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Association of *STAT4* With Rheumatoid Arthritis

A Replication Study in Three European Populations

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Objective. **This study was undertaken to investigate the previously reported association of the *STAT4* polymorphism rs7574865 with rheumatoid arthritis (RA) in 3 different European populations from Spain, Sweden, and The Netherlands, comprising a total of 2,072 patients and 2,474 controls.**

Methods. **Three different cohorts were included in the study: 923 RA patients and 1,296 healthy controls from Spain, 273 RA patients and 285 healthy controls from Sweden, and 876 RA patients and 893 healthy controls from The Netherlands. DNA from patients and controls was obtained from peripheral blood. Samples were genotyped for the *STAT4* single-nucleotide polymorphism rs7574865 using a TaqMan 5′-allele discrimination assay. The chi-square test was performed to compare allele and genotype distributions. Odds ratios (ORs) and 95% confidence intervals (95% CIs) were calculated.**

Results. **We observed a significantly increased frequency of the minor T allele in RA patients compared with healthy controls in the Spanish population (24.8% versus 20.8%; $P = 0.001$, OR 1.26 [95% CI 1.09–1.45]). This association was confirmed in both the Swedish population ($P = 0.03$, OR 1.35 [95% CI 1.03–1.77]) and the Dutch population ($P = 0.03$, OR 1.45 [95% CI 1.21–1.73]). The overall P value for all 3 populations was 9.79×10^{-6} (OR 1.25 [95% CI 1.13–1.37]). No association between rs7574865 and the presence of rheumatoid factor or anti–cyclic citrullinated peptide autoantibodies was observed. A meta-analysis of all published *STAT4* associations revealed an OR of 1.25 (95% CI 1.19–1.33) ($P = 1 \times 10^{-5}$).**

Conclusion. **Our findings indicate an association between the *STAT4* polymorphism rs7574865 and RA in 3 different populations, from Spain, Sweden, and The Netherlands, thereby confirming previous data.**

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Rheumatoid arthritis (RA) is the most common chronic autoimmune disease, affecting ~0.5–1% of the adult population worldwide. It is characterized by chronic inflammation and destruction of the synovial joints, leading to progressive joint damage, and it is associated with significant disability and early mortality (1). The etiology of RA, like that of other autoimmune disorders, is complex, and it is not completely understood. However, it is known that RA risk is probably influenced by an interaction between environmental and genetic factors.

Data obtained in family and twin studies suggest that up to 60% of disease susceptibility is due to genetic factors (2). The strongest and best-known genetic association with RA is that found for particular alleles of *HLA–DRB1* (3). Estimates suggest that the HLA locus probably accounts for no more than one-third of the total genetic component of susceptibility (4), so many genes that contribute to RA susceptibility remain to be discovered. To date, the association of the 1858 C/T (rs2476601) polymorphism of the *PTPN22* gene with RA is the most robust and reproducible one outside the HLA region in European populations (5–7).

Despite past efforts to discover the genetic basis of RA, only a few studies have yielded significant results. Fortunately, this situation may soon change, due to recent genome-wide association studies that have detected a relatively large number of new potential susceptibility loci (8,9). The next challenge is to examine these new putative susceptibility loci and determine which are indeed general risk factors for RA, which are specific for certain populations, and which are false-positives.

Recently, an RA linkage peak in chromosome 2q was detected in families of European ancestry (10). A followup study (11) identified several polymorphisms in the third intron of the signal transducer and activator of transcription 4 (*STAT4*) gene as the markers responsible for the signal in 2q. Four polymorphisms in tight linkage disequilibrium (rs11889341, rs7574865, rs8179673, and rs10181656; $r^2 > 0.97$ in Caucasians) form a susceptibility haplotype tagged by the T allele of rs7574865, which has been shown to have the most significant association with RA and systemic lupus erythematosus (SLE) (11). The association of *STAT4* with RA has recently been replicated in a Korean population (12). The present study was undertaken to determine whether the association of the *STAT4* polymorphism rs7574865 with RA was present in 3 different RA cohorts from Spain, Sweden, and The Netherlands.

