Lack of association between *RETN* rs1862513 polymorphism and cardiovascular disease in patients with rheumatoid arthritis

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Abstract

Objective

To assess the influence of the RETN rs1862513 polymorphism in the risk of cardiovascular (CV) disease and subclinical atherosclerosis in patients with rheumatoid arthritis (RA).

Methods

Six hundred and sixty-eight 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, were studied. Patients were genotyped for the RETN rs1862513 polymorphism using predesigned TaqMan single nucleotide polymorphism genotyping assay. Also, HLA-DRB1 genotyping was performed using molecular based methods. Carotid intima-media thickness (IMT), flow-mediated endothelium-dependent and endothelium independent vasodilatation, used as surrogate markers of subclinical atherosclerosis, were measured in a subgroup of patients.

Results

No significant differences in the genotypic or in the allelic distribution between RA patients with or without CV disease were found. In this regard, we only observed a slight increased frequency of homozygous and heterozygous for the minor allele G (CG+GG genotypes) among patients who experienced CV events compared to those without CV events (53.04% vs. 52.62%, p=0.94). A higher frequency of classic CV risk factors was observed among the carriers of the minor allele G. However, in the adjusted logistic regression model no association between the RETN variant and CV disease was found (p=0.50). Also, when surrogate markers of subclinical atherosclerosis were assessed, in the adjusted ANCOVA model only a trend towards a higher carotid IMT was found among allele G carriers (p=0.06).

Conclusion

RETN rs1862513 polymorphism does not seem to be a genetic risk factor for both clinically evident CV disease and subclinical atherosclerosis in patients with RA.

Key words

rheumatoid arthritis, atherosclerosis, cardiovascular disease, genetics, *RETN*, rs1862513

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Introduction

Rheumatoid arthritis (RA) is a chronic disease associated with an accelerated atherosclerosis (1-3), causing an increased cardiovascular (CV) morbidity and mortality (4). This accelerated atherosclerosis is a consequence of both traditional CV risk factors (5, 6) and the presence of a chronic systemic inflammatory status (7-9). With respect to this, a strong correlation between systemic inflammation and CV disease has been observed among RA subjects (8, 10).

Resistin is an adipokine that in humans is mainly expressed in monocytes and macrophages (11, 12). Unlike mice (13), human isolated primary adipocytes and preadipocytes do not express this adipokine (14). It is believed that resistin plays a role in inflammatory responses (15). Resistin expression and secretion is regulated by innate inflammatory signals such as endotoxins or LPS (16) and proinflammatory cytokines (17-21), such as TNF-α. Interestingly, high levels of resistin have been found in synovial fluid from patients with RA (22). Also, resistin serum levels are higher among RA patients compared to healthy controls (22). A positive correlation between C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR) with serum resistin has also been observed in RA patients (23-26). Also, higher DAS28 (23) and Larsen score (24) seem to be associated to higher resistin levels.

A *RETN* polymorphism located at -420 (rs1862513 C>G) has been associated to an enhanced *RETN* promoter activity, resulting in higher resistin plasma levels (25) in Asian populations. However, in Caucasians, this influence seems to be much weaker (26-29).

Taking into account the role of this adipokine in the inflammatory response and the emerging role of chronic inflammation in atherosclerosis (30), association between polymorphism of *RETN* rs1862513 and CV disease has been investigated. In nondiabetic Caucasian samples, no association between this polymorphism and coronary arterial calcification (29), angiographic coronary arterial disease (28), occurrence of myocardial infarction (31), CV mortality (28) or carotid atherosclerosis (31) was described. However, in Caucasian (32) and Japanese (33) diabetic patients, this polymorphism has been associated with cerebrovascular disease.

Taking into account the potential role played by this adipokine in RA and that *RETN* rs1862513 polymorphism may play a role in CV disease in those subject with an underlying chronic inflammatory diseases (34), we decided to analyse the potential role of this polymorphism to develop CV disease in RA patients.

