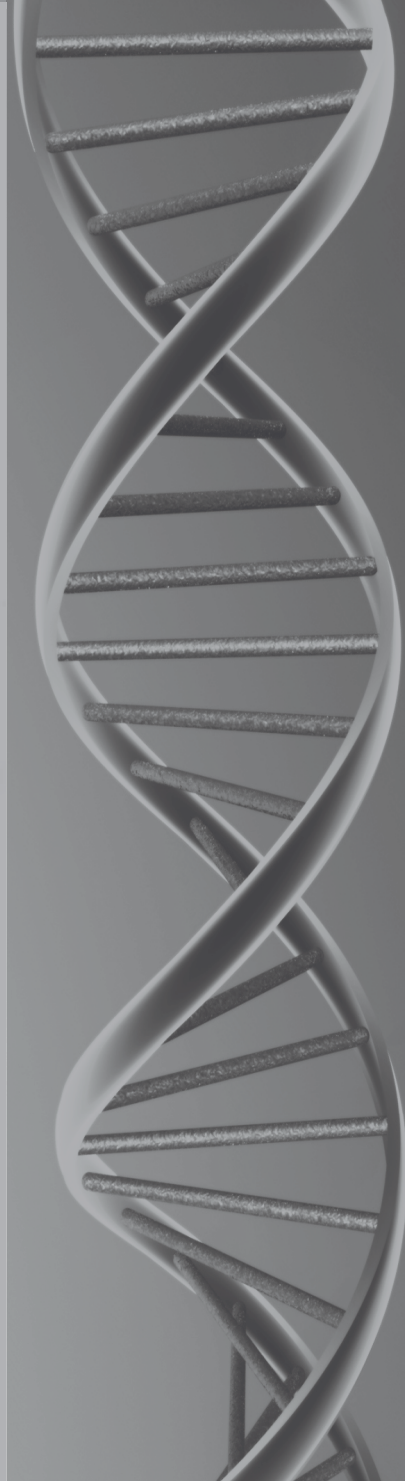
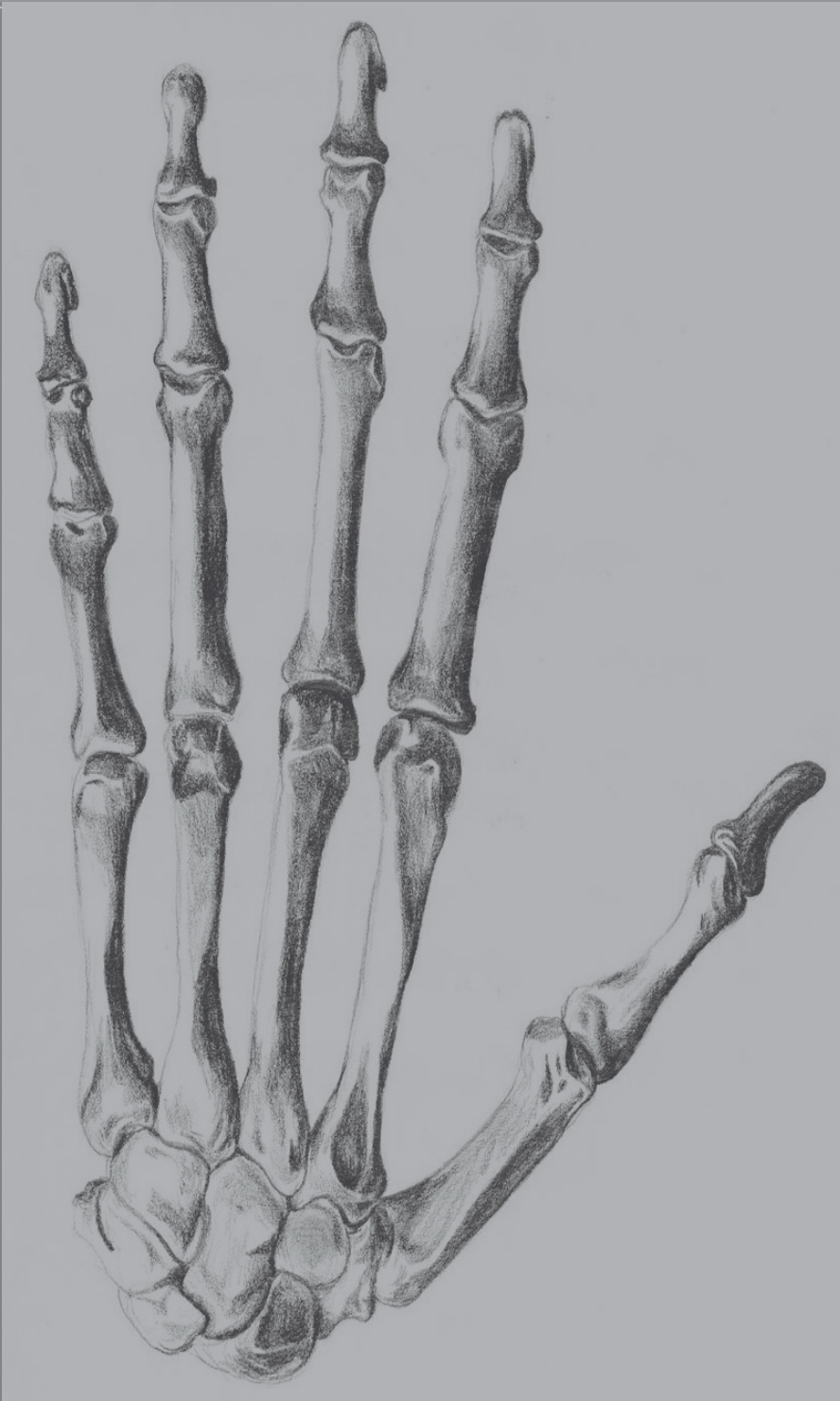
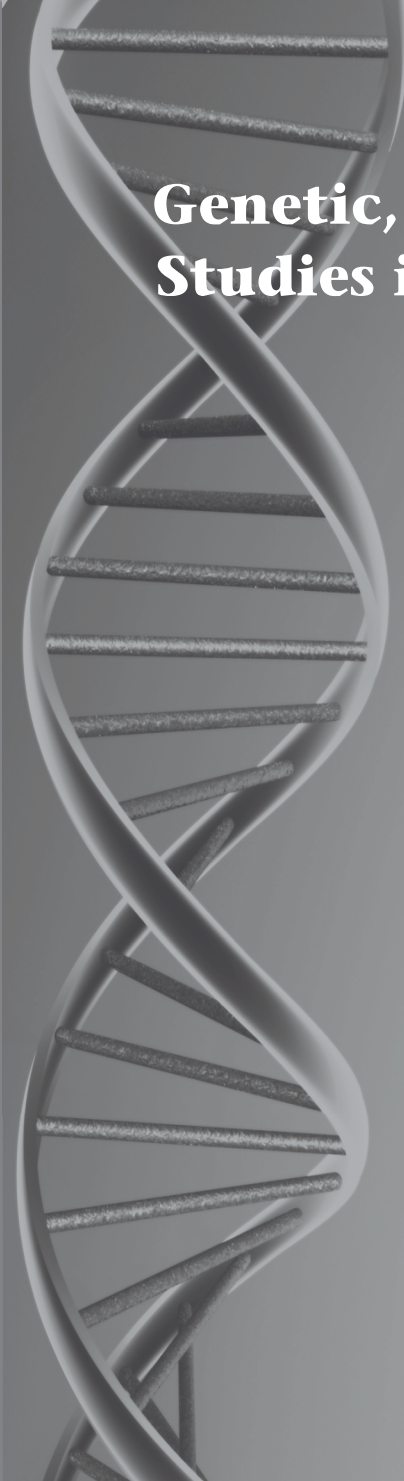
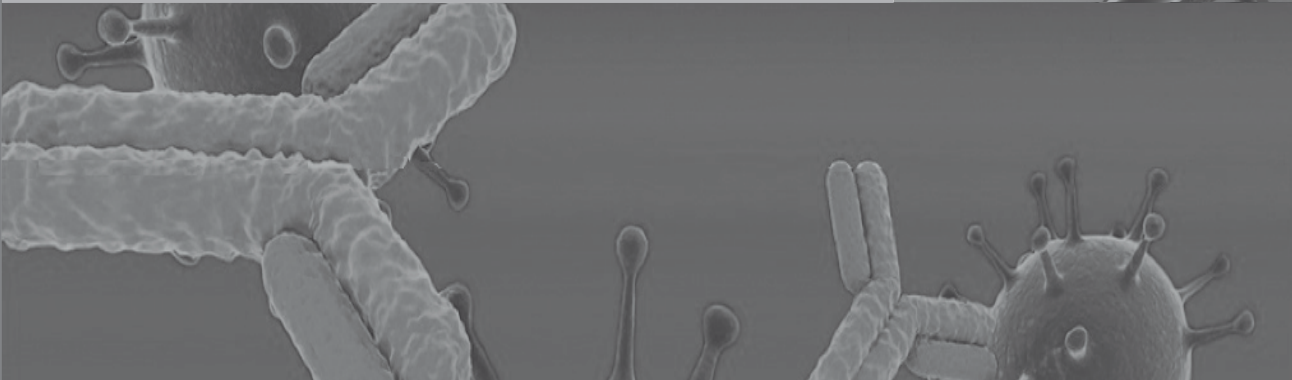




Genetic, Serological and Clinical Studies in Rheumatoid Arthritis



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Mohamed Okily



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Cover design and Layout: Mohamed Okily

Printing: SmartPrinting solutions

Genetic, Serological and Clinical Studies in Rheumatoid Arthritis

Proefschrift

ter verkrijging van
de graad van Doctor aan de Universiteit Leiden,
op gezag van Rector Magnificus prof. mr. C.J.J.M. Stolker,
volgens het besluit van het College voor Promoties
te verdedigen op donderdag 5 september 2013
klokke 11.15 uur

door

Mohamed Mohamed Thabet Okily

geboren te Abnüb
in 1975

PROMOTIECOMMISSIE

Promotor	Prof. dr. T.W.J. Huizinga
Copromotor	Dr. A.H.M. van der Helm- van Mil
Overige Leden	Prof. dr. D.M.F.M. van der Heijde Prof. dr. R.E.M. Toes Prof. dr. P.L.C.M. van Riel (UMC St. Radboud, Nijmegen)

Twenty years from now you will be more disappointed by the things that you didn't do than by the ones you did do. So throw off the bowlines. Sail away from the safe harbor. Catch the trade winds in your sails. Explore. Dream. Discover.

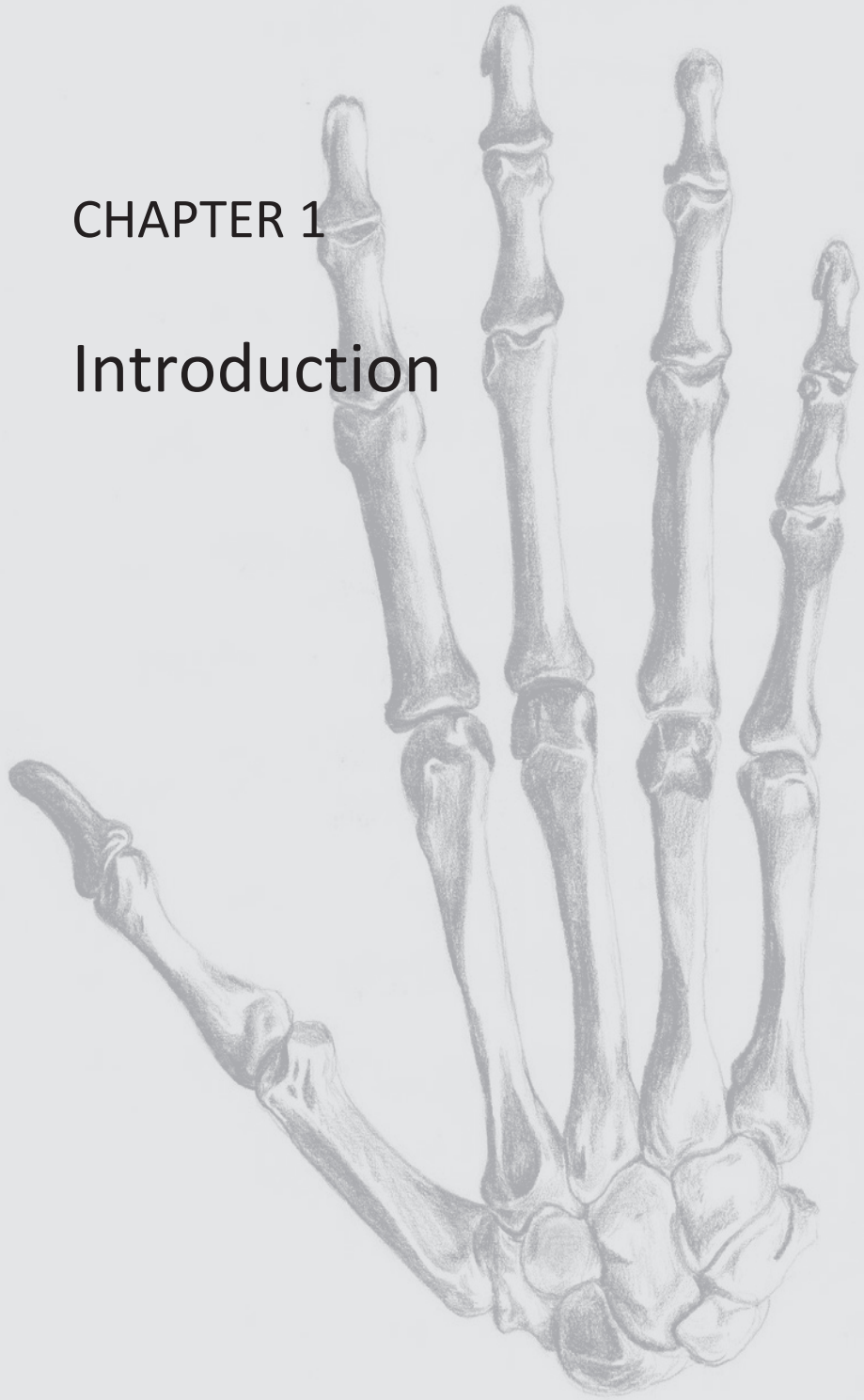
Mark Twain

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CHAPTER 1

Introduction



RHEUMATOID ARTHRITIS

Rheumatoid arthritis (RA) is a chronic, systemic inflammatory disease primarily affecting small peripheral joints in a symmetrical way, and may have extra-articular manifestations [1]. It is the most common inflammatory arthritis with a prevalence of 0.5 - 1.0% in the adult population worldwide [2]. The disease is approximately three times more frequent in women than in men, and the prevalence increases with age [3]. RA remains to be a major cause of disability, invalidity and reduced quality of life, and is associated with substantial economic costs [4].

CLASSIFICATION OF RA

The 1987 American College of Rheumatology (ACR) classification criteria [5] have been used for many years to identify RA, despite their poor sensitivity and specificity for classification of patients with early RA [6]. They fail to identify individuals with very early inflammatory arthritis who subsequently develop RA [7]. In order to, additionally, facilitate the identification of patients with very early stages of RA a joint initiative of the ACR and the European League Against Rheumatism (EULAR) recently developed the 2010 classification criteria for RA [8]. Those criteria reflect the shift towards earlier diagnosis and treatment of rheumatoid arthritis.

ACR 1987 Criteria	ACR/EULAR 2010 Criteria	Score
	1. Joint involvement	0 - 5
	• One medium-to-large joint	0
	• Two to ten medium-to-large joints	1
	• One to three small joints (large joints not counted)	2
	• Four to ten small joints (large joints not counted)	3
	• More than ten joints (at least one small joint)	5
1. Morning stiffness (at least 1h)	2. Serology	0 - 3
2. Arthritis of 3 or more joint areas	• Negative RF and negative ACPA	0
3. Arthritis of hand joints (≥ 1 swollen joints)	• Low positive RF or low positive ACPA	2
4. Symmetrical arthritis	• High positive RF or high positive ACPA	3
5. Rheumatoid nodules	3. Acute-phase reactants	0 - 1
6. Serum rheumatoid factor	• Normal CRP and Normal ESR	0
7. Radiographic changes (erosions)	• Abnormal CRP or abnormal ESR	1
	4. Duration of symptoms	0 - 1
	• Less than six weeks	0
	• Six weeks or more	1
➤ Four of these seven criteria must be present. ➤ Criteria 1-4 must have been present for at least weeks.		
➤ Cut-off point for RA is 6 points or more. ➤ Patients can also be classified as having RA if they have: (a) typical erosions; (b) long-standing disease previously satisfying the classification criteria		

Table 1: Overview of the 1987 ACR and the 2010 ACR/EULAR criteria

RISK FACTORS AND PATHOGENESIS

RA has a complex etiology; approximately 50% of the risk for developing RA is attributed to genetic factors. Environmental factors (mainly smoking), as well as autoimmunity (mainly Rheumatoid Factor (RF) and anti-citrullinated protein antibodies (ACPA)) contribute to the disease [3].

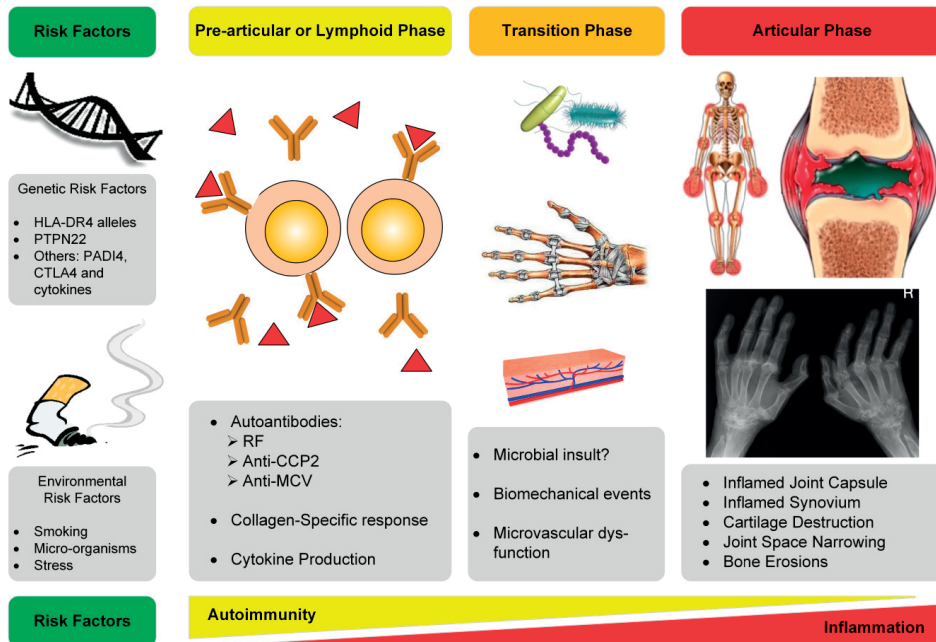


Figure 1: Overview of the pathophysiology and various stages of RA

Genetic risk factors

Genetic factors have a substantial impact on susceptibility to RA. The prevalence of RA in the general population is 0.5% to 1.0%, but it increases to 2% to 4% among siblings of RA probands [9]. The overall genetic contribution to the risk of developing RA has been estimated through studies of monozygotic and dizygotic twin pairs. Cross-sectional twin studies performed on a national scale in Finland and in the United Kingdom found concordance rates for RA of 12.3% and 15.4%, respectively, for monozygotic twins, compared to 3.5% and 3.6% for dizygotic twins [10].

Multiple loci contribute to the genetic risk for RA. The HLA (human leukocyte antigen) locus is the most important of these and accounts for 36% of overall genetic susceptibility to RA [11]. Within the HLA locus, the strongest association is with alleles of HLA-DRB1, which encodes the β -chain of the class II molecule HLA-DR, but recent evidence indicates that other HLA genes also contribute to genetic risk [12]. Outside the HLA locus, the strongest association identified to date is with a polymorphism in the protein tyrosine phosphatase non-receptor 22 (PTPN22) gene, which encodes the protein tyrosine phosphatase Lyp [13]. Advances in genotyping technology have facilitated the application of genome wide association studies (GWAS) to identify disease causal variants. This, coupled with the availability of large case and control collections has enabled the identification of low-to-moderate risk loci. These newer study designs combined with traditional linkage and association studies on single nucleotide polymorphisms (SNPs) level have accelerated the identification of novel RA risk loci (among others STAT4, 6q23 and TRAF1/C5) [11, 14, 15].

Fc γ -receptors (Fc γ Rs)

The Fc γ Rs play a crucial role in immunity by linking the IgG antibody mediated responses with cellular effector and regulatory functions [16]. These receptors are encoded by genes clustered on the long arm of chromosome 1 (1q21 - q24) in a complex region showing extensive nucleotide sequence homology that resulted from duplication and recombination events which occurred in this cluster during the evolution [17]. Such genetic complexity renders successful genotyping of different SNPs in that region using classical methods notoriously difficult [18].

Data from mice models revealed that, arthritis-susceptible, DBA/1 mice lacking FcR gamma chain (thus lacking Fc γ R1 and Fc γ R3 signaling) were protected from collagen-induced arthritis (CIA) [19]. In contrast, deletion of Fc γ RII can render arthritis-resistant 129/SvJ and C57BL/6 hybrid mice susceptible to CIA [20]. These observations suggest a crucial role of Fc γ Rs in triggering autoimmune arthritis [19]. Additionally, the balance of activating Fc γ Rs (Fc γ RI, Fc γ RIIa, Fc γ RIIc, Fc γ RIIIa, and Fc γ RIIIb) and inhibitory Fc γ Rs (Fc γ RIIb) might be important in regulating the chronic immune complex-mediated responses in patients with RA (figure 2) [21].

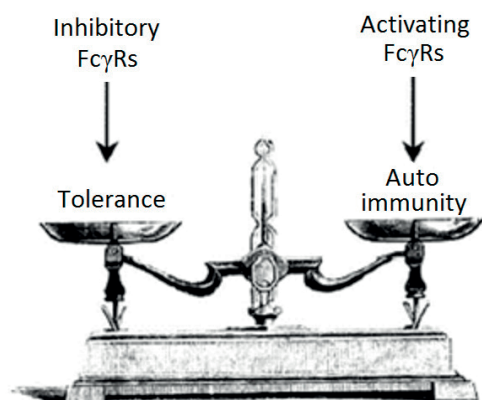


Figure 2: Immune reaction is a balance between the inhibitory (Fc γ RIIb) and activating Fc γ Rs (Fc γ RI, Fc γ RIIa, Fc γ RIIc, Fc γ RIIIa, and Fc γ RIIIb)

Environmental risk factors

In addition to genetic aspects, environmental risk factors are known to contribute to the etiology of RA. Many epidemiological studies have shown an association between smoking and the development of RA [22, 23]. Smoking was found to interact with the HLA SE (Shared Epitope) alleles in the predisposition to RF-positive RA [24] and ACPA-positive RA [25]. Smoking was associated with the development of citrullinated antigens in broncho-alveolar lavage fluid cells, providing a plausible pathogenic link between smoking and the development of ACPA-positive RA [25]. Other potential environmental risk factors include alcohol intake, coffee intake, vitamin D status, oral contraceptive use, and socioeconomic status although supporting evidence for these other factors is weak [26].

Auto-antibodies

Autoantibody formation is characteristic for RA and that is the reason it is considered an autoimmune disease. The positive predictive value of the presence of certain antibodies aims at another phase of the immune response that may play a role in the pathophysiology of RA. 50–80% of individuals with rheumatoid arthritis have rheumatoid factor, ACPA, or both [27].

Rheumatoid factor

The classical antibody in RA, rheumatoid factor (RF), is an antibody directed to the Fc part of immunoglobulins. The presence of IgM-RF is part of the 1987 ACR criteria for RA.

However, RF can be detected in other autoimmune diseases and healthy individuals. The sensitivity of RF for diagnosing RA ranges from 60 to 70%; and the specificity from 80 to 90% [28].

Anti-citrullinated peptide antibodies

Additional, and increasingly important, types of antibodies are those directed against citrullinated peptides (ACPA) [3]. Although most, but not all, ACPA-positive patients are also positive for RF, ACPA seem more specific and sensitive for diagnosis and seem to be better predictors of poor prognostic features such as progressive joint destruction [29]. Evidence from animal models and in-vivo data suggest that ACPA are pathogenic on the basis of induction of arthritis in rodent models and because immunological responses are present in ACPA-positive patients in a citrulline specific manner [30]. Findings of clinical studies show that patients with RA and both RF and ACPA autoantibody-positive disease) differ from individuals with so-called autoantibody-negative disease [3]. For example, histologically, people with ACPA-positive disease have more lymphocytes in synovial tissue, whereas those with ACPA-negative rheumatoid arthritis have more fibrosis and increased thickness of the synovial lining layer [31]. ACPA-positive disease is associated with increased joint damage and low remission rates [32]. ACPAs can be present up to 14 years upon diagnosing rheumatoid arthritis [33, 34].

Vascular factors

Recently, the role of the synovial vasculature in the pathogenesis of RA has gained more interest [3]. Data from mouse work lead to the hypothesis that changes in local vascular permeability might provide a route via which a systemic autoimmunity becomes focused on the synovium [35]. Early in RA angiogenesis within the hypertrophic synovial sub-lining occurs and is driven by the increased metabolic demands and hypoxia of the expanding inflammatory tissue [36]. New vessels in the RA pannus are of a characteristic branching morphology [37]. This is thought to result from the unfavorable co-expression by intimal fibroblast-like synoviocytes (FLSs) and vascular endothelial cells (ECs) of the angiogenic factor VEGF (vascular endothelial growth factor), and the angiopoietin-1/2–TIE2 complex [36]. VEGF and angiopoietin-2 together promote the invasive proliferation of ECs, and

inhibition of this process may be one mechanism of anti-TNF α drug activity [38]. It is hoped that the complex paracrine pathways that promote angiogenesis may yield additional therapeutic targets [39].

UNDIFFERENTIATED ARTHRITIS

Undifferentiated arthritis (UA) is defined as any arthritis of recent onset that cannot be classified according to the existing criteria for specific rheumatic disorders [40]. Patients with early UA form a heterogeneous group exhibiting a variable disease course. Of patients with early UA 40 to 50% remit spontaneously, whereas 30% subsequently fulfill the 1987 ACR criteria therefore classified as RA [41-43]. Since starting treatment with disease-modifying anti-rheumatic drugs (DMARDs) earlier leads to improved outcomes for patients with RA, initiating anti-rheumatic therapy already in the UA phase might result in sustained benefits and potentially remission [44-46]. That implies the need to identify which UA patients will have a chronic destructive disease course and those who will go into spontaneous remission or develop other diagnoses. Several prediction models have been made, yet none is accurate enough that all individual UA patients can be predicted correctly [47, 48].

ASSESSMENT OF RADIOGRAPHIC JOINT DAMAGE

Plain radiographs of hands and feet remain the gold standard of assessing joint damage in RA. Several methods are present for quantifying the amount of joint destruction visible on radiographs. Of these methods, the Sharp/van der Heijde scoring (SHS) is considered as the most sensitive widely used compared to the Larsen and the Ratingen scoring methods [49]. This method focuses on the small joints in the hands, the wrists and the feet. Joint space narrowing (JSN) is scored in 42 joints and is a measure for cartilage degradation. The erosion score (ES) is scored in 44 joints and is a measure of bone degradation **Figure 3**. The sum of JSN and ES is referred to as the total SHS and can be a maximum score of 448 [50]. In literature, evaluation of radiographic data is frequently performed on a single time point in a cross-sectional way, though, a more precise estimation of individual's progression rate can be ideally achieved with repeated evaluation of joint damage [51].

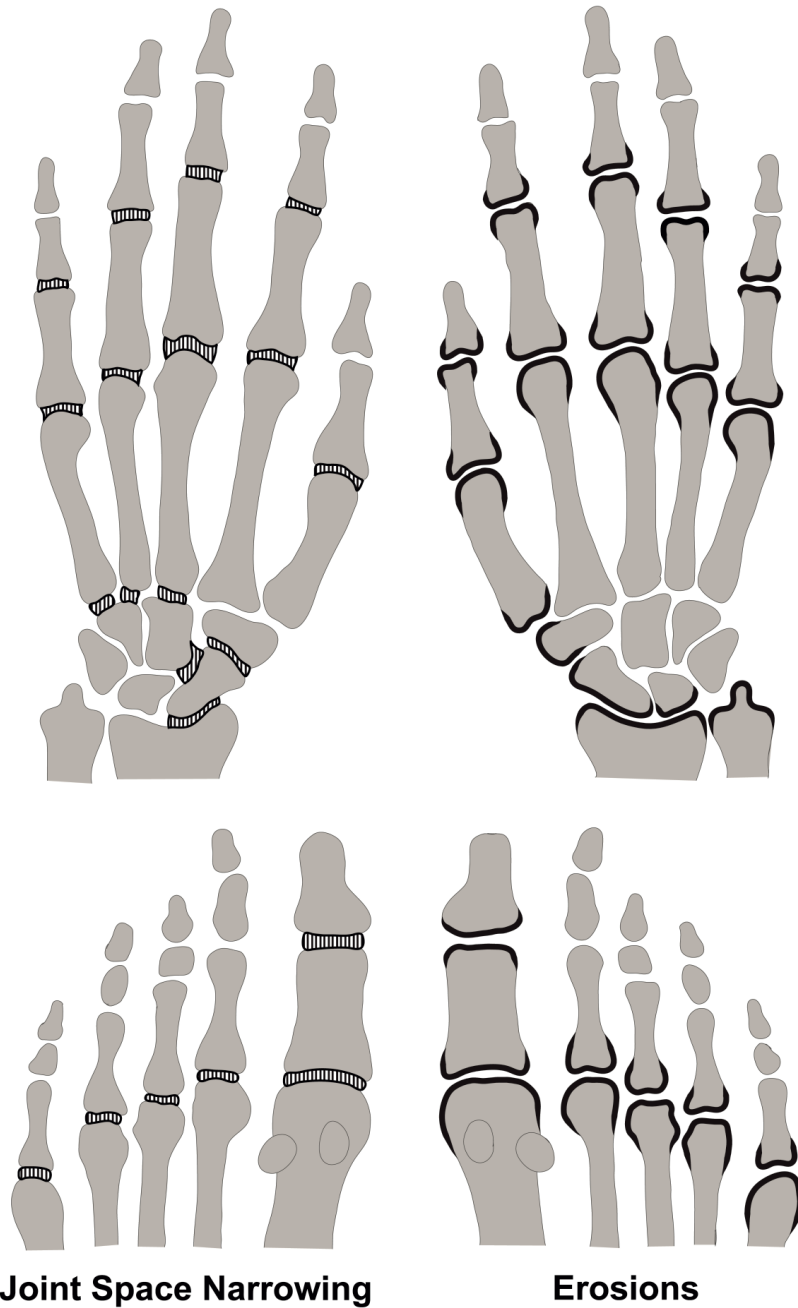


Figure 3: Scoring of hands and feet according to the SHS method. Joints and sites scored for joint space narrowing are shown on the left while joints and surfaces of the joints scored for erosions are shown on the right.

AIM AND OUTLINE OF THIS THESIS

The ultimate quest in RA is to fully understand the various risk factors and try to early identify or even predict RA before the criteria for diagnosis are fulfilled. This will permit the early start of DMARDs in identified RA cases or UA predicted to develop RA which was shown to have sustained beneficial effects.

The focus and objective of this thesis is to:

- 1- Add insight into genetic risk factors of RA in the context of antibody heterogeneity and interaction with environmental, racial and gender factors.
- 2- Study the diagnostic and prognostic value of the various autoantibodies in RA.
- 3- Define erosions and their value in prognosis of UA patients, prediction and early diagnosis of RA patients.

Consequently, the thesis is divided into three parts; each addressing one of the key objectives.

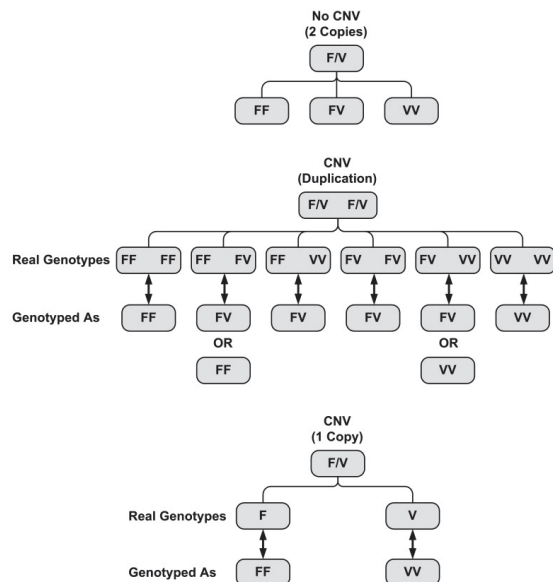
Part I of the thesis is devoted to thoroughly study various genetic risk factors of RA. The previously reported genetic risk factors have been explicitly shown to predispose for ACPA-positive RA but not for ACPA-negative RA emphasizing the need to systemically study genetic risk factors in ACPA- positive and ACPA-negative RA separately. The interaction of environmental factors with genetic risk factors should be taken into consideration. Additionally, we will try to approach the technical difficulties and contradicting results from genetic studies encompassing the FcγRs genes.

Even though PTPN22 R620W gene polymorphism has been consistently shown to be associated with RA in Caucasians, it is not found and thus is not disease associated in Asian populations [52]. In **chapter 2**, we investigated whether RA risk associated with PTPN22 genetic variants in Europeans was solely conveyed through the R620W allele, that is not polymorphic in Asians, or other SNPs and haplotypes play a role in that risk. Other genetic variants were shown to carry risk for RA in Asians but not in Europeans as FCRL3 and PADI4. We were interested to investigate those in Dutch Caucasians and explore whether stratifying for antibody status has an effect on the risk for RA. In **chapter 3**, we tested the

association of FCRL3 polymorphism with RA susceptibility and severity in Dutch Caucasian patients with RA. Additionally, we performed a meta-analysis to reveal the contribution of this gene to RA susceptibility. In **chapter 4**, we tried to elucidate the differential role of PADI4 polymorphism in RA risk between Asian and European populations. Moreover, we explored the possible gene-environmental interactions between the PADI4 polymorphism, sex and smoking status.

