA-DRβ1 molecule (26). *HLA-DRB1* typing was carried out using a reverse dot-blot kit with sequence-specific oligonucleotide (SSO) probes (Dynal RELITM SSO HLA-DRB1 typing kit; Dynal Biotech, Bromborough, UK).

Statistical analysis

All genotype data were checked for deviation from Hardy-Weinberg equilibrium using http://ihg.gsf.de/cgi-bin/hw/hwal.pl. Neither controls nor RA patients with CV disease or without CV disease had a deviation from Hardy-Weinberg equilibrium for the *LEP* rs2167270 polymorphism.

Comparison of proportions was carried out using χ^2 test or Fisher's exact test, when required. Strength of association between CV events and genotypes or alleles of *LEP* rs2167270 polymorphism was estimated using odds ratios (OR) and 95% confidence intervals (CI), via multiple logistic regression; estimates were further adjusted by gender, age at RA diagnosis, time of follow-up, presence or absence of rheumatoid shared epitope, and classical CV risk factors (hypertension, diabetes mellitus, dyslipidemia, obesity and smoking habit) as potential confounders.

The association between genotypes of the *LEP* rs2167270 polymorphism

Table I. Demographic characteristics of the patients with rheumatoid arthritis included in the study.

Variables	n=773
Females	569 (73.61)
Age of patients at the time of disease diagnosis, years, median (IQR)	56 (45–65)
Time follow-up, years, median (IQR)	13.0 (6.7–18.6)
anti-CCP positive (n=597)	337 (56.45)
Rheumatoid Factor positive (n=746)	545 (73.06)
Shared epitope, presence (n=697)	436 (62.55)
Cardiovascular events	118 (17.91)
Ischaemic heart disease	64 (9.79)
Cerebrovascular accidents	34 (5.24)
Heart failure	30 (4.64)
Peripheral arteriopathy	14 (2.16)
Hypertension (n=662)	276 (41.69)
Diabetes mellitus (n=656)	87 (13.26)
Dyslipidemia (n=743)	358 (48.18)
Obesity (n=616)	66 (10.71)
Smoking habit (n=628)	114 (18.15)

Except where indicated otherwise, values are n (%).
IQR: Interquartile range. Anti-CCP: anti-cyclic citrullinated peptide antibodies.

and surrogate markers of subclinical atherosclerosis: carotid IMT, FMD%-endothelium dependent vasodilatation or NTG%-endothelium independent vasodilatation was tested using unpaired *t*-test to compare between 2 groups, and by one-way analysis of variance (ANOVA) to compare among more than two groups. Moreover, we also tested association between these parameters and alleles using analysis of covariance (ANCOVA) adjusting for gender, age and duration of the disease at the time of the ultrasonographic study, and presence or absence of shared epitope and traditional CV risk factors. Statistical significance was defined as $p \leq 0.05$. Calculations were performed with STATA 10 (STATA Corporation, College Station, Texas).

In the comparative analysis of patients with RA *versus* controls a statistical power of 99.8% was reached to detect the effect of a polymorphism at an OR of 1.40 in a log additive model, and a power of 93.4% at an OR of 1.4 in a dominant model. The statistical power for the study which only involved RA patients with or without CV disease was 42.9% (log-additive model). Estimation of the power of the study was performed using the Quanto v. 1.2.3 software (Department of Preventive Medicine University of Southern California, California, USA) (27).

Results

Allele and genotype frequencies of the LEP rs2167270 polymorphism in RA patients and controls

As shown in Methods, genotype frequencies of the *LEP* rs2167270 variant studied were in Hardy-Weinberg equilibrium in patients and controls. No statistically significant differences in the genotype or allele frequency of the *LEP* rs2167270 (19 G>A) gene polymorphism between RA patients and controls were seen (Table II). Also, no significant differences in the age at the onset of the disease, rheumatoid factor, anti-cyclic citrullinated peptide antibodies, shared epitope, and age at the time of disease diagnosis were observed according to the different the *LEP* rs2167270 genotypes in this series of RA patients (data not shown).

