Candidate gene studies in rheumatoid arthritis

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Candidate gene studies in rheumatoid arthritis

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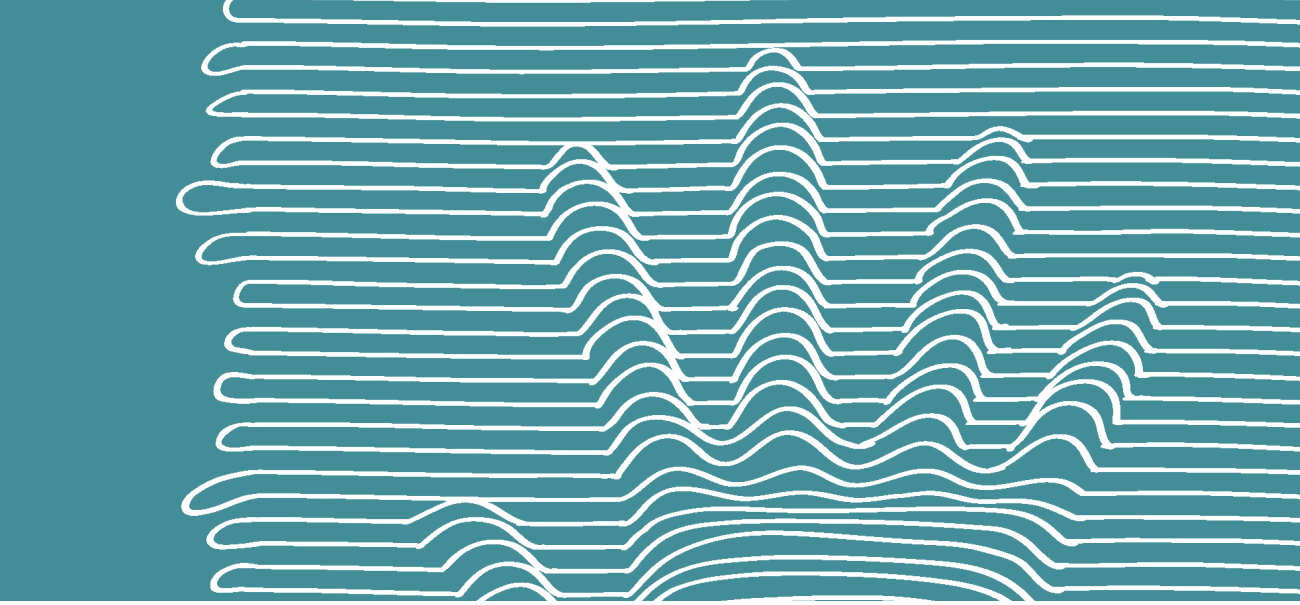
TABLE OF CONTENTS

Chapter 1	Introduction	7
Chapter 2	Confirmation of STAT4, IL2/IL21, and CTLA4 polymorphisms in rheumatoid arthritis. *Arthritis Rheum. 2009 May;60(5):1255-60.*	17
Chapter 3	Association of IL2RA and IL2RB with rheumatoid arthritis: a replication study in a Dutch population. *Ann Rheum Dis. 2009 Nov;68(11):1789-90.*	31
Chapter 4	Non-HLA genes modulate the risk of rheumatoid arthritis associated with HLA-DRB1 in a susceptible North American Native population. *Genes Immun. 2011 Oct;12(7):568-74.*	37
Chapter 5	Novel genetic association of the VTCN1 region with rheumatoid arthritis. *Ann Rheum Dis. 2012 Apr;71(4):567-71.*	53
Chapter 6	Variants of gene for microsomal prostaglandin E2 synthase show association with disease and severe inflammation in rheumatoid arthritis. *Eur J Hum Genet. 2011 Aug;19(8):908-14.*	65
Chapter 7	Genetic variants in the region of the C1q genes are associated with rheumatoid arthritis. *Clin Exp Immunol. 2013 Jul;173(1):76-83.*	81
Chapter 8	Complement activation by (auto-) antibodies. *Mol Immunol. 2011 Aug;48(14):1656-65.*	99
Chapter 9	Summary and discussion	129
Chapter 10	Nederlandse samenvatting	141
Appendix	Curriculum vitae	147
	List of publications	151
	Dankwoord	155



CHAPTER 1

Introduction

INTRODUCTION

Rheumatoid arthritis (RA) is an auto-immune disorder characterized by chronic inflammation of synovial joints. Although the precise etiology of the disease has not yet been established, it is known that both environmental and genetic factors play a pivotal role in development of the disease. In the current thesis, studies to unravel the genetic basis of RA and the putative functional consequences of these genetic variances are described.

Rheumatoid arthritis

RA is a chronic inflammatory disease characterized by persistent synovitis and destruction of synovial joints, leading to severe disability, decreased quality of life and premature mortality. In industrialized countries approximately 0.5-1.0% of the adult population is affected by the disease, with a dominance in females and elderly individuals. The disease reveals itself by joint swelling and joint tenderness, in which the small joints of the hands and feet are most commonly affected[1].

The definition of RA is phenotypic and is defined by use of classification criteria. These criteria were developed on a consensus procedure by clinical experts and resulted in the ACR 1987 criteria[2].

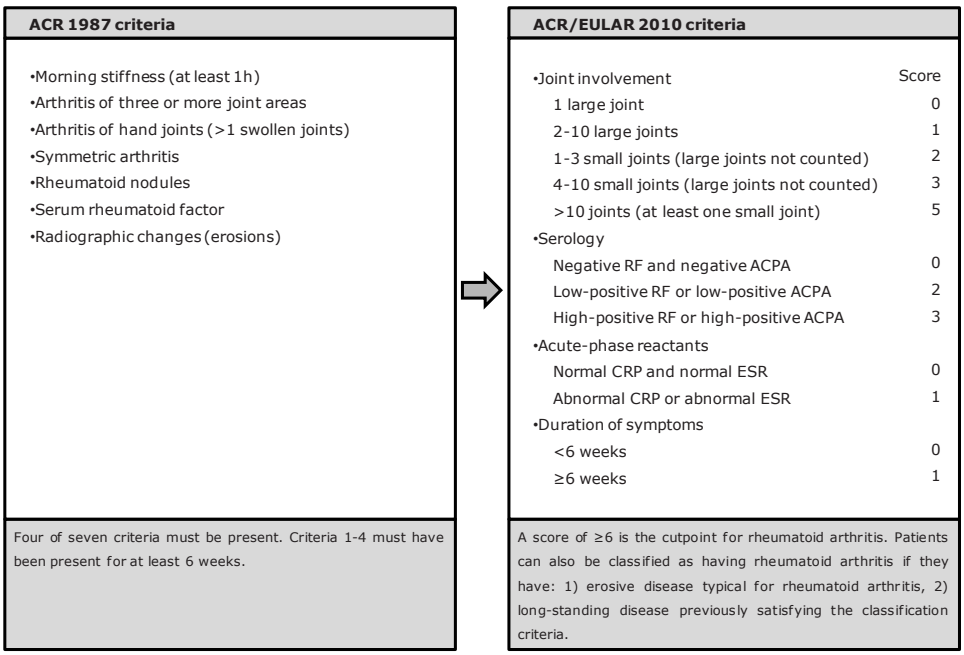


Figure 1. *ACR 1987 criteria and ACR/EULAR 2010 criteria. Classification criteria for rheumatoid arthritis. ACR = American College of Rheumatology. EULAR = European League Against Rheumatism. RF = rheumatoid factor. ACPA = Anti Citrullinated Peptide Antibodies. CRP = C-reactive protein. ESR = erythrocyte sedimentation rate.*

These criteria are well accepted for disease definition, but have proven to be of limited value in defining the earliest forms of RA. Therefore, recently, a new set of criteria has been developed (Figure 1)[3].

The mainstay of treatment in RA, are the Disease Modifying Anti Rheumatic Drugs (DMARDs), which are a heterogeneous collection of therapeutic agents of which the mechanisms of action are, largely, not well understood. When arthritis stays uncontrolled despite these agents, or when toxic effects arise upon administration of these drugs, biologic agents, such as tumor necrosis factor inhibitors can be used and have proven to be highly effective[1].

Pathophysiology

The inflamed synovial tissue is expanded by recruitment and retention of inflammatory cells, like macrophages, T- and B-cells. This leads to the formation of villous projections and the generation of pannus tissue, which is a feature seen in joints of RA patients. This pannus tissue results from proliferating synovium and is thought to mediate tissue destruction. Chronic synovitis is maintained by interaction of native and recruited inflammatory cells with subsequent establishment of cytokine networks. The combination of chronic inflammation and formation of pannus tissue will eventually lead to joint space narrowing and joint erosions[4,5].

RA is considered to have an auto-immune nature, because of the presence of autoantibodies and autoreactive T-cells in peripheral blood and synovial fluid. These autoantibodies are already present in the earliest stages of disease and can precede disease onset by several years[4].

The classic autoantibody in RA is rheumatoid factor (RF) and was first described in 1940 by Erik Waaler. This antibody is directed to the Fc portion of IgG, which is important for complement fixation and interaction with Fc receptors. The etiology of RF and its precise role in the pathogenesis of RA is still incompletely understood. RF is not unique for RA and is also present in other autoimmune diseases, infectious diseases and in healthy elderly individuals. The sensitivity varies between 60 and 70% and the specificity between 50 and 90%[6].

An additional type of autoantibody is directed against citrullinated peptides and is called Anti Citrullinated Peptide Antibody (ACPA). Citrullination is the post translational modification of protein-bound arginine into the non-standard amino acid citrulline (Figure 2). This process is mediated by an enzyme called peptidylarginine deiminase (PAD)[7,8]. The role of citrullination remains to be determined, however, it is known that the process of citrullination typically occurs in apoptotic cells. This results in a small change of molecular mass and loss of a positive charge. It has been proposed that this process prepares intracellular proteins for degradation during apoptosis[9,10].

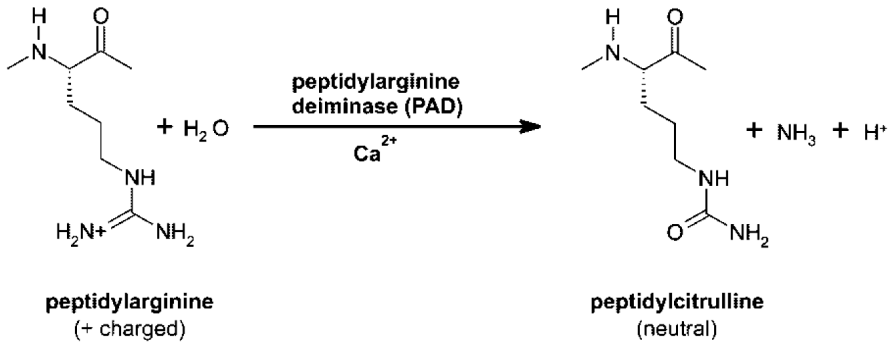


Figure 2. *Citrullination of an aminoacid. Posttranslational modification of arginine into citrulline, mediated by peptidylarginine deiminase (PAD).*

Although the presence of these antibodies has first been described in the early 1970's, it was not until the last decade that it became clear that these antibodies are highly predictive and specific for RA. The observations that these antibodies precede disease onset and that citrullinated antigens can be found in the inflamed joint, lead to the hypothesis that these antibodies play a role in the pathophysiology of RA[11,12].

Risk factors

Environmental factors

The major environmental risk factor for RA is smoking, which doubles the risk of developing the disease. This risk factor, however, is restricted to the ACPA positive disease subset[13]. Other factors that might influence the development of the disease have been proposed, but supporting evidence is weak and should be investigated more thoroughly.

Genetic factors

The first evidence for a genetic component in the susceptibility of RA came from twin and family studies. These studies showed that among siblings, the prevalence of RA was increased to 2-4% and that the concordance rate for monozygotic twins was 12-15%, as compared to 2-4% for dizygotic twins[14,15]. Additionally, the heritability of RA, which estimates the extent to which variation in liability to disease in a population can be explained by genetic variation, was calculated to be about 50%[16]. These studies demonstrate that genetic factors have a substantial impact on RA susceptibility.

The most important genetic risk factor was first described over 30 years ago and is confined to the human leukocyte antigen (HLA) locus[17]. Studies have shown that several HLA-DRB1 alleles are associated with the disease and that different alleles associate in different ethnic populations[18,19]. Interestingly, the association is confined to the ACPA positive

subset of the disease[20].

The HLA-DRB1 alleles encode the variable region of the HLA class II molecule. This variable region constitutes the binding groove for the peptide that is presented by the HLA molecule to T-cells. The product of the associated HLA-DRB1 alleles appears to share the same amino acid sequence at position 70-74 in the third hypervariable region of the HLA class II molecule. Therefore, the collection of different associated alleles are called the Shared Epitope (SE) alleles[21,22]. The SE hypothesis postulates that the SE motif is directly involved in RA pathogenesis by allowing presentation of certain (arthritogenic) peptides to T-cells[23]. To date, no specific peptide that is presented in this binding groove and subsequently activates autoreactive T-cells has been identified.

The total genetic contribution of the SE alleles to RA has been quantified in twin studies and is estimated to be 50-60% of the genetic contribution[16]. Therefore the HLA locus is thus far the most powerful genetic factor and accounts for approximately 30% of the genetic burden to RA[25].

It required almost 30 years before evidence of genetic associations outside the HLA locus were convincingly demonstrated by the discovery of *PADI4* and *PTPN22*. *PADI4* was discovered in a Japanese RA population by fine mapping of a linkage region on chromosome 1p36[26]. Interestingly, this gene encodes the PAD enzyme, which is needed for the posttranslational modification of arginine into the, by ACPAs recognized, citrulline[8]. The association has been convincingly replicated in other Asian populations, but could not be established the in Caucasian population[27,28].

The discovery of *PADI4* was followed by the identification of the genetic association with *PTPN22*. This gene was initially identified in a multi-tiered, case control study of putative functional single nucleotide polymorphisms (SNPs) in a Caucasian population from North America. The identified risk allele encodes an amino acid change, which is thought to alter the proteins normal function[29]. Well powered studies have successfully replicated the association and it is thought of as the most reproducible genetic association outside the HLA region[30]. The associated variant shows a decreasing frequency going north to south in European populations, with a minor allele frequency of approximately 15% in Scandinavia to 2.5% in the Italian population[29]. The risk allele is virtually absent in Asian populations and attempts of identifying additional *PTPN22* alleles in this population have not been successful[31]. Contrasting, in the case of *PADI4*, the risk allele is present in the Caucasian population, but has not proven to be associated with the disease.

After 2004 the identification of new genetic risk factors accelerated, due to advances in genotyping technology, available at reasonable costs. By candidate gene approach the CTLA4 gene was identified, followed by the identification of the C5/TRAF region[32,33]. This region was detected concurrently in a genome wide association study (GWAS)[34]. Linkage data led to the identification of *STAT4* as a genetic risk loci[35]. Large GWAS in ACPA positive individuals allowed the identification of new genetic risk factors to take enormous steps forward. In 2008 additional risk loci were identified by a meta-analysis of various GWAS,

of which the most significant finding is located near the CD40 gene[36]. Currently, over 100 genetic loci are considered as established genetic risk factors for RA, all with relatively weak effects with moderate ORs[37].

Aim and outline of this thesis

The aim of this thesis is to get more insight into the genetic contribution of loci located outside the HLA region to RA susceptibility and the functional role the associated variants play in disease pathology.

In recent years many genetic risk loci, located outside the HLA region, have been identified. Some of these loci represent true associations, while others will prove to be false positive findings. In **chapter 2** and **chapter 3** replication studies to identify the true genetic contribution of *STAT4, IL2/IL21, CTLA4* and *IL2RA* to RA susceptibility was investigated in the Leiden RA population. Subsequently, to establish the true genetic effect of these loci, a meta-analysis combining all previously published data was performed.

In **Chapter 4** we investigated the genetic contribution of the, then known, non HLA genes in a subset of North American Natives, that confer a higher risk to develop RA. Subsequently, we investigated whether an interaction between the genes was present and would confer a higher risk obtaining the disease.

To identify additional genetic risk factors for RA, a candidate gene study to investigate the genetic contribution of the *VTCN1* region to RA susceptibility was performed in **chapter 5.** Based on previously published genetic association data of this region with juvenile idiopathic arthritis and indications in mouse models, we hypothesized that the *VTCN1* region might play a role in the development of RA.

In **chapter 6** the genetic contribution of the PTGES gene was explored in relation to disease susceptibility and various disease outcomes, like gender, DAS28 and age of onset of RA. Gene expression was investigated in RA synovium and related to genotype.

Additionally, a candidate gene study on the genetic susceptibility of the C1Q region with RA was investigated in **chapter 7**. We hypothesized that C1Q, which plays a pivotal role in activation of the classical pathway of the complement system, is a genetic risk factor for RA susceptibility. Subsequently, gene expression profiles and C1q serum levels were investigated in relation to genotypes.

Subsequently, in **Chapter 8**, the role of the complement system to activate autoantibodies was summarized in a review that discusses the different pathways of activation.

The results of the studies performed in this thesis were summarized and discussed in **chapter 9.**

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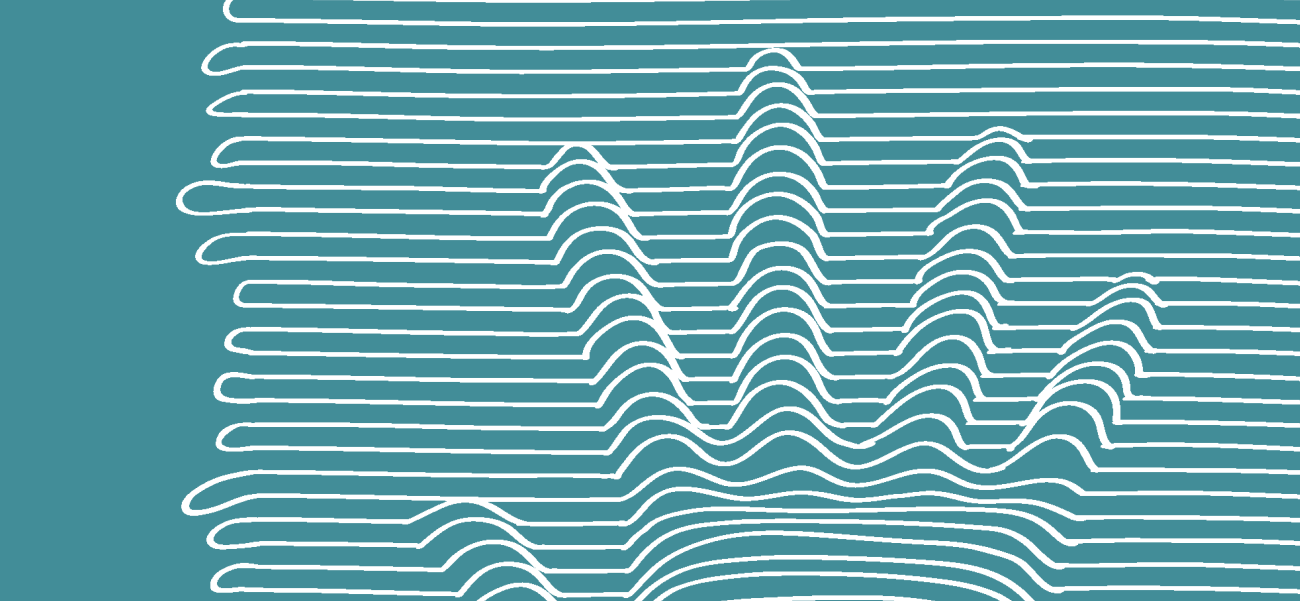

CHAPTER 2

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

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Arthritis Rheum. 2009 May;60(5):1255-60

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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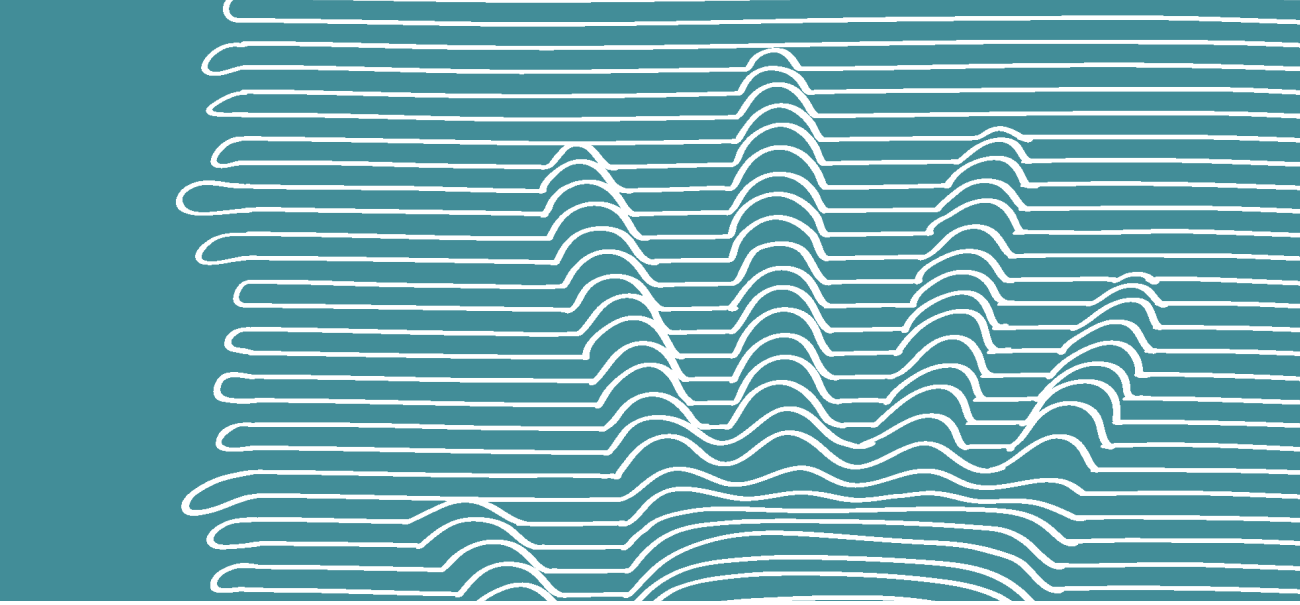

CHAPTER 3

Association of IL2RA and IL2RB with rheumatoid arthritis: a replication study in a Dutch population

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INTRODUCTION

Rheumatoid arthritis (RA) is an autoimmune disease with a worldwide prevalence of approximately 1%. The aetiology of RA is largely unknown, but it is thought that both genetic and environmental factors play a role in the pathogenesis of the disease. Genome-wide association studies (GWAS) and candidate gene approaches have led to the association of a number of genetic susceptibility loci[1-7]. The Wellcome Trust case-control consortium (WTCCC), the first GWAS in RA, identified a number of loci reaching genome-wide significance including the HLA region and the PTPN22 gene[5]. To identify new genetic risk factors, Thomson et al investigated whether tier 2 single nucleotide polymorphisms (SNPs) ($p = 1 \times 10^{-5}$-1×10^{-7}) in the WTCCC-GWAS showed an association with RA in an independent validation study of 5063 patients and 3849 healthy controls[8]. Of the nine loci investigated, a significant association was identified with rs6920220 in the TNFAIP3-OLIG3 region (odds ratio (OR) 1.23, 95% confidence interval (CI) 1.15 to 1.33, $p=1.1 \times 10^{-8}$). The association of RA with this region was independently identified by Plenge and co-workers[2], and a recent meta-analysis of GWAS data from three independent case-control populations of European descent confirmed these results[3].

To further investigate these tier 2 SNPs, the control group of the validation study was enlarged from 3849 to 11 487 individuals by including non-RA disease groups consisting of bipolar disorder, type 2 diabetes, hypertension and coronary artery disease[9]. In addition to an association with rs6920220, other statistically significant SNPs surfaced, located in MMEL1, IL2RA and IL2RB. Furthermore, investigation of 49 tier 3 loci ($p = 1 \times 10^{-4}$-1×10^{-5}) from the original WTCCC-GWAS identified significant RA-associated SNPs located in PRKCQ and KIF5A.8 Three of these regions, encoding MMEL1, KIF5A and PRKCQ, were also identified in a large independent study of RA samples of European descent[3], suggesting that these regions harbour true RA susceptibility loci.

In the present study we addressed the contribution of the two interleukin 2 (IL2) pathway SNPs - specifically, rs743777 located in IL2RB and rs2104286 in IL2RA - to the risk of RA in an independent Dutch case-control study. This is of relevance not only because additional replication would strengthen the putative contribution of IL2 receptor-positive cells to RA, but also because only a trend towards association for the IL2RA SNP was observed after correction for multiple testing using the Bonferroni method ($p<0.005$).

METHODS

A total of 616 Dutch patients and 545 healthy ethnically- and geographically-matched controls were genotyped by allele-specific kinetic PCR as previously described.[10]

RESULTS

In this study a significant association with RA was observed for both rs743777 and rs2104286 (OR 1.26, 95% CI 1.06 to 1.50, p = 0.009 and OR 0.81, 95% CI 0.67 to 0.98, p = 0.026 respectively; table 1). Combining the data from our study with the UK data strengthened the evidence for an association (rs743444: OR 1.12, 95% CI 1.06 to 1.18, p = $8.6x10^{-6}$; rs2104286: OR 0.92, 95% CI 0.87 to 0.97, p=$1.2x10^{-3}$; table 1). To bypass the phenomenon of the "winner's curse", in which effect sizes are often overestimated in the original study,13 we opted to analyse the data without the original WTCCC data to provide an estimate of the most likely effect size.

Table 1. *Analysis of rs743777 and rs2104286 with rheumatoid arthritis (RA) in two populations of northern European descent*

		Cases					Controls						HW-
	N	11	12	22	MAF	N	11	12	22	MAF	OR (95% CI)	p Value	controls
IL2RB rs743777													
This study	616	76 (0.12)	288 (0.47)	252 (0.41)	0.36	544	58 (0.11)	217 (0.40)	269 (0.49)	0.31	1.26 (1.06 to 1.50)	0.009	0.377
Validation study UK	4680	532 (0.11)	2031 (0.43)	2117 (0.45)	0.33	11200	1040 (0.09)	4832 (0.43)	5328 (0.48)	0.31	1.11 (1.05 to 1.17)	<0.001	0.501
Combined											1.12 (1.06 to 1.18)	8.6x10-6	
IL2RA rs2104286													
This study	616	36 (0.06)	226 (0.37)	354 (0.57)	0.24	545	54 (0.10)	200 (0.37)	291 (0.53)	0.29	0.81 (0.67 to 0.98)	0.026	0.100
Validation study UK	4660	312 (0.07)	1740 (0.37)	2608 (0.56)	0.24	11260	790 (0.07)	4464 (0.40)	6006 (0.53)	0.28	0.93 (0.88 to 0.98)	0.007	0.597
Combined											0.92 (0.87 to 0.97)	0.001	

Allele frequency data of RA cases versus controls was compared using a fixed effects (Mantel-Haenszel) meta-analysis. No significant heterogeneity or deviation from Hardy-Weinberg (HW) equilibrium was observed among the studies.

CONCLUSION

In conclusion, this study provides additional evidence for the association of IL2RB and IL-2RA with RA by independent replication in a Dutch population, underlining the importance of the IL2 pathway in RA. Recently, the IL2RA region was also found to be associated with other autoimmune diseases[11,12] - specifically, multiple sclerosis and type 1 diabetes - which suggests a possible common functional pathway.

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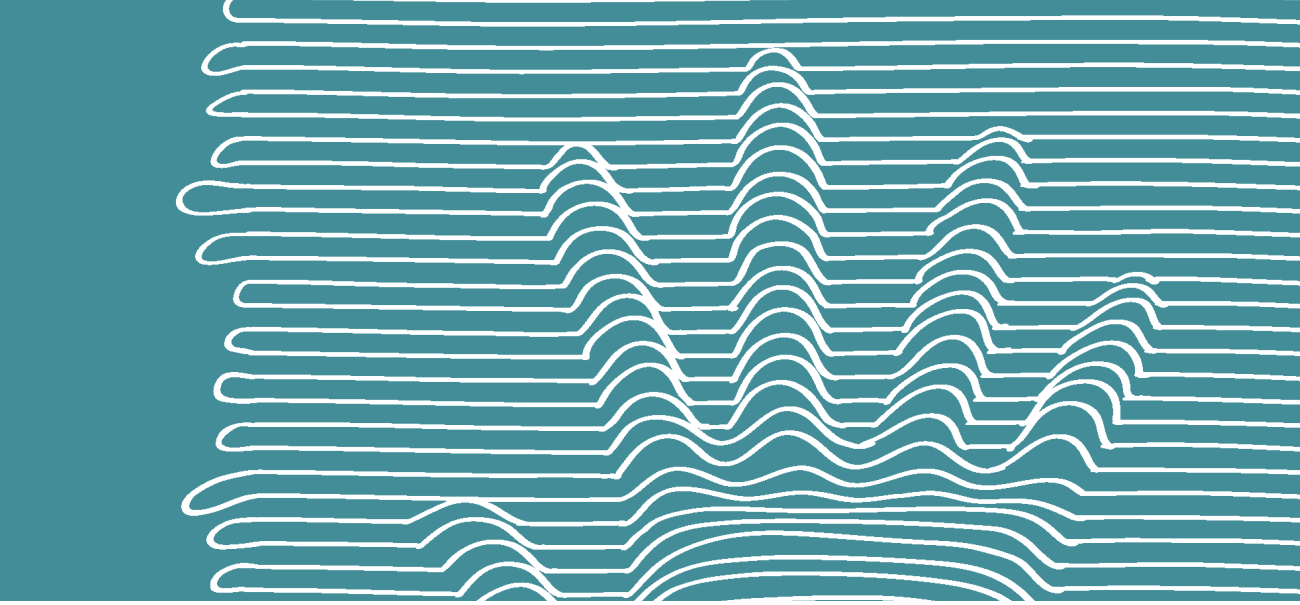

CHAPTER 4

Non-HLA genes modulate the risk of rheumatoid arthritis associated with HLA-DRB1 in a susceptible North American Native population

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ABSTRACT

Objective

Most of the genetic risk for rheumatoid arthritis (RA) is conferred by 'shared epitope' (SE), encoding alleles of HLA-DRB1. Specific North American Native (NAN) populations have RA prevalence rates of 2-5%, representing some of the highest rates estimated worldwide. As many NAN populations also demonstrate a high background frequency of SE, we sought to determine whether other genetic factors contribute to disease risk in this predisposed population.

