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Genes and Environmental factors associated with the severity of progression of Rheumatoid Arthritis

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Part I



Chapter 1

Introduction

RHEUMATOID ARTHRITIS

The first description of the symptom-complex nowadays known as Rheumatoid Arthritis (RA) was made in 1800 by Augustin Jacob Landré-Beauvais.¹ In 1859, Alfred Garrod was the first to differentiate gout from other arthritic conditions and to use the term “Rheumatoid Arthritis”. This disease entity was further studied and refined by one of his sons, Archibald Garrod.^{2;3}

The question how to define Rheumatoid Arthritis (RA) has bothered physicians ever since. In 1958, the American Rheumatism Association proposed the first set of diagnostic criteria.⁴ In 1987, these criteria were replaced by a new set of criteria.⁵ In 2010 the European League Against Rheumatism and the American College of Rheumatology jointly developed a new set of criteria, specifically aiming at early identification of patients.⁶

It has often been speculated that in Europe, RA did not exist prior to traveling to the America's in the late 15th century, but that by the trading of goods (e.g. smoking tobacco) or travelling of persons, an RA-inducing trigger was transported to Europe, suggesting that RA may be the result of interplay between constitutional and environmental factors.⁷⁻⁹

Alternatively, RA may have existed in Europe for a much longer time, but until the 19th century most people may have lived too short to be able to develop RA.¹⁰

The natural course of RA is often debilitating, and untreated, the disease leads to impaired function, disability and premature death.¹¹ In the last quarter of the 20th century, enormous progress was made in RA research and RA treatments. Although nowadays, RA should still be considered an incurable disease, its symptoms can be suppressed in the majority of patients and in a considerable group of patients, sustained remission from the disease without the use of medications can be achieved.¹²

Nevertheless, RA still has a huge impact on the life of most patients, and some patients still suffer a severe burden of the disease. RA is estimated to affect 0.5-1% of the people, predominantly middle-aged women.¹² For society, RA comes with large costs, both because of the reduced ability of patients to engage in work or other activities and of the direct costs of healthcare.^{13;14}

SUBGROUPS WITHIN RA

Rheumatoid arthritis (RA) has for long been considered a relatively homogeneous clinical syndrome, this view has changed since a number of studies suggested that RA can

Table 1. Comparison of the 1958, 1987 and 2010 criteria

ARA 1958 criteria	ACR 1987 criteria	ACR/EULAR 2010 criteria
Morning stiffness	Morning stiffness (at least 1 h)	1. Joint involvement (0-5)
Swelling of a joint	Arthritis in three or more joints	- One medium to large joint (0)
Swelling of another joint	Arthritis of hand joints (≥ 1 swollen joints)	- Two to ten medium-to-large joints (1)
Pain on movement or tenderness in a joint	Symmetrical arthritis	- One to three small joints (large joints not counted) (2)
Symmetric Swelling	Rheumatoid nodules	- Four to ten small joints (large joints not counted) (3)
Rheumatoid nodule	Serum rheumatoid factor	- More than ten joints (at least one small joint) (5)
Rheumatoid factor	Radiographic changes (erosions)	2. Serology (0-3)
Radiographic changes		- Negative RF and negative ACPA (0)
Mucin clot		- Low positive RF or low positive ACPA (2)
Synovial biopsy		- High positive RA of high positive ACPA (3)
Nodule biopsy		3. Acute phase reactants (0-1)
		- Normal CRP and normal ESR (0)
		- Abnormal CRP or abnormal ESR (1)
		4. Duration of symptoms (0-1)
		- Less than 6 weeks (0)
		- 6 weeks or more (1)
Classical RA: 7/11	Four of the seven criteria must be present. Criteria 1-4 must have been present for at least 6 weeks	Points are shown in parentheses. Cutpoint for RA 6 points or more. Patients can also be classified as having rheumatoid arthritis if they have: (a) typical erosions, (b) long-standing disease previously satisfying the classification criteria
Definite RA: 5/11		
Probable RA: 3/11		

be divided into two syndromes: anti-citrullinated peptide antibody (ACPA)-positive and ACPA-negative RA.¹⁵⁻¹⁷ This subdivision was based on differences in genetic risk factors, histopathologic differences, and differences in outcome of ACPA-positive and ACPA-negative RA.¹⁸ In general, patients with ACPA-positive disease have higher rates of joint destruction and are less likely to achieve remission. Many studies focus on ACPA-positive patients. One of the reasons for this is the fear of phenotypic misclassification, as ACPA-negative RA is often considered to be a heterogeneous disease¹⁵⁻¹⁹.

UNDIFFERENTIATED ARTHRITIS VS RHEUMATOID ARTHRITIS

Despite its typical characteristics, recent evidence indicates that in fact RA is only the end stage of a continuum from the asymptomatic presence of certain antibodies to a symmetric polyarthritis.²⁰

In this respect, the disease state referred to as 'Undifferentiated Arthritis' (UA), is of special interest. This is the presence of one or more arthritic joints in a patient without fulfilling the diagnostic criteria for RA.

The main purpose of the 2010-ACR/EULAR criteria was to classify patients as having RA in an earlier phase of the disease, making more patients eligible for RA-treatment at an earlier stage.⁶ Although officially, the 2010-criteria were only to be used for inclusion of patients in clinical trials, in practice they are also used for diagnosing RA in other settings.

For day to day clinical care, any set of criteria will only have one major purpose: to identify on the one side those patients in which the complaints will remit spontaneously and on the other side those patients that are prone to have a debilitating disease course and should receive treatment timely.

Some years ago, an important achievement was made by the prediction rule of van der Helm-van Mil et al.²¹ In a large number of patients, this prediction rule can adequately predict the development of RA in UA patients.²¹⁻²³ Compared to the 1987-criteria, the 2010-criteria classify more patients as having RA at an earlier stage, with acceptable discriminative ability.^{24;25}

RESEARCH ON THE SEVERITY OF RA

The burden of RA is highly variable between patients. Although effective treatment strategies exist, these are not without side-effects. Currently, treatment is not individualized, and treatment is certainly not based on the expected severity of the disease in the individual patient.²⁶

Also, research into the factors responsible for differences in the severity of RA between patients, may show new pathophysiological pathways and new opportunities for treatment.

It is difficult to judge whether someone has 'severe' RA because what someone regards as 'severe' is not just dependent on pathophysiological abnormalities, but also (or perhaps even mainly) on subjective disease perception and social and cultural factors. Patients will mostly complain of pain, fatigue or the inability to perform daily activities and to work. On the other hand, rheumatologists primarily treat inability to perform daily activities but this inability is poorly correlated to the "total diseases activity. For research purposes, using a measure that is stable over time and can be assessed more objectively, such as joint destruction or mortality, is preferable.²⁷

Another possibility is to study the severity of RA by assessing the radiographic joint destruction. This has the advantage that the phenotype is not fluctuating (repair of joint damage has only been described in a small minority of cases). The amount of joint damage RA-patients suffer can be considered the net result of the bone constitution and the severity of inflammation. For measuring joint destruction, validated scoring methods have been developed.

The most commonly used scoring method is the Sharp-van der Heijde Score (SHS). This scoring method analyzes erosions and joint space narrowing in joints of hands and feet.²⁸

Another commonly used method is the Larsen-score.²⁹ This method essentially measures the same, but it said to be less sensitive in detecting changes compared to the SHS. Still, the SHS and Larsen scores are comparable, and the relative progressions of joint destruction can be combined in meta-analyses.³⁰

Ideally, the severity of RA is assessed by serial X-rays over time, because the amount of joint damage on serial radiographs of the same patients is highly correlated. Scoring radiographs at subsequent time points substantially diminishes within patient variability in joint damage compared to scoring radiographs at one time-point only. Repeated radiological measurements therefore increase the power.^{27 31}

Sophisticated statistical models have been developed, allowing to take full advantage of repeated measurements.³²

GENETIC FACTORS AND THE SEVERITY OF RA

In the last years, several genetic variants that associate with a higher risk on developing RA have been described.³³⁻³⁶ One can study these genetic variants, referred to as so-called Single Nucleotide Polymorphisms (SNPs) in two ways. One way is by means of a candidate gene approach, whereby based on *a priori* knowledge of disturbances in function or homology to other diseases, the corresponding genetic regions are selectively typed for analysis. Another method is the Genome Wide Association Study (GWAS), which is an unbiased approach to the whole genome.

Of course, one can also take intermediate positions, for example by densely typing several very large pre-selected regions on the genome. The Immunochip is an example of this; recently, 186 risk loci for several auto-immune disease were densely typed and this showed 14 new risk loci for RA development.³⁶

By combining genealogic, genetic and radiographic severity data of RA-patients from Iceland, the heritability of the progression of joint destruction in RA-patients was estimated to be 45-58%, dependent on the method (assessment of genetic or genealogic kinship) used.³⁷

This finding underlines the importance of studying the genetic factors responsible for the severity of RA.

ENVIRONMENTAL RISK FACTORS FOR THE SEVERITY OF RA

Several environmental factors have been thought to inflict on the risk of RA-susceptibility. Some of these factors are thought to have interplay with genetic factors.

Smoking and the risk of RA

A large body of evidence on the effect of smoking on RA has been published. Smoking has been implicated as one of the most important environmental risk factors for the onset of Rheumatoid Arthritis (RA), especially for the ACPA-positive subgroup³⁸⁻⁴². It has been hypothesized that smoking is involved in the breaking of tolerance to citrullinated proteins⁴⁰. Whether smoking influences the severity of RA as well, is less clear. Smoking has been reported to influence radiographic joint destruction in a Swedish study,⁴³ but this relationship has not been established in other cohorts.⁴⁴⁻⁴⁶ Intriguingly, in a North-American cohort, smoking was shown to protect against joint replacement surgery,⁴⁷ and a Swiss study showed a trend towards less progression of radiographic joint destruction in heavy smokers.⁴⁶

Vitamin D and the risk of RA

Vitamin D is thought to influence the immune system by inhibiting dendritic cell T-cell activation.⁴⁸ Levels of vitamin D differ throughout the year, and these differences might explain observed seasonal differences in the onset of RA.⁴⁹⁻⁵² A recent study showed some protective effect of sunlight exposure on the risk of RA development.⁵³ Although several indications of an effect of low vitamin D levels on susceptibility to RA have been found, a definite conclusion on the role of vitamin D in RA cannot be drawn yet.⁵⁴⁻⁵⁶ A recent study found an effect of the season of first arthritis symptoms (which could be a proxy for different vitamin D levels) on the amount of joint damage after one year.⁵⁷

Other environmental factors

Many other factors have been thought to influence the severity of and the risk on developing RA, such as oral contraceptive use, air pollution and even dietary factors.⁵⁸⁻⁶¹ Although for some factors a definite influence on the risk of RA development has been found, research on environmental factors in relation to the severity of RA has not been very fruitful thus far. A major reason for this is that determining the exposure to environmental factors is much more complicated than for example genotyping patients. Also, exposure to environmental agents is likely to differ over time, whereas e.g. a genotype remains stable. As mentioned before, research into the (radiographic) severity of RA requires large cohorts with preferably repeated radiographs available. The availability of cohorts meeting these requirements is limited, and therefore, our knowledge of the environmental factors responsible for differences in RA severity, remains scarce.

Part I of this thesis is focused on describing the Leiden Early Arthritis Clinic, and predicting the progression from UA to RA and predicting the severity of RA by clinical information available early in the disease stage.

In **chapter 2**, an extensive description of the Leiden Early Arthritis Clinic (EAC) is provided. This chapter also describes the baseline variables related to the progression from UA to RA and to the radiographic severity of RA.

Although an excellent model for predicting which UA patients will go on to develop RA exists, some patients cannot be adequately predicted yet.²¹ New diagnostic tools may be helpful in predicting RA development in those patients difficult to classify. Already 50 years ago, assessments of Bone Mineral Density loss on standard hand X-rays have been described, and for some years a computerized tool is available for this.⁶²⁻⁶⁴

Bone Mineral Density loss measured by this method has been shown to associate with disease activity and the radiographic progression of RA in the Best-study.⁶⁵⁻⁶⁷ In **chapter 3**, we describe the use of hand Bone Mineral Density loss for the prediction RA-development in UA-patients.

According to the 2010-criteria, erosive disease typical of RA is sufficient to establish the diagnosis of RA. Recently, an EULAR-taskforce proposed a definition of erosive disease: erosive disease for use in the 2010-criteria is defined when an erosion (defined as a cortical break) is seen in at least three separate joints at any of the following sites: the proximal interphalangeal, the metacarpophalangeal, the wrist (counted as one joint) and the metatarsophalangeal joints on radiographs of both hands and feet.⁶⁸ In **chapter 4**, we examine whether a specific cut-off for the number of erosive joints at baseline, is predictive for rapid progression of radiographic joint damage.

For research into the factors responsible for the severity of RA, the more radiographic data are available, the higher the power. It is important to make optimal use of available radiographic data, and in **chapter 5**, we show that correlations in SHS scores between left and right hands and feet and correlations between hands and feet are rather weak, and that radiographing both hands and feet is preferable.

In **part II** of this thesis, several studies to genetic factors underlying the differences in the severity of joint destruction, are presented. In **chapter 6**, a candidate gene study to the genes encoding several proteins regulating the Wnt-signalling pathway is presented. We found that rs1896368 on *Dkk-1* is associated with higher rates of radiographic joint destruction and also with higher levels of Dkk-1, which is an inhibitor of the Wnt-signalling pathway.

Some years ago, SNPs on *TRAF1-C5* and *TNFAIP3-OLIG3* were reported to associate with the progression of joint destruction in RA.^{69,70} In **chapter 7**, we tried to replicate these findings in more cohorts and with more radiographic data available. This time, no significant findings were obtained.

Chapter 8 describes another candidate-gene study. Here, we tested SNPs encoding proteins regulating the OPG/RANK/RANKL/TRAF6 pathway in relation to the progression of joint destruction. In a meta-analysis of several cohorts, we found that RA-patients with the minor-allele of *OPG*-rs1485305 expressed higher rates of joint destruction compared to patients without the risk variants ($p=2.35 \times 10^{-4}$).

In **chapter 9**, we report the results of a multi-cohort study analyzing joint destruction in a total of 4,732 X-rays of 1,750 RA-patients with high quality phenotypic data that were collected longitudinally. RA-patients carrying the minor alleles of several SNPs in *IL2RA* have less severe progression of joint destruction. This genetic association is further substantiated by fine-mapping data as well as functional data. Patients carrying the minor alleles also have lower levels of soluble IL-2RA and hence may have less activation of CD4+ T cells, preventing severe inflammation and reducing long term joint destruction.

In **chapter 10**, we took a different approach. Several auto-immune diseases share genetic risk variants. For instance, some genetic variants that are associated with an increased risk on RA are also associated with susceptibility to other immune diseases, such as type-1 diabetes, celiac disease and systemic lupus erythematosus.⁷¹⁻⁷³ Such observations have led to the development of the Immunochip consortium and the Immunochip genotyping platform that includes dense mapping of markers across 186 loci associated with 12 auto-immune diseases (auto-immune thyroid disease, ankylosing spondylitis, Crohn's disease, celiac disease, IgA deficiency, multiple sclerosis, primary biliary cirrhosis, psoriasis, rheumatoid arthritis, systemic lupus erythematosus, type 1 diabetes and ulcerative colitis).⁷⁴ This platform allows fine-mapping of immune-mediating loci and replication of previously identified variants.^{36,75} Indeed, with regards to RA susceptibility, a recent study refined the association signals for 19 loci using the Immunochip.³⁶

Susceptibility factors may also influence disease progression; examples within RA are the HLA-DRB1 shared epitope alleles and the *IL2RA* locus.⁷⁶ This study was performed under the assumption that not only several diseases share key molecular processes, but that RA-development and RA-progression do so as well, which is reflected by shared genetic risk variants. We therefore took advantage of the Immunochip platform and studied progression of joint destruction in RA.

Part III of this thesis is devoted to ACPA-negative RA. Evidence is emerging that we should consider this a distinct pathophysiological entity from ACPA-positive RA. Several successful genome wide studies for genetic risk factors for ACPA-positive RA have been performed. Studies on genetic risk factors for ACPA-negative RA are thus far lacking. One of the reasons for this is the fear of phenotypical misclassification as ACPA-negative RA is often considered to be a heterogeneous disease.^{77,78} For future risk factor, translational and outcome studies on the subgroup of ACPA-negative RA patients, it is essential to provide

epidemiological and clinical evidence on whether or not ACPA-negative RA can be considered as one entity. In **chapter 11** we therefore aimed to determine whether the group of ACPA-negative RA patients can be separated in clinically distinguishable subphenotypes. Briefly, we could not identify such subgroups, and we concluded that, for risk factor studies, ACPA-negative RA can be studied as one group. Subsequently, in **chapter 12** we report the findings of a GWAS to the radiographic severity of ACPA-negative RA.

In **part IV** of this thesis, we describe two studies to non-genetic factors that could influence the severity of RA. In **chapter 13**, we analyzed whether the season of symptom onset influences the radiographic severity of RA in two cohorts. We did not find any clear seasonal differences. In **chapter 14**, we describe the effect of smoking on the severity of RA in six cohorts. We concluded that although smoking influences the long-term outcome of RA, the effect of smoking is not independent of ACPA-status.

Finally, in **chapter 15**, the findings reported in this thesis are summarized and discussed.

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Chapter 2

Predicting arthritis outcomes- what can be learned from the Leiden Early Arthritis Clinic?

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ABSTRACT

Introduction

In order to allow personalized medicine, adequate prediction of the disease outcome is required. In early undifferentiated arthritis (UA) prediction of the development of rheumatoid arthritis (RA) is crucial and in case of RA predicting the severity of the disease course may guide individualized treatment decisions.

Methods

570 UA patients and 676 RA-patients included in the Leiden Early Arthritis Clinic cohort were studied for baseline characteristics. The disease outcomes studied were fulfillment of the 1987 ACR-RA criteria and arthritis persistency in UA-patients and the rate of radiological joint destruction and achieving sustained DMARD-free remission in RA-patients.

Results

Predictive factors for fulfillment of the 1987 ACR-RA criteria and for persistent arthritis in UA were largely similar. Risk factors for a severe rate of joint destruction were: (P-value) older age (<0.001); male gender (<0.001); longer symptom duration at first visit (0.048), involvement of lower extremities (<0.001); BMI (<0.001); high acute phase reactants, presence of IgM-RF (<0.001); anti-CCP2 -antibodies (<0.001); anti-MCV-antibodies (<0.001) and HLA-SE-alleles (0.001). A high BMI was associated with a lower rate of joint destruction but with a higher risk on disease persistency. The proportion of variance in joint destruction explained was 32%

Conclusion

Predictors for RA-development, previously used to develop a prediction rule in UA-patients, are largely similar to predictors for arthritis persistency. Only part of the level of joint destruction in RA is explained by currently known risk factors. New factors need to be identified in order to guide pharmaceutical intervention at the level of individual RA patients.

INTRODUCTION

The outcome of early arthritis patients is highly variable. Approximately only one-third of the patients with a recent-onset Undifferentiated Arthritis (UA) progresses towards Rheumatoid Arthritis (RA). The severity of the progression of joint destruction in RA is highly variable as well, as only a minority will become severely destructed. In order to achieve individualized treatment decision making, the severity of the disease outcome needs to be estimated adequately. This is particularly relevant since it is widely acknowledged that early initiation of treatment of RA is effective in diminishing the level of joint destruction and disability¹⁻³. Fewer studies are performed on the effects of early intervention in recent-onset UA, but available data suggest that early treatment strategies hamper progression in UA as well⁴⁻⁶. Potent treatment strategies such as targeted therapies are generally not started in an early phase because of the risk of overtreatment. However, when the individuals who will have an unfavorable disease outcome can be identified at first presentation, the risk on overtreatment and undertreatment can be balanced, resulting in a personalized pharmaceutical regimen.

Observational studies of unselected patients are most appropriate to identify risk factors for a certain disease course. Following patients with and without risk factors allows direct assessments of absolute risks on a disease outcome. The Leiden Early Arthritis Clinic cohort is a population based inception cohort including early arthritis patients since 1993. Patients are being followed as long as they are seen at the rheumatologist and follow-up ends in case patients are discharged because of having a sustained DMARD-free remission or when patients die. During the past years several risk factors for a mild or progressive disease course, both in UA and RA, have been identified.

The present manuscript in this themed issue on Registries in Rheumatologic conditions reviews to what extent the disease outcome in early UA and early RA can be predicted, using data from the Leiden Early Arthritis Clinic cohort. The two disease outcomes studied in UA are fulfilling the 1987 ACR criteria for RA and having persistent arthritis. The disease outcomes studied in early RA-patients are the progression in joint destruction over time and disease persistency. These evaluations allow comparison of risk factors for joint destruction and RA persistency. Since it is thus far unclear to what extent the processes underlying joint destruction are similar to the processes that mediate disease persistency, evaluation of overlapping and dissimilar risk factors may increase understanding and the subsequent elucidation of the underlying biological pathways leading to these phenotypic characteristics. Finally, the fraction of explained variance of progression in joint destruction by the currently known risk factors is determined to assess how complete our current understanding is.

METHODS

Design of Leiden Early Arthritis Clinic

This Leiden EAC is a population-based prospective cohort that was started in 1993 in order to detect and treat inflammatory disorders early in the disease state, especially early RA. In order to obtain early referrals by general practitioners (GPs), a campaign was started among GPs to refer patients with suspected arthritis as soon as possible to the rheumatology department of the Leiden University Medical Center. This is the only center for rheumatic diseases in a semi-rural area with more than 400,000 inhabitants. Patients are seen within 2 weeks. 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 the first visit, the rheumatologist completed a questionnaire regarding the presenting symptoms, as reported by the patient: type, localization and distribution of initial joint symptoms, symptom duration, and course of the initial symptoms. The patient's smoking history and family history were assessed. Patients rated morning stiffness on a visual analog scale (VAS; range 0-100 mm); the duration of morning stiffness was also assessed. The Health Assessment Questionnaire (HAQ) was used to provide an index of disability. A 66-joint count for swollen joints (SJC) was performed. Blood samples were taken for routine diagnostic laboratory screening (including C-reactive protein (CRP), Erythrocyte sedimentation rate (ESR) and immunoglobulin (Ig)M-rheumatoid factor (RF)) and stored to determine other serum markers (amongst others antibodies against citrullinated peptide antibodies) at a later time. Blood samples were taken for DNA extraction as well. Follow-up visits with standard clinical assessments (including a SJC and a HAQ) were performed 3 months after the first presentation and yearly thereafter. Radiographs of the hands and feet were taken at baseline and yearly thereafter. Two weeks after inclusion, when results of laboratory investigations and radiography were known, patients that had a form of arthritis that could not be classified according to American College of Rheumatology (ACR; formerly, the American Rheumatism Association) criteria were documented as having UA. The diagnosis RA was established in case patients fulfilled the 1987 ACR criteria for RA. The initial treatment of RA-patients had changed in time and differed according to the inclusion period.⁷ Patients included between 1993 and 1995 were initially treated with analgesics and were subsequently treated with hydroxychloroquine or sulfasalazine if they had persistent active disease. Between 1996 and 1998, patients who were included were promptly treated with chloroquine or sulfasalazine, while after 1998, the initial treatment strategy consisted of either methotrexate or sulfasalazine.⁷ Treatment of UA-patients was not protocolized.

Definition of outcome measures

Patients with UA were assessed on two outcomes. First, after one year of follow up, the fulfillment of the 1987 ACR criteria for RA was evaluated. As previously described, 31% of UA patients progressed to RA during 1 year of follow-up. The majority of the patients (94%) had been followed up for more than 1 year (mean follow-up 8 years, SD 3 years) and 4.4% of UA patients developed RA later than one year after inclusion⁸. The second disease outcome was disease persistency. As a generally accepted definition for persistency is lacking, we defined persistent arthritis as the absence of sustained DMARD-free remission. 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. When remission was not obtained after 5 years of disease, a patient was classified as having persistent disease in the present study.

The RA-patients were studied for the rate of radiological joint destruction and for achieving sustained DMARD-free remission or having persistent RA, also during a five years period of follow-up. In order to study the progression rate in a sensitive way all serial radiographs were scored by one experienced reader (MvdL) according to the Sharp-van der Heijde method (SHS) in chronological order. Four hundred and nine radiographs belonging to 60 randomly selected RA-patients were rescored. The intraclass-observer correlation coefficient was 0.91 for all scored radiographs, and 0.97 for the radiographic progression rate. The means (+SEM) at the subsequent time points were 9.15 (0.43) at baseline; 15.65 (0.72) at one year follow up; 20.0 (0.93) at two years; 24.79 (1.36) at three years; 34.83 (2.14) at four years and 34.8 (2.14) at five years of follow-up. Persistent RA was defined as the absence of a sustained DMARD-free remission. A sustained DMARD free remission in RA was defined as the absence of swollen joints for at least one year after cessation of DMARDs and classification as DMARD-free remission by the rheumatologist. To ensure that remission was not temporary but rather sustained and long-lasting, the absence of swollen joints had to have been observed by a rheumatologist for at least 1 year after discontinuation of DMARD therapy. Corticosteroids were here considered to be equivalent to DMARDs. The majority of patients with disease in remission were discharged from the outpatient clinic at any time, however most patients who achieved remission were followed up longer than the minimum requirement of 1 year; the median time of observation after discontinuation of DMARDs in the absence of swollen joints was 2.5 years. Patients who had a recurrence of their arthritis after discharge could easily return to the Leiden University Medical Center. The frequency of disease relapse was 6%; these patients were included in the persistency group. We observed previously that sustained DMARD-free remission was obtained by 15% of RA patients after a median disease duration of 43 months⁹. Therefore, for the present study, patients that within the first 5 years did not achieve a sustained DMARD-free remission were classified as having persistent RA.

Statistical analysis

Predictors for RA-development and arthritis persistency were analyzed univariately with a logistic regression analysis. Since the aim of the present study was to review predictive factors and not to develop a prediction rule for the outcome of UA, which has been done before¹⁰, no multivariate regression analysis was performed in UA-patients.

Associations between baseline factors and rate of joint destruction were analyzed with a linear multivariate regression model see ref. 7 for detailed description.⁷ This was done for each variable separately, but all analyses were adjusted for the applied treatment strategy. In a previous study we showed that the inclusion period is an adequate proxy for the different treatments strategies that were applied over time⁷. The baseline characteristics were tested with an interaction term of a linear function of time. The risk estimate (β) resulting from these analyses reflected the relative difference in slopes between the groups over five years of follow-up. To test for a difference that is not progressive but stable over time, a model without interaction term was fitted; the overall effect of the risk factor then reflected a constant effect in time. This model does not exclude patients in case of missing radiographs and can deal with missingness provided that it is missingness at random⁷. Patients with complete datasets are weighted more heavily in the analysis than patients with missing radiographs.

All factors that were associated with the progression of joint destruction were entered in a multivariate analysis to determine the variance of joint destruction explained by these factors. This variance was defined by comparing the residual variance of the analysis including all risk factors with the residual variance of the analysis including only the adjustment factor for treatment strategy (inclusion period). The proportional reduction of the residual variance was the explained variance of the risk factors analyzed.

P-values <0.05 were considered significant. Since the aim was to review baseline characteristics in relation to the disease outcome, p values were presented without corrections for multiple testing. Analyses were performed using SPSS version 17.0 (SPSS Inc., Chicago, IL).

