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CARD8 rs2043211 (p.C10X) Polymorphism Is Not Associated with Disease Susceptibility or Cardiovascular Events in Spanish Rheumatoid Arthritis Patients

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Rheumatoid arthritis (RA) is a complex polygenic inflammatory disease associated with accelerated atherosclerosis, which is the main cause of increased cardiovascular (CV) morbidity and mortality in RA patients. CARD8 is a constituent of inflammasome, which regulates interleukin 1-beta production, and has been associated with a worse disease course in early RA. One thousand six hundred twenty-one patients fulfilling the 1987 ACR classification criteria for RA and 1300 matched controls, were genotyped for the *CARD8* rs2043211 (30T > A, p.C10X) single-nucleotide polymorphism (SNP) using predesigned TaqMan SNP genotyping assay. The genotyping success rate in our study was greater than 94%. We assessed *CARD8* rs2043211 gene polymorphism results in 1530 Spanish RA patients in whom information on CV disease and CV risk factors was available at the time of the study. Also, a subgroup of patients with no history of CV events ($n = 276$) was assessed for the potential influence of the rs2043211 variant in the development of subclinical atherosclerosis, by measurement of carotid intima-media thickness (IMT) and presence of carotid plaques. No statistically significant differences in allele or genotype frequencies for the rs2043211 *CARD8* gene variant between patients with RA and controls were seen. Similarly, *CARD8* rs2043211 (30T > A, p.C10X) SNP did not influence the development of CV events or the risk of CV events throughout the time. Likewise, no significant association between this gene variant and carotid IMT or the presence of plaques was found. In summary, our results do not support a role of the *CARD8* rs2043211 gene variant in susceptibility to RA or in the development of CV disease in patients with RA.

Introduction

Rheumatoid arthritis (RA) is a complex autoimmune disease associated with progressive disability, systemic complications, and early death. Mortality is higher among RA patients than among healthy people (González-Gay *et al.*, 2005a; Zinger *et al.*, 2009), and cardiovascular (CV) complications remain a major challenge (McInnes and Schett, 2011). Accelerated atherosclerosis is the main cause of increased CV morbidity and mortality in RA patients (Bartoloni *et al.*, 2011). Besides traditional CV risk factors, chronic systemic inflammation plays a pivotal role in the development of accelerated atherosclerosis observed in RA patients (Dessein *et al.*, 2005; González-Gay *et al.*, 2005b; Nurmohamed, 2009).

Fontalba *et al.* found that *CARD8* (also known as *TUCAN*) p.C10X polymorphism influenced the nuclear factor κB

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(NF-κB) transcriptional activity and was associated with RA severity (Fontalba *et al.*, 2007). Furthermore, epistatic interactions have been found between components of the inflammasome, such as NLRP3 and CARD8, and ACPA-positive RA and a more severe disease course (Kastbom *et al.*, 2008). The protein encoded by this gene (*CARD8*) belongs to the caspase recruitment domain (CARD)-containing family of proteins, which are involved in pathways leading to activation of caspases or NF-κB. This protein may be a component of the inflammasome, a protein complex that plays a role in the activation of proinflammatory caspases. It is thought that this protein acts as an adaptor molecule that negatively regulates NF-κB activation, CASP1-dependent IL1β secretion, and apoptosis. A single-nucleotide polymorphism (SNP) rs2043211 changing cysteine at codon 10 to a premature termination codon (c.30T > A; p.C10X) in this gene was found to be associated with inflammatory bowel disease (McGovern *et al.*, 2006; Fisher *et al.*, 2007) and RA severity and a worse disease course (Fontalba *et al.*, 2007; Kastbom *et al.*, 2010).

The present study aimed to assess the influence of *CARD8* rs2043211 (c.30T > A) gene variant in the susceptibility to and risk of CV disease in a large cohort of Spanish RA patients.

Patients and Methods

Patients and study protocol

A cohort of 1621 RA Spanish patients were included in the present study. Blood samples were obtained from patients recruited from Hospital Xeral-Calde, Hospital Universitario Marqués de Valdecilla, Hospital Universitario Bellvitge, and Hospital Universitario La Paz, Hospital de La Princesa and Hospital Clínico San Carlos from Madrid. The study was approved by the ethics committee of the corresponding hospitals and a subject's written consent was obtained in all the cases, according to the declaration of Helsinki. All the patients fulfilled the 1987 American College of Rheumatology criteria for the classification of RA (Arnett *et al.*, 1988).

