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Lack of Association Between *TLR4* rs4986790 Polymorphism and Risk of Cardiovascular Disease in Patients with Rheumatoid Arthritis

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Rheumatoid arthritis (RA) is a chronic inflammatory disease associated with increased cardiovascular (CV) mortality. Toll-like receptor-4 (*TLR4*) activates the innate immune response via NF-*k*B pathway and mitogen-activated protein kinase signaling, leading to expression of proinflammatory cytokines and chemokines. The G allele of *TLR4* rs4986790 (+896A>G, *Asp299Gly*) gene polymorphism has been implicated in reduction of risk of atherosclerosis. In this study, 1481 RA patients fulfilling the 1987 American College of Rheumatology (ACR) criteria were genotyped for the rs4986790 *TLR4* variant to determine the influence of this variant in the risk of CV events in these patients. Also, *HLA-DRB1* status was determined using molecular based methods. Moreover, potential influence of rs4986790 variant in the development of subclinical atherosclerosis was assessed in a subgroup of RA patients with no history of CV events by the measurement of surrogate markers of subclinical atherosclerosis. No statistically significant differences in allele or genotype frequencies for the rs4986790 variant between RA patients who experienced CV events or not were found. Likewise, no significant association between this gene variant and any of the surrogate markers of subclinical atherosclerosis was found. In summary, results in our study do not support the hypothesis that the rs4986790 (+896A>G, *Asp299Gly*) *TLR4* variant may influence predisposition for subclinical atherosclerosis and clinically evident CV disease in RA patients.

Introduction

Rheumatoid arthritis (RA) is a complex autoimmune disease associated with accelerated atherosclerosis (Sattar *et al.*, 2003; González-Gay *et al.*, 2005). Besides traditional cardiovascular (CV) risk factors, chronic inflammation (González-Gay *et al.*, 2007) and genetic factors (Farragher *et al.*, 2008; González-Gay *et al.*, 2009; Palomino-Morales *et al.*, 2009a, 2010a; Panoulas *et al.*, 2009; Rodriguez-Rodriguez *et al.*, 2011c) have been implicated in the increased CV mortality observed in RA patients.

Human toll-like receptors (TLRs) participate in the innate response and signal the activation of adaptive immunity. The toll-like receptor-4 (*TLR4*) plays an important role in the recognition of microbial components, particularly lipopolysaccharides. In addition, *TLR4* also interacts with endogenous proteins present during inflammation such as oxidized low-density lipoprotein, heat shock proteins 60 and 70, fibrinogen, and fibronectin (Akira *et al.*, 2001; Xu *et al.*, 2001). *TLR4* activates the innate immune response via the NF-*k*B pathway and mitogen-activated protein kinase signaling, through IRAK-1 and −4 and TRAF6 (Huang and Pope, 2010) leading to the expression of proinflammatory cytokines and chemokines. TLR4 downregulates disease severity in experimental autoimmune encephalomyelitis and Th17 cell responses, but promotes Th1 cell responses that may inhibit

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the differentiation of Th17 cells (Marta *et al.*, 2009). A single-nucleotide polymorphism (SNP) in the coding region of the human *TLR4* gene (rs4986790, +896A>G) at exon 3 results in amino acid exchange at position 299 *(Asp299Gly)*. The presence of *Asp299Gly* in Caucasian individuals alters the structure of the extracellular domain of *TLR4* (Akira *et al.*, 2001; Kiechl *et al.*, 2002) because it is a change in the ligand-binding site of the receptor (Rallabhandi *et al.*, 2006).

TLR4 has been shown to be expressed by macrophages and endothelial cells in human atherosclerotic lesions (Xu *et al.*, 2001; Edfeldt *et al.*, 2002). Kiechl *et al.* reported that carriers of the +896G allele had lower levels of proinflammatory cytokines, acute-phase reactants, and soluble adhesion molecules. They also found that these +896G allele carriers were more susceptible to severe bacterial infections but had a lower risk of atherosclerosis manifested by a smaller intima-media thickness in the common carotid (Kiechl *et al.*, 2002). Further, individuals with angiographically documented coronary atherosclerosis treated with pravastatin that carried the 299Gly were found to have lower risk of CV events during follow-up than noncarriers (Boekholdt *et al.*, 2003).