Methods

RA patients (n = 333) and controls (n = 490) from the Cree/Ojibway NAN population in Central Canada were HLA-DRB1 typed and tested for 21 single-nucleotide polymorphisms (SNPs) that have previously been associated with RA, including PTPN22, TRAF1-C5, CTLA4, PADI4, STAT4, FCRL3, CCL21, MMEL1-TNFRSF14, CDK6, PRKCQ, KIF5A-PIP-4K2C, IL2RB, TNFAIP3, IL10-1082G/A and REL.

Results

Our findings indicate that SE is prevalent and represents a major genetic risk factor for RA in this population (82% cases versus 68% controls, odds ratio = 2.2, 95% confidence interval 1.6-3.1, $P < 0.001$). We also demonstrate that in the presence of SE, the minor allele of MMEL1-TNFRSF14 significantly reduces RA risk in a dominant manner, whereas TRAF1-C5 increases the risk.

Conclusion

These findings point to the importance of non-HLA genes in determining RA risk in a population with a high frequency of disease predisposing HLA-DRB1 alleles.

INTRODUCTION

Rheumatoid arthritis (RA) affects populations worldwide, with prevalence rates of 0.5-1% typically estimated in European and United States populations, and lower rates in African, Chinese and Japanese populations[1]. However, several North American Native (NAN) populations have substantially higher prevalence rates, estimated as high as 5.3% in the Pima of Arizona and 2.4% in the Tlingit of Alaska[2-4]. The shared epitope (SE) hypothesis postulates that RA predisposing alleles of the HLA-DRB1 locus encode for a positively charged QK(R)RAA sequence in position 70-74 in the third hypervariable region of the molecule[5]. In most populations, the SE sequence is found in HLA-DRB1*04 alleles. In NAN populations, SE-bearing alleles are primarily HLA-DRB1*1402 and *0404[6-10]. High background population frequencies of the SE may, in part, explain the higher prevalence rates of RA in NAN populations, compared with other populations. For example, frequencies of the SE vary from 28 to 38% in United States and United Kingdom Caucasians, but frequencies as high as 60 to 95% have been reported in several NAN populations, where they have been studied[8,11].

In European populations, the contribution of HLA to genetic risk of RA has been estimated at 37%[12]. The availability of the human genome sequence, high through-put single-nucleotide polymorphisms (SNP) technologies and large cohorts of RA patients has allowed the identification of associations with 20-30 new non-HLA gene regions, using both candidate gene and genome wide association scans (GWAS). These have included PTPN22, CTLA4, TRAF1-C5, STAT4, TNFAIP3, CD40, MMEL1-TNFRSF14 and REL[13,14]. Interestingly, these studies have suggested ethnic differences in the patterns of genetic susceptibility, necessitating confirmation of associations in different populations[15-17]. Examples are a PTPN22-R620W polymorphism which is strongly associated with RA and other autoimmune diseases in Caucasian populations, but is absent in Orientals, and an association with PADI4 SNP found in Japanese populations which is not detected in most Caucasian populations, despite the presence of these gene polymorphisms[15-20]. Our own studies of the Cree/Ojibway population in Central Canada have suggested a 2-3% prevalence rate of diagnosed RA, and a 60-70% frequency of SE alleles in the background population[7,8,21]. We sought to determine additional non-HLA genes that may contribute to the high frequency of RA detected in this population. We, therefore, genotyped NAN RA patients and controls for a spectrum of SNPs that were shown to be disease associated, using candidate gene and GWAS approaches in other populations. Our results indicate that several of these SNPs may contribute to disease risk in this population, with an SNP in the MMEL1-TNFRSF14 region having a particularly strong protective effect in SE positive individuals.

PATIENTS AND METHODS

Study subjects

We recruited RA patients and controls of Cree, Ojibway or Ojicree ethnic background. To be included in the study, all patients and controls had at least three of four grandparents of this ethnic background by self-report. A total of 338 RA patients were recruited from rheumatology clinics in urban areas of Central Canada, Winnipeg and Saskatoon, as well as rural areas, Norway House and St Theresa Point, Manitoba. A total of 516 controls from the same geographic areas were recruited by one of three methods: an accompanying spouse of an RA proband, a participant in a Health Fair, or through random sampling of the study communities. The latter approach accounted for most of the controls recruited from the rural locations. All controls had no history of RA or other autoimmune diseases.

The study protocol was approved by the Research Ethics Board of the University of Manitoba and by the Tribal Band Councils of the study communities. All study subjects gave informed consent after having the study details explained in their language of choice.

HLA-DRB1 testing

HLA-DRB1 testing was performed by polymerase chain reaction, using sequence-specific oligonucleotide primers and sequence-based typing as previously described[8]. The following DRB1 alleles were included as SE-bearing alleles: DRB1*0101, 0102, 0401, 0404, 0405, 0408, 0410, 1001 and 1402. Other SE-bearing alleles were not found in this population.

SNP testing in non-HLA genes

The complete list of SNP evaluated in this study is shown in Tables 2 and 3. These were selected on the basis of previously published genome wide association data, indicating SNP association with RA[13,14]. Genotyping was undertaken in the laboratory of Dr Katherine Siminovitch at the University of Toronto, and the multiplexed SNP assays were performed on the Sequenom Mass Array iPLEX platform (Sequenom, Inc., San Diego, CA, USA). Allele-specific extension products were plated onto a SpectroCHIP (Sequenom, Inc.) subjected to mass spectrometric analysis, and the genotypes were identified using SpectroCALLER software (Sequenom, Inc.).

Data analysis

The genotyping data for all individuals tested were subjected to quality control and individuals with genotype call rates less than 95% were removed. For each marker, genotype data quality was verified by testing the Hardy-Weinberg equilibrium in the control samples. The SNPs included in the study met the minimum quality control standards, with call rate greater than 95%, MAFs more than 1% and deviation from the Hardy-Weinberg equilibrium at $P > 0.0001$.

The SNP associations with RA were tested using the Plink software version 1.06 (http://pngu.mgh.harvard. edu/~purcell/plink/summary.shtml) and SAS version 9.1.3 (SAS Institute Inc., Cary, NC, USA). As all the SNP associations with RA have been previously made in at least one population, no correction for multiple comparisons was made.

In total, there were 854 NAN individuals (338 RA cases and 516 controls). In all, 31 individuals were removed for low genotyping, and after quality control, 333 cases and 490 controls were retained for analysis. The statistical power was evaluated by the software 'The CaTS Power Calculator' (Center for Statistical Genetics, University of Michigan, Ann Arbor, MI, USA) for this study, with a total of 823 subjects consisting 333 cases and 490 controls. We assumed the estimated disease prevalence of 0.025, disease allele frequency 0.19, genotypic relative risk 1.5 and Type-1 error alpha 0.05.

Association analyses were performed using allelic, genotypic, minor allele dominant (major allele recessive) and minor allele recessive models. In analyzing the SE positive subjects 631 individuals (277 RA cases and 354 controls) were included. In all, 28 individuals were removed for low genotyping, and after quality control, 273 cases and 330 controls were retained. As with the entire population, association analyses were undertaken with all genetic models for this data set. Interaction between SE and MMEL1-TNFRSF14 was further tested using a multiplicative interaction model (risk factor 1 negative + risk factor 2 negative versus risk factor 1 positive + risk factor 2 positive), as previously described[34]. A logistic regression model was also tested, in which an interaction term between SE and the AA genotype of MMEL1-TNFRSF14 was added.35 The power to detect the association is 89% using an additive model and is 75% using a dominant model.

RESULTS

The demographic and clinical features of the patient and control populations are shown in Table 1. As is typical of other RA populations, this was a predominantly female population. Although there was also a female predominance in the control population, the frequency was significantly lower than in the RA patients (82 versus 63%, P<0.01). As we have previously shown in this population, the available RA autoantibody data indicated that, approximately 90% of this cohort was rheumatoid factor (RF) and anti-citrullinated protein antibody (ACPA) positive[22]. The overall prevalence of one or two copies of SE (SE/x) was 82 and 68% for RA patients and controls, respectively (P<0.01). The number of patients and controls with two copies of SE (SE/SE) was 32 and 23%, respectively. The two most common SE alleles were HLA-DRB1*0404 (43 and 32% in RA cases and controls, respectively) and *1402 (31 and 29% in RA cases and controls, respectively).

Table 1. *Clinical and immunogenetic characteristics of the study populations*

	RA (n = 333) %	Controls (n = 490) %	P-value
Females	82	63	<0.01
RF positive	88 (223/255)	-	-
Anti-CCP positive	91 (74/81)	-	-
SE/x genotype	82	68	< 0.01
SE/SE genotype	32	23	< 0.01

Abbreviations: Anti-CCP, anti-cyclical citrullinated protein; RA, rheumatoid arthritis; RF, rheumatoid factor; SE, shared epitope.

We sought to determine non-HLA genes that may contribute to the development of RA in the NAN population. We tested SNPs that have been previously shown to be associated with RA; using candidate gene and GWAS approaches, these studies having been performed primarily in Caucasian populations. Table 2 compares the minor allele frequencies (MAFs) detected in the NAN control population to that reported in the GWAS.23 The genotype distribution and MAF for the SNPs detected in the NAN RA cases and controls is shown in Table 3. The data in Table 3 indicate that genotypic distribution for MMEL1-TNFRSF14, FCRL3 and CCL21 differed significantly between controls and RA patients. The MAF for MMEL1-TNFRSF14 tended to be lower in RA patients (odds ratio (OR) 0.82, 95% confidence interval (CI) 0.7-1.0, P = 0.08) while being higher for TRAF1-C5 (OR 1.23, 95% CI 1.0-1.5, P = 0.06).

Table 2. *Comparison of the MAF for SNPs detected with >5% frequency in the NAN control population with the frequency previously published in GWAS of Caucasian populations.*

Gene	SNP	Population	Minor	Major	MAF	OR	P-value
MMEL1-TNFRSF14*	rs3890745	NAN	G	A	0.31	0.82	0.079
		Caucasian	C	T	0.32	0.89	3.6E—6
REL	rs13031237	NAN	T	G	0.23	0.91	0.393
		Caucasian[b]	T	G	0.37	1.13	7.9E—7
STAT4	rs7574865	NAN	T	G	0.29	1.01	0.981
		Caucasian[b]	T	G	0.22	1.16	2.9E—7
CTLA4	rs3087243	NAN	A	G	0.37	1.06	0.601
		Caucasian[b]	A	G	0.44	0.87	1.2E—8
TNFAIP4	rs10499194	NAN	T	C	0.12	1.22	0.199
		Caucasian[b]	T	C	0.27	1.91	7.4E—4
CCL21	rs2812378	NAN	C	T	0.38	0.92	0.994
		Caucasian[b]	G	A	0.34	1.10	1.0E—4
TRAF1-C5	rs3761847	NAN	G	A	0.29	1.23	0.062
		Caucasian[b]	G	A	0.43	1.13	2.1E—7
KIF5A-PIP4K2C	rs1678542	NAN	G	C	0.30	0.94	0.547
		Caucasian[b]	G	C	0.38	0.91	2.0E—4

Abbreviations: GWAS, genome wide association scans; MAF, minor allele frequencies; NAN, North American Natives; OR, odds ratio; SNP, single-nucleotide polymorphisms. [a]Bases used for genotyping the minor allele of rs3890745 are the complementary bases to those reported in GWAS, but the data indicate a comparable MAF in both populations.[b]Caucasian MAF data based on data published in Stahl et al.[23]

Table 3. *Genotype distribution and MAF for the panel of non-HLA SNP tested in the entire population of NAN RA patients (n = 333) and ontrols (n = 490)*

Gene	SNP	Minor	Major	Genotype distribution			MAF				
				Controls	Cases	P-value	Controls	Cases	OR	95% CI	P-value
MMEL1-TNFRSF14	rs3890745	G	A	43/232/215	33/123/177	0.012	0.32	0.28	0.82	0.7-1.0	0.079
PADI4	rs2240340	G	A	114/255/121	84/159/90	0.481	0.49	0.49	0.99	0.8-1.2	0.941
PTPN22	rs6679677	A	C	1/34/455	1/29/303	NA	0.04	0.05	1.28	0.8-2.2	0.323
PTPN22	rs2476601	A	G	1/32/457	1/27/305	NA	0.03	0.04	1.27	0.7-2.2	0.358
FCRL3	rs11264799	T	C	23/182/285	28/101/203	0.025	0.23	0.24	1.02	0.8-1.3	0.859
FCRL3	rs7528684	G	A	64/222/204	56/123/154	0.045	0.36	0.35	0.98	0.8-1.2	0.858
IL10-1082G/A	rs1800896	G	A	15/149/326	14/104/215	0.639	0.18	0.20	1.11	0.9-1.4	0.429
REL	rs702873	A	G	96/252/142	70/158/104	0.558	0.45	0.45	0.98	0.8-1.2	0.865
REL	rs13031237	T	G	21/186/283	16/111/206	0.395	0.23	0.21	0.91	0.7-1.2	0.393
STAT4	rs3024921	A	T	0/19/470	0/11/322	NA	0.02	0.02	0.85	0.4-1.9	0.665
STAT4	rs7574865	G	T	55/176/259	31/132/170	0.460	0.29	0.29	1.01	0.8-1.3	0.981
CTLA4	rs3087243	A	G	66/226/198	45/161/126	0.763	0.37	0.38	1.06	0.7-1.3	0.601
CTLA4	rs6748358	A	C	78/231/180	51/157/125	0.961	0.40	0.39	0.97	0.8-1.2	0.781
TNFAIP4	rs10499194	T	C	11/84/394	8/70/255	0.371	0.11	0.13	1.22	0.9-1.7	0.199
TNFAIP4	rs6920220	A	G	1/40/449	1/30/302	NA	0.04	0.05	1.13	0.7-1.9	0.618
CDK6	rs42041	G	C	3/56/431	8/41/284	NA	0.06	0.09	1.39	0.9-2.1	0.086
CCL21	rs2812378	C	T	82/207/201	41/170/122	0.031	0.38	0.38	0.92	0.7-1.1	0.994
TRAF1-C5	rs3761847	G	A	40/193/257	36/142/155	0.183	0.28	0.32	1.23	1.0—1.5	0.062
TRAF1-C5	rs10818488	A	G	40/193/257	36/142/155	0.183	0.28	0.32	1.23	1.0—1.5	0.062
PRKCQ	rs4750316	C	G	1/29/460	3/28/302	NA	0.03	0.05	1.65	1.0-2.8	0.047
KIF5A-PIP4K2C	rs1678542	G	C	51/189/249	26/137/170	0.414	0.30	0.28	0.94	0.8-1.2	0.547
IL2RB	rs3218253	T	C	2/28/460	0/33/300	NA	0.03	0.05	1.54	0.9-2.6	0.084

Abbreviations: CI, confidence interval; HLA, human leukocyte antigen; MAF, minor allele frequency; NAN, North American Natives; OR, odds ratio; RA, rheumatoid arthritis; SNP, single-nucleotide polymorphism. P-value for genotype distribution was tested using w2-test, df = 2.

We explored models other than the genotypic and allelic models that may potentially better explain the association between individual SNPs and RA, in particular minor allele dominant and minor allele recessive models. These models examine associations on the basis of the homozygous and heterozygous states for each SNP, as gene dosage may impact on risk. Figure 1 compares the OR detected for each SNP using an allelic model (Figure 1a) and a minor allele dominant model (Figure 1b). The latter model compares the heterozygous + minor allele homozygous states to the homozygous major allele state (Aa + aa versus AA). This analysis further demonstrates the significant protective effect of the MMEL1-TNFRSF14 in minor allele dominant manner.

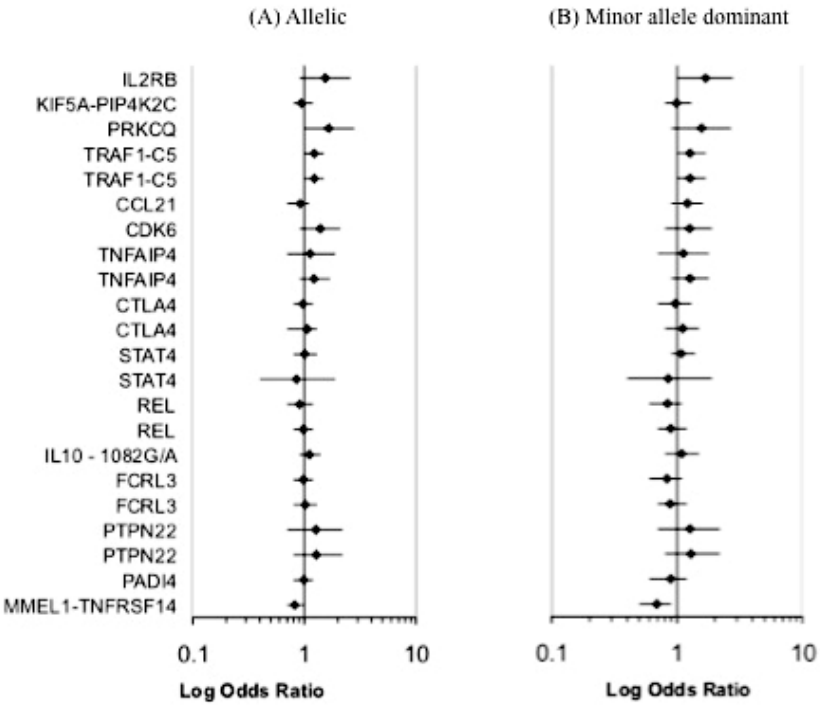

Figure 1. *Log odds ratio for association between a panel of non-HLA SNPs and RA in a NAN population. (a) Represents an allelic model; (b) represents a minor allele dominant model (AA versus Aa + aa). MMEL1-TNFRSF14 is significantly protective in the minor allele dominant model.*

Because of the high frequency of SE alleles in this population, we analyzed the panel of SNPs in the SE positive cases and controls. These data are shown in Table 4. On the basis of this analysis, we tested MMEL1-TNFRSF14 and TRAF1-C5 in minor allele dominant and recessive models. These data are shown in Table 5 and demonstrate the strength of the RA association in the minor allele dominant model, particularly when comparing only SE positive cases and controls. In the case of TRAF1-C5 minor allele, the association was positive, whereas the association with the MMEL1-TNFRSF14 minor allele was in the opposite direction.

Table 4. *Genotype distribution and MAF for the panel of non-HLA SNP, tested in the SE-positive population of NAN RA patients (n = 273) and controls (n = 330)*

Gene	SNP	Minor	Major	Genotype distribution			MAF				
				Controls	Cases	P	Controls	Cases	OR	95% CI	P-value
MMEL1-TNFRSF14	rs3890745	G	A	25/163/142	29/92/152	0.001	0.32	0.27	0.79	0.6-1.0	0.070
PADI4	rs2240340	G	A	71/178/81	70/125 /78	0.137	0.48	0.49	1.01	0.8-1.3	0.986
PTPN22	rs6679677	A	C	1/24/305	1/23/249	NA	0.04	0.05	1.17	0.6-2.1	0.583
PTPN22	rs2476601	A	G	1/24/305	1/22/250	NA	0.04	0.04	1.17	0.6-2.1	0.692
FCRL3	rs11264799	T	C	15/122/193	24/82/166	0.040	0.23	0.24	1.05	0.8-1.4	0.724
FCRL3	rs7528684	G	A	45/145/140	46/94/133	0.057	0.36	0.34	0.93	0.7-1.2	0.577
IL10—1082G/A	rs1800896	G	A	10/102/218	12/85/176	0.662	0.18	0.20	1.11	0.8-1.5	0.516
REL	rs702873	A	G	69/172/89	58/125/89	0.243	0.47	0.44	0.91	0.7-1.1	0.355
REL	rs13031237	T	G	17/118/195	12/89/172	0.611	0.23	0.21	0.85	0.6-1.1	0.330
STAT4	rs3024921	A	T	0/9/320	0/6/267	NA	0.01	0.01	0.81	0.3-2.5	0.675
STAT4	rs7574865	G	T	37/119/174	26/96/151	0.731	0.29	0.27	0.91	0.7-1.2	0.412
CTLA4	rs3087243	A	G	44/151/135	36/135/101	0.602	0.36	0.38	1.08	0.9-1.4	0.511
CTLA4	rs6748358	A	C	57/149/123	41/128/104	0.744	0.40	0.38	0.92	0.7-1.2	0.594
TNFAIP4	rs10499194	T	C	6/51/272	8/60/205	0.072	0.10	0.14	1.53	1.1-2.2	0.019
TNFAIP4	rs6920220	A	G	1/24/305	1/22/250	NA	0.04	0.04	1.12	0.6-2.1	0.692
CDK6	rs42041	G	C	0/33/297	3/34/236	NA	0.05	0.07	1.51	0.9-2.5	0.092
CCL21	rs2812378	C	T	57/141/132	35/143/95	0.051	0.39	0.39	1.02	0.8-1.3	0.894
TRAF1-C5	rs3761847	G	A	21/125/184	31/120/122	0.010	0.25	0.33	1.48	1.1-1.9	0.002
TRAF1-C5	rs10818488	A	G	21/125/184	31/120/122	0.010	0.25	0.33	1.48	1.1-1.9	0.002
PRKCQ	rs4750316	C	G	1/17/312	3/26/244	NA	0.03	0.06	2.11	1.1-3.9	0.010
KIF5A-PIP4K2C	rs1678542	G	C	36/127/166	21/113/139	0.375	0.30	0.28	0.91	0.7-1.2	0.482
IL2RB	rs3218253	T	C	1/17/312	0/25/248	NA	0.03	0.05	1.62	0.9-3.1	0.117

Abbreviations: CI, confidence interval; HLA, human leukocyte antigen; MAF, minor allele frequency; NAN, North American Natives; OR, odds ratio; RA, rheumatoid arthritis; SE, sharewd epitope; SNP, single-nucleotide polymorphism. P-value for genotype distribution was tested using w2-test, df = 2.

Table 5. *OR for RA association with MMEL1-TNFRSF14 and TRAF-C5, using a minor allele dominant (top panel) and minor allele recessive model (bottom panel), in the entire population of cases and controls (left panels) and SE-positive cases and controls (right panels)*

	All controls and cases			SE positive controls and cases		
	OR	95% CI	P-value	OR	95% CI	P-value
Minor allele dominant model (Aa+aa vs AA)						
MMEL1-TNFRSF14	0.69	0.5-0.9	0.009	0.61	0.4-0.8	0.002
TRAF1-C5	1.27	1.0-1.7	0.096	1.56	1.1-2.2	0.007
Minor allele recessive model (aa vs Aa+AA)						
MMEL1-TNFRSF14	1.14	0.7-1.8	0.620	1.45	0.8-2.5	0.200
TRAF1-C5	1.36	0.9-2.2	0.220	1.89	1.1-3.3	0.040

Abbreviations: CI, confidence interval; OR, odds ratio; RA, rheumatoid arthritis; SE, shared epitope.

Finally, we determined whether there was evidence of multiplicative interaction between SE and MMEL1-TNFRSF14. These data are shown in Table 6. As the minor allele is protective in a dominant manner, we tested the interaction between SE and the homozygous major allele state of the MMEL1-TNFRSF14 SNP (AA) as risk factors. The data indicate that, compared with the absence of both risk factors (OR = 1), having both SE and AA is associated with a substantial increase in risk, compared with having only one of these risk factors (OR 2.6, 95% CI 1.7-4.1, P< 0.0001). This interaction was further confirmed using a logistic regression model, where an interaction term SE*AA was added to a model that included both risk factors and gender. In this model, the interaction term was significantly associated with RA (OR 2.2, 95% CI 1.1-4.6, P = 0.03). An analysis of TRAF1-C5 as a risk factor showed a similar interaction with SE (OR 2.3, 95% CI 1.2-2.3, P<0.001).

Table 6. *Evidence for interaction between the homozygous major allele (AA) genotype of MMEL1-TNFRSF14 and SE*

MMEL1-TNFRSF14 AA genotype	SE allele	Cases/ controls	OR (95% CI)	P-value
0	0	35/87	1.0	
0	1	123/203	1.5 (1.0-2.4)	0.06
1	0	26/73	0.9 (0.5-1.6)	0.60
1	1	154/151	2.6 (1.7-4.1)	<0.0001

Abbreviations: CI, confidence interval; OR, odds ratio; SE, shared epitope. The reference genotype with both risk factors absent is shown in the first row. The presence of the individual risk factors alone and in combination is shown in the lower rows.

DISCUSSION

NAN populations have been shown to have a high prevalence of RA, compared with most other populations worldwide[4]. Moreover, as is the case with a number of the NAN populations that have been studied, we have shown that the Cree/Ojibway population in Central Canada has a high prevalence of SE alleles, specifically *0404 and *1402.8 Because of the high prevalence of SE in the population, we looked for SNP associations in non-HLA regions that may further predispose NAN individuals to RA, or alternatively, protect them from the risk associated with SE. In the current study, we show that several non-HLA SNP were marginally associated with RA risk in the NAN population, but that SNPs in the TRAF1-C5 and MMEL1-TNFRSF14 regions interacted significantly with SE, albeit in opposite directions with the former increasing risk and the latter being protective in the presence of the HLA-DRB1 risk.

In comparing the SNP allele frequencies in the NAN population with those reported in previous GWAS and candidate gene studies, some differences in SNP MAFs were apparent (Table 2). One potential confounder in such a comparison is that of ethnic admixture in the NAN population. However, to minimize admixture effects, inclusion in this study required each individual to have at least three of four grandparents of NAN background. Although this strategy does not eliminate the possibility of Caucasian (or other ethnic) admixture, our previously reported HLA data suggest that the frequency of the Caucasian HLA-A1 allele in this NAN population is <5%.7

The association with MMEL1-TNFRSF14 was initially demonstrated in a meta-analysis of three GWAS of approximately 4000 European patients and 12 000 European controls, whereas it had not been detected in any of the smaller individual GWAS[14]. In that study, the minor allele was also shown to have a protective effect on disease development (OR = 0.86), which is similar to what we detect in the current study. The modeling we performed suggested that the homozygous state for the major allele conferred the highest risk, and that the presence of the minor allele was indeed protective in a dominant manner. This finding is particularly relevant to a population, where most individuals carry one or more SE alleles, the primary risk factor for RA susceptibility. In such a population, it is likely that other genes have an important modulating effect on the HLA-DRB1 associated risk.

Recently, a study of Spanish RA patients and controls demonstrated an epistatic interaction between MMEL1-TNFRSF14 (as identified by rs6684865) and another TNFR superfamily member, TNFRSF6B (as identified by rs4809330), which increased the risk of RA[24]. TNFRSF6B alone did not affect RA risk. Moreover, although the genotypic distribution of MMEL1-TNFRSF14 was similar to that of NAN controls in our study, the association with MMEL1-TNFRSF14 alone was marginally demonstrable only in the replication study and not in the primary study, even after stratification for SE. The reasons for these differences remain unclear, but may relate to the distribution of the SE alleles in the Spanish population. The causal gene in the MMEL1-TNFRSF14 association has not been identified, as the SNP studied is not located in a genomic region where they would have a clear impact

on gene function. It is appealing to speculate that TNFRSF14 is indeed the RA associated gene as it is a key homeostatic molecule involved in T-cell activation[25]. Also known as herpes virus entry mediator for its role as a herpes virus receptor, TNFRSF14 is expressed on the surface of T cells and synovial fibroblasts and activates the canonical nuclear factor-kB (NF-KB) pathway upon ligation of LIGHT, a TNF super family member[26-28].

TRAF1-C5 was previously reported to be associated with RA in primarily Caucasian populations[29]. The OR for association with the minor allele of the rs3761847 SNP was reported at 1.32, which is comparable to an OR of 1.23 we report in the NAN population, using the same SNP. We further demonstrate that the OR is increased to 1.48 when analyzing SE positive cases and controls, and as with MMEL1-TNFRSF14, there is an indication of a significant interaction with SE. In both cases, the minor allele appeared to act in a dominant manner, albeit in opposite directions. This conclusion has to be interpreted cautiously however, in view of the challenges inherent to analysis of epistasis between gene loci in complex disorders such as RA[30].