The FcγRs genes region has been shown to exhibit copy number variation (CNV) in several large-scale whole genome and focused studies [53-56]. The presence of common CNVs can cause false SNP genotyping results **Figure 4**. The subsequent skewing of genotypes may lead them to fail the Hardy-Weinberg equation (HWE) (therefore excluding their results) and/or may blur the association of the studied SNPs with disease susceptibility. It may also limit the ability of the genome-wide SNP association studies to detect disease-associated SNPs in regions with CNV [57]. Such genetic complexity renders successful genotyping of different SNPs in that region using classical methods notoriously difficult. Therefore, an alternative approach is required to tackle the genetic variations in this region and its association with RA risk and/or severity.

Figure 4: Possible effect of copy number variation (CNV) on single nucleotide polymorphism (SNP) genotyping. This figure illustrates how the presence of CNV can cause false SNP genotyping results. A high copy number falsely enriches the heterozygotes, while the presence of low copy number falsely enriches the homozygotes. In this figure the use of F and V characters was only for illustration purpose but this kind of erroneous genotyping result can occur in every genetic region that harbours both SNPs and CNV.



In **chapter 5**, we explored whether FcγRIIIA 158V/F SNP associates differently with ACPA-positive and ACPA-negative RA. The CNV of FcγR genes was studied using Multiplex Ligation Dependent Probe Amplification (MLPA) and we tested whether the FcγRIIIA gene CNV itself confers risk for RA and if the CNV affects the association of the FcγRIIIA 158V/F SNP with RA. Similarly in **chapter 6**, the genetic variations of FcγRIIIB gene were studied using MLPA in RA patients.

Part II focuses on the autoantibodies formed in the course of RA; the best known of those is the rheumatoid factor. More recently, several tests have been developed that detect ACPA. These tests include the commercially available anti-CCP2 and anti-CCP3 assays as well as the anti-mutated citrullinated vimentin (anti-MCV) test. The first two assays use citrullinated peptide(s) for the detection of ACPA, whereas the later assay is based on an entire protein, citrullinated vimentin [58, 59]. The diagnostic performance of the anti-CCP2, anti-CCP3 and anti-MCV tests in differentiating RA from other forms of arthritis in a clinical setting of early arthritis is investigated in **chapter 7**.

Finally, **Part III** of the thesis focuses on bone erosions and its significance in the disease course of RA and UA. The presence of early bone erosions in RA patients is predictive of severe destructive disease course, while its prognostic value in UA is unknown. Additionally, the definition of erosive disease is unclear and different studies use different descriptions and cut-off values. Even though the presence of erosions is part of the 1987 ACR classification criteria for RA [5], it is not specified how many erosions are required and even more, mentions erosions in the hands and not in the feet. **Chapter 8** of this thesis aims to: study the predictive value of erosive joints in hands and feet for development of RA in UA-patients; define the optimal number of erosive joints to predict RA; define whether the predictive ability is different between erosive joints in hands and feet; determine whether information on erosive joints increases the discriminative ability of a recently developed prediction rule for RA-development [48, 60]; and investigate whether the results are different when disease persistency is studied instead of the development of RA according to the 1987 ACR criteria.

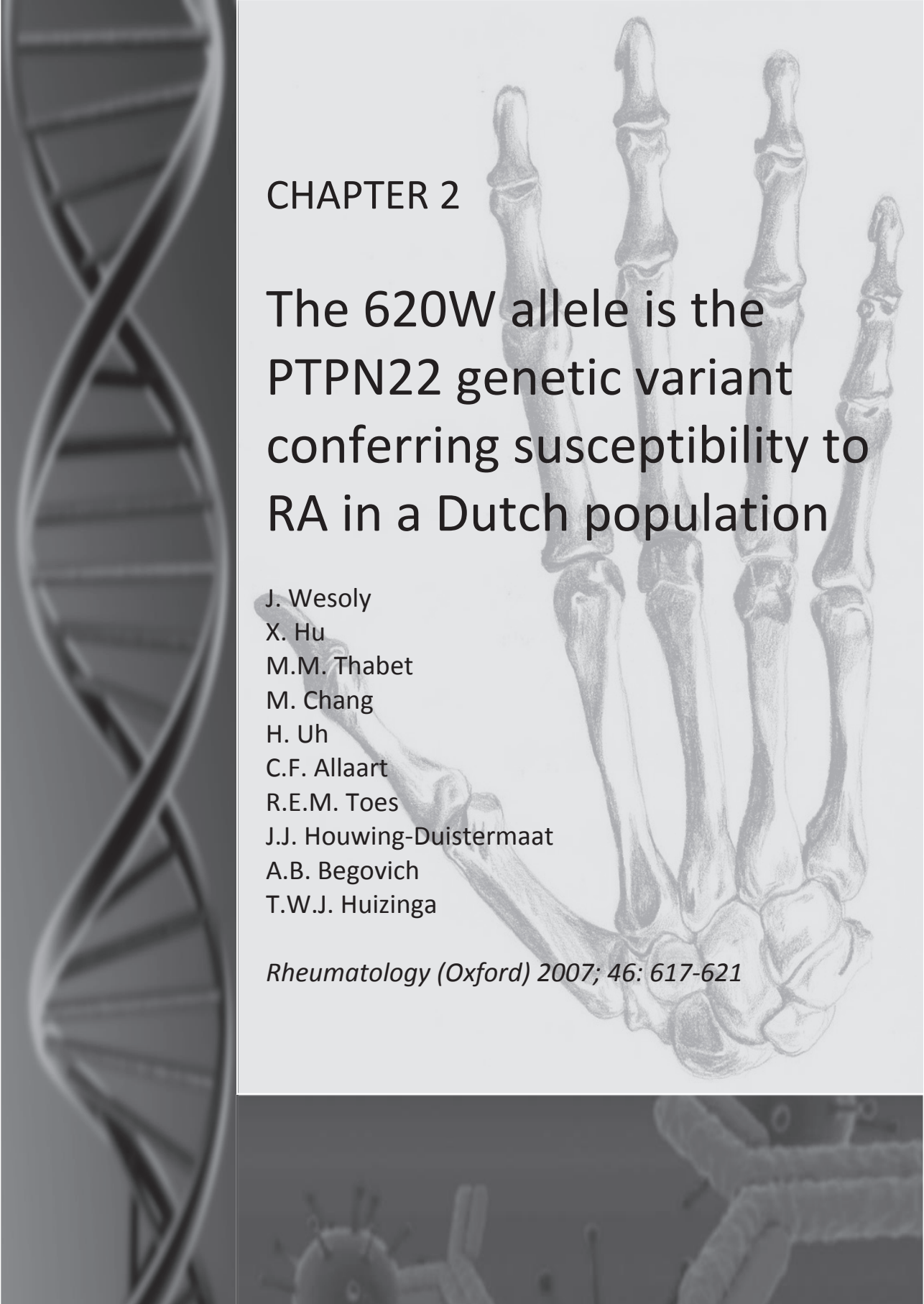
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CHAPTER 2

The 620W allele is the PTPN22 genetic variant conferring susceptibility to RA in a Dutch population

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Rheumatology (Oxford) 2007; 46: 617-621

ABSTRACT

Objectives. A missense SNP, C1858T, in PTPN22 has been identified as a genetic risk factor for rheumatoid arthritis (RA). Subsequent work has suggested that other variants in this gene, in particular a haplotype marked by the minor allele of rs3789604, are associated with RA in white North Americans independent of C1858T. We tested this hypothesis in an independent white Dutch study.

Methods. A total of 667 RA patients and 286 controls were genotyped for 13 PTPN22 single nucleotide polymorphisms (SNPs) by allele-specific kinetic polymerase chain reaction. rs3789604 was genotyped in an additional 410 RA and 270 UA patients participating in the Leiden early arthritis inception cohort. We conducted single-marker and haplotype association tests.

Results. The sole haplotype strongly associated with RA in our Dutch population carries the PTPN22 1858T allele. A second haplotype identical at all other SNPs tested except 1858 was not associated with disease. No significant association of the haplotype tagged by the 30 PTPN22 SNP, rs3789604, with RA susceptibility ($P = 0.134$) was observed in our sample set.

Conclusion. We conclude that C1858T is the sole PTPN22 variant predisposing to RA in our white Dutch sample set.

INTRODUCTION

A missense single nucleotide polymorphism (SNP), rs2476601, in the hematopoietic-specific protein tyrosine phosphatase gene, PTPN22, is the first non-HLA (non-human leucocyte antigen) genetic variant consistently associated with susceptibility to a number of autoimmune diseases including rheumatoid arthritis (RA), juvenile idiopathic arthritis (JIA), type 1 diabetes (T1D) and Graves disease (reviewed in [1]). The disease-associated C1858T SNP, encoding an arginine to tryptophan substitution at residue 620 (R620W), is located in the P1 proline-rich motif of PTPN22, which binds with high affinity to the Src homology 3 (SH3) domain of the tyrosine kinase, Csk. The minor 1858T (W620) allele disrupts the interaction of PTPN22 with Csk [2] and also encodes a significantly more active phosphatase, which suppresses TCR signaling more efficiently than the wild-type protein [3].

RA is the most common systemic autoimmune disease affecting up to 1% of general population, with a higher prevalence in women [4]. Persistent inflammations that may lead to bone destruction as well as the presence of autoantibodies are hallmarks of RA. Consequently, it is interesting that a prominent feature of PTPN22-associated autoimmune diseases appears to be the presence of autoantibodies. Two autoantibodies are commonly used as diagnostic indicators of RA: IgM rheumatoid factor (RF) directed to the Fc fragment of IgG and anti-citrullinated peptide antibodies (ACPAs) directed against peptides in which arginine residues have been post-translationally modified to citrulline [5]. However, not all RA patients have autoantibodies, and while some studies show exclusive association of the PTPN22 missense SNP with RF-positive RA, others do not [6-12].

Recently Carlton *et al* [13] resequenced the PTPN22 gene in 48 RA patients and identified a dense map of SNPs that were characterized in two independent white North American sample sets. Using the resulting genotype data, 10 distinct haplotypes with a frequency of $\geq 1\%$ were predicted, only one of which carried the 1858T risk allele. A second haplotype identical at all SNPs tested, except for 1858, was not associated with disease. This, together with the aforementioned biological data, provides strong evidence that 1858T is a causative variant. In addition, minor alleles of two SNPs, rs3811021 in the 3' UTR of PTPN22 and rs3789604 in a putative transcription factor binding site 3' of the gene, that are in strong

linkage disequilibrium (LD) with one another ($R^2 > 0.98$) tagged a second haplotype that appeared to contribute to RA predisposition independent of 1858T in both sample sets.

The aim of our study was 2-fold. First, we wanted to investigate association of the additional PTPN22 SNPs with RA in an independent white sample set, and second, we wanted to determine whether the 1858T allele was associated with RF-positive and/or RF-negative disease.

MATERIALS AND METHODS

Sample set 1 consisted of 667 white Dutch individuals with RA, all of whom fulfilled the ACR classification criteria for RA and have been described elsewhere [14, 15]. RF status was determined for 302 of the patients as previously described [16]. Sample set 2 consisted of 410 RA patients and 270 undifferentiated arthritis (UA) patients from a population-based inception cohort, the Leiden Early Arthritis Clinic (EAC) [17]. These RA patients also met the ACR classification criteria for RA. UA patients who had symptoms of arthritis that did not exceed 2 years, were aged 18 years or older and did not fulfill any classification criteria. Controls ($n = 286$) were unrelated white Dutch individuals with no history of RA [18]. Patients and control individuals were enrolled in the study with informed written consent obtained according to Declaration of Helsinki. Commissie Medische Ethiek, the Leiden institutional review board, approved all protocols.

A combination of 13 informative PTPN22 functional and tagging SNPs (selected from Carlton *et al* [13]) was genotyped in both sample set 1 and the controls (**Table 1**). rs3789604 was genotyped in sample 2; results for rs2476601 (C1858T) in this sample set have been reported previously [11]. Genotyping was done using allele-specific kinetic polymerase chain reaction PCR [19] and the data hand-curated prior to statistical analysis. Previous analyses suggest a genotyping accuracy of >99% [13]. An exact test of Hardy–Weinberg equilibrium (HWE) on the genotyping data for these 13 SNPs, performed separately for cases and controls, showed all SNPs were in HWE ($P > 0.05$) except for ss38346942 in the controls (expected AA = 276, AT = 9, TT = 0; observed: AA = 278, AT = 5, TT = 2; $P < 0.0001$). Consequently, ss38346942 was excluded from further analysis (this SNP uniquely marks one

of the 10 North American haplotypes; without it two haplotypes, haplotype 10, which is relatively infrequent, and 6, collapse into one haplotype [13]). A chi-square test was used for association analyses; haplotypes were predicted using the Haplo.stats package [20], and LD analysis was performed using Haploview [21]. The haplotype method of Thomson *et al* [22, 23] was used for conditional analyses. Regression analysis was done as described by Schaid *et al* [20] and power calculations were carried out according to the method of Purcell *et al* [24]. P-values <0.05 were considered significant.

TABLE 1. PTPN22 – genotype and allele frequencies in RA patients and controls

SNP Reference ^a	SNP ID ^b	Genotypes % (n)			P ^c	Allele frequency (%)		OR (95% CI) ^d	P
Sample Set 1									
1	rs1217414	CC	CT	TT		C	T		
	Cases (n¼ 667)	53.5 (357)	38.8 (259)	7.7 (51)		72.9	27.1		
2	rs2488458	GG	GA	AA	0.55	71.3	28.7	0.92 (0.74–1.15)	0.46
	Cases (n¼ 665)	48.6 (323)	43.3 (288)	8.1 (54)		70.2	29.8		
18	rs12760457	GG	GA	AA	0.024	75.7	24.3	1.31 (1.04–1.66)	0.017
	Cases (n¼ 666)	53.6(357)	40.4(269)	6.0(40)		73.8	26.2		
20	rs11102685	AA	AG	GG	0.88	73.3	26.7	0.98 (0.78–1.23)	0.83
	Cases (n¼ 658)	79.5 (523)	19.6 (130)	0.8 (5)		89.4	10.6		
21	rs12730735	AA	AG	GG	0.72	88.3	11.7	0.89 (0.65–1.24)	0.48
	Cases (n¼ 667)	53.2 (355)	40.8 (272)	6.0 (40)		73.6	26.4		
22 R620W	rs2476601	CC	CT	TT	0.8	72.5	27.5	0.8 (0.76–1.19)	0.63
	Cases (n¼ 661)	72.3 (478)	24.5 (162)	3.2 (21)		84.6	15.4		
23	rs38346944	GG	GT	TT	0.0014	90.3	9.7	1.7 (1.23–2.36)	0.0008
	Cases (n¼ 667)	92.8 (619)	7.0 (47)	0.2 (1)		96.3	3.7		
27	rs1310182	CC	CT	TT	0.25	97.7	2.3	1.64 (0.85–3.20)	0.11
	Cases (n¼ 667)	26.7 (178)	53.5 (357)	17.8		53.4	46.5		
28	rs38346943	TT	TC	CC	0.44	54.7	45.3	1.05 (0.86–1.29)	0.6
	Cases (n¼ 666)	96.4 (642)	3.3 (22)	0.3 (2)		98.05	1.95		
35	rs1217413	TT	TC	CC	0.52	97.9	2.1	0.93 (0.45–1.96)	0.83
	Cases (n¼ 664)	51.2 (340)	41.4 (275)	7.4 (49)		71.9	28.1		
36	rs3811021	TT	TC	CC	0.031	77.3	22.7	1.33 (1.05–1.68)	0.015
	Cases (n¼ 665)	69.3 (461)	28.3 (188)	2.4 (16)		83.4	16.5		
37	rs3789604	AA	AC	CC	0.10	79.4	20.6	0.76 (0.59–0.99)	0.03
	Cases (n¼ 666)	68.7 (458)	28.8 (192)	2.4 (16)		83.2	16.8		
	Controls (n¼ 284)	62.3 (177)	34.2 (97)	3.5 (10)	0.13	79.4	20.6	0.78 (0.60–1.01)	0.049
	Sample Set 2								
RA	rs3789604	AA	AC	CC		A	C		
37	Cases (n¼ 410)	68.3 (280)	28.0 (115)	3.7 (15)		82.3	17.7		
	Controls (n¼ 284)	62.3 (177)	34.2 (97)	3.5 (10)	0.23	79.4	20.6	0.83 (0.63–1.1)	0.17
UA	rs3789604	AA	AC	CC		A	C		
37	Cases (n¼ 270)	72.9 (192)	25.5 (71)	1.5 (7)		84.3	15.7		
	Controls (n¼ 284)	62.3 (177)	34.2 (97)	3.5 (10)	0.09	79.4	20.6	0.72 (0.52–0.99)	0.036

^aSNP ID based on Carlton *et al*. [13].

^bIndicates the dbSNP ID as well as the number of individuals successfully genotyped.

^cChi square test with 2 degrees of freedom.

^dOdds ratios (OR) and 95% confidence intervals are calculated for the minor allele of each marker.

RESULTS

Single-marker association

Genotype data for the 12 PTPN22 SNPs successfully typed in the Dutch cases (sample set 1) and controls were examined by single- marker analysis (**Table 1**). The results confirm association of the C1858T missense SNP (rs2476601, SNP 22) with RA in Dutch whites [allelic $P = 0.0008$, odds ratio (OR) = 1.7] [11]. Four additional markers also showed significant association with RA (SNP reference -2, 35, 36 and 37). SNP37, located downstream of PTPN22 and previously reported to tag a W620-independent risk haplotype, was also genotyped in sample set 2 (**Table 1**). In both sample sets, the minor allele of rs3789604 was less frequent in patients than in the controls (16.8% in sample set 1, 17.7% in sample set 2, 20.6% in controls); this difference between cases and controls was marginally significant in sample set 1 (allelic $P = 0.049$, OR = 0.78). The UA patients in sample set 2 were also genotyped for rs3789604; again the minor allele frequency was less in cases (15.7%) than in the controls. This is in contrast to the two North American sample sets where the rs3789604 minor allele was more common in cases than in controls (21 vs 16% and 20.6 vs 18.5%).

Association of SNP37 conditional on R620W

The known association of C1858T with RA in addition to the strong LD across the PTPN22 region can confound our ability to determine whether any other SNPs in this region are independently associated with disease. To address this issue, we assessed the association of SNP 37 conditional on 1858T using the haplotype method developed by Thomson and colleagues [22, 23] and used by Carlton *et al* [13] to help identify rs3789604 as a disease-associated SNP. In this analysis, two marker haplotypes were estimated using an expectation–maximization algorithm and the case and control chromosomes divided into two bins, those carrying the 1858T risk allele and those carrying the 1858C allele. Focusing on the 1858C chromosomes, we then determined whether the frequency of the rs3789604 minor allele was significantly different between case and control chromosomes. The results showed that SNP37 was not significantly associated with disease conditional on C1858T ($P = 0.82$), which was confirmed with a regression analysis for genotypes (OR = 0.826, $P = 0.15$) adjusting for C1858T genotypes.

Power analysis

Given that the effect of SNP 37 was relatively modest compared with the missense SNP (C1858T) in the original report [13], we calculated the power of our study to detect a similar effect using the method of Purcell *et al* [24]. Since the original association of SNP37 with RA was observed in patients and controls negative for 1858T, the sample size of our study for this calculation is 256 controls and 559 cases. Assuming an RA disease prevalence of 1%, a risk allele frequency of 0.19 for SNP 37 (derived from Dutch controls used in this study, **Table 2**), genotypic relative risks for AA and Aa of 1.78 and 1.15, respectively (derived from Carlton *et al* [13]), and a type 1 error rate of 0.05, we estimate we have 80.8% power to detect association in this sample set.

TABLE 2. PTPN22 haplotype frequencies^a

Reported haplotype ^b	Haplotype ^c	Frequency controls (%)	Frequency cases (%)	P
5	CGAAG CGCTTTA	26.74	26.13	0.772
4	CGGAACGTTTCC	18.97	16.22	0.134
1	CAGAA CGTTCTA	12.80	12.38	0.784
6	TGGAA CGCTTTA	11.92	10.70	0.479
8	TGGGA CGCTTTA	11.01	10.47	0.673
2	<u>CAGAA TGTCTA</u>	<u>9.51</u>	<u>15.46</u>	<u>0.00078</u>
3	CAGAA CGTTTTA	1.75	1.75	0.996
7	TGGAA CTCTTTA	2.00	3.7	0.048
9	TGGAA CGCCTTA	1.80	1.90	0.861

^aGenotype data from sample set 1 cases and the controls were used to predict haplotypes.

^bAccording to Carlton *et al.* [13].

^cSNPs included in haplotype analysis from left to right: rs1217414 (SNP 1), rs2488458 (SNP 2), rs12760457 (SNP 18), rs11102685 (SNP 20), rs12730735 (SNP 21), rs2476601 (SNP 22), rs38346944, rs1310182 (SNP 27), rs38346943 (SNP 28), rs1217413 (SNP 35), rs3811021 (SNP 36), rs3789604 (SNP 37). The C1858T polymorphism and the haplotype reported to be an additional independent susceptibility haplotype in North Americans (haplotype 4) are depicted in bold. The haplotype marked by the 1848T risk allele and corresponding data are underlined.

LD structure of the PTPN22 gene and haplotype analysis

Pairwise LD for the 12 PTPN22 SNPs was defined using the genotype data from sample set 1 cases and the controls separately, and the summary statistics D0 and R2 calculated using the HaploView program [21]. The LD structure for the PTPN22 region in the Dutch population was similar to the structure reported by the International HapMap project (data not shown).

SNPs 36 and 37 were in nearly complete LD ($R^2 = 0.98$ for cases and $R^2 = 1$ for controls), as previously reported in the North American samples [13].

Haplotypes were predicted for these 12 markers using the EM algorithm in the Haplo.stats package (**Table 2**). Nine haplotypes, each with a frequency $>1\%$, were predicted in both cases and controls accounting for $>96\%$ of all the haplotypes. Overall, association between the PTPN22 haplotypes and disease status was highly significant (global P-value = 0.00848; df = 9). The most common haplotype, haplotype 5, had a frequency of approximately 26% in both cases and controls. This was also the most common haplotype in the controls from the two white North American sample sets (frequencies of 29.8 and 35%) [13]. As expected from the single marker data, the sole haplotype carrying the 1858T risk allele, haplotype 2, was increased in patients compared with controls ($P = 0.00078$). Haplotype 1, identical at every SNP tested except 1858, is not associated with disease. Additionally, we observed a marginally significant increase in the frequency of haplotype 7 in the RA cases ($P = 0.048$). Haplotype 4, reported to be significantly increased in RA patients relative to controls independent of R620W [13], was actually more common in controls than cases in the Dutch sample set (19 and 16%, respectively); however, these frequencies were not significantly different ($P = 0.134$). The relative effect of each haplotype was also analysed using regression analysis with the most common haplotype, haplotype 5, serving as the reference group (haplo.stats). Relative to haplotype 5, the 620W-associated haplotype (haplotype 2) showed strong association with disease ($P = 0.006$, OR = 1.68), whereas haplotype 4 was not significant ($P = 0.4$, OR = 0.88).

Meta-analysis of PTPN22 30UT0R polymorphism rs3789604

We summarized the four available studies and performed a meta-analysis on the rs3789604 polymorphism. Using a random effects model, the common OR for the minor allele of SNP 37 was calculated as 1.08 (95% CI: 0.8–1.4) with $P_{\text{common}} = 0.6$, showing no overall association of the minor allele with RA (Cochran $Q = 5.91$, df = 2, $P = 0.0522$).

Association of the PTPN22 1858T allele with RF-positive and/or RF-negative disease

It has been postulated that the PTPN22 1858T allele is associated with autoimmunity characterized by the presence of autoantibodies [6]. We previously reported association of the

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PTPN22 risk allele with both RF-positive and ACPA-positive disease in a Dutch population of RA patients participating in our EAC—in this article referred to as sample set 2 [11]. Here we investigated whether the 1858T polymorphism was associated with RF-positive and/or RF-negative disease in 302 RA patients from sample set 1 (**Table 3**). ACPA-status was not known for this sample set. In line with our previous report [11] we observed a significant increase in the frequency of 1858T in 197 RF-positive RA patients compared with controls (0.142 in cases vs 0.097 in controls, $P = 0.03$, $OR = 1.55$). The frequency of the minor allele was also increased in the 105 RF-negative patients (0.133 in cases vs 0.097 in controls) although this difference was not statistically significant ($P = 0.14$; $OR = 1.43$). Comparison of allele frequencies of RF-positive and RF-negative revealed no significant differences between the two groups with $OR = 1.03$ and 95% CI: 0.87–1.21.

TABLE 3. Association of PTPN22 1858T allele with rheumatoid factor positive RA ^a

	Genotypes% (n)			P _{trend} ^b	Allele frequency (%)		OR (95% CI)	P ^c
	GG	AG	AA		G	A		
Controls (n¼ 284)	80.6 (229)	19.4 (55)	0 (0)		90.3	9.7		
RA cases								
RF-positive (n¼ 197)	75.6 (149)	20.3 (40)	4.06 (8)	0.03	85.7	14.2	1.55 (1.02–2.34)	0.03
RF-negative (n¼ 105)	76.2 (80)	20.95 (22)	2.8 (3)	0.136	86.7	13.3	1.43 (0.86–2.39)	0.14

^aThese data are from sample set 1 cases and the controls.
^bTest oflinear trend for genotypes.
^cSignificance of the differences in allele frequencies between cases and controls.

DISCUSSION

PTPN22 has been now unanimously accepted as a RA susceptibility gene due to robust replication of association with disease in multiple independent case-control and family based sample sets. However it is still a matter of debate whether the R620W polymorphism is the only PTPN22 disease-predisposing variant. In general, identification of independent genetic effects in a region of strong LD is difficult. A clear example is the HLA locus, the most significant RA-susceptibility region estimated to account for up to one-third of the total genetic effect. After nearly 30 years of research there is still no clear consensus on which genes, let alone alleles, in this region play a role in RA susceptibility/protection and/or disease severity.

We confirmed the PTPN22 haplotype structure reported by Carlton *et al* [13] for their two North American sample sets identifying 9 of the 10 reported haplotypes. We failed to detect the tenth haplotype for technical reasons; the SNP that uniquely marks this haplotype

was removed from this analysis because control genotypes were not in HWE. In addition, we confirmed that the 1858T risk allele was found on a single haplotype significantly associated with disease. As in the North American study, this haplotype was identical to a second haplotype (haplotype 1) at all positions genotyped except that it carried 1858C. Haplotype 1 was not associated with disease, confirming that 1858T is a disease-associated SNP. Although the same haplotypes were found in both sample sets, there were notable frequency differences between the two populations. In particular, haplotype 5, the most common haplotype in both populations, was more frequent in the North American controls while haplotype 8 was more frequent in the Dutch controls. We also found a marginally significant association of haplotype 7 with RA, marked by the minor allele of rs38346944 (SNP 23) ($P = 0.048$), which was not seen in the North American sample sets ($P = 0.23$ and $P = 0.3$).

The most striking difference, however, was that haplotype 4, tagged by SNP 37 and found to be associated with RA independent of the C1858T missense SNP in the North American study, was not associated with disease in the Dutch sample set. In fact, unlike the North American results, this particular SNP was actually more frequent in Dutch controls than cases. This discrepancy may be due to clinical heterogeneity of the groups studied, the relatively small size of the Dutch control population (although our calculations suggest 80% power to detect a similar effect), or population-based genetic diversity. Given the observation that the frequency of the 1858T risk allele increases in frequency as one travels north through Europe from Italy/Sardinia (2.3%) to Finland (15.5%) [1] and that North Americans are a composite of various European subpopulations, it is also possible that the original finding could be the result of population admixture that was consistent across both North American data sets.

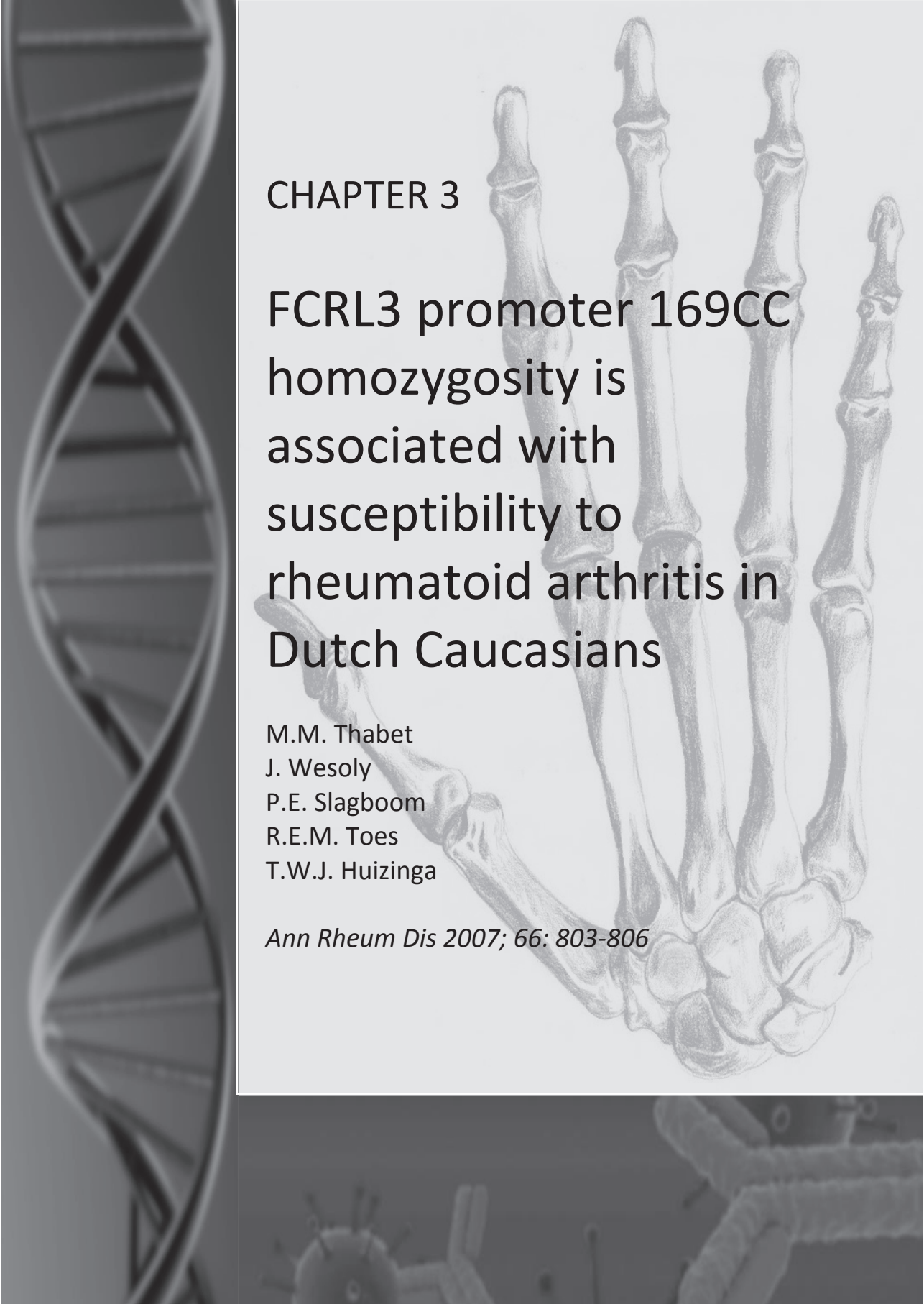
The PTPN22 1858T risk allele has been associated with a number of autoimmune diseases characterized by the presence of autoantibodies: SLE, T1D, Graves's disease and Wegener's Granulomatosis [1]. Given the well-defined role of T-cells in the production of autoantibodies and modulation of B-cell activity, it is plausible that subtle changes in T-cell function could affect the breakdown of tolerance leading to autoantibody production. However, association of the minor PTPN22 allele with autoantibody-positive RA remains

controversial. Four studies report association of the PTPN22 minor allele exclusively with RF-positive disease; however, in three studies the minor allele is associated with both RF-positive and RF-negative disease [6-12]. Although the power of the sample set reported here is limited, we see significant association of the 1858T risk allele with RF-positive disease ($P = 0.03$, $OR = 1.55$). Clinical heterogeneity could be the cause of the discrepancies between studies as misclassification of RF status can occur when different detection methods are used. In addition, fluctuations of RF titres can occur. A large adequately powered study is clearly necessary to unambiguously define the association of PTPN22 with autoantibody negative RA. There are additional indications that other PTPN22-independent mechanisms of antibody expression and regulation may be involved in autoantibody-mediated autoimmune disease. Vandiedonck *et al* [25] found association of the PTPN22 1858T allele with autoantibody-negative form of Myasthenia Gravis. Although this finding requires replication, it suggests that although autoimmune diseases share general features, such as the presence of autoantibodies, PTPN22 status may influence different pathways in different diseases.

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CHAPTER 3

FCRL3 promoter 169CC homozygosity is associated with susceptibility to rheumatoid arthritis in Dutch Caucasians

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Ann Rheum Dis 2007; 66: 803-806

ABSTRACT

Background: Human leucocyte antigen is the only genetic risk factor for rheumatoid arthritis (RA) that has been consistently observed in different populations. A number of other genes such as PTPN22 and PADI4 showed population-specific association with RA susceptibility. Recently, Fc receptor-like 3 (FCRL3) gene was found to be associated with RA susceptibility in Japanese, but with conflicting results in other populations.

