Lack of association of *PTPN22*, *STAT4* and *TRAF1/C5* gene polymorphisms with cardiovascular risk in rheumatoid arthritis

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Abstract

Objectives

To determine whether the PTPN22, STAT4 *and* TRAF1/C5 *gene polymorphisms may be implicated in the development of cardiovascular (CV) events and subclinical atherosclerosis manifested by the presence of endothelial dysfunction or increased carotid intima-media thickness (IMT) in a series of Spanish patients with rheumatoid arthritis (RA).*

Methods

Six hundred and twelve 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, were studied. Patients were genotyped using predesigned TaqMan single nucleotide polymorphism genotyping assays. Moreover, between March and December 2007, a subgroup of unselected RA patients with no history of CV events was studied for the presence of subclinical atherosclerosis by the assessment of the endothelial function (n=126) and the carotid artery IMT (n= 110) by ultrasonography studies.

Results

No significant differences in the allele or genotype frequencies for the PTPN22, STAT4 *and* TRAF1/C5 *gene polymorphisms between RA patients with or without CV events were found. It was also the case when we analysed the potential influence of the genotypes in the presence of endothelial dysfunction or increased carotid artery IMT of patients with RA.*

Conclusion

Our results do not show that the PTPN22, STAT4 *and* TRAF1/C5 *gene polymorphisms may confer a direct risk of CV disease in patients with RA.*

Key words

Rheumatoid arthritis, cardiovascular disease, *PTPN22*, *STAT4*, *TRAF1/C5*

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Introduction

Patients with rheumatoid arthritis (RA) have increased risk of cardiovascular (CV) disease due to accelerated atherosclerosis (1). Besides classic CV risk factors, a number of non-traditional CV risk factors have also been implicated in the elevated CV mortality observed in these patients (2). In this regard, chronic inflammation and the genetic background increase the risk of CV events in RA regardless of the presence of traditional CV risk factors (3).

Genetic studies clearly show a complex genetic component that predisposes to the susceptibility to RA (4, 5). The largest genetic contribution to genetic risk for RA clearly resides within the major histocompatibility complex (MHC) and it is mainly related to the *HLA–DRB1* gene and the group of alleles collectively referred to as the shared epitope (SE) (6). In the last years genome wide association studies and linkage have been undertaken to search for association with RA mediated by genes located outside MHC. The protein tyrosine phosphatase N22 (*PTPN22)*, the signal transducer and activator of transcription 4 (*STAT4*) and the tumour necrosis factor (TNF) receptor-associated factor 1/ C5 (*TRAF1/C5)* are the non-HLA genes that have been more convincingly associated with RA in independent Caucasians cohorts, including the Spanish population (4, 5). The most important genetic contribution in Caucasian populations outside of MHC comes from the *PTPN22* gene, in which a single-nucleotide polymorphism (SNP), due to an 1858 C/T polymorphism (rs2476601), encoding an arginine-to-tryptophan substitution at amino acid position 620 increases the risk of RA (7, 8). The *TRAF1-C5* region also appears to be associated with RA, but less strongly than *PTPN22* (9). Another important and consistent association with RA, although quite modest in comparison with *PTPN22*, has been found with the *STAT4* gene (10, 11). Interestingly, in previous studies we confirmed the association of some of these genes with RA in our Spanish cohort (12, 13).

In addition, we previously reported the presence of endothelial dysfunction, an early step in the atherogenesis process, in long-term treated patients with RA (14). We also observed an association between endothelial dysfunction and HLA-DRB1*04-SE+ alleles, in particular with HLA-DRB1*0404 (14). A further study of our group confirmed our previous results showing that HLA-DRB1 status may be a predictor of CV risk and CV mortality in these patients (3). The association of HLA-DRB1 with CV disease in RA was also confirmed in a British population (15, 16). These results supported the potential implication of a genetic component in the development of CV events and CV death in RA.

Several lines of evidence support a potential implication of *PTPN22*, *TRAF1/C5* and *STAT4* genes on atherosclerosis and CV risk. The *PTPN2* gene polymorphism linked to autoimmunity (4) seems also to enhance the development of atherosclerosis (17). TRAFs are overexpressed in atherosclerotic plaques, suggesting a role of these molecules in the CV disease in RA (18). Also, the *TRAF1/C5* locus has recently been associated with increased mortality in RA (19). Finally, STAT4 signalling may modulate the pro-inflammatory behavior of endothelial cells and the proliferation of vascular smooth muscle cells, contributing to the atherosclerosis process (20, 21).