TRAF1 and TNFRSF14 are both involved in the activation of NF-KB, a central pathway in the pathogenesis of RA. Indeed, many of the non-HLA SNPs that have been associated with RA are centered around the NF-KB pathway, and include TNFAIP3, CD40 and REL in addition to TRAF1 and TNFRSF14. The NF-KB pathway is activated by TNFa and IL-1P, two key cytokines in RA pathogenesis, and in turn, regulates the transcription of a gene signature that promotes both the chronic inflammatory response and the matrix degrading mechanisms that are a characteristic feature of the disease. SE encoding HLA-DRB1 alleles have been hypothesized to predispose to RA by efficiently presenting citrullinated antigens to T cells[31], and in turn, generating immune responses that target endogenous citrullinated antigens such as cit-vimentin, cit-fibrinogen and cit-enolase[32]. It is thus possible that in the context of such facilitated antigen presentation by SE encoding alleles, the subsequent downstream activation of immune effector cells such as T cells and macrophages is modulated by polymorphic variants of molecules in the NF-KB pathway, such as TRAF1 and TNFRSF14. To date, the functional consequences of these polymorphisms have not been demonstrated, nor is it clear that they are indeed the disease causal genes.

In addition to MMEL1-TNFRSF14 and TRAF1-C5, a number of other marginal associations were detected that perhaps would be more significant if the study had greater power. Perhaps, most surprising was the absence of a clear association with the R620W allele of PTPN22, this having been shown to predispose to RA in multiple populations. We found a frequency of 3% in controls for the R620W genotypes in the NAN population, as identified by rs2476601, and an almost identical genotypic distribution for a second SNP found in the PTPN22 gene, rs6679677. Although this frequency is somewhat lower than the 12-15% reported in Caucasian populations, it contrasts with that in several present-day East Asian populations in which this polymorphism is absent[15-17]. We were also unable to confirm a previously reported protective effect of an SNP in the IL10 promoter[33].

CONCLUSION

In summary, we have evaluated genomic RA risk in a NAN population with a high prevalence of RA. A spectrum of SNPs previously identified using GWAS and candidate gene approaches were tested. The data indicate that, in this population where RA-associated SE alleles are prevalent, there is a modulatory effect on disease risk from non-HLA genes, particularly MMEL1-TNFRSF14 and TRAF1-C5.

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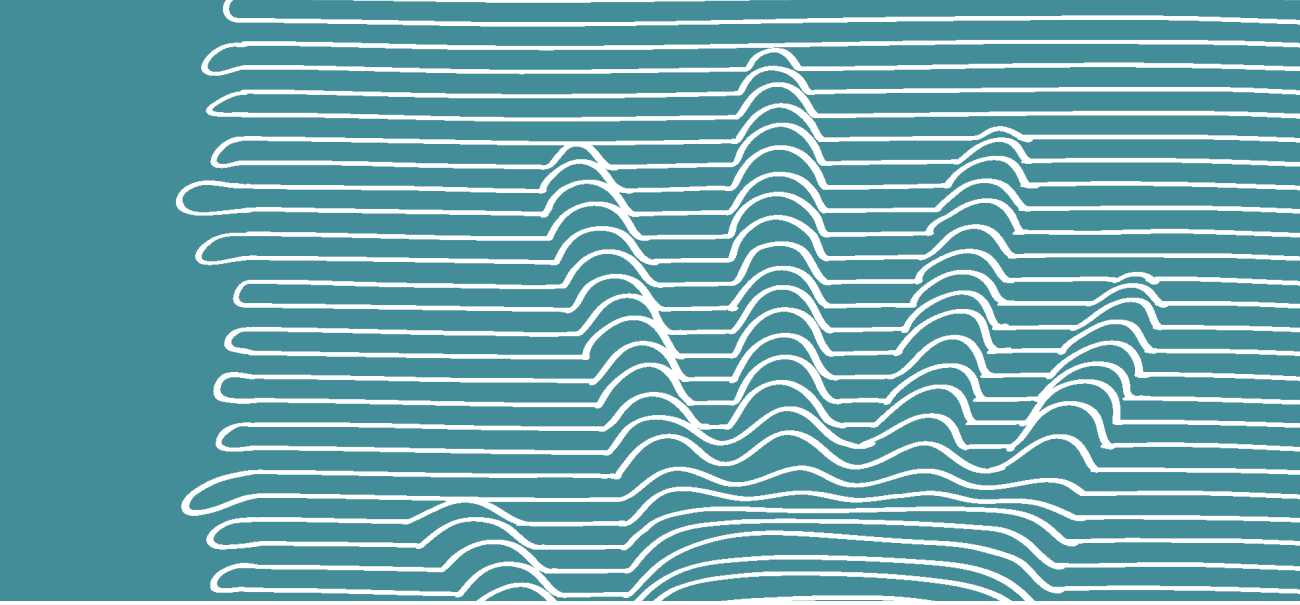

CHAPTER 5

Novel genetic association of the VTCN1 region with rheumatoid arthritis

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ABSTRACT

Objective

Based upon findings in juvenile idiopathic arthritis, the genetic contribution of the VTCN1 region to rheumatoid arthritis (RA) susceptibility and anticitrullinated protein antibody (ACPA) status was investigated. VTCN1 is known to play a pivotal role in regulation of the immune system and, in soluble form, has previously been associated with higher disease activity.

Methods

Ten VTCN1 polymorphisms were genotyped in 1237 Dutch patients with RA and 1055 healthy controls. Significant findings were replicated in two independent RA populations of northern European descent consisting of 2826 patients and 2122 healthy controls. Allele distribution was analysed using a χ^2 test and combined analysis of all studies was performed using the Mantel-Haenszel fixed effects method.

Results

A significant association with two polymorphisms was observed in the Dutch RA population. Replication of these findings showed an overall significant association with rs4376721 and rs10923217 (OR 1.13, 95% CI 1.03 to 1.24, p = 0.013 and OR 0.78, 95% CI 0.67 to 0.91, p = 0.0011, respectively). Stratification for ACPA status revealed an association in the ACPA-negative subset for rs4376721 (OR 1.19, 95% CI 1.05 to 1.35, p = 0.0071), while no overall significance could be observed in the ACPA-positive population. rs10923217 was associated with both subsets of the disease.

Conclusion

These results indicate a novel genetic association with the VTCN1 region in RA susceptibility.

INTRODUCTION

Although the aetiology of rheumatoid arthritis (RA) is largely unknown, it is thought that both environmental and genetic factors have a role in the development of the disease[1]. To date, over 30 genetic regions have been identified to associate with RA susceptibility. Most of these loci have been identified by genome-wide association studies (GWAS) performed in autoantibody-positive patient populations[2]. It is known, however, that RA is a heterogeneous disease and data indicate that distinct risk factors play a role in the anticitrullinated protein antibody (ACPA) subsets[3]. Further investigation in these subsets is needed to elucidate the genetic backgrounds.

Although many genes for complex diseases such as RA have been identified, it is thought that only part of the genetic susceptibility to these diseases can be explained by these loci. Part of this 'missing heritability' is speculated to be due to rare genetic variations which can be identified by resequencing genomes of patients[4]. Additionally, it is well recognised that GWAS are probably underpowered to detect all common disease variants and further investigation of these variants might lead to novel genetic associations[5].

As more autoimmune susceptibility loci are being identified, it has become clear that part of these genes overlap between autoimmune diseases. Recently, a GWAS in juvenile idiopathic arthritis (JIA) identified the VTCN1 region to be associated with JIA and observed this region as the strongest signal next to human leucocyte antigen[6].

VTCN1 encodes B7-H4 which has been reported as a negative regulator of T cell responses in vitro by inhibiting proliferation and cytokine production[7,8]. However, in B7-H4 deficient mice, no strong phenotype of hyperactivation of T and B cells was observed[9]. It has been shown that the protein is expressed in tumour-associated suppressive macrophages[8]. Recently, it was found that high levels of the soluble form of B7-H4 are seen more frequently in patients with RA than in healthy controls and are associated with higher disease activity as measured by the 28-joint disease activity score[10]. Likewise, in B7-H4 knockout mice the progression of inflammation in a collagen-induced arthritis model was accelerated compared with wild-type mice, which suggests a relevant role for B7-H4 in arthritis[10].

The aim of the present study was to identify the genetic contribution of the VTCN1 region to RA susceptibility and the clinically well-defined subsets, characterised by ACPA status.

METHODS

Patients

A total of 1257 patients with RA, all of whom met the American College of Rheumatology (ACR) 1987 criteria, were recruited from hospitals in the western part of The Netherlands. ACPA status was obtained for 940 patients. The characteristics of the patients have been described previously[11]. The control group consisted of 1060 healthy subjects randomly selected by the Immunogenetics and Transplantation Immunology section of the Leiden

University Medical Center.

For validation purposes, two independent western European cohorts were included. The first cohort consisted of 953 Norwegian patients with RA and 1121 healthy Norwegian controls. All patients fulfilled the ACR criteria and originated from two cohorts: the Oslo RA register (ORAR) and the Norwegian arm of the European Research on Incapacitating Disease and Social support. Follow-up has been described previously[12,13]. ACPA status was available for 893 patients. The second cohort consisted of 1908 Swedish patients with RA and 1061 healthy Swedish controls recruited from different parts of Sweden between May 1996 and December 2005. All patients originated from the Epidemiological Investigation of Rheumatoid Arthritis (EIRA) study and met the ACR criteria for RA. ACPA status was obtained for all patients. A more detailed description of the EIRA study has been presented previously[14]. The study was approved by the ethics committee at the Karolinska Institutet and by the Regional Stockholm ethics committee.

The power to detect an association within the combined population was >80%, calculated by Quanto, assuming an OR of 1.20.

Genotyping methods

Ten single nucleotide polymorphisms (SNPs) were selected on the basis of their previous significant association with JIA.6 rs6673837, rs2358817, rs10923217, rs6669320, rs10923223 and rs12046117 were genotyped in 985 patients and 865 controls using MassArray matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (Sequenom, San Diego, California, USA) with a success rate of >97%. rs2051047, rs4376721, rs12038533 and rs7415876 were genotyped in 1257 patients and 1060 controls using Taqman allelic discrimination assays (AppliedBiosystems, Bleiswijk, The Netherlands) with a success rate of >95%.

In the Norwegian dataset, rs4376721 and rs10923217 were genotyped using MassArray matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (Sequenom). The genotyping success rate was >95%.

Data from the Swedish EIRA study were retrieved from GWAS published previously[15]. After removing outliers, a total of 1147 ACPA-positive patients and 1079 controls were used for analysis.

Statistical analysis

Analysis of allele distribution for association in the Dutch population and carriage of the minor allele in the replication study was performed using a χ^2 test with one degree of freedom. ORs and 95% CI were calculated using the Statcalc module of EpiInfo Software (Centers for Disease Control and Prevention, Atlanta, Georgia, USA). P values <0.05 were considered significant and genotype frequencies in controls did not deviate from Hardy-Weinberg equilibrium ($p<0.05$). Combined analysis of the genotypes of all studies was performed using the Mantel-Haenszel fixed effects method. Heterogeneity across studies was analysed using the Breslow-Day test.

RESULTS

Ten polymorphisms which have previously been shown to be associated with JIA were genotyped in a Dutch RA population. Both rs4376721 and rs10923217 showed significant associations with RA (OR 1.21, 95% CI 1.07 to 1.38, p = 0.003 and OR 0.87, 95% CI 0.76 to 0.99, p = 0.031, respectively; table 1).

In this analysis we did not correct for multiple testing as we wished to replicate all the associations observed in a second stage. To validate the observed associations, the two significant polymorphisms rs4376721 and rs10923217 were replicated in two independent RA populations of western European descent. Since the genotype frequencies indicated a dominant inheritance, carriage of the minor allele was also analysed. A p value <0.025 was considered significant.

Table 1. *Analysis of 10 SNPs in the VTCN1 region in a Dutch rheumatoid arthritis population*

	Allele		Cases					Controls					1 vs 2		
SNP	Major	Minor	N	11	12	22	MAF	N	11	12	22	MAF	OR (95% CI)	p Value	HW controls
rs6673837	G	A	980	619	317	44	0.21	853	542	265	46	0.21	1.02 (0.86 to 1.20)	0.845	0.080
				63.1	32.35	4.5			63.54	31.07	5.4				
rs2358817	C	T	978	831	1 41	6	0.08	835	699	1 30	6	0.09	1.10 (0.86 to 1.40)	0.455	1.000
				85.0	14.42	0.6			83.71	15.57	0.7				
rs2051047	G	A	1 239	1 040	1 87	12	0.09	1057	885	164	8	0.09	1 .00 (0.81 to 1.24)	0.999	0.843
				83.94	1 5.09	1.0			83.73	15.52	0.8				
rs4376721	T	G	1 237	621	521	95	0.29	1055	481	455	119	0.33	1.21 (1.07 to 1.38)	0.003	0.490
				50.2	42.12	7.7			45.59	43.13	11.28				
rs10923217	G	C	968	230	495	243	0.51	836	245	395	196	0.47	0.87 (0.76 to 0.99)	0.031	0.145
				23.76	51.14	25.1			29.31	47.25	23.44				
rs6669320	G	A	976	706	250	20	0.15	845	615	203	27	0.15	1 .03 (0.85 to 1.24)	0.768	0.060
				72.34	25.6	2.0			72.7	24.0	3.195				
rs12038533	C	A	1 237	984	246	7	0.11	1057	839	203	15	0.11	1 .05 (0.87 to 1.28)	0.576	0.529
				79.55	1 9.89	0.6			79.3	19.2	1.4				
rs7415876	G	C	1 241	871	335	35	0.16	1057	742	279	36	0.17	1 .02 (0.87 to 1.20)	0.794	0.148
				70.19	26.99	2.8			70.2	26.4	3.4				
rs10923223	T	C	981	682	273	26	0.17	852	612	226	14	0.15	0.88 (0.73 to 1.06)	0.170	0.224
				69.52	27.83	2.7			71.83	26.53	1.6				
rs12046117	C	T	958	71 5	227	16	0.14	844	631	200	13	0.13	0.99 (0.81 to 1.20)	0.910	0.656
				74.63	23.7	1.7			74.76	23.7	1.5				

1, major (common) allele; 2, minor (rare) allele. MAF, minor allele frequency; SNP, single nucleotide polymorphism; HW, Hardy-Weinberg equilibrium.

Table 2. *Replication of rs4376721 and rs10923217 and stratification for anticitrullinated protein antibody status in populations of northern European descent*

			Cases				Controls					1 vs 2		11 vs 12+22	
SNP	Population		N	11	12	22	MAF	N	11	12	22	MAF	OR 95% CI)	p Value	OR (95% CI)	p Value
rs4376721	Dutch		1237	621	521	95	0.29	1055	481	455	119	0.33	1.21 (1.07 to 1.38)	0.0026	1.20 (1.02 to 1.42)	0.0277
				50.2	42.1	7.7			45.6	43.1	11.3					
		ACPA positive	531	261	224	46	0.30						1.15 (0.98 to 1.36)	0.0780	1.15 (0.93 to 1.43)	0.1799
				49.2	42.2	8.7										
		ACPA negative	404	207	164	33	0.28						1.23 (1.02 to 1.47)	0.0229	1.25 (0.99 to 1.59)	0.0533
				51.2	40.6	8.2										
	Norway		918	466	374	78	0.29	1061	491	471	99	0.32	1.13 (0.99 to 1.30)	0.0693	1.20 (1.00 to 1.43)	0.0465
				50.8	40.7	8.5			46.3	44.4	9.3					
		ACPA positive	533	270	219	44	0.29						1.14 (0.97 to 1.34)	0.1149	1.19 (0.96 to 1.48)	0.0986
				50.7	41.1	8.3										
		ACPA negative	327	172	123	32	0.29						1.15 (0.94 to 1.40)	0.1555	1.29 (1.00 to 1.66)	0.0454
				52.6	37.6	9.8										
	Sweden		1 908	952	767	189	0.30	1061	524	449	88	0.29	0.97 (0.87 to 1.10)	0.6559	1.02 (0.88 to 1.19)	0.7909
				49.9	40.2	9.9			49.4	42.3	8.3					
		ACPA positive	1143	557	470	116	0.31						0.94 (0.83 to 1.07)	0.3640	0.97 (0.82 to 1.16)	0.7582
				48.7	41.1	10.1										
		ACPA negative	765	395	297	73	0.29						1.02 (0.88 to 1.19)	0.7346	1.09 (0.90 to 1.32)	0.3435
				51.6	38.8	9.5										
	Combined		4063	2039	1662	362		3177	1496	1375	306		1.09 (1.02 to 1.17)	0.0158	1.13 (1.03 to 1.24	0.0131

Table 2. *Continued.*

		ACPA positive	2207	1088	913	206							1 .05 (0.97 to 1.15)	0.2365	1.07 (0.97 to 1.20)	0.1669
		ACPA negative	1496	774	584	138							1.11 (1.01 to 1.23)	0.0285	1.19 (1.05 to 1.35)	0.0071
rs1092321	Dutch		968	230	495	243	0.507	836	245	395	196	0.471	0.87 (0.76 to 0.99)	0.0309	0.75 (0.61 to 0.93)	0.0077
				23.8	51.1	25.1			29.3	47.2	23.4					
		ACPA positive	381	1 00	188	93	0.509						0.92 (0.77 to 1.10)	0.3567	0.86 (0.65 to 1.14)	0.2721
				26.2	49.3	24.4										
		ACPA negative	257	61	127	69	0.484						0.84 (0.68 to 1.02)	0.0750	0.75 (0.54 to 1.05)	0.0819
				23.7	49.4	26.8										
	Norway		910	193	491	226	0.518	1033	258	534	241	0.492	0.90 (0.79 to 1.02)	0.1010	0.81 (0.65 to 1.00)	0.0497
				21.2	54.0	24.8	25.0	51.7	23.3							
		ACPA positive	525	1 05	286	134	0.472						0.87 (0.74 to 1.01)	0.0585	0.75 (0.58 to 0.98)	0.0281
				20.0	54.5	25.5										
		ACPA negative	327	71	177	79	0.488						0.92 (0.77 to 1.10)	0.3617	0.83 (0.61 to 1.13)	0.2298
				21.7	54.0	24.2										
	Combined		1878	423	986	469		1869	503	929	437		0.91 (0.84 to 1.00)	0.0533	0.78 (0.67 to 0.91)	0.0011
		ACPA positive	906	205	474	227							0.92 (0.82 to 1.03)	0.1351	0.80 (0.66 to 0.96)	0.0185
		ACPA negative	584	132	304	148							0.91 (0.80 to 1.04)	0.1642	0.79 (0.64 to 0.99)	0.0393

A combined analysis of the genotypes of all studies was performed using the Mantel-Haenszel fixed effects method. 1, major (common) allele; 2, minor (rare) allele. ACPA, anticitrullinated protein antibody; SNP single nucleotide polymorphism.

A trend towards association was observed in the Norwegian cohort for both polymorphisms (rs4376721: p = 0.0456; rs10923217: p = 0.0497). This effect was in the same direction as that seen in the Dutch discovery cohort (table 2).

In the Swedish population only data on rs4376721 were available for analysis. Neither rs10923217 nor a proxy of any kind was included in the Swedish GWAS. In this study, rs4376721 did not indicate significance. Nonetheless, a combined analysis of all three independent populations did show a significant association (p=0.01). Likewise, for rs10923217, an overall significant association could be established in the combined Dutch and Norwegian cohort (p=0.001). Notably, no heterogeneity was observed between the different study populations (p>0.05) and in all studies the same allele contributed to disease (table 2). The linkage disequilibrium between the two polymorphisms was limited with a r2 of 0.30 and a D' of 0.85.

The contribution of the VTCN1 region to the ACPA subsets was also analysed. Stratification for ACPA status showed rs4376721 to be significantly associated in the ACPA-negative subset compared with healthy controls, but not in the ACPA- positive subset. The OR of the ACPA-negative subgroup was slightly higher than in the ACPA-positive subgroup, although no statistical difference was observed in the ACPA-negative subset compared with the ACPA-positive subset (OR 1.10, 95% CI 0.96 to 1.26, p=0.145). In ACPA-negative disease, all minor allele frequencies were biased in the same direction while, in ACPA-positive disease, more heterogeneity was observed (figure 1).

Stratifying for ACPA reactivity for rs10923217 demonstrated an overall association of this polymorphism for both subsets of the disease (table 2).

Expression data from the GenCord database[16] showed a significant correlation between rs6428686 (a perfect proxy for rs4376721) and VTCN1 transcript levels, with the risk allele G being associated with lower expression in primary T cells (p = 0.0069). A similar pattern was observed in lymphoblastoid cell lines (p = 0.067; see figure S1 in online supplement). These data indicate that the identified genetic variant has an impact on basal VTCN1 expression in non-progenitor immune cells.

DISCUSSION

In the present study a novel genetic risk factor for RA susceptibility was identified in a Dutch RA population and replicated in independent populations of northern European descent.

Previous studies in RA have suggested that associated loci predispose to specific subsets of the disease, characterised by ACPA status[3,17]. Also, non-genetic data indicate that ACPA-positive disease behaves differently from ACPA-negative disease[13,18]. It is therefore of relevance to analyse these potentially different forms of arthritis in a separate manner. Our results indicate that the VTCN1 region seems to associate mainly with an ACPA-negative disease subset and less with an ACPA-positive subset compared with healthy controls.

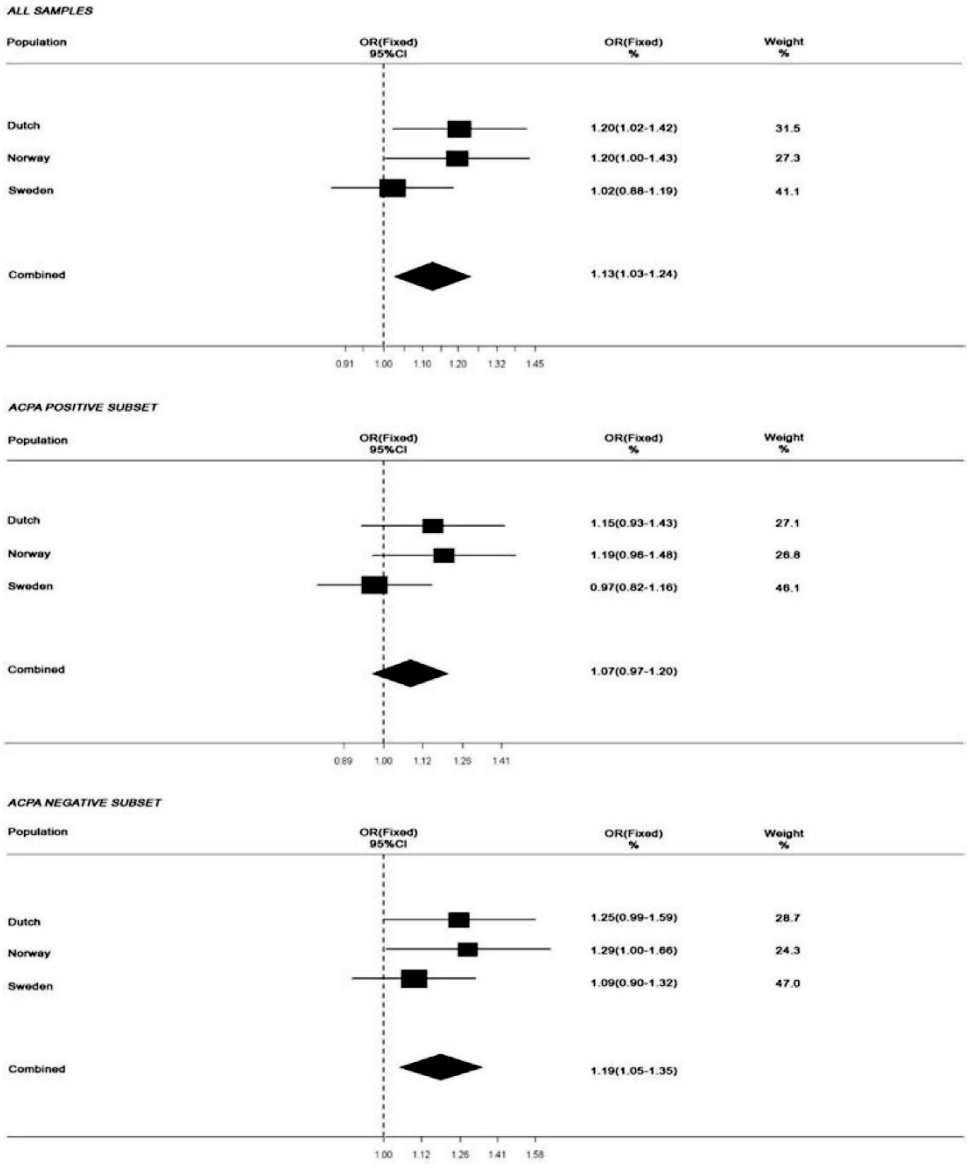

Figure 1. *Meta-analysis of association of rs4376721 for rheumatoid arthritis susceptibility and anti-citrullinated protein antibody status compared with healthy controls in three northern European rheumatoid arthritis populations. Squares represent OR values and lines represent 95% CI.*

Nonetheless, no significant difference was observed when the ACPA-negative subset was directly compared with the ACPA-positive subset. Although this lack of significance is possibly due to insufficient power (for the present sample numbers the power is ~72%), no final conclusion on this aspect can be drawn. Additional replication in independent cohorts is therefore necessary to identify a differential effect of the VTCN1 locus in the two subsets of RA.

The polymorphisms typed within this study were selected on the basis of their previous significant association with JIA[6]. It is notable that the association pattern of the variants seems to be different between the two diseases. First, the most significant SNP in JIA was not significantly associated within the Dutch cohort. Also, the distribution of the genotype frequencies is different between RA and JIA, which is mainly visible for rs4376721. Therefore, very subtle differences in minor allele frequency in the control or patient groups are prone to affect data and lead to different results, indicating the need for replication. One could also speculate that protein function or expression is affected differently by different genetic variants, which could explain the difference in disease outcome and disease course. Although indirect, and although it is not known whether VTCN1 is constitutively expressed, analysis of expression data from the GenCord database indeed shows differential expression for the different genotypes in lymphoid cells.

CONCLUSION

In conclusion, this study has identified the VTCN1 region as a new genetic region associated with susceptibility to RA.

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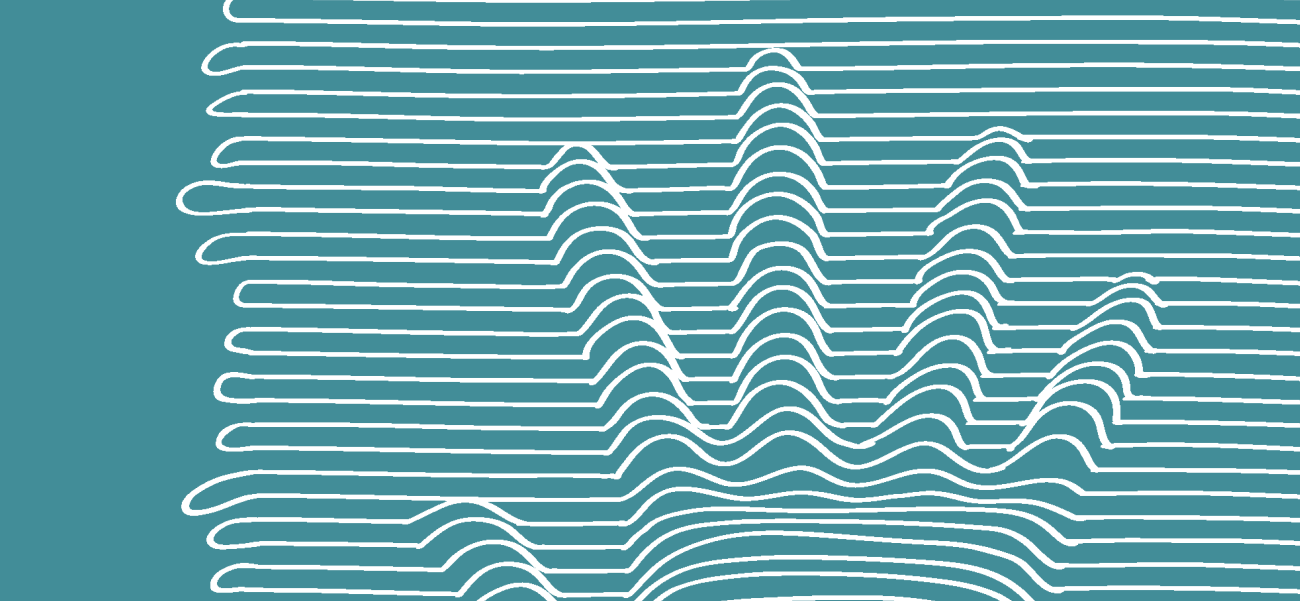

CHAPTER 6

Variants of gene for microsomal prostaglandin E2 synthase show association with disease and severe inflammation in rheumatoid arthritis

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ABSTRACT

Objective

Microsomal PGE synthase 1 (mPGES-1) is the terminal enzyme in the induced state of prostaglandin E2 (PGE2) synthesis and constitutes a therapeutic target for rheumatoid arthritis (RA) treatment. We examined the role of the prostaglandin E synthase (PTGES) gene polymorphism in susceptibility to and severity of RA and related variations in the gene to its function.