Objective: To investigate the association of FCRL3 polymorphism with RA susceptibility and severity in Dutch Caucasian patients with RA, as well as to perform a meta-analysis to reveal the contribution of this gene to RA susceptibility.

Methods: A total of 931 Dutch RA cases and 570 unrelated Dutch controls were genotyped for four FCRL3 single-nucleotide polymorphisms (SNPs). Genotyping was performed using the MassArray matrix-assisted laser desorption ionisation-time-of-flight mass spectrometry. Association of the FCRL3 SNPs with susceptibility to RA was examined by single-marker, carrier and haplotype analysis.

Results: Carrier analysis of the SNP (rs7528684) revealed the association of CC genotype with a higher risk of developing RA as compared with TT and TC carriers ($p = 0.039$ and $OR = 1.31$). There was no significant difference in the genotype and allele frequencies of all investigated SNPs between cases and controls. Meta-analysis of all studies comparing 9467 individuals showed that the OR for the CC genotype to develop RA was 1.2 and the p value <0.001 .

Conclusion: A promoter polymorphism of FCRL3 (rs7528684) is associated with an increased risk of developing RA in Dutch Caucasians, suggesting that this association is relevant for RA in both Japanese and Caucasian populations.

INTRODUCTION

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterised by chronic inflammation of the synovial joints and hyperplasia and overgrowth of synoviocytes, with consequent destruction. The aetiology and pathogenesis of this disease are not completely understood. Cumulative studies suggest that RA occurs in a genetic background in which multiple common genetic risk factors that may interact are inherited. The most thoroughly examined genes associated with RA are the human leucocyte antigen (HLA) class II gene particularly shared epitope (SE) alleles. However, HLA has been estimated to account for one-third of the genetic component in the disease, indicating that genes outside the HLA region also contribute to the disease [1]. By genome wide scanning and candidate approaches, new candidate susceptibility genes have been identified. Kochi *et al* [2] conducted linkage disequilibrium mapping in a Japanese population using 830 RA cases and 658 controls. An association between susceptibility to RA and a variant of the Fc receptor like 3 gene (FCRL3) was identified. The strongest evidence of association was derived from a polymorphism in the promoter region of FCRL3 (single-nucleotide polymorphism (SNP) rs7528684; 169T/C). The minor C allele was associated with susceptibility to RA, with an odds ratio (OR) of 2.15 (95% CI 1.58 to 2.93), $p < 0.001$.

The precise function of FCRL3 is unknown, but its predicted molecular structure suggests that it is a membrane protein that conveys signals into cells through a cytoplasmic domain containing an immunoreceptor-tyrosine activation motif and an immunoreceptor-tyrosine inhibitory motif [3]. Functional analysis showed that presence of the C allele alters a putative nuclear factor kB binding site, and, as a consequence, alters the expression of FCRL3. Moreover, higher expression of FCRL3 was observed in individuals carrying the susceptible allele. Likewise, augmented autoantibody production was associated with the susceptible genotypes, as Kochi *et al* [2] reported a significant association between FCRL3 genotypes and serum rheumatoid factor (RF) level.

In subsequent replication studies in the Japanese population 540 cases and 636 controls, and 748 cases and 934 controls a similar trend was observed regarding allele frequencies and carriership, but with lower p values. These findings did not allow for a conclusion to be

reached on whether the promoter SNP biological effect necessitates a carrier analysis of CC versus TC+TT or C allele analysis [4]. These findings were not replicated in other populations such as American and Spanish Caucasians. Therefore, it is not known whether the association observed in the Japanese is a population-specific effect that is confined to the Japanese or if it is also present in Caucasians [5, 6].

Here, we analysed allele frequencies and carriership in Dutch Caucasian patients with RA and controls. A meta-analysis of the results from Japanese and other populations was performed to gain a better appraisal of the contribution of FCRL3 in the pathogenesis of RA.

METHODS

Subjects

Patients were 975 Dutch Caucasian individuals with RA, all of whom fulfilled the American College of Rheumatology (ACR) classification criteria for RA and have been described elsewhere [7-9]. The main clinical characteristics of the patients were: mean (SD) age at onset 56 (16) years, male:female ratio 2:1, percentage of anticyclic citrullinated peptide antibody (anti-CCP) positivity 59.8%, percentage of shared epitope positivity 67.1% and percentage of erosive disease 56.4%.

Controls were 581 unrelated Dutch Caucasian individuals with no history of RA [10]. Patients and controls were enrolled in the study, with informed written consent obtained according to the Declaration of Helsinki. Commissie Medische Ethiek, the Leiden institutional review board, approved all protocols.

SNPs and genotyping

Two promoter SNPs (rs7528684, rs11264799), one exonic SNP (rs945635) and one intronic SNP (rs10489678) were genotyped using the MassArray matrix-assisted laser desorption ionisation-time-of-flight mass spectrometry, according to the protocols recommended by the manufacturer (Sequenom, San Diego, California, USA). SpectroCaller software supplied by the manufacturer was used to automatically call the genotypes. All doubtful calls were checked, and after manually evaluating their spectra, they were accepted, recalled or rejected.

Genotype frequencies for these SNPs were in Hardy–Weinberg equilibrium both in controls and in patients with *p* values .0.05. The percentage of successful genotyping was .97.1%.

Severity of RA

Radiographs of the hands and feet were scored at baseline, 1 year, 2 years and 4 years using the Sharp–van der Heijde method [11], and were used to assess disease severity and progression. The analysis of these scores was performed only for those patients whose radiographs are available for all time points, in order to avoid bias in the results by missing data.

Anti-CCP

Serum anti-CCP2 was determined by ELISA (Immunoscan RA Mark 2, Euro-diagnostica, Arnhem, The Netherlands) according to the manufacturer's instructions, with a cut-off value of 25 units.

Statistical analysis

A χ^2 test with 2 degrees of freedom was used for association analyses using standard free software package (Epi Info v6, CDC, Atlanta, Georgia, USA). Mann–Whitney U test was used to associate the severity of RA (assessed by radiograph scores) and different genotypes. SPSS V.12.0.1 was used to analyse the data. Haplotypes were predicted using the Chaplin software package v.1.1.3 [12, 13], and linkage disequilibrium (LD) analysis was performed using Haploview [14]. The Easy A 2001 free software package (designed by M Cucherat) was used for the meta-analysis, using the Mantel-Haenszel method for calculating ORs and Breslow and Day method to test for heterogeneity.

RESULTS

Four SNPs located in the FCRL3 gene were genotyped. No significant difference was observed between minor allele frequency and genotypes in patients and controls (**table 1**). Recessive trait comparison of the promoter SNP (rs7528684) (CC versus TT+TC carriers) revealed a significant difference between cases and controls (*p* = 0.039, OR = 1.31 (95% CI 1.00 to 1.72)) as shown in **table 2**. Comparison of the (TT) genotype carriers with the CC+TC

Table 1 FCRL3—genotype and allele frequencies in patients with Rheumatoid arthritis and controls

SNP reference *	SNP ID†	Genotypes, % (n)		p Value‡	Allele frequency (%)		OR (95% CI) 1	p Value
FCRL3_3	rs7528684	TT	TC	CC	T	C		
	Cases (n = 931)	30.9 (288)	46 (428)	23.1 (215)	53.9	46.1		
	Controls (n = 570)	31 (177)	50.4 (287)	18.6 (106)	56.2	43.8	1.1 (0.94 to 1.28)	0.22
	rs11264799	GG	GA	AA	G	A		
	Cases (n = 941)	52.7 (496)	39.6 (373)	7.7 (72)	72.5	27.5		
	Controls (n = 581)	55.9 (325)	37.4 (217)	6.7 (39)	74.6	25.4	1.11 (0.94 to 1.32)	0.2
	rs945635	CC	CG	GG	C	G		
	Cases (n = 939)	30.9 (290)	45.3 (425)	23.8 (224)	53.5	46.5		
	Controls (n = 568)	30.5 (173)	50.3 (286)	19.2 (109)	55.6	44.4	1.09 (0.94 to 1.27)	0.257
	rs10489678	GG	GA	AA	G	A		
	Cases (n = 932)	67.5 (629)	28.2 (263)	4.3 (40)	81.6	18.4		
	Controls (n = 571)	67.8 (378)	28.4 (162)	3.8 (22)	82	18	1.02 (0.84 to 1.25)	0.8

SNP, single-nucleotide polymorphism.

*SNP reference based on Kochi et al.³

†Indicates the dbSNP ID as well as the number of individuals successfully genotyped.

‡ χ^2 test with 2 degrees of freedom.

1OR and 95% CIs are calculated for the minor allele of each marker.

Table 2 Single-nucleotide polymorphism rs7528684 (169T/C) homozygote minor allele carrier analysis—patients and controls in different studies

Reference	SNP ID*	Genotypes, % (n)		OR (CC vs CT+TT) P Value†	Allele frequency (%)		OR (95% CI) 1	p Value
Current study	rs7528684	CC	CT+TT		C	T		
		Cases (n = 931)	23.1 (215)	76.9 (716)	46.1	53.9		
		Controls (n = 570)	18.6 (106)	81.4 (464)	43.8	56.2	1.1 (0.94 to 1.28)	0.22
Kochi et al (initial study)	rs7528684	CC	CT+TT		C	T		
		Cases (n = 824)	19.3 (159)	80.7 (665)	42	58		
		Controls (n = 649)	10 (65)	90 (584)	34.5	65.5	1.37 (1.18 to 1.60)	0.000 035
Kochi et al (replication)	rs7528684	CC	CT+TT		C	T		
		Cases (n = 540)	14.3 (77)	85.7 (463)	40.3	59.7		
		Controls (n = 636)	11.8 (75)	88.2 (561)	36	63.8	1.19 (1.00 to 1.41)	0.041
Ikari et al ⁶	rs7528684	CC	CT+TT		C	T		
		Cases (n = 748)	17.8 (133)	82.2 (615)	43	57		
		Controls (n = 934)	13.8 (129)	86.2 (805)	39.1	60.9	1.18 (1.02 to 1.35)	0.022
Hu et al ⁵ (Set 1)	rs7528684	CC	CT+TT		C	T		
		Cases (n = 467)	18.6 (87)	81.4 (380)	43.4	56.6		
		Controls (n = 473)	20.9 (99)	79.1 (374)	44.8	55.2	0.94 (0.78 to 1.14)	0.52
Hu et al (Set 2)	rs7528684	CC	CT+TT		C	T		
		Cases (n = 565)	20.4 (115)	79.6 (450)	45.7	54.3		
		Controls (n = 743)	23 (171)	77 (572)	46.8	53.2	0.95 (0.81 to 1.12)	0.55
Martínez et al ⁷ (Madrid)	rs7528684	CC	CT+TT		C	T		
		Cases (n = 448)	23(102)	77(346)	48	52		
		Controls (489)	21(103)	79(386)	45	55	1.15 (0.95 to 1.38)	0.14
Martínez et al (Granada)	rs7528684	CC	CT+TT		C	T		
		Cases (n = 221)	17(38)	83(183)	45	55		
		Controls (n = 229)	18(41)	82(188)	43	57	1.09 (0.83 to 1.44)	0.5
Meta-analysis of all studies	rs7528684	CC	CT+TT					
		Cases (n = 4794)	926	3868				
		Controls (n = 4502)	789	3713			1.2 (1.08 to 1.34)	0.001

SNP, single-nucleotide polymorphism.

*Indicates the dbSNP ID as well as the number of individuals successfully genotyped.

† χ^2 test with 2 degrees of freedom.

1OR and 95% CIs are calculated for the minor allele of each marker.

carriers (dominant model) showed no significant difference between patients and controls ($p = 0.96$ and $OR = 0.99$ (0.79 to 1.25)). No association of (rs7528684) CC genotype was found with SE status, anti-CCP positivity and severity of RA (data not shown). Moreover, we found a significant difference in homozygosity to minor allele between cases and controls in SNP (rs945635; $p = 0.034$, $OR = 1.32$ (1.01 to 1.72)). Pairwise LD for the four FCRL3 SNPs was defined using the genotype data from cases and controls separately. The four SNPs were in high LD with each other. Haplotypes were predicted for these four markers. Three haplotypes, each with a frequency $>1\%$, were predicted in both cases and controls, accounting for $>96\%$ of all the haplotypes (haplotypes 1 (TGCG), with frequency 53.6% in cases and 55.9% in controls, haplotypes 2 (CAGG), with frequency 27.5% in cases and 25.4% in controls, and haplotypes 3 (CGGA), with frequency 18.5% in cases and 18.4% in controls). Overall, there was no significant association between these FCRL3 haplotypes and disease status ($p = 0.2$ for haplotype 1, $p = 0.2$ for haplotype 2 and $p = 0.92$ for haplotype 3). The two haplotypes carrying the 169C risk allele (haplotypes 2 and 3) were more frequent in patients than in controls, but these differences in frequencies were not significant.

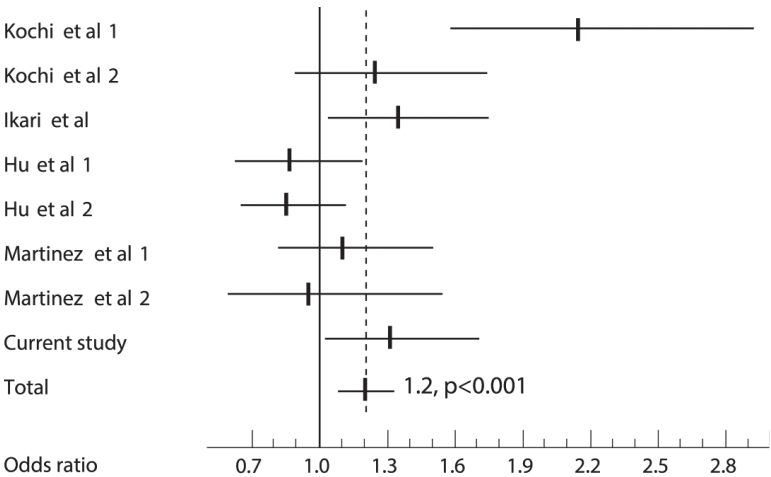


Figure 1 Meta-analysis of different studies on the promoter FCRL3 SNP (rs7528684) in RA.

As no significant differences in allele frequencies were observed between healthy controls in various replication study (Japanese and non-Japanese) compared with the original study of Kochi *et al* [2] (all p values were <0.05), we performed a meta- analysis for the carrier status and its association with RA using all the published studies. A significant difference was observed between CC against CT+TT carriers in 4744 cases and 4723 controls in the combined studied population ($p<0.001$ and OR = 1.2 (95% CI 1.08 to 1.34); **figure 1**). Meta-analysis of only the Caucasian studies (1600 cases and 1288 controls) similarly showed higher occurrence of CC carriers in patients with RA than in controls, but with a p value of 0.084 (OR = 1.18 (95% CI 0.98 to 1.42)).

DISCUSSION

In the current study, a meta-analysis of all the studies on FCRL3 promoter polymorphism in RA was performed. A total of 9467 patients were studied, and association between the CC carriers and susceptibility to RA was observed, with a p value <0.001 and an OR of 1.2.

Recently, Kochi *et al* [2] reported that an FCRL3 promoter SNP is associated with RA in the Japanese population, and this SNP was shown to alter the binding affinity of nuclear factor kB. Here, we investigated the association of four SNPs within the FCRL3 gene with RA. Our findings partially support the findings in Japanese patients with RA, as we found a significant association between RA and recessive genotype (CC) in the promoter 169T/C SNP. Although the C allele was more frequent in patients with RA than in controls in our study (similar to the findings of Kochi *et al* [2]), the difference was insignificant. Interestingly, a study performed in white North Americans revealed that the minor allele (C) was increased in controls as compared with patients with RA [5]. We also found a significant association between RA and homozygosity to the G allele in the exonic non-coding SNP (rs945635) C/G, but this association is most probably due to the very high LD between this SNP and 169T/C SNP ($r^2 = 0.99$).

Stratification of patients according to anti-CCP and SE status revealed no association between the promoter 169T/C SNP and RA susceptibility in anti-CCP and either SE-positive or SE- negative individuals. Also, no association was found between this promoter SNP and

the severity of RA. We inferred three common FCRL3 haplotypes, but their frequencies were not significantly different between cases and controls.

Our partial replication of the Japanese data, in addition to the failure of other American and Spanish groups to replicate these findings, cannot be explained by the difference in allele frequency between different ethnic groups, as we noticed no significant difference in allele frequency in controls between all the studied groups from different ethnic backgrounds. Likewise, we do not consider it likely that the difference in disease severity between different studied groups, in case this variant of FCRL3 is associated with severity, explains the variation found between different studied populations, because, in our study, no association between disease severity and the allele or genotype frequency in the promoter 169T/C SNP was observed; however, we could find an association between the recessive trait (CC) and RA.

A possible explanation for the difference in findings between different studies is related to the sizes of the studied groups. Until now, a total of 9467 individuals have been studied (in all the studies), and the meta-analysis indicates a significant difference between carriers of the recessive trait genotype in patients and controls ($p < 0.001$ and $OR = 1.2$). Additionally, even though the allele frequency of the promoter SNP was not significantly different between all populations, there might be other hidden factors influencing the effect of this SNP, which are specific for a particular ethnic group in the various studied populations. In our study, we analysed FCRL3 polymorphism in RA as a recessive trait (CC vs TC+TT) and we partially replicated the Japanese findings. This study is the first positive replication study in Caucasians, but, given the heterogeneity of results from different studies, additional well-powered studies in Caucasians are necessary to clear the remaining doubt regarding the validity of the association of FCRL3 with RA susceptibility. Taking into consideration that all the European studies on FCRL3 promoter polymorphism in RA, including ours, showed no differences in allele and genotype frequencies between patients and controls, future studies might consider the possible involvement of FCRL3 promoter polymorphism in RA in a recessive trait fashion rather than by allele frequency comparison.

CONCLUSIONS

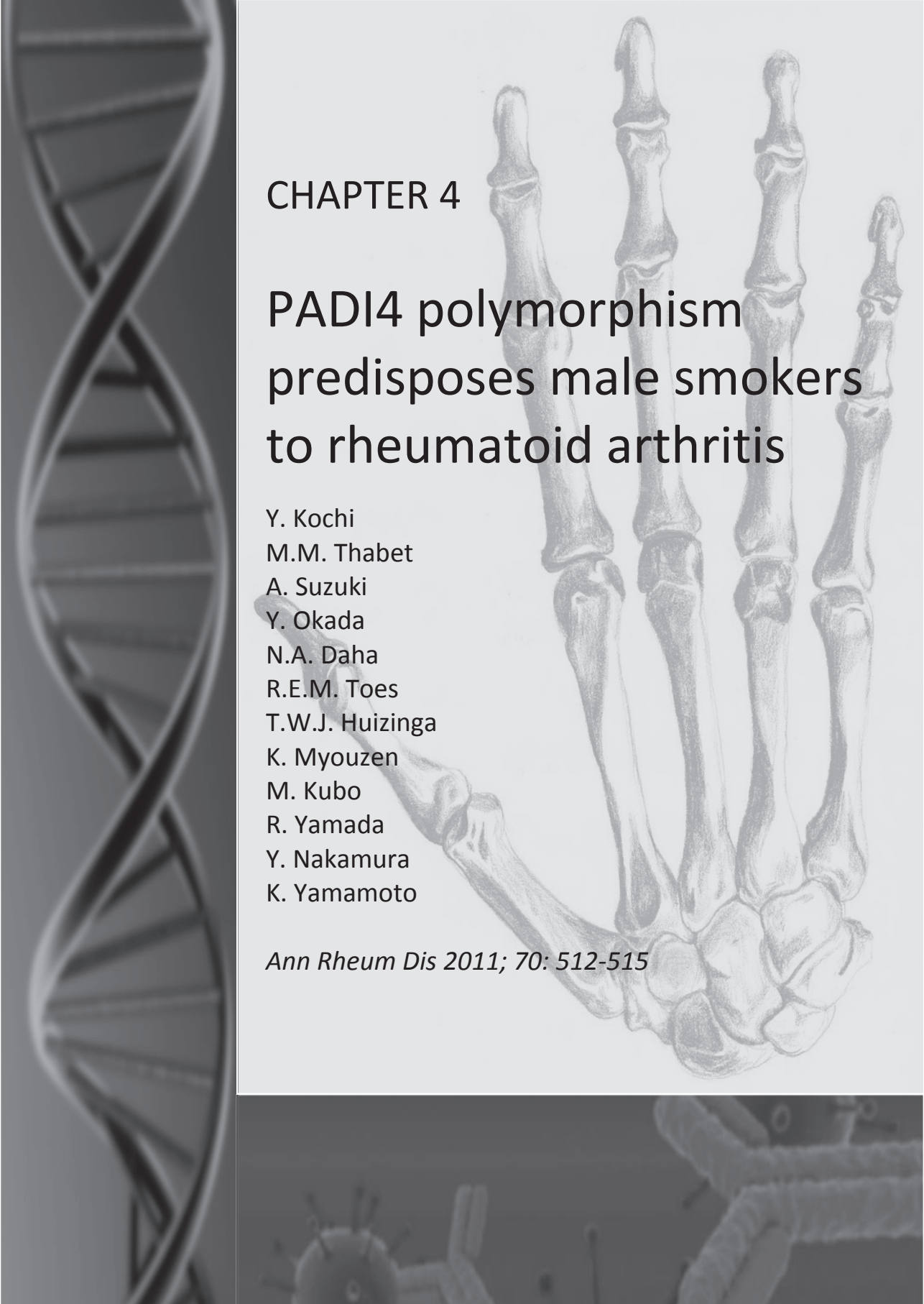
We conclude that the FCRL3 promoter SNP is associated with susceptibility to RA in individuals who carry the recessive genotype CC in Dutch Caucasians. These findings suggest that the association of FCRL3 with RA susceptibility might not be specific to the Japanese population, and that FCRL3 is possibly involved in the pathogenesis of RA in a recessive trait fashion. Further well-powered studies are needed to clarify this association in various ethnic groups.

Note added in proof: Shortly after this manuscript was accepted for publication, Newman *et al* [15] published a study that concluded that RA association with the FCRL32169C polymorphism is restricted to PTPN22 1858T-homozygous individuals in a Canadian population [15]. Accordingly, we performed a similar analysis in our cohort and found no statistically significant difference in FCRL3 169C variants between patients carrying and those lacking the PTPN22 1858T risk allele (data not shown). Hence, we conclude that, in our cohort, there is no interaction between FCRL3 and PTPN22 risk alleles in predisposing to RA.

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CHAPTER 4

PADI4 polymorphism predisposes male smokers to rheumatoid arthritis

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Ann Rheum Dis 2011; 70: 512-515

ABSTRACT

Objective: To elucidate the differential role of peptidyl arginine deiminase 4 (PADI4) polymorphism in rheumatoid arthritis (RA) between Asian and European populations, possible gene-environmental interactions among the PADI4 polymorphism, sex and smoking status were analysed.

Methods: Three independent sets of case-control samples were genotyped for single nucleotide polymorphisms in PADI4; Japanese samples (first set, 1019 RA patients, 907 controls; second set, 999 RA patients, 1128 controls) using TaqMan assays and Dutch samples (635 RA patients, 391 controls) using Sequenom MassARRAY platform. The association of PADI4 with RA susceptibility was evaluated by smoking status and sex in contingency tables and logistic regression models.

Results: In the first set of Japanese samples, PADI4 polymorphism (rs1748033) showed a greater risk in men (OR_{allele} 1.39; 95% CI 1.10 to 1.76; $p_{\text{trend}}=0.0054$) than in women and in ever-smokers (OR_{allele} 1.25; 95% CI 1.02 to 1.53; $p_{\text{trend}}=0.032$) than in never-smokers. Moreover, the highest risk was seen in male ever-smokers (OR_{allele} 1.46; 95% CI 1.12 to 1.90; $p_{\text{trend}}=0.0047$). Similar trends were observed in the second set of Japanese samples as well as in Dutch samples.

Conclusion: PADI4 polymorphism highly predisposes male smokers to RA, and the genetic heterogeneity observed between Asian and European populations may be partly explained by differences in smoking prevalence among men.

INTRODUCTION

Rheumatoid arthritis (RA) is a multigenic disease caused by interactions between genetic predispositions and environmental factors that result in abnormal immune response and joint destruction. The HLA-DRB1 region is considered to be the major genetic determinant of RA susceptibility, but recent genetic studies have revealed multiple non-human leucocyte antigen susceptibility genes for RA [1]. Among these, the peptidyl arginine deiminase 4 (PADI4) gene, which encodes a post-translational modification enzyme that converts arginine to citrulline residues in proteins, is thought to have significant relevance in RA pathogenesis as anti-citrullinated protein antibodies (ACPA) are specifically observed in the sera of patients [2, 3].

The association of the PADI4 polymorphism with RA susceptibility was first reported in a Japanese population [2] and has been replicated in several Asian populations [4, 5]. Conversely, inconsistent results have been observed in populations of European ancestry [6-8]. A meta-analysis confirmed the association in Asian populations, but not in European populations [6-7]. The genetic heterogeneity observed between different populations could be partly explained by the difference of disease severity between the study populations, as the PADI4 polymorphism was reported to influence erosive joint status [9]. However, it could also be explained by unknown gene-gene or gene-environmental interactions with PADI4, and the higher magnitude of risk with PADI4 in Asian populations suggests the presence of these interacting factors.

Smoking is one of the well-established environmental factors in RA [10], and several studies have described associations with the appearance of ACPA in RA patients [3]. Klareskog *et al* [11] first reported that citrullinated proteins were detected in bronchoalveolar lavage cells from smokers but not in those from non-smokers. A later study by Makrygiannakis *et al* [12] showed that a significantly increased PADI2 expression and a higher trend of PADI4 expression were observed in bronchoalveolar lavage cells from smokers compared with non-smokers. These lines of evidence suggest that the up-regulated expression of PADI enzymes provoked by smoking may promote the citrullination of proteins in the lung, leading to citrulline autoimmunity in RA [3].

The present study examined possible interactions between PADI4 polymorphism, sex and smoking status, and discusses the resulting influence on the genetic heterogeneity in PADI4 observed between Asian and European populations.

METHODS

Subjects

Japanese RA patients (first set n=1019, second set n=999) were provided by the Leading Project for Personalized Medicine in the Ministry of Education, Culture, Sports, Science and Technology, Japan (BioBank Japan) [13]. Unrelated Japanese controls (first set n=907, second set n=1128) were recruited through Midousuji Rotary club and several medical institutes in Japan. These Japanese case-control sets were independent from that used in the previous study [2]. Dutch cohorts and RA patients were previously described [14]. RA patients (n=635) were part of the Leiden Early Arthritis Clinic, which comprises an inception cohort of patients with recent-onset arthritis (duration of symptoms <2 years). Those patients were diagnosed with RA within the first year after their initial visit. All individuals with RA met the 1987 revised criteria of the American College of Rheumatology for RA [10]. The characteristics of the cohorts are described in detail in supplementary **table 1** (available online only). All subjects entered into this study provided informed consent prior to participation in the study, and all study protocols were preapproved by the ethics committees of each institute.

Smoking status

Smoking status was determined for each individual on the basis of self-reported information. An ever-smoker was defined as a person who had smoked tobacco, cigarettes or pipes at any stage in their life, whereas a never-smoker was defined as someone who had never smoked any of these. Smoking status was available for all the samples in the first Japanese case-control set, cases in the second Japanese set and a part of the Dutch RA patients (52.9%), but not for the control subjects in the second Japanese and Dutch sets.

SNP genotyping

The four exonic single-nucleotide polymorphisms (SNP) comprising two major transcripts of PADI4 (rs11203366=padi4_89, rs11203367=padi4_90, rs874881=padi4_92 and

Table 1 Association of the PADI4polymorphism and RA stratified with sex and smoking status in a Japanese population*

	Set	Sum		MAF		Per allele OR	p Value for trend test
		Case	Contol	Case	Contol		
s11203367							
All	1st	1019	907	0.43	0.40	1.14 (1.00 to 1.29)	0.045
	2nd	996	1124	0.42	0.40	1.09 (0.96 to 1.23)	0.16
Men	1st	190	672	0.48	0.39	1.44 (1.14 to 1.81)	0.0022
	2nd	185	448	0.44	0.40	1.18 (0.92 to 1.51)	0.19
Women	1st	829	235	0.42	0.41	1.02 (0.82 to 1.25)	0.84
	2nd	811	676	0.41	0.39	1.07 (0.92 to 1.24)	0.31
Ever-smoker	1st	337	488	0.47	0.39	1.35 (1.11 to 1.65)	0.0024
	2nd	302	1124	0.43	0.40	1.15 (0.95 to 1.38)	0.12
Never-smoker	1st	682	418	0.41	0.40	1.03 (0.86 to 1.23)	0.71
	2nd	694	1124	0.41	0.40	1.06 (0.92 to 1.21)	0.36
Male ever-smoker	1st	155	451	0.50	0.39	1.61 (1.24 to 2.09)	0.00031
	2nd	145	448	0.46	0.40	1.25 (0.96 to 1.63)	0.10
Male never-smoker	1st	35	221	0.39	0.40	0.92 (0.54 to 1.54)	0.77
	2nd	40	448	0.39	0.40	0.95 (0.59 to 1.52)	0.84
Female ever-smoker	1st	182	37	0.44	0.47	0.86 (0.52 to 1.42)	0.56
	2nd	157	676	0.41	0.39	1.05 (0.82 to 1.35)	0.64
Female never-smoker	1st	647	197	0.41	0.40	1.04 (0.83 to 1.32)	0.68
	2nd	654	676	0.41	0.39	1.08 (0.92 to 1.26)	0.31
s1748033							
All	1st	1018	904	0.37	0.35	1.12 (0.98 to 1.27)	0.089
	2nd	996	1125	0.36	0.34	1.08 (0.95 to 1.22)	0.20
Men	1st	190	669	0.42	0.34	1.39 (1.10 to 1.76)	0.0054
	2nd	185	448	0.40	0.34	1.25 (0.97 to 1.60)	0.08
Women	1st	828	235	0.36	0.36	1.00 (0.81 to 1.24)	0.96
	2nd	811	677	0.35	0.34	1.05 (0.90 to 1.22)	0.50
Ever-smoker	1st	336	485	0.40	0.35	1.25 (1.02 to 1.53)	0.032
	2nd	302	1125	0.38	0.34	1.19 (0.99 to 1.43)	0.055
Never-smoker	1st	682	418	0.36	0.34	1.07 (0.89 to 1.28)	0.47
	2nd	694	1125	0.35	0.34	1.03 (0.90 to 1.19)	0.59
Male ever-smoker	1st	155	448	0.44	0.34	1.46 (1.12 to 1.90)	0.0047
	2nd	145	448	0.41	0.34	1.34 (1.02 to 1.75)	0.039
Male never-smoker	1st	35	221	0.36	0.34	1.09 (0.64 to 1.85)	0.75
	2nd	40	448	0.34	0.34	0.96 (0.59 to 1.56)	0.90
Female ever-smoker	1st	181	37	0.37	0.41	0.87 (0.52 to 1.45)	0.60
	2nd	157	677	0.36	0.34	1.06 (0.82 to 1.37)	0.60
Female never-smoker	1st	647	197	0.36	0.35	1.03 (0.81 to 1.30)	0.79
	2nd	654	677	0.35	0.34	1.04 (0.89 to 1.22)	0.55

*rs112033673 (T/C, T is the minor allele) and rs174803 (T/C, T is the minor allele) were genotyped for the test. Both case and control subjects were stratified with smoking status in the first set, whereas only case subjects were stratified with smoking status in the second set.
MAF, minor allele frequency; PADI4, peptidyl arginine deiminase 4; RA, rheumatoid arthritis.

rs1748033=padi4_104) were genotyped [2]. Two of these SNP (rs11203367 and rs1748033) tag the three haplotypes (two common haplotypes and one rare haplotype, see supplementary table 2, available online only) and provide full information for PADI4. These were also tested in the Dutch population. In the Japanese population SNP were genotyped using predesigned TaqMan SNP genotyping assays (Applied Biosystems, Carlsbad, California, USA).

Fluorescence was detected using an ABI Prism 7900HT Sequence Detection System (Applied Biosystems). In the Dutch population SNP were genotyped using time-of-flight mass spectrometry-based Sequenom MassARRAY Platform (Sequenom, San Diego, California, USA). Genotyping assessment was made for over 95% of samples, for all of the polymorphisms genotyped. All SNP were in Hardy–Weinberg equilibrium in control subjects according to χ^2 statistics ($p > 0.01$).

Statistical analysis

The case–control association of each SNP was tested with the Cochran Armitage trend test and the χ^2 test. Genotype and allele frequencies for patients and controls were used to calculate the OR and the 95% CI using the method of Woolf [15]. Gene–environmental interactions were assessed by both ‘case-only’ analysis and logistic regression analysis [16]. All statistical analyses were performed using Plink software [17].

RESULTS AND DISCUSSION

A significant association between the PADI4 polymorphism and RA susceptibility was observed in the whole set of case–control subjects in the first Japanese set (rs11203367; per allele OR (OR_{allele}) 1.14; 95% CI 1.00 to 1.29; p value for a trend test (P_{trend})=0.045; **table 1**). In a stratified analysis with sex, the PADI4 polymorphism was significantly associated only in men (OR_{allele} 1.44; 95% CI 1.14 to 1.81; P_{trend} =0.0022), but not in women (OR_{allele} 1.02; 95% CI 0.82 to 1.25; P_{trend} =0.84). Similarly, when subjects in both cases and controls were stratified for smoking status, the PADI4 polymorphism had a greater effect in ever-smokers (OR_{allele} 1.35; 95% CI 1.11 to 1.65; P_{trend} =0.0024) compared with never-smokers (OR_{allele} 1.03; 95% CI 0.86 to 1.23; P_{trend} =0.71). Further stratification analysis with sex and smoking status revealed that the PADI4 polymorphism had the highest risk in the subpopulation of male ever-smokers (OR_{allele} 1.61; 95% CI 1.24 to 2.09; P_{trend} =0.00031). Similar findings were also observed, when only ACPA-positive patients were analysed (supplementary **table 3**, available online only).