Taking into account all these considerations, we studied whether the *PTPN22* (rs2476601) , *STAT4* (rs7574865) and *TRAF1/C5* (rs10818488) gene polymorphisms might be associated with an increased risk of CV events and subclinical atherosclerosis, manifested by the presence of endothelial dysfunction or increased carotid artery intima-media wall thickness (IMT), in patients with RA.

Patients and methods

Patients

Six-hundred and twelve consecutive patients who fulfilled the 1987 American College of Rheumatology classification criteria for RA (22), seen at the rheumatology outpatient clinics of Hospital Xeral-Calde, Lugo and Hospital San Carlos, Madrid between March 1996 and January 2006 were studied for the *PTPN22, STAT4* and *TRAF1/C5*

gene polymorphisms. Information on the main demographic characteristics of this Caucasian cohort is shown in Table I.

A CV event was considered to be present if the patient had ischemic heart disease, heart failure, cerebrovascular accident or peripheral arteriopathy. Eighty (13.1%) of the 612 patients with RA experienced CV events.

Study protocol

At the time of recruitment patients' data regarding traditional CV risk factors, previous history of CV events, clinical manifestations were registered. Patients were followed and assessed every 3-6 months and patients' medical records were screened for comorbidities.

Information on CV events over the extended follow-up was also registered. Plain radiographs of hands and feet were performed at least every 3 years.

Clinical definitions and patients' follow-up were performed as previously described (3, 23, 24).

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. Specific information on CV events was collected based on patients' medical records. Patients were prospectively followed and clinical records were examined until patient's death or January 1, 2008 to determine the potential implication of the *PTPN22, STAT4* and *TRAF1/C5* gene polymorphisms in the development of CV events.

Brachial artery reactivity and carotid artery ultrasonographic studies

To determine the potential association between these gene polymorphisms and the presence of subclinical atherosclerosis, between March and December 2007, a subgroup of patients from Lugo, randomly selected was assessed for the presence of endothelial dysfunction by brachial artery reactivity study and for the carotid IMT by ultrasonographic assessment of the carotid artery. However, for the purpose of the present study, patients with history of CV events at the time of the ultrasonographic studies were excluded.

Endothelium-dependent-FMD (post-ischemia) and endothelium independent- NTG (post-nitroglycerin) vasodilatation were measured in 126 patients with RA from this series by brachial ultrasonography as previously reported (14, 25, 26). In the laboratory of echocardiography of our centre, adults with FMD% values less than 7% are considered to have endothelial dysfunction (27).

Carotid IMT in the right common carotid artery was performed in 110 RA patients using high-resolution B-mode ultrasound as recently described (27, 28).

The subject's written consent was obtained and the design of the work was approved by the Ethics Committee of Galicia (Spain).

Genotyping

DNA from RA patients was obtained from peripheral blood, using standard methods. Patients were genotyped for the *PTPN22* (rs2476601), *STAT4* (rs7574865) and *TRAF1/C5* (rs10818488) gene polymorphisms using a PCR system with predesigned TaqMan allelic discrimination assay (Applied Biosystems, Foster City, CA) as previously described (12, 13, 29). Duplicate samples and negative controls were included to ensure accuracy of genotyping.

Statistical analysis

Strength of association between CV events in RA and alleles or genotypes of polymorphisms in these genes was estimated using odds ratios (OR) and 95% confidence intervals (CI), via multiple logistic regression; estimates were adjusted by age at diagnosis of the disease (continuous), gender, age at the time of study (continuous), rheumatoid factor and traditional (classic) CV risk factors (presence / absence) as potential confounders. The association between genotypes of these gene polymorphisms and FMD%-endothelium dependent vasodilatation, NTG%-endothelium independent vasodilatation and carotid IMT was tested using one-