Some studies have reported association between *TLR4* polymorphisms and susceptibility to RA (Radstake *et al.*, 2004). Although association of *TLR4* polymorphisms with other autoimmune diseases has also been described (Marshak-Rothstein, 2006), in keeping with data from a recent meta-analysis on association of *TLR4* (+896 A/G) gene polymorphism with susceptibility to giant cell arteritis (Alvarez-Rodriguez *et al.*, 2011), a disease that like RA is associated with HLA-DRB1*04 alleles (González-Gay *et al.*, 2003), Sánchez *et al.* (2004) did not confirm association of *TLR4* polymorphisms with RA in the Spanish population. Also, no association of the *TLR4* rs4986790 gene polymorphism with RA was found in Dutch (Emonts *et al.*, 2011) and French populations (Jaen *et al.*, 2009).

Despite those negative results, in the present study we aimed to determine whether the common SNP of the *TLR4* gene rs4986790 (+896A>G, *Asp299Gly*) may be associated with the risk to develop CV disease in patients with RA, given the crucial role of TLR4 in mediating inflammatory signaling.

Materials and Methods

Patients

Between March 1996 and September 2008, 1481 consecutive patients who fulfilled the 1987 American College of Rheumatology classification criteria for RA (Arnett *et al.*, 1988) were recruited from the Rheumatology Outpatient Clinics of Hospital Xeral-Calde (Lugo), Hospital Clínico San Carlos (Madrid), Hospital Universitario La Paz (Madrid), Hospital Universitario La Princesa (Madrid), Hospital Universitario Bellvitge (Barcelona), and Hospital Universitario Marqués de Valdecilla (Santander), Spain. Patients were assessed for differences in the *TLR4* rs4986790 gene variant.

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. Information on the main demographic data, clinical characteristics of the patients enrolled in the study, CV risk factors, and CV events of patients are shown in Table 1. Two hundred and sixty-one (17.62%) of these 1481 patients with RA experienced CV events. Clinical definitions for CV events (ischemic heart disease, heart failure, cerebrovascular accident, or peripheral arteriopathy) and classic CV risk factors were established as previously described (González-Gay *et al.*, 2007; González-Juanatey *et al.*, 2009).

To determine the potential association between the *TLR4* rs4986790 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 assessed. Presence of endothelial dysfunction was established by a brachial artery reactivity study in 134 patients. Flow-mediated endothelium-dependent vasodilatation-FMD (post-ischemia) and endothelium-independent-NTG (post-nitroglycerin) vasodilatation were measured by brachial ultrasonography as previously reported (González-Juanatey *et al.*, 2003b; González-Gay *et al.*, 2008). Also, carotid ultrasonography studies were performed in 112 patients to determine the carotid artery intima-media wall thickness (IMT). It was assessed in the right common carotid artery as earlier described (González-Juanatey *et al.*, 2006; González-Gay *et al.*, 2008).

A 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 Committees of the Hospital Clínico San Carlos (Madrid), Hospital La Paz (Madrid), Hospital de la Princesa (Madrid), Hospital Universitario Bellvitge (Barcelona), and Hospital Universitario Marqués de Valdecilla (Santander) also approved the study.

Genotyping

TLR4 genotyping. DNA was obtained from peripheral blood using standard methods. Subjects were genotyped to

Table 1. Demographic Characteristics and Genotype Distribution of Patients with Rheumatoid Arthritis Included in the Study