One thousand three hundred bone marrow and blood donors from the National Repository DNA Bank (University of Salamanca), matched by age and sex from the corresponding regions, were selected as healthy controls.

Information on the main demographical data, clinical characteristics of the patients enrolled in the study, CV risk factors, and CV events of patients is shown in Table 1.

When the study was performed, 337 (20.79%) of the patients had experienced clinically evident CV events: ischemic heart disease (154 patients, 9.54%), heart failure (90 patients, 5.90%), cerebrovascular accident (75 patients, 4.64%), or peripheral arteriopathy (38 patients, 2.42%). Traditional CV risk factors were established as previously described (González-Gay *et al.*, 2007; González-Juanatey *et al.*, 2009). Smoking habit encompassed to 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.

Patients with CV events were older at the time of disease diagnosis, were more likely to be men and more often had hypertension, diabetes mellitus, dyslipidemia, and obesity (Table 1). Moreover, smoking habit defined as above, was more frequent in men than in women RA patients (45.82% *vs.* 22.81%). Epidemiological studies have shown that among environmental factors, cigarette smoking is one of the most relevant causes involved in RA pathogenesis (Silman and Pearson, 2002; Stolt *et al.*, 2003; Klareskog *et al.*, 2006; Bang *et al.*, 2010; Innala *et al.*, 2011). It seems to play a relevant role in disease induction and progression, particularly in predisposed individuals (Bartoloni *et al.*, 2010).

To determine the potential association between the *CARD8* rs2043211 variant and the presence of subclinical atherosclerosis, between March 2007 and September 2010, a random subgroup of patients from Lugo and Santander ($n = 276$) with no previous history of CV events was selected. Carotid ultrasonography studies were performed to determine the carotid artery intima-media thickness (IMT) and plaques as described elsewhere (González-Juanatey *et al.*, 2006; González-Gay *et al.*, 2008).

Genotyping

DNA from patients was obtained from peripheral blood using standard methods. SNP rs2043211 consists in an A-to-T transversion in exon 5 of the *CARD8* gene predicted to

Table 1. Demographic Characteristics of Rheumatoid Arthritis Patients Included in the Study

Clinical feature	% (n/N)	*With CV events*, % (n)	*Without CV events*, % (n)	p-*value*
Main characteristics				
Women	74.09 (1201/1621)	62.61 (210)	77.06 (991)	$<10^{-6}$
Age of patients at the time of disease diagnosis, years, median (IQR)	54 (43–64)	62.4 (53.6–70)	52 (41–61)	$<10^{-3}$
Time follow-up, years, median (IQR)	10.4 (5–17)	11 (6–19)	10 (5–16)	0.30
Anti-CCP-positive	57.62 (760/1319)	51.39 (129)	59.08 (631)	0.017
Rheumatoid factor-positive	69.16 (1101/1592)	69.11 (217)	69.17 (884)	0.98
Cardiovascular risk factors				
Hypertension	40.87 (658/1610)	66.36 (219)	34.30 (439)	$<10^{-7}$
Diabetes mellitus	13.33 (214/1605)	23.40 (77)	10.74 (137)	$<10^{-7}$
Dyslipidemia	38.93 (626/1608)	55.39 (185)	34.62 (441)	$<10^{-7}$
Obesity	22.95 (350/1525)	28.66 (88)	21.51 (262)	0.008
Smoking habit	28.77 (443/1540)	34.07 (108)	27.39 (335)	0.019
Cardiovascular events	20.79 (337/1621)	100 (337)	NA	

Except where indicated otherwise, values are % (n/N).
CV, cardiovascular; IQR, interquartile range; Anti-CCP, anti-cyclic citrullinated peptide antibodies; NA, not applicable.

result in a Cys10-to-ter (C10X) substitution (Bagnall *et al.*, 2008). The p.C10X polymorphism in *CARD8* (c.30T→A, rs2043211) was analyzed using TaqMan Assays-on-Demand and TaqMan Genotyping Master Mix, and analyzed in the ABI 7900HT Fast Real-Time PCR System, according to the manufacturer's instructions (Applied Biosystems). Negative controls and duplicate samples were included to check the accuracy of genotyping.

Statistical analysis

All genotype data were checked for deviation from Hardy–Weinberg equilibrium (HWE) using http://ihg.gsf.de/cgi-bin/hw/hwa1.pl. Both allelic and genotypic frequencies were calculated and compared by the χ^2 or Fisher tests using the StatsDirect software V2.6.6 (StatsDirect; http://www.statsdirect.com: StatsDirect 2008). Strength of associations between CV events and genotypes or alleles were estimated using odds ratios (OR) and 95% confidence intervals (CI), via multiple logistic regression; estimates were further adjusted for sex, age at RA diagnosis, time of follow-up, and classic CV risk factors (hypertension, diabetes mellitus, dyslipidemia, obesity, and smoking habit).