To support these observations, we also analysed other case–control sets in the Japanese population and Dutch population (unstratified controls for smoking status were used in both

sets as no information was available). In the second Japanese set, the highest risk in the subpopulation of male ever-smokers was replicated in rs1748033 (OR_{allele} 1.34; 95% CI 1.02 to 1.75; $P_{\text{trend}}=0.039$; **table 1**). In the Dutch set, the association of the PADI4 polymorphism (rs1748033) was statistically significant in a dominant model (OR_{dom} 1.32; 95% CI 1.02 to 1.72; $P_{\text{dom}}=0.03$; **table 2**), but not in a trend test, when evaluated in total ($P_{\text{trend}}=0.14$). When patients were stratified by sex or/and smoking status and compared with control subjects, OR in the dominant model was higher for men (OR 1.36; 95% CI 0.90 to 2.06; $p=0.13$) than for women and was higher for ever-smokers (OR 1.56; 95% CI 1.06 to 2.31; $p=0.02$) than for never-smokers. Furthermore, it was highest in male ever-smokers (OR 1.79; 95% CI 0.98 to 3.27; $p=0.043$).

Table 2 Association of PADI4 polymorphism and RA stratified with sex and smoking status in a Dutch population*

	Sum		MAF		Genotype frequency test (dominant model)	
	Case	Contol	Case	Contol	OR	p Value
rs11203367						
All	646	385	0.44	0.42	1.19 (0.90 to 1.56)	0.2
Men	218	180	0.47	0.40	1.49 (0.96 to 2.33)	0.063
Women	398	188	0.42	0.43	1.06 (0.73 to 1.56)	0.7
Ever-smoker	174	385	0.43	0.42	1.14 (0.76 to 1.70)	0.5
Never-smoker	178	385	0.45	0.42	1.06 (0.72 to 1.57)	0.7
Male ever-smoker	76	180	0.45	0.40	1.46 (0.78 to 2.71)	0.2
Male never-smoker	40	180	0.53	0.40	1.38 (0.62 to 3.10)	0.4
Female ever-smoker	98	188	0.41	0.43	0.99 (0.58 to 1.72)	0.9
Female never-smoker	138	188	0.43	0.43	0.99 (0.61 to 1.61)	0.9
rs1748033						
All	635	391	0.34	0.30	1.32 (1.02 to 1.72)	0.03
Men	215	183	0.35	0.28	1.36 (0.90 to 2.06)	0.13
Women	389	191	0.32	0.31	1.33 (0.93 to 1.91)	0.11
Ever-smoker	158	391	0.36	0.30	1.56 (1.06 to 2.31)	0.02
Never-smoker	178	391	0.31	0.30	1.06 (0.73 to 1.53)	0.7
Male ever-smoker	70	183	0.38	0.28	1.79 (0.98 to 3.27)	0.043
Male never-smoker	41	183	0.35	0.28	1.35 (0.65 to 2.82)	0.4
Female ever-smoker	88	191	0.34	0.31	1.48 (0.86 to 2.55)	0.13
Female never-smoker	137	191	0.30	0.31	1.03 (0.65 to 1.64)	0.9

*rs112033673 (T/C, T is the minor allele) and rs174803 (T/C, T is the minor allele) were genotyped for the test. Only case subjects were stratified with smoking status. MAF, minor allele frequency; PADI4, peptidyl arginine deiminase 4; RA, rheumatoid arthritis.

These stratified analyses suggested gene–environmental inter-actions between PADI4 and sex, and/or between PADI4 and smoking status. We performed case-only analysis to test these interactions statistically, by comparing the allele frequency of the PADI4 polymorphism in the stratified subpopulation of patients (the first and second Japanese sets were combined). Allele frequency was significantly higher in men than in women (rs11203367; 0.48 vs 0.42; $P_{\text{trend}}=0.0016$) and in ever-smokers than in never-smokers (rs11203367; 0.47 vs 0.41; $P_{\text{trend}}=0.00077$), suggesting the presence of gene–environmental interactions for PADI4. Similar results were obtained for rs1748033. In addition to stratified analyses using the contingency tables, we analysed these gene–environmental interactions using logistic regression models. The first Japanese set was used for analysis because of the availability of smoking status. The PADI4 polymorphism was associated with RA susceptibility in an additive model, adjusted by sex and smoking status (rs11203367; OR_{add} 1.18; 95% CI 1.01 to 1.38; $P_{\text{add}}=0.035$). When an interaction term between SNP genotype and sex (a product term of genotype $\times$ sex) was introduced into the regression model, the logistic coefficient for the term was significant ($p=0.029$). Similarly, when an interaction term between SNP genotype and smoking status (a product term of genotype $\times$ smoking status) was introduced into the model, the coefficient for the term was again significant ($p=0.034$). We also added the age of subjects into the model, because it could be a confounding factor considering that smoking prevalence has been decreasing in recent decades, especially in Japanese men (OECD Health Data, 2009) [18]. The interaction term for SNP and smoking remained significant ($p=0.038$), whereas the significance level of the interaction term for SNP and sex became marginal ($p=0.075$).

Finally, we examined the association between the PADI4 polymorphism and ACPA status in the patients of Japanese sets. The allele frequency of PADI4 showed a higher trend in ACPA-positive patients compared with ACPA-negative patients (rs11203367; 0.43 vs 0.41; $P_{\text{trend}}=0.54$). When the genotype frequency was compared in a recessive model, the PADI4 polymorphism was significantly associated with the ACPA status in ever-smokers (rs11203367; OR_{rec} 2.33; 95% CI 1.23 to 4.39; $P_{\text{rec}}=0.0072$; **table 3**), suggesting that the PADI4 polymorphism may be involved in the appearance of ACPA in smokers.

Table 3 Association of PADI4 polymorphism and ACPA status in a Japanese population*

	Sum		MAF		Genotype frequency test (recessive model)	
	ACPA+	ACPA–	ACPA+	ACPA–	OR	p Value
rs11203367						
All	1614	401	0.43	0.41	1.25 (0.93 to 1.68)	0.14
Men	295	80	0.46	0.45	1.22 (0.65 to 2.28)	0.52
Women	1319	321	0.42	0.40	1.27 (0.90 to 1.78)	0.17
Ever-smoker	523	116	0.46	0.41	2.33 (1.23 to 4.39)	0.0072
Never-smoker	1091	285	0.41	0.41	0.99 (0.70 to 1.39)	0.96
Male ever-smoker	245	55	0.49	0.44	1.90 (0.85 to 4.25)	0.11
Male never-smoker	50	25	0.34	0.48	0.28 (0.08 to 1.01)	0.045
Female ever-smoker	278	61	0.43	0.39	3.20 (1.11 to 9.22)	0.024
Female never-smoker	1041	260	0.41	0.41	1.09 (0.75 to 1.56)	1
rs1748033						
All	1614	400	0.37	0.37	1.39 (0.98 to 1.98)	0.063
Men	295	80	0.41	0.42	1.40 (0.69 to 2.83)	0.34
Women	1319	320	0.36	0.36	1.41 (0.93 to 2.12)	0.10
Ever-smoker	523	115	0.40	0.38	2.15 (1.08 to 4.28)	0.026
Never-smoker	1091	285	0.35	0.37	1.13 (0.75 to 1.72)	0.54
Male ever-smoker	245	55	0.43	0.41	2.09 (0.84 to 5.16)	0.10
Male never-smoker	50	25	0.30	0.44	0.34 (0.08 to 1.43)	0.13
Female ever-smoker	278	60	0.37	0.36	2.28 (0.78 to 6.65)	0.12
Female never-smoker	1041	260	0.35	0.36	1.27 (0.81 to 1.98)	0.29

Anti-citrullinated protein antibody (ACPA)+ and ACPA–, ACPA-positive and ACPA-negative rheumatoid arthritis (RA) patients, respectively.

*rs112033673 (T/C, T is the minor allele) and rs174803 (T/C, T is the minor allele) were genotyped for the test. Case subjects of Japanese sets (first and second) were combined for analysis.

MAF, minor allele frequency; PADI4, peptidyl arginine deiminase 4.

Gene–environmental interactions in RA susceptibility have been well described between polymorphisms in HLA-DRB1 and PTPN22 genes and smoking habit in populations of European descent [19, 20]. Our observations here indicate that the PADI4 polymorphism is another genetic risk that would interact with smoking in RA susceptibility, although why this interaction is prominent in men remains to be solved. The status of sex hormones may influence the role of PADI4, as it is profoundly involved in the onset of RA [21]. Another possible explanation could be gender differences in smoking behaviour, which has also been argued in other smoking-related diseases [22]. Quantitative analysis of smoking history, such as pack-years smoked, may be needed to investigate further for the gender difference.

Smoking prevalence rates differed highly among the populations, and the attribution of smoking to the onset of RA may thus differ among populations. A recent epidemiological survey has shown that smoking prevalences are generally higher in men from Asian countries than in western European countries: Japan, 45.8%; Korea, 46.6%; UK, 25.0%; The

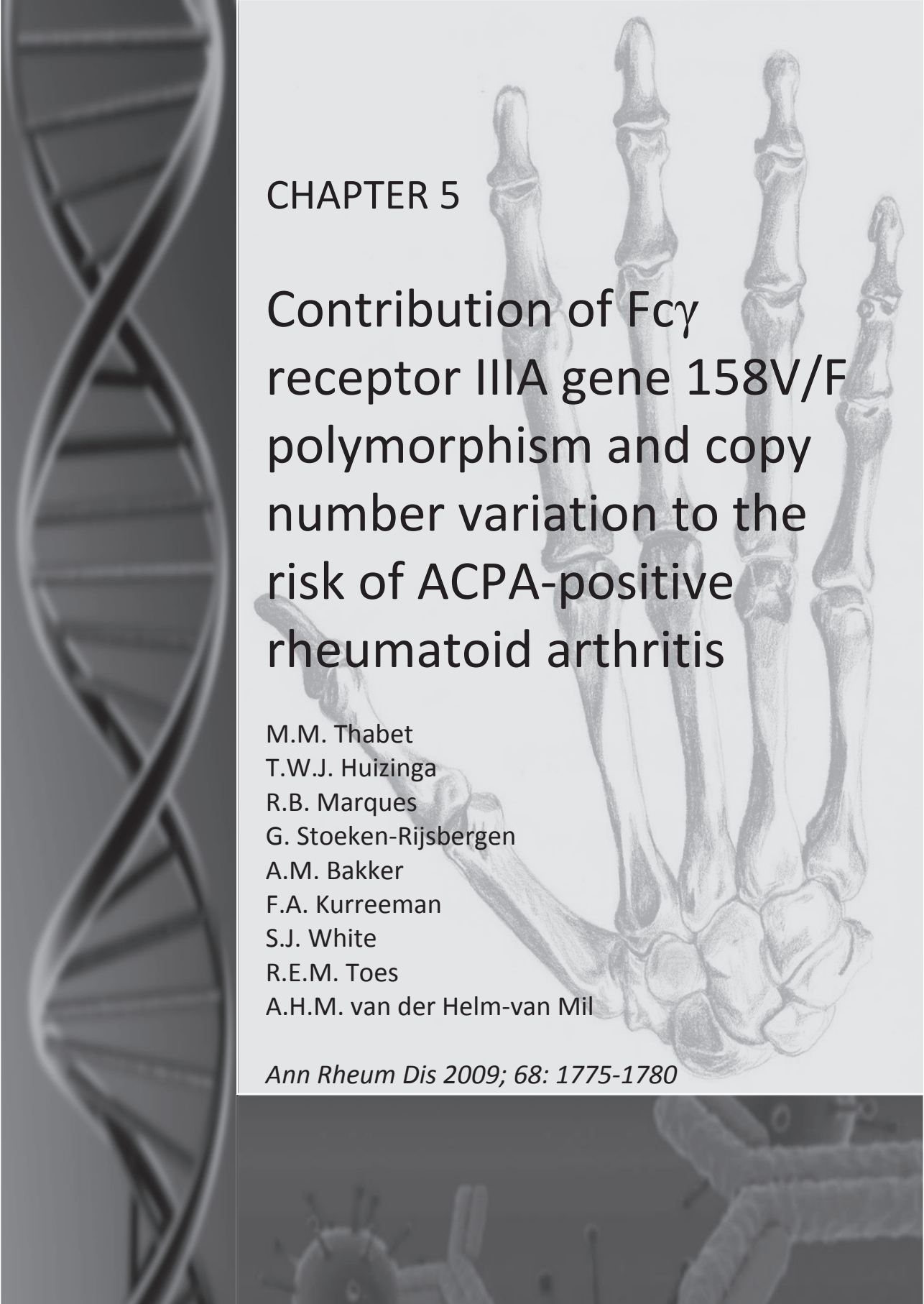
Netherlands, 35.0%; Sweden, 13.9%; and USA, 19.1% in 2005 [18]. Considering our observation that the PADI4 polymorphism has the highest risk in male ever-smokers, the attribution of the PADI4 polymorphism may be relatively high in populations with high smoking prevalences among men, such as Japan and Korea, corresponding to the positive results in association studies for PADI4 polymorphisms in these countries [2, 4, 5].

In conclusion, the PADI4 polymorphism highly predisposes male smokers to RA, and the genetic heterogeneity observed in the PADI4 polymorphism between populations of Asian and European countries may be partly explained by differences in smoking prevalences among men.

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CHAPTER 5

Contribution of Fcγ receptor IIIA gene 158V/F polymorphism and copy number variation to the risk of ACPA-positive rheumatoid arthritis

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Ann Rheum Dis 2009; 68: 1775-1780

ABSTRACT

Objectives: Fcγ receptors (FcγRs) are potent immune-modulators. FcγRs-genes encompass a complex region, polymorphic by both single nucleotide polymorphisms (SNPs) and copy number variation (CNV). The genetic complexity of FcγRs-genes, combined with the heterogeneity of rheumatoid arthritis (RA) may have caused inconsistent findings in previous studies on FcγR-SNPs in RA. Since there is increasing evidence that anti-citrullinated peptide autoantibodies (ACPA) positive RA and ACPA-negative RA have different genetic background, we investigated whether FcγRIIIA158V/F SNP differently associates with ACPA-positive and ACPA-negative RA. Moreover, this study is also the first to assess whether CNV of FcγRIIIA-gene affects the FcγRIIIA158V/F SNP genotyping and if CNV of FcγRIIIA-gene confers risk to RA.

Methods: This study comprises 945 RA patients and 388 healthy controls, all Dutch Caucasians. FcγRIIIA158V/F SNP was genotyped using Sequenom. The CNV of FcγRIIIA-gene was determined in 369 RA patients and 240 controls using Multiplex Ligation-dependent Probe Amplification (MLPA). Associations between the genotypes and RA were analysed stratifying for the presence/absence of ACPA and the presence/absence of CNV of the FcγRIIIA-gene.

Results: The FcγRIIIA-158V variant was associated with susceptibility to ACPA-positive RA (OR=1.3, 95%CI 1.01-1.6, p=0.034). In patients without CNV this association was also present (OR=1.6, 95%CI 1.2-2.4, p=0.005). FcγRIIIA-gene showed CNV that was not significantly different between patients and controls.

Conclusion: The FcγRIIIA-158V allele confers risk to ACPA-positive RA, before and after correcting for the presence of CNV. Although the FcγRIIIA-gene shows CNV, this was not associated with higher risk of RA.

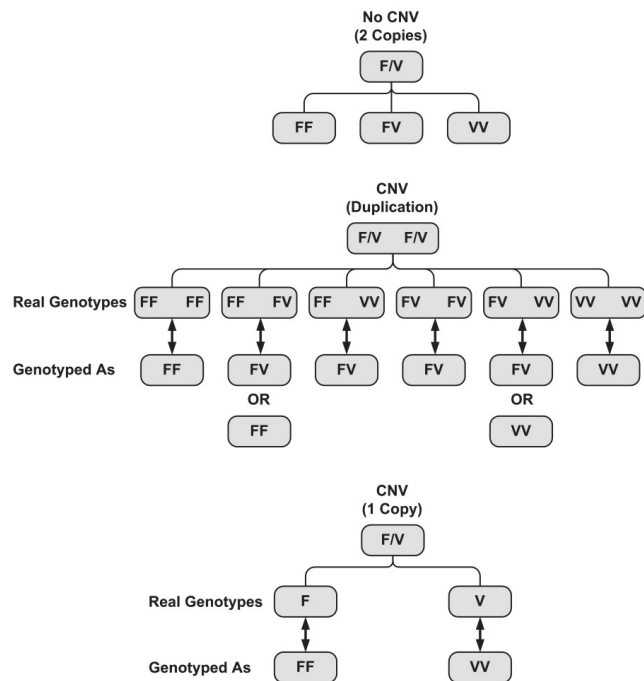
INTRODUCTION

Rheumatoid arthritis (RA) is a systemic autoimmune disease for which the aetiology and pathogenesis remain largely unclear. One of the characteristic features of part of the RA patients is the expression of auto-antibodies such as rheumatoid factors (RF) and ACPA [1]. Multiple genetic risk factors have been unequivocally shown to predispose for ACPA-positive RA but not for ACPA-negative RA, like *HLA* shared epitope (*SE*) [2], *PTPN22* [3], and recently *TRAF-C5* [4]. Also the results of HLA-association studies and genome wide SNP scans revealed that ACPA-positive RA has a different genetic background than ACPA-negative disease [5-7]. This emphasizes the need to systemically study genetic risk factors in ACPA-positive and in ACPA-negative RA separately.

The Fcγ receptors (FcγRs) play a crucial role in immunity by linking the IgG antibody mediated responses with cellular effector and regulatory functions [8]. FcγRIIIA is expressed by natural killer (NK) cells, macrophages [9] and a subset of T lymphocytes [10]. Additionally, this intermediate-affinity FcγR is believed to play a pivotal role in the clearance of immune complexes [11].

These receptors are encoded by genes clustered on the long arm of chromosome 1 (1q21-q24) in a complex region showing extensive nucleotide sequence homology that resulted from duplication and recombination events which occurred in this cluster during the evolution [12]. In addition, copy number variation (CNV) has been shown to be present in this region in several large scale whole genome studies and focused studies [13-16]. The presence of common CNVs can cause false SNP genotyping results. **Figure 1** summarizes the possible effects of CNV on SNP genotyping. A higher copy number (CN) may falsely enrich the heterozygotes, while the presence of a lower CN (a single copy) may falsely enrich the homozygotes (hemizygosity as one allele is absent). The subsequent skewing of genotypes may lead them to fail Hardy-Weinberg equation (HWE) and may blur the association of the studied SNPs with disease susceptibility. It may also limit the ability of the genome-wide SNP association studies to detect disease associated SNPs in regions with CNV [17]. Such genetic complexity renders successful genotyping of different SNPs in that region using classical methods notoriously difficult.

Figure 1: Possible effect of copy number variation (CNV) on single nucleotide polymorphism (SNP) genotyping. This figure illustrates how the presence of CNV can cause false SNP genotyping results. A high copy number falsely enriches the heterozygotes, while the presence of low copy number falsely enriches the homozygotes. In this figure the use of F and V characters was only for illustration purpose but this kind of erroneous genotyping result can occur in every genetic region that harbours both SNPs and CNV.



The presence of such a genetic complexity in the *FcγR* region, combined with the heterogeneity of RA, might be the cause of inconsistent findings in previous studies on *FcγR* SNPs in relation to RA. In particular, the functionally relevant, *FcγRIIIA* 158V/F polymorphism (rs396991) had been extensively studied in RA case-control studies, revealing remarkably contradicting results. The 158V allele was found to be associated with RA susceptibility in many studies [18-21], in another study the 158F allele was associated with RA [22], whereas in other studies no association with RA was observed [23-28]. These contradicting results can in part be caused by methodological difficulties due to the extreme homology to *FcγRIIIB* [29] but difficulties in genotyping due to the presence of CNV as well as the heterogeneity of RA regarding the ACPA status are likely causes that have never been addressed.

Given the important role of *FcγRIIIA* in auto-immunity, we specifically wanted to study the association of *FcγRIIIA* 158V/F polymorphism with ACPA-positive RA. The ACPA-

negative RA group was studied as well. Additionally, we investigated whether the presence of CNV of *FcγRIIIA* gene has any effect on the association between the FcγRIIIA 158V/F SNP and RA and also if the presence of CNV of the *FcγRIIIA* gene itself associates with susceptibility to RA.

PATIENTS AND METHODS

Subjects

Nine hundred and forty-five Dutch Caucasian individuals with RA, all of whom fulfilled the American College of Rheumatology (ACR) classification criteria for RA were studied and described elsewhere [30-32]. Controls were 388 unrelated Dutch Caucasians with no history of RA [33]. For both patients and controls an informed written consent according to the Declaration of Helsinki was obtained. The Commissie Medische Ethiek, the Leiden institutional review board, approved all protocols.

ACPA status was available for 619 patients, and was positive in 58.8% (N=364) of cases. Rheumatoid factor (RF) status was available for 899 patients, and was positive in 64.9% (N=583) of cases. Shared epitope (SE) status was available for 610 patients and was positive in 70.2% (N=428) of cases. Serum ACPA was determined by ELISA (CCP2, Immunoscan RA Mark 2, Euro-diagnostica, Arnhem, the Netherlands and Axis-Shield, Dundee, UK) and the cut-off level for ACPA positivity was set at 25 arbitrary units (AU), according to the manufacturer's instructions.

Genotyping

FcγRIIIA 158V/F (rs396991) was genotyped using the MassArray matrix-assisted laser desorption ionisation-time-of-flight mass spectrometry, according to the protocols recommended by the manufacturer (Sequenom, San Diego, California, USA). The sequences of PCR primers used in the assay were (ACGTTGGATGTTCACAGTCTCTGAAGACAC) and (ACGTTGGATGAAGCCACACTCAAAGACAGC) and the sequence of the extension primer was (ggagACTTCTGCAGGGGGCTT). SpectroCaller software supplied by the manufacturer was used to automatically call the genotypes. All doubtful calls were rechecked, and after manually evaluating their spectra, they were either accepted or recalled, and if still

doubtful the calls were rejected. Ten per cent of samples were genotyped in duplo. The error rate of genotyping was 0%.

CNV

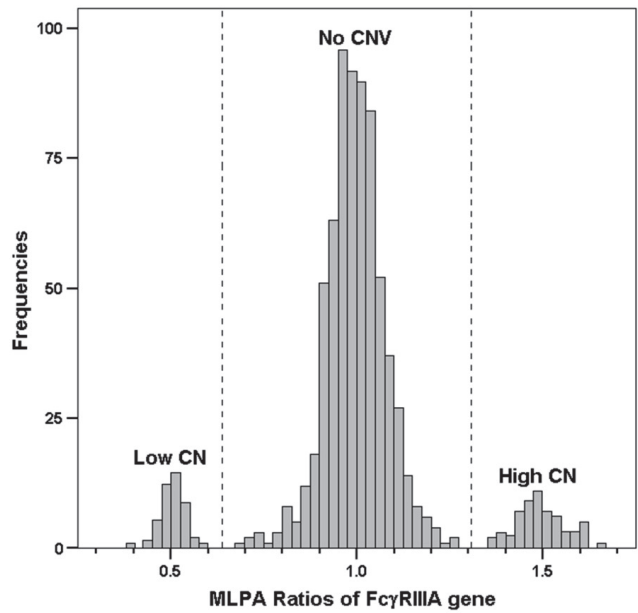
The CNV status of *FcγRIIIA* gene was assessed using Multiplex Ligation-dependent Probe Amplification (MLPA); which is a sensitive method for copy number quantification [34]. MLPA probe design and assay were performed as described by White *et al.* [35]. The MLPA probe sequences used were (GACTCCACCTTGAATCTCATCCCCAGGGTCTCA) and (CTGTCCCATTTCTTGGTGCTGGGTGGATCTAAATCCAGG). Because a relatively large amount of DNA is needed for MLPA (in our experiment 125 ng DNA per sample to get accurate and reliable results), enough DNA was not available from all the RA patients and controls genotyped for the *FcγRIIIA* 158V/F SNP. Additionally not all the DNA samples used for SNP genotyping were extracted using the same method. According to the manufacturer protocols, the usage of DNA samples extracted using different methods may influence the MLPA results. The presence of remnants of phenol in phenol-extracted DNA can inhibit MLPA-PCR and impede ligase enzyme activity and the use of old magnetic particles in automated DNA extraction devices may result in incomplete sample denaturation, subsequently influencing MLPA results and rendering them incomparable. Therefore DNA samples that were extracted using phenol were not used for MLPA. Consequently, the MLPA was performed on 456 RA patients and 285 controls for whom we had enough DNA that was extracted using the same method.

Statistical analysis

The χ^2 test with 2 degrees of freedom (Epi Info v6, CDC, Atlanta, Georgia, USA) was used to compare the relation between genotypes and ACPA+ve and ACPA-ve RA. The MLPA results were analysed as described by White *et al.* [36]. The height of each probe-specific peak was divided by the sum of three control peaks to give a ratio. The median ratio for *FcγRIIIA* across all samples within an assay was calculated and used to normalize the ratios around a value of 1. The normalized ratio for each individual was calculated and plotted in a scatter plot (**Figure 2**). Subgroups corresponding to different *FcγRIIIA* gene copy numbers were defined by eye and confirmed by cluster analysis (using R statistical software version 2.5.0),

and are delineated by vertical dotted lines. To minimize the possibility of mis-genotyping of FcγRIIIA158V/F polymorphism that can be caused by CNV (**Figure 1**), we performed the analysis on the subgroup of individuals with no CNV (The middle cluster in **Figure 2**), thus excluding genotypes from samples with either high or low copy number (the first and the third clusters in **Figure 2**). P-values were considered statistically significant if < 0.05 .

Figure 2: Copy number variation (CNV) of the FcγRIIIA gene. Cluster 1 represent samples with a low copy number and cluster 3 represents samples with a high copy number of FcγRIIIA compared with cluster 2 that represents samples without CNV of the FcγRIIIA gene. MLPA, multiplex ligation-dependent probe amplification.



RESULTS

This study included 945 RA patients and 388 healthy controls. The genotype frequencies of the FcγRIIIA 158V/F SNP in RA patients and controls are shown in **Table 1**. The genotype frequencies of FcγRIIIA-158V/F polymorphism (rs396991) were in accordance with HWE (P-value is 0.6 in cases and 0.4 in controls). No statistically significant differences in the genotype or allele frequencies between RA patients and controls were observed. However after stratifying for ACPA status, the 158VV genotype was more frequent in ACPA positive RA patients compared to controls (P=0.05, OR=1.5, 95%CI 0.99-2.27). Similarly, the frequency of the 158V allele in the ACPA positive RA group (N=358) was higher compared

to controls ($P=0.034$, $OR=1.3$, $95\%CI$ 1.01-1.55). No differences were found in the ACPA negative group ($N=252$) (**Table 1**).

Table 1: Comparison of the *FcyRIIIA* 158V/F genotype and allele frequencies in patients with rheumatoid arthritis (RA) and controls

	Total No	Genotypes			MAF			VV vs FV+FF	
		FF No (%)	FV No (%)	VV No (%)	%	OR (95% CI)	p Value	OR (95% CI)	p Value
Controls	388	148 (38.1)	189 (48.7)	51 (13.2)	37.5	–	–	–	–
All patients with RA	945	353 (37.3)	442 (46.8)	150 (15.9)	39.3	1.1 (0.9 to 1.28)	0.4	1.3 (0.87 to 1.78)	0.2
ACPA-positive RA	358	117 (32.7)	175 (48.9)	66 (18.4)	42.9	1.3 (1.01 to 1.55)	0.034	1.5 (0.99 to 2.27)	0.05
ACPA-negative RA	252	103 (40.9)	117 (46.4)	32 (12.7)	35.9	0.9 (0.73 to 1.19)	0.6	0.9 (0.58 to 1.58)	0.9

OR and p for each row represent comparison with the control group as reference ACPA, anti-citrullinated peptide antibodies; MAF, minor allele frequency.

Table 2: Copy Number Variation of the *FcyRIIIA* gene

	Total	Low CNV No (%)	Most common No (%)	High CNV No (%)	p Value*
Controls	285	11 (3.9)	258 (90.5)	16 (5.6)	–
Patients with RA	456	8 (1.8)	426 (93.4)	22 (4.8)	0.2
ACPA-positive	148	4 (2.7)	135 (91.2)	9 (6.1)	0.8
ACPA-negative	110	2 (1.8)	102 (92.7)	6 (5.5)	0.6

p Value compared with controls.

ACPA, anti-citrullinated peptide antibodies; CNV, copy number variation; RA, rheumatoid arthritis.

The *FcyRIIIA* gene shows CNV in 6.6% of RA patients and in 9.5% of controls (**Table 2**). Since the presence of CNV might lead to skewing of the genotype frequencies by causing genotyping errors (**Figure 1**), we assessed whether CNV of the *FcyRIIIA* gene has an influence on the association of *FcyRIIIA*-158V/F polymorphism and RA. Therefore, we determined the association between *FcyRIIIA* 158V/F and ACPA+ve and ACPA-ve RA in subjects with no CNV of *FcyRIIIA* gene. Genotypes from individuals with no CNV were selected (cluster 2 in **Figure 2**), thus excluding samples that showed either low or high CN of *FcyRIIIA* gene (cluster 1 & 3 respectively in **Figure 2**). Without stratifying for ACPA, the 158VV genotype was significantly more frequent in RA patients compared to controls (17.2 vs 11.7 respectively, $P=0.05$, $OR=1.6$, $95\%CI$ 0.97-2.6), but the frequency of the 158V allele was not significantly higher in RA patients compared to controls. Subsequently, stratifying for ACPA status showed an increased risk of RA as the 158VV genotype was more frequent in

the ACPA positive RA patients compared to controls (21.5 vs 11.7 respectively, $P=0.009$, OR= 2.1, 95%CI 1.2-3.8) also the presence of the 158V allele is associated with ACPA positive RA ($P=0.039$, OR=1.4, 95% CI 1-1.9). No association was found in the ACPA negative group (**Table 3**). Comparing the data without and with stratification for CNV (**Table 1** and **3** respectively) reveals that almost similar results were observed for the effect of the 158V allele on the risk of ACPA positive RA. In contrast the odds ratio for the effect of the 158VV genotype on the risk of RA became higher after correcting for the presence of CNV, although the confidence intervals were overlapping.

Table 3: Comparison of the FcγRIIIA 158V/F genotype and allele frequencies in patients with rheumatoid arthritis (RA) and controls with no copy number variation

	Total No	Genotypes			MAF		p Value	VV vs FV+FF	
		FF No (%)	FV No (%)	VV No (%)	%	OR (95% CI)		OR (95% CI)	p Value
Controls	258	94 (36.4)	134 (51.9)	30 (11.7)	37.6	–	–	–	–
All patients with RA	426	162 (38)	191 (44.8)	73 (17.2)	39.6	1.1 (0.9 to 1.4)	0.5	1.6 (0.97 to 2.6)	0.05
ACPA-positive RA	135	42 (31.1)	64 (47.4)	29 (21.5)	45.2	1.4 (1 to 1.9)	0.039	2.1 (1.2 to 3.8)	0.009
ACPA-negative RA	102	42 (41.1)	43 (42.2)	17 (16.7)	37.7	1 (0.7 to 1.4)	0.97	1.5 (0.8 to 3)	0.2

OR and p for each row represent comparison with the control group as reference.

The patients with RA and controls chosen for analysis in this table showed no evidence of CNV based on MLPA results (cluster 2)

ACPA, anti-citrullinated peptide antibodies; CNV, copy number variation; MAF, minor allele frequency; MLPA, multiplex ligation-dependent probe amplification

All the subjects identified as having low copy number (a single copy) were genotyped as homozygous for either the 158V or the 158F alleles (as suggested by **Figure 1**).

The third aim of this study was to investigate whether the difference in *FcγRIIIA* gene copy number confers risk to RA. The distribution of CNV was not significantly different between patients and controls with and without stratifying for ACPA status (**Table 2**).

DISCUSSION

In the current study we investigated the association between the FcγRIIIA 158V/F SNP and ACPA-positive as well as ACPA-negative RA and explored the effect of CNV of *FcγRIIIA* gene on the association of FcγRIIIA 158V/F SNP with RA. We observed that the association

between the FcγRIIIA-158V allele and RA is refined to the ACPA-positive group. In addition, after correction for the effect of the presence of CNV on genotypes, the strength of the association was slightly increased. To our knowledge this is the first study to consider the effect of disease heterogeneity (presence/absence of ACPA) and genetic heterogeneity (effect of CNV on SNP genotyping) on the association between FcγRIIIA-158V/F polymorphism and RA.

The FcγRIIIA is expressed on NK cells and on macrophages, the expression by macrophages is limited to only a few tissues which correlate with the sites of pathology seen in patients with rheumatoid arthritis (synovium, dermis under stress, lungs, pericardium and liver) [37]. The FcγRIIIA expression on NK cells and the number of FcγRIIIA-IgG binding sites per NK cell correlates with the antibody-dependent cell-mediated cytotoxicity (ADCC) function of these cells [38]. The presence of the FcγRIIIA 158V/F SNP, which is a T to G substitution at nucleotide 559 in *FcγRIIIA* gene that results in a switch from phenylalanine to valine at amino acid position 158 in the immunoglobulin binding domain, has functional consequences. It was shown that this 158V/F SNP affects the binding affinity of FcγRs to IgG: the 158V allele is associated with higher NK cells IgG binding affinity compared to the 158F allele, with a gene dosage effect [39]. In addition IgG stimulation of NK cells from 158VV individuals resulted in higher Ca²⁺ influx, higher concentrations of interleukin-2 (IL2) receptor (CD25) expression and reduced survival of NK cells after activation induced cell death when compared with 158FV or 158FF individuals [40].