Methods

The PTGES gene polymorphism was analyzed in 3081 RA patients and 1900 controls from two study populations: Swedish Epidemiological Investigation of Rheumatoid Arthritis (EIRA) and the Leiden Early Arthritis Clinic (Leiden EAC). Baseline disease activity score (DAS28) was employed as a disease severity measure. mPGES-1 expression was analyzed in synovial tissue from RA patients with known genotypes using immunohistochemistry.

Results

In the Swedish study population, among women a significant association with risk for RA was observed for PTGES single-nucleotide polymorphisms (SNPs) in univariate analysis and for the distinct haplotype. These results were substantiated by meta-analysis of data from EIRA and Leiden EAC studies with overall OR 1.31 (95% confidence interval 1.11-1.56). Several PTGES SNPs were associated with earlier onset of disease or with higher DAS28 in women with RA. Patients with the genotype associated with higher DAS28 exhibited significantly higher mPGES-1 expression in synovial tissue.

Conclusion

Our data reveal a possible influence of PTGES polymorphism on the pathogenesis of RA and on disease severity through upregulation of mPGES-1 at the sites of inflammation. Genetically predisposed individuals may develop earlier and more active disease owing to this mechanism.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by persistent synovial inflammation resulting in joint destruction and severe disability. It is one of the most common complex autoimmune diseases, with up to 1% prevalence, and a higher prevalence, activity and earlier disease onset in women. Genetic factors significantly contribute to the development of RA and its severity[1]. The major genetic risk factor for RA is the shared epitope alleles of the HLA-DRB1 gene locus[2,3]. Recently several common genetic variants have been discovered outside HLA-DRB1 locus, identifying important biological pathways predisposing to RA (for a review, see Raychaudhuri[4]).

Prostaglandin E2 (PGE2) is one of the key mediators of inflammation, pain and joint destruction in RA, and its production is an important target of anti-inflammatory drugs. Hence PGE2 biosynthetic pathway is of considerable interest to gene association and pharmacogenetic studies[5,6]. PGE2 is produced by conversion of arachidonic acid to PGH2 by cyclooxygenases (COX-1/COX-2) and subsequent conversion of PGH2 to PGE2 by terminal PGE synthases. Microsomal PGE synthase 1 (mPGES-1) is the terminal enzyme in the induced PGE2 production at the sites of inflammation[7,8] and has an important role in the pathogenesis of inflammatory arthritis[9,10]. In humans, mPGES-1 expression is induced by proinflammatory cytokines in RA synovial fibroblasts and synovial fluid monocytes[11,12] and markedly up-regulated in RA synovial tissue[13]. In mice with experimental arthritis, genetic deletion of mPGES-1 results in significantly reduced disease incidence, severity and pain[9,10]. mPGES-1 is recognized as an alternative target for anti-inflammatory treatment with improved selectivity and safety compared with COX-1/COX-2.[8]

The human mPGES-1 is encoded by prostaglandin E synthase (PTGES) gene, which is localized at chromosome 9q34.3, spans 14.8 kb and contains three exons. According to NCBI single-nucleotide polymorphisms (SNPs) database, there are 249 SNPs in the PTGES gene region, including four coding SNPs (two synonymous and two non-synonymous SNPs). Whereas none of the studied variants in PTGES gene had a significant effect on the risk of lymphoma,[14] non-fatal myocardian infarction or ischemic stroke,[15] and breast cancer susceptibility,[16] in a large-scale association study one SNP in the 5' region of the PTGES gene was associated with hypertension in women in the Japanese population[17]. SNPs in the PTGES gene could modify enzyme expression levels or activity and alter inducible PGE2 biosynthesis. Consequently, PTGES gene polymorphisms might contribute to inter-individual differences in susceptibility to or severity of RA, as well as clinical response to future mPGES-1 inhibitors. Hence, PTGES genetics may have clinical importance from disease risk assessment to choice of personalized treatment in RA.

Here, we studied the association between PTGES gene polymorphisms and risk and severity of RA. In addition, we examined whether PTGES gene polymorphism could contribute to altered mPGES-1 expression.

PATIENTS AND METHODS

Patients

In this study, two independent cohorts have been included: Swedish Epidemiological Investigation of Rheumatoid Arthritis (EIRA) and Leiden Early Arthritis Clinic (Leiden EAC). The first cohort consisted of 2012 RA patients and 1033 controls matched by age, sex and residential area from the EIRA, a population-based case-control study in Sweden (Table 1).A case was defined as a person who was diagnosed for the first time with RA based on the diagnostic criteria of the American College of Rheumatology[18]. Data regarding age of disease onset were available for all patients, and data regarding baseline disease activity score (DAS28) for 887 (44%) RA patients. Further characteristics of the EIRA study are described elsewhere[19].

At the replication stage, the second cohort consisted of 1069 Dutch Caucasian individuals with RA who fulfilled the ACR classification criteria for RA and 867 controls, who were unrelated Dutch Caucasians with no history of RA from EAC (Table 1). More details about the EAC study population have been described elsewhere[20]. Approval was granted by the regional ethics committees and all participants gave their informed consent to participate in the study.

SNP genotyping

In all, 11 SNPs were selected across the PTGES gene locus and flanking regions (Figure 1). The selection was based on the minor allele frequency being higher than 10% according to NCBI for all SNPs except for rs11792431 (minor allele frequency 2.5%). In the replication phase, we genotyped three SNPs in PTGES locus, which showed significant association with RA risk. Genotyping was performed using TaqMan SNP genotyping assays (Applied Biosystems, Europe BV, The Netherlands). The average genotyping call rate was 96.6%, varying from 95 to 98% for different SNPs.

In addition, we included in the analysis data from our previous genome-wide association scan21 for 14 SNPs in the PTGES locus and inter-gene regions with neighboring PRRX2 and TOR1B genes (Figure 1). Genotyping was performed on the Illumina HumanHap300 (version 1.0) array (Illumina, San Diego, CA, USA)[21].

Immunohistochemical analysis

Synovial tissue biopsy specimens from 24 RA patients from EIRA carrying different PTGES genotypes were obtained during arthroscopy. Synovial tissue samples were snap frozen and kept at -70°C until sectioned. Staining of cryostat sections with rabbit polyclonal anti-human mPGES-1 antiserum was performed as previously described[13]. Irrelevant rabbit IgG was used as a negative control. Stained tissue sections were examined using a Polyvar II microscope (Reichert-Jung, Vienna, Austria) and photographed with a digital Leica camera 300F (Leica, Cambridge, UK). The positive staining was assessed quantitatively using computer-assisted image analysis and expressed as the percentage of the total area of counterstained tissue.

Statistical analysis

Genotype and allele frequencies in the patient and the control groups were compared using the χ^2-test and/or Fisher's exact test when appropriate. Haplotype frequencies were estimated using HaploView 4.0 (http:// www.broad.mit.edu/mpg/haploview/index.php), and the permutation test was set to 1,000,000 permutations for the single markers and for the haplotypes, with $P<0.05$ for significance of empirical P-value. An odds ratio (OR) with 95% confidence interval (CI) was calculated for the association between different genetic factors and risk of RA. Evaluation of linkage disequilibrium and deviations from Hardy-Weinberg equilibrium were performed using HaploView. The distribution of genotypes for all studied SNPs within the RA group and within the control group was in agreement with Hardy-Weinberg equilibrium ($P>0.05$). Before combining for meta-analysis, the EIRA and Leiden EAC data were tested for the homogeneity in the ORs, using a Pearson χ^2 goodness-of-fit test as described elsewhere[22]. In the meta-analysis of haplotype-frequency data, the RA cases versus controls were compared in EIRA and Leiden EAC studies using Mantel-Haenszel analysis assuming random effects for the ORs[22].

In RA patients stratified by sex, the associations between PTGES polymorphisms and DAS28 and age at the first diagnosis of RA, as well as the results of functional study, were analyzed using Mann-Whitney's U-test with Bonferroni corrections for multiple comparisons. P-values <0.05 were considered statistically significant.

Table 1 *Clinical characteristics of EIRA and Leiden EAC studies*

Characteristics	EIRA	Leiden EAC
No. of patients	2012	1069
No. of controls	1033	867
Country of origin	Sweden	The Netherlands
Female sex, patients (%)	70.4	64
Female sex, controls (%)	74.6	21.9
Age of onset, years (mean ±SD)	51.7 ± 12.7	56.4±15.4
Disease duration, years	<1	<2
ACPA positive (%)	(1265/2012) 62.8	(375/625[a]) 60
HLA-DRB1 SE (patients) (%)	(1486/2012) 73.8	(590/842[b]) 70.1

Abbreviations: ACPA, anti-citrullinated peptide antibody; SE, shared epitope. Details on the EIRA study19 and the Leiden EAC study20 have been reported elsewhere. [a]The number of patients with defined ACPA status. [b]The number of patients with defined HLA-DRB1 SE status.

RESULTS

PTGES polymorphisms and risk of developing RA

In the discovery phase, we analyzed allelic and haplotype frequencies of 11 SNPs in the PTGES gene in the entire Swedish cohort, as well as in men and women separately. An association between the SNP rs16931419 and risk of RA was observed in the allelic model (women and men combined), but the difference was not significant after permutation test (Table 2). Among women three SNPs, rs10988484, rs11999368 and rs16931419, from a single linkage disequilibrium block in the gene locus downstream the PTGES gene showed higher minor allele frequencies in female RA patients compared with those in female controls (Table 2). The association of the SNP rs16931419 with the risk of RA was still apparent after permutation test (OR 1.38; 95% CI 1.14-1.67, empirical P-value = 0.0082). Among men, a trend toward an association between the SNP rs3844048 and risk of RA was observed, but this association was not statistically significant after permutation test (Table 2).

To exclude possible correlation with neighboring genes owing to extended linkage disequilibrium, we expanded the analysis by including 14 SNPs in the PTGES locus and intergenic areas with the PRPX2 or the TOR1B genes using data from our previous genome-wide association scan[21] (Figure 1). There was no evidence for an association between SNPs in proximity to the PRPX2 gene or the TOR1B gene and susceptibility to RA (data are not shown).

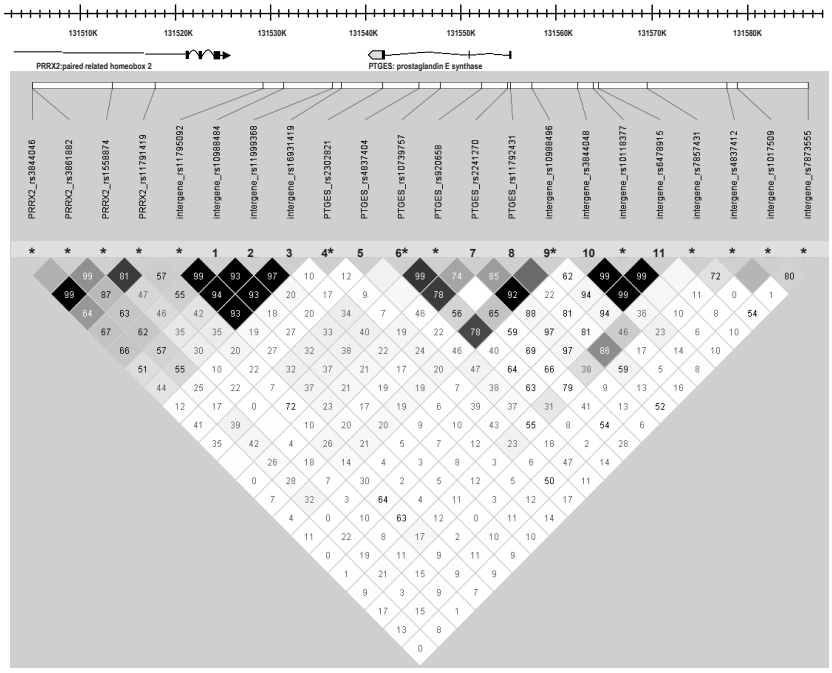

Figure 1 *Linkage disequilibrium map for the PTGES locus (r2). The SNPs marked with numbers were analyzed in the EIRA case-control study. The SNPs marked with * were analyzed using data from our previous genome-wide association scan.*

Table 2 *Minor allele frequencies of SNPs at PTGES locus in the Swedish and the Dutch populations*

No.	SNP	Position	MA		MAF, cases	MAF, controls	OR (95% CI)		P	P*
The Swedish population										
1	rs10988484	3' near gene	T	All	0.154	0.138	1.14	(0.98-1.33)	0.0966	NS
				F	0.160	0.129	1.28	(1.07-1.54)	0.0076	0.0591
				M	0.141	0.163	0.84	(0.63-1.12)	0.23	NS
2	rs11999368	3' near gene	T	All	0.148	0.134	1.12	(0.96-1.31)	0.15	NS
				F	0.152	0.124	1.26	(1.05-1.52)	0.0138	0.1035
				M	0.138	0.162	0.83	(0.62-1.10)	0.19	NS
3	rs16931419	3' near gene	C	All	0.149	0.127	1.2	(1.02-1.41)	0.02	NS
				F	0.153	0.116	1.38	(1.14-1.67)	0.0010	0.0082
				M	0.139	0.160	0.84	(0.63-1.14)	0.26	NS
4	rs2302821	3'-UNR	C	All	0.091	0.091	1.0	(0.83-1.21)	0.95	NS
				F	0.093	1.0	0.93	(0.75-1.15)	0.49	NS
				M	0.087	0.065	1.36	(0.91-2.04)	0.13	NS
5	rs4837404	Intron 2	G	All	0.333	0.325	1.04	(0.92-1.16)	0.54	NS
				F	0.334	0.323	1.05	(0.92-1.20)	0.44	NS
				M	0.330	0.331	0.99	(0.80-1.24)	0.93	NS
6	rs10739757	Intron 2	C	All	0.095	0.101	0.93	(0.78-1.12)	0.44	NS
				F	0.092	0.102	0.89	(0.72-1.1)	0.27	NS
				M	0.103	0.099	1.01	(0.91-1.12)	0.82	NS
7	rs2241270	Intron 1	T	All	0.096	0.097	1.00	(0.94-1.06)	0.93	NS
				F	0.096	0.096	1.00	(0.83-1.23)	0.95	NS
				M	0.098	0.100	0.99	(0.70-1.39)	0.93	NS
8	rs11792431	Exon 1	A	All	0.056	0.054	1.04	(0.82-1.32)	0.74	NS
				F	0.053	0.052	1.00	(0.76-1.33)	0.94	NS
				M	0.063	0.058	1.09	(0.71-1.69)	0.69	NS
9	rs10988496	5' near gene	G	All	0.104	0.104	1.00	(0.84-1.20)	0.96	NS
				F	0.101	0.103	0.97	(0.79-1.20)	0.80	NS
				M	0.101	0.107	1.00	(0.75-1.45)	0.81	NS
10	rs3844048	5' near gene	G	All	0.301	0.308	0.97	(0.86-1.09)	0.58	NS
				F	0.303	0.291	1.05	(0.92-1.22)	0.40	NS
				M	0.295	0.358	0.75	(0.60-0.93)	0.0114	NS
11	rs6478915	5' near gene	G	All	0.250	0.247	1.01	(0.89-1.15)	0.82	NS
				F	0.252	0.232	1.11	(0.96-1.29)	0.16	NS
				M	0.244	0.289	0.79	(0.63-1.01)	0.05	NS
The Dutch population										
1	rs10988484	3' near gene	T	All	0.152	0.152	1.00	(0.83-1.19)	0.95	NS
				F	0.151	0.129	1.20	(0.85-1.70)	0.28	NS
				M	0.152	0.159	0.95	(0.74-1.22)	0.72	NS
2	rs11999368	3' near gene	T	All	0.145	0.141	1.03	(0.86-1.24)	0.74	NS
				F	0.138	0.124	1.14	(0.82-1.62)	0.46	NS
				M	0.157	0.146	1.08	(0.85-1.39)	0.49	NS
3	rs16931419	3' near gene	C	All	0.139	0.138	1.01	(0.84-1.22)	0.93	NS
				F	0.131	0.116	1.16	(0.80-1.67)	0.42	NS
				M	0.153	0.145	1.06	(0.83-1.36)	0.59	NS

*Abbreviations: MAF, minor allele frequency; NS, not significant; F, females; M, males. *P-value after 1000000 permutations.*

Further, we examined the frequency of haplotypes (only with the frequency above 0.01) based on the three SNPs significantly associated with the risk of RA. The frequency of the haplotype TTC (susceptible haplotype) was significantly higher in women with RA than in women without RA (OR 1.34; 95% CI 1.11-1.63, empirical P-value = 0.0077) (Table 3). The frequency of the opposite haplotype CCT (protective haplotype) was significantly lower in women with RA compared with controls (OR 0.78; 95% CI 0.66-0.93, empirical P-value = 0.0172). We did not find significant associations between these haplotypes and RA risk among men (Table 3).

In the replication phase, we analyzed the association of the three SNPs, rs10988484, rs11999368 and rs16931419, with RA susceptibility in the Dutch cohort. Similarly to the Swedish cohort, these SNPs showed higher minor allelic frequencies in female patients

Table 3 *Haplotype frequencies of PTGES variations in female patients with RA and female controls from the Swedish and the Dutch populations*

Haplotype		HF	HF, cases	HF, controls	OR (95% CI)	P	p*
The Swedish population							
CCT	All	0.843	0.837	0.854	0.88 (0.76-1.02)	0.09	NS
	F	0.842	0.831	0.862	0.78 (0.66-0.93)	0.0066	0.0172
	M	0.844	0.851	0.828	1.19 (0.90-1.57)	0.22	NS
TTC	All	0.130	0.137	0.117	1.19 (1.01-1.40)	0.03	NS
	F	0.128	0.139	0.107	1.34 (1.11-1.63)	0.0030	0.0077
	M	0.136	0.131	0.146	0.88 (0.65-1.18)	0.39	NS
TCT	All	0.011	0.011	0.011	0.93 (0.56-1.57)	0.75	NS
	F	0.012	0.012	0.013	0.97 (0.55-1.71)	0.86	NS
	M	0.005	0.004	0.007	0.54 (0.16-1.8)	0.30	NS
The Dutch populations							
CCT	All	0.835	0.836	0.834	1.01 (0.85-1.20)	0.83	NS
	F	0.844	0.841	0.855	0.89 (0.65-1.24)	0.50	NS
	M	0.827	0.826	0.827	0.99 (0.78-1.25)	0.97	NS
TTC	All	0.126	0.128	0.125	1.01 (0.84-1.23)	0.82	NS
	F	0.12	0.125	0.105	1.21 (0.84-1.75)	0.29	NS
	M	0.131	0.132	0.131	1.00 (0.77-1.30)	0.97	NS
TCT	All	0.02	0.018	0.025	0.16 (0.46-1.13)	0.13	NS
	F	0.02	0.019	0.021	0.90 (0.40-2.00)	0.80	NS
	M	0.02	0.015	0.025	0.61 (0.31-1.19)	0.14	NS

*Abbreviations: NS, not significant; HF, haplotype frequency; F, females; M, males. Haplotypes are based on PTGES SNPs rs10988484, rs11999368 and rs16931419 and are considered only with the frequency above 0.01. *P-value after 1000000 permutations.*

with RA compared with the frequencies in female controls, though the difference was not statistically significant (Table 2). In the same way, the frequency of the susceptible haplotype TTC was higher in women with RA than in women without RA, though the difference was not significant (Table 3). The allelic frequencies among female controls in the two populations were identical, whereas the haplotype frequencies were highly similar.

Genetic variations with low or moderate effects on disease susceptibility may not be evident in an individual study owing to low statistical power. Thus, we performed a meta-analysis combining our results from the Swedish and the Dutch cohorts to verify the significance of PTGES polymorphisms for susceptibility to RA. Test for the heterogeneity between EIRA and EAC showed no significant differences (P>0.05). The overall statistical power in the combined analysis was >80% (in two-tailed test) to detect associations with OR ≥ 1.3, given the prevalence of studied genetic factors. The meta-analysis demonstrated a statistically significant association between the susceptible haplotype TTC and risk of developing RA in women with an overall OR of 1.31 (95% CI 1.11-1.56) and P = 0.002 (Figure 2).

PTGES polymorphisms and age of onset and activity of RA

RA is a heterogeneous syndrome with relatively broad variations in disease onset and activity. Therefore, we tested the correlation between these parameters and PTGES genetic markers in patients from EIRA. Baseline DAS28 and mPGES-1 expression in RA synovial tissue were used as disease activity measures.

	Cases			Controls				Odds Ratio
Study population	TTC	Total	Haplotype frequency	TTC	Total	Haplotype frequency	Weight	M-H, Fixed, (95%CI)
Sweden	388	2792	0.139	163	1520	0.107	76.8%	1.34 (1.11 -1.63)
The Netherlands	171	1368	0.125	40	380	0.105	23.2%	1.21 (0.84-1.75)
Total (95%CI)	559	4160	0.134	203	1900	0.107	100.0%	1.31 (1.11-1.56)

Test for heterogeneity: $X^2 = 0.23$, df = 1 (P = 0.63), $I^2 = 0\%$
Test for overall effect: Z = 3.11 (P = 0.002)

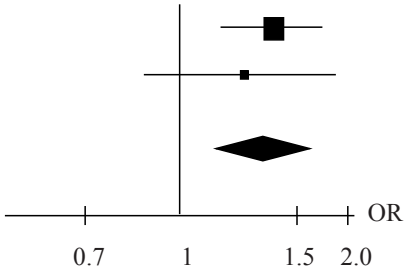

Figure 2 *The association of haplotype TTC with susceptibility to rheumatoid arthritis in women in two study populations and the meta-analysis. The squares represent odds ratios (ORs) and lines represent confidence intervals (95% CI).*

There was no association of RA risk haplotype TTC with baseline DAS28 scores or age of disease onset in women or men. The minor allele of the SNP rs10739757 corresponded to 3 years earlier onset of RA in female patients (median 51, range 19-74 vs median 54, range 16-82, P = 0.0065) (Table 4).

Table 4 *Association of PTGES genotypes with disease onset and activity in female patients with RA*

Disease parameters	SNP	Genotype	Median (range)	Counts	P -value
Disease onset, years	rs10739757	C	51 (19-74)	246	0.0065
		TT	54 (16-82)	1136	
DAS28, score	rs4837404	AA	5.57 (1.74-8.89)	266	0.0080
		G	5.28 (1.57-8.29)	321	
DAS28, score	rs2302821	C	5.57 (2.39-8.89)	114	0.0321
		AA	5.31 (1.57-8.37)	491	
DAS28, score	rs11792431	A	5.63 (3.82-8.89)	63	0.0369
		GG	5.32 (1.57-8.66)	536	

Abbreviation: DAS28, disease activity score.

Furthermore, we observed that the homozygous major allele in the SNP rs4837404 was associated with higher DAS28 scores in women with RA (median 5.57, range 1.74-8.89, vs median 5.28, range 1.57-8.29, P = 0.0080), but not in men. In addition, the minor allele in the SNP rs2302821 showed a trend toward association with higher DAS28 scores in women with RA (median 5.57, range 2.39-8.89 vs median 5.31, range 1.57-8.37, P = 0.0321). Also, the female RA patients carrying the minor allele of the SNP rs11792431 showed a trend toward higher DAS28 scores (median 5.63, range 3.82-8.89 vs median 5.32, range 1.57-8.66, P = 0.0369) (Table 4). All mentioned variations related to disease course were in low linkage disequilibrium (r2<0.34) with SNPs included in the susceptibility haplotype TTC.

Using immunohistochemical analysis, we evaluated the expression of mPGES-1 in RA synovial tissue in relation to PTGES genotypes. There was no difference in mPGES-1 expression in relation to RA risk haplotype TTC. mPGES-1 expression was significantly higher in RA synovial tissue from patients (mean ± SD age, 52.8 ± 14.4 years) carrying the minor allele C in SNP rs2302821 compared with the patients (mean ± SD age, 54.4 ± 14.1 years) who did not carry that allele (median 12.9% of the stained area, range 8.1-23.6 vs median 7.1%, range 0.9-10.5, P<0.001, n=9 and 15, respectively) (Figure 3). No differences were observed in mPGES-1 expression in relation to gender or treatment of patients with NSAIDs, MTX or prednisolone.

DISCUSSION

The present study demonstrates the association of variations in the PTGES gene, encoding mPGES-1, with susceptibility to RA in women. The data are consistent for single SNPs

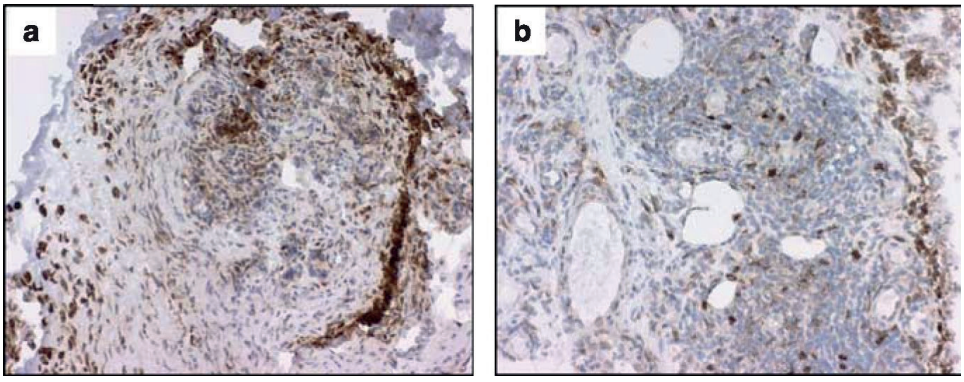

Figure 3 *The expression of mPGES-1 in synovial tissue of RA patients in relation to PTGES genotypes. Photographs illustrating brown immunoperoxidase staining of mPGES-1-positive cells in representative synovial tissue sections from RA patients. (a) Sample carrying the minor allele C in SNP rs2302821; (b) sample that dit not carry that allele, counterstained with hematoxylin (original magnification x250).*

and haplotypes in the large study from Sweden. Moreover, the finding was substantiated by a meta-analysis of data from the EIRA and Leiden EAC studies. We also found that the PTGES genotypes are likely to be related to earlier disease onset and disease severity, as they were associated with higher baseline disease activity scores and related to higher expression of mPGES-1 in synovial tissue biopsies from RA patients.

PGE2 is actively involved in the regulation of immune response, inflammation and pain, and polymorphisms in the genes in the PGE2 biosynthetic pathway are important risk factors for cardiovascular, neoplastic and inflammatory diseases[6,23]. The role of gene polymorphisms in the PGE2 pathway in relation to RA risk and severity is less investigated. Two case-control studies have revealed a possible protective influence of COX-2 - 765C allele on the susceptibility to RA and disease activity[24,25]. To our knowledge, the present study is the first one to examine an association between PTGES polymorphisms and the risk of developing RA.

We performed an association study in two independent populations. In a large sample from Sweden, we found the association between three SNPs from the same recombination block, which spread approximately within 9 kb downstream to PTGES, and RA risk in women. This significant association was apparent for the SNP rs16931419 (3kb downstream to PTGES) after 1,000,000 permutations. Moreover, the TTC haplotype based on the SNPs from this recombination block demonstrated a significant association with RA risk in women in the Swedish population. We expanded the analysis by including 14 SNPs in the PTGES locus and intergenic areas with the PRPX2 or the TOR1B genes to exclude possible association with neighboring genes. No evidence for the association between the SNPs in proximity to the PRPX2 and the TOR1B genes and RA risk was seen in our study population.

In order to replicate our finding, we analyzed the association between three PTGES SNPs included in the haplotype and risk of RA in a Dutch cohort. Though the respective case-

control frequencies had a similar pattern in RA females from Leiden EAC as in EIRA, there was no significant association between single SNPs or haplotype and susceptibility to RA in the Dutch cohort. The power of the replication study was though limited by the small size of the female control group. To increase the power of the study, we performed a meta-analysis of the data from EIRA and Leiden EAC studies and confirmed the association between TTC haplotype and increased risk to RA in women. This finding substantiates the evidence that PTGES polymorphisms might be important for susceptibility to RA in women.

The revealed genetic association was consistent for women but not detected for men. The higher prevalence of RA in women suggests that genetic risk of RA might vary with sex. Indeed, there are a number of associations that show genetic effects in only one sex, or reciprocal effects on risk of RA in males and females[26,27]. Thus, there is strong association of SNP rs2900180 in the TRAF-C5 locus with RA without obvious evidence of an effect in males[27]. Also, SNP rs11761231 has been reported in a large study to be associated with RA in females, but has no effect on disease status in males[26]. On the other hand, several SNPs in NCF4 (rs729749), NCF2 (rs789181) and RAC2 (rs1476002) genes were found to be mildly associated with RA only in men[28].

Moreover, the association of COX-2 polymorphisms with disease risk and response to NSAID varies between sexes,[29,30] most likely as a result of sexual dimorphism in PGE2 metabolism. Systemic biosynthesis of PGE2 differs markedly between males and females in human, as in mice[31,32]. In rodents, variations in COX-2 and mPGES-1 expression at basal and pathological conditions[33,34] and different patterns of EP receptor expression[35] have been reported in females and males. Thus, sex-dependent association of PTGES polymorphisms with RA susceptibility may reflect different response to this genetic trigger in females and males owing to differences in PGE2 metabolism. However, the absence of an association between PTGES polymorphism and RA risk in men might also reflect the small number of individuals and low study power, which needs further validation.