RESULTS

Predictors for disease outcome in UA

177 UA-patients developed RA (31%). An overview of baseline characteristics associating with RA-development and persistency of arthritis is presented in Table 1. Part of the variables predictive for the development of RA was described previously¹⁰⁻¹². Identified variables associating with the development of RA were patients' characteristics (age, gen-

Table 1. Baseline characteristics of patients with Undifferentiated Arthritis in relation to the outcome measures RA development and Persistency of Arthritis

Baseline characteristic	RA-development			Arthritis Persistency	
	Frequency	OR (95%CI)	P	OR (95%CI)	P
Age, years. mean (SD)	60.0 (16.8)	1.03 (1.01-1.04)	<0.001	1.00 (0.99-1.01)	0.90
Female gender. n (%)	329 (57.7)	2.00 (1.35-2.86)	<0.001	1.47 (1.03-2.08)	0.034
Pos family history for RA. n (%)	135 (23.7)	1.65 (1.11-2.45)	0.013	1.32 (0.87-1.98)	0.20
Chronic symptom (n (%) vs. (sub)acute	244 (42.8)	1.54 (1.11-2.23)	0.010	1.19 (0.84-1.69)	0.34
Symptom duration at fist visit. weeks. mean. (SD)	23.3 (23.6)	1.012 (1.004-1.019)	0.002	1.011(1.002-1.019)	0.012
Morning stiffness Severity- VAS (0-100). mean (SD)	41.3 (31.1)	1.02 (1.01-1.03)	<0.001	1.00 (1.00-1.01)	0.19
BMI. mean (SD)	26.0 (12.0)	1.03 (0.99-1.08)	0.18	1.07 (1.01-1.13)	0.013
Localization initial joint symptoms					
Small (n (%)) vs. large joints	266 (57.5)	2.48 (1.63-3.79)	<0.001	0.95 (0.65-1.40)	0.80
Large & small (n (%)) vs. large joints	107 (35.2)	4.18 (2.50-6.97)	<0.001	1.25 (0.76-2.06)	0.38
Upper (n (%)) vs. lower extremities	248 (43.5)	2.21 (1.36-3.57)	0.001	1.02 (0.68-1.53)	0.92
Upper & lower (n (%)) vs. lower extr.	161 (50.0)	6.07 (3.63-10.10)	<0.001	2.13 (1.31-3.46)	0.002
Symmetric (n (%)) vs. asymmetric	265 (46.5)	2.82 (1.98-4.03)	<0.001	1.20 (0.85-1.71)	0.29
Past or present smoker. n(%). vs. nonsmoker	271 (48)	1.0 (0.9-1.4)	0.98	0.7 (0.5-1.1)	0.10
SJC. mean (SD)	3.8 (4.0)	1.17 (1.11-1.23)	<0.001	1.07 (1.02-1.13)	0.01
CRP (mg/L). mean (SD)	21.4	1.01 (1.00-1.02)	0.001	1.01 (1.00-1.01)	0.03
ESR (mm/1hr). mean (SD)	29.5 (24.8)	1.02 (1.01-1.02)	<0.001	1.01 (1.00-1.02)	0.003
IgM-RF-positive. n (%)	140 (24.6)	5.10 (3.39-7.66)	<0.001	3.55 (2.18-5.76)	<0.001
Anti-CCP2-positive. n (%)	121 (21.2)	8.74 (5.51-13.84)	<0.001	5.97 (3.30-10.78)	<0.001
Anti-MCV (%)	172 (33.9)	6.48 (4.32-9.71)	<0.001	4.53 (2.87-7.17)	<0.0001
HLA-SE positive. n (%)	309 (55.9)	1.96 (1.36-2.81)	<0.001	1.76 (1.23-2.51)	0.002

Age, BMI, ESR, CRP, SJC, symptom duration at first visit and morning stiffness were analyzed as continuous variables; this means that the presented OR indicates the odds per unit. For instance, an OR of 1.03 for age in relation to the risk on RA development means that per year increase in age, the OR is 1.03. Morning stiffness is displayed in millimeters.

From all 570 patients data on RA-development was present, the remission/persistency state could be reliably determined in 538 patients and was not clear recorded in the medical file in 43 cases.

CRP C-reactive protein, ESR Erythrocyte sedimentation rate, BMI Body Mass Index, SJC swollen joint count, HLA-SE HLA-DRB1 shared epitope alleles, RF rheumatoid factor, anti-CCP2 anti-cyclic citrullinated peptide antibody, anti-MCV anti-modified citrullinated vimentin antibodies.

der, having a positive family history of RA), morning stiffness, inflammatory characteristics (CRP, ESR, number of swollen joints), localization of involved joints, and presence of auto-antibodies (RF, anti-CCP2, and anti-MCV). The environmental factors smoking and BMI were not associated with progression from UA to RA. The acuteness of the start of the complaints was associated with RA-development; UA-patients with a gradual onset of symptoms had a 1.5 higher odds ratio to develop RA than patients with a subacute symptom onset. A longer duration of symptoms at first presentation was associated with a higher risk on the development of RA as well.

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 these baseline characteristics in relation to arthritis persistency, defined as the absence of sustained remission. During the five year period of follow up, 210 UA patients achieved remission (39%). The median disease duration till remission was achieved was 17 months (IQR 6.3-37). Factors significantly associated with disease persistency were inflammatory markers (the number of swollen joints, CRP and ESR) and presence of auto-antibodies. Other characteristics such as the distribution of involved joints, the acuteness of the onset of the complaints, and morning stiffness were not predictive for having a persistent form of arthritis.

Predictors for outcome of RA

Baseline characteristics of RA-patients associated with the severity of joint destruction over time are presented in Table 2. The strongest association with the rate of joint destruction was seen for presence of (anti-Cyclic Citrullinated Peptide 2 (anti-CCP2). Anti-CCP positive RA-patients had over the 5 year period a 2.4 times higher progression rate than anti-CCP negative patients. A similar effect was seen for presence of IgM-RF. Higher levels of acute phase reactants at first presentation were also associated with more severe joint damage over time. RA-patients whom initial joint symptoms were located at the lower extremities had a higher rate of joint destruction. Interestingly, the severity of morning stiffness at first presentation was not associated with the severity of joint destruction over time. The body mass index (BMI) was inversely correlated with the progression of joint destruction over time. Few genetic factors are convincingly reported to associate with progression of joint destruction. Here we studied the HLA-Shared Epitope (SE) alleles and *CD40*, both are identified risk factors for anti-CCP-positive RA only^{11;13}. Although presence of the HLA-SE alleles associated with the progression of joint destruction in RA, *CD40* did not reveal such an association in a cohort consisting of both anti-CCP-positive and anti-CCP-negative RA-patients. All the analyses on the rate of joint destruction were adjusted for the treatment strategy applied; this variable was significantly associated with the rate of joint destruction in all performed analyses.

Table 2. Baseline characteristics of patients with Rheumatoid Arthritis in relation to the outcome measures rate of joint destruction and RA Persistency.

Baseline characteristic	Frequency	Rate of joint destruction over 5 years of follow-up		RA Persistency	
		β (95%CI)	P	OR (95%CI)	P
Age, years, mean (SD)	56.4 (15.7)	1.14 (1.11-1.16)	<0.001*	0.99 (0.97-1.00)	0.070
Female gender, n (%)	459 (67.9)	0.74 (0.63-0.86)	<0.001*	0.85 (0.50-1.45)	0.553
Pos family history for RA, n (%)	173 (26.5)	1.079 (0.92-1.27)	0.354	2.27 (1.18-4.36)	0.014
Chronic symptom (n (%) vs. (sub)acute	287 (44.6)	1.10 (0.94-1.27)	0.234	1.55 (0.93-2.59)	0.095
Symptom duration at fist visit, weeks, mean, (SD)	26.4 (22.4)	1.00 (1.00-1.00)	0.048	1.02 (1.01-1.03)	0.007
Morning stiffness					
Severity- VAS (0-100), mean (SD)	55.2 (28.7)	1.00 (1.00-1.00)	0.874	1.00 (0.99-1.01)	0.827
BMI, mean (SD)	25.8 (3.8)	0.96 (0.94-0.98)	<0.001	1.11 (1.01-1.23)	0.034
Localization initial joint symptoms					
Small (n (%)) vs. large joints	356 (75.7)	1.01 (0.83-1.24)	0.923	0.66 (0.34-1.28)	0.216
Large & small (n (%)) vs. large joints	177 (60.8)	0.92 (0.74-1.15)	0.470	0.96 (0.45-2.06)	0.911
Upper (n (%)) vs. lower extremities	268 (39.2)	0.62 (0.50-0.76)	<0.001	0.76 (0.35-1.62)	0.468
Upper & lower (n (%)) vs. lower extr.	222 (44.6)	0.72 (0.57-0.92)	0.009	1.01 (0.46-2.26)	0.972
Symmetric (n (%)) vs. asymmetric	415 (69.6)	0.93 (0.79-1.10)	0.396	0.89 (0.51-1.55)	0.687
SJC, mean (SD)	9.5 (7.4)	0.99 (0.98-1.00)	0.010	0.99 (0.96-1.02)	0.379
CRP (mg/L), mean (SD)	30.4 (34.7)	1.01 (1.00-1.01)	<0.001*	1.005 (1.997-1.013)	0.210
ESR (mm/1hr), mean (SD)	39.7 (27.4)	1.01 (1.01-1.01)	<0.001*	1.005 (0.995-1.015)	0.314
IgM-RF-positive, n (%)	378 (58.0)	1.76 (1.50-2.02)	<0.001	6.66 (3.69-12.02)	<0.001
Anti-CCP2-positive, n (%)	217 (32.1)	2.31 (2.00-2.67)	<0.001	11.46 (5.85-22.46)	<0.001
Anti-MCV-positive, n(%)	373 (54.6)	1.97 (1.68-2.30)	<0.001	6.13 (3.48-10.79)	<0.001
HLA-SE positive, n (%)	393 (63.8)	1.31 (1.12-1.52)	0.001	2.25 (1.35-3.74)	0.002
CD40 (rs4810485) non-G carrier, n (%)	22 (4.4)	1.02 (0.67-1.58)	0.915	0.78 (0.17-3.54)	0.751

* Outcome of analysis without interaction with time, evaluating whether a factor has an effect on the progression rate that is stable over time.

Age, BMI, ESR, SJC, CRP, symptom duration at first visit and morning stiffness were analyzed as continuous variables; this means that the presented OR indicates the odds per unit. For instance, a beta of 1.01 for CRP indicates a 1.01 times higher progression of SHS-score per mg/L CRP. From all 676 patients data on the rate of joint destruction was available, the remission/persistency state was reliably determined in 491 patients.

Since it is unclear whether the processes driving joint destruction are the same that drive RA persistency, predictive factors for both outcomes of RA were compared. The proportion of patients that achieved a sustained DMARD-free remission was 0.157, thus 84.3% of the patients were classified as having persistent RA. The median disease duration till remission was 40 months (IQR 25.5-66.5). The factors that were clearly associated with RA persistency were presence of auto-antibodies, the HLA-SE alleles and the duration of symptoms at the first visit. A high BMI was associated with a higher chance on RA persistency. Although the characteristics indicative for the level of inflammation (CRP, ESR, and SJC) were associated with severity of joint destruction, they were not predictive for having a persistent form of RA.

Fraction of variance of progression in joint destruction explained

The total variance of joint destruction at 5 years explained by the baseline characteristics studied was 32%. Subsequently we aimed to study the contribution of the individual risk

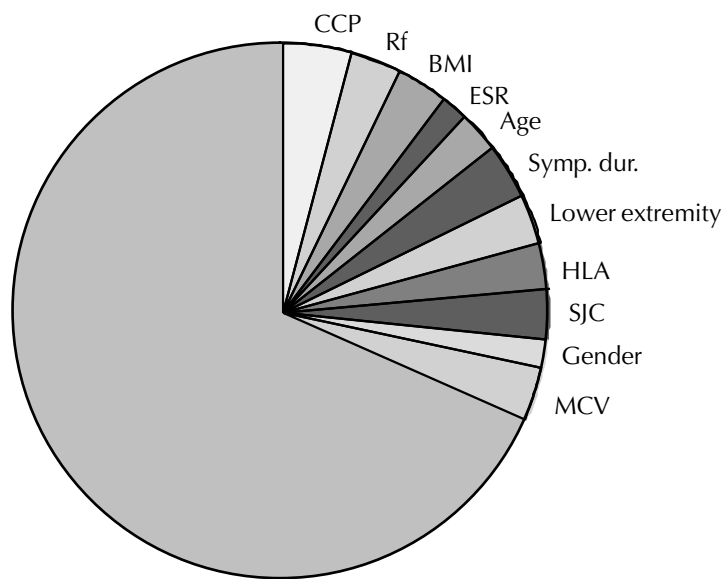


Figure 1. Contribution of baseline variables to the explained variance of Sharp-van der Heijde score over five years.

Presented is the explained variance at 5 years of baseline variables that were associated with the progression of joint destruction.

CCP anti-cyclic citrullinated peptide antibody; RF rheumatoid factor; BMI Body Mass Index; ESR Erythrocyte sedimentation rate, sympt dur symptom duration at first visit; lower extremity initial complaints at lower extremity versus upper extremities, HLA HLA-DRB1 shared epitope alleles, SJC swollen joint count, MCV anti-modified citrillunated vimentin antibodies.

All continuous variables were categorized in two groups in order to derive this figure; BMI was grouped in lower or higher than 25. Symptom duration at first visit (symp. dur.) was grouped in lower or higher than 12 weeks SJC was grouped lower and above 6 swollen joints. Age under and above the median of 57 years, ESR normal or elevated according to reference value.

factors to the explained variance. This was accomplished by calculating the proportion of the effect size of the individual factors in the multivariate analysis to the total effect. The proportional effect size of these variables is depicted in Figure 1.

DISCUSSION

Cumulating evidence supports the relevance of initiating DMARD therapy as early as possible. Individualized treatment decision making is hampered by the variability of the outcome of early arthritis. In case of early undifferentiated arthritis, the question is when DMARD therapy should be initiated. In early RA it would be beneficial to recognize the patients who will have a severe disease course, since in these patients the benefits of early combination therapy with potent targeted therapies will up weight the associated costs and risks on side effects. In this themed issue risk factors for the outcome of UA and RA patients are explored based on data of the Leiden EAC.

With regards to early UA it was observed that predictive factors for the fulfillment of the ACR87-RA criteria and for having a persistent arthritis were largely similar. A predictive tool for RA-development was derived before using a combination of identified risk factors¹⁰. This prediction rule is now well-validated¹⁴⁻¹⁶. Since the present study did not intend to re-derive or improve this predictive tool, no multivariate regression analyses were performed in UA-patients. Some studies tried to improve this prediction rule and assessed the additive value of baseline erosiveness and genetic markers^{8;17}. Unfortunately, these attempts did not result in an increased prognostic performance of this model. Further improvements of the model may be expected to come from ultrasound and MR imaging studies. Although at present not much data on US and MRI in unselected populations of UA-patients are available, initial results are promising¹⁸.

Fulfilling the 1987 ACR criteria as outcome of UA has the disadvantage that it may introduce some circle reasoning; in contrast the difficulty with the outcome measure disease persistency is that classification depends on the duration of follow-up. In UA-patients included in this study remission was achieved after a median period of 17 months, whereas in the RA patients the median disease duration till remission was 40 months. A too early comparison of disease outcomes may result in misclassification of potential remission patients into the persistent disease category. In order to diminish the risk on misclassification, in this study we chose to classify patients with ≥ 5 years of arthritis as being persistent. This follow-up duration is arbitrary and results may have been slightly different in case a shorter or longer follow-up period was chosen.

The most potent predictors for having a persistent course of arthritis in UA-patients and a persistent course of RA were the presence of auto-antibodies. Inflammatory markers (the number of swollen joints, ESR and CRP) were associated with the development of RA

and a persistent form of arthritis in UA-patients as well as the severity of joint destruction in RA-patients, which is in line with findings in older studies. However, no significant association between these inflammatory markers and disease persistency was found in RA-patients.^{19;20} This may be due to the fact that the number of patients with sustained DMARD-free remission in RA was low, thereby reducing the power to identify significant associations with this outcome measure.

It is interesting to note that morning stiffness is strongly associated with the development of RA but not with disease persistency or the severity of joint destruction. Several explanations may account for this feature. One of them is that morning stiffness is mainly related to RA according to the 1987 criteria because of circle reasoning. Morning stiffness is not part of the 2010 EULAR/ACR criteria for RA and it would be an interesting subject for further studies to see whether the association between morning stiffness and the risk on RA is still present when the new definition of RA is used.

Other intriguing findings concern the observations on BMI. Obese RA-patients are found to have less severe joint destruction. This observation was not only observed in the present study but also in other populations²¹⁻²³. The present study revealed that BMI was not associated with progression from UA to RA, but it was associated with having a persistent arthritis or persistent RA. Thus this indicates that obese patients have more often a persistent disease than non-obese arthritis patients. This observation is highly fascinating and may point to the notion that the role of fat tissue in rheumatoid arthritis is incompletely clear. Fat tissue secretes pro-inflammatory as well as anti-inflammatory adipocytokines²⁴. It is clear that some of the mechanisms of joint destruction like osteoclast activation are different than inflammatory pathways and as such it is tempting to speculate that diverse adipocytokines may have different preferential effects on arthritis persistency and on joint destruction.

The associations between disease outcomes and involvement of the joints of the lower or upper extremities were different for patients with UA and RA. Whereas within UA presence of arthritis on lower extremities was associated with a lower OR on RA, within RA-patients it was associated with a higher rate of joint destruction. This finding is in line with previous findings demonstrating that patients presenting with knee arthritis had a more severe rate of joint destruction compared to patients without knee arthritis, when measured using destruction of small feet and hands joint²⁵.

Emerging evidence indicates that Anti-CCP-positive and Anti-CCP-negative RA are subsets of RA with differences in the underlying pathologic mechanisms^{26;27}. The present study addressed all UA patients and RA patients; stratified analyses on anti-CCP-positive and Anti-CCP-negative patients were not performed. This may be an explanation why *CD40*, a genetic risk factor joint destruction in Anti-CCP positive RA is not associated with the rate of joint destruction in the whole RA population¹³.

The baseline characteristics associated with the severity of joint destruction in RA were mainly auto-antibodies and other patient characteristics and to a lower extend factors

expressing the level of inflammation. Although the present study did not evaluate the contribution of inflammation over time on the final level of joint destruction, such analyses have been performed before. Some of these studies also suggested that the largest part of joint destruction is not directly related to cumulative inflammatory markers²⁸.

The data presented are limited to data of the Leiden Early Arthritis Clinic cohort. However many of the associating risk factors for UA and RA are observed in individual studies originating from different early arthritis cohorts as well²⁹⁻³⁴.

The proportion of the explained variance in progression of joint destruction by the identified risk factors was 32%. Although no clear guidelines are available what the level of variance explained is required in order to derive a prediction model with an adequate discriminative performance, previous investigations and experience^{10;35} are highly suggestive that the explained variance is insufficient to proceed with a derivation of a prediction rule for the rate of joint destruction in RA. This notion is exemplified by recent attempts to derive at prediction models; with the current prediction rules about 50% of the RA-patients could not be adequately classified³⁵⁻³⁷.

In conclusion, although the processes determining the persistency and severity of arthritis are incompletely understood, the identification of risk factors may help in individualization of therapy in patients with recent-onset UA. In RA, in contrast, the currently known risk factors for a progressive destructive disease course explain only part of the individual differences in level of joint destruction and more risk factors need to be identified in order to achieve at individualized treatment decision making.

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Chapter 3

Loss of metacarpal bone density predicts RA development in recent onset arthritis

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ABSTRACT

Objective

Serum samples taken prior to the onset of Rheumatoid Arthritis (RA) suggest that one of the first features of RA is Bone Mineral Density (BMD) loss. We determined the ability of radiographic BMD loss to predict RA-development and arthritis persistency in patients with early Undifferentiated Arthritis (UA).

Methods

517 patients with early UA, included in the Leiden Early Arthritis Clinic, were assessed. Of these, 101 had hand radiographs made at first visit as well as after six months and were studied. BMD loss was measured using DXR-online. The outcome measures were fulfilling the 1987 ACR criteria for RA after 1 year and arthritis persistency during a mean follow-up of 7 years. Additionally it was assessed whether BMD measurements improved prediction making compared to a validated prediction rule.

Results

53.8% of UA-patients developed RA and 67.5% had persistent disease after 7 years follow-up. Highly elevated BMD loss (≥ 2.5 mg/cm² per month) was present in 16.3% of patients and associated with RA development (OR 6.1 (95%CI 1.2-29.2), PPV 85%, NPV 52%, sensitivity 26%, specificity 95%). BMD loss may have an independent effect of anti-CCP, when tested in a logistic regression analysis (OR 4.1, 95% CI 0.8-21.2), although the CI is large. All UA-patients that were unclassified with the prediction rule and had highly elevated BMD loss progressed to RA. BMD loss was not significantly associated with arthritis persistency (HR=0.56, 95% CI 0.14-2.29).

Conclusion

Present data suggest that BMD loss predicts RA development. These findings need to be verified in larger studies.

INTRODUCTION

The outcome of early arthritis patients is highly variable. Approximately only one-third of the patients with a recent-onset Undifferentiated Arthritis (UA) progresses towards Rheumatoid Arthritis (RA) as defined by the ACR-1987 criteria and 40-50% has a spontaneously remitting disease. In order to achieve individualized treatment decision making, the disease outcome needs to be estimated adequately. This is particularly relevant since it is widely acknowledged that early initiation of treatment of RA is effective in diminishing the level of joint destruction and disability¹. Initiating Disease Modifying Anti-Rheumatic (DMARD) Therapy in all UA patients induces overtreatment in about half of the patients, arguing for a 'wait-and-see strategy' for some time. On the other hand, studies in serum samples of patients that later have developed RA show that disease processes such as broadness of antigen recognition and isotype usage of anti-citrullinated antibodies mature very early in disease^{2,3}. Increased levels of the bone metabolism markers P1NP and osteoprotegerin have been shown in serum samples of patients years before the onset of disease⁴.

Moreover, intervention studies suggest that recent onset disease is more sensitive to current treatment than later stage disease. Therefore this early stage is sometimes referred to as the 'window of opportunity'. The current prediction models suggest that critical disease processes which drive the development of disease processes towards RA are autoantibody responses and inflammation^{5,6}. Metacarpal Bone Mineral Density (BMD) loss has been observed to be predictive for radiographic destruction in RA^{7,8}. Its predictive abilities in Undifferentiated Arthritis have not been investigated yet. Since current prediction models do not take local metacarpal or systemic bone loss into account, we hypothesized that bone loss in the three middle metacarpal bones may be of additional value to currently known predictive factors. An advantage of measuring hand BMD loss is that it is relatively easy to determine as it uses normal hand radiographs, which are part of the standard clinical care for most RA patients.

METHODS

567 patients with recent onset UA who participated in the Leiden Early Arthritis Clinic and were included between 1993 and 2006 were assessed¹. Written informed consent was obtained from all patients in this cohort and the cohort has been approved by the local medical ethical committee (ethics committee of the Leiden University Medical Center). Of the 567 patients with recent onset UA, 125 had hand radiographs made at first visit as well as after six months and were selected for further analysis.

Digital X-ray radiogrammetry online technology (DXR) (Sectra, Sweden) was used to measure cortical DXR-BMD (BMD)⁷. This technique is a computerized version of the

technique of radiogrammetry developed by Barnett and Nordin and has been shown to predict, among others, joint damage in recent onset rheumatoid arthritis⁷⁻⁹. The technique computes BMD loss by making use of the cortical thickness of the three middle metacarpal bones on hand radiographs.

The analogue radiographs were digitized with a Vidar VXR-12 Plus digitizer at 300 DPI and 12 bit. The DXR-BMD technique has been described in greater detail previously⁸. In short, BMD is estimated through an automated analysis of the cortical bone at the centers of metacarpals II, III and IV on a standard projection digital radiograph. Whenever possible, mean DXR-BMD of both hands was used for the analysis to maximize accuracy of the BMD loss measurement. The reproducibility of DXR-BMD when applied to analogue and digital X-rays acquired according to the DXR protocol has been assessed in previous studies^{10,11}.

BMD loss was calculated as the difference between BMD measured on the radiograph made at six months follow-up and the baseline radiograph. Cut-offs for the categories normal, elevated BMD loss and highly elevated BMD loss were established previously by Sectra Imtec AB and were by no means influenced by the findings of the present study (see also: dxr-online.com/ReportsWebTool/ManualChange.aspx). Elevated BMD loss was defined as a change in BMD ≥ 0.25 mg/cm²/month, highly elevated BMD loss was defined as a change in BMD ≥ 2.5 mg/cm²/month. Two main outcome measures were studied: fulfilling the 1987 ACR criteria for RA after one year and arthritis persistency during a mean follow-up period of 7 years. Persistent disease was defined as the absence of sustained DMARD-free remission, which was defined as the absence of synovitis for at least one year after cessation of DMARD-therapy, if any. It was also assessed whether patients that could not be adequately classified using a validated prediction rule consisting of nine clinical and serological variables (those patients had a prediction score > 6 and < 8), could be predicted correctly using DXR⁵. In addition, the value of BMD loss for the prediction of fulfilling the 2010 ACR criteria after 1 year follow-up was tested. The discriminative ability was expressed using an area under the receiver operator characteristic curve (AUC). The value of BMD loss for the prediction RA development after 1 year follow-up and arthritis persistency during a mean follow-up of 7 years was calculated using logistic regression and Cox regression respectively. Calculations were performed using SPSS version 17.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Of the 567 patients who visited the Leiden Early Arthritis Clinic between 1993 and 2006, 125 had radiographs of the hands made at first visit and at six months follow-up. Part of the radiographs could not be studied, mainly due to inadequate positioning of the hands

Table 1. Characteristics of the study population

	All patients (n=567)	Patients eligible (n=125)*	p-value	Patients analyzed (n=80)**	p-value
Female (%)	327 (57.7)	78 (62.4)	0.33	57 (71.3)	<0.01
Age, mean $\pm$ SD	50.9 $\pm$ 16.9	52.5 $\pm$ 14.2	0.37	53.2 $\pm$ 14.0	0.40
Symptom duration at baseline	63.1 $\pm$ 198.1	220.52 $\pm$ 405.2	<0.01	243.8 $\pm$ 175.9	0.01
Swollen Joint Count, mean $\pm$ SD	7.3 $\pm$ 7.8	9.0 $\pm$ 7.5	<0.01	8.9 $\pm$ 7.5	0.57
C-reactive protein mg/l, mean $\pm$ SD	22.8 $\pm$ 30.2	22.8 $\pm$ 26.6	0.34	22.3 $\pm$ 24.5	0.68
Anti-CCP antibody positive, n (%)	133 (24.4)	46 (37.4)	<0.01	30 (37.5)	0.98
RA development after 1 year, n (%)	176 (31.0)	65 (52.0)	<0.01	43 (53.8)	0.60

* UA-patients of whom a radiograph was made at first visit as well as at six months follow-up were compared to all UA-patients.

** UA-patients of whom the radiographs were used for BMD analyses were compared to all UA-patients with radiographs both at first visit and at six months follow-up.

on the radiographs. 160 radiographs of 80 patients were of sufficient quality to be studied. Characteristics of the remaining group of 80 patients, and the 125 patients with two radiographs and the whole group of 567 UA patients are depicted in Table 1. Patients with serial radiographs in 6 months had a higher number of swollen joints and were more often anti-CCP positive compared to patients of whom no radiograph at the 6 months time point was available. The patients whom pairs of radiographs could not be studied for technical reasons were not different from the patients who had two appropriate radiographs.

Thirteen patients (16.3%) had highly elevated BMD loss, 37 (46%) had elevated BMD loss and 30 (38%) had a stable or increasing BMD. 54% of patients fulfilled the ACR 1987 criteria for RA within the first year. Patients with highly elevated BMD loss (>2.5 mg/cm² per month) had a six-times increased odds to develop RA, which was significant (95% CI 1.24-29.24). The PPV was 85% (95% CI 54-97%), the NPV 52% (95% CI 40-64%), the sensitivity 26% (95% CI 14-41%), the specificity 95% (95% CI 80-99%) and the AUC 0.6 (95% CI 0.48-0.93). The OR's for RA-development of BMD loss were compared to the OR's of other known risk factors that were determined on the same dataset. The odds ratio's of these risk factors were lower than that of highly elevated BMD loss (see Figure 1).

When adjusting for the presence of anti-CCP antibodies in logistic regression analysis, BMD loss still tended to have an independent association with progression to RA (OR 4.08, (95% CI 0.78-21.22), though the confidence interval was too wide to draw definite conclusions.

27 patients could not be classified by the prediction rule because of a score >6 and <8 . All of these patients that had a highly elevated BMD loss developed RA, indicating a PPV of 100% (95% CI 85-100%) in this small subgroup. 60 of the 80 patients analyzed fulfilled

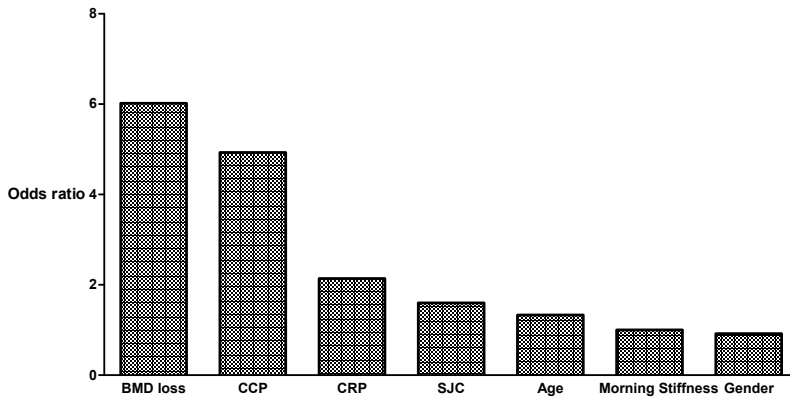


Figure 1

ORs of various risk factors for RA development. ORs for RA development according to the 1987 ACR criteria of various disease characteristics resulting from univariate analyses. BMD loss: highly elevated BMD loss as defined by Sectra; CCP: presence or absence of anti-cyclic citrullinated antibodies; CRP: C-reactive protein; SJC: 44-swollen joint count; morning stiffness: severity of morning stiffness on visual analogue scale; gender: female gender. In order to calculate ORs for the variables CRP, SJC, age and morning stiffness, patients were divided into two groups based on the mean. The means are derived from the data of the 80 people used for analysis; CRP 22.3 mg/l, SJC 8.9, age 53.2 years and morning stiffness 7.01 cm.

the ACR-EULAR 2010 RA criteria within one year of follow-up. All 13 patients with highly elevated BMD loss fulfilled the ACR-EULAR 2010 criteria, resulting in a PPV of 100% (95% CI 72-100%). However, 47 patients which fulfilled the ACR-EULAR 2010 criteria had no highly elevated BMD loss.