So far, the results of the published studies concerning the FcγRIIIA-158V/F SNP in RA vary markedly. They differ in the presence or absence of its association with RA as well as in the allele frequencies within similar ethnic populations. The results of these studies are summarised in **Table 4**. In our study, the genotype and minor allele frequency (MAF) in controls and RA patients were almost identical to those previously reported in Dutch Caucasians [23].

Table 4: The FcγRIIIA 158V/F polymorphism association with rheumatoid arthritis (RA) in different studies

Study	Ethnicity	Counts		MAF%		Alleles		VV vs FV+FF	
		RA	Controls	RA	Controls	p Value	OR (95% CI)	p Value	OR (95% CI)
V allele associated with RA									
Morganet al ⁸	UK	141	124	37	28	0.028	1.51 (1.03 to 2.21)	0.134	1.8 (0.78 to 4.18)
Morganet al ⁸	India	108	113	36	27	0.05	1.5 (0.98 to 2.28)	0.55	1.34 (0.46 to 3.91)
Morganet al ⁹	UK	828	581	35	31	0.02	1.21 (1.03 to 1.42)	0.029	1.45 (1.02 to 2.07)
Kastbomet al ⁹	Sweden	181	362	38	31	0.033	1.33 (1 to 1.75)	0.064	1.67 (0.93 to 2.99)
F allele associated with RA									
Nietoet al ²	Spain	117	142	32	41	0.039	0.68 (0.47 to 1)	0.77	0.9 (0.41 to 1.95)
No association with RA									
Matsumotoet al ⁶	Japan	187	158	28	24	0.19	1.25 (0.88 to 1.79)	0.36	1.52 (0.58 to 4.08)
Milicic et al ⁷	UK	401	420	35	34	0.5	1.07 (0.87 to 1.32)	0.1	1.46 (0.9 to 2.37)
Milicic et al ⁷	India	63	92	23	33	0.054	0.6 (0.35 to 1.04)	0.024	0.2 (0.03 to 0.98)
Kyogokuet al ⁸	Japan	382	303	27	30	0.15	0.84 (0.66 to 1.07)	0.55	0.84 (0.47 to 1.52)
Alizadehet al ³	Netherlands	601	1326	39	37	0.22	1.09 (0.95 to 1.26)	0.68	1.06 (0.8 to 1.4)
Brunet al ⁴	Norway	112	89	38	34	0.38	1.2 (0.78 to 1.85)	0.91	0.96 (0.42 to 2.21)
Stewart-Akerset al ¹⁵	USA	145	105	35	31	0.38	1.18 (0.8 to 1.76)	0.32	1.46 (0.65 to 3.3)
Chenet al ⁶	Taiwan	212	371	37	35	0.55	1.08 (0.83 to 1.39)	0.28	1.3 (0.78 to 2.17)

MAF, minor allele frequency.

It was suggested that these contradicting results originated from methodological difficulties, due to the extreme homology of *FcγRIIIA* to *FcγRIIIB* gene that might lead to falsely detecting an *FcγRIIIB* sequence as the *FcγRIIIA*-158V variant leading to false over-presentation of the 158V allele [29]. As indicated, there are two additional explanations that may have been overlooked. First, we now provide evidence that the *FcγRIIIA*-158VV genotype associates with ACPA positive subset of RA. A previous meta-analysis [23] showed an odds ratio of 1.3 for the *FcγRIIIA*-158VV genotype to increase the risk of RA; the percentage of ACPA positive RA patients in those studies is unknown. Our data suggest the need for an additional meta-analysis in ACPA positive RA specifically. Second, the presence of CNV in this gene cluster may previously have led to skewing of the genotype frequencies, subsequently affecting disease associations. Since the frequency of CNV was reported to vary significantly in different ethnic populations [36], CNV may be another cause of the different allele frequencies of *FcγRIIIA*-158V/F polymorphism and different associations with RA observed in different populations.

In our study, after controlling for the *FcγRIIIA* gene copy number, the association between RA patients in general (without considering the ACPA status) and presence of the 158VV genotype became borderline significant ($P=0.05$, $OR=1.6$ 95% CI 0.97-2.6 vs $P=0.2$, $OR=1.3$ 95% CI 0.87-1.78 without controlling for the CNV). Similarly in the ACPA positive group before correcting for the presence of CNV, presence of the 158VV genotype was associated with RA susceptibility with a borderline significant P-value (0.05) and an odds ratio of 1.5 (95% CI 0.99-2.27). In the ACPA positive group without CNV this association had an odds ratio of 2.1 (95% CI 1.2-3.8). Although these confidence intervals are overlapping and although the presence of CNV of *FcγRIIIA* didn't significantly change the genotype frequencies (probably because the frequency of CNV was relatively low in comparison to the 158V allele), correction for the presence of CNV affected the association between the 158VV genotype and RA. To our opinion, these data underline the need to take the CNV into consideration while performing analysis on SNPs.

The CNV of other *FcγRs* genes has been shown to associate with susceptibility to several auto-immune diseases such as lupus nephritis [16] and idiopathic thrombocytopenic purpura (ITP) [41]. The present study evaluated CNV in the *FcγRIIIA* gene. We confirmed the presence of CNV in the *FcγRIIIA* gene with a frequency of 9.5% in healthy controls and 6.6% in RA patients. A comparable frequency of CNV was recently reported in another study with a smaller sample size (116 ITP patients and 100 healthy controls) [41]. We did not find an association between CNV of *FcγRIIIA* gene and susceptibility to RA. Though, because of the low frequency of CNV in this gene, we were underpowered to formally conclude that CNV of *FcγRIIIA* gene is not associated with RA susceptibility.

In conclusion, the *FcγRIIIA*-gene shows CNV which was not differently distributed between RA patients and healthy controls. The analysis of association between the *FcγRIIIA*-158V/F polymorphism with RA, stratified for ACPA status and CNV of the *FcγRIIIA*-gene, revealed that the *FcγRIIIA*-158VV genotype confers risk to ACPA positive RA. The fact that a SNP in the *FcγRs* genes associates with ACPA positive RA points to the relevance of antibodies in the pathophysiology of ACPA+ve RA.

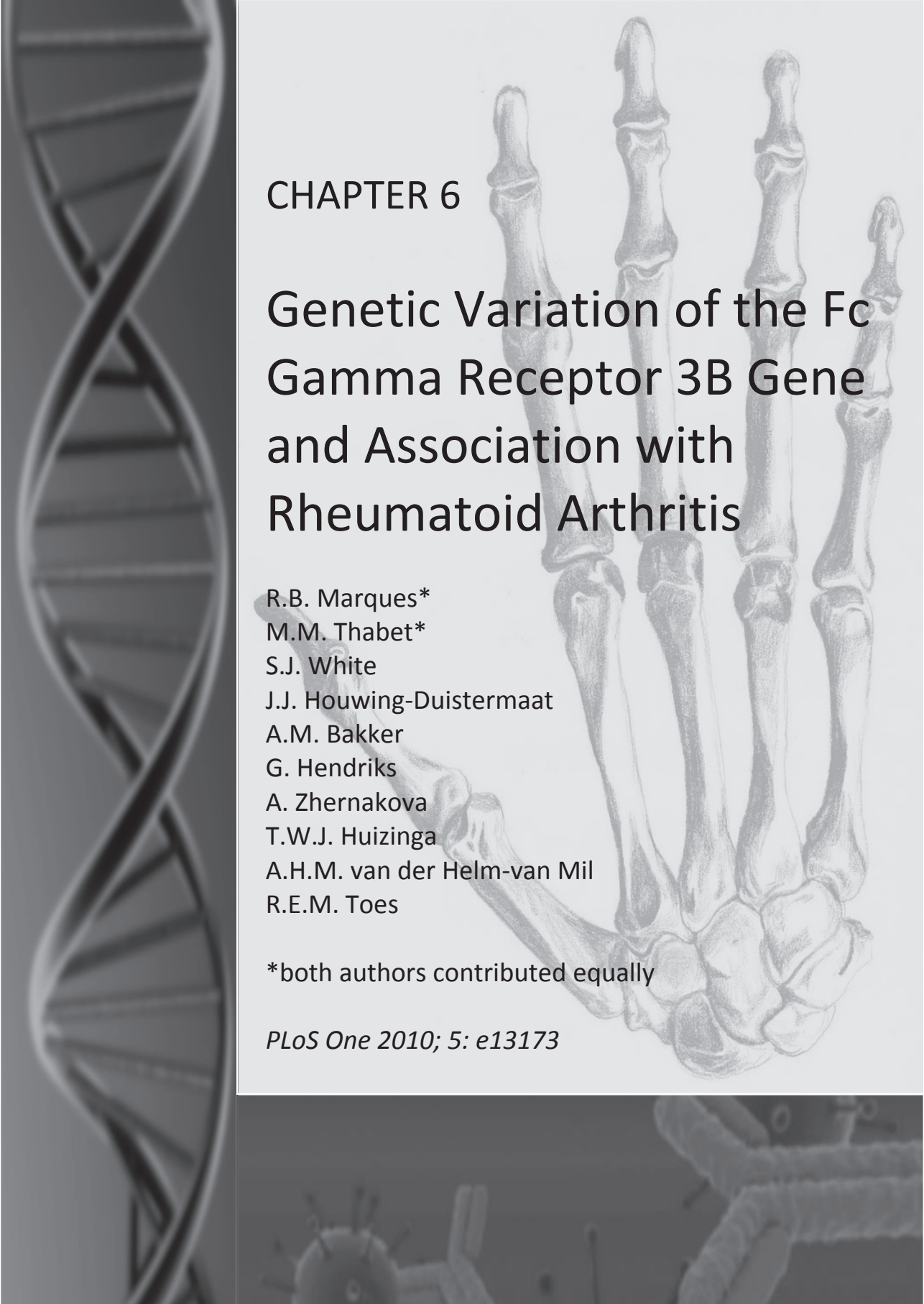
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CHAPTER 6

Genetic Variation of the Fc Gamma Receptor 3B Gene and Association with Rheumatoid Arthritis

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PLoS One 2010; 5: e13173

ABSTRACT

Background: Fc gamma receptors (FcγRs) play a crucial role in immunity by linking IgG antibody-mediated responses with cellular effector and regulatory functions. Genetic variants in these receptors have been previously identified as risk factors for several chronic inflammatory conditions. The present study aimed to investigate the presence of copy number variations (CNVs) in the *FCGR3B* gene and its potential association with the autoimmune disease rheumatoid arthritis (RA).

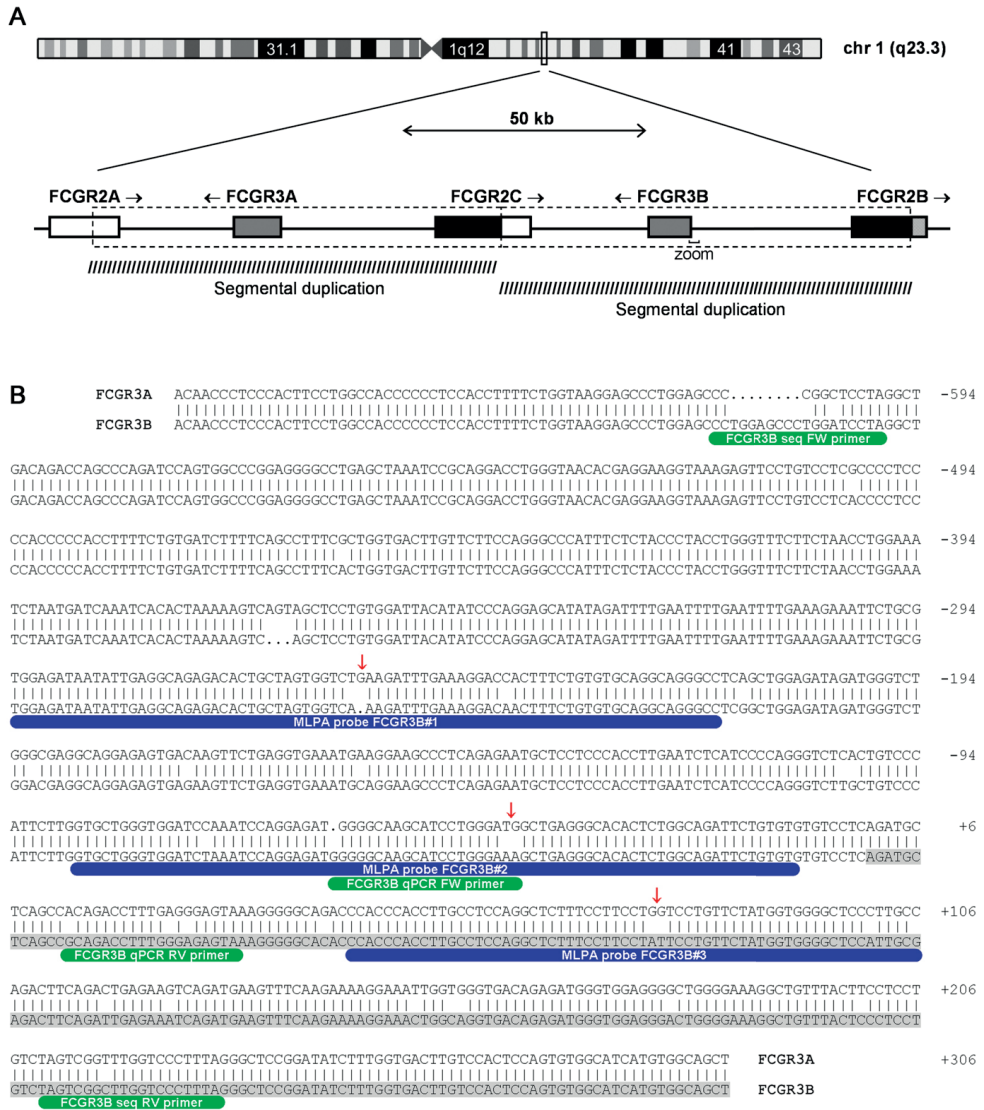
Methodology/Principal Findings: CNV of the *FCGR3B* gene was studied using Multiplex Ligation Dependent Probe Amplification (MLPA) in 518 Dutch RA patients and 304 healthy controls. Surprisingly, three independent MLPA probes targeting the *FCGR3B* promoter measured different CNV frequencies, with probe#1 and #2 measuring 0 to 5 gene copies and probe#3 showing little evidence of CNV. Quantitative-PCR correlated with the copy number results from MLPA probe#2, which detected low copy number (1 copy) in 6.7% and high copy number (≥ 3 copies) in 9.4% of the control population. No significant difference was observed between RA patients and the healthy controls, neither in the low copy nor the high copy number groups (p-values = 0.36 and 0.71, respectively). Sequencing of the *FCGR3B* promoter region revealed an insertion/deletion (indel) that explained the disparate CNV results of MLPA probe#1. Finally, a non-significant trend was found between the novel -256A>TG indel and RA (40.7% in healthy controls versus 35.9% in RA patients; $P = 0.08$).

Conclusions/Significance: The current study highlights the complexity and poor characterization of the *FCGR3B* gene sequence, indicating that the design and interpretation of genotyping assays based on specific probe sequences must be performed with caution. Nonetheless, we confirmed the presence of CNV and identified novel polymorphisms in the *FCGR3B* gene in the Dutch population. Although no association was found between RA and *FCGR3B* CNV, the possible protective effect of the -256A>TG indel polymorphism must be addressed in larger studies.

Introduction

Fc receptors are proteins expressed on the surface of immune cells, whose function is to help in the recognition and elimination of invading pathogens [1]. Fc receptors bind to antibodies attached on the surface of pathogens or infected cells, triggering immune effector responses, such as phagocytosis, antibody-dependent cellular cytotoxicity, cytokine release and antigen presentation. There are Fc receptors for each immunoglobulin (Ig) class: FcαR, FcδR, FcεR, FcγR and FcμR, for IgA, IgD, IgE, IgG and IgM, respectively. IgG antibodies are the most abundant serum immunoglobulins, are predominantly involved in the secondary immune response and increased amounts can occur upon infection, chronic inflammation and autoimmune diseases. Therefore, FcγRs are thought to play a crucial role in immunity, as well as in the pathogenesis of several autoimmune diseases, including rheumatoid arthritis (RA) [2]. FcγRs vary in their cellular distribution and affinity for different IgG isotypes and can be divided in three general classes: FcγRI (isoforms FcγRIA, IB and IC), FcγRII (isoforms FcγRIIA, IIB and IIC) and FcγRIII (isoforms FcγRIIIA and IIIB). These include activatory receptors, such as FcγRI, FcγRIIA and FcγRIIIA, and the inhibitory receptor FcγRIIB [3]. Furthermore, FcγRs can be distinguished between high-affinity receptors (FcγRI) and low-affinity receptors (FcγRII and FcγRIII). These low-affinity receptors are encoded by highly homologous FCGR genes, located in a genetically complex cluster on the long arm of chromosome 1 (**Figure 1**) [4]. It is believed that the different FCGR genes in this locus are the result of multiple duplication and recombination events during evolution [5]. Additionally, this region displays extensive genetic variation, which has been associated with susceptibility to various chronic inflammatory disorders [6- 8]. In particular, single nucleotide polymorphisms (SNPs) in FCGR2A (R131H), FCGR2B (I232T) FCGR3A (V158F) and FCGR3B (NA1/NA2), have been reported in association with systemic lupus erythematosus (SLE), rheumatoid arthritis (RA) and/or idiopathic thrombocytopenia purpura (ITP) [9- 17].

Although less studied than SNPs, copy number variants (CNVs) are also important sources of genetic variation. A CNV is defined as a sequence of DNA >1 kb that is present in altered copy number when compared with a reference genome [18]. Several recent studies have demonstrated that some genes or groups of genes can show variation in copy number [19- 24].

Figure 1: Genomic organization of the human FCGR locus in the chromosome 1q23.3.

A. FCGR2A, FCGR2B, FCGR2C, FCGR3A and FCGR3B are drawn in different shades of gray, representing the regions of homology between the genes. Arrows mark the direction of transcription. MLPA probes designed to measure FCGR3B copy number are located in the promoter region (zoom). **B.** Zoom of the promoter sequence of FCGR3B aligned against the homologous FCGR3A gene. The first exon of FCGR3B is highlighted in grey, MLPA probes in blue, quantitative PCR and sequencing primers in green. Red arrows mark the ligation site of the MLPA probes, which target paralogous sequence variations between the FCGR3A and FCGR3B genes to assure specificity.

In total, copy number variable regions may cover as much as 12% of the human genome, many of which exist with relatively high frequency (>5%) in general human populations and are also present at orthologous loci in other species [18, 20, 25]. The first evidence that copy-number alterations can influence human phenotypes came from sporadic diseases, termed ‘genomic disorders’, caused by de novo structural alterations [26]. The number of genomic disorders has grown, with several dozen reported to date [27]. In addition to such sporadic diseases, inherited CNVs have been found to underlie Mendelian diseases in several families [28- 30]. Nonetheless, CNVs have been implicated in only a few percent of the 2,000 or more Mendelian diseases so far explained at a molecular level. Little is known about the genetic basis of common, complex phenotypes, and it is premature to predict the relative proportion of complex disease explained by SNPs and CNVs. In principle, complex disease might be more susceptible to ‘soft’ forms of variation such as variation in non-coding sequences or copy number which alter gene dose without abolishing gene function. Common CNVs have been reported to be associated with several complex disease phenotypes including HIV acquisition and progression [31- 33]. Furthermore, CNVs have been implicated in several autoimmune diseases. For example, lower CN of FCGR3B and complement component C4 genes have been associated with SLE susceptibility [32- 35]. Higher CN of CCL3L1 has been suggested as a risk factor for developing RA [36]. Additionally, CNV of FCGR3B was associated with microscopic polyangiitis and Wegener's granulomatosis [35].

Given the important role of FcγRs in immunity, we are interested in whether genetic variation in these genes associates with susceptibility to rheumatoid arthritis (RA). In a previous study, we showed that FCGR3A 158V/V genotype but not CNV conferred risk to anti-citrullinated peptide antibodies (ACPA) positive RA [37]. In the present report, we focused on the FCGR3B gene.

MATERIALS AND METHODS

Patients and healthy controls

Patients were 518 Dutch Caucasian individuals with RA who fulfilled the American College of Rheumatology (ACR) classification criteria for RA, as described elsewhere [38, 39]. Controls were 304 unrelated healthy Dutch Caucasians with no history of RA [40]. An

informed written consent according to the Declaration of Helsinki was obtained for both patients and controls. The Leiden institutional review board, Commissie Medische Ethiek, approved all protocols. Anti-cyclic citrullinated peptide antibodies (ACPA) were positive in 59.6% of RA patients and was tested using Immunoscan CCPlus (CCP2 Euro-Diagnostica, Arnhem, the Netherlands).

Multiplex ligation-dependent probe amplification

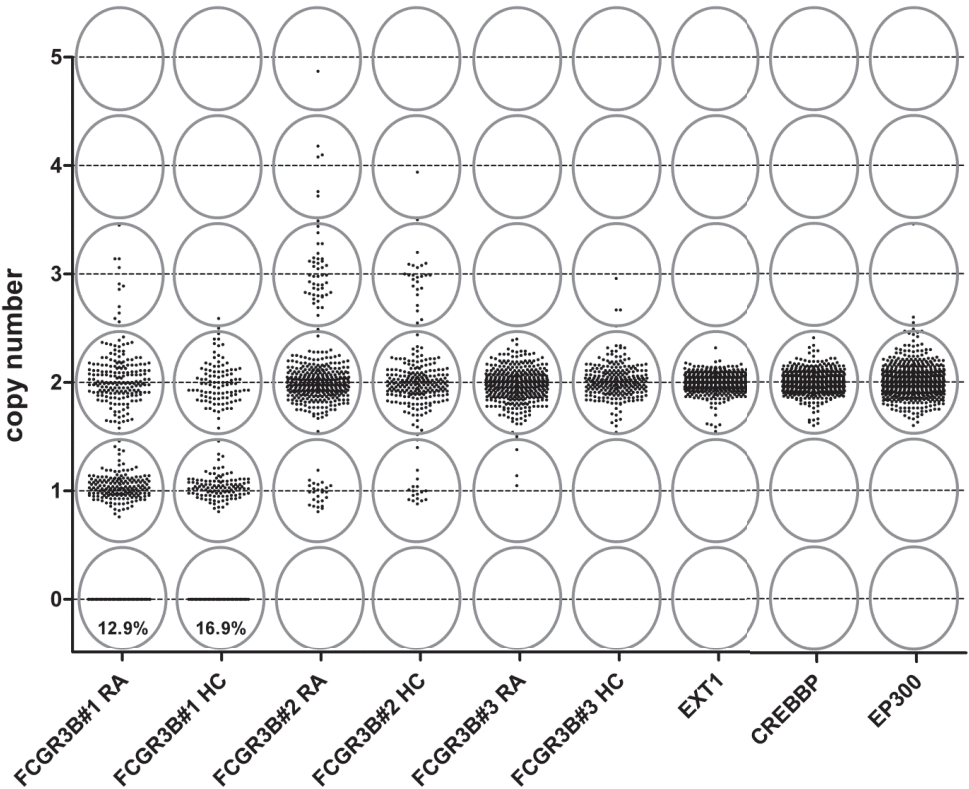
Copy number variation (CNV) of the FCGR3B gene was assessed using Multiplex Ligation-dependent Probe Amplification (MLPA), which is a sensitive method for copy number quantification [41]. MLPA probe design and assay were performed as described by White *et al.* [42, 43]. Due to the very high homology in nucleotide sequences of FCGRs genes, the choices for appropriate MLPA probes were limited. Three probe pairs were designed for FCGR3B, which locate to the promoter region of the gene, based on sequence information from NCBI build 36.1 and dbSNP built 126. The MLPA probe sequences used are shown in **Table 1**. Three genes CREBBP, EXT1 and EP300 were used as control genes in each assay. The MLPA results were analyzed as described by White *et al.* [42]. The height of each probe specific peak was divided by the sum of three control peaks (from CREBBP, EXT1 and EP300), to calculate the sample to control ratio.

Table 1: MLPA probes sequences used for CNV typing of FCGR3B and control genes.

Probe_Orientation	Sequence
FCGR3B#1_Forward	CGTGGAGATAATATTGAGGCAGAGACTGCTAGTGGTCA
FCGR3B#1_Reverse	AAGATTGAAAGGACAACTTTCTGTGTCAGGCAGGGC
FCGR3B#2_Forward	CCGACGTACGTATCTAAATCCAGGAGATGGGGCAAGCATCCTGGGAA
FCGR3B#2_Reverse	AGCTGAGGGCACACTCTGGCAGATTCTGTGTG
FCGR3B#3_Forward	CCCCACCTGCCTCCAGGCTCTTTCCTCCTA
FCGR3B#3_Reverse	TTCTGTCTATGGTGGGGCTCCATTGCGAGA
CREBBP_Forward	CCAGCTAGTGAATTCAAACACAATTGGTTCTGTGGCACA
CREBBP_Reverse	GGGCAACAGAATGCCACTCTTTAAGTAACCC
EP300_Forward	CCAACCTAAGCACTGTAGTCAGATTGATCCAGTCCAT
EP300_Reverse	AGAAAGAGCCTATGCAGCTCTTGGACTACCCATCA
EXT1_Forward	CCTGAAGAACATGGTGGACCAATTGCCAATTGTGAGGACAT
EXT1_Reverse	TCTCATGAACCTCCTGGTGTCTGTGTGACAAAATTCCTC

For each target gene, the normalized ratio was calculated by setting the median ratio across all samples within an assay at the value of 2. The normalized ratio for each individual was calculated and plotted (**Figure 2**). Subgroups corresponding to different gene copy numbers were defined by eye and confirmed by cluster analysis (using R statistical software version 2.5.0), and are delineated by elliptical lines.

Figure 2: Copy number of FCGR3B gene in RA patients versus healthy controls.



The normalized MLPA ratios measured with FCGR3B#1, FCGR3B#2 and FCGR3B#3 probe-sets are plotted for RA patients and healthy controls. Three genes EXT1, CREBBP and EP300 were used as reference for normalization (2 copies without CNV) and are shown here for all individuals. The clusters reflecting the copy numbers are illustrated by grey ovals. FCGR3B#1 and FCGR3B#2 showed clear clusters and copy numbers could be assigned, while FCGR3B#3 showed no CNV.

Quantitative PCR

To verify the results of the different MLPA probes for FCGR3B gene, a quantitative PCR (qPCR) assay was designed based on primers located within the promoter region of FCGR3B gene, the same regions where the MLPA probes for FCGR3B were located. 24 samples showing different copy numbers of FCGR3B in the MLPA assay were analysed by qPCR. Sequences of the primers used were, FCGR3B_qPCR_FW (CAA GCA TCC TGG GAA AGC T), FCGR3B_qPCR_RV (TAC TCT CCC AAA GGT CTG C), FOXP2_FW (TGA CAT GCC AGC TTA TCT GTT T) and FOXP2_RV (GAG AAA AGC AAT TTT CAC AGT CC). Quantitative PCR was conducted in triplicate on 25 ng genomic DNA, using iQ SYBR Green Supermix (Bio-Rad) and an iCycler Real-Time PCR Detection System (Bio-Rad). PCR mix contained per sample: 10 µl SYBR Green Supermix, 2 µl (20 pmol) each primer, 1 µl water and 5 µl DNA. QPCR cycling protocol was as follows: 3 min initial denaturation at 95°C, followed by 40 cycles at 95°C for 30 sec, 63°C for 30 sec and 72°C for 45 sec, and finalized by a melt-curve analysis (55°C to 95°C, with 0.5°C increments for 10 sec). FOXP2 was used as control for normalization (as it is known to have no CNV, reference). Copy number calculations were performed using the delta-delta Ct method, and the average copy number was set at 2. The results from qPCR were correlated with those from the MLPA assay using GraphPad Prism software v5.

Sequencing

The MLPA assay showed inconsistent results across the different probes used for FCGR3B gene. Therefore, the promoter region of the FCGR3B gene was sequenced to verify if any kind of genetic abnormalities, such as previously unreported SNPs or insertion/deletions (indels), were present within the regions targeted by the probes. Genomic DNA (25 ng) was amplified and sequenced using the following primers: FCGR3B_seq_FW (CCT GGA GCC CTG GAT CCT A) and FCGR3B_seq_RV (CTA AAG GGA CCA AGC CGA CTA). PCR amplification was performed using a Promega GoTaq PCR kit (according to the manufacturer's standard protocol) and an Applied Biosystems 9700 Thermal Cycler. The cycling protocol comprised an initial denaturation step at 94°C for 3 min, followed by 37 cycles at 94°C for 45 sec, 64°C for 45 sec and 72°C for 1 min, followed by a final elongation step at 72°C for 7 min. PCR products were purified using a QIAGEN PCR Purification Kit,

according to QIAGEN's manual. PCR products concentration and purity was determined using a Nanodrop spectrophotometer system. Sequencing was performed in an Applied Biosystems' ABI PRISM 3730 Analyzer and data was analysed with Chromas 2 (Technelysium).

Statistical analysis

The χ^2 test with 2 degrees of freedom (Epi Info v6, CDC, Atlanta, Georgia, USA) was used to compare the relation between genotypes and CNV and RA susceptibility. R statistical software (version 2.5.0) was used to cluster intensity ratios and validate the cut-offs of the discrete copy number genotypes. P-values were considered statistically significant if <0.05 .

RESULTS

FCGR3B copy number quantification and association with rheumatoid arthritis

The copy numbers of the FCGR3B gene were determined by MLPA using three independent probes. MLPA probe locations, targeting paralogous sequence variations between FCGR3A and FCGR3B, are depicted in Figure 1. Peak heights were normalized against 3 reference probes (EXT1, CREBBP and EP300), selected from known autosomal dominant disease genes, which would not be deleted or duplicated without an obvious phenotype. The normalized MLPA ratios were plotted to visualize the distinct copy numbers clusters (**Figure 2**). As expected, the reference genes showed no evidence of CNV. Surprisingly, the CNV frequency considerably differed between the three FCGR3B probes analyzed. While FCGR3B#1 and FCGR3B#2 probes revealed clear clustering indicating gene copies ranging from 0 to 5, the third probe FCGR3B#3 showed little evidence of CNV. The frequencies of copy number variations are shown in (**Table 2**).

FCGR3B#1 probe measured copy numbers ranging from 0 to 3 copies. One copy was the most frequent copy number, occurring in 44.3% of the controls and 41.9% of the RA patients. Furthermore, the FCGR3B#1 probe reported homozygous deletion (0 copies) in 16.9% of healthy controls versus 12.9% RA patients, and gene amplification (3 copies) in 0.4% of healthy controls versus 2.4% of RA patients (OR = 6.23, P = 0.05). In contrast, FCGR3B#2 probe showed that most individuals (>80%) contained 2 copies of FCGR3B.

High copy number (mainly 3 copies and few individuals with 4–5 copies) was present in 9.4% of controls and 11.7% of RA patients OR = 1.27, 95%CI = 0.74–2.2, P = 0.36. Conversely, low copy number (1 copy) was present in 6.7% of the controls compared to 6.0% of RA patients OR = 0.89, 95%CI = 0.45–1.76, P = 0.71.

Table 2: Copy number of FCGR3B determined with three independent MLPA probes.

MLPA Probe		RA		Controls		Low/high CN vs rest	
		Count	%	Count	%	P	OR (95% CI)
FCGR3B #1	0 Gene Copies	54	12.9%	43	16.9%	0.104	0.77 (0.55–1.07)
	1 Gene Copy	175	41.9%	113	44.3%		
	2 Gene Copies	179	42.8%	98	38.4%		
	3 Gene Copies	10	2.4%	1	0.4%	0.047	6.23 (0.82–131)
FCGR3B #2	Low CN	25	6.0%	17	6.7%	0.712	0.89 (0.45–1.76)
	2 Gene Copies	344	82.3%	213	83.9%		
	High CN	49	11.7%	24	9.4%	0.359	1.27 (0.74–2.2)
FCGR3B #3	Low CN	3	0.7%	0	0.0%	ND	ND
	2 Gene Copies	416	99.3%	252	98.8%		
	High CN	0	0.0%	3	1.2%	ND	ND

Mantel-Haenszel p-values were calculated. (ND) p-values and OR were not determined because the number of events was too low.

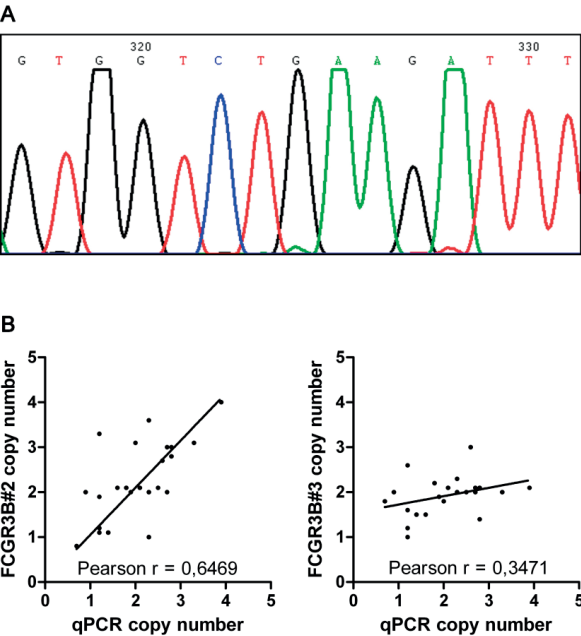
To investigate the reason why the three FCGR3B probes, located within 500 bp from each other, produced such different results we sequenced the promoter region of FCGR3B and measured the number of gene copies by quantitative PCR (qPCR).

