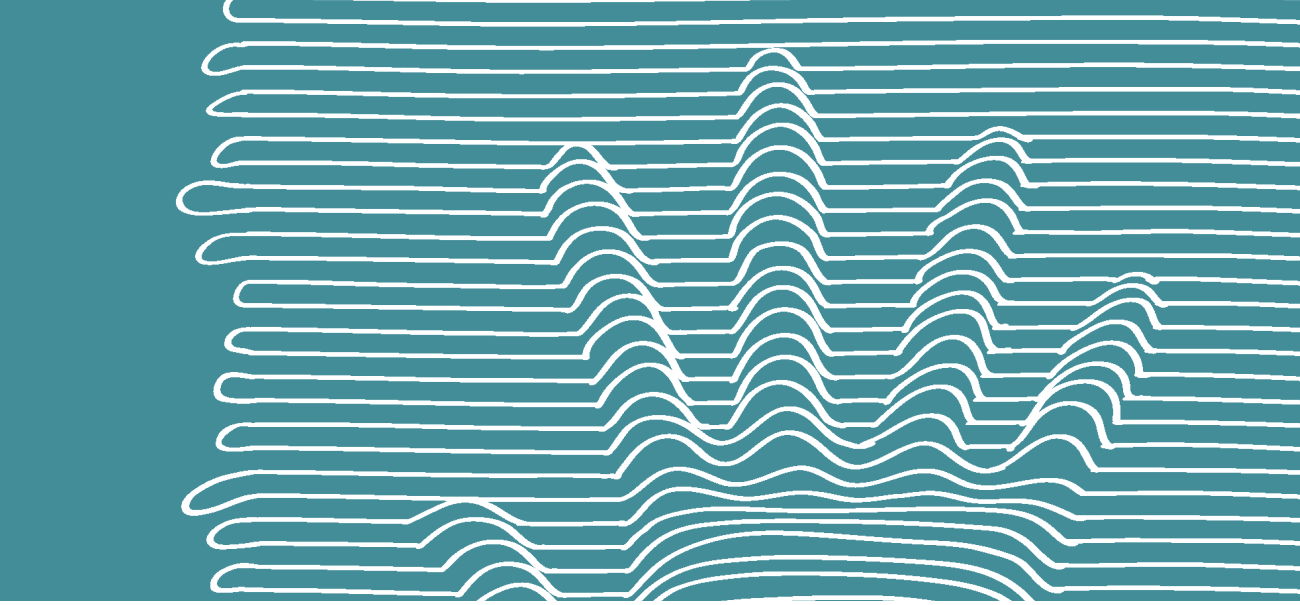

CHAPTER 2

Confirmation of STAT4, IL2/IL21, and CTLA4 Polymorphisms in Rheumatoid Arthritis

N.A. Daha*, F.A.S. Kurreeman*, R.B. Marques, G. Stoeken-Rijsbergen, W. Verduijn, T.W.J. Huizinga, and R.E.M. Toes

* Both authors contributed equally

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ABSTRACT

Objective

Recent advances have led to novel identification of genetic polymorphisms that are associated with susceptibility to rheumatoid arthritis (RA). Currently, 5 loci (HLA, PTPN22, TRAF1/C5, TNFAIP3, and STAT4) have been consistently reported, whereas others have been observed less systematically. The aim of the present study was to independently replicate 3 recently described RA susceptibility loci, STAT4, IL2/ IL21, and CTLA4, in a large Dutch case-control cohort, and to perform a meta-analysis of all published studies to date and investigate the relevance of the findings in clinically well-defined subgroups of RA patients with or without autoantibodies.

Methods

The STAT4, IL2/IL21, and CTLA4 gene polymorphisms (rs7574865, rs6822844, and rs3087243, respectively) were genotyped in 877 RA patients and 866 healthy individuals. A meta-analysis of all published studies of disease association with these polymorphisms was performed using the Mantel-Haenszel fixed-effects method.