Seventy-eight percent of the patients had persistent disease. Highly elevated BMD loss was not significantly associated with persistency of arthritis after a mean follow-up of seven years (HR=0.57, (95% CI 0.14-2.29)).

DISCUSSION

The concept of BMD loss is appealing as several lines of evidence indicate that bone metabolism activity occurs very early in RA. Increased concentrations of bone metabolism markers have been found in the sera of patients prior to the development of RA^{4,12}. In addition, within early RA BMD loss is predictive for future joint damage^{7,8}. Also, bone mineral density loss over 1 year in cortical and trabecular bones of the hand, measured with Dual Energy X-ray Absorptiometry, was demonstrated to be higher in RA patients compared to that of other inflammatory diseases¹³. It is interesting to speculate whether BMD loss is due to systemic or to local inflammatory factors. The presence of bone metabolism markers in the serum before disease onset at least points to the presence of systemically measurable phenomena.

Our study is the first to evaluate the predictive ability of measuring BMD loss using DXR in early UA. It indicates that in these patients, BMD loss may be a relevant predictor for the development of RA. From several inception cohort studies it is known that about 40-50% of these UA patients remit spontaneously, whereas one-third develops RA⁵. Ideally only the latter patients are treated with DMARDs. Our findings may be relevant for clinical practice as they may enhance the identification of UA patients that are in an early phase of RA⁵.

Nevertheless, this study has several limitations. First of all, the sample size is small; this may prevent definite conclusions to be drawn and points to the relevance of performing additional studies on BMD loss in UA patients. The sample size may be a concern for the total number of patients with BMD data (n=80), but in particular for the patients that were studied because of being unclassified by a clinical prediction rule (n=27). It was observed that all patients with highly elevated BMD loss that could not be classified according to the prediction rule, developed RA. Though this may indicate that DXR can importantly improve prediction making, these initial findings should be validated in larger, independent studies.

A second limitation is the fact that the UA patients that had a repeated radiograph after six months and therefore could be analyzed had more severe disease compared to the whole UA population. It is possible that some selection bias occurred here. This may limit the generalizability of our study to the standard UA population.

Third, treatment effects were not taken into consideration. Although corticosteroids were seldom prescribed in the UA patients studied, these data are incompletely registered since e.g. no records from GP's are available. However, supposed that a highly elevated BMD loss is not due to the disease but to any treatment, the true positive predictive value would be higher than observed now.

BMD loss measured by DXR did not significantly associate with arthritis persistency. It is undecided whether this is due to insufficient power, or whether there is truly no association between BMD-loss and arthritis persistency. More studies on this subject are needed. It was beyond the scope of the present study to determine the cost-effectiveness of DXR-BMD measurements for daily clinical care. This would require larger studies in unselected groups of UA-patients.

In conclusion, although more research into the value of BMD loss measurements in early UA is necessary, our study suggests that BMD loss may be an useful prognostic tool.

CONFLICTS OF INTEREST

J.K is an employee of Sectra Imtec AB. The other authors declare no conflicts of interest.

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Chapter 4

How to define baseline erosiveness to predict radiologic progression in Rheumatoid Arthritis?

LETTER TO THE EDITOR

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To the Editor,

Baseline erosiveness is one of the most potent predictive factors for a severe destructive disease course of Rheumatoid Arthritis (RA), that is also used in daily practice. There is however no uniform definition of erosive disease at disease onset. The definitions used in studies were highly variable; definitions used are amongst others any radiological evidence of erosions^{1,2}, a cortical break of ≥ 2 mm³ or presence of ≥ 2 or ≥ 3 erosions.^{4,5} The lack of a generally accepted definition makes studies on this subject incomparable. Moreover it prevents the use of radiological information at disease presentation in a consistent manner in clinical practice.

Recently, the radiologic criterion to classify RA according to the 2010-criteria was presented and defined as the presence of at least three erosive joints.^{6,7} Here an erosive joint was defined as a metacarpophalangeal (MCP), proximal interphalangeal (PIP), metatarsophalangeal (MTP) or wrist joint with a broken cortex on an X-ray. As such this measure is easy applicable in clinical practice. This prompted us to (I) evaluate the accuracy of baseline erosiveness expressed by the number of erosive joints to predict radiographic progression on the short term and long term and (II) to evaluate whether an optimal cut-off for the level of baseline erosiveness to predict severe joint damage progression can be determined.

Early arthritis patients included between 1993 and 2006 in the Leiden Early Arthritis Clinic that at first presentation were diagnosed with RA (according to the 1987 or the

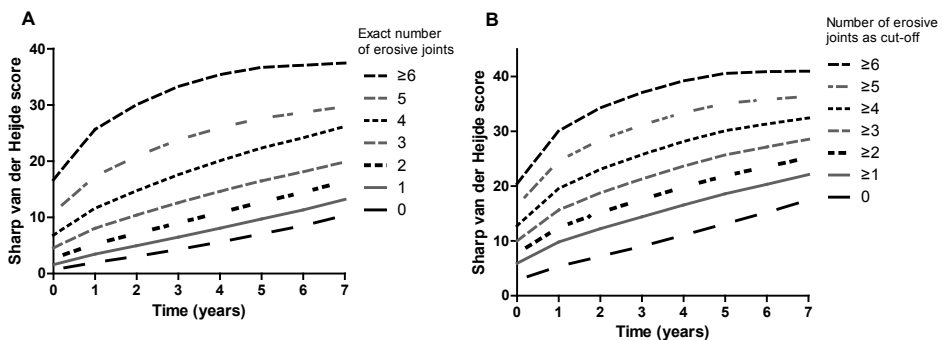


Figure 1. Progression in Sharp van der Heijde score over 7 years of follow-up for RA-patients with different numbers of erosive joints at first presentation (A) and with different numbers of erosive joints used as cut-off (B)

Depicted are the mean Sharp van der Heijde scores over 7 years of disease as predicted by multivariate normal regression analyses¹⁰ taking advantage of within patient correlations between serial radiologic measurements and adjusting for age, gender and inclusion period (as a proxy for different treatment strategies). In (A) SHS-data are presented for the absolute number of erosive joints, in (B) the cut-off for erosiveness is defined using different numbers of erosive joints.

Table 1. Characteristics of RA-patients with different numbers of erosive joints at first presentation (A) and the test characteristics and predictive values for developing rapid radiological progression during the first year of the disease (B)

(A) Exact number of erosive joints									
	No patients N(%)	Women N (%)	Age mean±SD	Smoker N (%)	ACPA+ N (%)	Sympt dur (wks) mean±SD	CRP mg/l mean±SD	RRP* N (%)	
none	137 (19.5)	108 (78.8)	45.4 (13.6)	32 (25.8)	61 (48.0)	24.1 (23.1)	20 (30)	27 (26.2)	
1	99 (14.1)	72 (72.7)	50.6 (15.7)	33 (34.7)	45 (47.4)	25.6 (19.5)	22 (22)	19 (22.9)	
2	78 (11.1)	52 (66.7)	53.1 (14.4)	25 (33.8)	44 (57.1)	27.3 (21.3)	31 (30)	23 (33.8)	
3	77 (11.0)	51 (66.2)	56.1 (15.2)	19 (26.0)	48 (64.0)	28.3 (25.3)	22 (26)	26 (40.0)	
4	69 (9.8)	45 (65.2)	57.7 (14.7)	17 (27.0)	39 (59.1)	25.6 (21.2)	33 (33)	24 (44.4)	
5	38 (5.4)	23 (60.5)	62.9 (12.9)	10 (28.6)	19 (51.4)	18.6 (11.8)	35 (40)	14 (45.2)	
≥6	189 (27.0)	116 (61.4)	67.4 (11.4)	42 (24.0)	92 (49.2)	27.8 (22.6)	37 (40)	78 (53.4)	
Total	687								
(B) Number of erosive joints used as cutoff									
	No patients N (%)	RRP N (%)	Sensitivity (95%CI)	Specificity (95%CI)	PPV (95%CI)	NPV (95%CI)	LR+ (95%CI)	LR – (95%CI)	AUC (SEM)
none	103 (18.7)	27 (26.2)							
≥1	447 (81.3)	184 (41.1)	0.87 (0.82-0.91)	0.22 (0.18-0.27)	0.41 (0.37-0.46)	0.74 (0.64-0.82)	1.12 (1.04-1.21)	0.57 (0.39-0.83)	0.55 (0.025)
≥2	364 (66.2)	165 (45.3)	0.78 (0.72-0.83)	0.41 (0.36-0.47)	0.45 (0.4-0.51)	0.75 (0.68-0.81)	1.33 (1.19-0.49)	0.52 (0.41-0.69)	0.60 (0.024)
≥3	296 (53.8)	142 (48.0)	0.67 (0.6-0.73)	0.55 (0.49-0.6)	0.48 (0.42-0.54)	0.73 (0.67-0.78)	1.48 (1.28-0.72)	0.60 (0.49-0.73)	0.61 (0.025)
≥4	231 (42)	116 (50.2)	0.55 (0.48-0.62)	0.66 (0.61-0.71)	0.50 (0.44-0.57)	0.70 (0.65-0.75)	1.62 (1.34-1.96)	0.68 (0.59-0.79)	0.61 (0.025)
≥5	177 (32.2)	92 (52.0)	0.44 (0.37-0.5)	0.75 (0.7-0.79)	0.52 (0.44-0.59)	0.68 (0.63-0.72)	1.73 (1.37-2.21)	0.75 (0.66-0.850)	0.59 (0.025)
≥6	146 (26.5)	78 (53.4)	0.37 (0.34-0.42)	0.80 (0.75-0.83)	0.53 (0.45-0.62)	0.67 (0.62-0.72)	1.84 (1.4-2.43)	0.79 (0.71-0.88)	0.59 (0.025)
Total	550	211 (38.4)							

In Table 1A, percentages or means±SDs are reported. The percentages indicate percentage of people within a specific group of patients having 0 - >6 erosions. *Not in all patients, a radiograph had been made after one year of follow-up. Therefore, in 550 patients, the presence of Rapid Radiological Progression could be calculated.

2010-criteria) were studied (n=701).⁸ Serial hand and foot radiographs were made at first presentation and on a yearly basis during follow-up. The number of joints with a cortex break was determined from the baseline radiographs, assessing bilateral MTP, PIP, MCP and wrist joints. Two outcomes were assessed: rapid radiological progression (RRP) on the short term and long term progression of joint destruction during 7-years of disease. RRP was defined as an increase of ≥5 Sharp-van der Heijde-points in 1 year, a change larger

than the smallest detectable difference that is also associated with functional decline.⁹ The serial X-rays were SHS-scored by two readers; the within reader-ICC was 0.87 and 0.91, the between-reader ICC was 0.89. Relations between the number of erosive joints and the progression of joint destruction over a maximum follow-up period of seven years were tested by multivariate normal regression analyses taking advantage of within patient correlations between serial radiologic measurements.¹⁰ Analyses were adjusted for age, gender and inclusion period, as a proxy for different treatment strategies. The treatment strategy applied was different in different periods; patients included in 1993-1995 were initially treated with NSAIDs, patients included in 1996-1998 were initially treated with chloroquine or sulphasalazine and patients included after 1999 were promptly treated with methotrexate. Only 4.7% of the patients was treated with anti-TNF at any moment during the follow-up period.¹⁰

687 patients had baseline radiographs. The baseline characteristics are shown in Table 1A. In 550 patients an X-ray was made at both baseline and year-1. In total, 211 (38.4%) patients had RRP, this frequency increased per category of erosive joints (Table 1A). Subsequently, patients were categorized using different numbers of erosive disease as cut-off for baseline erosiveness (Table 1B). With every increase in number of erosive joints as cut-off, the specificity and LR+ increased but the sensitivity and LR- decreased. The AUC was the highest for the cut-offs ≥ 3 or ≥ 4 joints, but the differences were small. Subsequently, the progression in Sharp van der Heijde score over 7 years of follow-up for RA-patients with different numbers of erosive joints at first presentation was studied. Additionally, the Sharp van der Heijde scores over 7 years were studied when different numbers of erosive joints were used as cut-off and patients were grouped accordingly (Figure 1). Every increase in cut-off was associated with more severe SHS-progression and no clustering of lines was observed.

As one may expect, every increase in the number of erosive joints at RA onset is associated with an increased chance on short term and long term progression of joint damage. Although further studies in other cohorts are required, we could not identify an obvious single cut-off point to define baseline erosiveness in relation to prediction of joint damage progression.

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Chapter 5

Evaluating joint destruction in Rheumatoid Arthritis, is it necessary to radiograph both hands and feet?

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ABSTRACT

Background

Radiological damage is an important outcome measure in rheumatoid arthritis (RA), both for research and clinical purposes. Depending on the setting, both hands and feet are radiographed, or only a part of these. It is unknown whether radiographing part of the four extremities gives comparable information to radiographing both hands and feet. We therefore aimed to compare the radiological information obtained both when evaluating single time-point radiographs and progression over time, in early and advanced RA.

Methods

6,261 sets of hands and feet X-rays of 2,193 RA-patients from Leiden, Groningen (both from Netherlands) and North America were studied. Correlations between joint damage at different regions were compared (unilateral versus bilateral and hands versus feet). Analyses were done at single time-points (cross-sectional) and for progression over time (longitudinal), both for continuous severity measures (Sharp-van der Heijde score, SHS) and binomial measures of erosiveness.

Results

When studying single time-points, the severity of joint damage (SHS) is highly correlated between left and right, but weakly correlated between hands and feet. Correlation coefficients were higher in advanced than early RA. These findings were comparable in the three datasets. When evaluating erosiveness using only unilateral X-rays or hands without feet, 19.3% and 24.0-40.4% is incorrectly classified as non-erosiveness. Similarly, when evaluating disease progression by imaging only unilateral X-rays or only hands X-rays, progression would have been missed in 11.6-16.2% and 21.2-31.0 % of the patients.

Conclusion

Performing X-rays of both hands and feet yields additive information compared to imaging only a part of these.

INTRODUCTION

Joint damage is a hallmark of Rheumatoid Arthritis (RA) that is commonly measured using X-rays of hands and feet. For scientific research purposes, X-rays provide an important outcome measure on the severity of RA. It is reflective of the cumulative burden of inflammation over time and can be quantified using validated scoring methods. In clinical practice X-rays are taken to evaluate treatment efficacy, since radiological joint damage may progress both in the presence and absence of low disease activity scores.

In some hospitals radiographic protocols are such that always complete sets of hands and feet are portrayed, whereas in other hospitals only the regions with most complaints are being depicted. In scientific studies it is common that bilateral extremities are radiographed; though this can concern both hands and feet¹⁻³ or only both hands.⁴ If there is sufficient proof that radiographing only one extremity is as informative as radiographing both hands and feet, this would imply that radiographing in daily practice can be done with less exposure to radiation and less costs. In addition this issue is relevant for scientific studies when patients are included that have radiographs made of only part of the extremities and the question rises whether missing radiologic data can be imputed.

Scientific data on the advantages of radiographing all hands and feet compared to radiographing only a part (either the most painful site or only the upper extremities) are, to the best of our knowledge, lacking. The results of recent MRI studies increased our interest in this issue. MRI evaluations are generally done on one hand and/or one foot and seldom on bilateral extremities because of practical concerns. Current data indicated that there are no major differences in joint damage between dominant and non-dominant hands measured with MRI,⁵ suggesting that imaging of a unilateral extremity may be sufficient. This led us to perform the present study in which we aimed to compare the radiological information that is obtained when all hands and feet, or only a part of these are radiographed. We studied this with regards to single time-point radiographing as well as with regards to evaluations of progression of joint damage over time, for continuous and binominal radiological outcome measures, and in early and advanced RA.

PATIENTS AND METHODS

Study population

Three datasets consisting of 6,261 sets of hand and feet X-rays belonging to 2,193 adult RA-patients in early and advanced disease stages were studied. RA was defined according to the 1987 American College of Rheumatology criteria.⁶

Leiden-Early Arthritis Clinic cohort (EAC)

678 early RA-patients originating from the western part of the Netherlands, whom were consecutively included in the Leiden-EAC between 1993-2006, were studied. Patients had less than two-years of symptoms. The median symptom duration at inclusion was 4.3 months. Mean age was 56.6 years and 457 (67.4%) were female. Hands and feet X-rays were taken at baseline and on yearly follow-up visits during 7-years. All X-rays were chronologically scored by one experienced reader using the Sharp-van der Heijde score (SHS).⁷ The correlation coefficient (ICC) within the reader was 0.91. Mean total SHS was 8.7 and mean hands SHS was 5.74 at first visit.

Groningen cohort

The second set of radiological data was obtained from 261 RA-patients from the Northern part of the Netherlands (Groningen). These patients were radiographed between 1965-2010. Mean age of diagnosis was 45.1 years and 177 (67.8%) were female. Hands and feet X-rays were available over a follow-up duration of at most 25-years with a mean number of X-ray per patient of 3.1 (with a maximum of eight X-rays per patient). The X-rays were scored chronologically by one of two readers using SHS. ICCs within readers were both >0.90 and between readers 0.96. Mean total SHS was 12.0 and mean hands SHS was 6.24 at first visit.

North American dataset

1,254 RA-patients deriving from different North American centres, whom were radiographed between 1975-2011 were studied. X-rays were made of hands but not of feet. The mean age at the time of the diagnosis was 48.9 years; 981 patients (78.2%) were female. X-rays were available over a follow-up duration of at most 25-years with a mean number of X-ray per patient of 2.0 (with a maximum of eight X-rays per patient). The X-rays were scored with known time order by one experienced reader; the within reader ICC was 0.99. The mean hands SHS at the first visit was 14.9.

Study Design

This study was divided into two parts. First we compared the severity of joint damage (expressed by the SHS) at different regions, both when evaluating single time-points cross-sectionally and when evaluating progression of joint damage over time. This was done to explore how well the quantity of radiological damage at one site was correlated to that at the other site.

Second it was studied whether classifying a patient as having erosive disease was different when both hands and feet X-rays were studied or only a part of them. Also here, X-rays obtained at single time-point were evaluated cross-sectionally and progression of erosiveness was evaluated longitudinally. Erosiveness was defined as having ≥ 2 erosive

joints in the cross sectional analysis. This cut-off was chosen based on data showing that the presence of ≥ 2 erosive joints is more predictive of RA than the presence of one erosive joint.⁸ In the longitudinal analyses, progression was expressed as a binomial outcome. Progression of erosiveness was defined as an increase in erosion score of ≥ 2 points in a one-year interval.⁹ Differences in classification of erosiveness if both hands and feet or a part of these are radiographed were evaluated. As sensitivity analyses, other cut-offs for progression were also studied.

Statistical analyses

The severity of joint damage at different regions on X-rays of single time points was compared using Pearson correlation coefficients. This was done for the individual follow-up years in the Leiden RA-patients. Since there was no yearly follow-up schedule the Groningen and North American datasets, follow-up years were combined into “year strata”, to include a larger number of patients per time-point; here X-rays taken at year 1-4, year 5-9 and year 10-25 were combined. X-rays taken at year 10-25 in the Leiden EAC were also combined. Since a high correlation coefficient does not by definition mean high agreement, the level of agreement was tested by plotting data in Bland-Altman plots.¹⁰

Analyses on progression of the severity of joint damage were performed in Leiden EAC only, since it contained sufficient radiological information for subsequent year of follow-up.

The presence of erosiveness at different sites was compared by cross-tabs. Patients that would be classified as having erosive disease when evaluating X-rays at all sides but classified as non-erosive when evaluating single locations were labeled as misclassified patients. Similar analyses were done with regards to progression of erosiveness.

All analyses were performed using SPSS version 17.0. P-values < 0.05 were considered significant.

RESULTS

Comparisons on severity of joint damage

First it was evaluated how well the severity of radiological damage (expressed by SHS) at one site was correlated to radiological damage at other sites. This was done both when radiological joint damage at single time-points was studied and when progression over time was evaluated.

Comparison at single time points

When evaluating the SHS of the Leiden RA-patients, it was observed that correlation coefficients were rather high between left and right comparisons (Table 1). In addition, with increasing follow-up duration the correlation coefficients increased. However, the

Table 1. Correlations between the severity of joint damage (according to the Sharp-van der Heijde method) at different sites and different disease durations in Leiden RA-patients.

	Baseline	Year 1	Year 4	Year 7	Year 10-15
Comparisons	N=678	N=580	N=408	N=254	N=157
<i>Hands & Feet</i>					
Right vs Left (total SHS)	0.73	0.79	0.90	0.94	0.92
Right vs Left (total ES)	0.77	0.82	0.89	0.93	0.92
Right vs Left (total JSN)	0.57	0.75	0.85	0.93	0.89
<i>Hands</i>					
Right vs Left (total SHS)	0.74	0.79	0.88	0.94	0.89
Right vs Left (total ES)	0.78	0.83	0.88	0.92	0.87
Right vs left (total JSN)	0.54	0.73	0.82	0.93	0.85
<i>Feet</i>					
Right vs Left (total SHS)	0.63	0.73	0.80	0.87	0.86
Right vs Left (total ES)	0.64	0.70	0.78	0.87	0.90
Right vs left (total JSN)	0.46	0.67	0.76	0.81	0.82
<i>Joint Groups</i>					
Right vs Left MCP (total SHS)	0.63	0.76	0.80	0.88	0.85
Right vs Left PIP (total SHS)	0.72	0.71	0.72	0.88	0.76
Right vs Left wrists (total SHS)	0.52	0.77	0.80	0.88	0.80
Hands vs Feet (total SHS)	0.34	0.19	0.50	0.35	0.50
Hands vs Feet (total ES)	0.35	0.41	0.45	0.39	0.51
Hands vs Feet (total JSN)	0.23	0.31	0.43	0.29	0.47

Presented are Pearson correlation coefficients. All correlation coefficients are statistically significant (all P values <0.01).

SHS – Sharp-van der Heijde Score, ES – Erosion Score, JSN – Joint Space Narrowing

MCP – Metacarpal Phalangeal Joints, PIP – Proximal Interphalangeal Joints

correlation coefficients of comparisons of hands with feet were moderate at all points in time (Table 1). Analyses in the Groningen and North American datasets also showed that the level of joint damage at the left and right sides were fairly comparable, although the correlation coefficients in Groningen did not increase during follow up. Similar to the Leiden data, in the Groningen data, the correlation of the severity of joint damage between hands and feet was moderate (Table 2).

Since the total SHS is composed of information on the severity of erosions and joint space narrowing, analyses were repeated for the total erosion score and total joint space narrowing score separately, leading to findings comparable to that of the total SHS (Tables 1&2).

Table 2. Correlations between the severity of joint damage (according to the Sharp-van der Heijde method) at different sites and different disease durations in Groningen and North-American RA-patients.

Groningen Dataset	Baseline	Year 1-4	Year 5-9	Year 10-25
Comparisons	N=87	N=116	N=136	N=129
<i>Hands & Feet</i>				
Right vs Left (total SHS)	0.92	0.92	0.90	0.93
Right vs Left (total ES)	0.95	0.92	0.91	0.92
Right vs Left (total JSN)	0.80	0.86	0.86	0.90
<i>Hands</i>				
Right vs Left (total SHS)	0.85	0.86	0.86	0.91
Right vs Left (total ES)	0.93	0.87	0.88	0.91
Right vs Left (total JSN)	0.70	0.79	0.79	0.87
<i>Feet</i>				
Right vs Left (total SHS)	0.90	0.87	0.90	0.82
Right vs Left (total ES)	0.87	0.80	0.88	0.80
Right vs Left (total JSN)	0.80	0.87	0.86	0.80
<i>Joint Groups</i>				
Right vs Left MCP (total SHS)	0.55	0.82	0.84	0.83
Right vs Left PIP (total SHS)	0.72	0.73	0.73	0.75
Right vs Left wrists (total SHS)	0.87	0.78	0.81	0.76
Hands vs Feet (total SHS)	0.83	0.74	0.58	0.52
Hands vs Feet (total ES)	0.84	0.69	0.58	0.54
Hands vs Feet (total JSN)	0.81	0.78	0.68	0.65
North American Dataset				
Comparisons	N=48	N=380	N=478	N=766
<i>Hands</i>				
Right vs Left (total SHS)	0.72	0.88	0.90	0.94
Right vs Left (total ES)	0.56	0.88	0.88	0.92
Right vs Left (total JSN)	0.74	0.84	0.87	0.92
<i>Joint Groups</i>				
Right vs Left MCP (total SHS)	0.52	0.83	0.83	0.87
Right vs Left PIP (total SHS)	0.69	0.80	0.81	0.85
Right vs Left Wrist (total SHS)	0.46	0.78	0.80	0.85

Presented are Pearson correlation coefficients. All correlation coefficients are statistically significant (all P values <0.01).

SHS – Sharp-van der Heijde Score, ES – Erosion Score, JSN – Joint Space Narrowing, MCP – Metacarpal Phalangeal Joints, PIP – Proximal Interphalangeal Joints

Next it was questioned whether the high correlations in the left and right comparisons may have been influenced by the magnitude of the SHS. To evaluate this, the agreement between left and right total SHS were portrayed with Bland-Altman plots for all three datasets. Good agreements were observed, indicating that the correlation between right and left total SHS was highly independent of the severity of joint damage.

Comparison of the severity of progression of joint damage

It was evaluated whether using only one hand or foot, two hands, two feet or both hands and feet provided comparable information to monitor progression of joint damage. Progression of joint damage was measured quantitatively using the total SHS.

In Leiden RA-patients two intervals were studied, progression during the first year of follow-up, and progression between year 6 and year 7. Correlation coefficients were determined (Table 3). These were fairly high for left and right comparisons, but lower for hands and feet comparisons. Also here, Bland-Altman plots were made for left and right comparisons, showing that the correlation coefficients were independent of the magnitude of the SHS scores.

Progression analyses could not be performed in the Groningen and North American datasets because too few patients had radiological data at pairs of subsequent follow-up years.

Table 3: Correlations between the severity of progression of joint damage at different sites at different intervals in Leiden RA-patients.

Comparisons	Correlation coefficients (r)	
	Delta year 1-0 N=580	Delta year 7-6 N=189
<i>Hands & Feet</i>		
Δ Right vs Δ Left total SHS	0.798	0.711
<i>Hands</i>		
Δ Right vs Δ Left total SHS	0.791	0.615
<i>Feet</i>		
Δ Right vs Δ Left total SHS	0.707	0.593
Δ Hands vs Δ Feet total SHS	0.294	0.417

Delta year1-0 denotes the difference in SHS score between baseline and year 1

Delta year 7-6 denotes the difference in SHS score between year 6 and year 7

Presented are Pearson correlation coefficients

All correlation coefficients are statistically significant (all P values <0.01).

Comparison of the presence of erosiveness

In the clinical setting, continuous measures for joint damage are seldom used and radio-graphs are often reported on the presence of erosiveness. Therefore we next evaluated to

what extend identification of erosiveness was different when X-rays of all hands and feet were considered compared to considering a part of these X-rays.

Comparison at single time points

First X-rays at individual time points were evaluated. Erosiveness was defined as having ≥ 2 joints with erosions. When studying the baseline X-rays of the Leiden RA patients, 67.4% of the patients had erosive disease. When only the hands were studied 54.9% of the patients had erosive disease. Evaluating one hand instead of both hands would led to incorrect classifying a patients as having non-erosive disease in 19.3% of cases. This proportion was also 19.3% when the X-rays at year 7 were studied. When X-rays of both hands were made but not of the feet, 40% and 24% of the patients were incorrectly classified as having non-erosive disease at baseline and 7 years of follow-up respectively (Table 4).

In the Groningen and North-American datasets, similar results were obtained. For instance, when evaluating the X-rays made during the first years of the disease, respectively

Table 4. Comparison of the frequency of erosive disease at hands and feet in Leiden RA-patients at baseline and after 7 years of follow-up.