Variables	n = *1481*
Females	1100 (74.27)
Age of patients at the time of disease diagnosis, years, median (IQR)	54.2 (43–64)
Time follow-up, years, median (IQR)	10.6 (5.5–17)
anti-CCP positive (*n* = 1154)	672 (58.23)
Rheumatoid Factor positive (*n* = 1449)	1012 (69.84)
Shared epitope, presence (*n* = 753)	471 (62.55)
Rheumatoid arthritis subjects with cardiovascular disease	261 (17.62)
Ischemic heart disease	141 (9.36)
Cerebrovascular accident	73 (4.85)
Heart failure	80 (5.32)
Peripheral arteriopathy	26 (1.73)
Hypertension (*n* = 1476)	579 (39.23)
Diabetes mellitus (*n* = 1470)	189 (12.86)
Dyslipidemia (*n* = 1445)	579 (40.07)
Obesity (*n* = 1401)	264 (18.84)
Smoking habit (*n* = 1427)	356 (24.95)

Except where indicated otherwise, values are *n* (%).
IQR, interquartile range; anti-CCP, anti-cyclic citrullinated peptide antibodies.

determine the *TLR4* rs4986790 (+896A>G) polymorphism located in exon 3, which leads to an aminoacid change *Asp299Gly* (Arbour *et al.*, 2000), using TaqMan Assays-on-Demand and analyzed using the ABI 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA), following manufacturer's instructions. Negative controls and duplicate samples were included to check the accuracy of genotyping.

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 (Gregersen *et al.*, 1987). *HLA-DRB1*-shared epitope alleles are also implicated in the severity of the disease (González-Gay *et al.*, 2002). *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, United Kingdom).

Statistical analysis

All genotype data were checked for deviation from Hardy–Weinberg equilibrium (HWE) using http://ihg.gsf.de/cgi-bin/hw/hwa1.pl. Comparison of proportions was carried out using χ^2 test or Fisher test, when required. Strength of associations between CV events and genotype or alleles of *TLR4* polymorphism were estimated using odds ratios (OR) and 95% confidence intervals, via multiple logistic regression; estimates were further adjusted for gender, age at RA diagnosis, time of follow-up, presence or absence of the rheumatoid shared epitope, and classic CV risk factors (hypertension, diabetes mellitus, dyslipidemia, obesity, and smoking habit) as potential confounders.

The association between genotypes of the *TLR4* polymorphism and surrogate markers of subclinical atherosclerosis: carotid IMT, FMD-endothelium dependent, or NTG-endothelium independent vasodilatation were tested using unpaired *t*-test, to compare between two groups, and one-way analysis of variance 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 the rheumatoid shared epitope and classic (traditional) CV risk factors. Statistical significance was defined as $p<0.05$. All analyses were performed with STATA statistical software 9.1 (Stata Corp., College Station, TX).

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

Results

Frequencies of the TLR4 *rs4986790 variant and CV events in RA patients*

The study had 74% statistical power to detect allelic ORs greater than 1.65, based on calculation done from the association study in RA (Radstake *et al.*, 2004) and reported in biopsy-proven giant cell arteritis association study (Palomino-Morales *et al.*, 2009b), at the stated significance level ($\alpha=0.05$), with a minor allele frequency of 0.06 and a prevalence of the disease in Spanish population 0.005 (Carmona *et al.*, 2002).

The study reached a genotyping success >95%. Genotype frequencies of the *TLR4* rs4986790 variant studied were in HWE equilibrium in the population under study. Minor allele frequency of rs4986790 SNP was in keeping with other studies previously reported (Radstake *et al.*, 2004; Sánchez *et al.*, 2004; Palomino-Morales *et al.*, 2009b), around 6% in most European populations.

Table 2 shows the genotype frequencies of the TLR4 rs4689790 gene polymorphism assessed in this cohort of RA patients stratified according to the presence or absence of CV events. No statistically significant differences in the genotype or allele frequency of the TLR4 rs4986790 (+896G>A) gene polymorphism between RA patients who experienced CV events or not were seen.

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 *TLR4* rs4986790 genotypes in this series of RA patients (data not shown).