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

Patients, n.	612
Main characteristics	
Age at disease onset, years	49.0 ± 15.1
Follow-up, years	14.3 ± 9.4
Women (%)	74.5
Rheumatoid factor positive (%)	68.5
Anti-CCP antibodies positive (%)	66.9
Shared epitope positive (%)	63.6
Extraarticular manifestations (%)	21.2
Radiographic erosions in hands and/or feet (%)	64.9
Medication	
Patients receiving DMARD (%)	90.1
Patients on treatment with methotrexate	84.6
Patients on treatment with TNF-alpha blockers	17.1
Cardiovascular risk factors	
Hypercholesterolemia and/or Hypertriglyceridemia (%)	19.2
Hypertension n. (%)	16.1
Diabetes mellitus n. (%)	2.8
Obesity n. (%)	5.4
Smoking (%)	9.1
Patients with cardiovascular events, n. (%)*	80 (13.1)
Ischemic heart disease	37 (4.6)
Heart failure	5 (2.3)
Cerebrovascular accidents	32 (5.1)
Peripheral arteriopathy	6 (1.0)

Except where indicated otherwise, values are the mean± standard deviation.
Anti-CCP: anti-cyclic citrullinated peptide; DMARD: disease-modifying antirheumatic drug.
*This category was defined considering the first type of cardiovascular event.

way analysis of variance (ANOVA) and the unpaired t test was used to compare variables between 2 groups. Moreover, we also tested the association between these parameters and genotypes using analysis of covariance (ANCOVA) adjusting by gender, age and duration of the disease at the time of the ultrasonographic study (continuous), rheumatoid factor and traditional (classic) CV risk factors (presence / absence). Statistical significance was defined as $p \leq 0.05$. Calculations were performed with the statistical package SPSS 15.0 for Windows.

Results

PTPN22, STAT4 *and* TRAF1/C5 *gene polymorphisms and CV events*

Table II shows the allele and genotype frequencies of the *PTPN22, STAT4* and *TRAF1/C5* gene polymorphisms assessed in this cohort of RA patients stratified by the presence of CV events. No significant associations between these gene polymorphisms and the development of CV events were found. In this regard, allele and genotype frequencies were similar in the whole group of RA patients when patients were stratified according to the presence or absence of CV events. It was also the case when patients were assessed according to follow-up duration, either after a 5- or 10-year follow-up (Table II).

PTPN22, STAT4 *and* TRAF1/C5 *gene polymorphisms and endothelial function or carotid IMT*

Endothelial function was studied in 126 RA patients that were stratified according to the genotypes. The mean value of FMD% in this series of RA patients was 5.22%. Values lower than 7% are considered abnormal in our echocardiography laboratory (27). Therefore, these results confirm the presence of endothelial dysfunction in long-standing RA patients from Northwest Spain. However, the mean values of FMD%-endothelium dependent and NTG%-endothelium independent vasodilatation stratified according to the *PTPN22, STAT4* and *TRAF1/C5* genotypes did not show statistically significant differences (Table III). Likewise, no significant differences according to the different genotypes were observed in the 110 patients assessed for the carotid IMT (Table III).

Discussion

RA is a complex polygenic disease (4, 5). Besides the strong association with the *HLA-DRB1* gene (30), other genes located outside the MHC region have been found to be associated with susceptibility to RA. In this regard, the *PTPN22, STAT4* and *TRAF1/C5* genes have shown to play a role in the genetic predisposition to RA (7-13). Since the outcome of patients with RA is strongly linked to the development of accelerated atherosclerosis and CV complications (31), an issue of major importance is to determine the genetic implication to the risk of CV morbidity and mortality in these patients. In this regard, we previously observed a contribution of the *HLA-DRB1* gene to the risk of endothelial dysfunction, CV events and CV mortality in Spanish individuals with RA (3, 14). Also, we have recently reported a contribution

Table II. Genotypic and allelic frequencies of the PTPN22 rs2476601, STAT4 rs7475845 and TRAF1/C5 rs10818488 gene polymorphisms in RA patients with (+) or without (-) cardiovascular events in the whole cohort and in patients with 5- and 10- year follow-up.*