	Frequency of erosiveness		Frequency of Misclassification		
	R Hand N (%)	L Hand N(%)	L+R- N (%)	L-R+ N (%)	Total N (%)
Baseline (n=678)	252 (37.2)	243 (35.8)	61 (8.9)	70 (10.3)	131 (19.3)
Year 7 (n=254)	178 (70.1)	171 (67.3)	21 (8.3)	28 (11.0)	49 (19.3)
	R Foot	L Foot			
Baseline (n=678)	116 (17.1)	113 (16.7)	52 (7.7)	55 (8.1)	107 (15.8)
Year 7 (n=254)	141 (55.5)	153 (60.2)	32 (12.6)	20 (7.8)	52 (20.5)
	Hands	Feet			
Baseline (n=678)	372 (54.9)	236 (34.8)	69 (10.2)	205 (30.2)	274 (40.4)
Year 7 (n=254)	214 (84.3)	191 (75.2)	19 (7.4)	42 (16.5)	61 (24.0)

L + R- denotes ≥ 2 erosive joints at the left side but not at the right side

L - R + denotes ≥ 2 erosive joints at the right side but not at the left side

F + H- denotes ≥ 2 erosive joints at the feet but not hands

F- H + denotes ≥ 2 erosive joints at the hands but not feet

Frequency of misclassification in erosiveness = sum of patients with erosions at one side but not at the other side to be divided by total number of patients

Erosiveness was defined as ≥ 2 erosive joints

19.8% and 23.2% of patients would incorrectly be classified as non-erosive if only one of the two hands was evaluated. In addition, evaluating hands without feet in the Groningen set of patients would result in misclassification of 37.1% of the patients.

Comparison of the severity of progression of joint damage

Progression of joint damage can be expressed quantitatively using scoring methods such as the Sharp-van der Heijde method. Although this quantitative outcome measure is more informative than qualitative outcome measures,¹¹ progression can also be expressed binomially. Here patients were identified as having progressive disease in case the erosion score increased for ≥ 2 points in a one-year interval. When studying the first one-year interval in the Leiden RA-patients, it was observed that 25.7% of patients had progression in the right hand, 24.0% in the left hand and 38.6% in both hands (Table 5). If only one hand was used to monitor progression for both hands, 16.2% of patients would be misclassified as having non-progressive disease. If X-rays of both hands without feet would be used to monitor progression, 31.0% of patients would be misclassified as having non-progressive disease. When studying the interval between year 6 and 7, it was observed that smaller percentages of patients progressed; 11.6% of patients progressed in the right hand, 10.6% in the left hand and 21.2% in both hands. If for instance only one hand was used to monitor progression in both hands in this disease phase, 14.8% of patients would be misclassified as non-progressors. Similarly monitoring both hands without feet would lead to such misclassification 21.2%.

Table 5. Comparison of the frequency of progression of erosiveness at hands and feet in Leiden RA-patients at baseline and after 7 years of follow-up.

Leiden EAC	Frequency of pts with progression of erosiveness			Percentages of patients misclassified		
	R Hand (R)	L Hand (L)	Both Hands (H)	L+R-N (%)	L-R+N (%)	Total N (%)
Delta year 1-0	25.7 (149/580)	24.0 (139/580)	38.6 (224/580)	42	52	94 (16.2)
Delta year 7-6	11.6 (22/189)	10.6 (20/189)	21.2 (40/189)	13	15	28 (14.8)
	Hands (H)	Feet (F)	Hands & Feet			
Delta year 1-0	38.6 (224/580)	31.0 (180/580)	52.9 (307/580)	68	112	180 (31.0)
Delta year 7-6	21.2 (40/189)	20.1 (38/189)	35.4 (67/189)	19	21	40 (21.2)

L + R- denotes progression of ≥ 2 erosion points at the left side but not at the right side

L - R + denotes progression of ≥ 2 erosion points at the right side but not at the left side

F + H- denotes progression of ≥ 2 erosion points at the feet but not hands

F- H + denotes progression of ≥ 2 erosion points at the hands but not feet

Frequency of misclassification in progression of erosiveness = sum of patients with progression at one side but not at the other side to be divided by total number

Progression of erosiveness was defined as more progression of ≥ 2 erosion points in the one year interval.

Delta year 1-0 denotes the period between baseline and year-1

Delta year 7-6 denotes period between year-6 and year-7

As sensitivity analyses, analyses were repeated when defining progression in erosiveness as an increase in erosion score of ≥ 3 or ≥ 1 in a one-year interval. This gave comparable results.

DISCUSSION

In the current study, we aimed to examine the difference in information content obtained when radiographing all hands and feet versus only a selection of these. To this end, radiological joint damage data of 6,261 sets of X-rays of 2,193 RA-patients in early and advanced phases of the disease were studied. It was observed that both absolute SHS scores and progression in SHS scores were highly correlated between left and right side X-rays, but that the correlations between hands and feet were much weaker. When evaluating qualitative outcome measures of joint damage (erosiveness), it was demonstrated that using only unilateral or only hands X-rays results in about 20% and 40% of patients that are incorrectly classified as non-erosive, as they had erosions on the non-radiographed site. Together these data suggest that feet X-rays importantly add to the information obtained by hand X-rays and that when joint damage is expressed qualitatively, the percentage of patients with erosive disease is underestimated.

A strength of this study is that it includes a large number of patients and X-rays that are scored by experienced readers. In addition, findings are done in datasets of two different populations and different levels of severity, which adds to the generalisability of the results.

The SHS, measuring the severity of joint damage quantitatively, were highly correlated between the left and right side, both at single time points and when evaluating progression over time. The Bland-Altman plots indicated that there is actual good agreement, which is independent of the level of joint damage. The correlation coefficients even improved at increasing disease durations in the Leiden and North American RA-patients. In the Groningen dataset this trend was not seen, presumably due to the already high correlation at baseline in this dataset. The symptom duration at the first visit of the Groningen and North American RA-patients are not known. It is therefore unclear whether baseline visits represent the same disease stage in the different cohorts.

The above mentioned findings are relevant for studies in which missing radiological data are imputed.¹² Our findings may support to impute missing SHS hands or feet data with information of SHS scores of the radiographed contralateral hand or foot. In contrast, based on these data it is not advocated to impute missing feet data with that of hands. It is important to note that this conclusion concerns missing SHS data in research studies.

However, the data in this study demonstrates that for monitoring purpose in clinical setting, it is better to X-ray both hands and both feet.

Two previous studies showed that patients in an early phase of RA had more feet than hand joints involved.^{13;14} We observed a higher frequency of erosive joints in hands than in feet joints though this may have been influenced by the fact that according to the SHS method, more hand than feet joints are evaluated (32 versus 12 respectively). Several studies have described the value of foot X-rays to diagnose RA¹⁴⁻¹⁸ and a recent study also concluded that both erosions and joint space narrowing should be assessed and that there are no redundant joints.¹⁵ Nonetheless, to the best of our knowledge, the present study is the first to evaluate the correlation between hands and feet joint damage.

In the clinical setting, continuous outcome measures such as the SHS are not used and radiographs are generally reported in terms of erosiveness. In an attempt to study erosiveness, continuous radiological data were categorized. It was observed that, when expressing the data in this way, radiographing a part of the extremities leads to a loss of information compared to radiographing both hands and both feet. Inherent to categorization is the question which cut-off defined erosiveness best. In the cross-sectional analyses a cutoff of ≥ 2 erosive joints was chosen as definition of erosive disease. This was done based on a previous study.¹⁹ In order to categorize progression of erosiveness, an increase of ≥ 2 erosive scores was used, which also was in line with previous studies.²⁰ Other cut-offs were evaluated as well and these analyses did not result in different findings.

Left and right comparisons were made, disregarding information on dexterity or the side of most complaints. Unfortunately, such data were lacking. Van der Heijde et al²¹ previously observed that there was no difference in SHS score between dominant and non-dominant hands.

The result of a recent study on MRI indicated that there is no major difference between dominant and non-dominant hands⁵. In this study joint involvement was evaluated with the RAMRIS score and hence quantitatively and not qualitatively. Our findings are partly in line with this as the total SHS between left and right hands were highly correlated.

In conclusion, evaluations on the presence/absence of erosive disease are preferably done by radiographing both hands and both feet because of the risk of false-negative classification. The severity of joint damage is highly correlated between left and right but not between hands and feet.

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Part II



Chapter 6

Genetic studies on components of the Wnt signaling pathway and the severity of joint destruction in Rheumatoid Arthritis

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ABSTRACT

Objective

Progression of joint destruction in Rheumatoid Arthritis (RA) is partly heritable; knowledge of genetic factors may enhance the comprehension of the mechanisms underlying joint destruction. The activity of the Wnt/ β -catenin pathway influences osteoblast differentiation. Dickkopf-1 (*Dkk-1*) and Sclerostin (*Sost*) are negative regulators and LRP-5 and Kremen-1 are transmembrane receptors involved in this pathway. We studied variants in the genes encoding these proteins in relation to progression of joint destruction.

Methods

1,418 RA-patients of four cohorts with 4,885 sets of hands and feet X-rays were studied. Explorative analyses were performed on 600 RA-patients from Leiden on SNPs tagging *Dkk-1*, *Sost*, *Kremen-1* and *LRP-5*. SNPs significantly associating with joint damage progression were subsequently genotyped in cohorts from Groningen (NL), Sheffield (UK) and Lund (Sweden). Data were summarized in meta-analyses. Serum levels of functional *Dkk-1* and Sclerostin were measured and studied in relation to genotypes.

Results

In the first cohort, 6 *Dkk-1*, 3 *Sost*, 1 *Kremen-1* and 10 *LRP-5* SNPs were significantly associated with radiological progression of joint destruction. Three *Dkk-1* SNPs associated significantly with progression of joint damage in the meta-analysis, also after correction for multiple testing (rs1896368, rs1896367 and rs1528873). Two *Sost* SNPs tended to significance (rs4792909 and rs6503475, $p=0.07$ after FDR-correction). Gene-gene interactions between SNPs on *Dkk-1* and *Sost* were observed. Serum levels of *Dkk-1* were significantly correlated with the genotypes in rs1896368 ($p=0.02$).

Conclusion

RA-patients carrying risk alleles of genetic variants in *Dkk-1* have higher serum levels of functional *Dkk-1* and more progressive joint destruction over time.

INTRODUCTION

Persistent inflammation and destruction of joints are hallmarks of Rheumatoid Arthritis (RA). The natural course of the disease is highly variable between patients. Disease severity is often measured by progression of joint destruction. Known risk factors mainly relate to inflammation and auto-antibodies and are estimated to explain 32% of the variance in joint destruction.¹ Recently, the heritability of the rate of joint destruction was estimated at 58%.² Identifying individual genetic risk factors may enhance the comprehension of mechanisms underlying differences in severity of joint destruction.

The Wnt/ β -catenin pathway is central to bone development and homeostasis. The original descriptions that causal mutations in low-density lipoprotein receptor related protein-5 (LRP-5) or genetic defects in Sclerostin are associated with syndromes characterized by high bone mass (High Bone Mass syndrome and Sclerostosis respectively) raised interest in this signaling pathway and in recent years understanding of the Wnt/ β -catenin pathway has progressed enormously. This pathway involves binding of Wnt proteins to a coreceptor complex, which includes LRP-5 or LRP-6 and a member of the frizzled family of proteins. This binding leads to a signaling cascade that results in intracellular release of β -catenin to the cytoplasm; ultimately osteoblast differentiation is stimulated.^{3,4} Wnt signaling is regulated by several proteins, among which Dickkopf-1 (Dkk-1) and Sclerostin (Sost). Sost binds to LRP-5 and prevents Wnt binding. Dkk-1 can bind LRP-5 and the cell-surface co-receptor Kremen-1; the ternary complex is then internalized and degraded. Hence Dkk-1 and Sost both negatively regulate the Wnt-signal.⁴

Aberrant regulation of Wnt/ β -catenin signaling pathway has been implicated in different types of cancer, cardiovascular and hematological diseases. Circulating levels of Dkk-1 have also been reported to be elevated in RA^{5,6} and have been related to radiographic joint damage in RA.⁷⁻⁹ Diarra et al recently demonstrated that TNF- α is a key inducer of Dkk-1 and that inhibiting Dkk-1 in a mouse model leads to reversal of bone destruction. Moreover, the expression of Sost seems to be positively correlated with Dkk-1 levels.¹⁰ Based on the known functions of Dkk-1, Sost, LRP-5 and Kremen-1 and the recent observation that a considerable part of the variance in joint destruction in RA is explained by genetic factors, we hypothesized that genetic variants in the genes encoding these four proteins that play a crucial role in the Wnt/ β -catenin pathway are associated with progression of joint destruction in RA. We performed a multi-cohort candidate gene study to evaluate this.

PATIENTS AND METHODS

Study population

Four cohorts of adult European RA-patients were studied. RA was defined according to the 1987-ACR criteria except for the Lund cohort where the 1958-criteria were used. X-rays of both hands and feet were available in all cohorts (Table 1).

Table 1. Characteristics of each cohort

	Cohort			
	Leiden-EAC (n=600)	Groningen (n=275)	Sheffield (n=391)	Lund (n=147)
Year of diagnosis	1993-2006	1945-2001	1938-2003	1985-1989
Total number of x-ray sets	2846	862	391	781
Follow-up years*	7 years	14 years	Not applicable*	5 years
Method of scoring	SHS	SHS	Larsen	Larsen
Female, n (%)	412 (69)	194 (71)	290 (73)	98 (67)
Age at diagnosis (years), mean±SD	56±16	49±13	46±13	51±12
Anti-CCP+, n (%)	323 (55)	160 (80)	302 (79)	114 (80)

*Data of Leiden-EAC, Groningen and Lund were collected from baseline onwards for 7, 14 and 5 years of follow-up, respectively. The data of Sheffield were collected during the disease period; the mean disease duration was 15 years (range 3-65 years). EAC: Early Arthritis Clinic, SHS: Sharp-van-der-Heijde score, anti-CCP: anti-citrullinated peptide antibodies.

Leiden Early Arthritis Clinic (Leiden-EAC)

600 early RA-patients included in the Leiden-EAC between 1993 and 2006 were studied.¹ X-rays were taken at baseline and yearly thereafter during 7-follow-up years (in total 2,846 sets of hands and feet X-rays). X-rays were chronologically scored by one reader using the Sharp-van der Heijde Score (SHS).¹¹ The correlation coefficient (ICC) within the reader was 0.91. Treatment strategies were different in three treatment-periods: (1993-1995, initial treatment with NSAIDs, 1996-1998, initial treatment with chloroquine or salazopyrine and 1999-2006, prompt treated with methotrexate or salazopyrine).

Groningen

The second cohort involved 275 RA-patients from the northern part of the Netherlands that were diagnosed between 1945-2001. Over at most 14 years of follow-up the mean number of X-ray sets (hands and feet) per patient was 3.1. The X-rays were scored chronologically using SHS (ICC 0.96). Patients included in the 1990's were treated with (mild) DMARD-therapy in contrast to patients included before 1990.

Sheffield

396 RA-patients diagnosed in 1938-2003 with X-rays available were recruited from the Rheumatology department of the Royal Hallamshire Hospital in Sheffield, UK in 1999-2006.¹² RA-patients were assessed once at a mean ($\pm$ SD) disease duration of 15 ± 11 years. Hands and feet X-rays were scored by one reader using a modification to Larsen's score.¹³ 10% of films were scored twice to quantify the intra-observer variation by a weighted kappa score which was 0.83.¹²

Lund

This cohort concerned 183 Swedish early RA-patients that were prospectively followed during 5 years, of which 147 had X-rays and DNA available.^{14;15} Patients were recruited during 1985-1989. X-rays of hands and feet were taken at baseline and annually for 5-years, resulting in a total of 781 sets of X-rays. X-rays were scored chronologically according to Larsen by one of two readers (ICC between readers 0.94).¹⁶ In the first years DMARD-therapy was uncommon and only half of the patients used any DMARD at 5-years follow-up, most commonly chloroquine, D-penicillamine, sodium aurothiomalate and auranofin.¹⁵

All patients gave informed consent. Approval was obtained from the local Medical Ethical Committee of each cohort.

SNP selection and genotyping

Pairwise tagging SNPs ($n=77$) (± 300 kb) were selected from the CEPH/CEU hapmap dataset (phase-II, release-21, NCBI build-35) using haploview software ($MAF > 0.05$, pairwise $r^2 > 0.8$). For *Dkk-1*, *Sost*, and *Kremen*, no amino acid changing SNPs were known. For *LRP-5*, rs3736228 is amino acid changing and has been associated with bone mineral density in various studies.^{17;18} This SNP was therefore forced into the selection. SNPs that were significantly associated with joint destruction in phase-1 were genotyped in the other three cohorts. Genotyping was done with an Illumina GoldenGate assay in phase-1 and Sequenom iPLEX in phase-2, as described previously.¹⁹ The overall error rate was $< 1\%$; the success rates were $> 95\%$.

Measuring serum levels of Dkk-1 and plasma levels of Sost

Levels of functional Dkk-1 in the baseline sera of 80 RA-patients from the Leiden-EAC were measured using ELISA (BI-20412, Biomedica, Austria). Levels of Sclerostin were determined in baseline plasma of a different set of 80 patients using ELISA (BI-20492, Biomedica, Austria). For both ELISA's, patients were selected on the genotype in the most significantly associating SNP.

Statistical analysis

In all cohorts, the radiological scores were log-transformed to obtain normal distributions (Supplementary Figure 1/2). Associations between genotypes and radiographic joint destruction were analyzed in two phases. First, an explorative analysis was performed; tagged SNPs were tested additively and recessively. Since this phase was explorative, no correction for multiple testing was applied yet and SNPs with a p-value <0.05 were studied in phase-2 using an additive or recessive model, based on the findings in phase-1.

Phase-1

A multivariate normal regression model for longitudinal data was used with the radiological score as a response variable. This method analyses all repeated measurements at once and takes advantage of the correlation between these measurements. To test for an association with the rate of joint destruction, an analysis with the SNP and its interaction with time in the model was conducted. The effect of time in the interaction term was linear. The resulting coefficient (beta) indicates how many fold the progression of joint destruction increased per year per minor allele when an additive effect was tested, and how many fold the progression of joint destruction increased per year in the presence of two minor alleles when a recessive model was tested. Adjustment variables (age, gender, treatment strategies) were entered based on their univariate association with joint destruction.

Phase-2

For the analyses in Groningen and Lund a multivariate normal regression analysis was used similar to that applied in the Leiden-EAC. Analyses in the Groningen cohort were adjusted for age and a proxy for DMARD-therapy (inclusion </>1990). The analyses on Lund were adjusted for age only, since gender and treatment were not associated with joint destruction in this dataset. In the Sheffield cohort, each patient had X-rays at one time point only. To make scores comparable to the other cohorts, the estimated yearly progression rate was calculated by dividing the total SHS by the number of disease years at the time of the X-ray.²⁰ The SNP association was tested in a linear regression analysis with the log-transformed estimated yearly progression rate as outcome. No adjustments were applied as none of the tested variables were associated with joint destruction. Also here, the resulting estimate reflects how many fold the rate of joint destruction increases per year in the presence of a risk variant. Analyses were done using SPSS version 17.0.

Meta-analysis

All three cohorts studied in phase-2 contained fewer patients and less X-rays than the initiation cohort. Consequently, the power was insufficient to replicate findings in each cohort individually, as well as in the three replication cohorts together. Because of differences in study designs, the separate cohorts could not be combined in one analysis directly. There-

fore, it was decided to test the SNPs in each cohort separately taking advantage of the specific dataset characteristics and to subsequently perform a fixed-effects meta-analysis with inverse variance weighting.²¹ Since the parameters in all datasets reflect the relative increase in the rate of joint destruction per year, it was allowed to pool the estimates of the individual cohorts in a meta-analysis.²² Stata, version 10.1 was used.

Multiple testing

To reduce the chance on false-positive findings, multiple testing correction using the Benjamini and Hochberg False Discovery Rate (FDR), was done in phase-2. P-values <0.05 after correction for multiple testing were considered significant.²³

Haplotype analysis

Haplotypes of Dkk-1 were studied. Blocks were defined by Gabriel's method.²⁴ Haplotypes were assigned to each individual using PLINK 1.06 requiring a probability >0.8. Analyses of the haplotypes were performed with methods similar to those used for the analyses of the individual SNPs by now testing the presence of a haplotype compared to the absence of the haplotype.

Gene-gene interactions

Multiplicative interactions between the significantly associating SNPs on Dkk-1 and Sost were tested by including the multiplication of the Dkk-1 SNP, Sost SNP and time into the additive model on the Leiden RA-patients. Also here correction for multiple testing was performed.

RESULTS

A complete overview of the results of all SNPs studied in phase 1 is presented in Supplementary Table 1.

Dkk-1

Phase-1

Dkk-1 was tagged by eight SNPs. Six of these associated significantly with the rate of joint destruction in the Leiden-EAC in an additive model: rs1896368, rs10762715, rs1528873, rs1441124, rs1194750 and rs1896367. Patients with one minor allele had respectively a 1.02 (95% CI 1.01-1.04), 1.03 (1.01-1.05), 1.02 (1.00-1.04), 1.03 (1.01-1.05), 1.04 (1.01-1.07) and 0.98 (0.96-0.99) fold progression rate per year per minor allele (Figure 1). An estimate of 1.03 per year equals a 23% (1.03^7) higher rate of joint destruction over seven

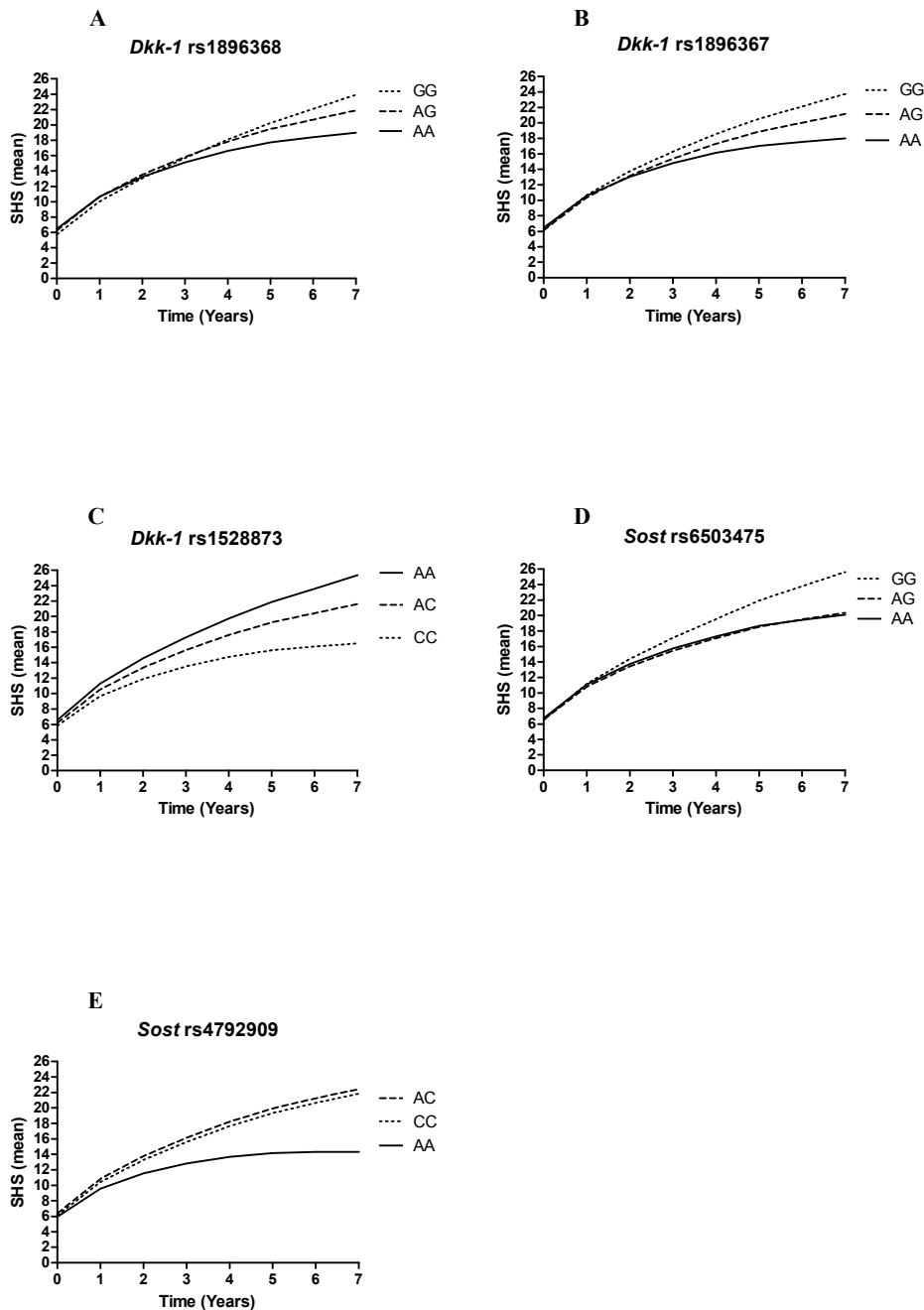


Figure 1. Radiographic joint damage progression in Leiden RA patients

Depicted are the predicted (by the multivariate normal regression model) SHS values (mean) during 7 years of follow-up of patients with different genotypes in phase 1 (Leiden-EAC) for the three SNPs in *Dkk-1* that remained significant after correction for multiple testing and the two SNPs in *Sost* that were significant in the meta-analysis. All *Dkk-1* SNPs have been analyzed in an additive model, all *Sost* SNPs in a recessive model.

years whereas a beta of 0.98 per year results in a 14% lower rate of joint destruction over seven years. Haplotype analyses were performed but did not yield additional information to the SNP analyses.

Phase-2

The significantly associating SNPs were typed in the cohorts from Sheffield, Groningen and Lund. As the numbers of X-rays of the individual cohorts were lower than of phase-1, it was not expected to achieve significant results in each cohort. We therefore performed a meta-analysis summarizing the results of the four cohorts in phase-2. All six SNPs associated significantly with radiographic joint damage progression (see Table 2, Figure 2). The strongest association was found for rs1896368 ($p < 0.001$). After correction for multiple testing, 3 SNPs remained significant (rs1896368, rs1896367 and rs1528873, Table 2).

Sost

Phase-1

Sost was tagged by nine SNPs. Three of these associated significantly with the rate of joint destruction in phase 1 using a recessive model. Patients carrying two minor alleles of rs4792909 had a 0.95 (0.91-0.99) fold higher progression rate per year compared to patients carrying one or no minor alleles. When patients carried two minor alleles of

Table 2. Results of inverse variance meta-analyses on the 4 cohorts

	Uncorrected p-value	FDR corrected p-value
Dkk-1		
rs1896368	0.001	0.013
rs10762715	0.034	0.074
rs1528873	0.009	0.039
rs1441124	0.033	0.086
rs1896367	0.003	0.020
rs11001702	0.84	-
rs1194750	0.031	0.1
Sost		
rs4792909*	0.010	0.065
rs6503475*	0.028	0.073
rs12600549*	0.071	-
LRP-5		
rs3736228	0.063	-
Kremen-1		
rs1322774	0.05	0.09

*Tested recessively. FDR-correction: correction according to Benjamini-Hochberg False Discovery Rate.