Results

An association of STAT4, IL2/IL21, and CTLA4 with RA was detected in Dutch patients (odds ratio [OR] 1.19 [P = 0.031], OR 0.84 [P = 0.051], and OR 0.87 [P = 0.041], respectively). Results from the meta-analysis confirmed an association of all 3 polymorphisms with RA in Caucasians (OR 1.24 [$P = 1.66 \times 10^{-11}$], OR 0.78 [$P = 5.6 \times 10^{-5}$], and OR 0.91[$P = 1.8 \times 10^{-3}$], respectively). The meta-analysis also revealed that STAT4 predisposed to disease development equally in patients with autoantibodies and those without autoantibodies, and that CTLA4 enhanced the development of anti-citrullinated protein antibody (ACPA)-positive RA as compared with ACPA-negative RA.

Conclusion

Our results replicate and firmly establish the association of STAT4 and CTLA4 with RA and provide highly suggestive evidence for IL2/IL21 loci as a risk factor for RA. Given the strong statistical power of our meta-analysis to confirm a true-positive association, these findings provide considerable support for the involvement of CTLA4 in distinct subsets of RA patients.

INTRODUCTION

Rheumatoid arthritis (RA) is a common auto-immune disease with unknown etiology. Nonetheless, it is known that both genetic and environmental factors play a role in the pathogenesis of the disease. The strongest known genetic association with RA is with particular alleles of the HLA locus[1]. In recent years, continuing advances in genotyping techniques have led to discovery of a large number of potential genetic associations outside this region[2-4]. Some of these newly identified susceptibility loci represent true associations, whereas others still remain to be conclusively investigated.

Followup replication studies in different populations are needed to resolve this issue. However, although some followup studies have shown robust associations, others have yielded encouraging, but inconsistent, results. This could be due to insufficient power to detect modest effects in some of these studies. To overcome this limitation, data from previously published studies can be systematically evaluated by a meta-analysis. Furthermore, since RA is a heterogeneous disease and data indicate that different risk factors predispose to autoantibody-positive disease as compared with autoantibody-negative disease[5], further investigation in these disease subsets remains to be performed in large data sets. In the present study, 3 previously described susceptibility loci in patients with RA, i.e., rs7574865 (for signal transducer and activator of transcription 4 [STAT4]), rs6822844 (for interleukin-2/interleukin-21 [IL2/IL21]), and rs3087243 (for cytotoxic T lymphocyte- associated antigen 4 [CTLA4]), were investigated for association with the disease and for association with autoantibody status.

The association of STAT4 with RA was first described in 2007, followed by a vast number of rep-lication studies in both Caucasian and East Asian populations, all of which yielded results that were consistent across the studies[3,6-11]. In contrast, the second polymorphism in this study, namely, rs6822844 in the IL2/IL21 region, has only been described in one study thus far, indicating that further replication is needed[4]. The third variant that we aimed to investigate maps to the CTLA4 region. Various replication studies have shown encouraging, yet inconsistent, results for this locus[12-17]. Plenge et al provided evidence that the differences between studies could be due to insufficient power in some of the studies[15].

Thus, the aim of our study was to replicate 3 previously described risk factors for RA and further study their association by a meta-analysis. Additionally, we examined whether the association was restricted to clinically relevant disease subsets that were characterized by autoantibody status.

PATIENTS AND METHODS

Patients

A total of 877 RA patients whose diagnosis met the American College of Rheumatology (formerly, the American Rheumatism Association) 1987 revised criteria for RA[18] were re-

cruited in 1994 from hospitals in the western part of The Netherlands, of whom 602 patients were from 2 independent cohorts of patients with early arthritis, the EAC and BeSt cohorts, and 275 were from the outpatient clinic of the Leiden University Medical Center. For both early arthritis cohorts, anti-citrullinated protein antibody (ACPA) status and rheumatoid factor (RF) status were obtained. For the patients from the outpatient clinic, only the RF status was obtained. Patients' characteristics have been described previously[19]. As healthy controls, 866 subjects were randomly selected from the Immunogenetics and Transplantation Immunology section of Leiden University Medical Center. All patients and controls gave their informed consent to participate in the study, and the study was approved by the local ethics committee of the participating hospitals.