PATIENTS AND METHODS

Patients. *Spanish cohort.* A total of 971 RA patients meeting the American College of Rheumatology (ACR; formerly, the American Rheumatism Association) 1987 revised criteria for RA (13) were recruited from Spanish hospitals. Patient characteristics have been described elsewhere (6). A total of 1,370 blood and bone marrow donors were used as healthy controls. All subjects were of Spanish Caucasian origin. Written informed consent was obtained from all subjects, and the study was approved by the local ethics committees of the participating hospitals.

Swedish cohort. A total of 288 Swedish patients were recruited from the Better Anti-Rheumatic Farmacotherapy (BARFOT) registry, which includes persons participating in a structured followup program for patients newly diagnosed as having RA between 1992 and 2005 in southern Sweden. This registry includes most of the adult patients with new-onset inflammatory polyarthritis who fulfilled the 1987 ACR criteria for RA (13) and were within the catchment areas of the 6 participating rheumatology centers in the BARFOT program (total population ~1.5 million) (14). All subjects gave written informed consent, and the study was approved by all regional ethics committees. We recruited 288 Swedish control subjects from the Uppsala Academic Hospital Blood Bank (Uppsala, Sweden). All individuals were of Swedish ancestry and had 4 grandparents born in Sweden. There was no overlap with the Swedish samples included in the previous study by Remmers et al (11).

Dutch cohort. The Dutch cohort comprised a total of 920 patients with RA, 635 from Nijmegen and 285 from Groningen. The patients from Nijmegen attended the outpatient clinic of the Department of Rheumatology at the Radboud University Nijmegen Medical Center or the outpatient clinics of the centers participating in the Dutch Rheumatoid Arthritis Monitoring registry. Patients were diagnosed according to the ACR criteria (13) and belonged to 2 prospective inception cohorts, which have been described previously (15). The RA patients from Groningen were recruited from the outpatient clinic of the Department of Rheumatology, University Medical Center Groningen; these patients were diagnosed according to the ACR criteria (13) and all were rheumatoid factor (RF) positive and/or had erosive disease. A total of 924 unrelated Dutch individuals were selected as controls. Controls were born in The Netherlands and had at least 3 grandparents born in The Netherlands (16). All patients and controls gave informed consent, and the medical ethics committees of the participating centers approved the study.

Genotyping methods. DNA from patients and controls was obtained from peripheral blood, using standard methods. Samples were genotyped for *STAT4* rs7574865 variants using a TaqMan 5′-allele discrimination assay (Applied Biosystems, Foster City, CA). Allele-specific probes were labeled with the fluorescent dyes VIC and FAM. Polymerase chain reaction (PCR) was carried out in a total reaction volume of 4 μl with the following amplification protocol: denaturation at 95°C for 10 minutes, followed by 40 cycles of denaturation at 92°C for 15 seconds and then annealing and extension at 60°C for 1 minute. After PCR, the genotype of each sample was attributed automatically by measuring the allele-specific fluorescence on an ABI Prism 7900 Sequence Detection System using

Table 1. Genotype and allele frequencies of the *STAT4* rs7574865 polymorphism in patients with RA and healthy controls in 3 populations*

Population	RA patients	Healthy controls	*P*	OR (95% CI)
Spanish				
GG	521 (56.4)	813 (62.7)	–	1.0 (referent)
GT	347 (37.6)	428 (33.0)	0.01	1.27 (1.06–1.51)
TT	55 (6.0)	55 (4.2)	0.02	1.56 (1.07–2.30)
G	1,389 (75.2)	2,054 (79.2)	–	1.0 (referent)
T	457 (24.8)	538 (20.8)	0.001	1.26 (1.09–1.45)
Swedish				
GG	134 (49)	169 (59.3)	–	1.0 (referent)
GT	120 (44)	100 (35.1)	0.02	1.51 (1.07–2.15)
TT	19 (7)	16 (5.6)	0.05	2.01 (1.00–4.08)
G	388 (71.1)	438 (76.8)	–	1.0 (referent)
T	158 (28.9)	132 (23.2)	0.03	1.35 (1.03–1.77)
Dutch				
GG	503 (57.4)	552 (61.8)	–	1.0 (referent)
GT	313 (35.7)	295 (33.0)	0.13	1.16 (0.95–1.42)
TT	60 (6.8)	46 (5.2)	0.08	1.43 (0.95–2.14)
G	1,319 (75.3)	1,399 (78.3)	–	1.0 (referent)
T	433 (24.7)	387 (21.7)	0.03	1.45 (1.21–1.73)

* Values are the number (%). *P* values for overall genotype associations were 0.007 for the Spanish cohort, 0.28 for the Swedish cohort, and 0.1 for the Dutch cohort. RA = rheumatoid arthritis; OR = odds ratio; 95% CI = 95% confidence interval.