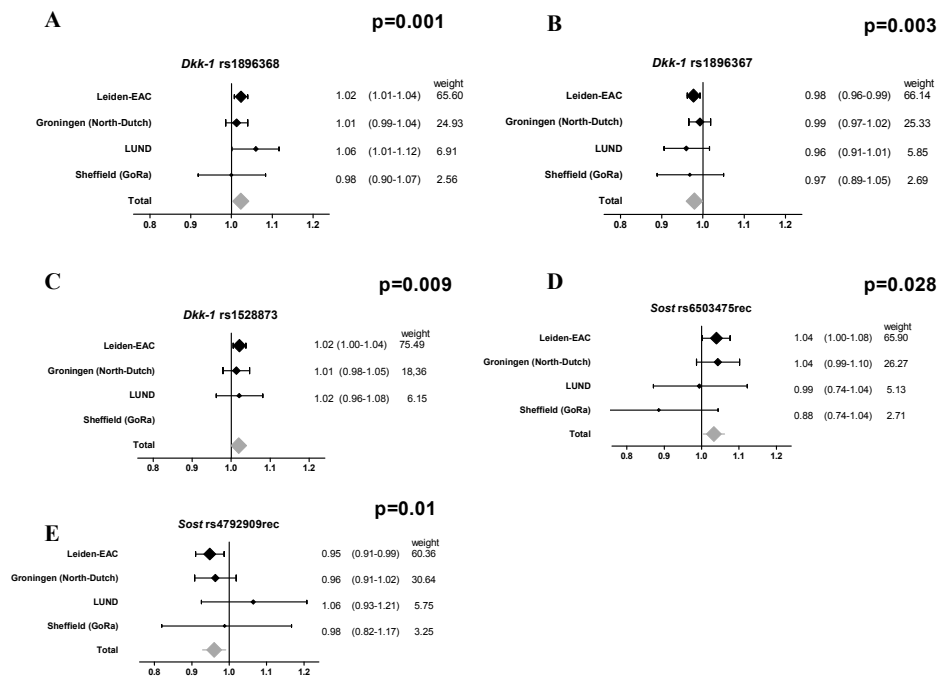


Figure 2. Results of the meta-analysis
Depicted are the results of the analyses of the individual cohorts and the results of the meta-analysis performed on the SNPs that were significant (after correction for multiple testing) in phase 2. The p-values in the graphs are FDR corrected for multiple testing. The meta-analyses are based on a fixed-effects model, which is applied to genetic studies to test whether there is a statistically significant effect; generalisability of the effect is of less importance. As a result of this choice, this method is less suitable to estimate the effect size overall. Therefore, the estimated effect of the meta-analysis is depicted in gray and without numbers. After FDR correction, rs1896368, rs1896367 and rs1528873 in *Dkk-1* were significant. The I-squares for these SNPs were all 0.0%, indicating little heterogeneity between the cohorts. The effect sizes of these three SNPs in the meta-analyses were 1.02 (95CI 1.01-1.04), 0.98 (95CI 0.97-0.99), and 1.02 (95CI 1.00-1.04), respectively. Rs1528873 could not be typed in the Sheffield patients. EAC, Early Arthritis Clinic; SNP, single-nucleotide polymorphism.

rs6503475, they had a 1.04 (1.00-1.08) fold higher progression rate per year. In addition, patients with one or two minor alleles of rs1260054 had a 0.95 (0.92-0.99) fold higher progression rate than patients carrying one or no minor allele.

Phase-2

These three SNPs were studied in the three other cohorts using a recessive model. In the meta-analysis two SNPs were significantly associated with joint damage progression: rs4792909 and rs6503475 (Table 2). However, after FDR-correction for multiple testing, only a trend towards significance remained ($p=0.07$ for both SNPs).

LRP-5

LRP-5 was tagged by 44 SNPs. Ten associated significantly with radiographic progression in phase-1, among which rs3736228 (beta 0.97 (0.95-0.99)). Due to technical difficulties, only one (rs3736228) could be typed in the three other cohorts. This SNP, which is amino acid changing, showed no significant association with radiographic joint damage progression in phase-2 assuming an additive model (Table 2).

Kremen-1

Kremen-1 was tagged by 16 SNPs. Three associated significantly with joint destruction: rs1322774 (beta 0.98 (0.96-0.99)). After typing in the three additional cohorts and analyzing the combined results in a meta-analysis, rs1322774 showed a borderline significant effect ($p=0.05$), which was lost after correction for multiple testing (Table 2).

Interactions between Dkk-1 and Sost

Subsequently it was analyzed whether gene-gene interactions between components of the Wnt/ β -catenin pathway exist. Several SNPs in *Dkk-1* ($n=6$) and *Sost* ($n=2$) associated with progression of joint destruction (phase-2 before multiple testing correction). The 12 possible *Dkk-1-Sost* combinations were analyzed for multiplicative interactions in Leiden RA-patients. Subsequently the resulting p-values were corrected for performing 12 tests, by both FDR and Bonferroni correction (Supplementary table 2). After FDR correction, three combinations of SNPs were significant: rs6503475 (*Sost*) and rs10762715 (*Dkk-1*) (corrected $p=0.05$), between rs4792909 (*Sost*) and rs10762715 (*Dkk-1*) (corrected $p=0.03$), and between rs6503475 (*Sost*) and rs1528873 (*Dkk-1*) (corrected $p=4.52 \times 10^{-9}$). The SHS data in the presence of a combination of risk alleles of these SNPs are presented in Figure 3.

Correlations between genotypes and levels of Dkk-1 and Sclerostin

Next we studied whether the genetic variants in the best associating SNP on *Dkk-1* (rs1896368) and on *Sost* (rs4792909 and rs6503475 were analyzed both because of comparable effect sizes) associated with serum levels of functional *Dkk-1* and plasma levels of Sclerostin respectively. Plasma levels of Sclerostin were not significantly associated with rs4792909 and rs6503475 genotypes (data not shown). However, patients carrying the rs1896368-GG genotype in *Dkk-1*, which is associated with higher radiographic progression rates, had significantly higher serum levels of functional *Dkk-1* than patients carrying the other genotypes ($p=0.023$, Figure 4).

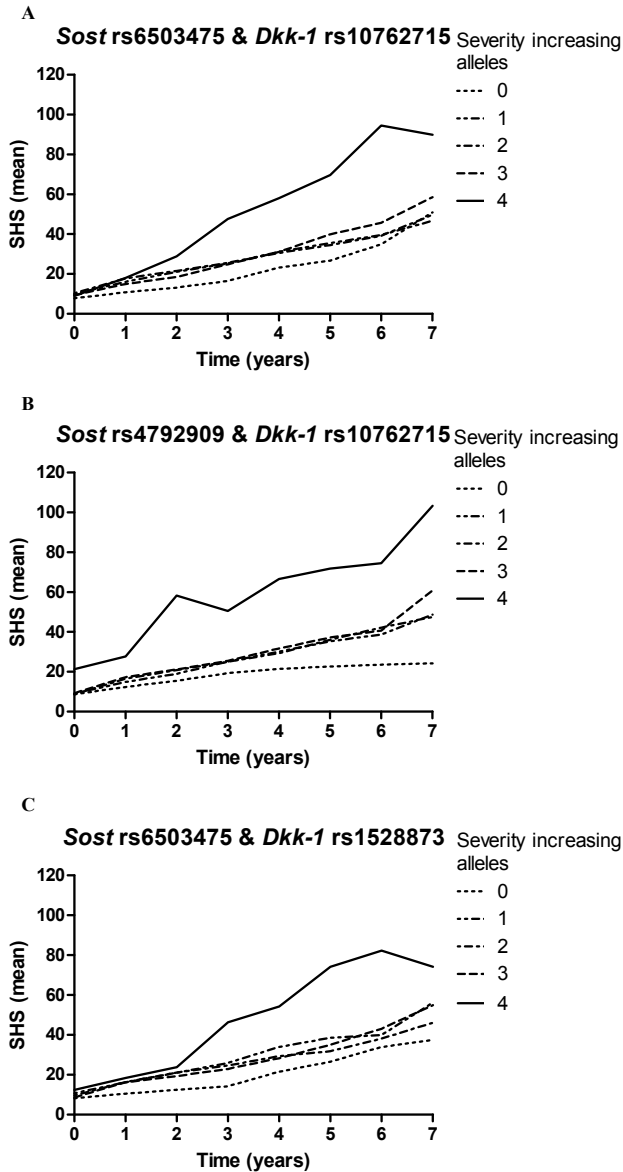


Figure 3. Radiological destruction data of SNPs on *Dkk-1* and *Sost* in which multiplicative interaction was observed

Depicted are the mean SHSs during 7 years of follow-up of patients with different genotypes of A. *Sost* rs6503475 and *Dkk-1* rs10762715, for patients with no risk alleles on one of these SNPs (n=69), one risk allele (n=158), two risk alleles (n=157), 3 risk alleles (n=53) and twice the risk allele for both SNPs (in total four risk alleles) (n=9), B. *Sost* rs4792909 and *Dkk-1* rs10762715, for patients with no risk alleles on one of these SNPs (n=108), one risk allele (n=263), two risk alleles (n=163), 3 risk alleles (n=49) and twice the risk allele for both SNPs (in total four risk alleles) (n=9), and C. *Sost* rs6503475 and *Dkk-1* rs1528873, for patients with no risk alleles on one of these SNPs (n=40), one risk allele (n=114), two risk alleles (n=170), 3 risk alleles (n=104) and twice the risk allele for both SNPs (in total four risk alleles) (n=18).

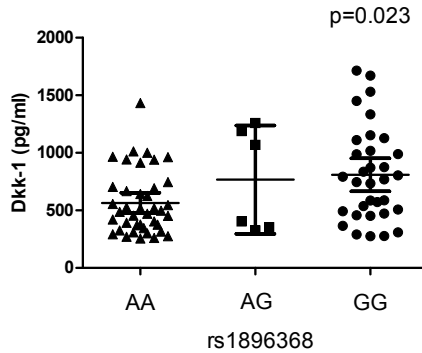


Figure 4. Levels of Dkk-1 in relation to various genotypes

Depicted are the levels of functional Dkk-1 in the sera of 80 patients and the corresponding genotypes in rs1896368 in Leiden RA-patients that were selected on their rs1896368 genotypes.

DISCUSSION

We evaluated four genes encoding proteins that are critically involved in the Wnt/ β catenin signaling pathway in relation to the severity of progression of joint destruction in RA. 4,885 X-ray sets of hands and feet radiographs of 1,418 RA-patients were studied to this end. The major finding is that RA-patients carrying the risk alleles of several genetic variants in *Dkk-1* have higher serum levels of functional Dkk-1 and more progressive joint destruction over time. These findings are in line with recent observations that Dkk-1 serum levels are associated with joint destruction.^{7,8} Together these data illustrate the relevance of *Dkk-1* to progression of joint destruction in RA. Based on these data it may also be hypothesized that blocking the binding of Dkk-1 to LRP-5 or LRP-6 may lead to new therapeutic strategies to protect patients against progression of joint damage.

Two genetic variants in *Sost* were suggestively associated with progression of joint destruction, but after correction for multiple testing only a tendency towards significance remained. Furthermore the results on *Sost* were obtained using a recessive model and a small number of patients homozygous for the minor allele. Altogether, definite conclusions on *Sost* in relation to joint damage cannot be drawn. Nonetheless, rs1513670, a full proxy of rs6503475 in *Sost*, was recently associated with bone mineral density in a genome-wide study, raising interest in this particular variant in relation to bone loss.²⁵ In order to search for functional consequences of carrying the risk genotypes of this SNP, we also studied plasma *Sost* levels, no association was detected. This does not preclude that carrying the risk genotypes is biologically relevant as *Sost* is mainly expressed by osteocytes, and local expression in bone may not be measurable in the systemic circulation.

Four Wnt signaling pathways have been described in the literature.²⁶ The Wnt/ β -catenin signaling pathway in particular is known for its pivotal role in bone homeostasis. We therefore performed the present candidate gene study, evaluating tagging SNPs of four relevant genes belonging to the Wnt signaling pathway. An advantage of analyzing several factors in one pathway is the ability to look for interactions. Hypothetically, carrying two different risk factors may have an enhancing effect that is larger than what would be expected than from combining the two. Additionally a cross-talk between *Dkk-1* and *Sost* has recently been observed in vitro.¹⁰ We studied interactions between the *Dkk-1* and *Sost* SNPs and observed significant multiplicative interactions. The most compelling effect on joint damage was present for patients carrying four risk alleles, which was a small group of patients. To evaluate whether the observed interaction was totally driven by these patients, we repeated the interaction analysis for rs1528873 and rs6503475 with exclusion of the patients carrying four risk alleles. Then, still a significant effect remained ($p=0.021$), indicating that the observed finding was not entirely based on this small patients group. Since the numbers of patients in the replication cohorts were smaller than that in phase-1 and data could not be easily combined, interactions were not analyzed in the cohorts of phase-2.

This study has limitations. The most relevant limitation is that the cohorts used in phase-2 contained less radiographs than the initial cohort. Hence the replication cohorts were insufficiently powered to achieve independent replications. However, since large prospective cohorts with high quality phenotypic data are scarcely available, it was decided to combine the data in a meta-analysis.

In the present study genetic variants in the *Dkk-1* gene were associated with increased joint destruction rates and with higher serum levels. The serological data are in line with findings of other studies where low serum levels were found in diseases characterized by bony depositions, such as ankylosing spondylitis and diffuse idiopathic skeletal hyperostosis.^{27;28} To evaluate whether the functional effects of carrying the *Dkk-1* risk genotype also influenced the *Dkk-1* serum levels, both SNP and serology data were included in one model with the radiographic progression rate as an outcome variable. Here, only the SNP effect was significant. This may be caused by the fact that the 80 patients who were studied on serum-level were selected on their genotypes.

In conclusion, this study evaluated phenotypic and genetic data of four longitudinal cohorts and showed that RA-patients carrying risk alleles of genetic variants in *Dkk-1* have higher serum levels of functional *Dkk-1* and more progressive joint destruction over time. These data illustrate the relevance of *Dkk-1* to progression of joint destruction in RA.

COMPETING INTERESTS

None declared.

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Supplementary table 1. Minor Allele Frequencies (MAF), P-values, beta's and their 95% CI for all SNPs tested in phase 1.

Name	MAF		P-value	Estimate	(95% CI)
Dkk-1					
rs1194750	0.10	Add	0.009	1.04	(1.01-1.07)
		Rec	0.849	1.01	(0.89-1.16)
rs1896368	0.14	Add	0.005	1.02	(1.01-1.04)
		Rec	2.55E-05	1.06	(1.03-1.09)
rs10762715	0.34	Add	0.001	1.03	(1.01-1.05)
		Rec	0.022	1.05	(1.01-1.09)
rs1528873	0.53	Add	0.019	1.02	(1.00-1.04)
		Rec	0.099	1.02	(1.00-1.05)
rs1441124	0.44	Add	0.003	1.03	(1.01-1.04)
		Rec	0.004	1.05	(1.01-1.08)
rs1896367	0.42	Add	0.008	0.98	(0.96-0.99)
		Rec	0.311	0.98	(0.96-1.01)
rs11001702	0.10	Add	0.254	0.98	(0.96-1.01)
		Rec	0.288	1.07	(0.95-1.21)
rs7083441	0.09	Add	0.387	0.99	(0.96-1.02)
		Rec	0.259	1.12	(0.92-1.36)
Kremen-1					
rs12166946	0.07	Add	0.081	0.97	(0.94-1.00)
		Rec	0.078	0.86	(0.72-1.02)
rs1322774	0.35	Add	0.019	0.98	(0.96-0.99)
		Rec	0.079	0.97	(0.94-1.00)
rs1540900	0.03	Add	0.371	0.98	(0.94-1.02)
		Rec	0.779	0.98	(0.83-1.15)
rs134615	0.48	Add	0.748	1.00	(0.99-1.02)
		Rec	0.795	1.00	(0.97-1.02)
rs134666	0.28	Add	0.540	1.01	(0.99-1.02)
		Rec	0.831	1.00	(0.96-1.05)
rs134619	0.35	Add	0.778	1.00	(0.99-1.02)
		Rec	0.597	0.99	(0.96-1.03)
rs6006029	0.25	Add	0.182	0.99	(0.97-1.01)
		Rec	0.116	0.97	(0.93-1.01)
rs134649	0.46	Add	0.681	1.00	(0.98-1.02)
		Rec	0.718	0.99	(0.96-1.03)
rs5762986	0.11	Add	0.883	1.00	(0.98-1.03)
		Rec	0.511	0.96	(0.86-1.08)
rs134656	0.41	Add	0.707	1.00	(0.99-1.02)
		Rec	0.776	1.00	(0.97-1.04)
rs2205771	0.17	Add	0.897	1.00	(0.98-1.02)

Name	MAF		P-value	Estimate	(95% CI)
rs132268	0.34	Rec	0.133	0.94	(0.87-1.02)
		Add	0.656	1.00	(0.98-1.01)
rs12167060	0.18	Rec	0.649	0.99	(0.95-1.03)
		Add	0.198	0.99	(0.97-1.01)
rs132275	0.34	Rec	0.822	1.01	(0.94-1.08)
		Add	0.027	0.98	(0.96-1.00)
rs132280	0.22	Rec	0.146	0.98	(0.94-1.01)
		Add	0.783	1.00	(0.98-1.02)
rs16987051	0.06	Rec	0.587	1.02	(0.96-1.07)
		Add	0.867	1.00	(0.96-1.03)
		Rec	0.032	1.26	(1.02-1.56)
Sost					
rs2741856	0.06	Add	0.652	0.99	(0.96-1.03)
		Rec	0.012	0.87	(0.78-0.97)
rs17531431	0.06	Add	0.733	0.99	(0.96-1.03)
		Rec	0.765	0.97	(0.80-1.17)
rs17610444	0.09	Add	0.586	1.01	(0.98-1.04)
		Rec	0.753	0.98	(0.85-1.13)
rs865429	0.11	Add	0.913	1.00	(0.97-1.03)
		Rec	0.317	0.92	(0.79-1.08)
rs9902563	0.42	Add	0.123	0.99	(0.97-1.00)
		Rec	0.039	0.97	(0.94-1.00)
rs4792909	0.31	Add	0.485	0.99	(0.98-1.01)
		Rec	0.011	0.95	(0.91-0.99)
rs2090019	0.52	Add	0.740	1.01	(0.97-1.05)
		Rec	na	na	na
rs6503475	0.41	Add	0.127	1.02	(1.00-1.04)
		Rec	0.033	1.04	(1.00-1.08)
rs12600549	0.33	Add	0.610	1.00	(0.98-1.01)
		Rec	0.023	0.95	(0.92-0.99)
LRP-5					
rs11826287	0.18	Add	0.510	0.99	(0.97-1.01)
		Rec	0.949	1.00	(0.94-1.07)
rs2512622	0.18	Add	0.216	0.99	(0.97-1.01)
		Rec	0.305	0.97	(0.91-1.03)
rs7117788	0.09	Add	0.802	1.00	(0.97-1.03)
		Rec	0.604	1.05	(0.87-1.27)
rs11228274	0.10	Add	0.420	1.01	(0.98-1.04)
		Rec	0.318	1.08	(0.93-1.25)
rs1996	0.09	Add	0.034	1.03	(1.00-1.06)
		Rec	0.001	1.26	(1.10-1.45)

Name	MAF		P-value	Estimate	(95% CI)
rs4988321	0.06	Add	0.003	0.94	(0.91-0.98)
		Rec	na	na	na
rs3781579	0.09	Add	0.075	1.03	(1.00-1.06)
		Rec	0.005	1.21	(1.06-1.37)
rs882979	0.22	Add	0.102	0.98	(0.96-1.00)
		Rec	0.975	1.00	(0.95-1.06)
rs624947	0.31	Add	0.301	1.01	(0.99-1.03)
		Rec	0.076	1.04	(1.00-1.08)
rs314756	0.08	Add	0.193	1.02	(0.99-1.05)
		Rec	0.993	1.00	(0.87-1.14)
rs7950900	0.34	Add	0.625	1.00	(0.98-1.01)
		Rec	0.717	1.01	(0.97-1.04)
rs676318	0.09	Add	0.446	0.99	(0.96-1.02)
		Rec	0.890	1.01	(0.90-1.13)
rs312016	0.31	Add	0.361	0.99	(0.97-1.01)
		Rec	0.426	0.98	(0.94-1.02)
rs3736228	0.14	Add	0.041	0.98	(0.95-1.00)
		Rec	0.627	0.97	(0.88-1.08)
rs606989	0.08	Add	0.353	1.02	(0.98-1.05)
		Rec	0.528	1.05	(0.90-1.23)
rs7946537	0.13	Add	0.543	1.01	(0.98-1.03)
		Rec	0.512	0.96	(0.85-1.08)
rs4930593	0.30	Add	0.540	0.99	(0.98-1.01)
		Rec	0.705	0.99	(0.95-1.03)
rs2510389	0.09	Add	0.059	1.03	(1.00-1.06)
		Rec	0.002	1.20	(1.07-1.35)
rs3781600	0.10	Add	0.286	0.99	(0.96-1.01)
		Rec	0.792	1.01	(0.92-1.12)
rs312781	0.28	Add	0.187	1.01	(0.99-1.03)
		Rec	0.704	1.01	(0.96-1.05)
rs4930590	0.36	Add	0.749	1.00	(0.98-1.01)
		Rec	0.914	1.00	(0.96-1.03)
rs312014	0.39	Add	0.092	0.99	(0.97-1.00)
		Rec	0.406	0.99	(0.95-1.02)
rs11228269	0.24	Add	0.729	1.00	(0.98-1.02)
		Rec	0.173	1.04	(0.98-1.09)
rs312023	0.45	Add	0.013	1.02	(1.00-1.04)
		Rec	0.148	1.02	(0.99-1.06)
rs312778	0.38	Add	0.378	1.01	(0.99-1.02)
		Rec	0.195	1.02	(0.99-1.05)
rs729635	0.10	Add	0.683	0.99	(0.97-1.02)

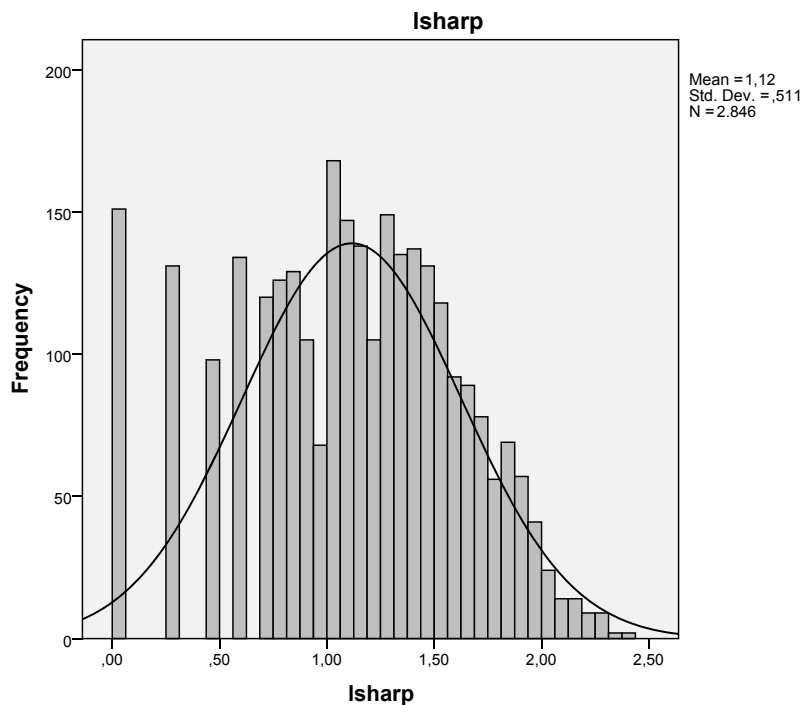
Name	MAF		P-value	Estimate	(95% CI)
rs4930573	0.25	Rec	0.631	1.03	(0.92-1.15)
		Add	0.091	0.98	(0.96-1.00)
rs638051	0.37	Rec	0.971	1.00	(0.95-1.05)
		Add	0.537	1.01	(0.99-1.02)
rs11600189	0.09	Rec	0.971	1.00	(0.97-1.03)
		Add	0.285	0.98	(0.96-1.01)
rs11606508	0.14	Rec	0.493	0.96	(0.87-1.07)
		Add	0.465	0.99	(0.97-1.01)
rs11602256	0.30	Rec	0.422	0.97	(0.89-1.05)
		Add	0.906	1.00	(0.98-1.02)
rs4988331	0.08	Rec	0.639	1.01	(0.97-1.05)
		Add	0.016	0.96	(0.94-0.99)
rs12804775	0.08	Rec	0.666	0.96	(0.81-1.14)
		Add	0.029	1.03	(1.00-1.07)
rs556442	0.41	Rec	0.993	1.00	(0.89-1.13)
		Add	0.627	1.00	(0.98-1.01)
rs546803	0.26	Rec	0.074	0.97	(0.95-1.00)
		Add	0.045	0.98	(0.96-1.00)
rs624003	0.33	Rec	0.030	0.95	(0.90-0.99)
		Add	0.476	0.99	(0.98-1.01)
rs12222446	0.26	Rec	0.574	1.01	(0.97-1.05)
		Add	0.249	1.01	(0.99-1.03)
rs7948782	0.14	Rec	0.820	0.99	(0.95-1.04)
		Add	0.211	0.98	(0.96-1.01)
rs17148984	0.20	Rec	0.097	0.90	(0.79-1.02)
		Add	0.483	0.99	(0.97-1.01)
rs2458266	0.41	Rec	0.996	1.00	(0.95-1.06)
		Add	0.271	1.01	(0.99-1.03)
rs2510404	0.05	Rec	0.165	1.02	(0.99-1.05)
		Add	0.452	0.98	(0.94-1.03)
rs948862	0.25	Rec	0.927	0.99	(0.86-1.14)
		Add	0.289	0.99	(0.97-1.01)
rs7123564	0.24	Rec	0.565	0.99	(0.94-1.03)
		Add	0.606	1.01	(0.98-1.03)
rs2277268	0.05	Rec	0.424	1.02	(0.97-1.08)
		Add	0.037	1.04	(1.00-1.09)
		Rec	na	na	na

na: not available. The beta indicates how many fold the progression of joint destruction increased per year per minor allele when an additive effect was tested, and how many fold the progression of joint destruction increased per year in the presence of two minor alleles when a recessive model was tested.