Material and methods

Patients and study protocol

Between March 1996 and March 2008, 696 consecutive patients, fulfilling the 1987 American College of Rheumatology classification criteria for RA (35), were recruited from the rheumatology outpatient clinics of Hospital Xeral-Calde, Lugo and Hospital Clínico San Carlos, Madrid, Spain. A DNA sample (see below) was extracted from these patients at the time of recruitment. 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, traditional CV risk factors and history of CV events were registered. Clinical definitions for CV events and risk factors were established as previously described (7, 36). 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 (7). 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 (7). Data regarding the clinical presentation of heart failure were also collected for all patients, based on the Framingham criteria (36). 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 (37). Peripheral arterial disease was considered to be present if it was confirmed by Doppler and arteriography (38). Information on their main demographic characteristics, CV risk factors and CV events are shown in Table I.

Since Hospital Xeral-Calde and Hospital Clínico San Carlos are the referral centres for the population of each respective area, the first CV event was defined as an event (case) of CV complication diagnosed at the hospital in a patient without a previous history of CV disease. Based on previously established protocols of management, all patients on methotrexate therapy were treated with folate supplementation.

To determine the potential association between *RETN* rs1862513 gene polymorphisms and the presence of subclinical atherosclerosis, between March 2007 and September 2009 a random subgroup of patients among 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 124 patients. Flow-mediated endothelium-dependent dilatation FMD (post-ischaemia) and endothelium independent- NTG (post-nitroglycerin) vasodilatation were measured by brachial ultrasonography as previously reported (39, 40). A value of FMD less than 7% was considered pathologic, indicating the presence of endothelial dysfunction (40). 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 those patients undergoing anti-TNF therapy was performed 24-48 hours before its administration. Also, carotid ultrasonography studies were performed in 104 patients to determine the carotid artery intima-media thickness (IMT). It was assessed in the right common carotid artery as previously reported (40, 41).

Informed consent was obtained from all patients. The local institutional committees approved the study.

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

Variables	n=668	
Females	497	(74.40)
Age of patients at the time of disease diagnosis, years, median [IQR]	56.0	(45.0–65.3)
Time follow-up, years, median [IQR]	13.3	(6.8–22.9)
anti-CCP positive (n=487)	285	(58.52)
Rheumatoid Factor positive (n=652)	480	(73.62)
Shared epitope (n=598)	375	(62.71)
Cardiovascular events	115	(17.22)
Ischaemic heart disease	62	(9.28)
Cerebrovascular accidents	32	(4.79)
Heart failure	30	(4.49)
Peripheral arteriopathy	13	(1.95)
Hypertension (n=663)	265	(39.97)
Diabetes mellitus (n=661)	84	(12.71)
Dyslipidemia (n=645)	299	(46.36)
Obesity (n=627)	67	(10.69)
Smoking habit (n=637)	112	(17.58)
RETN rs1862513		
CC	316	(47.31)
CG	276	(41.32)
GG	76	(11.38)
C	908	(67.96)
G	428	(32.04)

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

Genotyping

– RETN *genotyping*

DNA from patients was obtained from peripheral blood, using standard methods.

Six hundred and ninety-six subjects were genotyped to determine *RETN* rs1862513 status 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). The typing was successful in 668 patients (95.98%).

– *Shared epitope determination*

Several *HLA-DRB1* alleles (*HLA-DRB1**0401, *0404, *0405, *0408, *0101, *0102, *1001, *1402) are associated with susceptibility to rheumatoid arthritis. 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 (42).

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). When necessary, high-resolution typing of HLA-DRB1*03 samples was performed using Dynal AllSetTM SSP DRB1*03.

Statistical analysis

Comparison of means was performed using *t*-test. Comparison of proportion between 2 or more groups was carried out using χ^2 test or Fisher's exact test, when required.

Strength of association between CV events and genotypes of *RETN* rs1862513 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 shared epitope and traditional CV risk factors (hypertension, diabetes mellitus, dyslipidemia, obesity and smoking habit) as potential confounders. A dominant pattern of effect was considered for the RETN variant (CG+GG *vs*. CC).