Genotyping methods

Genotyping of STAT4 rs7574865, IL2/IL21 rs6822844, and CTLA4 rs3087243 was performed using MassArray matrix-assisted laser desorption ionization-time-of-flight mass spectrometry, according to the protocols recommended by the manufacturer (Sequenom, San Diego, CA). SpectroCaller software, which was supplied by the same manufacturer, was used to automatically identify, i.e., call, the genotypes. Each 384-well plate consisted of 8 positive controls and 8 negative controls, all of which were indeed shown to be positive or negative. Clusters were evaluated and all doubtful calls were checked; after manually evaluating the spectra of each cluster, the genotypes were accepted, recalled, or rejected. At least 10% of the genotypes were assessed in duplicate, with an error rate of <1%.

Statistical analysis

Allele distribution was analyzed for association with RA using a chi-square test with 1 degree of freedom. Odds ratios (ORs) and 95% confidence intervals (95% CIs) were calculated using the Statcalc module of Epi Info software (Centers for Disease Control and Prevention, Atlanta, GA). P values less than 0.05 were considered significant. Genotype frequencies in cases and controls did not deviate from Hardy-Weinberg equilibrium at a significance level of $P < 0.05$. A meta-analysis of published reports describing disease associations with STAT4, IL2/IL21, and CTLA4 was performed using the Mantel-Haenszel method of combining ORs. Reports published up to July 31, 2008 were included in the analysis. Heterogeneity of the ORs across sample sets was analyzed using the Breslow-Day test. Since no significant heterogeneity was observed among the studies, the ORs and 95% Cls were calculated using a fixed-effects model, and P values less than 0.05 were considered significant. (Genotype frequencies are available upon request from the corresponding author.) The meta-analyses had >80% power to detect allele associations both for association with

RA and for association with autoantibody status, at ORs of <1.18 (at a significance level of $P < 0.05$) for all 3 polymorphisms, except for the association of the IL2/IL21 locus with autoantibody status, in which the meta-analysis had 67% power to detect an OR of 1.20 (at a significance level of $P < 0.05$).

RESULTS

Replication of STAT4, IL2/IL21, and CTLA4 loci in an independent Dutch cohort

The polymorphisms STAT4 rs7574865, IL2/IL21 rs6822844, and CTLA4 rs3087243 were genotyped in 877 RA patients and 866 healthy controls. Both STAT4 and CTLA4 showed an association with RA in the Dutch cohort, while a clear trend toward association was observed for the IL2/IL21 locus (Table 1). The results had the same direction of association as has been reported in previous studies[3,4,6,9-12,14,15,17].

Table 1. *Results of association and stratification analysis of STAT4 rs7574865, IL2/IL21 rs6822844, and CTLA4 rs3087243 in a Dutch cohort**