The SNPs associated with RA are in a single linkage disequilibrium block and located downstream to the 3' untranslated region (3'UTR) of the PTGES gene. 3'UTR is involved in post-transcriptional gene regulation via mRNA stability, the mechanism for which is revealed in the regulation of mouse *Ptges* gene expression[36]. These SNPs may be regulatory or in linkage disequilibrium with other regulatory polymorphisms in 3'UTR that could similarly modulate the human mPGES-1 expression and predispose individuals to RA.

In our study, we found that gene polymorphisms in PTGES might have not only a disease-predisposing impact but also a disease¬modifying effect, as two SNPs within PTGES gene were related to early disease onset or baseline DAS28 scores. Revealing the relations between genetic variations and certain functions or phenotypes is currently the most challenging task for the genetics of complex diseases. We investigated whether gene variations associated with disease activity could relate to mPGES-1 expression. We previously demonstrated the expression of mPGES-1 in synovial biopsies as a reliable parameter of inflammatory activity in RA. Our data from the current study indicate that mPGES-1 expression seems to be dependent on the genotype, which was also reflected by genetic association

with the clinical measure of baseline DAS28. Individuals with the minor allele C in PTGES rs2302821 that showed a trend toward association with higher DAS28 had significantly higher mPGES-1 expression in RA synovial tissue, which represents additional phenotypic replication for this association. Although the total patient number in our experiment is small, it represents a unique collection of tissue samples from RA patients with distinct genotypes.

CONCLUSION

In conclusion, we provide the first evidence that PTGES gene polymorphisms are associated with susceptibility to RA in women. In addition, PTGES polymorphisms were related to earlier disease onset, higher baseline disease activity scores and might contribute to higher expression of mPGES-1 in synovial tissue biopsies from RA patients. These data suggest the importance of PTGES gene variants in the assessment of RA risk and severity.

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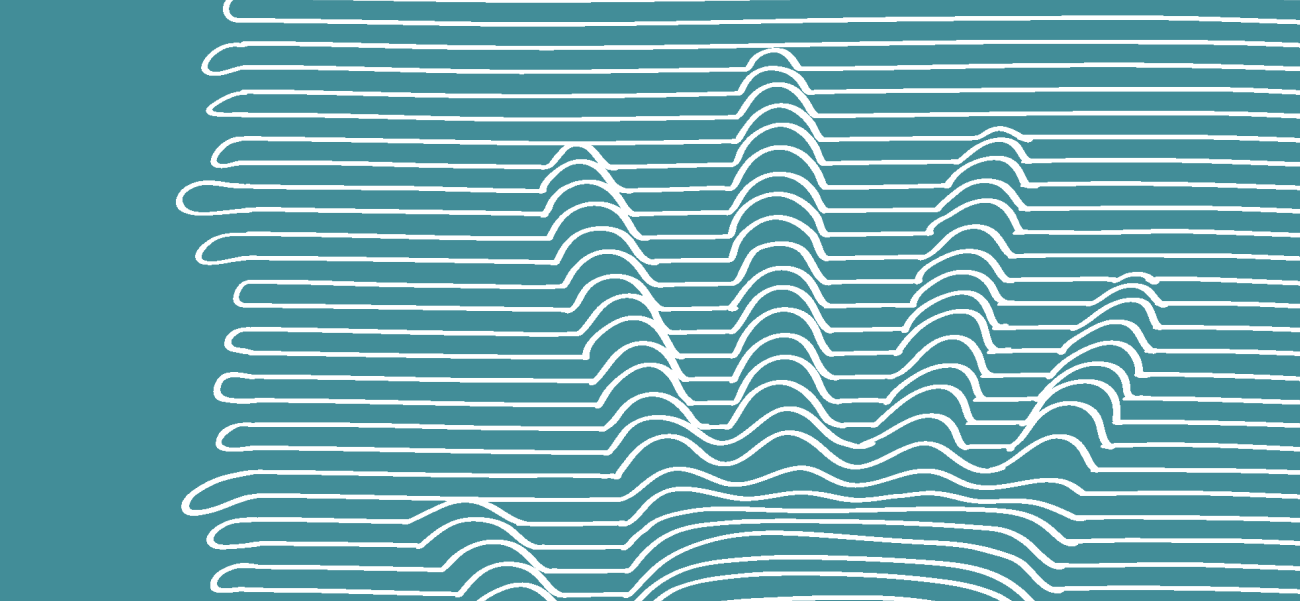

CHAPTER 7

Genetic variants in the region of the C1q genes are associated with rheumatoid arthritis

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ABSTRACT

Objective

Rodent models for arthritis implicate a role for complement in disease development and progression. In humans, complement deposition has been observed in inflamed synovia of rheumatoid arthritis (RA) patients. In this study we analysed whether genetic variants of complement component C1q predispose to RA.

Methods

We genotyped single nucleotide polymorphisms (SNPs) in and around the C1q genes, C1qA, C1qB and C1qC, in a Dutch set of 845 RA cases and 1046 controls. Replication was sought in a sample set from North America (868 cases/1193 controls), and a meta-analysis was performed in a combined samples set of 8000 cases and 23 262 controls of European descent. We determined C1q serum levels in relation to C1q genotypes.

Results

In the discovery phase, five of the 13 SNPs tested in the C1q genes showed a significant association with RA. Additional analysis of the genomic area around the C1q genes revealed that the strongest associating SNPs were confined to the C1q locus. Within the C1q locus we observed no additional signal independent of the strongest associating SNP, rs292001 [odds ratio (OR) = 0-72 (0.58-0.88), P = 0.0006]. The variants of this SNP were associated with different C1q serum levels in healthy controls (P = 0.006). Interestingly, this SNP was also associated significantly in genome-wide association studies (GWAS) from the North American Rheumatoid Arthritis Consortium study, confirming the association with RA [OR = 0.83 (0.69-1.00), P = 0.043]. Combined analysis, including integrated data from six GWAS studies, provides support for the genetic association.

Conclusion

Genetic variants in C1q are correlated with C1q levels and may be a risk for the development of RA.

INTRODUCTION

The recognition molecule of the classical pathway (CP) of complement, C1q, is essential in the initiation of the CP following its binding to ligands such as immune complexes[1], apoptotic cells[2] and C-reactive protein[3]. Initially, complement was thought to be involved only in innate immunity against pathogens. However, over the last decades a wealth of insight has been generated, showing that complement is also involved in many other processes, such as coagulation, tissue regeneration, clearance of dead cells and regulation of the adaptive immune system[1].

C1q is part of the C1 complex, which consists of one C1q molecule, two C1r and two C1s serine protease proenzymes[4]. Conformational changes in the C1q molecule induced by binding to one of its ligands result in the release of these enzymes[4]. Next to its traditional ligands such as immunoglobulin (Ig)G and IgM, C1q can also bind to dead cells, DNA and matrix components[5-7]. Structurally the C1q molecule (460 kDa) is composed of 18 polypeptide chains (6A, 6B and 6C). The A, B and C chains each have a short N-terminal region, followed by a collagen region (CLR) and a C-terminal globular region (gC1q domain). Three such structural units form the hexameric C1q molecule, that has a tulip-like structure via strong non-covalent bonds in the fibril-like central portion[4]. In contrast to most other complement factors, C1q is not made by hepatocytes, but by macrophages and immature dendritic cells[8,9]. Following their maturation, dendritic cells shut down C1q production completely[8,9], which is suggestive of a role in adaptive immune responses[10]. Indeed, a role for C1q in adaptive immunity can also be concluded from in-vivo studies regarding antigen presentation[11] and cellular activation[12-16].

Complete genetic deficiency of C1q is associated strongly with the development of systemic lupus erythematosus (SLE)[17]. Similarly, several studies, although small, have implicated C1q in the emergence of SLE, as several genetic variants located in the C1q region seem to associate with this disease[18-22]. In addition, two small studies indicated an effect of genetic variants of C1q on the progression of cancer and the efficacy of rituximab treatment for lymphoma[23,24].

These studies, and the observation that complement deposits are found in the rheumatoid arthritis (RA) synovium[25], as well as the described correlation between disease activity with the presence of activated complement fragments bound to C1q in sera of RA patients[26], point to a possible involvement of C1q in RA pathogenesis.

For these reasons, we studied whether single nucleotide polymorphisms (SNPs) present in the C1q region associate with RA, as this could provide further evidence for a role of the complement system in RA pathogenesis.

PATIENTS AND METHODS

Patients

SNPs have been genotyped in sets of controls and RA patients who met the American College of Rheumatology (ACR) 1987 revised criteria for RA. For the Leiden data set we analysed 845 RA patients who were recruited from hospitals in the western part of the Netherlands. As healthy controls, 1046 subjects were selected randomly by Immunogenetics and Transplantation Immunology section of the Leiden University Medical Center. These patient and control sets, as well as the patient characteristics, have been described previously[27]. Within this cohorts we obtained a statistical power of 0.7 to detect differences, with a $P < 0.0038$ for SNPs with a minor allele frequency of 0.20.

Replication sample sets consisted of (1) the North American Rheumatoid Arthritis Consortium (NARAC)[28], that comprised 868 patients and 1193 controls; (2) 277 RA patients and 387 healthy controls from Crete, Greece, the initial collection of these patients and controls has been described previously[29]; (3) samples from the Genomics Collaborative, Inc. (GCI; Cambridge, MA, USA), comprising 475 rheumatoid factor (RF)-positive RA patients and 475 individually matched controls from the United States, which has been described in detail elsewhere[30]; and (4) data from six genome-wide association studies (GWAS) comprising 5539 patients and 20 169 controls[31]. A summary of the demographic details of the cohorts used is provided in Table 1. 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

For the initial screening we selected tagging SNPs using Tagger[32] from HapMap Release II CEU data within a 54 kb region encompassing C1qA, C1qB and C1qC, with an $R2 < 0\text{-}8$, LOD score [logarithm (base 10) of odds] threshold = 3 and minor allele frequency (MAF) > 10%[33,34]. Genotyping of SNPs in the Dutch and Greek sample sets was performed using MassArray matrix- assisted laser absorption ionization time-of-flight mass spectrometry, according to the manufacturer's protocol (Sequenom, San Diego, CA, USA). At least 10% of the genotypes were assessed in duplicate, with an error rate of <1%.

The GWAS were run using different platforms, as described previously[31]. If the SNP of interest was not typed in a particular GWAS, then genotype imputation was performed using IMPUTE version 2 with the proposed default parameters[35]. Data were adjusted for population stratification, as described elsewhere[35].

Detection of circulating C1q levels

To analyse the effect of the different C1q genotypes on circulating levels of C1q we determined C1q levels in serum of 266 healthy controls by enzyme-linked immunosorbent assay (ELISA). We chose to use healthy controls to exclude potential C1q consumption due to disease activity in RA patients. The ELISA was performed essentially as described previously[8]. Briefly, 96-well Maxisorb plates (Nunc, Roskilde, Denmark) were coated with a C1q-

specific rabbit anti-human C1q antibody in coating buffer (100 mM Na2CO3/NaHCO3, pH 9-6) for 2 h at 37°C. A blocking step was performed using 3% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 1 h at 37°C. Highly purified serum C1q was used as a standard. After adding samples and incubating for 1 h at 37°C, purified rabbit IgG anti-human C1q-labelled with digoxigenin (DIG) (Boehringer Mannheim, Mannheim, Germany) was used for 1 h at 37°C, followed by horseradish peroxidase (HRP)-conjugated Fab anti-DIG (Boehringer Mannheim) for 1 h at 37°C; all these steps were performed in ELISA buffer (PBS, 1% BSA, 0-05% Tween 20). Each step was followed by three washes with PBS/0-05% Tween 20. Enzyme activity was assessed by the addition of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) (Sigma, St Louis, MO, USA) and H2O2. The absorbance at 415 nm was measured using a microplate biokinetics reader (EL312e; Bio-Tek Instruments, Winooski, VT, USA), as described previously[36].

Statistical analysis

Association of SNPs with RA was performed using a c2 test with one degree of freedom or a logistic regression. Odds ratios (OR) and 95% confidence intervals (95% CI) were calculated using the Statcalc module of Epi Info Software (Centers for Disease Control and Prevention, Atlanta, GA, USA). P-values less than 0.05 were considered significant and genotype frequencies in controls did not deviate from Hardy-Weinberg equilibrium at a significance level of $P < 0.05$. Bonferroni correction for multiple testing was applied in the discovery phase.

Combined analysis of the genotypes of all studies was performed using a random-effects meta-analysis on the estimated effect sizes (log OR) and their standard error. The test for heterogeneity was not statistically significant ($P = 0.1$).

Conditional analysis

To distinguish independent effects in the region we performed an adjusted analysis for associations with RA. In order to adjust the effect of rs292001 with effects of other SNPs in the region, we compared a two-locus model with rs292001 and each of the other SNPs with the model including rs292001 alone, using a likelihood ratio test. The likelihood ratio tests were based on the comparison of two generalized linear models. We chose a recessive model for rs292001 based on the data of the discovery cohort and an additive model for other SNPs, as no prior information was available.

Sliding window-based haplotype analysis

In order to analyse which haplotypes would confer most risk, the SNPs used in this study were subjected to a sliding window-based haplotype analysis. Adjacent groups of SNPs of sizes 2, 3 and 4 were analysed using a generalized linear model. The function haplo.cc of the R-package haplo.stats was used with the simulation option to obtain empirical P-values. Rare haplotypes were grouped together using the default of minimally five expected haplotypes in the sample, as suggested in the package.

Global permuted P-values

Global P-values for genetic association with RA were obtained by applying the tail-strength statistics to P-values from individual SNP associations and permuting phenotype status 5 x 10^3 in order to obtain empirical P-values[37]. Permutation was necessary, as P-values were not independent due to linkage disequilibrium between SNPs.

Correction for population stratification

Effect sizes and P-values for the NARAC data set were corrected by a stringent inflation factor of 1.3 prior to performing a meta-analysis or the tail-strength measure used for computing global P-values. No other data set showed significant inflation factors and therefore did not require any inflation correction.

Table 1. *Study subjects.*

Cohort	Cases/controls	Genetic ancestry	RA criteria, autoantibody	Use
Leiden EAC	845/1-046	Caucasian/Dutch	ACR 1987, unselected	Discovery
NARAC- I	868/1-193	Caucasian/USA	ACR 1987, CCP+	Replication
Crete	277/387	Caucasian/Cretan	ACR 1987, unselected	Replication
GCI	475/475	Caucasian/USA	ACR 1987, RF+	Replication
WTCCC	1525/10-608	Caucasian/UK	ACR 1987, CCP+/RF+	Meta-GWAS*
NARAC- I+III	1769/5551	Caucasian/USA	ACR 1987, CCP+	Meta-GWAS
EIRA	1173/1089	Caucasian/Swedish	ACR 1987, CCP+	Meta-GWAS
Canada	589/1472	Caucasian/Canada	ACR 1987, CCP+	Meta-GWAS
BRASS	483/1449	Caucasian/USA	Rheumatologist,* CCP+	Meta-GWAS

*The number of individuals available for analyses is shown for each cohort as well as the genetic ancestry, the criteria used to define rheumatoid arthritis (RA), the selection of patients on the basis of their autoantibody status and the use of the particular cohorts in this manuscript. *Detailed information in references [31,35]. *The diagnosis of RA was made by a board-certified rheumatologist. GWAS: genome-wide association studies; BRASS: Brigham and Women's Rheumatoid Arthritis Sequential Study; NARAC: North American Rheumatoid Arthritis Consortium; WTCCC: Well-come Trust Case Control Consortium; GCI: Genomics Collaborative, Inc.; Leiden EAC: Leiden early arthritis clinic; EIRA: Epidemiologic Investigation of Rheumatoid Arthritis; ACR: American College of Rheumatology; CCP: cyclic citrullinated peptide; RF: rheumatoid factor.*

RESULTS

Genetic variants in the C1q genes predispose to RA

We first analysed whether genetic variants in the genes encoding C1q (C1qA, C1qB and C1qC) are a risk for the development of RA in the Dutch population. For this purpose we genotyped a set of 13 tagging SNPs in a discovery cohort of 845 RA patients and 1046 healthy controls from the Leiden area, the Netherlands. SNPs were selected from a 54 kb haplotype block on chromosome 1, with an R2 < 0.8, LOD score threshold = 3 and MAF > 10%. We captured 94% of the variation in this region. In this discovery set we observed a

statistically significant association for five SNPs (Table 2). Four of these five SNPs remained significant after Bonferroni correction for multiple testing ($P < 0.0038$). We observed the strongest association when analysing the data using a recessive model. We also analysed these data for global significance using a permutation procedure[37]; this analysis revealed a global significance for C1q in the Leiden data set with a $P = 0.003$.

Table 2. *Genotypes of 13 single nucleotide polymorphisms (SNPs) across the C1q genes reveal association with rheumatoid arthritis (RA) in the Dutch population.*

	Patients				Controls						
SNP	n	11	12	22	n	11	12	22	OR	(95% CI)	P
rs186037_AG	826	0-03	0-25	0-72	839	0-02	0-25	0-73	0-95	(0-76-1-19)	0-677
rs158762_AG	834	0-06	0-36	0-58	1023	0-05	0-35	0-6	0-92	(0-76-1-11)	0-386
rs587585_CT	829	0-02	0-26	0-72	1019	0-02	0-24	0-74	0-93	(0-84-1-10)	0-255
rs665691_CG	822	0-20	0-54	0-26	1022	0-19	0-48	0-33	0-71	(0-58-0-88)	0-0006
rs292001_GA	845	0-17	0-52	0-31	979	0-16	0-45	0-39	0-72	(0-58-0-88)	0-0006
rs682658_GT	821	0-25	0-53	0-21	1018	0-22	0-49	0-28	0-68	(0-55-0-85)	0-0009
rs12404537_CT	832	0-04	0-31	0-65	1018	0-04	0-29	0-67	0-92	(0-75-1-12)	0-38
rs294185_AG	820	0-14	0-48	0-38	1023	0-10	0-48	0-42	0-83	(0-68-1-00)	0-047
rs294179_CT	832	0-22	0-53	0-25	1019	0-18	0-51	0-31	0-73	(0-59-0-90)	0-0024
rs17486657_CT	835	0-08	0-40	0-52	837	0-10	0-42	0-48	1-18	(0-97-1-43)	0-09
rs629409_CT	836	0-02	0-28	0-70	834	0-02	0-25	0-73	0-86	(0-69-1-08)	0-168
rs12040131_CG	820	0-05	0-36	0-59	837	0-05	0-33	0-62	0-88	(0-85-1-09)	0-225
rs292007_CT	837	0-15	0-49	0-36	836	0-12	0-48	0-40	0-85	(0-70-1-05)	0-125

A total of 13 SNPs across the C1q genes were genotyped in 845 patients and 1046 Dutch controls from the Leiden area. Shown are genotype frequencies, odds ratios (OR), confidence intervals (CI) and P-values using the recessive model; 11 represents the minor alleles, 22 represents the major alleles and 12 represents the heterozygous state with the minor allele defined with the allele frequencies in the controls. The P-values of significantly associating SNPs are shown in bold.

Next, we investigated to what extent the signal we observed was limited to the C1q genes, and therefore we genotyped an additional 40 SNPs covering a region of 400 kb, including the genes EPHA8, EPHB2 and the C1q genes. In this additional analysis we identified two additional SNPs in the C1q genes and four SNPs in the EPHA8 region that are associated significantly with RA (Fig. 1). We used conditional analysis to study whether the observed signals represent one signal or may represent two independent signals. Effect sizes (ORs) for rs292001 (0.72 when analysed alone) varied between 0.77 and 0.66 when effects of other SNPs on RA were taken into account by analysing two-locus models, where one SNP was always rs292001. Four SNPs from EPHA8 were significant, most notably rs606002, with a P-value of 0.005. Models including EPHA8 SNPs together with rs292001 could explain the data set significantly more effectively than the model including rs292001 alone, indicating an independent effect of EPHA8, which is located relatively far away from

rs292001 in a region with low linkage disequilibrium. This indicates that the observed effect in the C1q genes is not mediated via other linked genes, but represents a true effect of the C1q genes themselves. We also performed sliding window-based haplotype analysis and observed, for a window size of two, nominal P-values < 0.05 for five SNP windows around rs209749 and rs521570 in EPHA8 and 10 SNP windows around rs292001. For a window size of three a similar pattern is observed, and a window size of four leads to significant associations only around rs292001. The strongest associations were observed for windows starting at rs294179, rs6690827 and rs12404537 for window sizes two, three and four, respectively. All these SNPs are, at most, five SNPs away from rs292001 based on physical ordering of SNPs. Collectively, these data suggest that the main signal is driven by the C1q genes, with a small and independent contribution from EPHA8. Within the C1q region we did not observe other signals independent from rs292001 and therefore we focused on this SNP in further analysis.

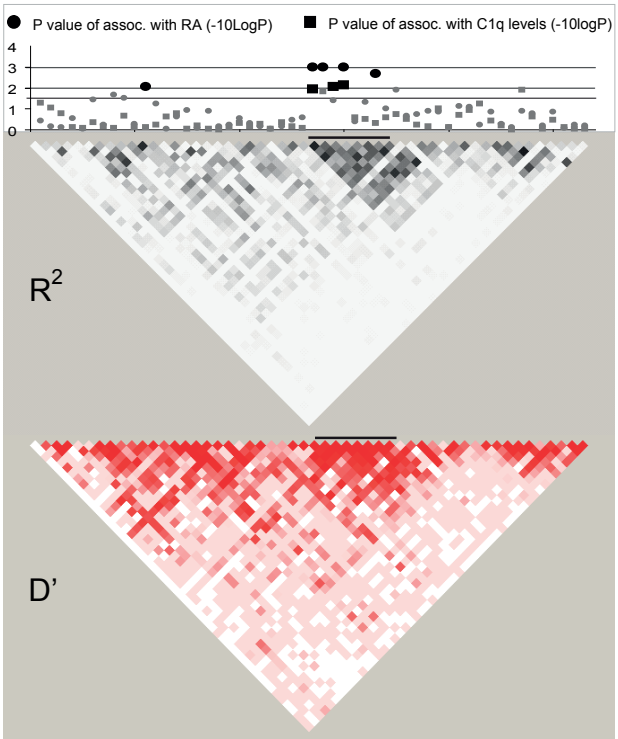

Figure 1. *Association plots across the genomic area containing EPHA8, C1qA, C1qC, C1qB and EPHB2. We calculate the D-prime and R2 between all the single nucleotide polymorphisms (SNPs) analysed in this study in the Dutch cohort. Solid horizontal lines indicate the location of the three C1q genes as well as EPHA8 and EPHB2. The top part of the figure depicts the P-value of association with rheumatoid arthritis (RA) (circles) and with C1q serum levels in healthy controls (squares) for each SNP as -log10 P-values. Horizontal lines indicate the P-values of 0.05, 0.01 and 0.001. The P-values shown in black are smaller than 0.001; other values are shown in grey.*

Genetic variants in C1q associate with C1q serum levels

We next analysed specifically the impact of the genetic variants of rs292001 on the protein levels of C1q. In order to exclude possible effects of disease activity and treatment on C1q production and/or consumption we have analysed the circulating levels of C1q in sera from 266 healthy controls. This analysis revealed a significant correlation between the presence of the 'protective' rs292001 G-genotype and higher C1q levels in serum (Fig. 2). These data indicate that genetic variants in the C1q locus have an impact upon circulating levels of C1q, providing a possible explanation as to how genetic variants present in the C1q-region may contribute to RA.

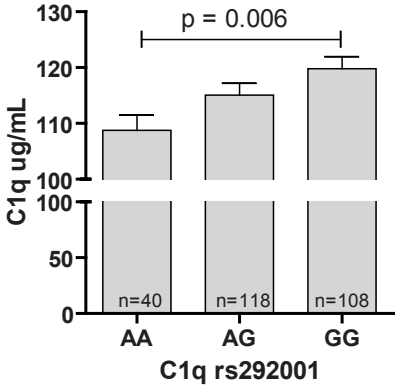

Figure 2. *Genetic variants in the C1q genes affect circulating protein levels of C1q. Circulating levels of C1q were analysed by enzyme-linked immunosorbent assay (ELISA) in 266 healthy, genotyped controls. Data are plotted in relation to their genotype for rs292001 as mg/ml.*

Replication of the C1q association

The finding that genetic variants of C1q are associated with C1q protein and mRNA levels provided further support for a contribution of C1q to RA. We next confirmed this genetic association by independent replication. Similar to our first analysis of the Leiden data set, we analysed the 13 SNPs in the NARAC data set for global significance[37]. In this replication data set we observed a global significance (P = 0.036), confirming that in the NARAC data set genetic variants in the C1q genes are also associated with RA. Similarly, an OR of 0.83 (95% CI: 0.69-1.00, P = 0.043) was observed for rs292001.

To further confirm our findings, rs292001 was typed in two additional cohorts, one from Crete and one from North America (GCI). The data from these two cohorts consisting of 269 patients/369 controls and 469 patients/464 controls, respectively, revealed an effect in the same direction as observed in the Leiden and NARAC data set, although not significant on its own (Table 3). A meta-analysis on these non-imputed data sets confirmed an association between genetic variation at rs292001 and susceptibility to RA (P = 0.0001, OR = 0.80; 95% CI: 0.72-0.89).

Table 3. *Analysis of association with rheumatoid arthritis (RA) in four cohorts of non-imputed genotypes of C1q single nucleotide polymorphism (SNP) rs292001.*

Cohort	Nationality	Patients				Controls				OR (95% CI)	P
		AA	AG	GG	n	AA	AG	GG	n		
Leiden	Dutch	0-17	0-52	0-31	845	0-16	0-45	0-39	979	0-72 (0-75-0-88)	0-0006
NARACI	USA	0-17	0-46	0-37	868	0-13	0-46	0-41	1193	0-83 (0-69-1-00)	0-043
GCI	USA	0-16	0-47	0-37	469	0-15	0-46	0-39	464	0-93 (0-70-1-20)	0-548
Crete	Greek	0-11	0-51	0-38	269	0-14	0-46	0-41	369	0-89 (0-64-1-25)	0-502
Meta										0-80 (0-72-0-89)	0-0001

Genotype counts of the non-imputed data for the cohorts from Leiden, North American Rheumatoid Arthritis Consortium (NARAC), Genomics Collaborative, Inc. (GCI) and Crete are depicted, including odds ratios (OR), confidence intervals (CI) and P-value. Also a meta-analysis was performed on these non-imputed data sets.

Supportive evidence from GWAS data

As the GWAS data for the RA studies are now publically available, we also further replicated our findings obtained for rs292001. Unfortunately, most GWAS data sets did not include this SNP and have been generated using a variety of genotyping platforms. Therefore, to obtain an impression of the effect of rs292001 genotypes, imputation was performed. The quality of imputation was different for each GWAS, with imputation scores ranging from 0.76 to 0.98 (Table 4; mean maximum posterior probability).

Table 4. *Meta-analysis of the six genome-wide association studies (GWAS) for rs292001.*

SNP rs292001						
	WTCCC	NARACI	NARAC III	EIRA	Canada	BRASS
Imputation score	0-950	g-1-000	0-766	0-904	0-901	0-976
Z-score	0-44	1-96	1-2	1-76	0-28	-0-82
	GG	GA	AA		Meta OR	0-95 (0-91-1-00)
Cases	1961-8	2683-8	890-2		Meta z	1-908
Controls	7489-6	9605-1	3066-6		Meta 2tP	0-0563

The data that are available for rs292001 from the six GWAS are the composite of six individual GWAS, some of which used imputation to obtain data for this single nucleotide polymorphism (SNP); data on imputation score and Z-score for each study are given as well as the overall genotype counts for cases and controls. The imputation score provides information on the quality of imputation and the Z-score is indicative of the effect size, with a score > 0 indicating a positive and a score < 0 indicating a negative effect. BRASS: Brigham and Women's Rheumatoid Arthritis Sequential Study; EIRA: Epidemiologic Investigation of Rheumatoid Arthritis; NARAC: North American Rheumatoid Arthritis Consortium; WTCCC: Wellcome Trust Case Control Consortium.

All Z-scores, except for the Brigham and Women's Rheumatoid Arthritis Sequential Study (BRASS), revealed an effect in the same direction as observed in the Leiden data set. The data from these six GWAS studies, combined with the other data sets studied, now excluding NARAC from the non-imputed cohorts, revealed a small but significant (P = 0.025) contribution to the susceptibility of RA (OR = 0.85; 95% CI: 0.73-0.98) when tested using a recessive model, as also used for the individual analyses (Fig. 3).