The relationship between *CARD8* rs2043211 genotypes and CV events was analyzed via multivariate Cox regression adjusting for age at RA diagnosis, sex, and classic CV risk factors (hypertension, diabetes mellitus, dyslipidemia, obesity, and smoking habit); patients that had not experienced a CV event at the end of follow-up were considered as censored. Results were expressed as the hazard ratio (HR) with its 95% CI.

The association between genotypes of the *CARD8* rs2043211 variant and carotid IMT was tested using the unpaired *t* test to compare between two groups, and one-way analysis of variance (ANOVA) to compare among more than two groups. Moreover, we also tested the association between this parameter and alleles using analysis of covariance (ANCOVA) adjusting for sex, age, and duration of the disease at the time of the ultrasonography study, anticyclic citrullinated peptide (CCP) antibody status, and traditional CV risk factors.

All *p*-values refer to two-tailed tests, with $p \leq 0.05$ considered statistically significant. All analyses were performed with STATA statistical software 9.1 (Stata Corp.).

Statistical power for the study was calculated using CaTS - Power Calculator for Two Stage Association Studies (http://sph.umich.edu/csg/abecasis/CaTS/) (Skol *et al.*, 2006).

Results

No deviation from HWE ($p > 0.5$) in controls was detected for *CARD8* rs2043211 polymorphism; however, cases were slightly out of HWE ($p = 0.041$). The genotyping success rate in our study was greater than 94%.

The distributions of genotypes or alleles between patients and controls revealed no significant differences as shown in Table 2, nor were there any differences in distribution in individuals stratified for the presence or absence of anti-CCP antibodies (data not shown). Anti-CCP positivity status has been reported to be a risk factor for the development of ischemic heart disease in RA patients (López-Longo *et al.*, 2009), and epistatic interactions have been found between components of the inflammasome, such as NLRP3 and CARD8, and anti-CCP-positive RA and a more severe disease course (Kastbom *et al.*, 2008).

Allele frequencies were in agreement with the ones previously published in other studies (Fontalba *et al.*, 2007; Schoultz *et al.*, 2009; Pontillo *et al.*, 2012) for European populations.

The overall study (case–control association) has statistical power equal or higher 98% to detect modest OR equal to 1.25 or higher, at the stated significance level ($\alpha = 0.05$), and a prevalence of RA in Spanish population 0.5% (Carmona *et al.*, 2002); in the case of the association with CV disease, the statistical power reached 67%.

As shown in Table 2, no significant differences in the genotype and allele frequencies were found when patients who had experienced CV disease were compared with those who had not suffered any kind of CV events.

In a further step, we constructed a logistic regression model to explain the presence of CV disease according to the *CARD8* rs2043211 allele distribution, which was adjusted for sex, age at the time of RA diagnosis, follow-up time from the disease diagnosis, anti-CCP status, and traditional CV risk factors (hypertension, diabetes mellitus, dyslipidemia, obesity, and smoking habit) as potential confounders. However, no significant association was found in the crude ($p = 0.67$, OR 0.96, 95% CI [0.80–1.16]) or adjusted model ($p = 0.55$, OR 1.08, 95% CI [0.83–1.42]). Also, no association was detected for the risk of developing each one of the CV disease complications described before—heart failure, ischemic heart disease, cerebrovascular accidents, or peripheral arteriopathy (data not shown).

Likewise, no significant associations were detected when we analyzed our cohort of patients by presence or not of hypertension, diabetes mellitus, dyslipidemia, or anti-CCP status (data not shown). Interestingly, in smokers patients—that is individuals who smoked at the time of RA diagnosis, during the follow-up or who had smoked within the 10 years before the onset of RA symptoms or the disease diagnosis—when we performed a logistic regression model adjusted for sex, age at the time of RA diagnosis, follow-up time from the disease diagnosis, anti-CCP status, and long-standing CV risk factors, results disclosed a marginally significant association: $p = 0.051$, OR 1.61, 95% CI [0.99–2.63]. Results of smokers T allele carriers in the adjusted logistic regression model were as follows: $p = 0.014$, OR 2.60, 95% CI [1.21–5.60]. However, these results should be interpreted with caution due to the small number of patients involved.