	Cardiovascular events occurrence in RA patients					
	Overall		5-year follow-up		10-year follow-up	
PTPN22 rs2476601 C>T	+ n = 75 (%)	(-) n = 525 (%)	+ n = 71 (%)	(-) n = 488 (%)	+ n = 60 (%)	(-) n = 362 (%)
CC	60 (80.0)	407 (75.5)	57 (80.3)	377 (73.3)	47 (78.3)	281 (77.6)
CT	14 (18.7)	106 (20.2)	13 (18.3)	103 (21.1)	12 (20.0)	71 (19.6)
TT	1 (1.3)	12 (2.3)	1 (1.4)	8 (1.6)	1 (1.7)	10 (2.8)
C	134 (89.3)	920 (87.6)	127 (89.4)	857 (87.8)	106 (88.3)	633 (87.4)
T	16 (10.7)	130 (12.4)	15 (10.6)	119 (12.2)	14 (11.7)	91 (12.6)
STAT4 rs7574865 G>T	+ n = 75 (%)	(-) n = 520 (%)	+ n = 72 (%)	(-) n = 481 (%)	+ n = 60 (%)	(-) n = 355 (%)
GG	41 (54.7)	283 (54.4)	38 (52.8)	258 (53.6)	32 (53.3)	185 (52.1)
GT	26 (34.7)	202 (38.8)	26 (36.1)	189 (39.3)	23 (38.3)	145 (40.8)
TT	8 (10.7)	35 (6.7)	8 (11.1)	34 (7.1)	5 (8.3)	25 (7.0)
G	108 (72)	768 (73.8)	102 (70.8)	705 (73.3)	87 (72.5)	515 (72.5)
T	42 (28)	272 (26.2)	42 (29.2)	257 (26.7)	33 (27.5)	195 (27.5)
TRAF1/C5 rs10818488 G>A	+ n = 74 (%)	(-) n = 508 (%)	+ n = 69 (%)	(-) n = 468 (%)	+ n = 60 (%)	(-) n = 349 (%)
GG	24 (32.4)	208 (40.9)	22 (31.9)	190 (40.6)	20 (33.3)	141 (40.4)
AG	42 (56.8)	225 (44.3)	40 (58.0)	210 (44.9)	35 (58.3)	156 (44.7)
AA	8 (10.8)	75 (14.8)	7 (10.1)	68 (14.5)	5 (8.3)	52 (14.9)
G	90 (60.8)	641 (63.1)	84 (60.9)	580 (63.0)	75 (62.5)	438 (62.8)
A	58 (39.2)	375 (36.9)	54 (39.1)	346 (37)	45 (37.5)	260 (37.2)

*No statistically significant differences between patients with (+) or without (-) cardiovascular events were found.

Table III. Distribution of the PTPN22 rs2476601, STAT4 rs7475845 and TRAF1/C5 rs10818488 genotypes according to endothelial function and carotid IMT values in patients with RA.

	FMD%			NGD%			Carotid IMT	
PTPN22 rs2476601 C>T	n. patients	Mean ± SEM	*p*-value	n. patients	Mean ±SEM	*p*-value	n. patients	Mean ±SEM
CC	88	5.49 ± 4.73	reference	8	16.37 ± 6.68	reference	78	0.77 0.18
CT	35	4.35 ± 4.37		3	15.33 ± 7.07		29	0.73 ± 0.17
TT	3	7.57 ± 4.13	0.61	3	19.03 ± 2.87	0.82	3	0.70 ± 0.22
CT+TT	38	4.59 ± 4.38	0.75	38	15.62 ± 6.89	0.90	32	0.73 ± 0.18
STAT4 rs7574865 G>T								
GG	61	4.74 ± 4.76	reference	61	15.39 ± 6.59	reference	58	0.78 ± 0.18
GT	54	5.57 ± 4.47		54	16.78 ± 7.33		47	0.76 ± 0.20
TT	5	3.94 ± 2.51	0.52	5	14.66 ± 4.10	0.55	4	0.74 ± 0.27
GT+TT	59	5.43 ± 4.35	0.95	59	16.60 ± 7.11	0.65	51	0.75 ± 0.20
TRAF1/C5 rs10818488 G>A								
GG	51	4.88 ± 3.73	reference	51	16.21 ± 6.38	reference	45	0.75 ± 0.18
AG	55	5.69 ± 4.76		55	16.76 ± 7.05		51	0.76 ± 0.17
AA	15	3.65 ± 6.03	0.17	15	14.15 ± 7.43	0.60	13	0.79 ± 0.28
AG + AA	70	5.25 ± 5.08	0.16	70	16.20 ± 7.16	0.89	64	0.77 ± 0.20