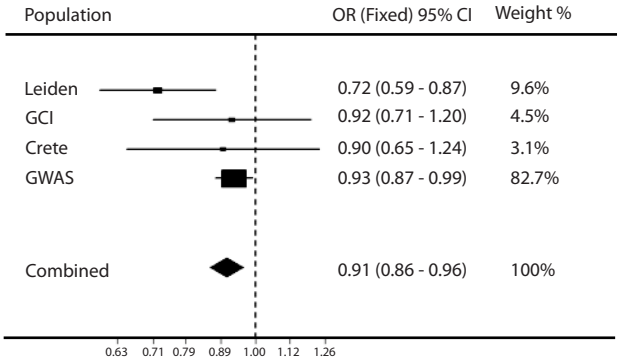

Figure 3. *Meta-analysis of all available data for C1q single nucleotide polymorphism (SNP) rs292001. Meta-analysis of the genotyped data from Crete, the Genomics Collaborative, Inc. (GCI), Leiden and the combined six genome-wide association studies (GWAS) using a random-effects model. Significance of the meta-analysis was P = 0.025.*

DISCUSSION

In the current study, we have obtained evidence that genetic variants of C1q associate with the susceptibility for RA. The observation that several of these genetic variants also associate with circulating C1q protein levels suggests that differences in C1q quantity are involved in the onset of RA. C1q, the initiation molecule of the classical pathway of complement, can trigger complement activation following binding to its ligands, such as immune complexes, matrix molecules and apoptotic cells[1,6,38], all conceivable targets in the context of RA. Immune complexes, formed, for example, by anti-citrullinated protein antibodies (ACPA), can trigger the classical pathway[39]. However, this would occur after the induction of such antibodies[40,41], which would put genetic variants of C1q at the effector-phase of RA rather than at the onset of RA. We have divided the patient group on the basis of their ACPA status and found the effect of C1q genetic variants in both strata, although the P-values were stronger in the larger ACPA-positive group (data not shown). However, next to its role in activation of the complement system, C1q is thought to have a direct effect on adaptive immunity and autoimmunity[11-15] and as recently suggested on Wnt signalling[42].

The observed relationship between SNPs in the C1q genes and C1q serum levels might

point to a role for the cells that produce C1q, predominantly macrophages and immature dendritic cells in the onset of RA[8,9]. These two cell types are well known to be instrumental in shaping the adaptive immune response[43]. It is therefore conceivable that the basal expression or the induction of enhanced C1q production by these cells would modulate differentially the immune response to foreign antigens and possibly also against self-antigens. Dedicated experiments will need to show how C1q, either intracellularly or excreted by such cells, impacts upon the adaptive immune response.

None of the SNPs tested in this study or in high LD with rs292001 (1000genomes.org, pilot 1 data, R2 > 0-8) have an amino acid-changing effect. Therefore, it seems likely that the associating SNPs, or SNPs in LD, have an impact upon the basal expression of C1q or on its induction following specific triggers. A genetic locus was also identified in the mouse that had an impact upon the C1q expression, secretion and autoimmune organ damage[44]. We observed that the rs292001 G allele, which provides protection against development of RA, associates with a higher C1q serum concentration. This is in line with earlier observations in SLE that low C1q levels predispose to autoimmunity[44,45]. Although we observed an association of genetic variants of C1q with circulating levels of C1q, we did not observe an effect on the activity of the CP (data not shown), which is in line with the observation that not C1q, but rather C2, is the rate-limiting factor of the CP[46].

Although complete genetic deficiency of C1q is associated with development of SLE[45], it is not yet clear to what extent smaller genetic differences such as represented by SNPs are also associated with development of autoimmunity[18-21]. In the case of complete genetic C1q deficiency, it is thought that the absence of circulating C1q may have a direct effect on autoimmunity because of defective clearance of apoptotic cells[47]. However, many other processes may (in concert) also play a role, e.g. effects on cytokine production[48] or Wnt signalling[42].

The observed association between genetic variants of C1q and RA do not achieve stringent genome-wide significance. Nevertheless, we believe these data are important, as we confirmed our observations in the NARAC data set and observed a similar trend in other smaller cohorts, as well as the GWAS studies. We also believe that the associations between genetic variants of C1q with C1q protein levels provide additional support for a true association between genetic variants of C1q and RA. It is possible that deep sequencing of these genes would provide additional insight and may reveal the truly causal, functional variant.

CONCLUSION

Collectively, our data show that genetic variants in the region of the C1q genes are associated with the susceptibility for RA, and that this may potentially be explained by an effect of these genetic variants on basal or induced C1q production.

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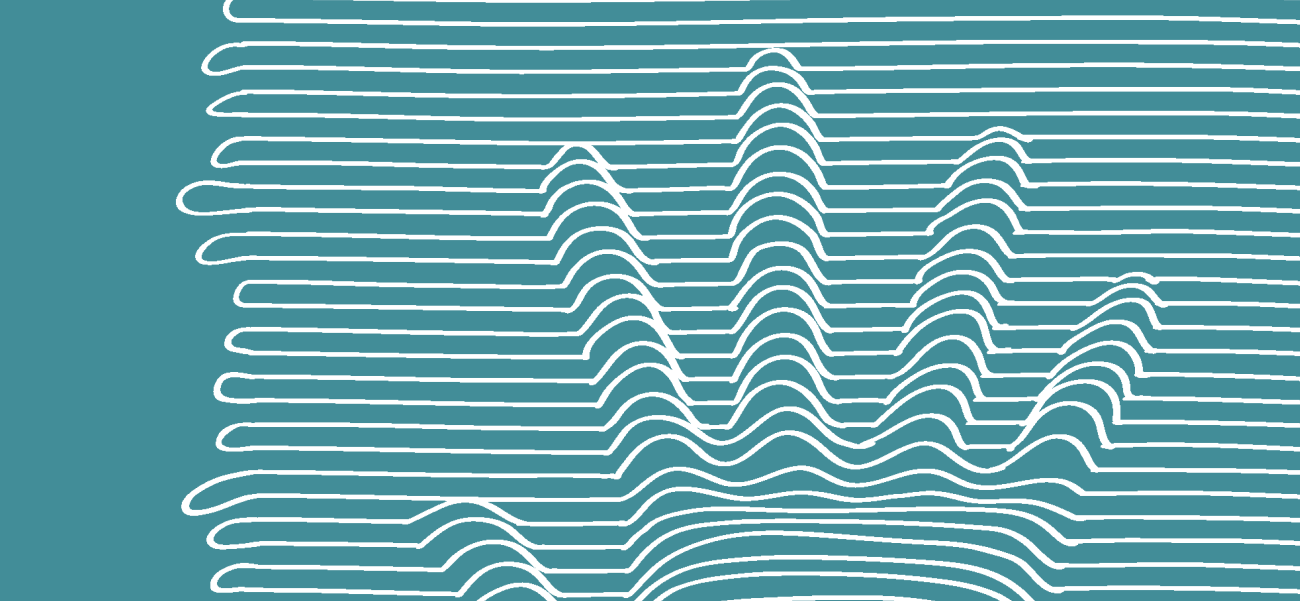

CHAPTER 8

Complement activation by (auto)antibodies

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ABSTRACT

The complement system is a key part of the innate immune system and plays an important role in the clearance of pathogens and apoptotic cells upon its activation. It is well known that both IgG and IgM can activate complement via the classical pathway by binding of C1q to the Fc regions of these immunoglobulins. Recent advances have shown that also IgA is capable of activating the complement system. Besides, more insight is gained into an additional role for antibodies in the activation of both the alternative and the lectin pathways. Mouse models have shown that autoantibodies can activate the alternative pathway and induce in cell lysis and tissue damage.

Besides the role of antibodies in complement activation, complement may also be a target for recognition by antibodies directed against autologous complement components. These autoantibodies play a role in several diseases, especially vascular diseases. Understanding how antibodies interact with the complement system will allow the manipulation of this interaction to diminish pathological consequences of autoantibodies and optimize the effect of therapeutic antibodies.

In the current review, we discuss complement activation by (auto)antibodies by the different pathways.

INTRODUCTION

As part of the innate immune system, complement is important in the clearance of pathogens and apoptotic cells. The activation of complement can occur by three different pathways, the classical pathway (CP), the lectin pathway (LP) and the alternative pathway (AP). All three pathways comprise different components and are activated in different fashions but use a common terminal pathway. The CP is activated when C1q interacts with its ligands such as immune-complexes (ICs), the LP is activated when MBL or ficolins binds to certain carbohydrates and the AP can be activated by spontaneous C3 hydrolysis or via properdin, thereby generating C3(H2O) which can bind factor B (fB). Activation of each of these pathways generates C3-convertases resulting in activation of the common terminal pathway and generating several effector processes, such as chemotaxis by C3a and C5a, opsonization by C3b, and lysis by the membrane attack complex (Ricklin et al., 2010). Additionally, the complement system provides a critical link to the adaptive immune system by serving as an effector arm of the acquired immune system supplementing antibodies. Complement activation by this manner is part of the physiologic immune response and plays an important role in fighting infections; however, it is not always beneficial and can play a role in different debilitating diseases.

In the current review, an overview of complement activation by (auto)antibodies in both human and mouse models is given and the role of this process in disease is discussed.

Complement activation by antibodies IgG and IgM

It is well known that antibodies can activate the complement system through activation of the classical pathway. However, new insights indicate that antibodies also play a role in activating and influencing both the lectin and alternative pathways.

The classical pathway is activated when C1q binds to the Fc portions of immunoglobulins. In humans the different immunoglobulin isotypes differ in their ability to bind C1q and cause subsequent activation of the classical pathway. In this respect IgM, IgG1 and IgG3 are very effective in this, while IgG2 fixes complement relatively poorly and IgG4, IgA, IgD and IgE are not capable of activating the classical pathway (Bindon et al., 1988).

Binding of C1q to IgG occurs within the CH2 domain of the immunoglobulin and is sensitive to ionic strength (Duncan and Winter, 1988; Hughes-Jones and Gardner, 1978). The C1q binding site is localized to three side chains, namely, Glu 318, Lys 320 and Lys 322. These sequence motifs seem highly conserved through evolution and are found on IgGs in all species, indicating this binding site to be universal (Burton et al., 1980; Duncan and Winter, 1988). However, this motif is also present on the IgG4 isotype, which is not able to bind C1q and activate the CP, suggesting that other features in or near the CH2 domain are important for C1q binding. It was shown that the amino acid residue at position 331, which is in close proximity to the indicated binding site of C1q, is important for proper binding and subsequent activation. Both IgG1 and IgG3 carry a proline residue at this location, in contrast to IgG4, which carries a serine residue (Tao et al., 1991). In IgM the CH3 domain is

important for C1q binding and Asp 417, Glu 418 and Hys 420 are thought to be involved in this process. Additionally, proline residues at position 436 seem to be critical for CP activation. Since the structure of the C1q binding sites between IgG and IgM are not identical, it is thought that the recognition structure of C1q may vary between different molecules (Miletic and Frank, 1995).

Additionally, comparative analysis has shown that hexameric IgM is ten to twenty times more cytolitically active than pentameric IgM, which may be due to a better symmetry of the IgM molecule to bind a C1q molecule (Collins et al., 2002; Davis et al., 1988).

Upon binding of C1q to either IgG- or IgM-containing ICs, auto-activation of C1r occurs, followed by activation of C1s. This subsequently leads to activation of C4 and C2 and to the formation of the CP C3 convertase.

Next to the well known and well studied activation by IgG and IgM of the CP, more recent studies indicate a role for these antibodies in both the AP, as well as in the LP of the complement system.

Activation of the LP occurs when mannose-binding lectin (MBL) binds to certain sugar residues. Upon binding of MBL, MBL-associated serine proteases (MASP-1 and MASP-2) are activated, which subsequently leads to activation of C4 and C2, similar to the CP. Immunoglobulins are present in the human body in different glycosylated variants and it has been shown that MBL can bind especially to antibodies with certain patterns of glycosylation.

IgG contains a single N-linked glycosylation site on each heavy chain of the antibody and the glycans that occupy this site vary in the number of terminal galactoses attached to them. MBL can bind the IgG-G0 form, in which the glycans lack a terminal galactose residue, but instead have terminal GlcNAc residues. Interaction of one carbohydrate recognition domain head of the MBL molecule with a glycan is relatively poor and therefore, most likely, only transient in serum. However, in immune complexes containing multiple IgG-G0 glycans, numerous recognition domains of MBL can engage these antibodies, generate high avidity interactions and subsequently activate complement through the LP (Arnold et al., 2005; Malhotra et al., 1995).

Although IgM has five N-linked glycosylation sites it is not able to bind MBL and activate the LP, because the GlcNAc-structures are not exposed when IgM is bound to antigen (Arnold et al., 2005).

In the AP, activation is not necessarily dependent on immunoglobulins and the first complement components are bypassed in this process. Antibodies however, do play a role and influence the activation of this pathway in different ways. IgG heavy chains can bind nascent C3b in a covalent manner, thereby enable the generation of C3-convertases on the surface of sensitized targets. Additionally, the rate of cleavage of IgG bound C3b by factor H and factor I is slowed remarkably, when compared to unbound C3b. This reduced degree of cleavage is mainly due to a diminished affinity for factor H and therefore the amplification loop can be sustained for a longer period of time (Fries et al., 1984). Additionally, it

has been shown that C3b(2)-IgG complexes in plasma are the major substrate of C3-convertase and properdin provides these complexes with partial protection from inactivation, maintaining the amplification loop longer as compared to that of free C3b (Jelezarova and Lutz, 1999; Jelezarova et al., 2000).

Complement activation by IgA

Not only in traditional antibody-mediated diseases such as systemic lupus erythematosus (SLE) and poststreptococcal glomerulonephritis, but also in IgA-dominated diseases such as primary IgA nephropathy and Henoch Schonlein purpura (Endo et al., 1998, 2000; Hisano et al., 2001; Roos et al., 2006), complement has been clearly recognized as a component in the inflammatory process. These findings have led to numerous studies into mechanisms of complement activation by IgA, revealing that IgA can activate the AP (Hiemstra et al., 1987) and LP (Roos et al., 2001) but not the CP.

AP activation by IgA has been shown with immobilized IgA purified from human plasma, resulting in activation of complement in a calcium-independent way (Hiemstra et al., 1987,1988). Moreover, IgA bound to a bacterial surface in an antigen-specific way can also mediate activation of C3 (Fasching et al., 2007; Janoff et al., 1999). The mechanism of AP activation has not been precisely defined. Most likely it involves stabilization of the C3 convertase on the IgA molecule. Since properdin has recently been recognized as a recognition molecule of the AP (Spitzer et al., 2007), it cannot be excluded that properdin may directly interact with IgA.

IgA-mediated complement activation, both via the AP and via the LP, is a function of polymeric but not monomeric IgA (Hiemstra et al., 1987; Roos et al., 2001). Both IgA subclasses in the human circulation, IgA1 and IgA2, consist of polymeric and monomeric IgA. From circulating human IgA, the majority is IgA1 and generally less than 20% is polymeric. Whereas monomeric IgA is a clearly defined molecule, polymeric IgA may consist of different molecular complexes which are incompletely defined (Papista et al., 2011).

Purified human polymeric serum IgA has been shown to interact with human MBL resulting in activation of the LP (Roos et al., 2001). This interaction involves the lectin domain of MBL and likely involves a carbohydrate ligand. Whereas IgG, IgM and IgA2 are glycosylated via N-linked sugars only, the IgA1 heavy chain also has O-linked glycosylation sites. The structure of IgA heavy chain glycans is highly variable and is determined by the cellular source of IgA. Glycosylation may be different between secretory IgA and plasma IgA (Royle et al., 2003), and between monomeric- and polymeric IgA (Oortwijn et al., 2006). Moreover, also components present in polymeric IgA complexes may be (heavily) glycosylated, such as J-chain and secretory component (Royle et al., 2003). In secretory IgA, the glycans of secretory component mask MBL-binding sites on the IgA heavy chain (Royle et al., 2003). Until now, the precise identity of the ligand involved in interaction between MBL and polymeric IgA has not been established.

In a recent study from Terai et al. (2006) purified polymeric serum IgA did not bind to MBL unless the glycan structure was modified or disrupted (Terai et al., 2006). As also stated

by these authors, it is impossible to explain this discrepancy, but most likely it is related to the isolation procedure.

The pathophysiological significance of the interaction between IgA and MBL is underlined by studies in patients with IgA nephropathy. This renal disease is characterized by deposition of IgA in the mesangial area of the glomerulus, which is accompanied by complement activation, activation of the inflammatory cascade, renal injury, and, on the longer term, end stage renal disease. Several reports showed deposition of MBL together with IgA in the mesangium (Endo et al., 1998, 2000; Hisano et al., 2001; Matsuda et al., 1998; Roos et al., 2006). This MBL deposition was associated with deposition of MASP-2 and C4 (Roos et al., 2006), supporting its role in glomerular complement activation. Moreover, the subpopulation of IgA nephropathy patients with mesangial MBL deposition has a more severe glomerular disease than IgA nephropathy patients who only showed evidence for complement activation via the AP (Roos et al., 2006). These data were confirmed in a later study reporting that C4d-positive IgA nephropathy patients have a significantly more rapid progression to end-stage renal disease than C4d-negative patients (Espinosa et al., 2009). Taken together, complement activation by IgA may support humoral host defense, but, in diseased conditions, may also amplify inflammation and tissue injury. There is evidence for involvement of the AP and the LP, and not only MBL but also ficolins may be involved (Roos et al., 2006). Since MBL deficiency is the most common human complement deficiency, this effector function of the IgA immune system differs between individuals. Furthermore, given the large variety of molecular forms of human IgA, the molecular interactions are complex and need multidisciplinary studies, with respect to the identity of the protein components as well as the structure of the glycans on these molecules.

Complement activation by murine autoantibodies

Several mouse models are available in which autoantibodies play a clear role. Using these established models it was possible to unravel the complex contribution of complement activation into the overall process of tissue damage. Although many informative models are available, in this review we have focused on mouse models of rheumatoid arthritis (RA), SLE and subepidermal blistering diseases. The role of autoantibodies in complement activation in mouse models of auto-immune diseases suggests a prominent and in some cases essential role of the AP in their pathogenesis.

Murine models of arthritis

Several auto-antibody-mediated mouse models of arthritis are available to study RA. Initially, the role of complement in Collagen Induced Arthritis (CIA) was suggested from studies where mice treated with cobra venom to deplete complement were found to be refractory to arthritis until their complement levels were restored (Backlund et al., 2002). Two additional mouse models have been extensively used to explore the role of complement in arthritis: the anti-G6PI (K/B x N) and CAIA (anti-collagen antibody induced) models. Interestingly, in the K/B x N model disease develops spontaneously in the offspring of TCR-transgenic KRN mice crossed with NOD mice carrying the MHC class II Ag7 allele (Ji

et al., 2002). Disease is dependent on KRN TCR recognition of G6PI (residues 282-294) when presented by MHC class II Ag7. It has been shown that G6PI autoantibodies are present in these mice and transfer of either serum or affinity-purified G6PI autoantibodies will induce arthritis in healthy mice in a lymphocyte independent fashion (Ji et al., 2002; Matsumoto et al., 1999). These antibodies act through FcγIII receptors and C5a (Ji et al., 2002). Importantly, the AP and not the CP is required for the development of arthritis. Furthermore, in mice it has been shown that anti-G6PI IgG localizes specifically to the joints where arthritis occurs and that localization is dependent on mast cells, neutrophils, FcRs, and ICs (Mandik-Nayak and Allen, 2005).

Traditional views regarding the role of properdin in the activation of the AP has also been changed, since it has now been shown that properdin can bind to target surfaces and initiate AP activation (Hourcade, 2006). It was shown that Cfp-/- mice were significantly protected from development of arthritis induced by serum transfer from K/B x N mice (Kimura et al., 2010).

Complement activation is also critical for the effector phase of arthritis in the CAIA model (Hietala et al., 2004). It was demonstrated that have shown, using this model, that the AP is necessary and sufficient for the development of arthritis (Banda et al., 2006). Consistent with this observation, it was found that fD-/-, C1q-/-/Df-/-, and MBL-/-/Df-/- mice were also resistant to CAIA while C1q-/-/MBL-/- were highly susceptible (Banda et al., 2007, 2010a) (Fig. 1). Interestingly MASP-1/3-/- mice are resistant to CAIA for these mice only have an inactive form of factor D called pro-factor D (pro-Df), and MASP-1 protein is required for the cleavage of inactive pro-Df into active factor D (Banda et al., 2010b; Takahashi et al., 2010). These studies indicate that the AP can be activated by anti-collagen autoantibodies directly and independently from the CP and LP.

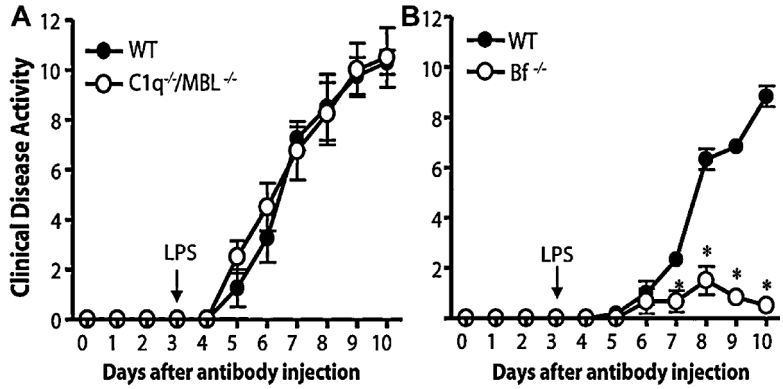

Fig. 1. Antibody mediated arthritis is depending on the AP and not the CP or LP. Clinical disease activity of anti-collagen type II mAb-induced arthritis in WT and Clq-/-/MBL-/- and Bf-/— C57 BL/6 mice (Banda et al., 2006, 2007). Mice were given a mixture of anti-collagen type II mAbs at day 0. All mice were injected with LPS at day 3. The clinical disease activity was assessed every day using a 3-point score for each paw with four paws per animal (maximum score of 12). The data are expressed as the clinical disease activity scores. (A) WT (solid black circles) *(n* = 4) and C1q-/-/MBL-/- (empty white circles) *(n* = 4) mice vs. days after the initial injection of the anti-collagen type II mAb mixture. (B) WT (solid black circles) (n = 6) and Bf-/- (empty white circles) (n = 6) mice vs. days after the initial injection of the anti-collagen type II mAb mixture. These data are represented as mean ± SEM. *All of the p-values for clinical disease activity in Bf-/- mice in comparison with WT were statistically significant at $p < 0.001$.

About a decade ago it has been reported that anti-citrullinated protein antibodies (ACPA) are present in RA patients that can be detected years before disease onset (Rantapaa-Dahlqvist et al., 2003). ACPA can be of IgG, IgM, IgE or IgA isotype (Schuerwegh et al., 2010; Verpoort et al., 2006) and recognize proteins that are post-translationally modified by the conversion of arginine to citrulline by the enzyme peptidyl arginine deiminase (PAD) (Vossenaar et al., 2003). As ACPA are found long before disease onset, it was hypothesized that they could be pathogenic. Human ACPA when tested in vitro have the capacity to activate the CP and AP but not the LP (Trouw et al., 2009).

Injection of one purified mouse IgM ACPA monoclonal antibody against citrullinated fibrinogen was not pathogenic by itself but exacerbated established disease induced by anti-collagen antibodies (Kuhn et al., 2006). In another study five monoclonal autoantibodies to citrullinated collagen type (CII), ACC1-5, were generated (Uysal et al., 2009). Two of these five autoantibodies, ACCI (IgG2c) and ACC5 (IgM), when injected into B10.RIII mice, directly induced arthritis and the severity of disease was increased after the injection of LPS. Importantly, another clone, AAC4 (IgG1), did not induce arthritis. However, when this AAC4 antibody was mixed with an anti-collagen antibody M2139 (IgG2b) it did cause severe arthritis. Since in mice IgG1 does not activate complement while IgG2b is a strong activator of complement this may explain the difference between these monoclonal antibodies in inducing arthritis.

Banda et al. (2008) have shown that the AP of complement is capable of initiating C3 activation induced by ICs of collagen and anti-collagen autoantibodies, and that it requires the presence of N-glycans on the IgG. IgG G0 anti-collagen antibodies activated both the CP and AP more than the LP. The glycosylation status of IgG has also been implicated in the pathogenesis of RA. EndoS, an endoglycosidase isolated from Streptococcus pyogenes, specifically cleaves the terminal sialic acid residues from glycan groups and has been used extensively in recent years as a tool to modify the glycosylation status of pathogenic antibodies. For one EndoS-treated anti-collagen autoantibody, M2139-IgG2b, it was shown to have lost its capacity to induce arthritis in B10.RIII mice (Nandakumar et al., 2007). Interestingly, in these studies EndoS treatment did not affect the binding of anti-collagen antibodies to CII and their capacity to activate complement as there was no difference in C1q deposition on anti-collagen antibodies with and without EndoS treatment bound to CII, but it reduced IgG binding to FcγR and the formation of stable ICs (Table 1). Collectively, these mouse models show that the AP is essential for tissue damage induced by auto-antibodies.

Murine models of SLE

SLE is an auto-immune disease in which complement plays a prominent role. It is characterized by autoantibody production, immune complex formation, complement activation and systemic tissue damage. The deposition of ICs in glomeruli of patients with SLE causes lupus-nephritis via complement activation, generation of C3a and C5a, and MAC formation (Biesecker et al., 1981; Falk et al., 1983; Wyatt et al., 1979). Complement activation increases clearance of ICs but deposition of complement activation products contributes to inflammation, fibrosis and local tissue injury (Couser et al., 1985).

Table 1. *Overview of auto-antibodies directed towards complement components.*

Antibody	Disease association
Anti-C1q	Hypocomplementaemic Urticarial Vasculitis Syndrome; Felty's syndrome; SLE; Rheumatoid vasculitis; Classic polyarthritis nodosa; Sjogren's syndrome; Mixed connective tissue disease; Polygondritis; Temporal arthritis; Mixed cryoglobulinaemia; MPGN; IgA nephropathy; Anti-GBM glomerulonephritis; Membranous glomerulopathy
Anti-C1s	SLE
Anti-C1-INH	SLE
Anti-MBL	SLE
Anti-H ficolin	SLE
Anti-factor B	Dense deposit disease
Anti-factor H	MPGN
Anti-C4	SLE
C3NeF	Post streptococcal glomerulonephritis; MPGN; partial lipodystrophy; SLE
C4NeF	MPGN
Immunoconglutinins	SLE; rheumatoid arthritis; paroxysmal nocturnal hemoglobinuria; chronic liver disease
Anti-CR1	Colitis; SLE; liver cirrhosis
Anti-CR2	HIV
Anti-CR3	Rheumatoid arthritis; auto-immune neutropenia
Anti-CD46	HIV
Anti-CD59	Multiple sclerosis

Adapted from (Norsworthy and Davies, 2003; Seelen et al., 2003a; Trouw et al., 2001), with addition of anti-H ficolin (Yae et al., 1991), anti-MBL (Seelen et al., 2003b), anti-factor H (Skerka et al., 2009) and anti-factor B (Strobel et al., 2010).

Antibodies to double-stranded DNA (dsDNA) represent the hall-mark of SLE and their capacity to fix complement has been associated with disease activity (Mackworth-Young et al., 1986; Rothfield and Stollar, 1967). By using antigen microarray technology to analyze serum derived from lupus prone MRL/lpr mice it has been shown that C3 deposition on nucleic acids is profoundly increased by the presence of mAbs specific for dsDNA (Papp et al., 2010). Observational studies have shown that the inflamed glomeruli of MRL-lpr mice stain positive for markers of CP, LP and AP activation (Trouw et al., 2004b, 2005). MRL/lpr mice lacking either fB or fD developed less renal disease than WT mice (Elliott et al., 2004; Watanabe et al., 2000). Auto-antibodies to dsDNA, did not differ between fD+/+, fD+/- and fD -/- MRL/lpr mice at any age. Thus lack of AP activation protects against proliferative renal disease in MRL/lpr mice. However, C3-/- MRL/lpr mice are not protected from renal disease and there is no difference in autoantibody titer compared with WT mice. This indicates that complement activation is not required for the production of auto-antibodies in MRL/lpr mice. Nonetheless inhibition of the AP is beneficial in lupus nephritis despite glomerular IgG deposition mediated effects. Blocking the AP has profound effects next

to SLE also in many inflammatory and renal diseases underscoring the generality of this pathogenic mechanism (Fung et al., 2001; Kalli et al., 1994; Moore, 1994; Pascual et al., 1993; Tanhehco et al., 1999). In these mouse models of SLE also anti-C1q autoantibodies play a role, which will be discussed in below.

Murine models of subepidermal blistering disease

Epidermolysis bullosa acquisita (EBA) is an auto-immune disease with chronic subepidermal blisters of skin and mucous membranes and is characterized by autoantibodies to type VII collagen (COL7) (Lapiere et al., 1993; Woodley et al., 1988). Experimental EBA can be induced in mice by injecting rabbit or human anti-mouse COL7 IgG (Sitaru et al., 2006; Woodley et al., 2005, 2006). The pathogenic relevance of COL7 auto-antibodies has been shown by passively transferring these antibodies in mice and by injecting mice with autologous COL7 (Sitaru et al., 2006). Anti-COL7 antibodies induced blisters in normal mice but failed to induce blisters in C5-deficient mice, supporting the role of complement activation in the pathogenesis of EBA. Additionally, using genetic manipulations similar to those described above, it has been shown that complement activation by the AP is required to elicit disease (Mihai et al., 2007).

Bullous pemphigoid (BP) is another example of an auto-immune subepidermal blistering disease (Gudi et al., 2005; Langan et al., 2008). Serum anti-human COL17 autoantibodies target human type XVII collagen (Robson et al., 2003). Deposition of anti-human COL17 auto-antibodies at the basement membrane zone triggers a sequence of inflammatory cascades including complement activation, infiltration of neutrophils and eosinphils, and degranulation of dermal mast cells (Chen et al., 2001; Liu et al., 2008; Nishie et al., 2007; Oikarinen et al., 1983; Robson et al., 2003; Stahle-Backdahl et al., 1994). Recently, an anti-hCOL17 IgG1 monoclonal antibody was shown to effectively reproduce a BP-like phenotype in mice (Li et al., 2010). Interestingly, a human IgG1 monoclonal antibody, mutated at residue P331 in the Fc region, lost its capacity to activate complement and also lost its pathogenicity. This is the first report that IgG1 antibodies to hCOL17 can induce blister formation in mice and it raises the possibility that an IgG1 monoclonal with an Fc modification may be used to block pathogenic epitopes in auto-immune disease (Li et al., 2010).