SDS version 2.3 software for allele discrimination (Applied Biosystems). Duplicate samples and negative controls were included to check the accuracy of genotyping. In the Spanish cohort, genotyping was successful for 923 (call rate 95%) of the RA patients, and for 1,296 (95%) of the control subjects. In the Swedish cohort, genotyping was successful for 273 (call rate 95%) of the RA patients, and for 285 (99%) of the control subjects. In the Dutch cohort, genotyping was successful for 599 (call rate 94%) of the RA patients from Nijmegen, 277 (97%) of the RA patients from Groningen, and 893 (97%) of the control subjects.

Statistical analysis. The chi-square test was used to compare allele and genotype distributions. Odds ratios (ORs) and 95% confidence intervals (95% CIs) were calculated according to Woolf's method using the Statcalc modules implemented in Epi Info software, version 2002 for Windows (Centers for Disease Control and Prevention, Atlanta, GA). *P* values less than 0.05 were considered significant.

The meta-analysis of all published *STAT4* associations was conducted using the Mantel-Haenszel test to calculate pooled ORs. To accommodate the effect of different ethnic backgrounds on the association between *STAT4* and RA, heterogeneity between studies was analyzed using the chi-square test, and the 95% CI for the OR was estimated using a random-effects model. Meta-analysis was conducted using Cochrane Review Manager software, version 4.1.2.

RESULTS

Table 1 shows the *STAT4* rs7574865 genotype and allele frequencies in RA patients and controls in our 3 independent cohorts of Spanish, Swedish, and Dutch origin. Genotype frequencies were in Hardy-Weinberg equilibrium in patients and controls in all 3 populations.

In the Spanish cohort, rs7574865 GT and TT genotypes were present in RA patients at significantly higher frequencies than in controls ($P = 0.01$, OR 1.27 [95% CI 1.06–1.51] and $P = 0.02$, OR 1.56 [95% CI 1.07–2.30], respectively) (P for trend = 0.002). Accordingly, we observed a statistically significant increase in the frequency of the minor T allele in RA patients compared with healthy controls (24.8% versus 20.8%; $P = 0.001$, OR 1.26 [95% CI 1.09–1.45]) (Table 1).

A similar effect was found in the Swedish cohort (P for trend = 0.026). Carriers of the rs7574865 T allele were more frequently RA patients than controls ($P = 0.02$, OR 1.51 [95% CI 1.07–2.15] and $P = 0.05$, OR 2.01 [95% CI 1.00–4.08], for the GT and TT genotypes, respectively), and the overall frequency of the rs7574865 T allele was significantly increased in patients versus controls (28.9% versus 23.2%; $P = 0.03$, OR 1.35 [95% CI 1.03–1.77]) (Table 1).

This trend was further confirmed in the Dutch cohort, with a similar increase in the frequency of the rs7574865 T allele in RA patients ($P = 0.03$, OR 1.45 [95% CI 1.21–1.73]) and in the GT and TT genotypes. Of the 2 genotypes, however, only the difference in frequency of the TT genotype was borderline statistically significant ($P = 0.13$, OR 1.16 [95% CI 0.95–1.42] for