To investigate a potential role of the *TLR4* rs4986790 SNP in the development of CV events, we assembled a logistic regression model to explain the presence of CV disease in patients with RA according to *TLR4* rs4986790 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, which did not disclose statistically significant differences: $p=0.86$, adjusted $p=0.68$. Moreover, no association with the *TLR4* rs4986790 was found when we

Table 2. Differences in Genotype and Allele Frequencies of *TLR*4 rs4986790 Polymorphism Between Rheumatoid Arthritis Patients with Cardiovascular Events or Without Cardiovascular Events

TLR4 *rs4986790*	*With CV events*	*Without CV events*	p-Value	*OR [95% CI]*
AA	219 (87.95)	1039 (88.58)	—	Ref.
AG	29 (11.65)	127 (10.82)	0.71	1.08 (0.69–1.70)
GG	1 (0.40)	7 (0.60)	0.71	0.68 (0.03–5.50)
AG+GG	30 (12.05)	134 (11.42)	0.78	1.06 (0.68–1.65)
A	467 (93.78)	2205 (93.99)	—	Ref.
G	31 (6.22)	141 (6.01)	0.85	1.04 (0.68–1.58)

CV, cardiovascular; OR (95% CI), odds ratio with 95% confidence interval.

specifically assessed the subtypes of CV events studied (ischemic heart disease, cerebrovascular accidents, peripheral arteriopathy, or heart failure) (data not shown).

Since some studies suggested a potential role of *TLR4* gene in the risk of metabolic syndrome (Cuda *et al.*, 2011) or type 1 diabetes mellitus (Kolek *et al.*, 2004), we also sought for potential association of the *TLR4* rs4986790 polymorphism with diabetes mellitus or dyslipidemia in our series. However, no association was disclosed (data not shown).

TLR4 *rs4986790 gene polymorphism and subclinical atherosclerosis markers*

Previous studies have shown an increased frequency of subclinical atherosclerosis in RA patients without clinically evident CV disease (González-Juanatey *et al.*, 2003a, 2003b). Because of that, we also aimed to establish the possible influence of this *TLR4* polymorphism in the development of subclinical atherosclerosis using two well-defined surrogate markers of atherosclerosis, endothelial function, and the carotid IMT (González-Juanatey *et al.*, 2006; González-Gay *et al.*, 2008), which have been proved to be predictors of future CV events in asymptomatic stages of the atherosclerotic disease (González-Gay *et al.*, 2006; Full *et al.*, 2009; Emonts *et al.*, 2011).

Although the results described in the present study confirmed the presence of endothelial dysfunction in patients with long-standing RA (González-Gay *et al.*, 2008), no association between *TLR4* rs4986790 polymorphism and markers of subclinical atherosclerosis was found in this series of RA patients. Data from this series of patients with RA without clinically evident CV disease stratified according to the genotype distribution were the following:

Carotid IMT—mean (standard deviation [SD]): AA ($n=104$) 0.74 mm (0.18), AG ($n=8$) 0.65 mm (0.12), GG ($n=0$); $p=0.20$.

FMD%—mean (SD): AA ($n=124$) 5.60 (4.93), AG ($n=10$) 7.05 (4.72), GG ($n=0$); $p=0.38$.

No significant association in genotype distribution was found for NTG% ($p=0.34$).

This study had >90% statistical power to detect a difference in carotid IMT of 0.15 mm or higher. Statistical power was >60% to detect a variation of 3.5% or higher in FMD-endothelium-dependent vasodilatation.

In the ANCOVA model adjusted for gender, age at the time of the ultrasonography assessment, follow-up time, absence or presence of shared epitope, and traditional CV risk factors, no significant differences were found according to *TLR4* rs4986790 alleles (*TLR4* rs4986790 carotid IMT: $p=0.93$; FMD: $p=0.71$; NTG: $p=0.94$).

Discussion

Previous studies have described expression of TLR4 by macrophages and endothelial cells within human atherosclerotic lesions (Xu *et al.*, 2001; Edfeldt *et al.*, 2002).

Association of *TLR4* rs4986790 allele G polymorphism with reduction of CRP levels and with decreased risk of angiographic coronary artery disease and clinical diabetes was observed in 1894 patients without acute myocardial infarction (MI) undergoing coronary angiography (Kolek *et al.*, 2004). In keeping with this observation, a case-control study on 183 patients with acute coronary syndromes and 216 controls disclosed that the *TLR4* rs4986790 allele G was associated with a decreased risk of coronary events independent of the presence of classic CV risk factors (Ameziane *et al.*, 2003). Also, association of this variant with MI has been described (Balistreri *et al.*, 2004) in older subjects.