Although the initiation of the complement activation normally takes place by the CP or by the LP, potentially it is the AP amplification that causes most tissue damage. In addition the AP itself can be activated by auto-antibodies in the absence of CP and LP to cause tissue damage.

Why is the absence of any component of the AP so protective for tissue damage induced by autoantibodies? The major part of the pro-inflammatory C5a is generated by the AP (Banda et al., 2010a) and each component of the AP uniquely participates in this activation process. Whether properdin binds to autoantibodies and initiates AP of the complement is a subject of future research.

To what extent this is also true for humans is not clear. One in vitro study, compared the C3a and C5a generation by either the CP or AP induced by anti-collagen antibodies using

mouse and human sera. The level of C5a generated by the AP alone (79.1%) in mouse sera was significantly higher than the level generated by the CP alone (46.6%). However, the opposite was observed with human sera (Banda et al., 2010a,b). Also of note is the observation that C3 deficient persons can develop SLE, indicating that in the absence of complement activation, immunecomplex mediated damage can occur. However, in complement sufficient SLE patients complement activation clearly contributes to damage.

Autoantibodies to complement components

Next to the fact that several complement components like MBL, C1q and Properdin can interact with antibodies and result in complement activation, complement itself may be a target of autoantibodies (Norsworthy and Davies, 2003; Trouw et al., 2001). In this regard the best known autoantibodies are directed against for instance C1q and the amplification convertase C3bBb. In this section we provide a very brief overview of autoantibodies against complement.

Autoantibodies against several complement components have been described including autoantibodies against C1q and MBL(Seelen et al., 2005; Siegert et al., 1991; Trendelenburg et al., 1999; Trouw and Daha, 2005). Both of these autoantibodies have been described in patients with systemic lupus erythematodus (SLE) but may also be found in other diseases. Generally the auto-antibodies are directed against neoepitopes and this is evident especially concerning autoantibodies against C1q that react mainly with immune-complex-bound C1q.

Anti-C1q autoantibodies are present in 30-50% of SLE patients (Monova et al., 2002; Siegert et al., 1991), and are strongly associated with the development of lupus nephritis. The absence of these antibodies seems to exclude active renal disease (Trendelenburg et al., 1999). However high titers of anti-C1q autoantibodies have also been reported in hypocomplementaemic urticarial vasculitis syndrome (HUVS), suggesting that there may be a contextual element to the deposition of this antibody (Wisnieski and Naff, 1989).

There is strong evidence that anti-C1q autoantibodies may result in direct interaction with C1q that is transiently present in the glomerular basement membrane (GBM) leading to a low degree of inflammation accompanied by infiltration of polymorphonuclear leukocytes. However for a pronounced induction of renal injury and proteinuria more than one hit seems to be required (Trouw et al., 2004a). Anti-C1q autoantibodies are present in many strains of auto-immune mice (Hogarth et al., 1996; Trinder et al., 1995). In MRL-lpr mice a temporal association between the occurrence of anti-C1q autoantibodies and nephritis was observed (Trouw et al., 2004b) whereas in another model of lupus nephritis no such correlation was found (Bigler et al., 2010). In search of the involved mechanistics, mice were pretreated with a subnephritogenic dose of anti-GBM antibody followed by an injection of anti-C1q, and a complement-dependent glomerular inflammation was induced (Trouw et al., 2004a). Mice treated with anti-GBM antibody or anti-C1q antibody alone developed no renal disease. Anti-C1q antibodies, were dependent on their antigen C1q, as this effect was not seen in C1q-/- mice. The combination of anti-GBM and anti-C1q

antibodies caused no glomerular damage in C4-/-, C3-/- and FcɣR receptor-deficient mice, demonstrating that the mechanism of glomerular damage was dependent on the CP and on FcɣR receptors.

Auto-antibodies against MBL have been described also in patients with SLE (Seelen et al., 2003b; Trendelenburg et al., 1999; Trouw and Daha, 2005) but seem to occur only in patient that are not deficient for MBL. In SLE patients, MBL deficiency, is associated with increased presence of autoantibodies against cardiolipin and against C1q (Seelen et al., 2005). The hypothesis is that an enhanced presence of autoantibodies may be related to disturbed clearance of apoptotic cells due to impaired MBL function.

Several autoantibodies against complement regulators have been reported including autoantibodies against factor H and C1-inhibitor. Anti-factor H autoantibodies are strongly related with the occurrence of atypical hemolytic uremic syndrome and C3-nephropathies (Dragon-Durey et al., 2010a). The presence of anti factor H autoantibodies induces systemic AP activation resulting in low plasma levels of C3 and factor B. The antigenic level of factor H may be subnormal but more importantly the binding of these autoantibodies causes a functional defect in factor H imparing its capacity to interact with endothelial surfaces (Dragon-Durey et al., 2010b). The age at onset of the anti factor H auto-antibodies varies between the very young of less than 1 year up to 13 years in age (Dragon-Durey et al., 2010a).

The presence of anti-C1-inhibitor autoantibodies is a hallmark of acquired C1-inhibitor deficiency (Donaldson et al., 1996; Meszaros et al., 2010). A recent study in a large cohort of

SLE patients revealed that the level of anti-Cl-inhibitor-autoantibodies was significantly higher in SLE patients than in controls, which was associated with disease activity. Acquired Cl-inhibitor deficiency in elderly patients is in most cases associated with clonal B cell diseases, most likely due to production of monoclonal antibodies against C1 inhibitor. Several studies have indicated that the anti-Cl-inhibitor-autoantibodies result both in inhibition of functional activity of Cl-inhibitor and in reduction in Cl-inhibitor levels and as a consequence in deregulation of both the CP and LP.

Two convertases determine the degree of activation of the central component of complement C3. These are the CP/LP induced C3-convertase C4b2a and the amplification convertase C3bBb. Autoantibodies against C4b2a (C4NeF) were described in patients with SLE (Daha et al., l980; Gigli et al., l985) and shown to functionally stabilize the otherwise labile enzyme resulting in enhanced activation of C3, low levels of C3 and potentially in inflammation. Unfortunately C4NeF had received little attention in later years.

An activity present in serum of patients with membranoproliferative glomerulonephritis (MPGN) that was able to induce activation of complement in normal human serum via the AP was recognized for the first time by West et al. (l965) in the early sixties. This activity was indicated as C3-nephritic factor (C3NeF). Since then C3NeF has received broad attention. It was demonstrated that C3NeF is an auto-antibody directed against C3bBb and that

it stabilizes the otherwise labile C3bBb convertase giving it a longer half-life and thereby the potential to activate more C3 resulting in reduced circulating levels of C3. C3NeF is highly associated with the occurrence of MPGN. Stabilization of the C3bBb convertase can also occur by Properdin (Fearon and Austen, l975) but there is a main difference between Properdin and C3NeF. While the stabilization by C3NeF gives a biphasic decay characteristic of the C3bBb convertase sites (Daha et al., l976), the Properdin stabilization results in a linear decay pattern. This suggests that C3NeF only stabilizes the C3bBb convertase sites to which it is bound while Properdin seems to interact with all convertase sites dependent on the concentration of properdin used and presumably by moving from one site to another.

Results obtained with a large number of C3NeF preparations suggest that C3NeF is an auto-antibody directed against antigenic determinants on Bb, which are exposed after interaction of B with C3b (Daha and Van Es, l98l). Taken together C3NeF seems to be responsible for hyper catabolism of C3 with the consequence that activated C3 is deposited in the kidney resulting subsequently in injury to the kidney. One study provided the proof-of-concept showing that three mouse IgM monoclonal antibodies had C3Nef-like activity (Daha et al., l984). More recent studies indicate that several mechanisms including deregulation of convertase function as can be seen in patients with atypical hemolytic syndrome, gain of function mutations in C3 and B, or the occurrence of anti-H and anti-B auto-antibodies are all associated with hypercatabolism of C3 and induction of vascular injury (Skerka et al., 2009).

Therapeutical antibodies and complement activation

With the approval by the US Food and Drug Administration of 28 monoclonal antibodies for use in humans in 20ll, these antibodies have become a critical component of clinical treatment regimens in a large variety of indications. Many therapeutic monoclonal antibodies exert their killing activity through several mechanisms of action. Whereas several antibodies kill via apoptosis, receptor down modulation or by altering signal transduction, others use the immune system to induce efficient killing of target cells. Indeed, successful therapeutic antibodies like alemtuzumab and rituximab seem to be well able to eradicate target cells via antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) (Teeling et al., 2004, 2006; Waldmann and Hale, 2005). Alemtuzumab targets CD52 on B- and T-cell cancers, while rituximab targets CD20 on B-cells and is used in the treatment of many lymphomas, leukemias, transplant rejection and some autoimmune disorders (Olszewski and Grossbard, 2004). A large body of evidence supports the role of complement activation in antibody immunotherapy in patients (Cragg et al., 2003; Di Gaetano et al., 2003; Golay et al., 2000, 2006; Idusogie et al., 2000; Manches et al., 2003; Reff et al., l994; Zent et al., 2008). Furthermore, under certain complement limiting conditions, rituximab can become ineffective (Glennie et al., 2007; Taylor and Lindorfer, 2008). Fresh frozen plasma could enhance or restore complement titers and rituximab therapy in CLL patients, which was probably due to the exhaustion of the limiting factor C2 (Kennedy et al., 2004; Klepfish et al., 2008; Taylor, 2007).

The role of complement in immunotherapy is further supported by the observation that polymorphisms in C1q were associated with response rates to rituximab treatment in follicular lymphoma (Racila et al., 2008).

The fact that therapeutic monoclonal antibodies are able to use the complement system to induce target cell killing is intriguing. First of all, they need to overcome the inhibitory effects of a set of plasma proteins and membrane-bound complement regulatory proteins, which prevent unintended injury by our own activated complement (Atkinson et al., l99l; Junnikkala et al., 2000; Kim and Song, 2006; Kirkitadze et al., l999; Liszewski and Atkinson, l996; Morgan et al., l998). Especially tumor cells, in comparison to normal cells, can be difficult to kill since complement inhibitors may be upregulated such as via cytokines, streamers or be the result of selection (Beum et al., 2008; Blok et al., 2003; Cruz et al., 2007; Teeling et al., 2004).

Secondly, binding to very small or compact surface proteins, like CD20, CD52 and CD38 seems to be a prerequisite for killing via CDC (de Weers et al., 20ll; Polyak et al., l998; Xia et al., l993). Possibly, binding close to the cell surface facilitates effective capture and concentration of active complement components proximating to the cell membrane, potentially shielding activated complement components (C4 and C3) from inactivation by fluid phase inhibitors, overcoming inhibition by mCRPs and promoting efficient generation of the MAC complex (Beum et al., 2008; Bindon et al., l988; Michaelsen et al., l990). This is further exemplified by atumumab that binds an epitope encompassing both the small and large loop on the CD20 molecule very close to the cell membrane, which results in killing of B cells with low CD20 densities, and reduced sensitivity to complement inhibitors (Beum et al., 2008; Pawluczkowycz et al., 2009; Teeling et al., 2004, 2006). Also other factors, like binding avidity to C1q and clustering of surface antigens into lipid rafts may further influence CDC and subsequent therapeutic activity (Cragg et al., 2003; Mone et al., 2006; Pawluczkowycz et al., 2009; Taylor and Lindorfer, 20l0; Teeling et al., 2006; Wierda et al., 20l0).

Recent work has shed light on the role of complement factors, C1q, and C3 in opsonization of target cells (Gelderman et al., 2004; Racila et al., 2008). A split product of the initiation of the MAC complex, C5a, is thought to contribute to the complement mechanism of action. C5a is a potent chemoattractant, stimulator and survival factor of effector cells which are involved in the elimination of malignant cells (Monk et al., l994; Perianayagam et al., 2002). It also modulates IgG Fc receptor expression via upregulation of activating and downregulation of inhibitory IgG Fc receptors (Shushakova et al., 2002). By using fusion proteins containing C5a, enhanced ADCC of breast cancer cells was shown (Fuenmayor et al., 20l0).The importance ofCR3 in tumor cell killing was underscored by Boross et al. (20ll) who recently showed that antibodies can kill tumor cells in vivo after binding of the complement receptor CR3 to C3 on target cells.

Recent evidence shows that CDC can be further augmented by conferring more potent CDC activity of IgG. This was done by exchanging segments between IgG isotypes to

generate a variety of chimeric IgG molecules or by amino acid substitutions (Dall'Acqua et al., 2006; Idusogie et al., 2001; Michaelsen et al., 2009; Moore et al., 2010; Natsume et al., 2009). Optimization of IgG1 antibodies for complement has enormous potential for improving the next generation of therapeutic antibodies which could bear great promise to increase the chances to cure patients in the future.

CONCLUSIONS

Collectively we conclude that antibodies, as a traditional hallmark of adaptive immunity, contribute to host protection and to tissue damage by utilizing additional mediators of the innate immune system and especially complement activation. All three pathways of complement can be initiated directly by antibodies. However, clearly not all antibodies activate each pathway. Different isotypes have a different capacity to trigger complement activation in vitro. In-vivo however, the relative contribution of each isotype is less clear. Even the relatively weak activators may still activate sufficient complement as to result in tissue pathology as for instance seen in IgA nephropathy. The molecular composition, sequence, density and glycosylation status of the different antibody isotypes determine, to a certain extend, their ability to activate each of the pathways. Such modifications provide us with the possibility to interfere with the pathogenic effects of autoantibodies and serve as a tool for optimization of therapeutic antibodies. Obviously complement activation by antibodies is not just bad news, it provides an important effector mechanism to clear pathogens and may be used therapeutically to kill tumor cells or autoreactive immune cells.

The fact that autoantibodies not directed against a tissue antigen but rather against a complement factor or a convertase may still induce a tissue specific inflammatory response, makes it difficult to dissect the relative contribution of each autoantibody to the overall disease process. The traditional view that only the CP is directly activated by antibodies and that only IgG and IgM can activate complement are both replaced by the concept that all pathways of complement can be activated by antibodies directly and that no general rules apply to isotypes and or subtypes regarding their in vivo complement activating potential. Additionally, once tissue injury has been initiated, the tissue itself, as exemplified by the activation of all three pathways by cell debris, may directly contribute to enhanced tissue damage.

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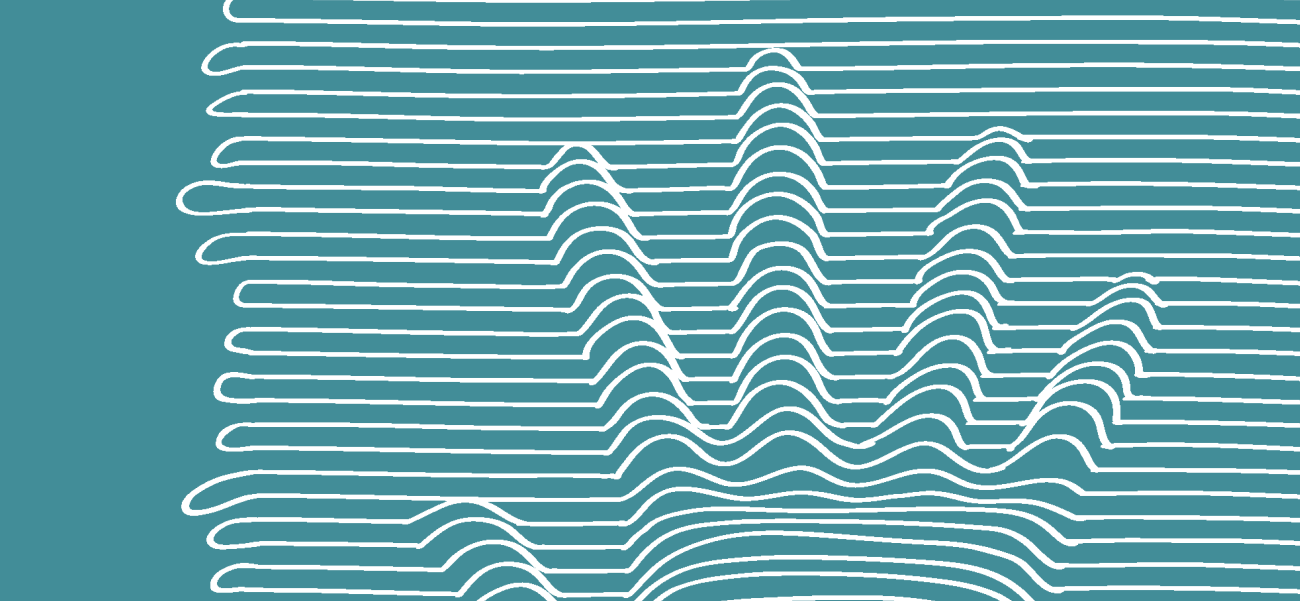

CHAPTER 9

Summary and discussion

Rheumatoid arthritis is a chronic auto-immune disorder, of which persistent synovitis, bone erosions and auto-antibody formation are characteristic features[1]. Although the etiology of the disease remains largely unknown, it is established that genetic risk factors play a pivotal role in disease pathology. Both family and twin studies have shown that the genetic contribution to the disease can be estimated around 50%[2-4].

The most important genetic risk factor for RA was first described in the 1970's and is located in the HLA region. The associated HLA-DRB1 alleles all encode a similar amino-acid sequence at a particular position of the hyper variable region of the HLA class II molecule. This finding resulted in the SE hypothesis, in which was proposed that the SE motif is directly involved in RA pathogenesis. It was postulated that in this binding groove, the HLA class II molecule could present a specific arthritogenic peptide to auto-reactive T-cells[5, 6]. Although it has been shown that epitopes derived from human citrullinated vimentin can be recognized by HLA-DRB1*0401 restricted T-cells, the hypothesis still remains to be elucidated[7].

After the identification of the HLA-DRB1 alleles, it was not until 2004 that the first non-HLA locus that associated with RA susceptibility was identified[8]. Due to advances in genotyping technologies, which could be performed at reasonable costs, the identification of novel genetic risk factors took a flight[9].

In the current thesis the genetic contribution of non-HLA genes to RA susceptibility was further investigated and the functional relevance of these loci in disease pathology was explored.

Establishing genetic risk factors

The recent burst in genetic association studies for disease susceptibility has led to the identification of many risk loci associated with RA susceptibility. Although part of these newly identified loci will truly be associated with RA, part will show to be type I errors, in which the associations appear to be false positive findings. To separate the true findings from the false positive ones, it is of utmost importance to replicate newly identified genes in independent populations of similar origin. In both chapter 2 and chapter 3, such replication studies for recently identified genes were performed.

In chapter 2 three SNPs in STAT4, IL2/IL21 and CTLA4 genes, were investigated in the Leiden RA population. Subsequently, a meta-analysis of all publicly available genotype data was performed to identify the overall genetic association with RA susceptibility. The association with the STAT4 gene was first described in 2007 and replicated consistently in several Caucasian and Asian populations[10-12]. The IL2/IL21 region, on the other hand, had only been described in one study thus far and the CTLA4 gene had shown inconsistent results in several replication studies[13-16]. In the present study, both the SNPs in the STAT4 gene and the CTLA4 gene could be independently replicated in our Dutch RA population, while the SNP in the IL2/IL21 region showed a clear trend towards association. More importantly, all three loci were further confirmed in a well powered meta-analysis, indicating these loci as true genetic risk factors for RA susceptibility.

In chapter 3 a replication study of two SNPs located in the interleukin 2 pathway is described. A recent GWAS in the WTCCC population had indicated the IL2RA and IL2RB regions, located in this pathway, to be novel genetic risk factors for RA[17]. These findings were replicated in our independent Dutch RA population, thereby providing further evidence for true association.

Missing heritability

In addition to the in chapter 2 and chapter 3 described loci, currently over 46 genetic regions have been identified as true genetic risk factors with statistically robust associations. These associations however, confer relatively small increments in risk and thereby contributing only marginally to disease susceptibility[9, 18]. It is thought therefore, that a great part of the genetic risk for RA has not yet been explained. Even including the HLA region, it is likely that more than 50% of the genetic risk for RA remains to be identified. Many explanations for this 'missing heritability' have been suggested, including structural variants that were poorly captured by the existing genotyping arrays, gene-gene interactions and inadequate account of the shared environment among relatives. An additional possibility, on which most speculations have been focused, is that multiple rare variants contribute to disease risk in a significant subset of the RA population. Rare variants in this matter are defined as variants with an allele frequency less than 5%, or even less than 1%, but not rarer than 0.5%. Such variants are not sufficiently frequent to be captured by the current GWAS arrays, nor do they have large enough effect sizes to be identified by linkage analysis in family studies. These variants can be identified by resequencing genomes of patients and healthy individuals[19]. These low frequency variants will have higher effect sizes than the currently identified non-HLA risk loci and could thereby contribute substantially to the current 'missing heritability'.

Next to the investigation of rare variants, structural variants and gene-gene interactions to explore the 'missing heritability', additional common variants can be identified by candidate gene approach. Although the contribution of these variants to disease risk have shown to be marginal and the candidate gene approach has its disadvantages compared to GWAS, a candidate gene study might identify a risk factor that has simply not been typed or identified in the large scale assays.

In chapter 5 a study is described, in which common variants located in the VTCN1 region were investigated for association with RA susceptibility by candidate gene approach. VTCN1 encodes a protein, which has been reported to be a negative regulator of T-cell responses in vitro by inhibiting proliferation and cytokine production[20, 21]. In RA patients the soluble form of the protein is more frequently observed than in healthy individuals and is correlated with disease activity as measured by DAS 28[22]. In knock-out mice, the progression of inflammation in a collagen induced arthritis model was accelerated compared to wild type mice[22]. In addition to these functional indications that VTCN1 plays a role in RA pathology, genetic studies in juvenile idiopathic arthritis identified this region to associate with this disease[23]. Since both functional and genetic data indicated a possible role of VTCN1 in RA, the genetic contribution to RA susceptibility was investigated by a candidate gene approach in our Leiden RA population.

In the discovery phase of this study, a significant association for two SNPs was observed. Replication of these findings in two independent populations from northern-European descent, showed an overall significant association for both variants. These data indicate that this is a novel genetic risk factor for RA susceptibility, although further replication in independent cohorts is necessary to tease apart the role of VTCN1 genetics in RA.

In chapter 7 another novel genetic risk factor is identified by candidate gene approach. In this study the genetic contribution of the C1Q genes was investigated in relation to RA susceptibility. As well as in chapter 5, this region was chosen upon strong indications of both functional and genetic data. It was observed that a complete genetic deficiency of C1Q is strongly associated with the development of Systemic Lupus Erythematosus[24]. Moreover, complement deposits can be found in the synovium of RA patients and a correlation between disease activity and the presence of activated complement fragments bound to C1q in sera of RA patients has been described[25, 26].

In the Leiden RA population five SNPs in the C1Q genes were significantly associated with disease. Subsequent replication of the most significant SNP in three independent populations supported the initial finding. Additionally, in a meta-analysis of six GWAS, containing a substantial number of patients and controls, the association was maintained, even though with borderline significance.

Disease subsets

The identification of additional variants, being rare variants, structural variants, gene-gene interactions or common variants, will probably lead to the identification of a substantial part of the genetic risk conferred to RA. It can be expected however, that part of the genetic background of RA, as described currently, will not be elucidated. RA is a phenotypic disease and is defined by use of classification criteria. In these criteria, points are scored for certain disease features and an individual is classified as having RA when a minimum score is obtained[27]. This results in a heterogeneous disease, with highly variable disease outcomes between patients. It can be expected that the current classification embraces several disease subgroups, each with their own distinct risk factors and pathophysiology. This implies that a more precise definition is needed and further research towards specific immunological or other biologic features that distinct subgroups is desired.

To date, a feature that has been shown to identify specific subgroups of the disease is ACPA. It is known that, to a certain extent, presence or absence of ACPAs can predict disease outcome and these antibodies are one of the best clinical predictors of radiological progression[28]. Moreover, it has been shown that patients with ACPA-negative disease are more likely to achieve drug-free remission than those who test positive for these autoantibodies[29]. Interestingly, the most prominent genetic risk factor for RA, the HLA shared epitope region, predisposes to ACPA positive RA, whereas another haplotype in this region, *HLA-DRB1*03*, predisposes to ACPA-negative disease[30, 31]. The majority of loci that correlate with RA have, however, been identified in ACPA-positive patient populations, and little is known about the genetic contribution to the ACPA-negative subset[32]. Small

studies have suggested a role for *IRF5* and genes encoding C-type lectin-like receptors in ACPA-negative disease, and, in chapter 2 of this thesis, it was shown that a polymorphism of *STAT4* is associated with both disease subsets, whereas a variant of *CTLA4* is associated with ACPA-positive RA only[33, 34]. Together, these studies reveal that ACPA-positive and ACPA-negative RA have different genetic association patterns.

To gain further insight into these association patterns, a genome-wide association study in ACPA-positive and ACPA-negative patient populations was performed recently[35]. More than 1.7 million SNPs were studied for association with disease, in 774 ACPA-negative and 1,147 ACPA-positive patients with RA. The ACPA-positive findings were subsequently replicated in two RA cohorts of Western European descent. When the ACPA-positive subset was compared with the ACPA-negative subset, genome-wide significant differences between the two groups (P <2.9 × 10–8) could be established for several SNPs within the HLA region. No significant associations of loci located outside the HLA region could be identified, which is likely to be due to the insufficient power provided by the relatively small ACPA-negative study population. Nonetheless, these data support the idea that genetic backgrounds contribute differently to the two RA disease subsets characterized by ACPA status. Expansion of the ACPA-negative study population is needed to elucidate the genetic contribution of loci outside the HLA region.

The data of Padyukov *et al.*[35] further suggest, in combination with the results of previous studies, that distinct risk factors operate in the two ACPA subsets. This divergence implies that different pathophysiology underlies ACPA-positive and ACPA-negative RA, and therefore, that these subsets should be considered as two separate diseases, and studied separately in both genetic and functional studies of RA pathophysiology. Previous functional studies have shown that in ACPA-positive RA, immunological responses occur in a citrulline-specific manner, and, in mouse models, it has been shown that citrulline-specific antibodies can induce and promote arthritis[36, 37]. Furthermore, activation of basophils from ACPA-positive patients with RA, in contrast to those from ACPA-negative patients, occurs upon exposure to citrullinated antigens[36]. These findings thus indicate a differential response of immune cells to citrullinated antigens.

Diseases with a distinct pathogenesis might, logically, benefit from different treatment strategies. In RA the mainstays of treatment are DMARDs, which are a heterogeneous collection of therapeutic agents for which mechanisms of action are, largely, not well understood. Methotrexate is the most prominent DMARD and is widely used for the treatment of RA and other inflammatory diseases. ACPA-positive patients with undifferentiated arthritis treated with methotrexate are less likely to progress to RA, and do so at a later time point than a placebo control group[38]. By contrast, no effect of methotrexate therapy on progression to RA could be observed in an ACPA negative patient population, indicating that the two ACPA subgroups respond differently to methotrexate treatment[38].

It is not just methotrexate therapy that produces different outcomes in the two disease subsets. In RA refractory to therapy with tumor necrosis factor blockers, rituximab—a

monoclonal antibody directed towards the B cell marker CD20—has proven to be an effective therapy. Upon binding of rituximab, circulating B cell populations are depleted for periods of at least 3 months. Recently, in a clinical study of rituximab in 208 patients with refractory RA, it was shown that the presence of ACPA predicted a better EULAR (European League Against Rheumatism) response at 24 weeks, indicating that this drug might have a greater role in the ACPA-positive subset than in ACPA-negative patients[39].

Thus, although little is known about the genetic contribution to the development of ACPA-negative RA, data suggests contrasting genetic backgrounds for the disease subsets characterized by ACPA status. This genetic divergence lends further support that distinct genetic risk factors play a part in specific subsets of the disease. To fully identify the genetic risk conferred to RA, genetic risk factors that contribute only to certain disease subgroups should be identified.

In chapter 6, by a candidate gene approach, polymorphisms in the PTGES gene were investigated for association with RA susceptibility and disease characteristics. The PTGES gene is involved in prostaglandin synthesis, a key mediator of inflammation. The prostaglandin production is an important target for non-steroid-anti-inflammatory drugs (NSAIDs), which are key painkillers in the therapy of RA patients[40, 41]. The initial study in a Swedish RA population indicated that no genetic association could be identified in the RA population as a whole. However, upon investigation of disease subgroups it seemed that the association was mainly conferred to female RA patients. Although this finding could not be replicated independently in our Leiden RA population, the minor allele frequencies were biased in the same direction. Further replication of this finding is needed to tease apart the exact role of the PTGES gene in female RA patients, however, it does indicate that genetic risk factors might only contribute to a subset of the disease and further investigation into these subsets is desirable.

Linking genotype to biological pathways

The identification of variants that make up the genetic risk conferred to RA is thought to provide valuable insight into the pathophysiology that underlies the disease. The next step is to translate the genetic associations into biological pathways and use this knowledge for the invention of curative therapies for RA.