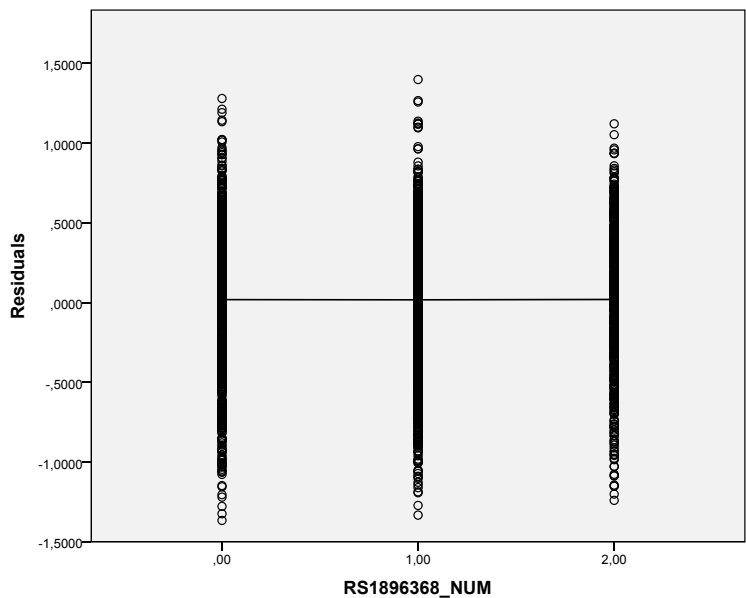
Supplementary table 2. P-values for the tested interactions between SNPs in Dkk-1 and Sost, p-values are presented uncorrected as well as after FDR- or Bonferroni

correction			
Interaction	P	FDR-corrected	Bonferroni-corrected
rs6503475 * rs1528873	3.77x10-10	4.52x10-9	4.52x10-9
rs4792909 * rs10762715	0.005	0.029	n.s.
rs6503475 * rs10762715	0.013	0.05	n.s.
rs4792909 * rs1194750	0.034	n.s.	n.s.
rs4792909 * rs1896367	0.051	n.s.	n.s.
rs6503475 * rs1194750	0.074	n.s.	n.s.
rs6503475 * rs1896367	0.44	n.s.	n.s.
rs6503475 * rs1896368	0.44	n.s.	n.s.
rs4792909 * rs1441124	0.66	n.s.	n.s.
rs4792909 * rs1528873	0.85	n.s.	n.s.
rs6503475 * rs1441124	0.86	n.s.	n.s.
rs4792909 * rs1896368	0.88	n.s.	n.s.

n.s: not significant



Supplementary Figure 1. Data after log-transformation



Supplementary Figure 2. Residuals plot

Chapter 7

Studying associations between variants in *TRAF1-C5* and *TNFAIP3-OLIG3* and the progression of joint destruction in rheumatoid arthritis in multiple cohorts

LETTER TO THE EDITOR

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The severity of joint destruction in Rheumatoid Arthritis (RA) is highly variable between patients. Recent twin and population studies indicated that the severity of joint destruction is influenced by genetic factors.^{1,2} Previously we reported associations of rs10818488 (*TRAF1-C5*) and rs675520 (*TNFAIP3-OLIG3*) with progression of joint destruction.^{3,4} The genes near these loci encode for tumor necrosis factor receptor-associated factor-1 (*TRAF1*), complement component-5 (*C5*) and tumor necroses factor alpha-induced protein-3 (*TNFAIP3*); a protein that inhibits NF-kappa B-activation. A basic principle in genetic association studies is to evaluate multiple cohorts to validate observed findings. We therefore studied both SNPs in several RA-cohorts with radiological follow-up data

6,282 X-rays of 2,666 RA-patients were studied: 147 patients from Lund (Sweden), 385 patients from Sheffield (UK), 285 patients from Iceland, 384 patients from the North American Rheumatoid Arthritis Consortium (NARAC), 756 patients from the National Databank of Rheumatic Diseases (NDB), 113 patients from Wichita and 596 patients from the Leiden-EAC (Table 1). Detailed information on these datasets is provided elsewhere.^{2,5-10} Genotyping of Lund and Sheffield DNA was performed using Sequenom analysis, genotyping results of the Icelandic and NARAC patients were retrieved from genome-wide-association studies^{2,7} and those of the Wichita and Leiden cohorts were done with the Illumina Immunochip.¹¹ Radiographs were scored according to the Sharp-van der Heijde method (Iceland, Wichita, NDB, Leiden-EAC) or Larsen method (Lund, Sheffield). Per dataset the relative increase in progression rate per year of follow-up in comparison to the reference genotype was estimated, using multivariate normal regression (cohorts with repeated radiological measures – Leiden-EAC, Lund, Groningen and Wichita) or linear regression (datasets with single measurements per patient – Sheffield, NARAC, NDB and Iceland), adjusting for age and gender. The RA-patients studied were treated in era when treatment strategies were not as intensive as nowadays. In the Groningen and

Table 1 Characteristics of the sets of RA patients studied

Cohort	N patients	Total no. of X-ray sets	Year of diagnosis	Year of X-ray	Anti-CCP+ n (%)	Tested SNP <i>Traf1-C5</i> (r2*)	Tested SNP <i>TNFAIP3-OLIG3</i> (r2*)
Lund	147	781	1985-1990	1985-1995	114 (78)	rs10818488	rs525977 (1)
Sheffield	385	385	1938-2003	1999-2006	302 (79)	rs10818488	rs525997
Iceland	285	285	1942-2008	1989-2010	148 (52)	rs3761847 (0.97)	rs525997
NARAC	384	384	1953-2002	1985-2002	385 (100)	rs3761847	rs666619 (1)
Wichita	113	555	1963-1999	1976-2006	110 (97)	rs10818488	rs525977
NBD	756	756	1980-1999	1989-2006	490 (72)	rs10818488	rs525977
Leiden-EAC	596	3,136	1993-2006	1993-2006	302 (51)	rs10818488	rs525997
Total	2666	6282					

* r2 = correlation of the tested SNP with the originally published SNP: for *Traf1-C5* rs10818488 and for *TNFAIP3-OLIG3* rs675520

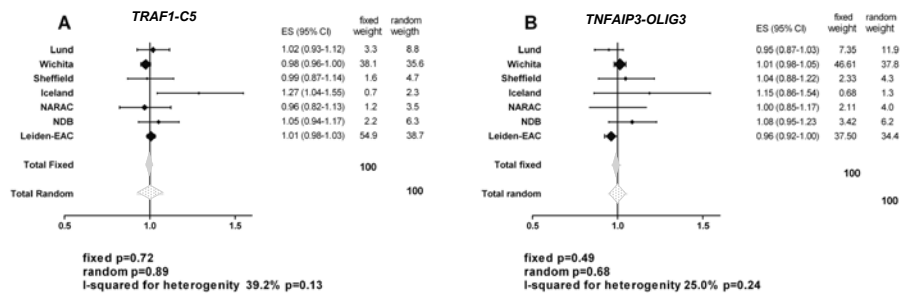


Figure 1 Results of the analyses of (A) *Traf1-C5* (rs10818488) and on (B) *TNFAIP3-OLIG3* (rs675520) with progression of joint destruction in 7 sets of RA-patients

Rs10818488 was tested in all RA-patients (without selection on anti-CCP status) under a dominant model; patients homozygous for the minor allele together with patients heterozygous were compared to the reference group of patients homozygous for the common allele. Rs675520 was tested in anti-CCP-positive patients under a recessive model; patients homozygous for the minor allele were compared to the reference group of heterozygous patients together with patients homozygous for the common allele. The effect sizes are the estimated relative progression rates per year for the tested genotype compared reference group. To illustrate the value of the beta, a beta of 1.02 (rs10818488-Lund) per year equals to a 1.15 higher rate of joint destruction over 7 years, which is similar to 15% higher increase in rate of joint destruction for patients with genotype AA versus AG and GG.

Leiden-cohorts adjustment for treatment strategies were applied¹²; in the other datasets no treatment effects on the radiological progression rates were observed.

Considering the number of patients and X-rays studied, it was not expected to obtain statistical significance in individual datasets. Therefore the estimates of the individual datasets, reflecting the relative increase in rate of joint destruction per year, were summarized in a meta-analysis with inverse variance weighting. Rs10818488 was previously associated with joint destruction in a dominant analysis performed on all RA-patients and rs675520 in a recessive analysis on anti-CCP-positive RA-patients. In line with this, analyses were primarily performed using a dominant model on all patients (rs10818488) and recessive model on anti-CCP positive patients (rs675520). SPSS version 17.0 and Stata version 10.1 were used.

Figure 1 summarizes the results. The effect estimates of rs10818488 were not consistent in their direction. Statistical significance was not obtained in the meta-analysis. Analysing the subgroup of anti-CCP-positive patients did also not result in significant findings (data not shown). Similarly, rs675520 was not associated with progression in joint destruction in anti-CCP-positive RA (figure 1) or in all patients (data not shown).

In the Leiden-cohort, rs10818488 was previously found associated with joint destruction over the first 2-years in 278 RA-patients and rs675520 on 181 anti-CCP-positive patients with progression over 5 years. Extending the Leiden dataset to 596 RA-patients and yearly follow-up over 7-years resulted in a different finding for rs10818488 but not for rs675520 (Figure 1).^{3,4} Rs2900180 in *TRAF1/C5* is observed to associate with erosiveness in a UK study, this variant is in low LD ($R^2=0.67$) with rs10818488.¹³

A drawback of the current study is that the studied datasets differed in designs; this heterogeneity may affect findings. Nonetheless, the present data do not support the initial findings that rs10818488 and rs675520 are associated with the severity of joint destruction in RA.

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Chapter 8

Genetic variants in *OPG* are associated with progression of joint destruction in Rheumatoid Arthritis

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Submitted

ABSTRACT

Background

Progression of joint destruction in Rheumatoid Arthritis (RA) is partly heritably; 45-58% of the variance in joint destruction is estimated to be explained by genetic factors. The binding of RANKL (Receptor Activator for Nuclear Factor κ B Ligand) to RANK results in the activation of TRAF6 (TNF receptor associated factor-6), and osteoclast formation ultimately leading to enhanced bone resorption. This bone resorption is inhibited by osteoprotegerin (OPG) which prevents RANKL – RANK interactions. The OPG/RANK/RANKL/TRAF6 pathway plays an important role in bone remodeling. Therefore, we investigated whether genetic variants in *OPG*, *RANK*, *RANKL* and *TRAF6* are associated with the rate of joint destruction in RA.

Method

1,418 patients with 4,885 X-rays of hands and feet derived from four independent data-sets were studied. In each data-set the relative increase of the progression rate per year in the presence of a genotype was assessed. First, explorative analyses were performed on 600 RA-patients from Leiden. 109 SNPs, tagging *OPG*, *RANK*, *RANKL* and *TRAF6*, were tested. SNPs significantly associated in phase-1 were genotyped in data-sets from Groningen (NL), Sheffield (UK) and Lund (Sw). Data were summarized in an inverse weighted variance meta-analysis. Bonferonni correction for multiple testing was applied.

Results

33 SNPs were significantly associated with the rate of joint destruction in phase-1. In phase-2, six SNPs in *OPG* and four SNPs in *RANK* were associated with progression of joint destruction. However, after correction for multiple testing one SNP remained significant. In the meta-analyses of all four data-sets RA-patients with the minor allele of *OPG*-rs1485305 expressed higher rates of joint destruction compared to patients without these risk variants ($P=2.35 \times 10^{-4}$).

Conclusion

These results indicate that a genetic variant in *OPG* is associated with a more severe rate of joint destruction in Rheumatoid Arthritis.

INTRODUCTION

Rheumatoid Arthritis (RA) is an autoimmune disorder that affects 0.5-1% of the population and is associated with significant morbidity, disability and costs for society. Radiographic joint destruction reflects the cumulative burden of inflammation and is conceived as an objective measure of RA severity.¹ The degree of joint destruction varies significantly between patients. The processes behind this difference are incompletely understood. Inflammatory markers and auto-antibodies are known risk factors for joint destruction but explain approximately 30% of the total variance in joint destruction.² A twin study suggested that genetic factors influence the severity of joint destruction in RA and a recent study in the Icelandic RA-population estimated the heritability of the rate of joint destruction around 45-58%.^{3,4} Hence, to increase the understanding of progression mediating disease processes, it seems valuable to study genetic variants that could predispose to joint destruction in RA.

The balance between osteoblast and osteoclast activity is crucial for healthy bone and is disturbed in systemic or local conditions that affect the skeleton such as osteoporosis or RA. Figure 1 schematically depicts the OPG/RANK/RANKL/TRAF6 pathway which mediates osteoclast related bone loss. RANKL (Receptor Activator for Nuclear Factor κ B Ligand) is expressed and released by osteoblasts and activated T lymphocytes.⁵ RANKL promotes osteoclast formation and perpetuate their function and survival through binding of RANK (Receptor Activator of Nuclear Factor κ B). Subsequently, the signal of RANK is mediated by TRAF6, a member of the TNF receptor associated factor (TRAF) protein family, which functions as a signal transducer in the NF κ β family.⁶ The process of osteoclast formation

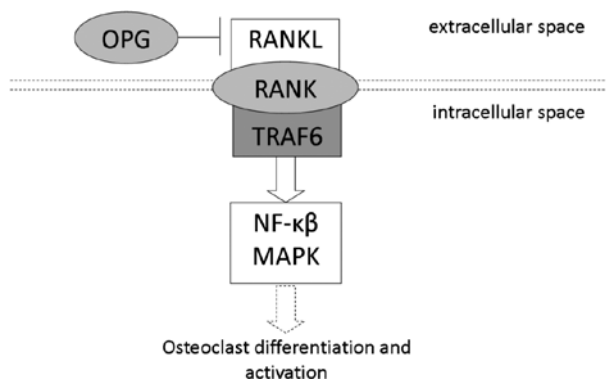


Figure 1 Schematic presentation of the OPG/RANK/RANKL/TRAF6 pathway in osteoclasts

The RANK signaling cascade is initiated upon the binding of RANKL to the extracellular domain of RANK which panes the signal along to TRAF6. The activation of TRAF6 initiates pathways leading to the activation of several transcription factors (among which NF κ β and MAP kinase mediators), which contribute to osteoclast differentiation, activation and survival. OPG is able to prevent the interaction between RANKL and RANK.

and bone resorption is also regulated by OPG (osteoprotegerin), which is secreted by osteoblasts. By binding of OPG to RANKL, activation of the RANK receptor is inhibited.

The net bone loss in RA suggests that there is an imbalance in the OPG-RANKL axis favoring bone resorption and resulting in erosions.⁶⁻⁸ Recent studies showed that in RA RANKL is expressed in cultured synovial fibroblasts and by CD4+ and CD8+ T lymphocytes.^{5,9,10} In addition, the ratio of OPG/RANKL serum levels is associated with joint destruction in RA.¹¹ Furthermore, several studies have observed an association of genetic variants in *OPG*, *RANK* or *RANKL* with bone mineral density and osteoporosis.¹²⁻¹⁵ Together, these data led us to hypothesize that genetic variants in *OPG*, *RANK*, *RANKL* and *TRAF6* are associated with the severity of joint destruction in RA. We tested this hypothesis using four data-sets of European RA-patients with longitudinal radiological data on joint destruction. All data-sets included patients that were diagnosed in a period when treatment strategies were less aggressive and disease activity was less controlled than today. These conservative treatment strategies made these data-sets suitable for the present study as the natural course of disease was less inhibited.

PATIENTS AND METHODS

Study population

Four data-sets consisting of adult European RA-patients were studied. RA was defined according to the 1987 ACR criteria in all data-sets except for the Lund data-set where the 1958 ACR-criteria were used. X-rays of both hands and feet were available for all patients (Table 1). All patients gave their informed consent and approval was obtained from the local Ethical Committee of each study.

Table 1: Characteristics for each data-set.

Cohort	Leiden-EAC	Groningen	Sheffield	Lund
	(n=600)	(n=275)	(n=391)	(n=147)
Year of diagnosis	1993-2006	1945-2001	1938-2003	1985-1990
Follow-up years*	7 years	14 years	Not applicable*	5 years
Total no. of X-ray sets	2,846	862	391	781
Method of scoring	SHS	SHS	Larsen	Larsen
Female n (%)	412 (69)	194 (71)	290 (73)	98 (67)
Age at diagnosis, mean $\pm$ SD	56 $\pm$ 16	49 $\pm$ 13	46 $\pm$ 13	51 $\pm$ 12
ACPA+ n (%)	323 (55)	160 (80)	302 (79)	114 (80)

SHS= Sharp-van der Heijde score

*Data of Leiden-EAC, Groningen and Lund were from baseline onwards during respectively 7, 14 and 5 years of follow-up. The data of Sheffield were collected once during the disease period, the mean disease duration was 15 years (range 3-65 years).

Leiden-Early Arthritis Clinic cohort (Leiden-EAC)

This cohort contained 600 early RA-patients from the western part of the Netherlands, who were included in the Leiden-EAC between 1993 and 2006.² Arthritis patients were included at the first visit at the outpatient clinic and yearly followed. X-rays were taken at baseline and on yearly follow-up visits during 7-years. In total, 2,846 sets of hands and feet X-rays were available. All X-rays were chronologically scored by one experienced reader who was unaware of genetic or clinical data using the Sharp-van der Heijde scoring method (SHS) on hands and feet.¹⁶ 499 randomly selected X-rays were scored twice. The correlation coefficient (ICC) within the reader was 0.91. The treatment of these patients could be divided into three treatment periods. Patients included in 1993-1995 were initially treated with NSAIDs, patients included in 1996-1998 were initially treated with chloroquine or sulphasalazine and patients included after 1999 were promptly treated with methotrexate or sulphasalazine.

Groningen

The second set of data involved 275 RA-patients from the Northern part of the Netherlands that were diagnosed in 1945-2001. The follow-up duration after diagnosis was limited to 14-years. The mean number of X-ray sets (hands and feet) per patient was 3.1 (with a maximum of eight X-rays per patient). The total number of sets of X-rays was 862. The X-rays were scored chronologically by one of two readers using SHS. ICCs within readers were >0.90 and between readers 0.96. The development of joint destruction was significantly different for patients included after 1990 compared to patients included before 1990. This observation is in line with the introduction of early initiation of DMARD-therapy after 1990.

Sheffield

The third set of patients concerned 391 RA-patients from the area of Sheffield, UK. RA-patients with X-rays available were recruited from the Rheumatology department of the Royal Hallamshire Hospital in Sheffield between 1999 and 2006.¹⁷ RA-patients were assessed once during their disease course. The mean ($\pm$ SD) disease duration at assessment was 15 ± 11 years (range 3-65 years). X-rays of hands and feet were scored by one reader using a modification to Larsen's score.¹⁸ 10% of films were scored twice to quantify the intra-observer variation by a weighted kappa score which was 0.83.¹⁷

Lund

This cohort concerned 183 Swedish early RA-patients that were prospectively followed yearly during 5-years, of which 147 had X-rays and DNA available.^{19,20} Patients were recruited from primary care units in the area of Lund during 1985-1989. X-rays of hands and feet were taken at study start and annually for 5-years, resulting in a total of 781 sets

of X-rays. X-rays were scored chronologically according to Larsen by one of two readers.²¹ The ICC between the readers determined on 105 X-rays was 0.94. In the inclusion period, immediate DMARD-therapy was not common and at 5-years follow-up still a substantial proportion of the patients were not treated with a DMARD. The most commonly used DMARDs were chloroquine, D-penicillamin, sodium aurothiomalate and auranofin.¹⁹

SNP selection and genotyping

The region of *OPG*, *RANK*, *RANKL* and *TRAF6* plus the haplotype blocks up- and downstream these genes were tagged by the algorithm of HaploView.²² One SNP in *OPG*, two in *RANK* and one in *TRAF6* were known to be amino acid changing SNPs; respectively rs2073618, rs1805034, rs8092336 and rs3740958. Eight SNPs were associated with bone mineral density in the hip or spine in previous studies; *OPG* rs6993813¹², rs646980¹², rs4355801¹³ and rs2073618¹⁴; *RANK* rs3018362¹² and rs884205¹⁵; *RANKL* rs9594738¹² and rs9594759¹². All these SNPs were forced to include. Pairwise tagging SNPs were selected from the CEPH/CEU hapmap data-set (phase II, release 21, NCBI build 35) using haploview software (MAF >0.05, pairwise r^2 >0.8). In total 109 SNPs captured *OPG* (34), *RANK* (54), *RANKL* (21) and *TRAF6* (17). Multiplex SNPs arrays were designed using Illumina Golden Gate platform, according to the protocols recommended by the manufacturer [Illumina, San Diego, CA]. Three SNPs could not be designed; rs10505348, rs7239667 and rs9951012. Proxies were sought and found for rs10505348:rs4355801 ($r^2=0.80$), for the other two SNPs no good proxy existed.

Software supplied by Illumina was used to automatically identify the genotypes. Each 96-wells plate consisted of 1 positive and 1 negative control, which were all indeed tested positive and negative. Clusters were evaluated and all doubtful calls were checked; after manually evaluating the spectra of each cluster, the genotypes were accepted, recalled or rejected. At least 12% of the genotypes were assessed in duplicate, with an error rate of <2.5% for all SNPs. SNPs were selected if the success rate were $\geq 95\%$ and the Hardy-Weinberg equilibrium (HWE) p -value >0.001.

SNPs that had a clear and significant association with joint destruction in the first cohort were selected to be genotyped in the other three data-sets. The SNPs were genotyped as a part of multiplex SNPs arrays designed with Sequenom iPLEX, according to the protocols recommended by the manufacturer [Sequenom, San Diego, California]. Software supplied by the same manufacturer was used to automatically identify the genotypes. Two SNPs could not be designed but full proxies ($r^2=1.0$) were typed instead (rs17666267:rs9959310 and rs1564861:rs3134057). Each iPLEX consisted of at least 9 positive and 9 negative controls, which were indeed tested positive and negative. All doubtful calls were checked manually, DNA samples with >30% failed SNPs were excluded from analysis ($n=31$). At least 5% of the genotypes were assessed in duplicate, with an error rate of <1%. SNPs were selected if the success rate were $\geq 95\%$ and HWE p -value >0.001.

Statistical analysis

Associations between genotypes and radiographic joint destruction were analyzed. Two phases were carried out. First, an explorative analysis was performed in the Leiden-EAC. In this data-set the tagged SNPs were tested in two ways; additively and recessively. Since phase-1 was an explorative phase no correction for multiple testing was applied yet and SNPs with a p-value <0.05 were studied in phase-2.

Significant SNPs from phase-1 were proceeded in phase-2 if they had a high enough minor allele frequency and if the effect was independent of other SNPs. In phase-2 SNPs were analyzed in three independent data-sets and meta-analyses of all four data-sets. In the present study the power to detect genetic effects is a function of the number of patients and the number of measurements per patient studied.²⁴ All three data-sets studied to verify the results of phase-1 contained (individually and combined) less X-rays than the initial data-set. Consequently, the power to replicate findings in each data-set individually as well as in the three replication data-sets together was expected to be limited due to a lower number of X-rays than in the discovery data-set. Therefore it was decided to test the SNPs in each data-set separately taking advantage of the specific data-set characteristics and to subsequently perform a meta-analysis on the results to determine the association of the SNPs with the rate of joint destruction. An inverse variance weighting meta-analysis testing for fixed effect^{25,26} was performed in Stata, version 10.1. In phase-2 analyses were performed either additively or recessively depending of the findings of phase-1.

For the analyses in Leiden-EAC, Groningen and Lund a multivariate normal regression model for longitudinal data was used with radiological score as response variable (see detailed descriptions elsewhere²⁷). Adjustment variables were entered based on their association with joint destruction; in Leiden-EAC age, gender and the described treatment periods, in Groningen age and inclusion $\leq/\geq 1990$, as proxy for DMARD-therapy and in Lund adjustments were made for age.

In the Sheffield data-set, each patient had a set of hands and feet X-rays at one time-point. To make the scores comparable to the other data-sets, the estimated yearly progression rate was calculated, by dividing the total Larsen by the number of disease years at time of X-ray.²⁸ The SNP association was tested in a linear regression analysis with log-transformed estimated yearly progression rate as outcome variable. No adjustments were applied as none of the tested variables was significantly associated with joint destruction.

In all data-sets, the radiological scores were log-transformed to obtain a normal distribution. Since the analyses were performed on the log-scale, the resulting coefficient on the original scale indicates how many fold the joint destruction increased per year of follow-up. Over a follow-up period of n years the coefficient increases to the power of n .

Testing multiple SNPs on one data-set leads to inflation of the p-value. It is debatable which multiple testing correction is best to use. In the current study the most conservative method was applied, the Bonferroni method, to reduce the chance on false-positive find-

ings as much as possible. Since phase-1 was used as identification phase, this correction was applied to the number of variants tested in phase-2.

Haplotype analyses

Haplotypes in *OPG*, *RANK*, *RANKL* and *TRAF6* were studied. Haplotype blocks for the tag-SNPs were defined with Gabriel's method.²⁹ Haplotypes were assigned to each individual using PLINK 1.06 requiring a probability >0.8. Analyses of the haplotypes were performed with methods similar to those used for the analyses of the individual SNPs by now testing the presence of a haplotype compared to the absence of the haplotype.

RESULTS

Phase-1; SNP identification

123 tagging SNPs in *OPG* (n=33), *RANK* (n=52), *RANKL* (n=21) and *TRAF6* (n=17) were genotyped. Eleven SNPs were not analyzed because of a low typing success rates and three were out of HWE. From the 109 analyzed SNPs, 33 SNPs were significantly associated with joint destruction; eighteen SNPs were located in *OPG*, nine in *RANK*, four in *RANKL* and one in *TRAF6*. The associations of *OPG* SNPs were most prominent in the additive analyses. For *RANK*, *RANKL* and *TRAF6* mainly recessive associations were observed. The effect sizes observed represent the estimated relative progression rates per year. Consequently over a follow-up of a certain number of years, the effect sizes increases by the power of the number of follow-up years. For example, the estimate of 1.03 fold rate of joint destruction per year of the minor variant of *OPG*-1485305 (T) compared to patients with the common genotypes equals 1.23 (1.03^7) fold rate of joint destruction over 7-years. In other words patients carrying one minor allele had over 7-years a 23% higher rate of joint destruction (Figure 2).

Haplotype analyses were performed in addition to SNPs analyses. In phase-1, four haplotypes from one haplotype block in *OPG* were identified as possible more informative than the individual SNPs located in these haplotypes. To tag these haplotypes in phase-2, one non-significant SNP of phase-1 was also typed in phase-2, rs1905785.

Phase-2; meta-analysis

Of the 33 significant SNPs from phase-1 19 were subsequently typed and analyzed in phase-2 since they had a high enough minor allele frequency and their associations were independent of other SNPs. Therefore these nineteen SNPs plus one SNP to allow haplotype analysis were studied in phase-2. As expected due to insufficient power of the replication cohorts, the 95% confidence intervals in each of the three cohorts separately all included 1. The SNPs were subsequently analyzed in all 1,418 patients in an inverse

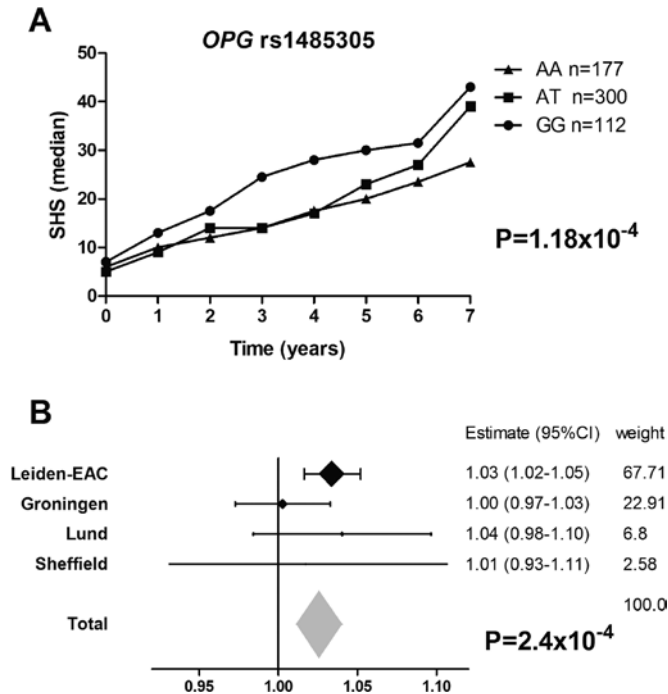


Figure 2 Depicted is *OPG*-rs1485305 in the Leiden-EAC (A) and in the meta-analysis on all data-sets (B).

SHS = Sharp-van der Heijde score. The effect sizes are the estimated relative progression rates per year for the presence of the minor allele for *OPG* compared to patients without the minor allele. The presence of the minor variant of *OPG*-1485305 (T) is associated with a 1.03 fold rate of joint destruction per year compared to patients with the common genotypes. Since the effect sizes increases by the power of the number of follow-up years, these patients have a 1.23 (1.03^7) fold rate of joint destruction over 7-years, in other words a 23% higher rate of joint destruction. The meta-analysis is based on a fixed effect model, which is applied to genetic studies to test whether there is statistically significant effect; generalisability of the effect is of less importance. Consequently, this method is less suitable to estimate the effect size overall. Therefore, the estimated effect of the meta-analysis is depicted in gray.

variance weighting meta-analysis. Here, ten SNPs (six located in *OPG* and four in *RANK*) were significantly associated with rate of joint destruction ($0.04 > P > 2.4 \times 10^{-4}$). After correction for testing 20 SNPs using the Bonferroni method, one SNP was significantly associated with the rate of joint destruction; *OPG*-rs1485305 (Figure 2). Patients carrying at least one minor allele of *OPG*-rs1485305 (T) had a higher rate of joint destruction as compared to patients without this minor allele ($P=2.35 \times 10^{-4}$, uncorrected P -value).

When the association of *OPG*-rs1485305 with the rate of joint destruction was studied in ACPA-negative and ACPA-positive patients separately in the Leiden-EAC, rs1485305 was significantly associated with progression of joint destruction in ACPA-negative patients (1.29 95%CI 1.10-1.50, $P=0.001$) but not in ACPA-positive patients although a similar trend was observed (1.14 95%CI 0.97-1.34, $P=0.111$).

In phase-2, none of the tested haplotypes provided additional information to the results of the individual SNPs.

DISCUSSION

The variance in joint destruction between RA-patients is considerable and the mechanisms driving these differences are thus far scarcely understood. Part of the severity of joint damage is explained by the cumulative levels of inflammation³⁰⁾ though this explanation is incomplete. We reasoned that individual susceptibility of bones to erode may affect the severity of joint destruction in RA as well. We therefore studied the association of genetic variants in *OPG*, *RANK*, *RANKL* and *TRAF6* with joint destruction as the products of these genes together constitute a pathway that is crucial in osteoclastogenesis and bone resorption. One SNP, *OPG*-rs14085305, was observed to significantly associate with progression of joint destruction in RA.

OPG is expressed in several cells and tissues among which osteoblasts and bone marrow. Serum *OPG* is decreased in the synovium and serum of RA-patients³¹ and a low serum *OPG*/*RANKL* ratio¹¹ has been associated with progression of joint destruction. Rs1485305 is situated in the 5-UTR flanking region of *OPG*. Thus far no functional data on this variant exist, hence the mechanism by which rs1485305 affects *OPG* expression or function is yet unknown. Nonetheless, *OPG*-rs1485305 was recently also observed to associate with bone mineral density loss, which strengthens the relevance of this SNP in relation to bone and joint disease.³²

Our group recently performed a genome-wide study on joint damage progression in ACPA-positive RA-patients³³ and evaluated the genetic variants included on the Immunochip in relation to joint damage progression in RA (unpublished data). In these studies the current variant *OPG*-rs1485305 was not identified as a risk factor for joint damage progression as this variant was not (also no proxies) included in both these genotyping platforms.

An advantage of the four studied data-sets is that the evaluated RA-patients were treated in an era when treatment was not as aggressive as nowadays. Hence, the radiologic progression rates of the studied patients are more reflective of the natural course of RA than that of recently treated patients. Some data-sets included patients from different periods that had received different treatment regimens potentially affecting an association with progression of joint destruction. Therefore, the analyses in these data-sets were adjusted for inclusion period as proxy for treatment.