	STAT4 rs7574865					IL2/IL21 rs6822844					CTLA4 rs3087243				
	Allele					Allele					Allele				
	G	T	MAF	OR (95% CI)	P	G	T	MAF	OR (95% CI)	P	A	G	MAF	OR (95% CI)	P
RA															
Cases	1,276	432	0.25	1.19 (1.01-1.40)	0.031	1,469	285	0.16	0.84 (0.70-1.0)	0.0506	729	1,005	0.42	0.87 (0.76-1.00)	0.041
Controls	1,348	384	0.22			1,407	325	0.19			785	941	0.45		
RF status															
RF+	711	239	0.25	1.18 (0.98-1.43)	0.080	811	163	0.17	0.87 (0.70-1.08)	0.188	406	554	0.42	0.88 (1.75-1.03)	0.111
RF-	362	116	0.24	1.12 (0.88-1.44)	0.332	422	78	0.16	0.80 (0.61-1.06)	0.105	207	287	0.42	0.86 (0.70-1.06)	0.159
RF+ vs. RF-				1.05 (0.81-1.37)	0.713				1.09 (0.80-1.48)	0.577				1.02 (0.81-1.27)	0.887
ACPA status															
ACPA+	478	158	0.25	1.16 (0.93-1.44)	0.170	552	102	0.16	0.80 (0.62-1.03)	0.072	257	387	0.40	0.80 (0.66-0.96)	0.015
ACPA-	325	111	0.25	1.20 (0.93-1.54)	0.144	380	76	0.17	0.87 (0.65-1.15)	0.303	193	257	0.43	0.90 (0.70-1.12)	0.325
ACPA+ vs. ACPA-				0.97 (0.72-1.29)	0.819				0.92 (0.66-1.29)	0.633				0.88 (0.69-1.14)	0.324

** Values for alleles G, T, and A are the allele frequency. MAF = minor allele frequency; OR = odds ratio; 95% CI = 95% confidence interval;*
RA = rheumatoid arthritis; RF = rheumatoid factor; ACPA = anti-citrullinated protein antibody.

Stratification by autoantibody status

For a better understanding of disease etiology, we investigated whether the associations were restricted to a specific subset of disease, defined by either ACPA positivity or RF positivity. In these subsets, no differential association could be observed for either the STAT4 polymorphism or the IL2/IL21 polymorphism (Table 1). The CTLA4 polymorphism, however, did show a significant association with ACPA-positive RA in patients as compared with healthy controls (OR 0.80, 95% CI 0.66-0.96, P = 0.015), but showed no association with ACPA- negative RA in patients as compared with healthy controls (OR 0.90, 95% CI 0.70-1.12, P = 0.325). Furthermore, an increase in frequency of the G allele was observed in patients with ACPA-positive RA (60%) as compared with patients with ACPA-negative RA (57%), but the difference was not significant.

Meta-analysis of STAT4, IL2/IL21, and CTLA4 loci.

Association with RA overall.

To systematically assess the contribution of the 3 studied polymorphisms in RA, a meta-analysis of all published studies to date was performed. This analysis provided an overall OR for the widely and consistently replicated STAT4 locus in the Caucasian population (OR 1.24, 95% CI 1.17-1.33, P = 1.66 X 10^{-11}) (Table 2). Evaluation of both studies dealing with IL2/IL21 provided additional evidence of an association of IL2/IL21 with RA in Caucasians (OR 0.78, 95% CI 0.69-0.88, P = 5.6 X 10^{-5}). Examination of the 6 previously published studies on rs3087243 in CTLA4 confirmed an overall association of this region with RA in Caucasians (OR 0.91, 95% CI 0.85-0.96, P = 1.8 X 10^{-3}) (Table 2).

Association with RA stratified by autoantibody status.

Results from our meta-analysis indicated that STAT4 was associated with both autoantibody-positive and autoantibody-negative disease in the Caucasian population (OR 1.00, 95% CI 0.89-1.14, P = 0.97) (Table 3). Moreover, IL2/IL21 showed a significant association with RF-positive disease in Caucasian patients as compared with healthy controls (OR 0.78, 95% CI 0.68-0.90, P = 6.9 X 10^{-4}), but IL2/IL21 showed no significant association with RF-negative disease in Caucasian patients as compared with controls (OR 0.82, 95% CI 0.66-1.03, P = 0.083). However, the effect sizes of both associations were of the same extent. Furthermore, the effect size of RF-positive disease compared with RF-negative disease was limited, indicating an association of IL2/IL21 in both disease subsets (OR 1.02, 95% CI 0.81-1.29, P = 0.86) (Table 3). Interestingly, in the meta-analysis, CTLA4 in the Caucasian population was found to predispose to ACPA-positive disease only, and not to ACPA-negative disease (OR 0.86, 95% CI 0.78-0.96, P = 4.7 X 10^{-3}) (Table 3).