As more auto-immune susceptibility loci are being identified, it becomes clear that part of these genes overlap between auto-immune diseases. Family based epidemiology studies have suggested there is a shared genetic basis underlying auto-immune diseases in general, which can explain this overlap[42]. The great number of overlapping genes, however, might partly be due to the way genetic association studies are currently performed. In both GWAS and candidate gene studies, genetic risk factors are identified by comparing patients to healthy individuals. Although this approach is considered best practice, it does not distinguish between RA and inflammation in general. Therefore, in functional studies to identify the biological relevance of a genetic risk factor this should be taken into account.

To date, over 46 genetic risk factors have been established to associate with RA suscep-

tibility[18]. Interestingly, all identified genes are located within immunological pathways, as was expected before hand. However, all the identified genes are possible candidates for further functional studies and their relevance in disease pathology is not yet elucidated. It should be noted however, that the significantly associated variants are generally named after known immune genes that are located in the region. Sometimes only one gene is located nearby, making it a reasonable assumption this gene is related to the associated variant. Other times, however, the variant might be located in a region harboring several genes. An association can also be due to a genetic feature, like a micro-RNA or a regulatory element, which has not been described yet. Although this is for a big part due to the fact that the actual causative variant has not been identified, studies towards exploring the functional consequence of a variant are difficult to perform. Thereby, to translate the genetic associations into biological pathways, great hurdles will have to be taken.

In the candidate gene studies described in this thesis, an attempt to identify the functional relevance of the associated genetic variant is made. Although mRNA expression levels and serum levels of the associated proteins and genes could be significantly related to the genotype, these studies all identified further associations and no functional pathways were observed, further indicating that the identification of the disease pathology by translating the genetic association into a biological pathway involved in disease remains a difficult task to perform.

Concluding remarks

In the current thesis the genetic contribution of non-HLA genes to RA susceptibility was further investigated and the functional relevance of these loci was explored. The studies described were able to establish several previously identified risk factors in a statistical robust manner. Also novel genetic risk factors that are associated with RA susceptibility could be identified, as well as risk factors that are conferred to specific subgroups of the disease. The next step towards understanding the pathophysiology of RA will be to identify the relevance of these risk factors, which might prove to be of great challenge.

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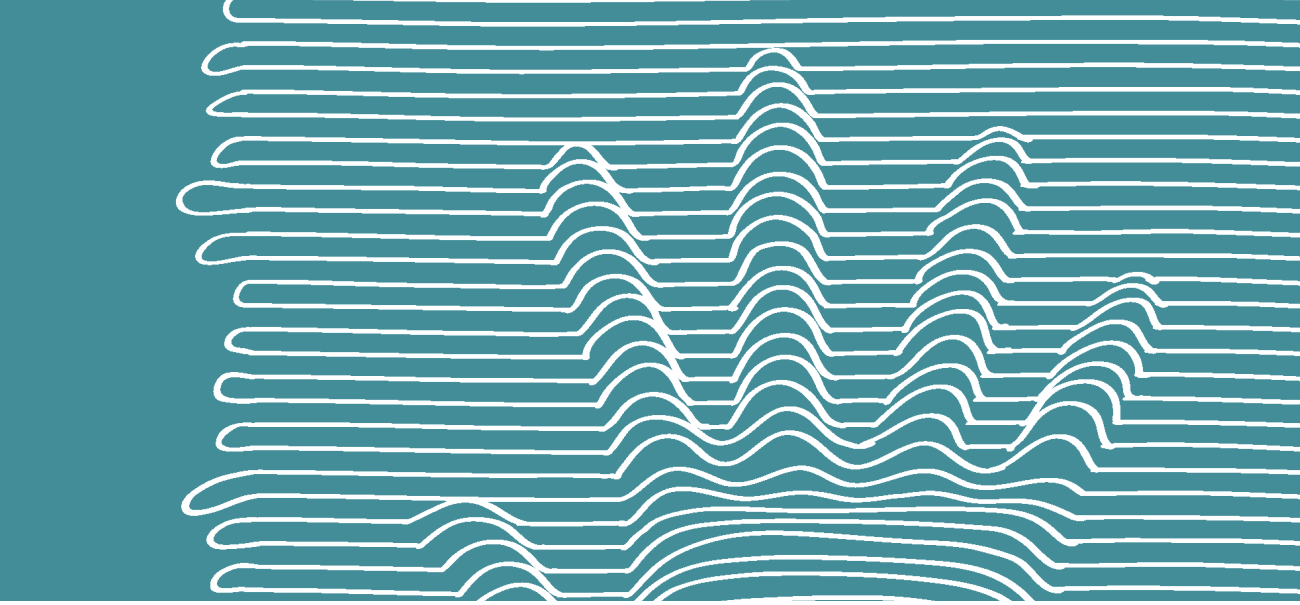


CHAPTER 10

Nederlandse samenvatting

Reumatoïde artritis (RA) is een veel voorkomende auto-immuun aandoening waarbij een chronische ontsteking van de gewrichten een centrale rol speelt. Ondanks een nog grotendeels onbekende etiologie, is het bekend dat zowel genetische- als omgevingsfactoren een rol spelen bij het ontstaan van de ziekte.

In dit proefschrift worden verscheidene studies beschreven, die de rol van genetica binnen de etiologie van RA bestuderen.

Reumatoïde artritis

RA is een auto-immuun aandoening die gekarakteriseerd wordt door een chronische ontsteking van het synovium, wat kan lijden tot beschadiging van het kraakbeen en subchondraal bot met als gevolg misvormingen en invaliditeit. In de westerse wereld is ongeveer 0,5-1% van de volwassen populatie gediagnosticeerd met deze aandoening. Het komt vaker voor bij vrouwen dan bij mannen en de prevalentie neemt toe met de leeftijd.

Patiënten presenteren zich vaak met gezwollen en pijnlijke gewrichten, waarbij voornamelijk de kleine hand- en voetgewrichten zijn aangedaan. Karakteristiek voor een beginnende RA is een symmetrische ontsteking van deze gewrichten.

De belangrijkste pijler binnen de behandeling van RA is de zogenaamde Disease Modifying Anti Rheumatic Drugs (DMARDs), wat een heterogene groep van therapeutische middelen omvat waarvan het werkingsmechanisme grotendeels onbekend is. Daarnaast speelt de groep van biologicals, zoals tumor necrosis factor (TNF) blokkers een belangrijke rol in het effectief onderdrukken van de ontsteking. Tegenwoordig kan hierdoor, bij het grootste deel van de patiënten, onderdrukking van de chronische ontsteking worden bewerkstelligd met als gevolg het beperken van gewrichtsschade en invaliditeit.

Pathofysiologie

Door infiltratie van inflammatoire cellen, zoals macrofagen, T- en B-cellen in het synoviale weefsel van RA patiënten vindt een sterke toename van dit weefsel plaats. Ter plaatse van de overgang van dit synoviale weefsel naar kraakbeen kan het ontstoken weefsel lokaal ingroeien en ontstaat het zogenoemde pannus weefsel, wat schade aan kraakbeen en bot en vorming van erosies tot gevolg heeft. Daarnaast zorgt deze chronische ontsteking voor een diffuse afbraak van kraakbeen, wat leidt tot gewrichtsspleet versmalling.

RA wordt algemeen beschouwd als een auto-immuunziekte, gezien de aanwezigheid van auto-antilichamen en auto-reactieve T-cellen in zowel het perifere bloed, als in synoviaal vocht. Deze anti-lichamen zijn reeds aanwezig in de eerste stadia van de ziekte en kunnen enkele jaren voor het ontstaan van de ziekte al worden aangetoond.

Het klassieke auto-antilichaam binnen RA is reuma factor (RF). Dit antilichaam bindt aan het Fc gedeelte van immuunglobuline G, waarbij het een belangrijke rol speelt in complement activatie en interactie met Fc receptoren. De precieze rol van RF binnen de pathofysiologie van RA is nog grotendeels onbekend. De sensitiviteit varieert tussen de 60 en 70% en de specificiteit tussen de 50 en 90%.

Een ander belangrijk auto-antilichaam binnen RA is Anti Citrullinated Peptide Antibody (ACPA) wat gericht is tegen gecitrullineerde eiwitten. Deze eiwitten ontstaan na post-translationele modificatie van eiwit gebonden arginine.

De rol van deze antilichamen binnen de pathofysiologie van RA is nog grotendeels onbekend, maar mogelijk spelen deze antilichamen een belangrijke rol in de etiologie van de ziekte.

Risicofactoren

Bij de ontwikkeling van RA spelen zowel omgevings- als genetische factoren een belangrijke rol. Uit studies is gebleken dat ongeveer 50% toebedeeld kan worden aan erfelijke factoren, waarvan het human leukocyte antigen (HLA) locus het belangrijkste is. Als gevolg van nieuwe genotyperingstechnieken is de identificatie van genetische risicofactoren die geassocieerd zijn met de ziekte de afgelopen jaren in een sneltreinvaart verlopen. Momenteel zijn ruim honderd genetische variaties geïdentificeerd die associëren met het hebben RA.

In dit proefschrift onderzochten we de contributie van verschillende genetische varianten die buiten het HLA locus zijn gelegen.

Genetische risicofactoren

Replicatiestudies

Nadat aangetoond is dat een bepaalde genetische variatie associeert met het hebben van RA, is het van belang deze associatie ook in andere cohorten te onderzoeken en zo de vals positieve bevindingen te identificeren. In zowel hoofdstuk twee als drie worden twee van deze replicatiestudies beschreven. In **hoofdstuk twee** worden drie, eerder geïdentificeerde genetische variaties, respectievelijk STAT4, CTLA4 en IL2/IL21, onderzocht op associatie binnen een Nederlands cohort van RA patiënten. Voor alle drie de varianten kon een associatie worden aangetoond zowel in het Nederlandse cohort, als in een meta-analyse van alle tot dan toe gepubliceerde studies. Binnen de gedachte dat ACPA positieve RA mogelijk een andere pathofysiologie kent dan ACPA negatieve RA, is ook onderzocht of deze varianten verschillend associeerden binnen deze twee patiënten groepen. Gezien het feit dat er onvoldoende data beschikbaar was voor een gedegen uitspraak over de associatie met IL2/IL21 is dit locus hiervoor buiten beschouwing gelaten. Wel kon worden aangetoond dat de associatie van CTLA4 met name lijkt bij te dragen in de ACPA positieve patiëntengroep, in tegenstelling tot STAT4, die in beide patiëntengroepen associeert met het hebben van RA.

In **hoofdstuk drie** werd een andere replicatie studie uitgevoerd, waarbij onderzocht werd of twee genetische varianten, gelegen in de IL2RA en IL2RB loci, geassocieerd waren met RA in een Nederlands cohort. Voor beide variaties kon deze associatie worden aangetoond, een aanwijzing dat de IL2 pathway een rol speelt in de pathofysiologie van RA.

Interactie

Een bepaalde genetische variatie gelegen in het HLA locus, de zogenaamde shared epitope (SE) allelen, komen vaker voor bij patiënten met RA in vergelijk met gezonde controles. Dit zijn de eerst aangetoonde genetische variaties die associëren met RA en zijn

nog steeds de belangrijkste erfelijke component. Het is bekend dat binnen een Noord-Amerikaanse inheemse bevolkingsgroep uit centraal Canada deze SE allelen frequenter voorkomen, waarbij binnen deze groep ook een hogere prevalentie van RA bestaat. In **hoofdstuk vier** is onderzocht of het hebben van deze SE allelen in combinatie met een van de andere, tot dan toe bekende, associërende genetische variaties een hogere kans gaf op het hebben van RA. Hierbij is gezien dat de aanwezigheid van HLA-DRB1 in combinatie met de genetische variatie gelegen in het C5-TRAF locus, sterker associeerde met het hebben van RA dan beide genetische variaties apart van elkaar. De reeds bekende associatie met MMEL-1 liet echter een verminderde associatie met RA zien binnen de HLA-DRB1 positieve patiënten groep. Deze studie toont hiermee een modulerend effect van de genen, gelegen buiten het HLA gebied, aan.

Kandidaat gen studies

Naast het onderzoeken van de reeds bekende associërende genetische variaties is het van groot belang om nieuwe associaties te ontdekken. Dit kan gedaan worden door uitgebreide, niet hypothese gedreven, studies uit te voeren, waarbij genen gelegen over het gehele genoom worden getest in grote patiënt cohorten. Nadelig hiervan zijn de hoge kosten die dit met zich meebrengt, met name in de eerste dagen van introductie van deze nieuwe technieken. Een andere benadering is om hypothese gedreven een specifiek gen van interesse te onderzoeken. In **hoofdstuk vijf** is op laatstgenoemde wijze een kandidaat gen onderzoek gedaan naar genetische variaties gelegen op het VTCN1 locus. Uit eerder onderzoek was gebleken dat VTCN1, wat codeert voor B7-H4, een negatieve regulator van T-cel activiteit, associeert met juveniele idiopathische artritis. Kort voor start van het onderzoek was een studie gepubliceerd, waarin werd beschreven dat hoge levels B7-H4 frequenter werden waargenomen in RA patiënten dan in gezonde controles en dat dit geassocieerd was met een hogere ziekte activiteit. In dit hoofdstuk werd een significante associatie aangetoond met het hebben van RA in vergelijk met gezonde controles in een Nederlands cohort. Replicatie van onze bevindingen in twee Scandinavische cohorten liet een vergelijkbare associatie zien. Na een stratificatie voor ACPA status, kon worden aangetoond dat deze genetische associatie met name was toe te schrijven aan een associatie van VTCN1 met de ACPA negatieve groep van RA.

In **hoofdstuk zes** wordt een andere kandidaat gen studie besproken, waarbij de associatie van het prostaglandine E synthase (PTGES) gen met RA werd onderzocht. PTGES codeert voor microsomaal prostaglandine E2, wat een belangrijke rol speelt bij ontsteking. In zowel een Zweeds als een Nederlands cohort is gekeken naar de associatie van verschillende genetische variaties met RA. Hierbij werd in het Zweedse cohort gevonden dat een bepaalde genetische variatie vaker voorkwam bij vrouwen met RA, dan bij gezonde vrouwelijke controles. In een meta-analyse, waarbij het Nederlandse cohort werd geïncludeerd, kon deze associatie ook worden aangetoond. Tenslotte werd gezien dat het genotype dat associeerde met een hogere DAS28, een maat voor de ziekte activiteit, een hogere expressie van mPGES-1 in synoviaal weefsel gaf. Deze bevindingen laten zien dat dit gen mogelijk een rol speelt in de pathofysiologie van ontsteking bij RA.

In **hoofdstuk zeven** wordt een studie beschreven waar de genetische associatie tussen het C1q gen en RA wordt onderzocht. Uit eerdere studies was gebleken dat complement mogelijk een rol speelt in RA. Zo zijn in het synovium van RA patienten complement depositie gevonden en bestaat een correlatie tussen ziekte activiteit en de aanwezigheid van aan C1q gebonden, geactiveerde complement fragmenten.

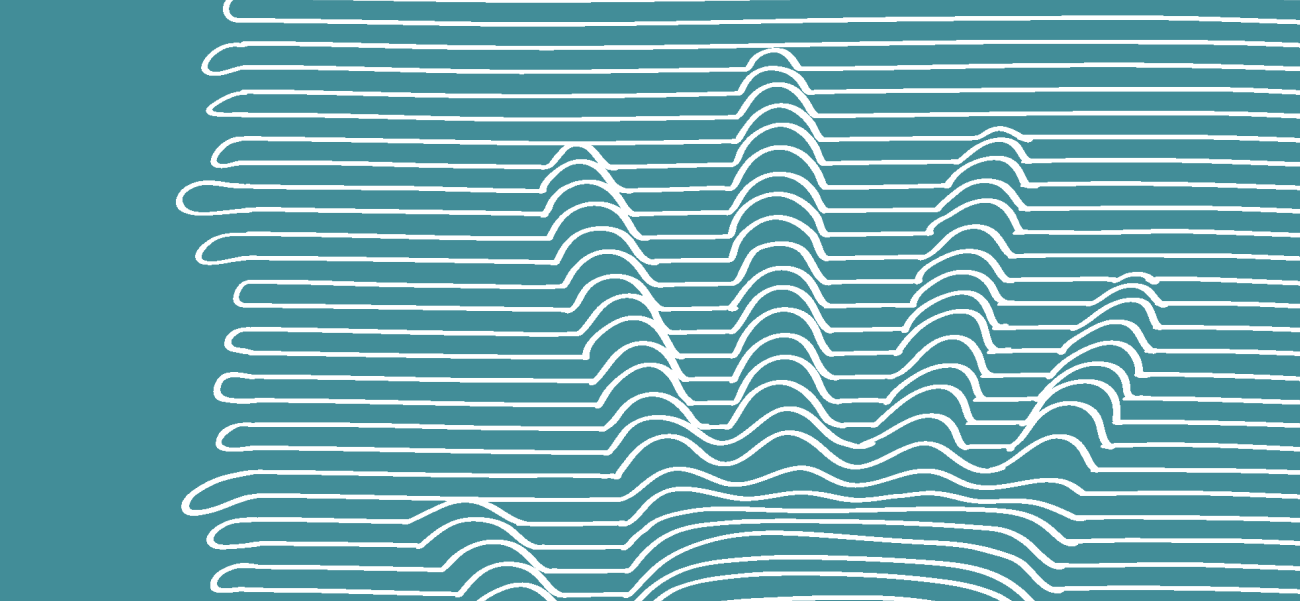
In de studie in hoofdstuk zeven zijn meerdere genetische variaties bepaald in een Nederlands cohort van RA patiënten en gezonde controles en bleek een van de geteste variaties te associëren met het hebben van RA. Deze genetische variatie is vervolgens in verschillende andere cohorten en in een meta-analyse onderzocht, waarbij deze bevinding werd gerepliceerd. Tevens werd gevonden dat deze genetische variatie gecorreleerd is aan C1q levels in gezonde controles, wat impliceert dat deze variatie van invloed is op de C1q productie.

Het complementsysteem

Zoals boven genoemd spelen auto-antilichamen een belangrijke rol in de pathofysiologie van RA. Gezien in hoofdstuk zeven een aanwijzing is gevonden dat C1q mogelijk een rol speelt in de pathofysiologie van RA en de associërende genetische variatie van invloed is op de productie van C1q, is het in het laatste hoofdstuk, **hoofdstuk acht**, een studie ingevoegd waarin de bestaande literatuur over complement activatie door (auto-)antilichamen op een rij is gezet. Hierin wordt een overzicht gegeven van de huidige kennis op dit gebied en wordt beschreven dat complement activatie via de drie verschillende pathways van activatie kan verlopen en niet alleen, zoals bekend, via de klassieke route middels C1q binding aan de Fc regio's van IgG en IgM.

Conclusie

In het huidige proefschrift is de bijdrage van genetische variaties gelegen in non-HLA genen onderzocht op hun rol in de pathofysiologie van RA. In de beschreven studies is een aantal eerder geïdentificeerde risicofactoren verder onderzocht en kon op deze wijze een statisch robuuste onderbouwing worden geleverd voor de associatie met RA. Ook zijn nieuwe genetische risicofactoren beschreven, waarbij enkele specifiek geassocieerd zijn met subgroepen van de ziekte. In navolging van de beschreven studies zal onderzocht moeten worden wat de betekenis van de beschreven genetische variaties is voor het ontstaan en het chronische karakter van RA.



APPENDIX A

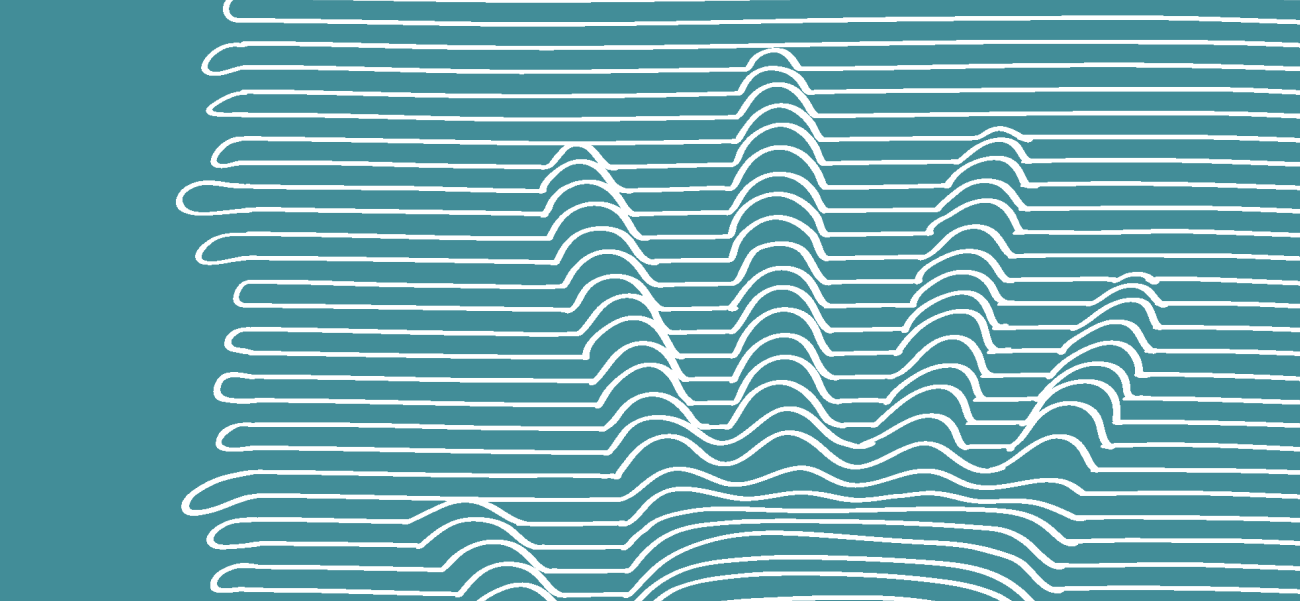
Curriculum Vitae

CURRICULUM VITAE

Op 22 november 1982 werd Nina Daha geboren te Leiderdorp. In 2001, na het behalen van haar VWO diploma aan het Stedelijk Gymnasium Leiden, startte zij met haar studie geneeskunde aan de Universiteit Leiden. Tijdens haar studie deed zij onderzoek op de afdeling pathologie van het Leids Universitair Medisch Centrum (LUMC). Haar wetenschapsstage deed zij vervolgens in het Beth Israel Deaconess Medical Center in Boston, Massachusetts in de Verenigde Staten. Hier deed zij ruim tien maanden onderzoek naar de rol van de ligatie van CD47-SIRPα in immuuncomplextransfer.

In 2008 behaalde zij haar arts-examen en vervolgens startte zij in hetzelfde jaar met haar promotieonderzoek op de afdeling Reumatologie van het LUMC onder begeleiding van prof. dr. R.E.M. Toes en prof. dr. T.W.J. Huizinga.

In september 2011 is zij gestart met haar opleiding tot reumatoloog en na het succesvol afronden van haar vooropleiding interne geneeskunde, is zij nu wederom werkzaam op de afdeling reumatologie van het LUMC (opleider prof. dr. T.W.J. Huizinga). Naar verwachting zal haar opleiding tot reumatoloog worden afgerond in december 2018.



APPENDIX B

List of Publications

LIST OF PUBLICATIONS

Daha NA, Kurreeman FA, Marques RB, Stoeken-Rijsbergen G, Verduijn W, Huizinga TW, Toes RE. Confirmation of STAT4, IL2/IL21, and CTLA4 polymorphisms in rheumatoid arthritis. Arthritis Rheum. 2009 May;60(5):1255-60.

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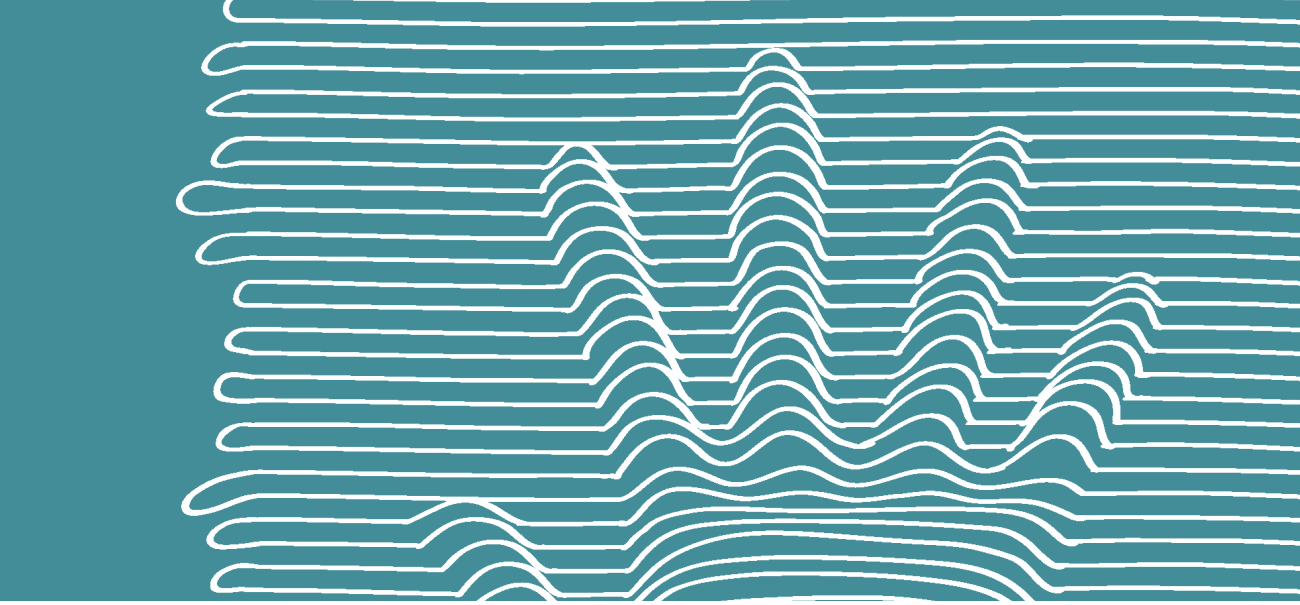

APPENDIX C

Dankwoord

Het is zo ver, ik mag mijn dankwoord schrijven. De afgelopen jaren heb ik met veel plezier en bij tijde ook zeker mopperend, aan dit proefschrift mogen werken. Mijn dank gaat dan ook uit naar een grote groep mensen, waarvan ik enkele op deze plaats extra wil bedanken.

Ten eerste natuurlijk mijn promotores Tom en René. Geachte professor Huizinga, beste Tom, jouw enthousiasme heeft me over de streep getrokken om voor geneeskunde en later voor de reumatologie te kiezen. Ik was van mening dat als iemand zo enthousiast is over zijn vak, het wel goed moet zijn. Ik wil je dan ook bijzonder bedanken voor je enthousiasme en je positieve kijk op alle aspecten van onderzoek doen.

Tijdens mijn promotietraject speelde de kritische noot ook zeker een belangrijke rol. Geachte professor Toes, beste René, ik zal altijd kritisch blijven kijken naar informatie die me voorgeschoteld wordt en de zin 'denk je dat of weet je dat' gonst daarbij nog altijd in mijn achterhoofd. Mijn welgemeende dank hiervoor.

Onderzoek doe je niet alleen en genetica onderzoek al helemaal niet. Mijn dank voor alle samenwerkingen in binnen- en buitenland met mooie resultaten tot gevolg.

Leendert, wat had ik zonder je gemoeten. Veel dank voor je begeleiding, samenwerking, gezelligheid en me het gevoel geven dat ik altijd welkom was om langs te lopen.

Dan natuurlijk de collega's van het lab. Aleida, Gerrie, Nivine, Joris, Ellen, Linda en Annemarie. Jullie wil ik bij deze bijzonder bedanken voor al jullie geduld, de uitleg van de 'labdingen' en het even kunnen kletsen en klagen. Jozé en Cedric, fijn dat jullie alle data die we wisten te generen in een mooie database hebben weten te krijgen en de vragen die ik er vervolgens over had heel geduldig wilden beantwoorden, dank jullie wel.

Ook al mijn collega kamergenootjes en promovendi van zowel de vijfde als de eerste wil ik op deze plaats bedanken voor de gezelligheid en de discussies. Mijn eilandgenootjes van de geneticagroep, Fina, Sasha en Rute, dank voor de samenwerking, het overleg en begeleiding bij de artikelen die we samen hebben geschreven. En natuurlijk ook dank aan de reuma-meisjes voor het kunnen mopperen over vooropleidingen en het benadrukken dat de reumatologie echt een mooi vak is.

Op deze plaats wil ik ook Joyce, Nancy en Hughine bedanken. Fijn dat ik altijd bij jullie kon aankloppen voor vragen, regel dingen en een luisterend oor. Hughine, zonder jou was dit boekje er letterlijk niet geweest, dank je wel voor de hulp en het regelen van alle bureaucratische rompslomp in de laatste fase van mijn promotietraject.

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Ilse, de afgelopen jaren zijn anders gelopen dan we dachten toen we met zijn allen een cordial aan het vormen waren. Je hebt me aangemoedigd bij het schrijven van mijn eerste artikel, toen ik geen idee had wat ik moest doen. Ook toen wist je iets slims te zeggen, waardoor ik me niet meer volledig verloren voelde. Ik mis je.