LEP rs2167270 polymorphism and CV events in patients with RA

Table II shows the genotype frequencies of the *LEP* rs2167270 gene polymorphism assessed in this cohort of RA patients stratified according to the presence or absence of CV events. However, as observed when genotype and allele frequencies of this series of RA patients were compared with matched controls, *LEP* rs2167270 polymorphism did not influence the development of CV events. In this regard, only a mild reduction of the minor allele A of the *LEP* rs2167270 gene polymorphism was observed in the subgroup of RA patients who experienced CV events (31.36%) compared with those who did not have such complications (32.35%); p=0.77; OR: 0.96 (95% CI 0.71–1.30) (Table II). With respect to this, a logistic regression model to explain the presence of CV disease in patients with RA according to *LEP* rs2167270 allele distribution adjusted for gender, age at the time of RA diagnosis, follow-up time, presence or absence of shared epitope, and classic CV risk factors did not disclose statistically significant differences (*LEP* rs2167270 A *vs*. G: p=0.79; OR: 1.05 [95% CI: 0.72–1.53]).

LEP rs2167270 gene polymorphism and subclinical atherosclerosis

Endothelial function was studied in 133 RA patients with no history of CV events at the time of the brachial ultrasonographic assessment. Patients were stratified according to the *LEP* rs2167270 genotypes and alleles. The mean value of FMD% in this series of RA patients was lower than 7%. This result confirmed the previously reported presence of endothelial dysfunction in long-standing RA patients from Northwest Spain (7, 23).

However, as shown in Tables IIIA and IIIC, no significant differences according to the genotype or the allele distribution were found. It was also the case when we assessed potential differences in the carotid artery IMT in 113 patients without history of CV events stratified according to the genotype and allele distribution (Tables IIIB and IIIC).

Discussion

Leptin has been demonstrated to potentiate agonist-induced platelet aggregation as well as arterial and venous thrombosis, endothelial and smooth muscle cell proliferation, osteogenic differentiation or the activation of inflammatory cells, all of which may be relevant for the development of CV disease. Furthermore, expression of the leptin receptor by monocytes/macrophages within human atherosclerotic lesions has been found. Since atherosclerosis is an inflammatory disease, this effect of leptin may

Table II. Genotype and allele frequencies of the rs2167270 polymorphism of the *LEP* gene in healthy controls and patients with rheumatoid arthritis. Differences in the genotype and allele frequencies between rheumatoid arthritis patients who experienced cardiovascular events (RA patients with CV events) or not (RA patients without CV events).

	RA Patients n (%)	Controls n (%)	*p*-value	OR (95% CI)	RA patients without CV events n (%)	RA patients with CV events n (%)	*p*-value	OR (95% CI)
GG	358 (46.31)	427 (44.62)	---	1	250 (46.21)	58 (49.15)	---	1
GA	321 (41.53)	409 (42.74)	0.52	0.94 (0.76-1.15)	232 (42.88)	46 (38.98)	0.47	0.85 (0.55–1.34)
AA	94 (12.16)	121 (12.64)	0.62	0.93 (0.68-1.27)	59 (10.91)	14 (11.86)	0.94	1.02 (0.51–2.04)
G	1037 (67.08)	1263 (65.99)	---	1	732 (67.65)	162 (68.64)	---	1
A	509 (32.92)	651 (34.01)	0.50	0.95 (0.83-1.10)	350 (32.35)	74 (31.36)	0.77	0.96 (0.71–1.30)

CV: Cardiovascular. OR [95% CI]: Odds Ratio with 95% Confidence Interval.

also be important for disease progression and contribute to plaque instability and rupture (28). On the other hand, an association of *LEP* rs2167270 (19 G>A) with obesity and body mass index in severely obese compared with non-obese Caucasian women was reported (16). This gene variant was associated with leptin levels in morbidly obese individuals suggesting that *LEP* rs2167270 (19 G>A) may play a role in leptin regulation (17). Since we observed an association between leptin levels and body mass index in patients with severe RA, refractory to conventional therapy (19), in the present study we aimed to determine whether *LEP* rs2167270 variant might also account for increased incidence of CV events in white individuals with RA. However, our data do not confirm an association of this gene variant with susceptibility to RA or with the increased risk of CV events previously reported in Spanish RA patients (3).