DISCUSSION

In the present study, 2 genetic risk factors for RA were replicated in an independent Dutch population, with a third genetic risk factor showing a clear trend toward association. All 3 loci were further confirmed in a well-powered meta-analysis. Interestingly, these polymor-

phisms have been described in several autoimmune diseases, varying from type 1 diabetes to systemic lupus erythematosus[3,4], which further emphasizes their role in autoimmunity. In RA, previous studies have suggested that genetic risk factors predispose to specific subsets of the disease, characterized by autoantibody status. For example, both the HLA shared epitope and PTPN22 loci have been shown to be associated with a clear predisposition to ACPA-positive disease only. At a biologic level, classifying these genetic risk factors will ultimately enable a better understanding of the disease processes involved. Although STAT4, IL2/IL21, and CTLA4 have been found in association with ACPA-positive disease[20], they have not been investigated extensively in autoantibody-negative patients.

In this study, we did not observe a difference in effect size between the autoantibody strata for either STAT4 or IL2/IL21. Our results are consistent with recent findings for STAT4. Zhernakova and colleagues[4] have shown an association of IL2/IL21 in RF-positive patients as compared with controls, but no conclusive difference could be established between the 2 subgroups. In support of these findings, our combined data sets also indicated that IL2/IL21 predisposes individuals to both autoantibody-positive and autoantibody negative disease. However, additional replication in independent cohorts will still be necessary to tease apart the precise role of IL2/IL21 in these disease subsets. The results from several studies previously suggested that CTLA4 is associated with RA in an autoantibody-dependent manner. However, we provided, for the first time, conclusive evidence that CTLA4 is associated with ACPA-positive RA, but not with ACPA-negative RA.

CONCLUSION

In conclusion, this study provides independent replication of an association of STAT4, IL2/IL21, and CTLA4 with RA, as well as substantial evidence of the involvement of CTLA4 in ACPA-positive disease only, as compared with the involvement of STAT4 and IL2/ IL21, which predisposes to both disease subsets.

***Table 2**. Meta-analysis of the association of STAT4 (rs7574865), IL2/IL21 (rs6822844), and CTLA4 (rs3087243) with RA in Caucasian and East Asian populations**

	RA cases					Controls						
	No. of subjects	Allele G	Allele T	Allele A	MAF	No. Of subjects	Allele G	Allele T	Allele A	MAF	OR (95% CI)	P
STAT4 rs7574865												
Caucasian populations												
This study	854	1,276	432		0.25	866	1,348	384		0.22	1.19 (1.01-1.40)	0.031
Remmers et al 2007												
NARAC	606	872	340		0.28	1,309	2,042	576		0.22	1.38 (1.18-1.62)	4.4 X 10~5
Replication study, US	1,013	1,499	527		0.26	1,326	2,069	583		0.22	1.25 (1.09-1.43)	1.3 X 10~3
EIRA	1,529	2,293	765		0.25	881	1,374	388		0.22	1.18 (1.03-1.36)	0.018
Barton et al 2008												
WTCCC	1,858	2,835	881		0.24	2,934	4,580	1,288		0.22	1.11 (1.00-1.22)	0.045
Replication study, UK	3,399	5,140	1,658		0.24	3,024	4,744	1,304		0.22	1.17 (1.08-1.28)	1.4 X 10~4
Orozco et al 2008												
Spanish	923	1,389	457		0.24	1,296	2,054	538		0.21	1.26 (1.09-1.45)	1.6 X 10~3
Dutch	876	1,319	433		0.25	893	1,399	387		0.22	1.19 (1.01-1.39)	0.031
Swedish	273	388	158		0.29	285	438	132		0.23	1.35 (1.03-1.77)	0.028
Palomino-Morales et al 2008	257	316	198		0.38	410	562	258		0.31	1.36 (1.08-1.73)	8.1 X 10~3
Zervou et al 2008	311	451	171		0.27	344	574	114		0.17	1.91 (1.46-2.49)	1.7 X 10~6
Pooled Caucasian	11,899					13,568					1.24 (1.17-1.33)	1.66 X 10“11
East Asian populations												
Lee et al 2007	1,032	1,269	795		0.38	908	1,215	601		0.33	1.27 (1.11-1.45)	4.5 X 10~4
Kobayashi et al 2008												
Tokyo, Japan	1,481	1,870	1,092		0.37	745	1,026	464		0.31	1.29 (1.13-1.48)	1.6 X 10~4
Biobank Project, Japan	1,109	1,396	822		0.37	938	1,295	581		0.31	1.31 (1.15-1.50)	4.3 X 10~5
Tokushima, Japan	941	1,178	704		0.37	500	662	338		0.34	1.17 (0.99-1.38)	0.055
Pooled East Asian	4,563					3,091					1.27 (1.18-1.36)	1.4 X 10-11
Pooled Caucasian + East Asian	16,462					16,659					1.24 (1.19-1.31)	<1 X 10~15