Replication data-sets are ideally larger than the initial data-set, since effects sizes are generally smaller at a replication stage. A limitation of the present study is that we were not able to include replication data-sets that contained more X-rays than the initial data-set and of which the RA-patients were “conventionally” treated. Most likely, few of such longitudinal data-sets exist. The number of patients and the number X-rays of each data-set separate were insufficient to allow well powered analyses. Also the replication data-sets combined contained less radiological measurements than in phase-1. Consequently, the data available for phase-2 were expected to be underpowered to replicate findings individually. Therefore, data were summarized in an inverse variance weighting meta-analysis. Importantly, the effects of *OPG*-rs1485305 went into the same direction in each dataset supporting the validity of the results.

To prevent false positive findings due to performing multiple comparisons, data were corrected for multiple testing using the Bonferroni method. This was done in phase-2, since phase-1 was used as discovery phase. However, the association of *OPG*-rs1485305 with the rate of joint destruction would also have remained significant when Bonferroni correction would have been applied in phase-1 correcting for 109 SNPs. This further consolidates the validity on the results on *OPG*-rs1485305.

Interestingly, several other studies, among which genome-wide association studies, have revealed several genetic variants in *RANKL/RANK/OPG* to associate with bone mineral density or osteoporosis. Therefore during the tagging and SNP selection phase, such variants were forced into the selection. Interestingly (except for rs1485305) none of these variants were significantly associated with progression of joint destruction in phase-2. Conceptually, the balance between osteoblast and osteoclast activity is crucial both in osteoporosis and joint destruction in RA but the individual genetic variants predisposing to such systemic or local bone loss are largely dissimilar.

During the SNP selection phase, three coding SNPs were also prioritized; these three were also not significantly associated after correction for multiple testing in phase-2. A coding variant in *RANK*, rs8092336, was significantly associated with joint destruction in phase-1 and phase-2, but did not remain significant after correction for 20 tests. The same was observed for a coding SNP in *OPG*, rs2073618, which has also been associated with bone mineral density.¹⁴ These two SNPs could potentially be associated with progression of joint destruction, but the chance of a Type-I error is too large to conclude this on the basis of current data.

Finally, *TRAF6* was chosen as candidate gene because it is a relevant signal transducer in the Nuclear Factor κ B pathway. Rs540386 in *TRAF6* was previously also identified as a

risk locus for RA susceptibility, though *TRAF6* was not genome-wide significant in a recent study on more than 11,000 cases and 15,000 controls.[34, 35] Our candidate gene study had started a year before the first report of *TRAF6* and RA susceptibility was published, hence this report had not affected the choice of *TRAF6* as candidate gene. The susceptibility SNP rs540386 is in close LD ($r^2=0.94$) with one of the tag SNP evaluated in the current study (rs11033647). This SNP was not associated with the rate of joint destruction in our analyses. Although this could be a false-negative finding, it is also possible that different genetic variants are involved in RA-susceptibility and the progression of joint destruction.

In conclusion, with a candidate gene approach evaluating patients of four different cohorts, we found association of a genetic variant in *OPG* with an increased rate of joint destruction in RA. The present data support the role of *OPG* in joint destruction in RA by indicating that the risk allele of rs1485305 may affect the homeostasis in bone.

LIST OF ABBREVIATIONS

OPG – osteoprotegerin

RANK – Receptor Activator for Nuclear Factor κ B

RANKL – Receptor Activator for Nuclear Factor κ B Ligand

TRAF6 – Tumour necrosis Receptor Associated Factor-6

RA – Rheumatoid Arthritis

NF – Nuclear Factor

EAC – Early Arthritis Clinic

SHS – Sharp-van der Heijde

NSAIDs – Non-steroid anti-inflammatory drugs

DMARDs – Disease modifying anti-rheumatic drugs

ICC – intra-correlation coefficient

SD – standard deviation

MAF – minor allele frequency

SNP – single nucleotide polymorphism

HwE – Hardy-Weinberg equilibrium

COMPETING INTERESTS

None of the authors has any financial conflicts of interest to declare.

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Chapter 9

Association of variants in *IL2RA* with progression of joint destruction in Rheumatoid Arthritis

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ABSTRACT

Background

Heritability studies have suggested an important role of genetic predisposition to progression of joint destruction in Rheumatoid Arthritis (RA); the heritability is estimated at 45-58%. Several SNPs have been identified to associate with RA susceptibility. We studied the association of several of these loci with progression of joint destruction.

Method

In total 1,750 RA-patients with 4,732 Sharp-van der Heijde scored X-rays of four independent data-sets were studied. Thirteen susceptibility SNPs that were not associated with joint destruction before were tested in 596 Dutch RA-patients. Subsequently, significant SNPs were studied in RA data-sets from North-America and Iceland. Data were summarized in inverse weighted variance meta-analyses. Further, the association with circulating protein levels was studied and the associated region was fine-mapped.

Results

In stage-1, three loci (AFF3, IL2RA and BLK) were significantly associated with rate of joint destruction and were further analyzed in the additional data-sets. In the combined meta-analyses, the minor allele of IL2RA-rs2104286(C) was associated with less progression of joint destruction ($P=7.2 \times 10^{-4}$). Furthermore, the IL2RA-rs2104286 protective genotype was associated with lower circulating levels of soluble IL-2RA (0.85 95%CI 0.77-0.93, $P=1.4 \times 10^{-3}$). Additionally, lower sIL-2RA levels were associated with a lower rate of joint destruction ($P=4.2 \times 10^{-3}$). The association of IL2RA with rate of joint destruction was further focused to a region of 40kb encompassing the IL2RA intron 1 and the 5' region of IL2RA and RBM17.

Conclusion

Present genetic and serologic data suggest that inherited altered genetic constitution at IL2RA locus may predispose to a less destructive course of RA.

INTRODUCTION

Rheumatoid Arthritis (RA) is an autoimmune disorder that affects approximately 1% of the population and is characterized by inflammation and subsequent destruction of joints.¹ The disease is associated with significant morbidity, disability and costs for society.² The severity of RA progression is objectively measured by radiographic joint destruction scores of the hand and foot joints,³ which is also reflective of the cumulative burden of inflammation.⁴ In the past decades, genetic studies have identified many loci involved in disease pathogenesis of autoimmune diseases; several of these have led to the identification of new pathways and targets for therapy.⁵ For RA susceptibility, 32 risk factors have been identified. Since RA is a chronic disease, interrogation of the genome with disease severity as an outcome is attractive since this could increase the understanding of processes that are fundamental to RA progression and it may provide new therapy opportunities. Heritability studies have recently suggested an important role of genetic predisposition to progression of joint destruction in RA; the heritability of the radiologic progression rate is estimated at 45-58%.⁶ However, thus far, only a few genetic variants have been reported to associate with joint destruction in RA.⁷⁻¹⁰ To date, 20 of the 32 loci that are known to associate with RA susceptibility¹¹⁻²⁴ have been tested for an association with joint destruction.^{7-9,25,26} In the current study, we investigated the remaining 12 susceptibility loci for their association with rate of joint destruction (AFF3, ANKRD55, BLK, CCL21, CTLA4, IL21, IL2RA, IL2RB, IRF5, REL, SPRED2 and STAT4, Table 1).

For this, a multistage approach was conducted to detect an association between the selected SNPs and the rate of joint destruction in four data-sets RA-patients with longitudinal radiological data on joint destruction. All data-sets included patients that were diagnosed in a period when treatment strategies were less aggressive and less controlled than today. These conservative treatment strategies made these data-sets suitable for the present study as the natural course was less inhibited.

We will show an association of *IL2RA*-rs2104286 with the severity of joint damage progression. We further explored this association by fine mapping of this *IL2RA* region and evaluating the *IL2RA* serum levels in relation to both genotypes and the radiologic progression rate.

PATIENTS AND METHODS

Study population

Four data-sets consisting of adult European RA-patients were studied (Table 2). RA was defined according to the 1987 ACR criteria in all data-sets. To obtain high-quality phenotypic radiological data, all X-rays studied in the current project were scored by experienced

Table 1: Schematic overview of the SNPs selection for the analyses with rate of joint destruction.

							¹² REL	¹⁴ AFF3
							¹² BLK	¹⁴ ANKRD55/IL6ST
							¹³ TAGAP ^e	¹⁴ C5orf30 ^e
				^{3,4} TNFAIP3 ^b	¹¹ CD40 ^d	¹³ CD28 ^e		¹⁴ CCR6 ^e
				⁵ STAT4	¹¹ CCL21 ^d	¹³ TRAF6		¹⁴ IRF5
				^{6,7} CS-Traf1 ^c	¹¹ MMEL ^d	¹³ PTPC ^e		¹⁴ PXK ^e
				⁹ IL2RA	^{9,11} PRKCCQ ^d	¹³ FCGR2A ^e		¹⁴ RBJP ^e
				^{8,9} IL2RB	^{9,11} KIF5 ^d	¹³ PRDM1 ^e		¹⁴ SPRED2
								¹⁴ CCL21
HLA-DR1		¹ PTPN22 ^a	² CTLA4	¹⁰ IL21	¹¹ CDK6 ^d	¹³ CD2/CD58 ^e		¹⁴ IL2RA
1978	1987	2004	2005	2007	2008	2009	2010	
References discovered susceptibility SNPs in white RA-patients					References tested susceptibility SNPs with rate of joint destruction			
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14. Stahl, Nat Genet 2010;42:508-514								
					Bold SNPs were tested in the current manuscript			

readers from one center (LUMC) who were blinded for the clinical and genetic data using the Sharp- van der Heijde score (SHS).³ All patients gave their informed consent and approval was obtained from the local Ethical Committee of each study.

Table 2: Characteristics of the individual data-sets.

Cohort	N patients	Total no. of X-ray sets	Year of diagnosis	Year of X-ray	Anti-CCP+n (%)
Leiden-EAC	596	3,136	1993-2006	1993-2006	302 (51)
Iceland	285	285	1942-2008	1989-2010	148 (52)
Wichita	113	555	1963-1999	1976-2006	110 (97)
NBD	756	756	1980-1999	1989-2006	490 (72)
Total	1,750	4,732			

Leiden-RA cohort (Leiden-EAC)

This cohort concerned 596 early RA-patients from the western part of the Netherlands, who were included in the Leiden Early Arthritis Clinic between 1993 and 2006.²⁸ Patients were included at time of diagnosis and followed yearly. X-rays were taken at baseline and on yearly follow-up visits during 7-years. In total, 3,136 sets of hands and feet X-rays were available. All X-rays were chronologically scored with SHS by one experienced reader who was unaware of genetic or clinical data. 499 randomly selected X-rays were scored twice. The correlation coefficient (ICC) within the reader was 0.91. The treatment of these patients could be divided into three treatment periods. Patients included in 1993-1995 were initially treated with NSAIDs, patients included in 1996-1998 were initially treated with chloroquine or sulphasalazine and patients included after 1999 were promptly treated with methotrexate or sulphasalazine.

Iceland data-set

325 RA patients that were referred to Landspítali hospital or the private clinic of Reykjavik with X-rays available of both hands and both feet at one similar time-point were studied. 285 patients had also genotypes available. Joint destruction was determined using SHS by two trained readers. Twelve percent of the X-rays were scored by both readers and each scorer rescored 15% of their own scored X-rays. The ICCs between and within readers were all >0.95.

Wichita

These patients from one practice in Wichita (Kansas, USA) were recruited between 1973 and 1993.²⁸ Because of the rather low minor allele frequency in some variants and the low number of X-rays at a longer disease duration of these patients, the follow-up duration was restricted to a maximum of 10 years. Collection of laboratory and radiographic data was not protocolized but obtained when needed for clinical care. In total, 461 sets of hands X-rays were available of 90 patients. All hand X-rays were chronologically scored with SHS by one experienced reader, the within reader ICC was 0.98.

NDB patients

These patients are included in the National Databank for Rheumatic diseases, a databank that consists of patients with rheumatic diseases from the USA and Canada.²⁹ 756 patients had a single time-point X-ray of the hands available and scored using SHS by one the same reader who scored the Wichita X-rays.

SNP selection and genotyping

Of the 32 regions that are known to associate with RA susceptibility in patients of European origin, 12 loci have thus far not been tested for an association with joint destruction

and were investigated in the current study; AFF3, ANKRD55, BLK, CCL21, CTLA4, IL21, IL2RA, IL2RB, IRF5, REL, SPRED2 and STAT4. The thirteen reported susceptibility SNPs within these twelve loci were studied (Table 1). These twelve loci were not in LD with each other.

Leiden, Wichita and NDB SNPs were typed with the Immunochip, Illumina Infinium High-Density array (Illumina IScan Platform), which has recently been designed to densely genotype immune-mediated disease loci identified by GWAS of common variants using data.³⁰ Genotypic data was accepted after quality control, requiring MAF>0.0001, HWE $p>0.001$ and genotyping success rate (% of samples that were successful) >0.98. Genetic outliers and relatives (both defined by principal component analysis) and patients with a gender mismatch between data-file and DNA were excluded. In Wichita and NDB, BLK had a genotyping success rate of 0.977 and therefore did not pass the initial quality control. After manually checking the genotyping quality, it was concluded that the SNP data could be used for analyses.

The Icelandic chip-typed samples were assayed with the Illumina Human Hap300, Hap CNV370, Hap 610, 1M or Omni-1 Quad bead chips at deCODE genetics. Only the 317,503 SNPs from the Human Hap300 chip were used in the long range phasing and the subsequent SNP imputations.³¹ SNPs were excluded if they had (i) yield lower than 95%, (ii) minor allele frequency less than 1% in the population or (iii) significant deviation from Hardy-Weinberg equilibrium in the controls ($P < 0.001$), (iv) if they produced an excessive inheritance error rate (over 0.001), (v) if there was substantial difference in allele frequency between chip types (from just a single chip if that resolved all differences, but from all chips otherwise). All samples with a success rate below 97% were excluded from the analysis.

Soluble IL-2RA

Soluble IL-2RA (sIL-2RA) serum levels were evaluated of 159 Leiden RA-patients. Patients were selected based on their SHS progression over 1 year, to include 1/3 without SHS progression, 1/3 with mild SHS progression (1-5 SHS) and 1/3 with severe SHS progression (>5 SHS). Samples were taken several years after diagnosis of RA (mean disease duration 4.2 ± 2.2 , range 1-9 years). Standard sandwich ELISA for sIL-2RA was performed according to the manufacturer's recommendations (BD Biosciences).

Fine-mapping

The IL2RA region was fine-mapped to seek stronger associations with rate of joint destruction than the initial susceptibility variant rs2104286. Patients included in the discovery cohort (Leiden-EAC) and in the North-American replication data-sets (NDB and Wichita) were genotyped with the Immunochip which densely types 186 auto-immune loci among which IL2RA. Previous sequencing by Lowe et al. has demonstrated that the flanking

gene downstream of *IL2RA*, *IL15RA*, is clearly genetically separated from *IL2RA* and that there is no clear breakdown in LD in the 21kb region between *IL2RA* and *RBM17*, the upstream flanking gene (see Entrez Gene URLs).³² Therefore, data of genetic variants from 23kb downstream *IL2RA* to 11kb downstream *RBM17*, including the haplotypes at the end of *IL2RA* and *RBM17* (Chr10:6070000-6210000) were retrieved. In this way, 495 variants were obtained, 473 of which were polymorphic. These variants were analyzed in the discovery data-set for their association with joint destruction in a manner similar as the SNP analyses. Genetic variants that were stronger associated with rate of joint destruction than rs2104286 in the discovery data-set were subsequently analyzed in the North-American samples.

Statistical analyses

Associations between genotypes and radiographic joint destruction were analyzed, applying a multiple stage approach. First, all thirteen SNPs were analyzed in 596 Leiden RA-patients with 3,136 sets of X-rays. Genotypes were tested additively assuming a greater impact on rate of joint destruction for two minor variants than for one. Since this concerned a discovery stage no correction for multiple testing was applied and SNPs with a p-value = 0.05 were studied in stage-2. For the second stage three data-sets were used, one from Iceland and two from the USA (Wichita and the National Databank (NDB)), with a total of 1,154 patients and 1,596 X-rays. In all data-sets the radiological scores were log-transformed to approximate a normal distribution. For the analyses in the cohorts with multiple measurements per patient (Leiden-EAC and Wichita) a multivariate normal regression analysis was used with radiological damage as response variable. This method analyzes all repeated measurements at once and takes advantage of the correlation between these measurements. This model is similar to a linear mixed model, only no random effects is added.³³ To model the correlation over time, the heterogeneous first-order autoregressive (ARH1) matrix was used. It assumes a stronger correlation for measurements taken in a shorter period than taken over a longer period in time. The effect of time was entered as factor in the model, to properly capture a mean response profile over time. For each tested SNP an analysis with the SNP and its interaction with time in the model were conducted, thereby testing the progression of joint destruction, which was the outcome of interest. The effect of time in the interaction term was linear. Since the analyses were performed on the log-scale, the resulting effect estimate were back transformed to the original scale (β_{org}). The β_{org} indicates how much higher the rate of joint destruction is per year for each minor allele compared to the reference genotype; the β_{org} changes for each follow-up year by the power of the number of years.

For the data-sets in which patients were radiographed once during follow-up (Iceland and NDB), the estimated yearly progression rate (dividing the total SHS by the number of

disease years at time of X-ray) was calculated in order make the scores comparable to the other data-sets. Then the SNP association was tested in a linear regression analysis with log-transformed estimated yearly progression rate as outcome variable. Also here, the resulted estimate reflects how many fold the rate of joint destruction increases per year in the presence of a minor allele compared to the absence of this allele.

In all data-sets adjustments for age and sex were made. In the analyses on the discovery data- sets, consisting of the most recently diagnosed patients, an additional adjustment for the described treatment periods was made.

Due to the differences in study designs, the separate data-sets could not be combined in one analysis directly. To test the overall association, the effect sizes and standard deviations of the individual analyses were combined in an inverse-weighting meta-analyses. This method weights the results with a low standard deviation stronger than the results with a higher standard deviation, hence preventing an overrepresentation of less precise data on the outcome. The analysis was conducted in STATA, version 10.1.³⁴ In this stage, correction for multiple testing was performed using the Bonferroni method.

Haplotype analysis was performed on the top fourteen significant SNPs in an attempt to further differentiate between the significant findings. When a haplotype has a stronger association with joint destruction a combined effect of the SNPs on joint destruction is suggested. Haplotype blocks were defined by Gabriels method. Haplotypes were assigned to each individual using PLINK 1.06. Analyses of the haplotypes were performed with methods similar to those used for the analyses of the individual SNPs, now testing the once or twice presence of a haplotype compared to the absence of the haplotype.

The association between IL2RA-rs2104286 and sIL-2RA was tested with a linear regression analysis with $\log_e(\text{sIL-2RA}+1)$ as outcome variable and rs2104286 tested additively as independent variable. Adjustments were made for covariates that were associated with the response variable in simple analysis, which were age at diagnosis and disease duration at the time of serum collection.

All analyses were performed in SPSS 17.0 (see URLs) unless stated otherwise.

RESULTS

In the discovery stage, the analysis of the 13 susceptibility SNPs (at 12 loci) in the discovery data-set resulted in three significant findings: AFF3 (rs11676922) $\beta_{\text{org}}=1.02$ (95%CI 1.002-1.03, $P=2.8 \times 10^{-2}$; BLK (rs13277113) $\beta_{\text{org}}=0.98$ (95%CI 0.97-1.00) $P=3.1 \times 10^{-2}$; IL2RA (rs2104286) $\beta_{\text{org}}=0.97$ (95%CI 0.96-0.99) $P=4.4 \times 10^{-3}$.

In stage-2, these three associations were further examined in one Icelandic and two USA datasets (NDB and Wichita). Each of these cohorts contained fewer X-rays per patient

compared to the discovery cohort, influencing the precision with which an individual patient's progression rate is estimated. The results of the individual cohorts as well as of the meta-analyses are presented in Figure 1. BLK was not significant in any of the

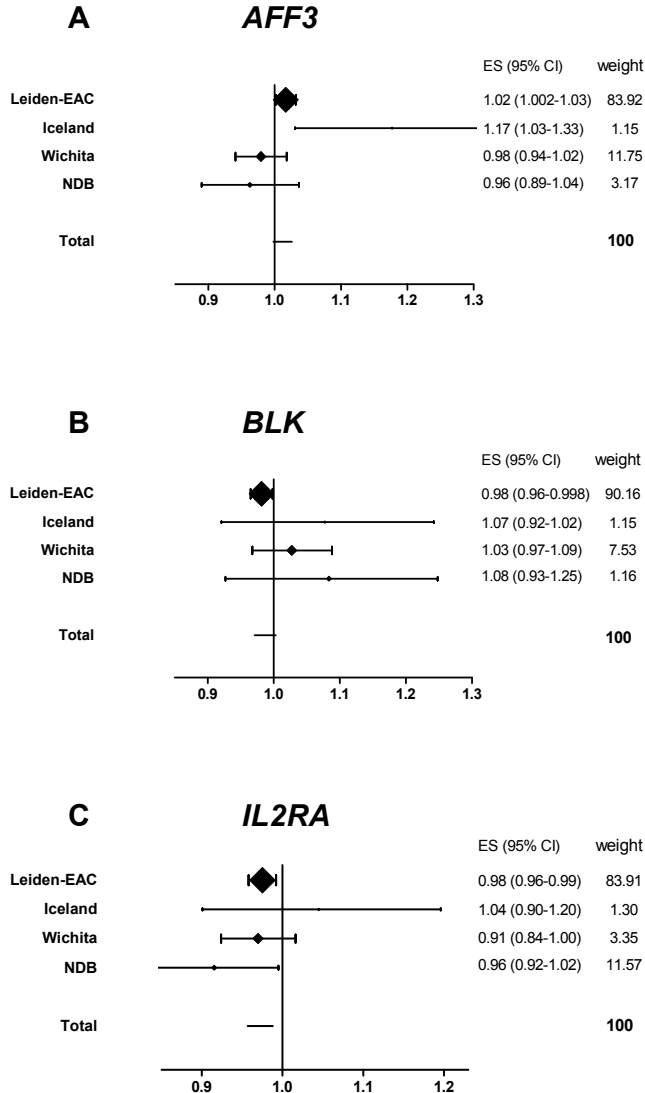


Figure 1 Results for the three SNPs associated with joint destruction in the discovery cohort.

Three susceptibility SNPs were associated with rate of joint destruction in the discovery cohort: A) *AFF3*-rs11676922, B) *BLK*-rs2736340 and C) *IL2RA*-rs2104286. The estimates are the coefficients back-transformed onto the original scale (β_{org}). The meta-analyses are based on a fixed effect model, which is applied to genetic studies to test whether there is a statistically significant effect but which may yield effect size estimates that do not generalize well. The effect size of the meta-analyses should thus be interpreted with caution and is therefore depicted in grey.

replication data-sets or in the meta-analysis ($P=0.11$). AFF3 was independently significant in Iceland ($P=0.015$), but was not significant in the meta-analysis ($P=0.08$). The result of IL2RA-rs2104286 was supported by a significant finding in NDB ($P=0.045$), an a suggestive association when the three data-sets of stage-2 were combined ($P=0.056$), and a significant association in the meta-analysis of all four data-sets ($P=7.2 \times 10^{-4}$, Figure 1). This association was strong enough to survive the conservative Bonferroni correction.

sIL-2RA

Supported by these findings, we studied whether IL2RA-rs2104286 was associated with sIL-2RA concentrations in RA-patients. The minor variant of rs2104286 (C), which is associated with lower rate of joint destruction, was associated with 0.85 (95%CI 0.77-0.93) fold sIL-2RA levels ($P=1.4 \times 10^{-3}$, Figure 2C). Accordingly, we determined whether sIL-2RA was associated with the rate of joint destruction. A decrease of 1,000 pg/mL sIL-2RA was associated with a 0.57 (95%CI 0.39-0.83) fold rate of joint destruction per year ($P=3.4 \times 10^{-3}$, Figure 2D). When the association of both IL2RA-rs2104286 and sIL-2RA were tested in one analysis, only sIL-2RA was significant ($P=8.0 \times 10^{-3}$). This suggests that the association between IL2RA-rs2104286 and joint destruction is mediated by a mechanism affecting sIL-2RA concentrations.

Fine-mapping

To further localize the association of IL2RA with rate of joint destruction, the IL2RA region was fine-mapped and stronger associations with rate of joint destruction than rs2104286 were sought.

In the Leiden-EAC cohort, fifteen variants had a statistically stronger association with rate of joint destruction than rs2104286 (Figure 3). Thirteen of these top hits were situated in one LD block of 40kb ($1.3 \times 10^{-4} > p < 4.4 \times 10^{-3}$, $0.94 = \beta_{\text{org}} = .97$), in which rs2104286 is also situated. This region encompasses IL2RA intron 1 and the 5' region of IL2RA and RBM17 (Chr10:6118559-6158117). Two variants were outside this region; seq-NOVEL-50 (Chr10:6074516) ($\beta_{\text{org}}=1.79$ (95%CI 1.40-2.28), $P=3.2 \times 10^{-6}$), which lies 19kb downstream of IL2RA, and rs41295367 in RBM17 ($\beta_{\text{org}}=1.22$ (95%CI 1.09-1.36), $P=3.4 \times 10^{-4}$). These two variants both had an effect opposite to that of rs2104286. To assess whether these effects were independent, the genetic analysis was repeated including the three SNPs, rs2104286, rs41295367 and seq-NOVEL-50, in one analysis. In this analysis, all three SNPs were independently significant (data not shown).

We further focused on the rs2104286 region by studying the fourteen top hits of this region (rs2104286 and the thirteen additional variants in the IL2RA region) in the two USA data-sets (NDB and Wichita). Six SNPs were significantly associated with progression of joint destruction in the USA data-sets. In the meta-analysis combining the Dutch and USA data-sets (Figure 4A), the association of all fourteen SNPs were significant and

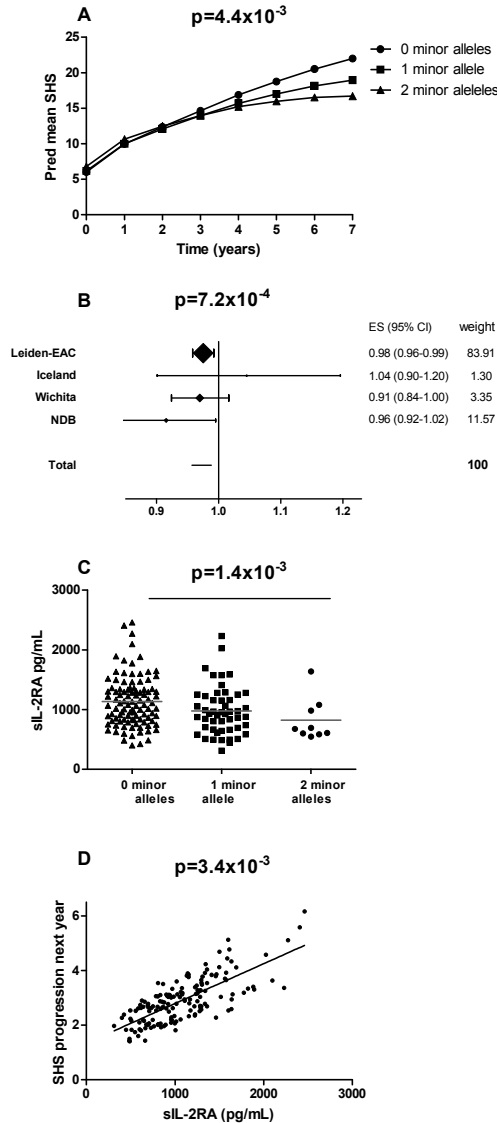


Figure 2 IL-2RA-rs2104286 is associated with rate of joint destruction and sIL-2RA serum levels.

(A) A significant association was found for the *IL2RA* (rs2104286) genotype with rate of joint destruction in the discovery cohort, the Leiden-EAC ($\beta_{\text{adj}}=0.97$ (0.96-0.99), $P=4.4 \times 10^{-3}$), the minor variant or rs2104286 was associated with 0.97 fold lower rate of joint destruction per year, corresponding to 0.65 fold lower rate of joint destruction over 7 years in the presence of 2 minor alleles ($0.97^7 \approx 0.65$). The plotted data are the result of the multivariate normal regression model adjusted for age, sex and treatment. (B) The association was in the meta-analyses of 1,750 RA-patients with 4,732 X-rays ($P=7.2 \times 10^{-4}$). The depicted effect sizes (ES) indicate that the rate of joint destruction is lower in the presence of a minor allele compared to the common genotype. (C) The minor variant of rs2104286 was associated with lower sIL-2RA levels in 159 Leiden-RA patients. The green line indicates the mean. (D) Lower sIL-2RA levels are associated with less progression of joint destruction in the next year ($P=7.2 \times 10^{-4}$). Plotted data are the results of the linear regression analysis adjusted for disease duration at time of serum sample and for age at inclusion.