Taking into account these results that suggested a potential influence of the *TLR4* rs4986790 gene polymorphism in the risk of atherosclerosis, we conducted a study in a large series of patients with RA, a disease associated with accelerated atherogenesis and increased incidence of CV events (González-Gay *et al.*, 2005, 2006), to determine whether the *TLR4* rs4986790 gene variant might also account for a decreased incidence of CV events in white individuals with RA. However, our data did not confirm any association of the *TLR4* rs4986790 gene polymorphism with the risk of CV events in RA patients. It was also the case when we stratified RA patients according to the presence of specific CV events such as coronary heart disease or cerebrovascular accidents. Interestingly, our results in RA are in accordance with a study on 3657 patients with MI and 1211 controls with angiographically normal coronary arteries and without signs or symptoms of MI that did not show influence of the *TLR4* gene polymorphism in the risk of MI in Caucasian individuals (Koch *et al.*, 2006). Likewise, another study did not disclose differences in the prevalence of the *TLR4* rs4986790 genotypes in patients with cerebral ischemia and control subjects (Reismann *et al.*, 2004). Moreover, when the *TLR4* rs4986790 gene polymorphism was assessed in 1400 patients from the Southampton Atherosclerosis Study that were stratified according to the presence of 0, 1, 2, or 3 coronary arteries with >50% stenosis, no significant differences in the genotype distribution were observed (Yang *et al.*, 2003). Further, to the best of our knowledge, genome-wide association studies (GWAS) have not shown associations between *TLR4* gene and RA disease susceptibility or CV traits (www.genome.gov/gwastudies, accessed November 30th, 2011).

With regard to surrogate markers of atherosclerosis, a study performed in 287 Dominicans from the Northern Manhattan Study showed an association of carotid plaques with genes involved in inflammation, including the *TLR4* gene variant (Gardener *et al.*, 2011). Also, the G allele of the *TLR4* gene—Asp299Gly- polymorphism was found to be associated with increased carotid artery compliance in young adults (Hernesniemi *et al.*, 2008). Regrettably, in accordance with these negative results in terms of CV events, no associations of surrogate markers of subclinical atherosclerosis in RA patients without clinically evident CV disease (carotid IMT or endothelial function) with the *TLR4* rs4986790 (A/G) gene polymorphism were found in our study.

Our negative results are in line with previous reports of our group on several gene polymorphisms implicated in the inflammatory response that failed to establish an association of gene polymorphisms with clinically evident CV disease or subclinical atherosclerosis in Spanish RA patients: other gene variants located outside the major histocompatibility complex region (MHC) (*PTPN22*, *STAT4*, and *TRAF1/C5*), which are also associated with increased disease susceptibility to RA (Palomino-Morales *et al.*, 2010b); polymorphisms implicated in inflammation such as *MIF-173* (Palomino-Morales *et al.*, 2010c) or vascular endothelial growth factor A *(VEGFA)* polymorphisms (Rodriguez-Rodriguez *et al.*, 2011a), as well. Neither association with CV disease was observed

when distinct adipokines polymorphisms implicated in the development of metabolic syndrome nor association with the degree of inflammation in RA patients with severe disease was assessed (García-Bermúdez *et al.*, 2011; Rodriguez-Rodriguez *et al.*, 2011b).

In conclusion, findings shown in our study do not support the hypothesis that *TLR4* rs4986790 (+896A>G, *Asp299Gly*) polymorphism would be a risk factor for CV disease in patients with RA. However, since TLR4 is a mediator of inflammatory reactions (Akira *et al.*, 2001) and atherosclerosis is also an inflammatory disease, further studies in diverse populations with different genetic backgrounds are needed to completely exclude the role of the *TLR4* rs4986790 gene polymorphism in the development of the accelerated atherosclerosis found in patients with RA.

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Disclosure Statement

The authors declare that no competing financial interests exist.

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