***Table 2**. Continued*

IL2/IL21 rs6822844											
Caucasian populations											
This study	877	1,469	285		0.16	866	1,407	325	0.19	0.84 (0.70-1.00)	0.051
Zhernakova et al 2007	1,012	1,739	285		0.14	924	1,506	342	0.19	0.72 (0.61-0.86)	1.9 X 10~4
Pooled Caucasian	1,889					1,790				0.78 (0.69-0.88)	5.6 X 10~5
CTLA4 rs3087243											
Caucasian populations											
This study	867	1,005		729	0.42	863	941		785	0.45	0.87 (0.76-1.00)	0.045
Plenge et al 2005											
EIRA	1,505	1,870		1,140	0.38	878	1,070		686	0.39	0.95 (0.84-1.08)	0.410
NARAC	828	1,003		653	0.39	845	934		756	0.45	0.80 (0.70-0.93)	0.001
Barton et al 2004	719	820		618	0.43	755	848		662	0.44	0.97 (0.83-1.12)	0.636
Orozco et al 2004	433	432		434	0.50	398	401		395	0.50	1.02 (0.84-1.24)	0.841
Zhernakova et al 2005	153	173		133	0.43	900	959		841	0.47	0.88 (0.68-1.13)	0.291
Pooled Caucasian	4,505					4,639					0.91 (0.85-0.96)	0.0018
East Asian populations											
Lei et al 2005	326	449		203	0.31	250	305		195	0.39	0.71 (0.55-0.91)	0.005
Tsukahara et al 2008	1,498	2,284		712	0.24	441	653		229	0.26	0.89 (0.75-1.06)	0.181
Pooled East Asian	1,824					691					0.82 (0.72-0.95)	0.007
Pooled Caucasian + East Asian	6,329					5,330					0.89 (0.85-0.95)	8.3 X 10~5

** Values for alleles G, T, and A are the allele frequency. Rheumatoid arthritis (RA) cases and controls were compared using a fixed-effects (Mantel-Haenszel) meta-analysis. No significant heterogeneity was observed among the studies. MAF = minor allele frequency; OR = odds ratio; 95% CI = 95% confidence interval; NARAC = North American Rheumatoid Arthritis Consortium; EIRA = Epidemiological Investigation of Rheumatoid Arthritis; WTCCC = Wellcome Trust Case Control Consortium.*

Table 3. *Meta-analysis of STAT4 (rs7574865), IL2/IL21 (rs6822844), and CTLA4 (rs3087243) stratified by autoantibody status**