A Cox regression model was used to estimate the influence of the *RETN* rs1862513 polymorphism on CV disease. We used the occurrence of at least one CV event as the outcome and the survival time when the first CV event occurred. The survival time of individuals without CV events was the age at patient's death, loss to follow-up or December 1st, 2009. Patients who died of any other causes different from CV events were considered as not having CV events. Proportional hazard assumption was tested using Schoenfeld residuals. Results were expressed as hazard ratios (HRs) with 95% confidence intervals [95% CIs] and were computed as both crude and adjusted for age at RA diagnosis, gender and classic CV risk factors.

The association between genotypes and alleles of the *RETN* rs1862513 gene polymorphism and carotid IMT, FMD%-endothelium dependent vasodilatation and NTG%-endothelium independent vasodilatation was tested using unpaired *t*-test, to compare between 2 groups, and 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 by 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≤0.05. Calculations were performed with STATA 10 (STATA Corporation, College Station, Texas).

Table II. Differences between RA patients with CV events or without CV events according to the *RETN* rs1862513 polymorphism.

RETN rs1862513	with CV events	without CV events	*p*-value	OR [95% CI]
Genotype n (%)				
CC	54 (46.96)	262 (47.38)		1
CG	50 (43.48)	226 (40.87)	0.74	1.07 [0.69–1.68]
GG	11 (9.57)	65 (11.75)	0.58	0.82 [0.38–1.73]
CG+GG	61 (53.04)	291 (52.62)	0.94	1.02 [0.68–1.52]
Allele 2n (%)				
C	158 (68.70)	750 (67.81)		1
G	72 (31.30)	356 (32.19)	0.79	0.96 [0.70–1.32]

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

Table III. Demographic characteristics and CV risk factor distribution in carriers and non carriers of the minor allele G of the *RETN* rs1862513 polymorphism.

	Variables		
RETN rs1862513	CC	CG+GG	*p*-value
Females	238 (75.32)	259 (73.58)	0.61
Age of patients at the time of disease diagnosis, years, median [IQR]	56 (46-66)	56.4 (45-65)	0.66
Time follow up, years, median [IQR]	13 (7.7-19.0)	14.4 (6.4-19.3)	0.27
anti-CCP positive (n=487)	132 (58.15)	153 (58.85)	0.88
Rheumatoid Factor positive (n=652)	226 (72.67)	254 (74.49)	0.60
Shared epitope (n=598)	173 (61.79)	202 (63.52)	0.66
Hypertension (n=663)	112 (35.90)	153 (43.59)	0.04
Diabetes mellitus (n=661)	32 (10.26)	52 (14.90)	0.07
Dyslipidemia (n=645)	130 (42.90)	169 (49.42)	0.10
Obesity (n=627)	33 (11.07)	34 (10.33)	0.77
Smoking habit (n=637)	45 (14.90)	67 (20.00)	0.09

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

Results

Influence of RETN rs1862513 gene polymorphism in the risk of CV disease in patients with RA

After the examination of all patients' clinical records, we observed that 115 (17.22%) patients had experienced CV events after de diagnosis of RA.

We compared the genotypic and allelic frequencies of *RETN* rs1862513 polymorphism between the subgroup of patients who experienced CV disease and the remaining patients with RA. No statistically significant difference between both groups was observed (Table II). In this regard, we only observed a slight increased frequency of homozygous and heterozygous for the minor allele G (CG+GG genotypes) among patients who experienced CV events compared to those without CV events (53.04% *vs*. 52.62%, *p*=0.94).

In a further step we analysed the distribution of the clinical characteristics and CV risk factors between carriers and non carriers of the minor allele G (Table III). We observed a higher frequency of classic CV risk factors among the carriers of the minor allele G (hypertension in non G carriers 35.90% *vs*. G carriers 43.59%, *p*=0.04, diabetes mellitus 10.26% *vs*. 14.90%, *p*=0.07, dyslipidemia 42.90% *vs*. 49.42%, *p*=0.10, smoking habit 14.90% *vs*. 20.00%, *p*=0.09), except for obesity (*p*=0.77).