In a further step, we performed a Cox regression model to account for the variation of risk of CV event through time according to the *CARD8* polymorphism. According to this model, *CARD8* genotypes are not associated with high risk of CV event when adjusting for age at RA diagnosis, sex, and CV risk factors. However, when analyzing nonsmoker patients, those carrying the T allele had a lower risk of CV events than AA homozygous patients (AT genotype: $p = 0.49$, HR = 0.66, 95% CI [0.46–1.00]; TT genotype: $p = 0.24$, HR = 0.66, 95% CI [0.37–1.31]). It is noteworthy that T being the minor allele, the number of patients with the TT genotype is lower, so it is more difficult to reach statistical significance for this genotype. In smoker patients, the T allele did not yield a decrease in the risk of CV event (AT genotype: $p = 0.36$, HR = 1.39, 95% CI [0.68–2.85]; TT genotype: $p = 0.27$, HR = 1.66, 95% CI [0.68–4.05]). However, since the

Table 2. Genotypes and Minor Allele Frequencies of *CARD8* rs2043211 Genetic Variant in Healthy Controls and RA Patients, as Well as in RA Patients Stratified According to the Presence (With) or Absence (Without) of CV Events

	Genotypes					
Subgroup	*AA*	*AT*	*TT*	*MAF (%)*	p-*value*[a]	*OR [95% CI]*[b]
Controls ($N=1292$)	577 (44.66)	582 (45.05)	133 (10.29)	32.82		
RA patients ($N=1530$)	669 (43.73)	712 (46.54)	149 (9.74)	33.01	0.88[c]	1.01 [0.90–1.13][c]
Without CV events ($n=1208$)	524 (43.38)	566 (46.85)	118 (9.77)	33.20		
With CV events ($n=322$)	145 (45.03)	146 (45.34)	31 (9.63)	32.30	0.67[d]	0.96 [0.79–1.16][d]

Values are the number (percentage) unless otherwise indicated.
[a]*p*-Value for the allelic model.
[b]Odds ratio for the minor allele.
[c]With respect to controls.
[d]Comparing RA patients with RA patients without CV events.
RA, rheumatoid arthritis; CARD, caspase recruitment domain; MAF, minor allele frequency; OR, odds ratio; CI, confidence interval.

number of smoker patients was relatively small, no strong conclusions could be reached about the trend to increased risk displayed in this study.

Since carotid IMT and the presence of carotid plaques have been found to be well predictors of the risk of CV events in the extended follow-up of patients with RA (González-Juanatey *et al.*, 2009; Evans *et al.*, 2011), in a further step, we analyzed potential differences in the carotid IMT and presence of carotid plaques in 276 RA patients with no history of CV events stratified according to the rs2043211 *CARD8* genotype and allele distribution.

Results of the comparison between the different genotypes and allele frequencies of the *CARD8* rs2043211 polymorphism according to carotid IMT were as follows:

Carotid IMT-mean (standard deviation): AA ($n=108$) 0.73 mm (0.15), AT ($n=144$) 0.74 mm (0.18), TT ($n=24$) 0.75 (0.19); $p=0.85$. Carriers of T allele ($n=168$): carotid IMT: 0.74 mm (0.18); $p=0.57$.

No statistically significant differences were found when allele frequencies of *CARD8* rs2043211 gene variant were analyzed according to this parameter in the ANOVA model: A ($n=360$) 0.74 (0.16), T ($n=192$) 0.74 (0.18); $p=0.61$. It was also the case when association between the *CARD8* rs2043211 polymorphism and results of carotid IMT were adjusted for potential confounders like sex, age at the time of the ultrasonography assessment, follow-up time, anti-CCP status, and traditional CV risk factors (hypertension, diabetes mellitus, dyslipidemia, obesity, and smoking habit) in the ANCOVA model ($p=0.43$). Additionally, no association was found when a logistic regression model was constructed to explain the presence of carotid plaques according to the *CARD8* rs2043211 allele distribution ($p=0.29$, OR 0.82, 95% CI [0.57–1.18]), which was adjusted for sex, age at the time of RA diagnosis, follow-up time from the disease diagnosis, anti-CCP status, and classic CV risk factors as potential confounders: $p=0.82$, OR 0.95, 95% CI [0.58–1.53].

Discussion

In this large Spanish cohort of RA patients, we were not able to detect the influence of the rs2043211 *CARD8* deleterious gene variant in the susceptibility to the disease, despite data previously reported regarding involvement of this genetic variant in disease severity (Fontalba *et al.*, 2007) and worse disease course in patients with RA (Kastbom *et al.*, 2010).