Sequencing of the FCGR3B promoter region

A 843 base-pair segment of the FCGR3B promoter encompassing the binding sites for the three MLPA probes was amplified and sequenced, to control for eventual unreported genomic variations that could affect probe ligation. Sequencing results revealed the presence of a 2

nucleotides insertion/deletion (indel) polymorphism, located exactly at the ligation site for the probe FCGR3B#1 (**Figures 3A**). This polymorphism consisted of the deletion of the A nucleotide at position 256 upstream of the transcription starting site, followed by TG insertion. This FCGR3B indel polymorphism has not been previously reported in the literature or public SNP databases, and will be referred throughout the manuscript as -256A>TG indel. The TG insertion prevents the ligation of the two FCGR3B#1 half-probes on the target sequence and subsequently, the absence of signal is misread as a lowered copy number of the FCGR3B gene. It is important to note that the -256A>TG indel is not a rare polymorphism, judging from the high frequency of low copy numbers measured with the FCGR3B#1 probe. Finally, these results showed that the FCGR3B#1 probe simultaneously measures CNV and the -256A>TG indel genotype of the FCGR3B gene, thus explaining the discordant results between that probe and the FCGR3B#2.

Figure 3: Validation of the FCGR3B MLPA probes.



A. Example of the sequencing results for an individual homozygous for the novel FCGR3B-256A>TG indel at the ligation site of MLPA probe FCGR3B#1. **B.** Validation of the results of probe FCGR3B#2 (but not FCGR3B#3) by quantitative RT-PCR.

FCGR3B copy number determination by quantitative PCR

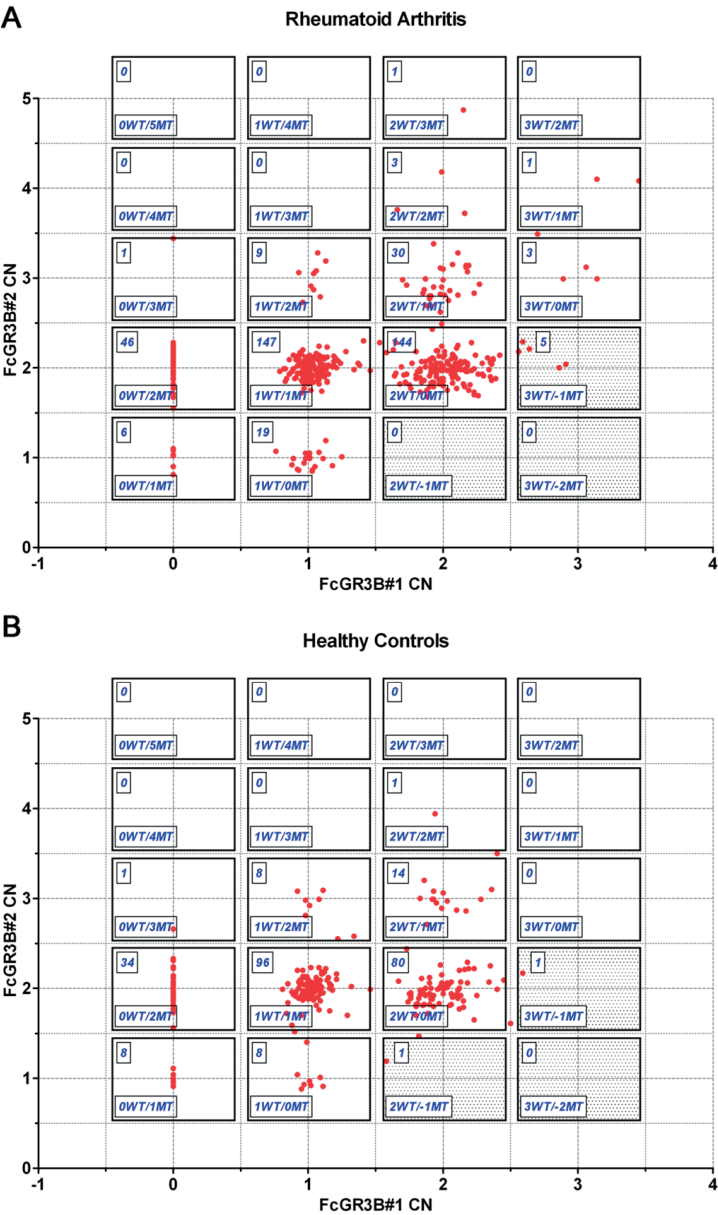
The sequencing results explain the discrepancy between the FCGR3B#1 probe and FCGR3B#2 probe but not with FCGR3B#3. Quantitative PCR was chosen as an independent method to measure the copy number of FCGR3B and was performed on 24 samples (**Figure 3B**). The results from qPCR were correlated with those from probe FCGR3B#2, with a Pearson R of 0.65 ($P = 0.0009$). In contrast, the copy numbers measured with FCGR3B#3 probe did not correlate with those from qPCR Pearson R = 0.35, $P = 0.1$. In conclusion, FCGR3B#2 showed to be a reliable probe for CNV quantification, whereas FCGR3B#3 appeared to be not reliable for this purpose.

Combining the data from MLPA and sequencing of FCGR3B

The data described above indicate the presence of an additional genetic variant in the FCGR3B promoter. The finding of the -256A>TG indel raised the question whether this novel variant was associated with susceptibility to RA. This genetic analysis was complicated by the presence of CNV next to the -256A>TG polymorphism in the FCGR3B gene. Therefore, we combined the MLPA data from FCGR3B#2 probe (providing information about CNV) with the data from FCGR3B#1 probe (information about CNV and -256A>TG indel genotype). The MLPA normalized ratios were plotted in **Figure 4A** for the RA patients and **Figure 4B** for the healthy controls.

The presence of the -256A>TG indel disrupts the binding and ligation of the FCGR3B#1 MLPA probe. Therefore, this probe only measures the number of copies of the wild-type allele (without the indel). On the other hand, the FCGR3B#2 probe measures the total number of copies of the FCGR3B gene. The difference between the signals of FCGR3B#2 and FCGR3B#1 probes equals the number of -256A>TG alleles. For example, an individual showing 2 copies with FCGR3B#2 and 2 copies with FCGR3B#1 has 2 copies of the wild-type (WT) FCGR3B gene; but an individual showing 2 copies with FCGR3B#2 but only 1 copy with FCGR3B#1 has one WT allele and one -256A>TG allele. Obviously, the copy number obtained with probe FCGR3B#1 can never be higher than that determined with FCGR3B#2 probe. For this reason, 7 samples were excluded from further analysis as they measured 2–3 copies with FCGR3B#1 probe and 1–2 copies with probe FCGR3B#2,

Figure 4: FCGR3B MLPA probes FCGR3B#1 versus FCGR3B#2.



MLPA normalized ratios were cross-plot in RA patients (A) and healthy controls (B). Boxes delineate the clusters and the number of individuals within each cluster is shown on the upper left. WT: wild-type allele of FCGR3B (without the -256A>TG indel). MT: mutant-type (-256A>TG indel) allele of the FCGR3B gene.

respectively, which represents a technical error rate <1% (see grey areas of the graphs on **Figure 4**).

Table 3 shows the frequencies of the FCGR3B polymorphisms, as detected by the 2 probes and stratified for the presence (mutant type: MT) or absence (wild-type: WT) of the -256A>TG indel. The WT>MT genotype was more frequent in RA patients than healthy controls (48% versus 40.8%, $P = 0.1$). Conversely, the WT<MT genotype was more frequent in healthy controls than RA patients (20.4% versus 15.4%). In other words, RA patients tended to have less copies of the “mutated” -256A>TG FCGR3B allele (35.9 versus 40.7%, $P = 0.08$), although this trend did not reach statistical significance. Stratification of the RA patients for the presence of ACPA antibodies did not change the results (data not shown).

Table 3: FCGR3B gene stratified for the -256A>TG indel polymorphism.

	RA		Controls	
	Count	(%)	Count	(%)
WT>MT	197	(48%)	102	(40.8%)
WT = MT	150	(36.6%)	97	(38.8%)
WT<MT	63	(15.4%)	51	(20.4%)
MT allele	306	(35.9%)	207	(40.7%)
WT allele	546	(64.1%)	302	(59.3%)
OR (95% CI)	0.82 (0.65–1.03)			
Allelic P-Value	0.08			
Genotypic P-value	0.12			

WT = Wild-type FCGR3B allele (adenine at position –256 of FCGR3B gene).
 MT = Mutant type of the FCGR3B gene (deletion of the adenine nucleotide at position –256, followed by the insertion of thymine/guanine dinucleotide).

DISCUSSION

DNA segments in sizes ranging from kilobases to megabases can vary in copy number between different individuals [18, 20, 25]. These CNVs are an important source of genetic variation, which are not only responsible for population diversity but may also contribute to the inter-individual variability in disease susceptibility. A recent study of gene expression variation as a model of complex phenotype measured the fraction of gene expression ‘traits’ that could be associated with either single nucleotide polymorphisms (SNPs) or CNVs [44]. In this study, SNP genotypes and CNV measurements were associated with 83% and 18%, respectively, of those gene expression traits for which statistically significant associations were found. This may still underestimate the role of CNVs, given the higher frequency of SNPs as well as the greater completeness and accuracy with which SNPs can be queried at present [43, 45].

Curiously, there appears to be an enrichment of CNVs in genomic regions encoding immune response genes and secreted molecules [18, 46]. This finding suggests that CNVs affecting expression of immunity genes may play a functional role in infectious and autoimmune diseases, in particular. Supporting this hypothesis are previous publications describing association of CNVs in FCGR3B and complement component 4 (C4A/C4B) with systemic lupus erythematosus (SLE), FCGR2C with idiopathic thrombocytopenic purpura (ITP), defensin beta (DEFB) with psoriasis and Crohn disease, CCL3L1 with HIV-AIDS infection, SLE, rheumatoid arthritis (RA) and type 1 diabetes (reviewed in [47]). On the other hand, two recent genome wide association studies (GWAS) of CNVs suggest that this type of variation is unlikely to identify novel genetic loci associating with common human diseases [48, 49]. This conclusion was based on two key findings: (i) most of the common CNVs (about 75% with MAF>10%) are well-tagged by SNPs ($r^2>0.8$) and have already been indirectly screened in existing SNP GWAS studies; (ii) the number of confirmed disease associating loci in the CNV GWAS was much smaller than the number of loci identified in SNP GWAS, and consisted of already known associations (HLA for RA, T1D and Crohn's disease; TSPAN8 for T2D; IRGM for Crohn's disease) [47]. However, rare CNVs (MAF<10%) are poorly tagged by SNPs and may have been missed in SNP-based GWAS,

which encourages further CNV genotyping studies. Additionally, regions that have increased recombination, e.g. duplicons that include the FCGR3B gene, are unlikely to be tagged well.

In the current study we investigated the association of CNVs of the FCGRs genes with RA susceptibility. Several methods can be used to quantify CNVs, including fluorescence in situ hybridization, PCR, sequencing and genome-wide array based approaches [50]. In the present study we have chosen for multiplex ligation-dependent probe amplification (MLPA), a simple high-throughput method, which has the advantages of consuming relatively low amounts of genomic DNA and a cost-effective multiplexing potential. MLPA utilizes a ligation step to join two separate oligonucleotide half-probes after hybridization [41]. This provides increased sensitivity to distinguish highly homologous sequences, by designing the half-probes such that a difference in sequence is located under or near the ligation site [51]. However, unsuspected sequence changes at or near the ligation site may lead to erroneous interpretations, as these disturb the ligation reaction and may result in the absence of signal, which is consequently misunderstood as a deletion [52, 53]. This limitation is not restricted to the MLPA assay but to most primer/probe based assays. Thus a deletion detected with a single probe set needs to be confirmed with another assay, for example with quantitative PCR.

The FCGR locus on chromosome 1q23 originated from segmental duplication and cross-over events, which resulted in high homology between the five low affinity FCGR genes [4]. This high homology, together with the presence of multiple (uncharacterized) SNPs, makes it extremely difficult to distinguish between these genes and to design probes for genotyping assays, which may lead to erroneous interpretations [54]. During the preparation of this manuscript, three independent studies report on the FCGR3B gene copy number in association with the risk of developing autoimmune diseases, including RA [48, 55, 56]. The results of these studies are conflicting: while McKinney *et al.* observed a significant association of low FCGR3B gene copy number ($CN < 2$) with RA, that was not the case in the studies by Mantani *et al.* and the CNV GWAS from the Wellcome Trust Consortium [48, 55, 56]. These conflicting reports further strengthen the notion that, due to a high degree of homology, CNV in this FCGR locus is difficult to measure and results are sometimes difficult to interpret. It could be argued that, having a smaller sample size, our

present study (518 RA patients and 304 healthy controls) and that of Mantani *et al.* (158 cases and 409 controls) had limited power to detect a significant association, compared to the study of McKinney *et al.* (in total, 1661 cases and 1374 controls from New Zealand, the Netherlands and the UK) [55, 56]. Nonetheless, our present study had a power of 90% to detect an OR = 2.01 and a power of 70% for OR = 1.67, which are the effect sizes reported by McKinney *et al.* in the “Dutch RA” and “Combined RA” populations, respectively [55]. Also, it should be noticed that the OR we detected (0.89, confidence interval 0.45–1.76) is not in the same direction as the effect reported by McKinney *et al.* Finally, the study from the Wellcome Trust Case Control Consortium was the largest CNV study reported to date, including 2000 RA cases and 3000 controls [48]. Therefore, we believe that the lack of association cannot merely be explained by an insufficient statistical power of these studies. Nevertheless, it remains difficult to explain why two Dutch cohorts (ours and that of McKinney *et al.*) yield different results. We feel that the most likely explanation is found in the different methods and probes used in each study to target this complex region. The quantitative real-time PCR assay used by Mantani *et al.* to measure FCGR3B CNV targeted the same sequence as our FCGR3B#2 probe and yielded similar results [56]. McKinney *et al.* also used a quantitative PCR based technique, but the assay used may not be specific for FCGR3B, due to the presence of FCGR3A SNPs (rs57263253 and rs60966737) in one of the TaqMan primers [55]. Since the frequency of these potential SNPs is unknown, it is difficult to ascertain the possible effect they could have on the CNV measurement. Finally, the GWAS case control study from the Wellcome Trust Consortium used a comparative genomic hybridization (CGH) microarray approach, but the probe used in this region was not specific for the FCGR3B and could not distinguish from the FCGR3A gene [48]. Thus, it is possible that the disparate results between the different studies are due to technical issues that hamper accurate CNV measurement in this locus. Additionally, we identified a novel insertion/deletion polymorphism (-256A>TG) in the promoter region of the FCGR3B gene. The finding of FCGR CNVs and novel polymorphisms adds an extra level of complexity to this already intricate region. To illustrate this problem we describe here the example of our three FCGR3B probes, which all showed different copy number results in the MLPA assay. Using the human reference FCGR sequences (NCBI Build 36.1) and SNP information from dbSNP build 126, these probes were designed to be specific for FCGR3B gene, avoiding SNPs in FCGR3A that

would make it “look” like FCGR3B and, obviously, in the absence of SNPs at the probe ligation site. However, failure of FCGR3B#1 probe in 15% of the individuals, which seemed too high a frequency for deletion, prompted us to sequence the promoter region of FCGR3B gene in 24 individuals. This led to the discovery of the -256A>TG indel, a novel polymorphism at the ligation site of the FCGR3B#1 probe. The data from FCGR3B#3 probe could not be explained by sequencing, as this probe lacked unreported SNPs or indels. Therefore, real time PCR was used to quantify FCGR3B copy number in 24 individuals, which validated the data from FCGR3B#2 probe and excluded the results from FCGR3B#3. The strength of our study resides in the combination of these three techniques: MLPA, real-time PCR and sequencing, reaching a depth that cannot be achieved by large-scale GWAS studies.

A problem of another kind for genetic studies in copy number variable regions is caused by the presence of CNVs resulting in low SNP call rates, so that validated SNPs tend to occur with low densities in regions harboring CNVs. This is due to the fact that deletions and amplifications can lead to a skewing in the pattern of marker genotypes [20, 37]. The subsequent Hardy-Weinberg disequilibrium limits the ability of the genome-wide SNP association studies to detect disease associated SNPs in regions with CNV [45]. Additionally the presence of CNVs may blur the association of the studied SNPs with disease susceptibility [37]. Accurate quantification of counts of CNV repeats, which can be thought of as allele sizes, is not yet routinely possible; most technologies are able to quantify only the total phenotype or the sum of all alleles detected. The accurate assignment of the copy number (integer count) in an individual will present new challenges to assays, and proposals to use quantitative SNP genotypes to infer CNVs will require more-refined calling algorithms [20, 57].

In conclusion, CNV of the FCGR3B gene was detected in the Dutch population but did not associate with the risk of developing RA. However, a trend of association was also found for a newly identified polymorphism in the promoter region of this FCGR3B gene, the -256A>TG indel. Larger studies will be needed to convincingly validate these findings. Finally, the conflicting results found for the different FCGR3B probes illustrate the complexity of this very homologous region and the urgent need for a thorough

characterization of this locus. Only so will future genetic studies of these crucial immunity genes yield consistent and reliable results.

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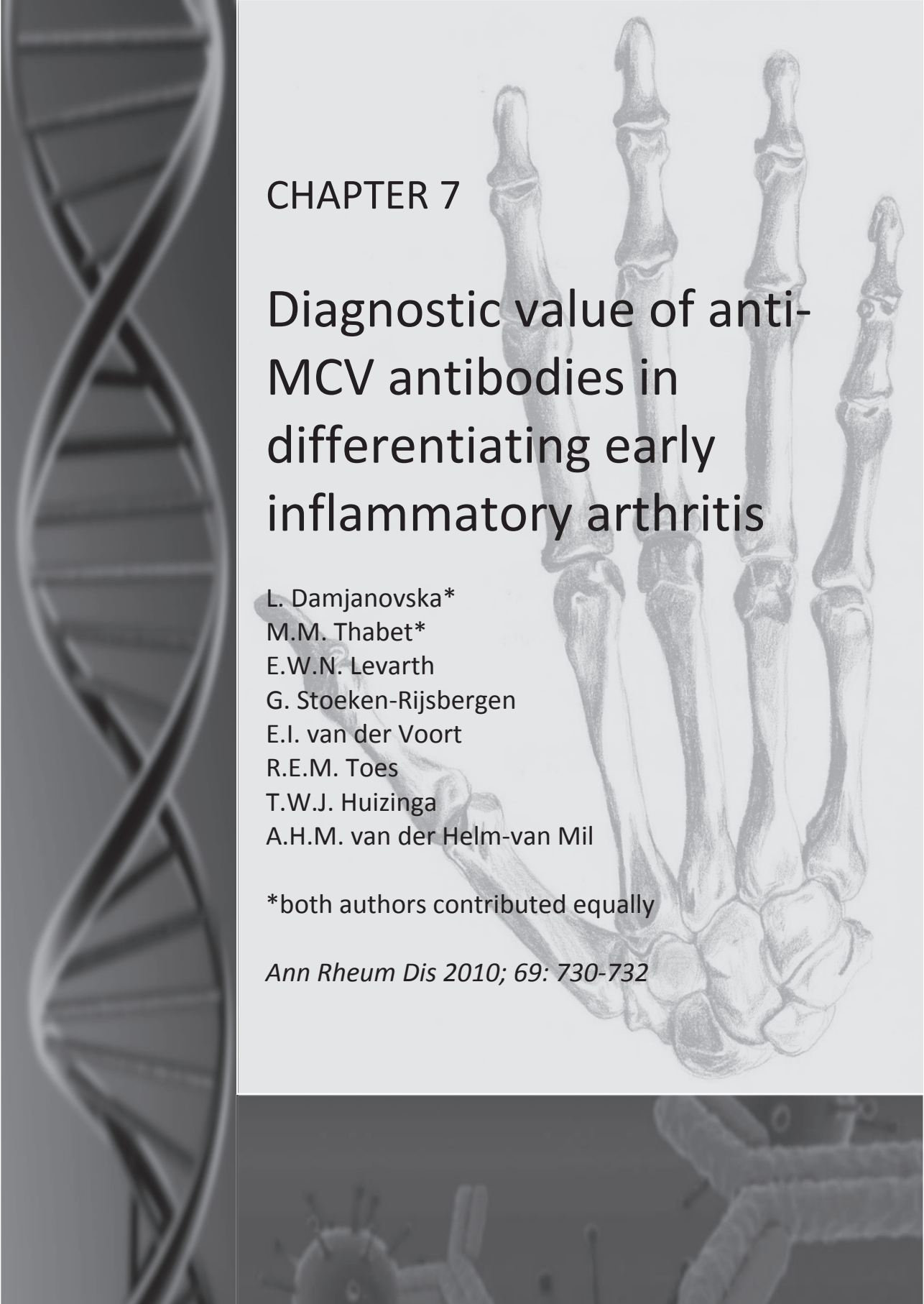
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CHAPTER 7

Diagnostic value of anti-MCV antibodies in differentiating early inflammatory arthritis

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Ann Rheum Dis 2010; 69: 730-732

ABSTRACT

Objectives: To evaluate the diagnostic performance of the anti-CCP2, anti-CCP3 and anti-mutated citrullinated vimentin (anti-MCV) tests in differentiating rheumatoid arthritis (RA) from other forms of arthritis in a clinical setting of early arthritis.

Methods: In 917 patients with recent-onset arthritis (566 RA, 351 other diseases) and in 99 healthy-controls the anti-MCV, anti-CCP2 and anti-CCP3.1 tests were performed and the test characteristics compared.

Results: Comparison of the tests for differentiating RA from other causes of arthritis showed a lower specificity for anti-MCV (82.9%) than for anti-CCP2 (93.4%) and anti-CCP3.1 (90.0%). Similarly, the positive likelihood ratio for anti-MCV was also lower (3.6, compared with 8.7, 5.8 for anti-CCP2 and anti-CCP3.1). The anti-MCV test had a higher sensitivity (62% vs 56.9% and 58.1%, respectively). In psoriatic arthritis, spondyloarthropathy and other arthritis anti-MCV antibodies had a prevalence of 15.2%, 13.9% and 19.4%.

Conclusion: The diagnostic performance of the anti-MCV test in the differential diagnosis of early arthritis is lower than that of the anti-CCP tests.

Introduction

Autoantibody formation is characteristic for rheumatoid arthritis (RA). The best-known autoantibody is rheumatoid factor, which however lacks specificity. More recently, several tests have been developed that detect anti-citrullinated protein antibodies (ACPA). These tests include the commercially available anti-CCP2 and anti-CCP3 assays as well as the anti-mutated citrullinated vimentin (anti-MCV) test. The first two assays use citrullinated peptide(s) for detection of ACPA, whereas the later assay is based upon an entire protein, citrullinated vimentin [1, 2].

Several recent studies studied the test characteristics of the anti-MCV test and the anti-CCP2 [1, 3-9] and anti-CCP3 tests [3, 7], showing conflicting results (online supplementary table). This might be partly due to a limited sample size [3- 9]. In addition, in the majority of these studies RA patients were compared with healthy controls, which entails that the results cannot be easily extrapolated to clinical practice where these tests are used to differentiate early RA from other forms of early inflammatory arthritis. Therefore this study aims to evaluate the diagnostic performance of the anti-MCV test in the differentiation of RA from other forms of inflammatory arthritis in a large inception cohort of early arthritis patients and to compare these test characteristics with those of the anti-CCP2 and anti-CCP 3.1 tests.

PATIENTS AND METHODS

Patients

A total of 917 patients with early arthritis who were part of the Leiden Early Arthritis Clinic (EAC) were studied. The Leiden EAC is an inception cohort that started in 1993 at the Department of Rheumatology, Leiden University Medical Center, the Netherlands and includes patients with recent-onset arthritis (symptom duration <2 years) [10]. All patients with RA, psoriatic arthritis (PsA), spondylo-arthropathy (SpA) and baseline serum available, as well as a random selection of patients with other forms of arthritis were evaluated. The 917 patients had the following diagnoses: 566 RA according to the 1987 revised ACR classification criteria for RA [11]. 99 PsA, 72 SpA, 38 patients reactive arthritis, 32 inflammatory osteoarthritis, 30 remitting seronegative symmetrical synovitis with pitting edema (RS3PE), 21 connective tissue diseases, 26 sarcoidosis, 11 paramalignant arthritis, 6

gout and pseudo-gout, 6 Lyme disease, 2 juvenile idiopathic arthritis and 8 patients with other inflammatory arthritis. RA was diagnosed at baseline (75%) or within the first year of follow-up (25%). The other patients were diagnosed at baseline in 73% and within the first year in 27%. The diagnoses made by the treating physician were checked by an independent rheumatologist using chart review and existing criteria. 99 anonymous blood donors who served as healthy controls were also studied.

ACPA measurement

From all patients and controls, stored baseline serum samples were used to perform the anti-MCV, anti-CCP2 and anti-CCP3.1 tests. Anti-MCV antibodies were measured by a commercially available anti-MCV ELISA (Orgentec Diagnostika, Mainz, Germany). The assay was performed according to the manufacturer's instructions and values >20 U/ml were considered positive. Anti-CCP2 antibodies were measured by Immunoscan CCPlus (Euro-Diagnostica, Arnhem, the Netherlands) and anti-CCP3.1 antibodies using Quanta Lite CCP 3.1 IgG/IgA (Inova, California, USA). The cut-off points were used as determined by manufacturers, 25 U/ml for CCP2 and 20 U/ml for CCP3.1.

Statistical analysis

Sensitivity and specificity with 95% confidence interval (CI) as well as the positive and negative likelihood ratios (LR+, LR-) were calculated for each of the tests. Receiver operating characteristic curves as (ROC) were constructed by plotting sensitivity (true positive rate) against 1-specificity (false positive rate) and the areas under the curve (AUC) were compared. For analysis SPSS version 16 (SPSS 16 Chicago, IL, USA) was used. In all tests, p values <0.05 were considered statistically significant.

RESULTS

Of the 917 early arthritis patients 566 patients had RA, 99 PsA, 72 AS and 180 other forms of inflammatory arthritis. To evaluate the diagnostic performance of the tests in the differentiation of RA from other forms of arthritis, the patients with PsA, AS and other diagnosis were grouped. Amongst the RA-patients 379 (67%) were female; the mean age was

56.9 +/- 15 years. In the group with all other early arthritis patients the mean age was 45.9 ± 16.8 years and 184 (52%) were female (both p-values <0.001 versus RA).

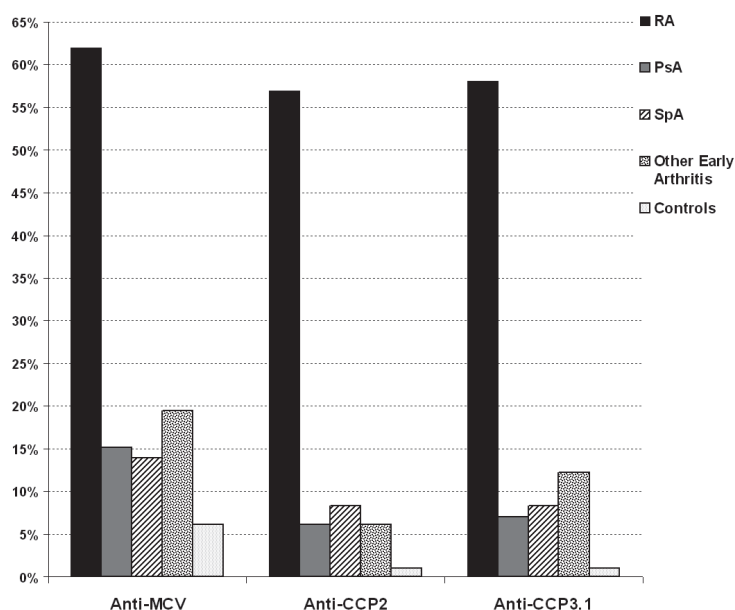


Figure 1: Prevalence of autoantibody positivity in early arthritis and in healthy controls. CCP, cyclic citrullinated peptide; MCV, anti-mutated citrullinated vimentin; PsA, psoriatic arthritis; RA, rheumatoid arthritis; SpA, spondyloarthritis.

Anti-MCV antibodies were present in 351 out of 566 (62%) RA patients and in 60 out of 351 (17.1 %) of all other forms of inflammatory arthritis. Dividing this group into disease categories revealed that anti-MCV antibodies were present in 13.9% of SpA, 15.2% of PsA and 19.4% of patients with other forms of arthritis. As depicted in **Figure 1** the prevalence of anti-CCP2 and anti-CCP3.1 antibody positivity in these conditions were significantly lower. **Figure 2** shows the prevalence of autoantibodies in RA, AS, PsA, and other forms of early inflammatory arthritis given they were positive in the anti-CCP2/3, respectively anti-MCV assay. This figure indicates that most anti-MCV positive RA patients (82%) are also positive in the anti-CCP assays. More importantly, the results depicted in **figure 2** illustrate that only 40% of anti-MCV positive patients with other inflammatory arthritides are anti-CCP positive.

These results indicate that the anti-MCV assay is less specific for RA compared to the two anti-CCP assays.

Figure 2: Prevalence of anti-MCV and anti-CCP2/3 antibodies in different diagnoses. AB+, antibody positive; AB-, antibody negative; CCP+, patients tested positive for either anti-CCP2 and/or anti-CCP3; MCV+, patients tested positive for anti-MCV; CCP+ MCV+, patients tested positive for (either anti-CCP2 and/or anti-CCP3) and positive for anti-MCV. CCP, cyclic citrullinated peptide; MCV, anti-mutated citrullinated vimentin; PsA, psoriatic arthritis; RA, rheumatoid arthritis; SpA, spondyloarthritis.

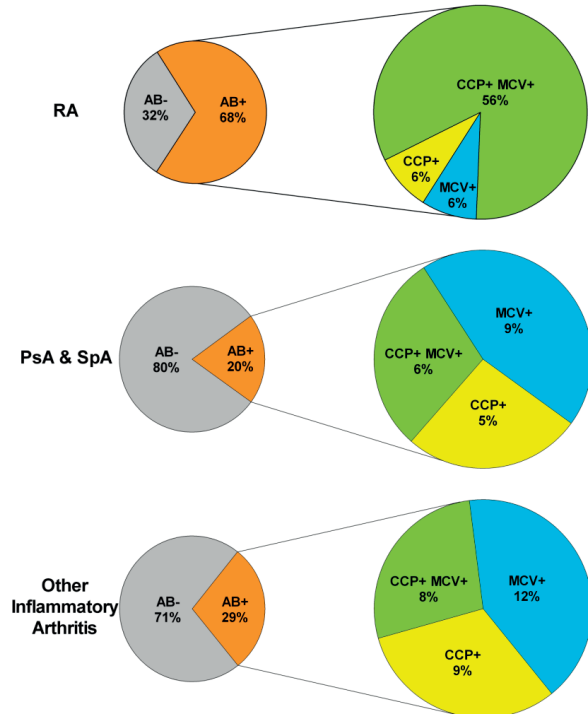


Table 1: Diagnostic performance of anti-MCV, anti-CCP2 and anti-CCP3.1 in differentiating RA from other early arthritis and from healthy controls

	Specificity (95% CI)	True negative/all other early arthritis or controls	Sensitivity (95% CI)	True positives/all RALR+ (95% CI)	LR– (95% CI)	AUC (SEM)	
RA vs other early arthritis							
Anti-MCV	82.9 (78.5 to 86.6)	291/351	62.0 (57.9 to 66.0)	351/566	3.6 (2.8 to 4.6)	0.46 (0.41 to 0.51)	0.725 (0.017)
Anti-CCP2	93.4 (90.2 to 95.7)	328/351	56.9 (52.7 to 61)	322/566	8.7 (5.8 to 13)	0.46 (0.42 to 0.51)	0.752 (0.016)
Anti-CCP3.1	90.0 (86.3 to 92.9)	316/351	58.1 (53.9 to 62.2)	329/566	5.8 (4.2 to 8)	0.47 (0.42 to 0.51)	0.741 (0.016)
RA vs healthy controls							
Anti-MCV	93.9 (86.6 to 97.5)	93/99	62.0 (57.9 to 66.0)	351/566	10.2 (4.7 to 22.3)	0.40 (0.36 to 0.45)	0.789 (0.020)
Anti-CCP2	99.0 (93.7 to 99.9)	98/99	56.9 (52.7 to 61)	322/566	56.3 (8 to 396)	0.43 (0.39 to 0.48)	0.78 (0.019)
Anti-CCP3.1	99.0 (93.7 to 99.9)	98/99	58.1 (53.9 to 62.2)	329/566	57.5 (8.2 to 405)	0.42 (0.38 to 0.47)	0.785 (0.019)

LR+, positive likelihood ratio; LR-, negative likelihood ratio. The LR are measures of the degree to which a positive test result increases the probability of a disease (LR+) and a negative test result decreases the probability of a disease (LR-).

AUC, area under the receiver operator characteristic curve; CCP, cyclic citrullinated peptide; MCV, anti-mutated citrullinated vimentin; RA, rheumatoid arthritis; SEM, standard error of the mean.

In the 99 healthy controls 6 individuals had anti-MCV antibodies (6.1%), compared to 1 individual positive for anti-CCP2 and 1 individual positive for anti-CCP3.1. The test characteristics in differentiating RA from healthy controls showed that the anti-MCV test had the highest sensitivity but the lowest specificity (93.9% compared to 99% for anti-CCP2 and anti-CCP3.1) (**Table 1**). In addition, the LR+ was markedly higher for anti-CCP2 and anti-CCP3.1 compared to anti-MCV (56.3, 57.5 and 10.2 respectively).

DISCUSSION

The present study evaluated the diagnostic performance of a novel commercially available anti-MCV ELISA test for the detection of anti-MCV antibodies in differentiating RA patients from other forms of early inflammatory arthritis, taking advantage of a large inception cohort. Using the cut-off values as indicated by the manufacturer, the anti-MCV assay displayed a higher sensitivity but a lower specificity compared to a second and third generation anti-CCP test. In addition, the LR+ was higher for anti-CCP2 and anti-CCP3.1 than for anti-MCV, suggesting that a positive anti-CCP2 or anti-CCP3.1 test indicates a larger increase in the probability on RA compared to a positive anti-MCV test. This finding is further supported by the observation of a high prevalence of anti-MCV antibodies in other forms of arthritis, reducing its discriminative ability in an early arthritis setting.