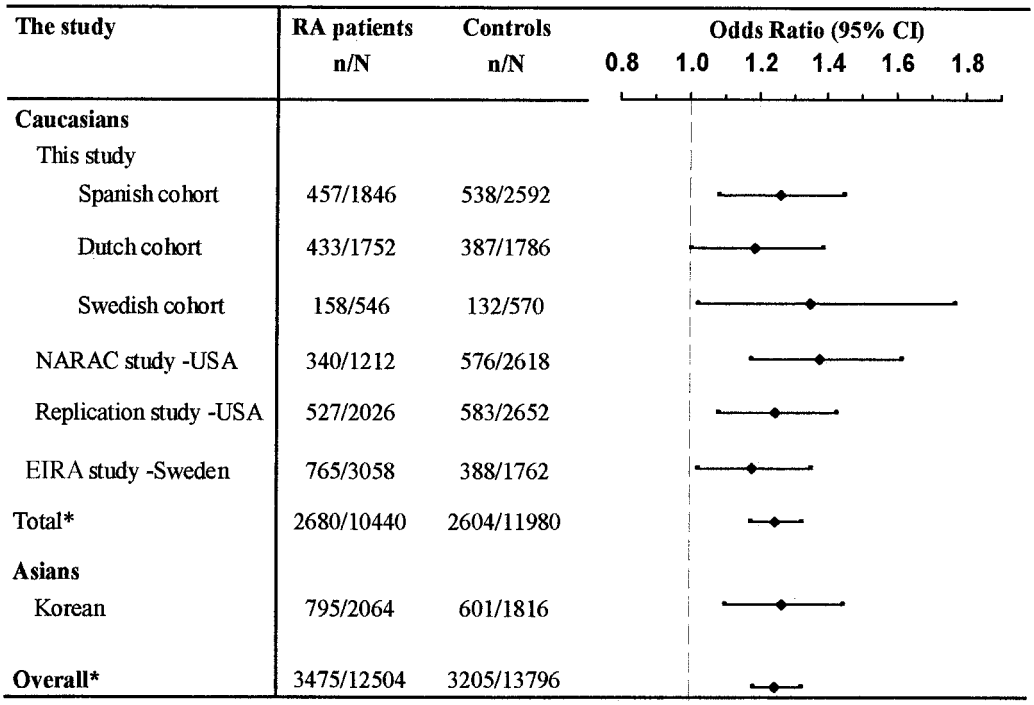

Figure 1. Meta-analysis of the association of the *STAT4* rs7574865 T allele with rheumatoid arthritis (RA) in Caucasian and Asian populations. Data from the North American Rheumatoid Arthritis Consortium (NARAC), a US replication study, and the Epidemiological Investigation of Rheumatoid Arthritis (EIRA) study were obtained from ref. 11, and data from the study of a Korean population were obtained from ref. 12. The vertical broken line represents no effect (i.e., disease risk 1.0). The 95% confidence interval (95% CI) was estimated using a random-effects model. * = $P = 0.00001$ for overall association of the T allele with RA, by Mantel-Haenszel test. In tests for heterogeneity for the overall analysis of data from all studies, $\chi^2 = 3.01$, 6 df, $P = 0.80$, and for the overall analysis of data from studies of Caucasians, $\chi^2 = 2.98$, 5 df, $P = 0.70$. N = total number of chromosomes analyzed; n = number of chromosomes carrying the T allele.

GT and $P = 0.08$, OR 1.43 [95% CI 0.95–2.14] for TT) (Table 1). Given these similar results for the 3 population cohorts, we combined them for an overall analysis, which showed strong evidence of an association of RA both with genotype frequencies ($P = 6.05 \times 10^{-5}$) and with allele frequencies ($P = 9.79 \times 10^{-6}$, OR 1.25 [95% CI 1.13–1.37] for the rs7574865 T allele). Interestingly, *STAT4* rs7574865 minor allele frequencies in all 3 populations in the present study were similar to those previously found in other Caucasian populations (11).

A meta-analysis of all of the Caucasian populations studied to date showed an overall association between the *STAT4* polymorphism and RA ($P = 1 \times 10^{-5}$, OR 1.25 [95% CI 1.18–1.33]) (Figure 1). The analysis included the Spanish, Swedish, and Dutch populations from our study, North American populations (from the North American Rheumatoid Arthritis Consortium and replication cohorts), and the Epidemiological Investigation of Rheumatoid Arthritis Swedish cohort (11). This association remained unchanged when we included data from a study of a Korean population (12) ($P = 1 \times 10^{-5}$, OR 1.25 [95% CI 1.19–1.33]). Of note, the magnitude of risk from the *STAT4* rs7574865 T allele did not differ significantly among the study populations, implying that the *STAT4* rs7574865 T allele may predispose to RA with a similar effect across different populations.