We wanted to take into account this different distribution of classic CV risk

Table IV. Logistic regression model to explain the presence of cardiovascular disease in rheumatoid arthritis patients according to *RETN* rs1862513 genotype, adjusted for classic CV risk factors.

	p-value*	OR [95% CI]*
G carriers *vs*. non carriers	0.50	0.83 [0.50-1.40]

*Analyses adjusted for gender, age at rheumatoid arthritis diagnosis, follow-up time, presence or absence of shared epitope, hypertension, diabetes mellitus, dyslipidemia, obesity and smoking habit.
OR [95% CI]: Odds Ratio with 95% Confidence Interval.

factors between carriers and non carriers of the minor allele in the influence of the *RETN* rs1862513 polymorphism on the risk of CV disease in RA patients. Therefore we constructed a logistic regression model to explain the presence of CV disease according to *RETN* rs1862513 polymorphism. However, the adjusted model showed no association between the *RETN* variant and CV disease (p=0.50) (Table IV).

In a further step, we specifically assessed the influence of this polymorphism in the occurrence of cardiac ischaemic events or cerebral ischaemic events. Again, no significant associations were found in both the adjusted (p=0.96, p=0.73 respectively) and in the unadjusted (p=0.25, p=0.25 respectively) analyses.

We analysed the occurrence of CV events over a median of 13.4 years (interquartile range 6.8-18.8). We calculated the HR for the *RETN* variant, assuming a dominant pattern of effect, in a crude and adjusted model. However, we did not observe any significant association (unadjusted p=0.75, adjusted p=0.44).

RETN *rs1862513 polymorphism and the presence of subclinical atherosclerosis*

When we studied the carotid IMT according the *RETN* rs1862513 genotypes, we observed, a trend for a higher carotid IMT (p=0.09) and a significantly lower EDV% values among allele G carriers (p=0.03). No significant differences were found regarding EIV (p=0.20) (Table V A). However, in the adjusted ANCOVA model, only a trend towards a higher carotid IMT was found among allele G carriers (p=0.06) (Table V B).

Table V. A. Comparison of carotid artery intima-media thickness, Flow-mediated endothelium dependent (post-ischaemia) vasodilatation (FMD) and endothelium independent (post-nitroglycerin) vasodilatation (NTG), according to *RETN* rs1862513 polymorphism. **B**. Comparison of carotid artery intima-media thicknesss, Flow-mediated endothelium dependent (post-ischaemia) vasodilatation (FMD) and endothelium independent (post-nitroglycerin) vasodilatation (NTG), according to *RETN* rs1862513 polymorphism in an adjusted ANCOVA model.

A

	IMT mm, mean (SD)	*p*-value		
CC (n=51)	0.71 (0.14)			
CG+GG (n=53)	0.76 (0.20)			
Model		0.09		
C	0.74 (0.17)			
G	0.74 (0.19)			
		0.84		
	FMD%, mean (SD)	*p*-value	NTG% mean (SD)	*p*-value
CC (n=63)	6.75 (5.39)		18.00 (8.19)	
CG+GG (n=61)	4.79 (4.58)		16.27 (6.71)	
Model		0.03		0.20
C	6.20 (5.29)		17.60 (7.77)	
G	4.87 (4.46)		16.14 (6.82)	
		0.06		0.16

B

	IMT	FMD	NTG
p carrier G *vs*. non carrier G*	0.06	0.20	0.59

* Analyses adjusted for gender, age at brachial ultrasonography performance, follow-up time, presence or absence of shared epitope, hypertension, diabetes, dyslipidemia, obesity and smoking habit. FMD: Flow-mediated endothelium-dependent Vasodilatation. NTG: Endothelium independent (post nitroglycerin) vasodilatation. IMT: Carotid artery intima-media thickness. SD: Standard Deviation.