An increased prevalence (6-10%) of anti-CCP2 antibodies has been reported previously in PsA [12- 14]. We observed a prevalence of anti-CCP2 of 6.1% in PsA and approximately three times higher percentage of anti-MCV positivity. The antibodies detected by the anti-MCV test were not only relatively frequently present in PsA but also in SpA and other forms of inflammatory arthritis, suggesting that these antibodies are not highly specific for RA.

The current observation of a higher sensitivity but a lower specificity in differentiating RA from other inflammatory arthritis is in line with a previous study comparing the test characteristics in 92 RA-patients and 150 patients with other forms of arthritis [8]. The strengths of the present study are the sample size (n=917) and the early arthritis setting representing the situation in clinical practice where these auto-antibody tests are used.

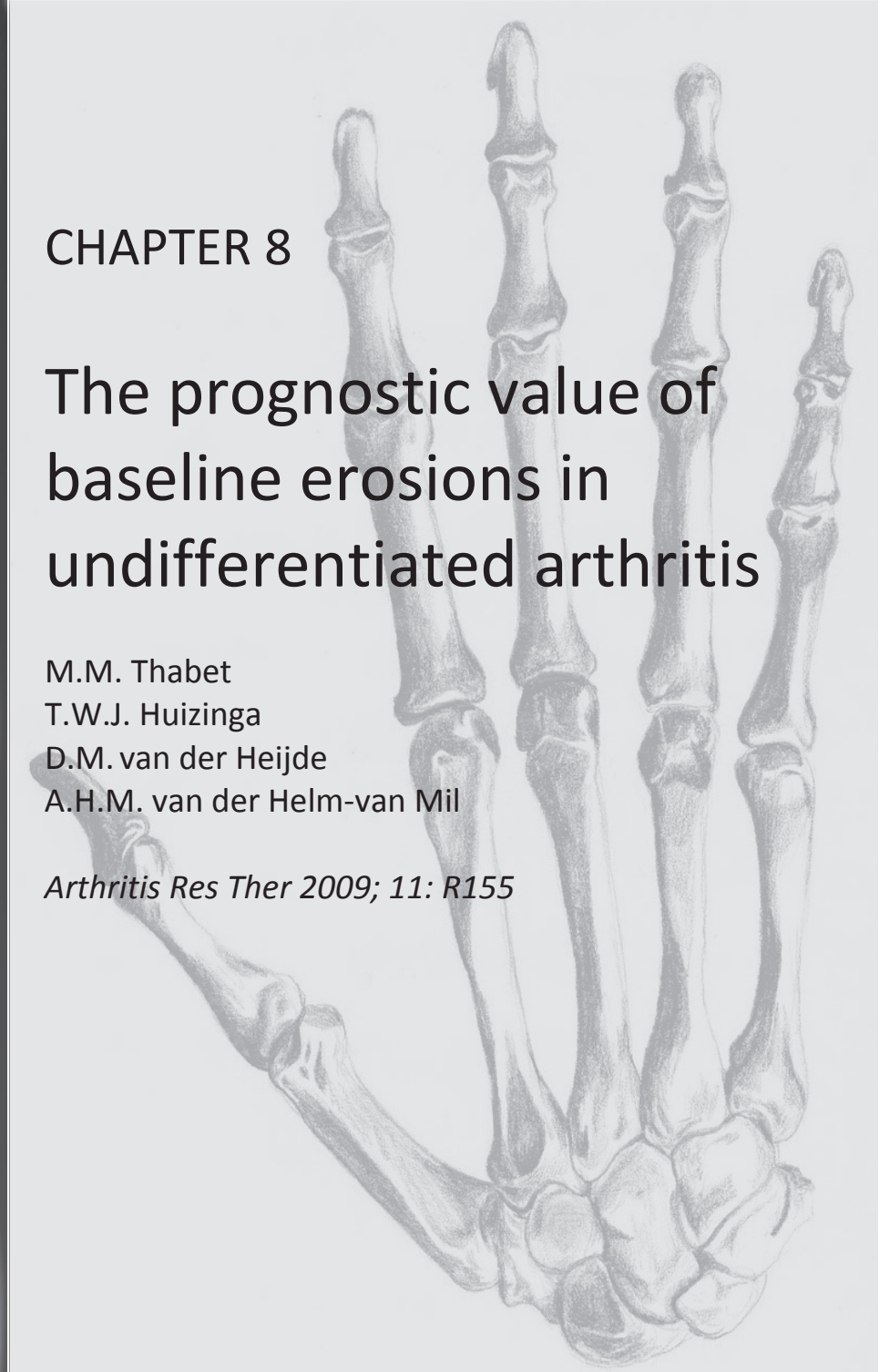
Generally, the autoantibody tests are performed in patients with recent-onset arthritis in order to differentiate between different diagnoses.

In conclusion, the diagnostic performance of the anti-MCV test in the differential diagnosis of early arthritis is lower compared to the anti-CCP tests. Anti-MCV antibodies are present in 13.9-19.4% of non-RA arthritis. It will be interesting to know whether the presence of the antibodies detected by the anti-MCV assay is related to arthritis only or whether they will be found in other inflammatory disorders as well.

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CHAPTER 8

The prognostic value of baseline erosions in undifferentiated arthritis

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Arthritis Res Ther 2009; 11: R155



ABSTRACT

Introduction: Undifferentiated arthritis (UA) has a variable disease course; 40 to 50% of UA patients remit spontaneously, while 30% develop rheumatoid arthritis (RA). Identifying the UA patients who will develop RA is essential to initiate early disease-modifying anti-rheumatic drug (DMARD) therapy. Although the presence of bone erosions at baseline is predictive for a severe destructive disease course in RA, the prognostic importance of erosive joints for disease outcome in UA is unknown. This study evaluates the predictive value of erosive joints for the disease outcome in UA as measured by RA development and disease persistency.

Methods: Baseline hands and feet radiographs of 518 UA patients were evaluated for erosions using a clinical definition as well as the Sharp/van der Heijde method. After 1 year follow-up, patients were re-assessed for the fulfilment of the 1987 ACR classification criteria for RA. Disease persistency was defined as the absence of sustained remission during all available follow-up (mean 8 ± 3 years).

Results: At baseline, 28.6% of UA patients had erosive joints. Presence of ≥ 2 erosive joints showed a positive predictive value for RA development of 53% and for persistent disease of 68%. Patients with erosions that did not develop RA were less often anticyclic citrullinated peptide antibody (ACPA)+ve, rheumatoid factor (RF)+ve and had lower C-reactive protein (CRP), erythrocytic sedimentation rate (ESR) and number of swollen joints compared to those who developed RA. Feet erosions are equally predictive compared to erosions at hands.

Conclusions: Presence of ≥ 2 erosive joints at baseline in UA patients gives a risk for RA development of 53% and for persistent disease of 68%, indicating that erosions in UA are not always predictive for unfavorable disease outcomes.

INTRODUCTION

Early undifferentiated arthritis (UA) is defined as any arthritis of recent onset that cannot be classified according to the existing criteria for specific rheumatic disorders [1]. Patients with early UA form a heterogeneous group exhibiting a variable disease course. Of patients with early UA, 40 to 50% remit spontaneously, whereas 30% develop rheumatoid arthritis (RA) [2- 4]. Recent data indicate that initiation of disease-modifying anti-rheumatic drug (DMARD) therapy in an early stage is beneficial and thus underlines the necessity to recognize those UA patients that will develop RA [5].

In the clinical setting generally much impact is given to clinical predictors of the disease outcome. In RA the early occurrence of erosions is one of the most significant predictors for a severe destructive disease course. In contrast, the prognostic value of baseline erosions for the disease outcome in UA is unknown. Even more, the definition of erosive disease is unclear and different studies use different descriptions and cut-off values. Interestingly, the presence of erosions is part of the 1987 American College of Rheumatology (ACR) classification criteria for RA, but it is not specified how many erosions are required and it only includes erosions in the hands and not in the feet [6].

Considering the lack of knowledge on the prognostic value of erosions in UA, the present study aims to: study the predictive value of erosive joints in hands and feet for development of RA in UA-patients; define the optimal number of erosive joints to predict RA; define whether the predictive ability is different between erosive joints in hands and feet; determine whether information on erosive joints increases the discriminative ability of a recently developed prediction rule for RA-development [7,8]; and investigate whether the results are different when disease persistency is studied instead of the development of RA according to the 1987 ACR criteria.

The presence of erosions was assessed using two methods. First, as the present study aims to have results that are useful for clinical practice, we defined an erosive joint as a joint with at least one erosion, defined as a lesion with an interrupted cortex. The number of erosive joints was counted. As such, this definition is the same as the erosion score in the

Simplified Erosion Narrowing Score (SENS) and is in line with common clinical practice [9]. Several other scoring methods quantify the radiological joint destruction in more detail [10]. Commonly used are the Sharp and the Larsen methods with their modifications [11,12]. Although these methods are very valuable for research purposes, they are difficult to use in clinical practice [10] because they require specialized training and are time-consuming [13,14]. We primarily studied the predictive value of the number of erosive joints but also performed the analyses using the erosion score of the Sharp/van der Heijde scoring (SHS) method in which the size and number of erosions per joint are weighted [15].

MATERIALS AND METHODS

Patients

The present study includes 518 early arthritis patients who were included between 1993 and 2005 in the Leiden Early Arthritis Cohort (EAC). This EAC is a prospective cohort started in 1993 [16]. Patients were referred by general practitioners when arthritis was suspected and inclusion took place when arthritis was confirmed at physical examination and symptom duration was less than two years. Written informed consent was obtained from all participants. The study was approved by the local Medical Ethical Committee. At inclusion, patients were asked about their joint symptoms, disease duration and subjected to a physical examination. Blood samples were taken for routine diagnostic laboratory screening (including immunoglobulin (Ig)M-rheumatoid factor (RF)) and stored to determine other autoantibodies at a later time. Follow-up visits were performed on a yearly basis and included radiographs of hands and feet. Patients who at baseline did not fulfil the criteria for known rheumatic disorders and thus were referred to as having UA (518 patients).

Radiographs

Baseline radiographs of the hands (anterioposterior view) and feet (posteroanterior view) were available for all the 518 UA patients and were evaluated by the same person (MT). Erosions were defined according to the erosive score of the SENS method that was developed for use in clinical practice, by the presence of a joint with at least one erosion. Subsequently, the number of erosive joints was counted in the following joints: in hands, the proximal interphalangeal (PIP) joint in digits 1 to 5, the metacarpo-phalangeal (MCP) joint in digits 1 to 5

and 6 radio-carpal sites (base of metacarpal bone digit 1, trapezium, lunate, scaphoid, distal ulna and distal radius) and in feet, the inter-phalangeal (IP) joint digit 1 and metatarsophalangeal (MTP) joint in digits 1 to 5. In addition, the radiographs were scored according to the SHS method [15] assessing the same hand and feet joints as the SENS method. In the present study, only the erosion scores were studied while the joint space narrowing scores were omitted. Of the radiographs, 10% were scored twice to determine the intra-class correlation coefficient (ICC). With 10% rescoring, the erosive joint count according to the SENS definition showed an ICC of 0.91 and a smallest detectable change (SDC) of 0.92, while the erosion SHS scores showed an ICC of 0.94 and an SDC of 1.04.

Disease outcome

All patients were followed prospectively. After one year of follow up, the fulfilment of the 1987 ACR criteria for RA was evaluated. The second disease outcome was disease persistency. As a generally accepted definition for persistency is lacking, we defined disease persistency as the absence of sustained remission during all available follow up (mean 8 ± 3 years). Sustained remission was diagnosed when patients had no swollen joints for at least one year, after cessation of eventual DMARD therapy. The absence of swollen joints had to have been observed by a rheumatologist for at least one year to ensure that remission was not temporary, but rather sustained. Most patients identified with remission were discharged from the outpatient clinic. Although patients should have absence of arthritis for at least one year according to the definition, most patients with remission had a longer follow up after the identification of remission (median of 16 months). Patients who had a recurrence of their arthritis after discharge, could easily return to the Leiden University Medical Center, the only referral center for rheumatology in a health care region of approximately 400,000 inhabitants. This occurred in six patients and these patients were not classified as sustained remission.

Statistical analysis

Proportions were compared using the chi-squared test with two degrees of freedom (Epi Info v6, CDC, Atlanta, Georgia, USA). Differences in mean values between groups were analyzed with the Mann-Whitney U test. The positive predictive values (PPV), negative predictive values (NPV), specificity, sensitivity, and positive and negative likelihood ratios (LR) were

determined for several cut-off values of the erosive joint count and erosion scores according to SHS method. Receiver operating characteristic (ROC) curves for the different cut-off values were constructed and the area under the curve (AUC) provided a measure of the overall discriminative ability. SPSS software, version 14.0 (Chicago, IL, USA), was used for data analyses. *P* values less than 0.05 were considered statistically significant.

RESULTS

Predictive accuracy for RA development

From the 518 UA patients, 31% (*n* = 160) fulfilled the 1987 ACR classification criteria for RA after one year of follow-up. Baseline characteristics of UA patients that did and did not progress to RA are summarized in **Table 1**. Overall, 148 of 518 (28.6%) UA patients had erosive joints at baseline. Seventy-six of the 160 (42%) patients who developed RA had baseline erosive joints, compared with 81 of the 358 (22.6%) who did not develop RA (*P* ≤ 0.001).

Table 1

Characteristic	Total (<i>n</i> = 518)	Developed RA (<i>n</i> = 160)	Did not develop RA (<i>n</i> = 358)	<i>P</i>	LR+	LR-
Female gender <i>n</i> (%)	305 (59)	111 (69)	194 (54)	0.001	1.3	0.67
Age in years (Mean ± SD)	50.6 ± 16.5	56.3 ± 15	48 ± 16.5	< 0.001	-	-
Symptom duration at inclusion in months (Mean ± SD)	4.9 ± 5.5	5.9 ± 6	4.4 ± 5.1	0.002	-	-
Morning stiffness (Mean ± SD)	55.7 ± 89.9	82.9 ± 111.4	43.6 ± 75.6	< 0.001	-	-
Tender joint count (Mean ± SD)	6.5 ± 6.3	9.5 ± 7.4	5.1 ± 5.3	< 0.001	-	-
Swollen joint count (Mean ± SD)	3.8 ± 4	5.7 ± 5.1	2.9 ± 3.1	< 0.001	-	-
HAQ score (Mean ± SD)	0.76 ± 0.6	0.9 ± 0.6	0.7 ± 0.6	< 0.001	-	-
ESR (mm/hr) (Mean ± SD)	28.9 ± 24.4	36.8 ± 23.7	25.3 ± 23.9	< 0.001	-	-
CRP (mg/L) (Mean ± SD)	20.6 ± 28.2	26.5 ± 29.1	17.9 ± 27.4	< 0.001	-	-
RF positivity <i>n</i> (%)	125 (24.2)	76 (47.5)	49 (13.7)	< 0.001	3.5	0.61
ACPA positivity <i>n</i> (%)	114 (23.4)	76 (50)	38 (11.3)	< 0.001	4.4	0.56
**Having erosive joints in hands and/or feet <i>n</i> (%)	148 (28.6)	67 (41.9)	81 (22.6)	< 0.001*	1.8	0.75
**Hands only <i>n</i> (%)	58 (11.2)	23 (14.4)	35 (9.8)	0.1	-	-
**Feet only <i>n</i> (%)	46 (8.9)	22 (13.8)	24 (6.7)	0.009	-	-
**Hands and feet <i>n</i> (%)	44 (8.5)	22 (13.8)	22 (6.2)	0.004	-	-
SHS erosion score (mean ± SD)	0.8 ± 2	1.5 ± 3	0.5 ± 1.3	< 0.001	-	-

ACPA = anticyclic citrullinated peptide antibody; CRP = C-reactive protein; ESR = erythrocytic sedimentation rate; HAQ = health assessment questionnaire; LR+ = positive likelihood ratio; LR- = negative likelihood ratio; RA = rheumatoid arthritis; RF = rheumatoid factor; SD = standard deviation; SHS = Sharp/van der Heijde scoring; UA = undifferentiated arthritis. Morning stiffness score on a 100 mm visual analogue scale.

P value is calculated in reference to the no-erosion group; ** Defined as a broken cortex in at least 1 joint.

Different cut-off values for erosive joint count were tested for their PPV and NPV regardless the localization of erosive joints (**Table 2**). The PPV of having one or more erosive joint was 45%. With higher cut-offs the PPV gradually increased. In the presence of two or more, three or more and four or more erosive joints the PPVs were roughly similar: 53 to 54%. For the cut-off of having five or more erosive joints, the PPV was 73%. As the 95% confidence interval (CI) were overlapping, it could not be concluded that the PPV of one of these cut-off values is statistically superior to the others. The specificity was significantly different between the cut-off of two or more erosive joints (89%) compared with the cut-off of one or more erosive joints (77%). Using higher cut-off values, the specificity gradually increased but the 95% CI overlapped. Evaluating the positive and negative LR revealed the cut-off of two or more erosive joints had the best balance between the positive and negative LRs.

Table 2

Predictive value for the progression from UA to RA within one year using different cut-off values for erosions in hands and/or feet												
Number of erosive joints	n	PP	(95% CI)	NPV	(95% CI)	Specificity	(95% CI)	Sensitivity	(95% CI)	LR+	LR-	AUC (SEM)
≥1	148	45	37-53	75	70-79	77	73-82	42	34-50	1.8	0.75	0.60 (0.028)
≥2	83	53	42-64	73	69-78	89	86-92	28	21-34	2.5	0.81	0.58 (0.028)
≥3	50	54	40-68	72	68-76	94	91-96	17	11-23	2.6	0.89	0.55 (0.028)
≥4	24	54	34-74	70	66-74	97	95-99	8	4-12	2.6	0.95	0.53 (0.028)
≥5	15	73	51-96	70	66-74	99	98-100	7	3-11	6.2	0.94	0.53 (0.028)

n = number of UA patients positive for this cut off.

AUC = area under the curve; CI = confidence interval; LR+ = positive likelihood ratio; LR- = negative likelihood ratio; NPV = negative predictive value; PPV = positive predictive value; RA = rheumatoid arthritis; SEM = standard error of measurement; UA = undifferentiated arthritis.

Additionally, the predictive values of the SHS erosion score were investigated in a similar way, using different cut-off values (**Table 3**). In case of an erosion score of one or more, 45% of UA patients developed RA, which increased to 51 and 61% of UA patients in the presence of an erosion score of two or more and five or more, respectively. As such, the resulting PPVs and NPVs were comparable with those obtained using the erosive joint count. Also, here the cut off of an erosion score of two had the best balance between the positive and negative LRs.

Table 3

Predictive value for the progression from UA to RA within one year using the erosion score of the Sharp van der Heijde method with different cut-off values for erosions												
SHS erosion score	n	PPV	(95% CI)	NPV	(95% CI)	Specificity	(95% CI)	Sensitivity	(95% CI)	LR+	LR-	AUC (SEM)
≥1	148	45	37-53	75	70-79	77	73-82	42	34-50	1.9	0.75	0.60 (0.028)
≥2	92	51	41-61	73	69-78	87	84-91	29	22-36	2.3	0.80	0.58 (0.028)
≥3	61	52	40-65	72	68-76	92	89-95	20	14-26	2.5	0.87	0.56 (0.028)
≥4	39	56	41-72	71	67-75	95	93-98	14	8-19	2.9	0.90	0.55 (0.028)
≥5	23	61	41-81	71	67-75	97	96-99	9	4-13	3.5	0.94	0.53 (0.028)

n = number of UA patients positive for this cutoff.

AUC = area under the curve; CI = confidence interval; LR+ = positive likelihood ratio; LR- = negative likelihood ratio; NPV = negative predictive value; PPV = positive predictive value; RA = rheumatoid arthritis; SEM = standard error of measurement; SHS = Sharp/van der Heijde scoring; UA = undifferentiated arthritis.

Assessing data on the total available duration of follow up, implying that patients with differences in the duration of follow up are compared, revealed that 23 UA patients (4.4%) developed RA later than one year after inclusion. Categorizing these patients in the RA group and using the cut off of having at least two erosive joints revealed a slightly higher PPV of 60% (95% CI 50% to 71%) and a slightly lower NPV of 69% (95% CI 65% to 74%).

Effect of localization of erosion

Baseline erosions were present in the hands in 11.2% of UA patients, in the feet in 8.9% of UA patients, and both in hands and feet in 8.5% of UA patients (**Table 1**). From these data, it cannot be concluded that the small joints in the feet are less often erosive than the small joints in the hands because the number of assessed joints in feet and hands are different, 12 versus 26, respectively. Moreover, the LR+ for erosive joints in the feet is for all cut-off points somewhat higher as compared with the LR+ for erosive joints in the hands with a similar LR- (**Tables 4 and 5**). These data suggest that presence of erosions in the joints of the feet is slightly more predictive than erosions in the hand joints.

The frequency of erosions (with a cut off of two or more erosive joints) in the MCP joints was 3.1% in those who developed RA compared with 2.2% in those who did not develop RA ($P = 0.6$, PPV = 38.5%, LR+ = 1.4, LR- = 0.99). Erosive PIP joints were present in 3.1% of the RA group compared with 1.1% of the non-RA group ($P = 0.1$, PPV = 55.6%, LR+ = 2.8, LR- = 0.98). The frequency of erosions in the wrist joints in the RA group was 4.4%

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compared with 1.1% in the non-RA group ($P = 0.04$, PPV = 63.6%, LR+ = 3.9, LR- = 0.97). Although the frequency of erosions in the MTP joints in the RA group was 14.4% compared with 3.4% in the non-RA group ($P < 0.001$, PPV = 67.6%, LR+ = 4.3, LR- = 0.88; **Table 6**).

Table 4

Predictive value for the progression from UA to RA within one year using different cut-off values for erosions in hands												
Number of erosive joints	n	PPV	(95% CI)	NPV	(95% CI)	Specificity	(95% CI)	Sensitivity	(95% CI)	LR+	LR-	AUC (SEM)
≥1	103	44	34-53	72	68-77	84	80-88	28	21-35	1.7	0.86	0.56 (0.028)
≥2	38	55	40-71	71	67-75	95	93-98	13	8-18	2.8	0.91	0.54 (0.028)
≥3	18	56	33-79	70	66-74	98	96-99	6	3-10	2.8	0.96	0.52 (0.028)
≥4	9	67	36-98	70	66-74	99	98-100	4	1-7	4.5	0.97	0.52 (0.028)
≥5	7	71	38-100	70	66-74	99	99-100	3	0-6	5.6	0.97	0.51 (0.028)

Table 5

Predictive value for the progression from UA to RA within one year using different cut-off values for erosions in feet												
Number of erosive joints	n	PPV	(95% CI)	NPV	(95% CI)	Specificity	(95% CI)	Sensitivity	(95% CI)	LR+	LR-	AUC (SEM)
≥1	89	49	39-60	73	69-77	87	84-91	28	21-34	2.2	0.83	0.57 (0.028)
≥2	42	60	48-74	72	68-76	95	93-98	16	10-21	3.3	0.89	0.55 (0.028)
≥3	18	67	45-88	70	66-74	98	97-100	8	3-12	4.5	0.94	0.53 (0.028)
≥4	9	67	36-98	70	66-74	99	98-100	4	1-7	4.5	0.97	0.52 (0.028)
≥5	5	80	45-115	70	66-74	100	99-100	3	0-5	8.9	0.98	0.51 (0.028)

n = number of UA patients positive for this cutoff.

AUC = area under the curve; CI = confidence interval; LR+ = positive likelihood ratio; LR- = negative likelihood ratio; NPV = negative predictive value; PPV = positive predictive value; RA = rheumatoid arthritis; SEM = standard error of measurement; UA = undifferentiated arthritis.

Table 6

Predictive value for the progression from UA to RA within one year by location of erosive joints and cutoff of at least two erosive joints												
Location of erosions	n	PPV	(95% CI)	NPV	(95% CI)	Specificity	(95% CI)	Sensitivity	(95% CI)	LR+	LR-	AUC (SEM)
Wrist	7	63.6	32-88	69.8	66-74	98.9	97-100	4.4	2-9	3.9	0.97	0.52 (0.028)
MCP	5	38.5	15-68	69.3	65-73	97.8	95-99	3.1	1-8	1.4	0.99	0.50 (0.028)
PIP	5	55.6	23-85	69.5	65-73	98.9	97-100	3.1	1-8	2.8	0.98	0.51 (0.028)
MTP	23	67.6	5-9	71.6	67-76	96.6	94-98	14.4	10-21	4.3	0.88	0.56 (0.028)

n = number of UA patients positive for this cutoff.

AUC = area under the curve; CI = confidence interval; LR+ = positive likelihood ratio; LR- = negative likelihood ratio; MCP = metacarpo-phalangeal joint; MTP = metatarso-phalangeal joint; NPV = negative predictive value; PIP = proximal inter-phalangeal joint; PPV = positive predictive value; RA = rheumatoid arthritis; SEM = standard error of measurement; UA = undifferentiated arthritis.

Characteristics of erosive UA patients that did not develop RA

As we observed that 47% of the UA patients with at least two erosive joints did not fulfil the 1987 ACR classification criteria for RA after one year of follow up, we compared the baseline characteristics of the patients who had at least two erosive joints that did and did not progress to RA (**Table 7**). The erosive patients that did not develop RA were less often anticyclic citrullinated peptide antibody positive ($P < 0.001$), less often RF positive ($P = 0.01$), had a lower erythrocytic sedimentation rate ($P = 0.004$), a lower C-reactive protein ($P = 0.005$), a lower tender joint count ($P < 0.001$), a lower swollen joint count ($P < 0.001$) and experienced less severe morning stiffness as recorded on a visual analogue scale ($P = 0.04$) compared with the patients that progressed to RA.

Table 7

Baseline characteristics of the UA patients with erosions (≥ 2 erosive joints) that did and did not progress to RA within one year				
Characteristic	Total (n = 83)	Developed RA (n = 44)	Did not develop RA (n = 39)	P
Female gender n (%)	44 (53)	26 (53.8)	18 (46.2)	0.2
Age in years (Mean $\pm$ SD)	61.5 $\pm$ 16	60.7 $\pm$ 16.4	62.5 $\pm$ 15.6	0.6
Symptom duration at inclusion in months (Mean $\pm$ SD)	6.5 $\pm$ 6.3	7.1 $\pm$ 7.4	5.7 $\pm$ 4.8	0.9
Morning stiffness (Mean $\pm$ SD)	74.9 $\pm$ 100.5	92.2 $\pm$ 114.4	55.4 $\pm$ 78.9	0.04
Tender joint count (Mean $\pm$ SD)	6.9 $\pm$ 5.8	9 $\pm$ 6.7	4.6 $\pm$ 3.7	<0.001
Swollen joint count (Mean $\pm$ SD)	4.7 $\pm$ 4	6 $\pm$ 4	3.2 $\pm$ 3.4	<0.001
HAQ score (Mean $\pm$ SD)	0.89 $\pm$ 0.74	0.9 $\pm$ 0.73	0.87 $\pm$ 0.75	0.9
ESR (mm/hr) (Mean $\pm$ SD)	34.7 $\pm$ 24.7	41.3 $\pm$ 24	27.2 $\pm$ 23.6	0.004
CRP (mg/L) (Mean $\pm$ SD)	27.8 $\pm$ 31.4	33.4 $\pm$ 30.4	21.5 $\pm$ 31.7	0.005
RF positivity n (%)	31 (37.3)	22 (50)	9 (23.1)	0.01
ACPA positivity n (%)	25 (32.5)	20 (51.3)	5 (13.2)	< 0.001
SHS erosion score (mean $\pm$ SD)	4.2 $\pm$ 3.4	4.7 $\pm$ 4.3	3.7 $\pm$ 1.8	0.4

ACPA = anticyclic citrullinated peptide antibody; CRP = C-reactive protein; ESR = erythrocytic sedimentation rate; HAQ = health assessment questionnaire; RA = rheumatoid arthritis; SD = standard deviation; SHS = Sharp/van der Heijde scoring; UA = undifferentiated arthritis. Morning stiffness score on a 100-mm visual analogue score.

Of the UA patients with baseline erosions who developed RA, 74% were treated with DMARDs in their first year (compared with 34% of the erosive UA patients who did not develop RA) and 66% of non-erosive UA patients who developed RA were treated with DMARDs (compared with 21% of non-erosive UA patients that did not develop RA). This indicates that rheumatologists did not treat erosive patients more aggressively than non-erosive patients. DMARDs used were methotrexate (34.8%), hydroxychloroquine (29.3%),

sulphasalazine (25%), combination of methotrexate and hydroxychloroquine or sulphasalazine (8.7%), or other DMARDs (2.2%).

Contribution to the prediction rule for RA development

Recently, a prediction rule was published that estimates the risk for individual UA patients to progress to RA using nine commonly assessed clinical and serological variables [7]. Presence of erosions is not part of this rule. As we hypothesized that using different cut offs or a different definition for erosive joints (according to the SENS method) may affect the predictive ability of the prediction rule, data on the number of erosive joints were added to the logistic regression model with a backward selection procedure that was used to derive the prediction rule [7]. For all the different cut-off values used, the presence of erosive joints was not an independent predictor for RA development (data not shown), and thus this information did not improve the predictive ability of the model.

When using the prediction rule, there is a group of patients (25% of all UA patients) for whom no accurate prediction of RA development can be made. These patients have a prediction score between 6.0 and 8.0 and for these patients the PPV for RA development is 48% and the NPV is 52%. It was tested if the radiological data on erosions can serve as an extra predictive tool, improving the NPV and PPV for this specific group of UA patients (n = 139). When using the cut off of at least two erosive joints, the PPV was 56% and the NPV was 63% [see Additional data file 1]. Together these results indicate that using data on joint erosion disease does not result in an important further differentiation of the risk estimation for RA in patients with UA additive to the predictive ability of the known clinical and serological factors.

Predictive accuracy for disease persistency

As the outcome measure of fulfilling the 1987 ACR criteria for RA might be subject to discussion (because these criteria were not designed to identify RA in an early phase) and to circular reasoning (because the presence of hand erosions are part of the ACR criteria), we also tested the ability of the presence of erosive joints to predict disease persistency, defined as the absence of sustained remission. For this analysis we used all available follow-up data,

implying that the follow up was not similar for all patients studied. During the whole period of follow up, 39.6% (n = 205) of UA patients achieved clinical remission, while the remaining 60.4% (n = 313) had a persistent disease course (remained UA, developed RA or developed a disease other than RA). The PPV for having a persistent disease course gradually increased with higher cut-off values (**Table 8**). When using the cut-off value of having two or more erosive joints, the PPV was 68% and the NPV was 41%. These PPV are somewhat higher compared with the data on RA development according to the ACR criteria, but because of overlapping 95% CIs the differences were not significant. In addition to the finding that from all UA patients with two or more erosive joints 32.5% achieved sustained remission, it was observed that from all UA patients that achieved sustained remission, 13.2% already had at baseline at least two erosive joints. For all cut-off values for the number of erosive joints the positive and negative LR_s were around one (**Table 8**).

Table 8

Predictive value for having persistent disease in UA patients using different cut-off values for number of erosive joints												
Number of erosive joints	n	PPV	(95% CI)	NPV	(95% CI)	Specificity	(95% CI)	Sensitivity	(95% CI)	LR+	LR-	AUC (SEM)
≥1	148	65	57-73	41	36-46	75	69-81	31	26-36	1.2	0.93	53 (0.026)
≥2	83	68	57-78	41	36-46	87	82-92	18	14-22	1.4	0.95	52 (0.026)
≥3	50	70	57-83	41	36-45	93	89-96	11	8-15	1.5	0.96	52 (0.026)
≥4	24	68	48-86	40	36-44	96	93-99	5	3-8	1.3	0.99	51 (0.026)
≥5	15	73	51-96	40	36-44	98	96-100	4	2-6	1.8	0.98	51 (0.026)

n = number of UA patients positive for this cutoff.

Patients with remission 205 (39.6%).

Patients with persistent disease 313 (60.4%).

AUC = area under the curve; CI = confidence interval; LR+ = positive likelihood ratio; LR- = negative likelihood ratio; NPV = negative predictive value; PPV = positive predictive value; SEM = standard error of measurement; UA = undifferentiated arthritis.

DISCUSSION

The current study explored the prognostic value of the presence of erosive joints for RA-development (defined by fulfilment of the 1987 ACR classification criteria) and for having a persistent disease course in patients with recent-onset UA. As the primary aim was to determine the risk on these disease outcomes for individuals, so that the results are useful for clinical practice, we concentrated on the PPV and NPV and put less emphasis on the sensitivity and specificity, which provide information on the quality of the test. The LR_s

compare probabilities of true results to false results and as such also provide information on the test but not on absolute probabilities for individuals. The PPV and NPV are dependent on the prevalence of the disease in the population and therefore the results of the present study apply to recent-onset UA patients seen by rheumatologists.

We observed that in the presence of at least two erosive joints, the PPV for RA-development within one year was 53% and the PPV for persistent disease was 68%. The presence of one or more erosive joint had a lower PPV (45%) and the presence of three or more or four or more erosive joints was not more predictive compared with the presence of two or more erosive joints (PPV 54% versus 53%). Thus, a considerable proportion of UA patients with erosive joints do not progress to RA. Additionally, the LRs for disease persistency were around one, illustrating the low impact of the quantity of erosions for the likelihood of persistent disease. Although in patients with RA, the presence of baseline erosions is a potent predictor for a severe destructive disease course, the present data implicate that for personalized medicine in UA, information on the presence of erosions alone is inadequate to obtain optimal treatment decision making.