of the inducible and endothelial nitric oxide synthase (*NOS2A* and *NOS3*) gene polymorphisms to CV event risk in patients with RA (32). With respect to this, we found interactions between *NOS2A* promoter *CCTTT* repeat microsatellite or *NOS3 gene* polymorphisms and *HLA-DRB1* for the risk of developing CV events in patients with RA (32). We observed an increased frequency of CV events in patients with RA who carried the *HLA-DRB1*0404* allele and were homozygous for the *NOS3 (-786) TT* genotype (OR: 9.06 (95% CI: 1.29–63.37); *p*=0.03). It was also the case for RA patients who were homozygous for the presence of long *NOS2A* alleles and carried the *HLA-DRB1*0404* allele (OR: 11.7 (95% CI: 1.53–88.4); *p*=0.02) (32).

Taking into account all these considerations, due to the implication *of* the *PTPN22, STAT4* and *TRAF1/C5* gene polymorphisms in the susceptibility to RA, in the present study we assessed whether these polymorphisms might be also implicated in the CV risk of these patients.

Endothelial function and the carotid IMT are two useful non-invasive surrogate markers of subclinical atherosclerosis that have been proved to be predictors of future CV events in asymptomatic stages of the atherosclerotic disease (27, 33). In the present study we sought for the presence of subclinical atherosclerosis in our patients with RA. We also tried to determine the possible influence of the *PTPN22, STAT4* and *TRAF1/C5* gene polymorphisms in the development of subclinical atherosclerosis. Although the results derived from the present study confirmed the presence of endothelial dysfunction in patients with RA, we did not observe association between the endothelial dysfunction or carotid IMT and *PTPN22, STAT4* and *TRAF1/C5* genotypes in Spanish RA patients. Likewise, our results did not confirm an association between the development of CV events in patients with RA and these gene polymorphisms.

In assessing data from a population-based prospective multicenter cohort study of atherosclerosis risk factors in children and adolescents from five university hospital cities in Finland, Pertovaara *et al*. disclosed that polymorphism of PTPN22 (rs2476601) +1858 C/T had an effect on the carotid IMT. In this regard, in young males carriage of the T allele of PTPN22+1858 was associated significantly with IMT in univariate and multivariate analyses (17). Interestingly, it occurred in males without affecting the presence of classical risk factors of atherosclerosis (LDL cholesterol level was even lower in T allele carriers of the PTPN22 gene in males). Therefore according to these data, it seems that this autoimmune gene polymorphism may be an independent predictor of atherosclerosis in males (17). However, although RA is also associated with early development of atherosclerosis, this gene polymorphism was not associated with subclinical markers of atherosclerosis such as the presence of endothelial dysfunction or the carotid IMT in our series of patients with RA. Likewise, we did not find an increased incidence of CV complications when patients with RA were assessed for this PTPN22 (rs2476601)+1858 C/T polymorphism.

STAT4 has also been proposed to influence the development of atherosclerosis. Activation of STAT4 in endothelial cells may be a potential mechanism leading to increased inflammatory response that may play a role in the vascular disease (20). In the experimental model of atherosclerosis that occurs in ApoE (-/-) mice, suppressive oligodeoxynucleotides down-regulated the phosphorylation of STAT1 and STAT4 leading to inhibition of the development of atherosclerosis (34).

An association of the TRAF1/C5 rs3761847 polymorphism with increased mortality, particularly from

malignancy or sepsis, was reported in English patients with RA (19). However, no association between death from CV disease and TARF1/C5 rs3761847 genotypes was observed in this population (19). More recently, Van Nies et al assessed the influence of the TRAF1/C5 rs10818488 polymorphism in the CV mortality of 615 patients with RA from the Leiden Early Arthritis Clinic (35). Seventy-two of them died, 37.7% due to CV complications. However, in keeping with our data, van Nies *et al*. did not observe an association of the TRAF1/C5 rs10818488 polymorphism with CV mortality (35). It was also the case when a potential association of the TRAF1/C5 rs10818488 with all-cause mortality was assessed (35).

In conclusion, our study does not support a major contribution of *PTPN22*, *STAT4* and *TRAF1/C5* gene polymorphisms in the development of subclinical atherosclerosis and the risk of CV events in patients with RA. However, further studies in individuals with different genetic backgrounds are needed to confirm our negative findings. Also, the search for additional genes that may increase the risk of accelerated atherogenesis in patients with RA is warranted.

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