In addition, we tested whether the *STAT4* polymorphism was associated with the presence of serum RF or anti–cyclic citrullinated peptide (anti-CCP) autoantibodies. For this we used the Spanish cohort, since autoantibody data was available for this population. We observed a statistically significant association with the T allele of the *STAT4* single-nucleotide polymorphism in RA patient groups, both positive and negative for RF, compared with healthy controls ($P = 0.0003$, OR 1.36 [95% CI 1.15–1.60] for RF-positive patients and $P = 0.01$, OR 1.36 [95% CI 1.07–1.74] for RF-negative

Table 2. Genotype and allele frequencies of the *STAT4* rs7574865 polymorphism in healthy controls and in Spanish patients with RA, stratified by the presence or absence of serum autoantibodies*

	GG	GT	TT	G	T
Controls (n = 1,296)	813 (62.7)	428 (33.0)	55 (4.2)	2,054 (79.2)	538 (20.8)
RA patients					
RF+ (n = 549)	299 (54.5)	212 (38.6)	38 (6.9)	810 (73.8)	288 (26.2)
P†	–	0.005	0.003	–	0.0003
OR (95% CI)	1.00 (referent)	1.35 (1.08–1.67)	1.88 (1.19–2.96)	1.00 (referent)	1.36 (1.15–1.60)
RF− (n = 202)	109 (54.0)	80 (39.6)	13 (6.4)	298 (73.8)	106 (26.2)
P†	–	0.03	0.07	–	0.01
OR (95% CI)	1.00 (referent)	1.39 (1.01–1.93)	1.76 (0.89–3.45)	1.00 (referent)	1.36 (1.07–1.74)
Anti-CCP+ (n = 288)	152 (52.8)	117 (40.6)	19 (6.6)	421 (73.1)	155 (26.9)
P†	–	0.005	0.03	–	0.001
OR (95% CI)	1.00 (referent)	1.46 (1.11–93.0)	1.85 (1.03–3.30)	1.00 (referent)	1.41 (1.15–1.73)
Anti-CCP− (n = 187)	106 (56.7)	66 (35.3)	15 (8.0)	278 (74.3)	96 (25.7)
P†	–	0.32	0.01	–	0.03
OR (95% CI)	1.00 (referent)	1.18 (0.84–1.66)	2.09 (1.09–3.97)	1.00 (referent)	1.33 (1.03–1.70)

* Values are the number (%). Rheumatoid factor (RF) status was available for 751 patients, and anti–cyclic citrullinated peptide (anti-CCP) status was available for 475 patients. *P* values for overall genotype associations were 0.001 for RF-positive patients, 0.04 for RF-negative patients, 0.005 for anti-CCP antibody–positive patients, and 0.04 for anti-CCP antibody–negative patients. See Table 1 for other definitions.
† *P* values indicate the difference between each patient group and the healthy control group.

patients) (Table 2). Similarly, we found a statistically significant association with the T allele in anti-CCP–positive and anti-CCP–negative RA patients compared with controls ($P = 0.001$, OR 1.41 [95% CI 1.15–1.73] for anti-CCP–positive patients and $P = 0.03$, OR 1.33 [95% CI 1.03–1.70] for anti-CCP–negative patients) (Table 2).

To further investigate the effect of serum autoantibodies on the association of *STAT4* with RA, we analyzed the distribution of the rs7574865 genotypes and alleles among RA patients stratified according to autoantibody status. We found a similar trend of distribution between RF-positive and RF-negative patient groups and between anti-CCP–positive and anti-CCP–negative patient groups. This finding suggests that the susceptibility to RA conferred by the rs7574865 T allele does not differ according to RF or anti-CCP status in patients with RA.