The present data revealed that the PPVs and NPVs for having erosions in the feet joints were at least equal to the predictive values for having erosions in the small joints of the hands, and from all joints studied the LR+ was the highest for the MTP joints. This is notable as in the 1987 ACR classification criteria for RA [6], the presence of erosions in hands are included but feet erosions are not. In line with a previous study [17], the current study suggests that the presence of feet erosions should form part of the classification criteria as well.

Recently, a prediction rule for RA development was developed and validated [7,8,18], and presence of erosions was not part of this rule. The data presented revealed that radiological data on erosions, regardless of its definition, does not importantly improve the predictive ability of the prediction rule that is based on common clinical and serological risk factors.

In order to have a similar duration of follow up for all studied UA patients, progression to RA was evaluated after one year. This might have introduced misclassification because patients may have fulfilled the ACR criteria later on in the disease. Therefore RA development was also recorded in the total available follow up (mean 8 ± 3 years). Of UA patients, 4.4% developed RA later than one year after inclusion. Taking these patients into consideration led to a slight increase of the PPV and decrease of the NPV although 95% CIs overlapped.

In the current study we evaluated erosions in anteroposterior x-rays of the hands and posteroanterior x-rays of the feet without additional planes. This is in accordance with clinical practice in the Netherlands and many other countries. The use of an extra plane could have increased the sensitivity to detect erosions, but at the costs of extra irradiation and financial costs.

Data about the value of erosions detected by other new imaging modalities such as sonography or magnetic resonance imaging (MRI) in UA population are scarce. Duer and colleagues investigated the value of MRI erosions in UA patients without erosions at conventional radiography and found that the presence of MRI erosions has a PPV of 50%, a NPV of 85%, a LR+ of 2.7 and a LR- of 0.5 for prediction of RA development [19]. Tamai and colleagues studied the predictive value of MRI erosions (without knowledge if these were radiographically visible) and observed a PPV of 81.5%, a NPV of 48%, a LR+ of 3.18 and a LR- of 0.78 [20].

Several studies have shown that the current ACR criteria are not well suited for establishing early RA [21-24]. In the current study, many UA patients had arthritis in few joints, were seropositive and had erosive joints. Because they did not fulfil the 1987 ACR criteria for RA at baseline they were classified as UA but not as RA. This may also illustrate that the 1987 ACR criteria are not sensitive to diagnose RA at an early stage and further implies the need for new classification criteria suitable for early RA. Moreover, determining the predictive values of baseline erosions for the development of RA according to the ACR criteria can lead to circular reasoning as hand erosions are part of the criteria. Therefore, we

studied the predictive accuracy for having persistent disease as well. This is in line with previous studies which used persistent disease as an outcome measure in UA patients [24,25]. Although thorough investigation of the medical files taught us that the group of patients with persistent disease is heterogeneous and the available duration of follow up in which persistency/remission is assessed differed between patients, the predictive values for RA development and disease persistency were in the same range.

CONCLUSIONS

In conclusion, the presence of erosions in small joints of the hands and feet in UA patients at first presentation gives a risk for RA development of 53% after one year and a risk for persistent disease of 68%. These data imply that in early UA baseline erosions are not always predictive for a poor disease outcome and are on their own insufficient to found treatment decisions.

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CHAPTER 9

Summary and discussion



RA has a complex multifactorial etiology, of which many elements remain unknown. Genetic and environmental risk factors play a major role in disease development. An important characteristic of RA is the production of autoantibodies. It was shown that ACPA-positive differs from ACPA- negative RA in phenotype (having more severe and persistent disease) as well as having different genetic and environmental risk factors [1, 2]. The most prominent example being the HLA shared epitope (SE) alleles, which accounts for 36% of overall genetic susceptibility, specifically predispose to ACPA-positive disease [3-5].

Part I

Several genetic risk factors associate differently with RA susceptibility in various populations. In **Chapter 2, 3 and 4** of this thesis, part of the genetic variants that were reported to differentially associate with RA risk were investigated in a Dutch Caucasian population. Furthermore, these data were analysed within the context of autoantibody status besides considering the interaction with various environmental factors and gender in a trial to, hopefully, explain the ethnic disparity in those risk factors.

The functional polymorphism in the PTPN22 gene associated with RA susceptibility in Caucasians is extremely rare in Asian populations, and other polymorphisms within the gene show no evidence for association [6, 7]. In **Chapter 2**, we confirmed the association of C1858T variant of PTPN22 with RA susceptibility in Dutch Caucasians. This association was more evident and only statistically significant in autoantibody positive (rheumatoid factor (RF) positive) patients when compared to RF negative patients. Additionally we showed that C1858T is the sole PTPN22 variant predisposing to RA in Dutch Caucasians in contrast to other reports finding other associated PTPN22 variants [8].

FCRL3 promoter SNP (rs7528684) was shown to associate with RA risk in Japanese [9, 10] but not in Americans [11] and Spanish [12]. We investigated the FCRL3 promoter SNP association with RA in a Dutch Caucasian population in **Chapter 3**. Carrier analysis revealed that the recessive CC genotype carriers are at higher risk of developing RA compared to TT+TC genotype carriers. Additionally, stratification for autoantibodies (ACPA) didn't reveal association with ACPA+ve or ACPA-ve disease. Similarly, no association of FCRL3

genotypes with RA severity was found. Meta-analysis of the previous studies in Japanese [9, 10], Americans [11], Spanish [12] and with the our data included confirmed that CC carriers are RA susceptible across various populations. Consequently, our findings suggest that the association of FCRL3 with RA susceptibility might not be specific to the Japanese population, and that FCRL3 is possibly involved in the pathogenesis of RA in a recessive trait fashion.

PADI4 gene polymorphisms found associated with RA susceptibility in multiple Asian populations but lack the association in Caucasians [13]. In **Chapter 4**, we tried to elucidate the genetic heterogeneity and differential role of PADI4 in RA observed between Asian and European populations. Additionally, we examined possible gene-environmental interactions between PADI4 polymorphism, sex and smoking status in 2 Japanese and 1 Dutch patients-controls sample sets. In the first Japanese sample set, PADI4 polymorphism (rs1748033) showed a greater risk for RA in men than in women and in those who ever smoked compared to never-smokers. Moreover, the highest risk was seen in males who ever-smoked. Similar trends were observed in the second Japanese as well as the Dutch sample sets. Those data suggest that PADI4 polymorphism highly predisposes male smokers to RA. Furthermore, the genetic heterogeneity observed between Asian and European populations may be partly explained by differences in smoking prevalence among men and gender of patients and controls studied for PADI4 genotypes.

Genetic susceptibility loci are usually identified via three main approaches: candidate gene, linkage, and GWAS. GWAS have become the most powerful and extensively employed approach in discovering susceptibility variants for complex disease traits. Advances in technology, reduced costs, and the ascertainment of large well-characterized case cohorts and panels of controls have made GWAS widely available. Despite its high-throughput and feasibility, GWAS has important limitations; the potential for false-positive results, lack of information on gene function, insensitivity to rare variants and structural variants (therefore, potential for false-negative results), requirement for large sample sizes, and possible biases due to case and control selection and genotyping errors [14]. Structural variants can be in the form of insertion deletion or copy number variation.

Variation in the copy number (CN) of genes within the human genome is an important source of genetic variation, and is defined as a sequence of DNA > 1 kb present in altered CN when compared with a reference genome [15]. In the diploid human genome, autosomal genes are normally present in two copies (one on each chromosome). However, when CNV is present the number of copies can vary from zero to greater than two. Studies have demonstrated a large number of genes within the human genome that display CNV in a relatively high frequency. CNV may be a major source of quantitative variation in expression, and has been proposed to contribute to phenotypic diversity and disease susceptibility [16-18].

Fc γ Rs are found on most effector cells of the immune system where they are frequently co-expressed [19]. They interact with the Fc portion of the IgG molecule, therefore, link the IgG antibody mediated responses with cellular effector and regulatory functions [20]. They can be either stimulatory or inhibitory and play an integral role in the identification and destruction of both endogenous and foreign opsonised material [21]. The balance between the stimulating and inhibiting signal is crucial to maintaining immune homeostasis [19]. Functionally relevant polymorphisms have been identified in low-affinity Fc γ Rs which affect the binding with IgG subclasses and have been shown to play an important role in immune complex mediated autoimmune diseases such as SLE [22, 23], RA [24], and immunocytopenic purpura [25]. The initial suggestion that there might be CNV at the Fc γ Rs locus came from an isolated case report of serological surveys revealing rare individuals with complete Fc γ RIIIb deficiency and either neonatal autoimmune neutropenia or lupus nephritis. The deletion of Fc γ R3B was previously associated with SLE and with neonatal autoimmune neutropenia in offspring from Fc γ RIIIb-negative mothers [26, 27].

In **Chapter 5 and 6** of this thesis we studied Fc γ R gene polymorphisms. The technical difficulties arising from the high homology of Fc γ Rs and the presence of structural variations as CNV warranted the use of a different approach to investigate Fc γ Rs polymorphisms. Therefore, besides genotyping SNPs, we used MLPA to test for CNV and combined the analyses to explore whether the presence or absence of CNV has any effect on SNPs genotyping and its association with RA risk.

A functional polymorphism in FcγR3A results in an amino acid substitution at position 158 of phenylalanine for valine which reduces the affinity of IgG1, IgG3 and IgG4 binding to the receptor. These variants, which may lead to a reduced clearance of immune complexes, have been shown in previous studies to contribute to the pathogenesis of autoimmune disorders [28]. This FcγRIIIA 158V/F polymorphism has been extensively studied in RA but revealed remarkably contradictory results. Some studies showed the 158V allele was associated with RA susceptibility [29-32], in another study the 158F allele was associated with RA [33], while in other studies no association with RA was observed [24, 28, 34-37]. Those, strikingly, contradicting results in the same ethnic group might be explained with the genetic complexity of the FcγR region combined with the heterogeneity of RA. Therefore, in **Chapter 5** we investigated whether FcγRIIIA 158V/F SNP associates differently with ACPA-positive and ACPA-negative RA and assessed if the FcγRIIIA gene CNV affects the association of the FcγRIIIA 158V/F SNP with RA and whether the FcγRIIIA gene CNV confers risk for RA. In our cohort we found that FcγRIIIA 158VV genotype only confers risk for ACPA-positive RA. After stratifying for the presence of CNV, this association became slightly more evident. Though, FcγRIIIA CNV by itself is not associated with RA risk. Our data suggest that ACPA status and the presence of CNV can affect the FcγRIIIA association with RA and must be taken into consideration when studying FcγRIIIA polymorphisms.

FcγRIIIB is most likely involved in the uptake of immune complexes; it has been found to express two isoforms, NA1 and NA2, which differ by four amino acids in the membrane-distal Ig-like domain. Differential glycosylation of these isoforms affects their interaction with IgGs. The NA2 isoform has been shown to have a reduced power in the stimulation of phagocytosis and this has functional consequences in development of blood transfusion reactions, autoimmune neutropenia and renal disease in systemic vasculitis [38]. **Chapter 6** focuses on investigating the CNV of FcγRIIIB gene and if it has an association with RA risk. CNV was studied with 3 different MLPA probes. Probes 1 and 2 showed CNV with copy numbers ranging from 0 to 5 but with different frequencies. Probe 3 showed almost no evidence of CNV. Quantitative-PCR was done for confirmation and its results correlated with those of probe 2. Thus, CNV was observed both in healthy controls as well RA patients but not significantly different between them. In healthy controls low copy number (1 copy) was

observed in 6.7% compared to 9.4% of individuals with high copy number (≥ 3 copies). Furthermore, sequencing of the FCGR3B promoter region revealed an insertion/deletion (indel) that explained the disparate CNV results of MLPA probe 1. This novel -256A.TG indel was found in 40.7% of healthy controls versus 35.9% of RA patients but not significantly different. Those data highlight the complexity and poor characterization of the FCGR3B gene sequence and indicate that design and interpretation of genotyping assays in this region must be performed with caution. The possible protective effect of the -256A.TG indel polymorphism must be addressed in larger studies.

Data from **Chapter 5 and 6** denote that analysis of the associations of CNV results and disease susceptibility or severity is rather complicated. First, because of its low frequency and second, because of the existing SNPs within those gene copies with subsequent functionality and expression effects which might result in obscuring the real effect of CNV.

Part II

Although autoantibody formation is characteristic for RA and can precede RA manifestations by several years [39], the range of antibodies formed in RA are not entirely specific for RA and can be detected in other forms of inflammatory arthritis as well. Additionally, the majority of studies exploring the test characteristics of RF, anti-CCP and anti-MCV compare RA patients to healthy controls while in clinical practice those tests are used to differentiate early RA from other forms of early inflammatory arthritis. In **chapter 7**, we evaluated the diagnostic performance of the anti-MCV test in differentiating of RA from other forms of inflammatory arthritis in a large inception cohort of patients with early arthritis. Additionally, the anti-MCV test characteristics were compared with those of the anti-CCP2 and anti-CCP3.1 tests. In our cohort, anti-MCV antibodies were present in 13.9-19.4% of non-RA arthritides and it showed lower specificity to differentiate RA from other arthritides (82.9%) compared to anti-CCP2 (93.4%) and anti-CCP3.1 (90.0%), although it had a higher sensitivity (62% vs 56.9% and 58.1%, respectively). Therefore, our data suggest that the diagnostic performance of anti-MCV test is lower than anti-CCP tests in the differential diagnosis of early arthritis.

Part III

Albeit the 1987 ACR classification criteria for RA had been widely used for many years to identify RA patients, they lack the sensitivity to identify patients with early RA and fail identify individuals with very early inflammatory arthritis who subsequently develop RA [40, 41]. Because the focus now is shifted towards early therapy a new tool to identify early RA was warranted. Recently, the ACR and EULAR developed the 2010 new classification criteria for RA aiming at identification of patients with very early stages of RA [42]. Before the new classification criteria were developed, we wanted to explore if the 1987 ACR criteria can be improved. Even though bone erosions were part of the 1987 ACR criteria [43] no clear description of the quantity and location of erosions was given. Additionally, the prognostic value of bone erosions in UA patients was unknown while in RA patients it is predictive of a severe destructive disease course. The focus of **Chapter 8** is to explore the predictive value of erosions for RA development in UA patients, define optimal erosions cut-off to predict RA, differential value of erosions in hands and feet and investigate whether information on erosions improves the discriminative ability of a recently developed prediction rule of RA-development [44, 45]. In **Chapter 8**, the presence of erosions was assessed using two methods. First method is erosive joint count. An erosive joint is defined as a joint with at least one erosion which in turn is defined as a lesion with an interrupted cortex. As such, this definition is the same as the erosion score in the Simplified Erosion Narrowing Score (SENS) and is in line with common clinical practice [46]. Second method is the erosion score of the Sharp/van der Heijde scoring (SHS) method in which the size and number of erosions per joint are weighted [47].

At baseline, 28.6% of UA patients had bone erosions. Presence of ≥ 2 erosive joints showed a positive predictive value of 53% for RA development and 68% for persistent disease. Additionally, the presence of feet erosions was equally predictive compared to erosions at hands. Incorporating the erosions data (SENS method) into the prediction rule of RA-development [44, 45] using all the different cut-off values, did not improve the predictive ability of the model. Similarly, using data on joint erosions in UA patients who cannot be accurately predicted to develop RA by the rule (scoring between 6.0 and 8.0) does not significantly add to the predictive ability of the known clinical and serological factors. Those

data indicate that the presence of erosions in UA patients is not always predictive for unfavourable disease outcomes.

In conclusion, over the past few years, understanding of the genetic basis of the susceptibility to RA has increased dramatically with new susceptibility genes now confirmed. Recently, Eyre *et al.* [5] confirmed the identified risk loci to account for 51% of the total genetic effect of which 36% explained by HLA in addition to 15% non-HLA genetic factors. The challenge remains now to identify the rest of those genetic effects and explore how these variants interact with each other as well as environmental factors to induce the development of RA. Translating the genetic information to help clinically defining subgroups of patients and aid clinical management is an interesting target, hopefully, in the near future. The ultimate goal of personalized treatment decision making incorporates genetic, serological as well as clinical factors.

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CHAPTER 10

Nederlandse samenvatting



RA heeft een complexe multifactoriële etiologie, waarvan veel elementen tot nu toe onbekend zijn. Wel zijn er duidelijke aanwijzingen dat genetische en omgevingsfactoren een belangrijke rol spelen in de ontwikkeling van de ziekte. Een belangrijk kenmerk van RA is de productie van autoantilichamen. De pathogenese van RA-patiënten met en zonder ACPA verschilt. Deze groepen hebben verschillende genetische en omgevingsfactoren. Het meest prominente voorbeeld hiervan zijn de HLA-DRB1 shared epitope allelen (SE). Zij predisponeren specifiek voor ACPA-positieve ziekten en zijn verantwoordelijk voor 36% van de totale genetische gevoeligheid. Fenotypisch wordt ACPA-positieve RA gekenmerkt door een ernstiger ziektebeloop.

Deel I

Sommige genetische risicofactoren associëren alleen met RA in bepaalde populaties. In **hoofdstuk 2, 3 en 4** van deze thesis, wordt onderzocht hoe en of bepaalde genetische varianten in de Nederlandse bevolking leiden tot RA. Bovendien werden deze gegevens geanalyseerd in het kader van de aanwezigheid van andere risicofactoren zoals bepaalde autoantilichamen, verschillende omgevingsfactoren en geslacht.

Het functionele polymorfisme in het PTPN22 gen dat predisponeert voor RA in de Kaukasische bevolking, is uiterst zeldzaam in Aziatische populaties. In **hoofdstuk 2** bevestigden wij de associatie tussen C1858T variant van PTPN22 met RA susceptibiliteit in Nederlanders. Deze associatie was duidelijk en alleen statistisch significant aanwezig in autoantistof positieve (reumatoïde factor (RF) positieve) patiënten in vergelijking met RF negatieve patiënten. We vonden geen aanwijzingen voor associaties met andere PTPN22 varianten.

Een FCRL3 promotor SNP (rs7528684) geeft een verhoogd risico op RA in Japan maar niet in Amerika en in Spanje. We onderzochten de FCRL3 promotor SNP associatie met RA in de Nederlandse populatie in **hoofdstuk 3**. Het bleek dat de recessief CC genotype dragers een groter risico hebben op het ontwikkelen van RA in vergelijking met TT + TC genotype dragers. Bovendien liet een analyse gestratificeerd voor autoantilichamen (ACPA) geen specifieke associatie met ACPA positieve of ACPA negatieve ziekte zien. Ook werd geen

associatie tussen FCRL3 genotypen met ernst van RA gevonden. Vervolgens liet een meta-analyse van data uit Japan, Amerika, Spanje en Nederland zien dat CC dragers een hogere kans hebben op RA. Derhalve suggereren onze bevindingen dat de associatie van FCRL3 met RA mogelijk niet specifiek is voor de Japanse bevolking, en dat FCRL3 mogelijk bij de oorzaak van RA ongeacht het ras betrokken is.

Ook het PADI4 polymorfisme dat predisponeert tot RA in meerdere Aziatische populaties lijkt geen risico voor RA in de west Europese bevolking. In **hoofdstuk 4**, hebben we geprobeerd deze verschillen te verhelderen. Mogelijk speelt roken hier een rol, en predisponeert het PADI4 polymorfisme met name in rokende mannen. Mogelijk kan de genetische heterogeniteit tussen Aziatische en Europese bevolking ten dele worden verklaard door verschillen in de prevalentie van roken in mannen.

Genetische studies zijn meestal kandidaat gen studies of genome wijde studies (GWAs). GWAs onderzoeken minstens 30.000 genen tegelijk terwijl kandidaatgen studies een klein aantal genen tegelijk beïnvloeden. Vooruitgang in technologie, en met name lagere kosten, hebben GWAS wereld wijd beschikbaar gemaakt. Desalniettemin zijn er ook nadelen zoals de kans op vals-positieve resultaten, gebrek aan informatie over genfunctie, ongevoeligheid voor zeldzame varianten en structurele varianten (dus een potentieel voor vals-negatieve resultaten. Structurele varianten kunnen bijvoorbeeld een ‘copy number variatie’ (CNV) betreffen.

Variatie in het exemplaraantal (CN) van genen in het menselijk genoom is een belangrijke bron van genetische variatie. Het is gedefinieerd als een opeenvolging van DNA aanwezig in in vergelijking met een referentie-genoom. In het diploïde menselijk genoom zijn van autosomale genen normaal gesproken twee exemplaren aanwezig (een op elk chromosoom). Echter, wanneer CNV aanwezig is, kan het aantal kopieën variëren van nul tot meer dan twee. Studies hebben aangetoond dat een groot aantal genen in het menselijk genoom relatief veel CNV hebben. CNV kan een belangrijke bron zijn van kwantitatieve variatie in expressie, en bijdragen aan fenotypische verscheidenheid.

FcγRs zijn te vinden op de meeste effector cellen van het immuunsysteem. Ze interacteren met het Fc-gedeelte van het IgG molecuul IgG. Na interactie kunnen de Fc-receptoren de cellen activeren. FcReceptor activatie kan een stimulerende of remmende ontstekingsremmende rol hebben. Het evenwicht tussen het stimulerende en remmende signaal is van cruciaal belang voor het behoud van immuun homeostase.

In **hoofdstuk 5 en 6** van dit proefschrift bestudeerden we FcγR gen polymorfismen. De technische moeilijkheden die voortvloeien uit de hoge homologie van FcγRs en de aanwezigheid van structurele variaties als CNV vereisten speciale technieken. Derhalve is als methode van genotypering zowel SNPs als MLPA gebruikt om te onderzoeken of de aanwezigheid of afwezigheid van CNV effect heeft op SNPs genotypering en de associatie met RA.

Een functionele polymorfisme in FcγR3A resulteert in een aminozuur vervanging op positie 158 van fenylalanine naar valine. Hierdoor verandert de affiniteit van IgG1, IgG3 en IgG4 binding aan de receptor. Deze varianten, die tot een verminderde klaring van de immuuncomplexen kunnen leiden, zijn in eerdere studies aangetoond als relevant voor de pathogenese van auto-immune aandoeningen. Dit FcγRIIIA 158V/F polymorfisme is uitgebreid bestudeerd in RA maar dit gaf tegenstrijdige resultaten. Sommige studies vonden dat het allel 158V werd geassocieerd met RA terwijl in een andere studie juist het tegenovergestelde resultaat gevonden werd. We hebben daarom in hoofdstuk 5 onderzocht of de FcγR158V/F IIIA SNP geassocieerd is met ACPA-positieve en negatieve ACPA RA. Ook hebben we geëvalueerd of FcγRIIIA gene CNV hier van invloed is. In onze cohort vonden we dat FcγRIIIA 158VV genotype alleen geassocieerd is met een verhoogde kans op ACPA-positieve RA. Deze associatie werd na de stratificatie voor de aanwezigheid van CNV, nog duidelijker. FcγRIIIA CNV zelf is echter niet geassocieerd met het risico op RA. Onze data tonen dat ACPA status en de aanwezigheid van CNV invloed kunnen hebben op de associatie van FcγRIIIA met RA.

FcγRIIIB is waarschijnlijk betrokken bij de opname van immuuncomplexen; twee isoforms komen tot expressie, NA1 en NA2, die onderling vier aminozuren in het membraan-distale Ig-

achtige domein verschillen. Differentiële glycosylatie van deze isoforms heeft invloed op hun interactie met IgGs. De NA2 isovorm is geassocieerd met verandering in efficiëntie van fagocytose en met het in een verschillende frequentie voorkomen van bloedtransfusie reacties, auto-immune neutropenie en nierbetrokkenheid in systeemvasculitiden. Hoofdstuk 6 richt zich op onderzoek naar het CNV van FcγRIIIB gen en een associatie met RA. CNV werd bestudeerd met 3 verschillende MLPA probes. 1 En 2 toonde een aantal CNV-kopies variërend van 0 tot en met 5, maar met verschillende frequenties. Probe 3 toonde geen CNV. Kwantitatieve PCR werd gebruikt om de bevindingen te bevestigingen, en de resultaten hiervan correleerden met de gegevens van probe 2. Het bleek dat CNV evenveel voorkomt in gezonden en RA patiënten. Bovendien liet sequencing van de regio FCGR3B een indel zien in de promotor regio, welke de uiteenlopende CNV resultaten van MLPA probe 1 kon verklaren. Deze nieuwe - 256A.TG indel werd gevonden in 40,7% van gezonde controles tegenover 35,9% van RA patiënten, wat geen significant verschil is. Of deze - 256A.TG indel polymorfisme beschermend werkt, kan in grotere studies bestudeerd worden. Deze gegevens wijzen op de complexiteit en de tot nu toe slechte karakterisering van het Fc-gamma Receptor gebied, en met name het FCGR3B gebied. Dit maakt dat men met de interpretatie van de tot nu toe gepubliceerde genotyperings resultaten in deze regio voorzichtig moet zijn.

Ook laten de gegevens van hoofdstuk 5 en 6 zien dat de associatie studies naar CNV ook ingewikkeld zijn. Issues hier zijn de lage frequentie van copy number variaties en ten tweede is het voorkomen van SNPs binnen deze gen kopieën een complicerende factor.

Deel II

Hoewel de aanwezigheid van auto-antistoffen karakteristiek is voor RA en deze zich al ontwikkelen voor er klinische manifestaties zijn, zijn ze niet volledig specifiek voor RA en kunnen ook voorkomen in andere vormen van inflammatoire artritiden. Veel studies die test karakteristieken analyseren worden RA patiënten met gezonde controles vergeleken. In de klinische praktijk echter worden de antistof testen gebruikt om mensen met RA te differentiëren van andere early arthritis patiënten. In hoofdstuk 7, hebben we de diagnostische eigenschappen van de anti-MCV test onderzocht om RA van andere vormen van inflammatoire artritiden te onderscheiden. Ook werd de anti-MCV test vergeleken met die van

de anti-CCP2 en anti-CCP3.1 proeven. In ons cohort, waren anti-MCV antilichamen aanwezig in 13,9-19,4% van de niet-RA arthritiden. De specificiteit was lager (82,9%) dan die van de anti-CCP2 (93,4%) en anti-CCP3.1 (90,0%) testen. De sensitiviteit was hoger (62% versus 56,9% en 58,1%, respectievelijk). Alles bij elkaar zijn de test karakteristieken in vroege artritis patiënten van de anti-MCV test lager dan van de anti-CCP testen.

Deel III

Hoewel de 1987 ACR classificatiecriteria voor RA op grote schaal gebruikt zijn voor het identificeren van RA patiënten, zijn ze niet geschikt om RA in een vroege fase te detecteren. Omdat de focus nu naar vroege therapie verschoven is, waren nieuwe criteria noodzakelijk. Onlangs, zijn daarom de ACR/EULAR de 2010 criteria voor RA ontwikkeld. Voordat de nieuwe indelingscriteria werden ontwikkeld, wilden we verkennen of de 1987 ACR criteria konden worden verbeterd. We richtten ons daarom op het item erosies. Hoewel bot erosies een van de zeven criteria van de ACR 1987 criteria vormden, was er geen duidelijke beschrijving van de hoeveelheid en de locatie van erosies die aanwezig moest zijn voor een positieve score. Bovendien was de voorspellende waarde van bot erosies bij UA patiënten onbekend, terwijl bij RA patiënten bekend was dat het een goede voorspeller is voor een ernstig destructief beloop van de ziekte. Het focus van hoofdstuk 8 was daarom het verkennen de voorspellende waarde van erosies voor het ontwikkelen van RA in UA patiënten. Dit werd gedaan met behulp van twee methoden. Eerst werd een erosieve laesie gedefinieerd als een gewricht met een onderbroken cortex. Als zodanig, is deze definitie gelijk aan de vereenvoudigd erosie vernauwing Score (SENS) en in overeenstemming met de gangbare klinische praktijk. Tweede methode is de score van de erosie van de Sharp/van der Heijde scoren (SHS) methode gebruikt waarin de grootte en aantal erosies per gewricht gewogen zijn.

Bij het eerste bezoek had 28,6% van de UA patiënten een erosie. Aanwezigheid van ≥ 2 erosieve gewrichten toonde een positieve voorspellende waarde van 53% voor RA ontwikkeling en 68% voor permanente ziekte. Bovendien, was de aanwezigheid van voet erosies even voorspellend als hand erosies. Ook werd de toegevoegde waarde van het beoordelen van erosies op röntgen foto's voor een klinische voorspelregel op RA te

ontwikkelen beoordeeld. Het bleek dat dit voorspel model niet beter functioneerde als radiologische informatie meegenomen werd. Opvallend was verder dat lang niet alle patiënten met UA die erosief waren een ongunstige ziekte uitkomst hadden. Inmiddels zijn deze data verder geëxtrapoleerd en is er een definitie gevormd voor erosiviteit in RA, wat nu een onderdeel is van de 2010 ACR/EULAR criteria. De definitie luidt de aanwezigheid van 3 of meer kleine hand of voet gewrichten met een cortex onderbreking.

Tot slot is in de afgelopen paar jaar, de kennis van de genetische factoren die een rol spelen bij het ontwikkelen van RA fors toegenomen. Eyre *et al.* liet onlangs zien dat 51% van de erfelijke factoren bekend zijn; hiervan wordt 36% van het totale genetische effect bepaald door HLA en 15% door niet-HLA genetische factoren. De uitdaging die nu overblijft, is om de rest van die genetische effecten te identificeren en te ontdekken hoe deze varianten met elkaar en de omgevingsfactoren interacteren tijdens de ontwikkeling van RA. Mogelijk helpt deze kennis ook om mensen met RA vroeger te kunnen identificeren en draagt het bij aan geïndividualiseerde behandeling van RA.

CURRICULUM VITAE

Mohamed Mohamed Thabet Okily was born in the 24th of May 1975 in Abnüb, Egypt. In 1992 he was awarded his high school diploma from Abnoub high school, Assiut, Egypt (A+ level). Between 1992 and 1999 the author studied medicine in the Faculty of Medicine, Assiut University, Egypt where he graduated after finishing his internship year (cum laude).

In March 2000 the author began his residency training in Internal Medicine in the department of Internal Medicine, Assiut University Hospital, Egypt and in 2004 he obtained his Master degree in Internal Medicine from Assiut University after defending his Master thesis “Anticardiolipin antibodies in acute rheumatic fever and chronic rheumatic heart disease” under supervision of professors N.Tawfik and M.Kamel. Following that, he was included in the Egyptian Specialist Register for Internal Medicine. From June 2004 he has been working as a Specialist and Associate Lecturer of Internal Medicine in Assiut University Hospital, Egypt. Since 2000 the author was deeply involved in clinical research, medical education and clinical tasks. The author showed special interest in Rheumatology.

Between 2006 and 2010 the author worked as a clinical research fellow in Leiden University Medical Center, the Netherlands. Currently the author is still affiliated with and working for the department of Internal Medicine & Rheumatology Unit, Assiut University Hospital, Egypt as an Associate Lecturer and Specialist of Internal Medicine.

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Master's Thesis (2004): "Anticardiolipin antibodies in acute rheumatic fever and chronic rheumatic heart disease" Supervisors Prof. Dr. N. Tawfik & Prof. Dr M. Kamel.

DANKWOORD

I would like to sincerely thank everyone who, in one way or another, contributed to the creation and completion of this thesis.

To start with, I would like to thank Professor Tom Huizinga, Professor Rene Toes, and Dr. Annette van der Helm for being my mentors. Tom, I admire your marvellous enthusiasm for work and collaboration which gave me the chance to be here in the LUMC to start my fellowship and I do appreciate your continuous support which led me to complete my thesis. Rene, it was always a pleasure to have you around during all the meetings and discussions. Your critical approach and valuable remarks were always a good way of brain storming and always improved our research. Annette, thanks for always being available to reply my queries and your meticulous yet very quick and remarkable feedback. I want to express my gratitude to my Egyptian professors who always have been supporting, teaching and guiding me especially Professor Nashwa Tawfik and Professor Mahmoud Ashry.

Moving to the Netherlands to start my fellowship in the Department of Rheumatology in LUMC was a great adventure. I have spent some great years and gained a lot of scientific and life experiences. I must admit that life was not easy at sometimes, but what made it fun to be here was the good company of my colleagues especially those of room C1-45 and C1-46. I will never forget our funny yet productive conversations and discussions, support for each other both in work as well as personal related matters, and the best of all our lunch outs. Hopefully I won't forget anybody, Michael, Onno, Robert, Sjoerd, Melek, Naomi, Jessica, Karen, Emalie, Rosanne, Linda, Diane, Rosaline, Angga, and Wing-Yee.

The lab group, thank you all, I have learned a lot from you all. Rute, thanks for your active and thorough collaboration and help. Joris, Andrea, Gerrie, Aleida, Nivine, Ellen, Hanan, and everyone else thank you very much for your help and very friendly attitude. Stefan, you were really helpful in the methodology and design of gene copy number articles even when you moved to Australia, thanks for that. Jeanine, thank you, your help with statistics were pivotal

to many of the manuscripts in this thesis. Not to forget the people of the staff-centrum, Hanny, Joyce, Hugline and José thank you all for helping with all the official and paper work.

I would like to express my sincere gratitude and thanks to my friends in Leiden and their families who were and still are like a family to me; Bakr Morsy, Attia Abdelbaki, Ali Marashdeh, Mahmoud Al Saify, Mohamed Ghaly, Umar Ryad, Mohamed Myqias, Ayman Khairy, Ibrahim Bayoumi, Khalid Younes, Taleb Alkurdi and Mohamed Alhissi.

I am also indebted to my entire family in Egypt for always being there for me even during the most difficult times especially my parents for their endless wisdom and continuous support.

Last but not least, my wife Randa, I cannot express how wonderful, supportive and caring you have been. Thanks for always being there for me and thanks for bearing with me through those times. None of this would have been possible without you. And kids, although you were distracting me, I have to admit I enjoyed and still enjoying it, you could always draw smiles on my face.