Several studies have shown lack of association of this *CARD8* genetic variant with several autoimmune diseases, such as Crohn's disease (Buning *et al.*, 2008), type-1 diabetes (Pontillo *et al.*, 2010), or systemic lupus erythematosus (Pontillo *et al.*, 2012). Although some other studies have detected positive associations (McGovern *et al.*, 2006; Schoultz *et al.*, 2009; Roberts *et al.*, 2010), to the best of our knowledge, genome-wide association studies (GWAS) have not shown associations between the *CARD8* gene and autoimmune diseases in general, and RA disease susceptibility or CV traits in particular (www.genome.gov/gwastudies, accessed July 5th, 2012).

RA is a chronic inflammatory disease associated with increased mortality, mainly due to accelerated coronary artery and cerebrovascular atherosclerosis (Kaplan, 2006). Chronic inflammation is a key factor in the atherogenesis process observed in RA (González-Gay *et al.*, 2007). Even in inactive phases of the disease, dysregulated levels of cytokines are present and in this way, promote vascular disease in RA (Sattar *et al.*, 2003). Our results do not support a role of *CARD8* rs2043211 gene variant in the development of CV events, nor in subclinical atherosclerosis disease, assessed by carotid IMT and presence of carotid plaques, in Spanish RA patients. In keeping with our findings, a study in the New Zealand population disclosed that the *CARD8* SNP rs2043211 minor allele only conferred a modest protective effect against abdominal aortic aneurysm (Roberts *et al.*, 2011).

We have recently described that the NF-κB rs28362491 (−94ATTG ins/del) gene variant influences the risk of CV events in RA patients (López-Mejias *et al.*, 2012). NF-κB transactivates the *CARD8* gene, which may lead to a regulatory loop that controls NF-κB activation in response to inflammatory stimuli (Fontalba *et al.*, 2007). The activation and translocation of NF-κB to the nucleus induces gene expression of TNF-α, pro-ILβ and matrix metalloproteinases, prototypic mediators of inflammation, and tissue degradation in RA (Makarov, 2001). In the Fontalba study with 91 RA patients, those who were homozygous carriers of the T allele had a higher disease activity score, more extra-articular manifestations comprising nodules, secondary Sjögren syndrome, episcleritis, pericardial or pleural effusion, interstitial

lung disease, and systemic vasculitis, as well as a lower probability of clinical remission (Fontalba *et al.*, 2007).

Limitations of the study include, as in many other human genetic studies, that sample size is fixed, and the phenotypes studied are complex and involve multiple small-to-modest effects of multiple genes and environmental factors. Therefore, additional studies are needed to clarify the role of inflammasome components and interactions between environmental, hormonal, and genetic factors in the risk of CV disease in RA patients.

Increased risk of CV disease is observed not only in RA, but also in other chronic inflammatory rheumatic diseases, such as ankylosing spondilitis, psoriatic arthritis, systemic lupus erythematosus, and systemic sclerosis, among others (Murdaca *et al.*, 2012). Due to the potential implication of *CARD8* in inflammatory mechanisms that might potentially influence or modulate the development of atherosclerosis in these patients, further investigation is warranted to enlighten the possible role of the inflammasome components in the development of atherosclerosis, which drive out to the elevated CV morbidity and mortality seen in those patients. However, given the clearly pleiotropic nature of this SNP, it is difficult to predict the direction of effect of any association.

In summary, our results do not support an association between the *CARD8* rs2043211 gene polymorphism and susceptibility to disease or risk of development of CV disease in RA. Further studies are warranted to shed light on the complex genetic influence in the development of the accelerated atherosclerosis and the increased morbidity and mortality observed in patients with this disease.

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Authors' Contributions

M.G.B. and R.L.-M. carried out genotyping, participated in the design of the study, data analysis, and drafted the manuscript. J.A.M.-F., C.G.-V., A.B., D.P.-S., and R.B. participated in the acquisition and interpretation of data. C.G.-J., A.C., S.C., A.M.O., and B.F.-G. have been involved in the acquisition and interpretation of data and in revising it critically for important intellectual content. J.L. carried out the analysis and interpretation of the data. J.M. and M.A.G.-G. have made substantial contributions to conception and design of the study, acquisition of data, coordination, and helped to draft the manuscript and have given final approval of the version to be published.

Disclosure Statement

The authors declare that they have no conflicts of interest.

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