Discussion

Data from the present study show that the *RETN* rs1862513 polymorphism does not seem to be a genetic risk factor for CV disease in patients with RA. Previous studies on gene polymorphisms associated with susceptibility to RA have shown contradictory results in terms of genetic association with the increased risk of CV disease observed in patients with this condition. In this regard, an association of *HLA-DRB1**04 shared epitope alleles with increased incidence of CV events (7), CV mortality (7) and endothelial dysfunction (39) has been reported in Spanish individuals with RA. This association of *HLA-DRB1* alleles with CV disease in patients with RA was also confirmed in British individuals (43, 44). However, we could not establish an association with clinically evident CV disease or subclinical atherosclerosis in Spaniards when the influence of other gene variants located outside the MHC region (*PTPN22*, *STAT4* and *TRAF1/C5*) which are also associated with increased disease susceptibility to RA was studied (45). Although an association of endothelial dysfunction with genes implicated in the inflammatory response such as *IL6* was observed in patients with RA (46), no association between subclinical atherosclerosis or CV events with other gene polymorphisms such as *MIF-173* was found (47). In contrast, we recently observed that the *methylene tetrahydrofolate reductase* 1298 A>C gene polymorphism confers an increased risk for subclinical atherosclerosis and CV events in patients with RA (48). Therefore, the search for potential gene candidates that may influence the development of CV disease in patients with RA needs further investigation. Interestingly, an association of the *RETN* rs1862513

polymorphism with CV disease (specifically cerebrovascular ischaemic disease) in Caucasian (32) and Japanese (33) diabetic patients has been reported. However, it is important to highlight that this *RETN* rs1862513 polymorphism does not seem to be associated with susceptibility to diabetes mellitus in Caucasian subjects (25, 32, 49, 50).

Taking together all these observations we feel that the lack of association of this *RETN* rs1862513 polymorphism with CV disease (28, 29, 31), except when CV disease was specifically assessed in diabetic patients (32, 33), suggests that the *RETN* variant may increase the risk of CV disease only if another underlying predisposing disease is present. RA can also be considered a predisposing condition for CV disease (4, 7). A chronic inflammatory status seems to be responsible for the higher risk of CV disease observed in patients with this RA (7, 8). Nevertheless, in the present study we could not find a significant association between the *RETN* rs1862513 polymorphism and the presence of clinically evident CV disease in patients with RA. Regarding subclinical atherosclerosis, carriers of the minor allele G seemed to be associated with greater carotid IMT, even after adjustment for classic CV risk factors, although this potential association did not reach statistical significance (p=0.06). Therefore, this result would require confirmation in a larger patients' sample. On the other hand, no strong association between the *RETN* rs1862513 polymorphism and endothelial function was observed.

A potential limitation of this work was the lack of determination of serum resistin concentration in all the RA patients assessed in the present study. However, this *RETN* variant seems to exert only a small influence in the serum levels of resistin (26-29). In this regard, as previously described (51), serum resisting levels were assessed in a representative subsample of 39 patients with RA included in the present study. However, no statistically significant differences in the serum resistin concentrations were found when these 39 patients were stratified according to the *RETN* rs1862513 genotypes (data not shown).

Resistin seems to play a role in the pathophysiology of RA. Its production is induced by and in turn induces cytokine synthesis such as TNF-α and IL-6 (16-21), both playing a pivotal role in RA (52-53) and atherosclerosis pathogenesis (54-55). In this regard, a strong correlation between serum resistin levels and inflammatory markers such as C-reactive protein has been observed in patients with RA undergoing TNF-alpha antagonist therapy due to severe disease refractory to conventional disease modifying anti-rheumatic drugs (51). Moreover, resistin seems to exert a deleterious effect on the human cartilage by altering the proteoglycan synthesis (56).

In conclusion, the *RETN* rs1862513 polymorphism does not seem to be a genetic risk factor for CV disease in RA.

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