In a cross-sectional sample of 137 normal-weight and obese men and women, plasma leptin levels were found to be associated with the carotid IMT at the common carotid artery (29). In that study, the IMT of the common carotid artery showed a significant relationship with leptin concentrations in both male and female subjects, irrespective of age, insulin sensitivity, smoking habits, blood pressure, fasting blood glucose and plasma lipid pattern (29). Moreover, leptin levels were found to be significantly higher in a series of 120 patients with coronary heart disease undergoing coronary stenting than in 58 non-diabetic and non-coronary heart disease matched control subjects (30). In addition, among the stented patients, leptin levels were higher in patients with restenosis than in patients without restenosis (30). However, no association between *LEP* rs2167270 polymorphism and surrogate markers of atherosclerosis such as carotid IMT or endothelial dysfunction was observed in our series of RA patients without clinically evident CV disease.

RA is a complex polygenic disease and a number of genes have been implicated in both disease susceptibility and the increased risk of CV mortality. In our population we previously observed an association of *HLA-DRB1**04 shared epitope alleles with increased incidence of CV events (3), CV mortality (3) and endothelial dysfunction (7). This association of *HLA-DRB1* alleles with CV disease was also confirmed in British patients with RA (10, 11). However, we failed to establish an association with clinically evident CV disease or subclinical atherosclerosis in Spanish RA patients when we analysed the potential influence of other gene variants located

Table III. A. Comparison of brachial flow-mediated endothelium dependent (post-ischaemia) vasodilatation (FMD) and endothelial independent (post-nitroglycerin) vasodilatation (NTG), according to *LEP* rs2167270 polymorphism. **B**: Comparison of carotid artery intima-media thickness (IMT) according to *LEP* rs2167270 polymorphism. **C**. Comparison of carotid artery IMT, FMD and NTG vasodilatation, according to *LEP* rs2167270 alleles in an adjusted ANCOVA model.

A.

LEP rs2167270	FMD%, mean (SD)	*p*-value	NTG% mean (SD)	*p*-value
GG (n=60)	5.94 (5.65)		17.37 (8.67)	
GA (n=55)	5.41 (4.69)		16.37 (7.27)	
AA (n=18)	5.33 (4.30)		17.39 (5.26)	
Model		0.82		0.76
G (n=175)	5.69 (5.20)		16.89 (8.01)	
A (n=91)	5.33 (4.30)	0.78	17.39 (5.26)	0.80

B.

LEP rs2167270	IMT mm, mean (SD)	*p*-value
GG (n=54)	0.73 (0.20)	
GA (n=43)	0.78 (0.18)	
AA (n=16)	0.75 (0.16)	
Model		0.41
G (n=151)	0.74 (0.16)	
A (n=75)	0.76 (0.19)	0.85

C.

	IMT	FMD	NTG
LEP rs2167270, p A *vs*. G*	0.38	0.82	0.94

*Analyses adjusted for gender, age at the time of the ultrasonography study, follow-up time, presence or absence of shared epitope, hypertension, diabetes, dyslipidemia, obesity and smoking habit.

outside the MHC region (*PTPN22*, *STAT4* and *TRAF1/C5*), which are also associated with increased disease susceptibility to RA (31). Moreover, although we found an association of endothelial dysfunction with *IL6* gene in patients with RA (8), we did not observe an association between subclinical atherosclerosis or CV events with other gene polymorphisms implicated in the inflammatory response such as *MIF-173* (32).

With regard to adipokine polymorphisms, we did not find an association of *ADIPOQ* rs266729 and rs1501299 polymorphisms, either isolated or in combination, with the risk of clinically evident CV disease or with any surrogate marker of subclinical atherosclerosis in RA patients (33). In keeping with these negative data on adiponectin gene variants, the present results do not show a role of *LEP* rs2167270 (19 G>A) polymorphism in the development of atherosclerosis in RA.

In conclusion, *LEP* rs2167270 (G>A) polymorphism was not found to be associated with susceptibility to RA or with the risk of CV disease in RA. However, given that this is not a comprehensive study, the possible role of *LEP* in RA or CV disease susceptibility cannot be completely ruled out. In this regard, we cannot exclude that other polymorphisms located in the *LEP* gen receptor might have an influence in the development of atherosclerosis in RA. The search for potential gene candidates that may influence the development of CV disease in patients with RA needs further investigation.

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