DISCUSSION

In this study, we confirmed the reported association of the *STAT4* rs7574865 polymorphism with RA in 3 independent cohorts of Spanish, Swedish, and Dutch origin (11). Moreover, the overall association of the polymorphism with RA in 2,072 RA patients and 2,474 healthy controls was highly significant ($P = 1 \times 10^{-4}$, OR 1.24 [95% CI 1.12–1.37]). The association of rs7574865 with RA was initially reported in individuals of European ancestry from the US and Sweden and was replicated in a Korean population (12). These findings, together with the data presented here, suggest that *STAT4* is a common RA susceptibility marker in European and Asian populations. Although rs7574865 is clearly involved in RA predisposition in populations of European ancestry, further replication in different cohorts will be required to definitively establish *STAT4* as an RA susceptibility marker in Asian populations. The hypothesis that *STAT4* is also a risk factor for other common autoimmune diseases, such as SLE, remains to be tested (11). Additional studies are needed to clarify the role of *STAT4* as a marker for novel common pathways involved in autoimmune diseases.

The JAK/STAT pathway is the signaling target of a multitude of cytokines that are thought to play biologically significant roles in rheumatoid synovial inflammation (17). In particular, STAT4 transmits signals induced by interleukin-12 (IL-12), IL-23, and type I interferons (IFNs) (18). A major action of IL-12 through STAT4 signaling is to promote the differentiation of naive CD4+ T cells into Th1 cells, which produce IFNγ. These Th1 cells are thought to drive the chronic autoimmune response (19). STAT4 is also important for the development of the recently identified IL-17–secreting T helper cells, which are stimulated by IL-23 (20). These Th17 cells play critical roles in autoimmune diseases such as RA through the production of IL-17 (21–23). Furthermore, IL-17 expression is increased in serum, synovial fluid, and synovial biopsy samples from RA patients (24–28). Therefore, *STAT4* plays a key role in the regulation of Th1 and Th17 cell responses. Since

both lineages are master regulators of RA pathogenesis in humans, *STAT4* may exert its influence in RA through defective signaling in these pathways. *STAT4* is also highly expressed in RA synovium compared with normal tissue (29–31).

Studies using animal models of autoimmunity have provided further evidence that *STAT4* is involved in these pathologic conditions. Interestingly, Stat4$^{-/-}$ mice are resistant to proteoglycan-induced arthritis (32) and develop significantly less severe collagen-induced arthritis (CIA) than do wild-type control mice (33). Moreover, the specific targeting of *STAT4* expression by antisense phosphorothioate oligonucleotide suppresses CIA (33). This suggests that *STAT4* may be a possible therapeutic target. Although it seems clear that *STAT4* plays a key role in several pathways involved in RA pathogenesis, the functional roles of the associated polymorphisms remain to be elucidated.

Finally, a unique mature dendritic cell subset, apparently specific to patients with seropositive RA who strongly express *STAT4*, has been identified (31). In view of the fact that these cells are correlated with the presence of serum RF, we investigated whether the association of *STAT4* with RA was dependent on RF and anti-CCP seropositivity in the Spanish cohort. Our results suggest that *STAT4* is a risk factor for RA, independently of the presence of serum autoantibody. A previous study demonstrated similar results for anti-CCP in a Korean population (12). However, verification of this finding in larger populations is needed, since the autoantibody study in the Korean population and the present study both lacked sufficient power to convincingly assess the effect of RF and anti-CCP status on the contribution of *STAT4* to RA risk. In conclusion, we found an association of the *STAT4* polymorphism rs7574865 with RA in 3 different European cohorts, from Spain, Sweden, and The Netherlands, thereby confirming previously reported data (11).

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AUTHOR CONTRIBUTIONS

Dr. Martín had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study design. Orozco, Alarcón-Riquelme, Martín.

Acquisition of data. Orozco, Alizadeh, Delgado-Vega, González-Escribano, Barrera, Coenen.

Analysis and interpretation of data. Orozco, Alizadeh, Delgado-Vega.

Manuscript preparation. Orozco, Alizadeh, Radstake, Alarcón-Riquelme, Martín.

Statistical analysis. Orozco, Alizadeh, Delgado-Vega, Koeleman.

Sample provision. González-Gay, Balsa, Pascual-Salcedo, Fernández-Gutierrez, González-Escribano, Petersson, van Riel, Barrera, Radstake, van Leeuwen, Wijmenga.

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