Study	No. of subjects	Allele				Cases vs. controls		ACPA+ vs. ACPA-		RF+ vs. RF-
		G	T	A	MAF	OR (95% CI)	P	OR (95% CI)	P	OR (95% CI) P
STAT4 rs7574865										
Caucasian populations										
This study								1.03 (0.77-1.38)	0.819	
ACPA+	318	478	158		0.25	1.16 (0.93-1.44)	0.170			
ACPA-	218	325	111		0.25	1.20 (0.93-1.54)	0.144			
Barton et al 2008								1.01 (0.86-1.91)	0.880	
ACPA+	1,211	1,823	599		0.25	1.20 (1.07-1.34)	0.001			
ACPA-	617	926	308		0.25	1.21 (1.05-1.40)	0.009			
Orozco et al 2008								0.94 (0.69-1.27)	0.672	
ACPA+	288	421	155		0.27	1.41 (1.14-1.74)	0.001			
ACPA-	187	278	96		0.27	1.32 (1.02-1.71)	0.030			
Pooled Caucasian								1.00 (0.89-1.14)	0.97	
ACPA+	1,817					1.22 (1.12-1.33)	8.1 X 10-6			
ACPA-	1,022					1.23 (1.10-1.37)	2.8 X 10-4			
East Asian populations										
Lee et al 2007								1.00 (0.74-1.35)	0.985	
ACPA+	612	749	475		0.39	1.01 (0.87-1.17)	0.869			
ACPA-	111	136	86		0.39	1.01 (0.75-1.35)	0.949			
Pooled Caucasian + East Asian								1.00 (0.89-1.12)	0.98	
IL2/IL21 rs6822844										
Caucasian populations										
This study										1.09 (0.80-1.48) 0.577
RF+	487	811	163		0.17	0.87 (0.70-1.07)	0.188			
RF-	250	422	78		0.16	0.80 (0.81-1.06)	0.105			

Table 3. *Continued.*

Zhernakova et al 2007									1.22(0.82-1.83) 0.306
RF+	664	1,143	185	0.14	0.71 (0.58-0.87)	0.0006			
RF-	112	187	37	0.17	0.87 (0.59-1.28)	0.467			
Pooled Caucasian									1.02(0.81-1.29) 0.86
RF+	1,151				0.78 (0.68-0.90)	6.9 X 10-4			
RF-	362				0.82 (0.66-1.03)	0.083			
CTLA4 rs3087243									
Caucasian populations									
This study							0.88 (0.69-1.13)	0.324	
ACPA+	322	387	257	0.40	0.80 (0.66-0.96)	0.015			
ACPA-	225	257	193	0.43	0.90 (0.73-1.11)	0.325			
Plenge et al 2005		644							
EIRA		450					0.84 (0.73-0.98)	0.03	
ACPA+	904	1,152	656	0.36	0.89 (0.78-1.02)	0.08			
ACPA-	581	694	468	0.40	1.05 (0.90-1.22)	0.51			
NARAC							0.87 (0.67-1.11)	0.26	
ACPA+	572	697	447	0.39	0.79 (0.68-0.92)	0.003			
ACPA-	161	185	137	0.43	0.91 (0.72-1.16)	0.48			
Karlson et al 2008							0.88 (0.70-1.11)	0.293	
ACPA+	436	514	358	0.41					
ACPA-	220	246	194	0.44					
Pooled Caucasian							0.86 (0.78-0.96)	0.0047	
ACPA+	2,234				0.83 (0.76-0.91)	5.4 X 10-5			
ACPA-	1,187				0.98 (0.88-1.09)	0.709			

** Values for alleles G, T, and A are the allele frequency. Data were compared using a fixed effects (Mantel-Haenszel) meta-analysis. No significant heterogeneity was observed. A significant (P < 0.05) association could be observed only for CTLA4 rs3087243 in anti-citrullinated protein antibody (ACPA)-positive patients. RF = rheumatoid factor (see Table 2 for other definitions).*

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