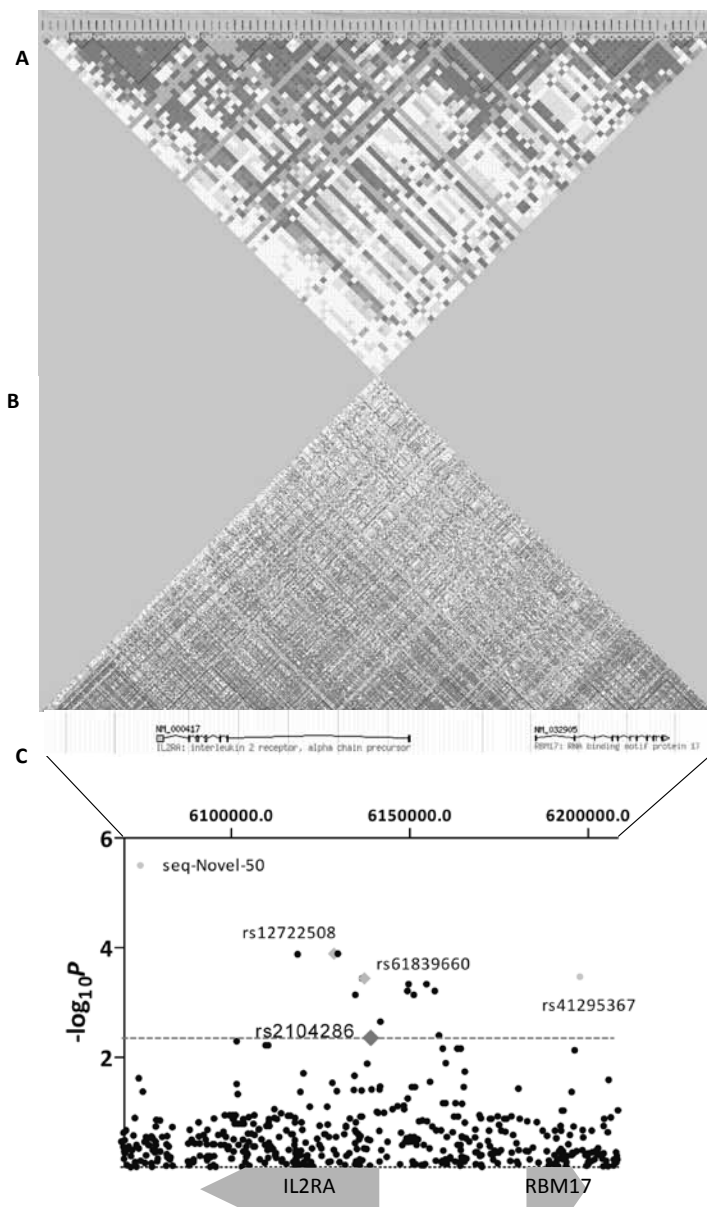


Figure 3 LD plots of the IL2RA-RBM17 region

(A) Total region in hapmap CEU patients and (B) fine-mapped in Leiden-EAC. (C) Results of the multivariate normal regression analysis for 495 genetic variants in the IL2RA-RBM17 region in Leiden-EAC. The colors reflect the D' between the SNPs. Coordinates relate to NCBI36 hg18 release 2006.

the strongest association was found for rs12722508 ($P=2.6 \times 10^{-5}$). This SNP was highly correlated ($r^2 > 0.80$, Figure 4B) to ten other top hits in the region, making it difficult to distinguish which of these variants is of most importance.

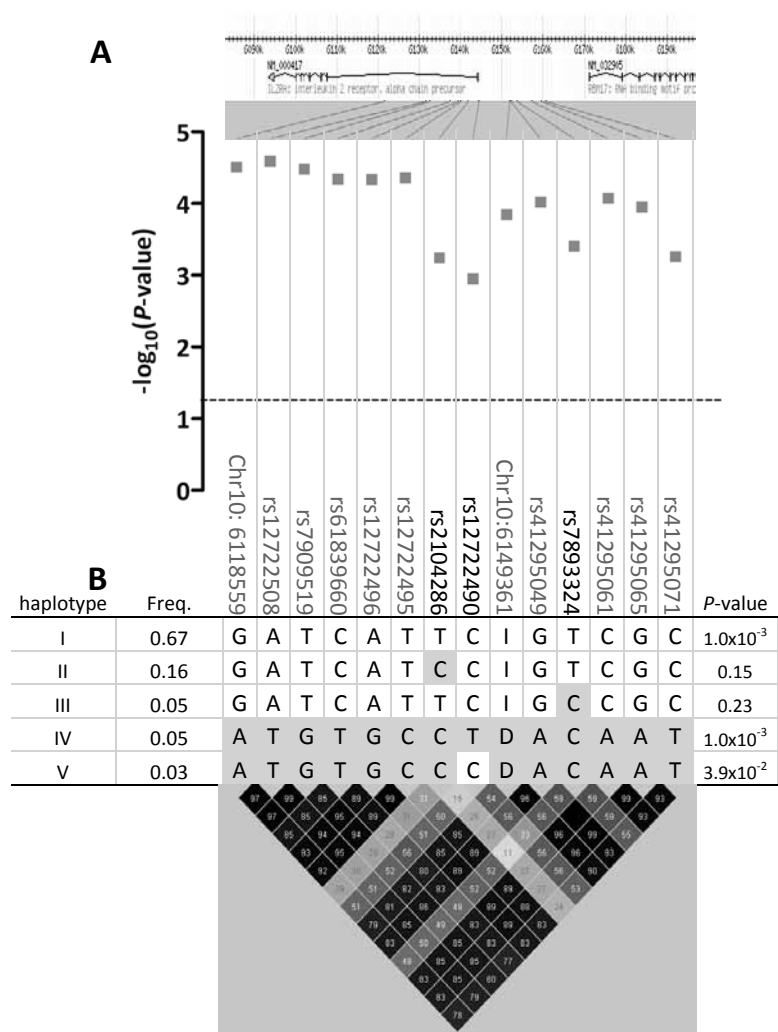


Figure 4 Exploration of the *IL2RA* region
 The results presented here are the combined results of the Leiden and the USA samples (1,465 patients with 4,447 X-rays). (A) In total fourteen variants (including rs2104286) in a 40kb region were strongly associated with rate of joint destruction (dotted line presents the cut-of for significance at 0.05) (B) Haplotype analysis for these fourteen SNPs. Three haplotypes were significantly associated with joint destruction, the most common with risk and two minor haplotypes with protection for joint destruction. Minor alleles are highlighted in grey. SNPs with an $r^2 > 0.8$ with each other are depicted in the same color (orange, green, purple and blue). The LD between the strong variants in RA-patients is depicted with HaploView (see URLS). The color and the numbers in the graph represent the r^2 between the genetic variants.
 Since the presented results are from the fixed effect meta-analysis only p-values are given.

Subsequently haplotype analyses were performed in an attempt to further differentiate between the fourteen genetic variants. To enlarge power, the data of the Leiden-EAC and the North-American samples were combined in inverse weighting meta-analyses

The haplotype that associated most strongly with rate of joint destruction (haplotype IV, $P=1.0 \times 10^{-3}$, Figure 4B) was characterized by one SNP, rs12722490. When the haplotypes were analyzed in the Dutch and the North-American samples separately, similar results were observed (data not shown).

Four SNPs together tag the fourteen top SNPs in the 40kb region; rs12722508 (the strongest associating SNP), rs2104286 (the initial SNP), rs12722490 (the haplotype-tagging SNP) and rs7893324 (in low LD with all other SNPs in 40kb region) (see Figure 4B). The correlation between these four SNPs was modest (r^2 0.15-0.50).

DISCUSSION

The variance in joint destruction between patients is considerable and the mechanisms driving these differences are thus far scarcely understood. In the past decades, many genetic variants have been identified to associate with the susceptibility of RA. We hypothesized that similar variants could be involved in disease severity as well. Therefore, a candidate gene study was performed to investigate the association of identified RA-susceptibility SNPs with rate of joint destruction. We observed that RA-patients with the minor genotype of IL2RA-rs2104286 had lower rate of radiographic joint destruction than patients with the common genotype. This association was further supported by the observations that the minor genotype of rs2104286 were associated with lower circulating soluble IL-2RA levels and that lower sIL-2RA levels was associated with lower rate of joint destruction. Finally, the association was localized to a region of 40kb encompassing the IL2RA intron 1 and the 5' region of IL2RA and RBM17.

The present study uniquely combines data-sets of patients who started treatment in a time when treatment was not as aggressive as nowadays. Hence, the radiologic progression rate of the patients studied here are more reflective of the natural course of RA than that of recently treated patients. Replication data-sets are ideally larger than the discovery data-set, since at a replication stage effects sizes are generally smaller. Unfortunately, relatively few large prospective data-sets exists with both X-rays and DNA available in conventionally treated patients. The number of patients and the number of X-rays of each separate data-set were insufficient to expect well powered analyses. However, the combination of the data in an inverse variance weighting analysis supported the association of IL2RA-rs2104286 with rate of joint destruction in the discovery data-set. In addition, independent replication of the association of rs2104286 was obtained in the NDB. Furthermore six hits in the 40 kb IL2RA region were significantly associated with progression of joint destruction in the Dutch and the North-American RA-patients.

IL2RA (CD25) encodes the high affinity alpha chain of the IL-2 receptor. It is expressed by numerous immune cell types, including several subsets of CD4+ T-cells, and it is

upregulated in inflammatory conditions.^{35,36} Besides the known association with RA susceptibility,^{20,24} *IL2RA* is also associated with the risk on other auto-immune diseases such as Juvenile Idiopathic Arthritis³⁷, Type-I-Diabetes (T1D)^{38,39}, Multiple Sclerosis (MS)⁴⁰, Systemic Lupus Erythematosus⁴¹ and Grave's disease⁴². In most of these associations, the minor allele rs2104286 was associated with a protective effect on disease susceptibility. Closer studies on the *IL2RA* region in T1D and MS have demonstrated that other genetic variants in the region of rs2104286 play a supplementary role, which is sometimes in the opposite direction of rs2104286.^{38,43,44} Some studies in T1D, in MS and in healthy individuals also revealed that carrying the rs2104286 minor allele is associated with lower circulating levels of soluble IL-2RA (sIL-2RA)^{43,44} sIL-2RA is produced by the proteolytic cleavage of membrane-bound IL-2RA after the induction of growth and cell division.^{43,45-473} and hence considered as a marker for T-cell proliferation.

The association of *IL2RA*-rs2104286 with susceptibility to RA was initially discovered by Barton et al.²⁰ and afterwards supported by Stahl et al.²⁴ In the latter study another variant, rs706778, was more strongly associated with RA susceptibility. The association was strongest for the haplotype defined by rs706778-rs2104286-rs11594656. In the current study evaluating the severity of RA, rs760778 was analyzed in the discovery cohort; no association of rs760778 with rate of joint destruction was observed. Also rs11594656 was not significantly associated in the analyses on the fine-mapped data.

The RA susceptibility SNPs analyzed in this study were identified in previous GWAs studies. GWAs platforms are designed to type SNPs throughout the genome but genes are not fine-mapped, making it possible that other, non-typed variants that are linked to the observed variant, are the cause of phenotypic variation. To further localize the association of *IL2RA* with rate of joint destruction, we fine-mapped the *IL2RA* region and sought stronger associations with rate of joint destruction. Here we found SNPs that had a stronger association with rate of joint destruction than rs2104286 and several were independently replicated in the North-American samples.

In our study, fourteen SNPs in a 40 kb region of *IL2RA* associated with progression of joint destruction. It cannot be concluded which one of these are the most important. Fine-mapping of *IL2RA* in case-control studies revealed an association of the same set of linked SNPs (linked to rs12722508) found in studies of T1D and MS.

Thus far, no studies have investigated genetic variants in *IL2RA* with long-term disease outcome. The present study provides evidence for a role of *IL2RA* in disease progression in RA. Dendrou et al. observed in healthy individuals that presence of the rs2104286 minor allele reduced the probability of CD4+ T-cells expressing CD25, thereby reducing the likelihood of CD4+ T cell activation.³⁶ Notably, an effect of the *IL2RA* genotypes on the expression of CD25+ in T-regulatory cells was not observed.³⁶ Those results are consistent with our observation of lower sIL-2RA and lower rate of joint destruction in the presence of the rs2104286 minor allele. Possibly, the protective *IL2RA* genotypes mediate a reduced

expression of CD25 and less activation of CD4+ T cells, preventing severe inflammation and reducing long term joint destruction.

Hampering the expansion of T cells with daclizumab, an antibody against IL-2RA, has shown to be beneficial as treatment for MS.⁴⁸ The effects of daclizumab on specific immune cells are not completely characterized. Since T cell activation is very probably involved in disease development and progression in RA, it would be interesting to assess the efficacy of daclizumab in RA.

The present study is one of the few large scale studies evaluating genetics in relation to the severity of RA. The associations of candidate gene loci with progression of joint destruction were studied using longitudinal data-sets with high quality phenotypic data. The genetic and serologic data provide evidence for a role of IL2RA in progression of RA, making it an interesting target for future therapeutic studies.

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WEB RESOURCES

The URLs for data presented here are as follows: SPSS version 17.0, www.spss.com

Stata version 10.0, www.stata.com

Entrez Gene, <http://www.ncbi.nlm.nih.gov/gene> <http://genome.ucsc.edu/cgi-bin/hgGateway>

Plink version 1.07, <http://pngu.mgh.harvard.edu/~purcell/plink/> Haploview version 4.2, www.broad.mit.edu/mpg/haploview/

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Chapter 10

A genetic variant in the region of *MMP-9* is associated with serum levels and progression of joint damage in Rheumatoid Arthritis

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ABSTRACT

Objectives

The severity of joint destruction is highly variable between Rheumatoid Arthritis (RA) patients. The majority of its heritability is still unexplained. Several auto-immune diseases share genetic risk variants that may also influence disease progression. We aimed to identify genetic risk factors for the severity of joint damage in RA by studying genetic susceptibility loci of several auto-immune diseases.

Methods

In phase-1, 3143 sets of X-rays of 646 Dutch RA-patients taken over 7-years (Sharp-van der Heijde (SHS) scored) were studied. Genotyping was done by Immunochip. Associations of SNPs with MAF >0.01 and joint destruction were analyzed. In phase-2, 686 North-American RA-patients with 926 SHS-scored X-rays over 15 years of follow-up were evaluated. In both phases multiple testing corrections were done for the number of uncorrelated SNPs; the thresholds for significance were $p < 1.1 \times 10^{-6}$ and $p < 0.0036$. MMP-9 levels were measured with ELISA in baseline serum samples.

Results

In phase-1, 109 SNPs associated significantly with joint destruction ($p < 1.1 \times 10^{-6}$). Of these, 76 were located in the HLA-region; the 33 non-HLA variants were studied in phase-2. Here two variants were associated with the severity of joint destruction: rs451066 on chromosome 14 ($p = 0.002$, MAF=0.20) and rs11908352 on chromosome 20 ($p = 0.002$, MAF=0.21). Rs11908352 is located near the gene encoding Matrix Metalloproteinase-9 (MMP-9). Serum levels of MMP-9 were significantly associated with the rs11908352 genotypes ($p = 0.007$).

Conclusion

These data indicate that two loci that confer risk to other autoimmune diseases also affect the severity of joint destruction in RA. Rs11908352 may influence joint destruction via MMP-9 production.

INTRODUCTION

Persistent inflammation and destruction of joints are hallmarks of Rheumatoid Arthritis (RA). The severity of RA is reflected by the severity of joint destruction and is highly variable between patients. The processes underlying these inter-individual differences are incompletely understood. Known risk factors mainly relate to inflammation and auto-antibodies and are estimated to explain 32% of the variance in joint destruction.¹ In a recent Icelandic study the heritability of the rate of joint destruction was estimated at 58%.² Identifying the individual genetic risk factors may enhance the comprehension of mechanisms underlying the differences in severity of joint destruction between patients. Recently, some risk factors for progression of joint damage were identified³⁻⁵ but the majority of the genetic risk factors underlying the estimated heritability are still to be discovered.

Several auto-immune diseases share genetic risk variants. For instance, some genetic variants that are associated with an increased risk on RA are also associated with susceptibility to other immune diseases, such as type-1 diabetes, celiac disease and systemic lupus erythematosus.⁶⁻⁸ Such observations have led to the development of the Immunochip consortium and the Immunochip genotyping platform that includes dense mapping of markers across 186 loci associated with 12 auto-immune diseases.⁹ This platform allows fine-mapping of immune-mediating loci and replication of previously identified variants.^{10,11} Indeed, with regards to RA susceptibility, a recent study refined the association signals for 19 loci using the Immunochip.¹¹

Susceptibility factors may also influence disease progression; examples within RA are the HLA-DRB1 shared epitope alleles and the IL2RA locus.^{12,13} This study was performed under the assumption that not only several diseases share key molecular processes, but that RA-development and RA-progression do so as well, which is reflected by shared genetic risk variants. We therefore took advantage of the Immunochip platform and studied progression of joint destruction in RA.

PATIENTS AND METHODS

Patients

Phase-1

646 consecutive RA-patients (according to the 1987 ACR-criteria), included in the Leiden-Early Arthritis Clinic between 1993-2006, were studied. X-rays were taken at baseline and yearly thereafter during 7 years. In total, 3143 sets of hands and feet X-rays were

chronologically scored by one experienced reader who was unaware of genetic or clinical data using the Sharp-van der Heijde scoring method (SHS). The correlation coefficient (ICC) within the reader was 0.91. Treatment strategies differed in three treatment periods: (1993-1995, initial treatment with NSAIDs, 1996-1998, initial treatment with chloroquine or salazopyrine and 1999-2006 prompt treatment with methotrexate or sulfasalazine).¹

Phase-2

Here, 686 North American 1987-ACR-criteria-positive RA-patients were studied. 118 were recruited from a practice in Wichita¹⁴, Kansas and 568 were recruited from the National Databank of Rheumatic Diseases (NDB).¹⁵ Serial hands X-rays were available in the patients recruited from Wichita. In the NDB-patients, hands X-rays were made at a single time-point. All X-rays were SHS-scored by one experienced reader (ICC=0.98). All patients developed RA in eras when early, tailored treatment and use of biologics were uncommon.¹⁶

Patients gave informed consent. Approval was obtained from the local medical ethical committees.

Genotyping

Phase-1

Genotyping was done using the Immunochip according to Illumina's protocols.⁹ The Illumina Genome Studio software was used to perform the SNP clustering. The SNP clustering and quality control of samples was performed as described in Trynka et al.⁹ Samples were excluded for call rate <99.5% across 172,242 markers. We also excluded samples for incompatible recorded and genotype-inferred gender, duplicates and first- or second-degree relatives. Potential ethnic outliers were identified by multi-dimensional scaling plots of samples merged with HapMap3 data.

Initially, 704 RA cases were genotyped. 14 patients were excluded due to call rates below 99.5%, 16 because they were relatives or duplicates, 26 because they were population outliers and 1 patient was excluded because of a gender mismatch. Finally, one patient was excluded because no clinical data were available. After quality control 646 Dutch RA cases were included in the analysis. After removing non-polymorphic markers, 148,880 SNPs remained for further analysis. In this study SNPs with MAF>0.01 (130,841 SNPs) were analyzed.

Phase-2

Genotyping of phase-2 samples was done by Immunochip. Quality control was similar as in phase-1. SNPs that were significantly associated with the severity of joint damage in phase-1 were selected and analyzed.

Statistical analysis

Phase-1

In both phases the radiological scores were log-transformed in order to obtain a normal distribution. An additive model was used. SNPs can have a constant effect over time, an effect on progression, or both. In the present study, evaluating >100k SNPs, it was not possible to study the raw data per SNP to choose the effect to be tested. Therefore, a method that analyzed both constant and main effects together was used, this resulted in an “overall p-value”. This concerned a marginal regression model for longitudinal data with the radiological score as a response variable. This method takes advantage of the within-person correlation between repeated measurements. The null hypothesis was that the coefficients for both the main SNP effect and its interaction with time equal zero, the alternative hypothesis was that at least one differed. This hypothesis has been tested using the likelihood ratio test, which is assumed to follow under the null the chi-squared distribution with 2 degrees of freedom. The effect of time was entered as a factor in the model, to capture the mean response profile over time properly. The effect of time in the interaction term was linear. Adjustment variables (age, gender and the described treatment strategies) were entered based on their univariate association with joint destruction. The Bonferroni correction for multiple testing was done by the number of uncorrelated SNPs. In the phase-1, multiple testing correction was based on $R^2 > 0.5$ (44,544 uncorrelated SNPs). Given the explorative character of this phase, that would be followed by a replication phase, we have chosen to correct for the number of SNPs with $R^2 > 0.5$; hence the cut-off for significance was set at 1.1×10^{-6} .

Phase-2

An additive model was used. In the Wichita-patients, associations between genetic variances and joint destruction over time were analyzed using a marginal regression model as described above. For the NDB-patients, the estimated yearly progression rate was calculated (total SHS divided by disease years at time of X-ray). The mean disease duration at the time of the X-ray was 10 years. The SNP association was tested in a linear regression model with $\log_e(\text{estimated progression} + 1)$ as outcome variable. Analyses were corrected for gender and age. All patients were included (long) before 2000, when treatment was relatively mild and treatment strategies did not change between the inclusion years. Treatment adjustments were therefore not made. As both cohorts evaluated in phase-2 had less patients and less X-rays than the cohort of phase-1, the power was expected to be insufficient to obtain significant results in the individual cohorts. Therefore, the coefficients for the main SNP effect and its interaction with time observed in both data-sets of phase-2 were combined into an inverse variance weighted fixed-effects meta-analysis. Then the combined estimates and their corresponding variance-covariance matrix were

used in a multivariate Wald test to test for the significance of both the main SNP effect and its interaction with time. In this case, the multivariate Wald test is assumed to follow under the null hypothesis, the Chi-squared distribution with 2 degrees of freedom. Also here the Bonferroni correction for the number of uncorrelated SNPs (now based on the more stringent $R^2=0.8$) tested in phase-2 was applied, yielding a threshold for significance of 3.5×10^{-3} .

Testing of serum-MMP-9 levels

Levels of MMP-9 in the baseline serum samples of 105 patients with RA from the Leiden EAC were measured using ELISA (eBioscience BMS2016/2). Patients were selected on their genotype (homozygous major versus homozygous minor) in rs11908352, the most significantly associating SNP on chromosome 20.

Software

All analyses were done using the R statistical software package.¹⁷ P-values of the SNPs of the region from 68238574 Kb to 68387815 Kb on chromosome 14 and of the region from 44027635 to 44217743 Kb on chromosome 20, their linkage and the genes located in this region were made using snap software.¹⁸

RESULTS

Phase-1

The characteristics of the patients studied are presented in Table 1. In the first phase, 109 SNPs passed the threshold for significance ($P < 1.1 \times 10^{-6}$, Supplementary Table 1). Of these, 76 were located in the HLA-region on chromosome 6; a region of which the association with severity of joint damage was already known.¹⁹ These variants were not studied in

Table 1. Characteristics of the populations studied

	Phase-1	Phase 2	
	Leiden-EAC (n=600)	Wichita (n=118)	NDB (n=568)
Total number of x-ray sets	3143	358	568
Year of diagnosis	1993-2006	1955-1999	1980-1999
Follow-up years*	7 years	1-15 years	1-20 years
Method of scoring	SHS	SHS	SHS
Female, n (%)	431 (66.5)	80 (67.8)	444 (78.2)
Age at diagnosis (years), mean $\pm$ SD	56.9 (15.6)	48.1 (12.2)	48.6 (12.7)
ACPA-positivity (%)	325 (52.2)	113 (96.6)	453 (79.8)

*Data of Leiden-EAC for 7 of follow-up, respectively. In the Wichita and NDB patients, the maximum duration of follow-up was 15 and 20 years respectively. EAC: Early Arthritis Clinic, SHS: Sharp-van-der-Heijde score.

phase-2. Among the other 33 non-HLA genetic variants (presented in Table 2), several were in high LD. A Q-Q plot of the p-value distribution is depicted in Supplementary Figure 1.

Table 2. Results of phase-1 and phase- 2.

Chr	Position	SNP	Locus/loci	P phase-1	P phase-2
1	92,917,314	kg_1_92917314		2.32x10 ⁻⁰⁹	NA
2	43,183,613	rs4567998	<i>CR600703, ZFP36L2</i>	4.00x10 ⁻⁰⁹	0.53
2	43,183,644	rs4575770	<i>CR600703, ZFP36L2</i>	4.08x10 ⁻⁰⁹	0.73
2	43,183,824	rs6709667	<i>CR600703, ZFP36L2</i>	4.23x10 ⁻⁰⁹	0.70
2	43,185,647	rs4952660	<i>CR600703, ZFP36L2</i>	4.86x10 ⁻⁰⁹	0.85
2	43,188,295	rs11306948	<i>CR600703, ZFP36L2</i>	2.22x10 ⁻⁰⁷	0.98
2	196,806,195	rs4241188	<i>HECW2</i>	7.70x10 ⁻⁰⁷	0.42
3	24,899,499	rs6550923	<i>THRB</i>	9.17x10 ⁻⁰⁷	0.08
5	176,542,618	rs6872021	<i>NSD1</i>	1.59x10 ⁻⁰⁷	0.16
5	176,592,176	rs7724098	<i>NSD1</i>	3.43x10 ⁻⁰⁷	0.28
5	176,717,045	rs4976688	<i>RGS14</i>	2.75x10 ⁻⁰⁷	0.28
5	176,717,118	rs4075958	<i>LMAN2, RGS14</i>	1.75x10 ⁻⁰⁷	0.18
5	176,721,176	rs4976646	<i>RGS14</i>	1.70x10 ⁻⁰⁷	NA
5	176,730,912	rs11746443	<i>RGS14</i>	1.07x10 ⁻⁰⁶	0.75
9	74,710,880	rs348472	<i>ALDH1A1</i>	4.22x10 ⁻⁰⁷	0.007
9	100,689,774	rs2038621	<i>GALNT12, COL15A1</i>	4.73x10 ⁻⁰⁷	0.38
9	100,702,907	rs732726	<i>GALNT12, COL15A1</i>	4.43x10 ⁻⁰⁷	0.43
10	16,966,739	rs780849	<i>CUBN</i>	9.27x10 ⁻⁰⁸	0.28
10	61,970,935	rs1938525	<i>ANK3</i>	1.07x10 ⁻⁰⁶	0.12
11	70,877,597	rs10898200	<i>NADSYN1</i>	1.76x10 ⁻⁰⁷	0.52
12	26,514,209	rs1565190	<i>ITPR2</i>	8.35x10 ⁻⁰⁷	0.31
12	54,990,419	rs10783780	<i>CNPY2</i>	3.09x10 ⁻⁰⁸	0.58
12	55,027,965	rs12422499	<i>STAT2</i>	3.09x10 ⁻⁰⁸	0.58
12	112,687,229	rs7305258	<i>AK096932/BC007399</i>	5.33x10 ⁻⁰⁷	0.39
13	43,277,896	rs9590793	<i>ENOX1</i>	7.95x10 ⁻⁰⁷	NA
14	68,295,438	rs451066	<i>RAD51L1, ZFP36L1</i>	1.09x10 ⁻⁰⁶	0.002
14	68,296,852	rs189866	<i>RAD51L1, ZFP36L1</i>	1.09x10 ⁻⁰⁶	0.002
14	68,386,268	rs57418859	<i>ZFP36L1, c14orf181, ACTN1</i>	4.30x10 ⁻⁰⁷	NA
18	10,401,194	rs206514	<i>AX747048, APCDD1</i>	2.66x10 ⁻⁰⁷	0.036
19	59,543,040	rs8102662	<i>LILRA4</i>	2.40x10 ⁻⁰⁸	0.254
20	44,085,804	rs62215576	<i>SLC12A5, MMP-9, CD40</i>	1.75x10 ⁻⁰⁷	0.003
20	44,089,547	rs11908352	<i>SLC12A5, MMP-9, CD40</i>	1.75x10 ⁻⁰⁷	0.002

Depicted are the results of the SNPs that showed a significant association in phase 1 and the p-values obtained in phase-2. For technical reasons four variants that were significant in phase-1 were not assessed in phase-2 (NA). Both rs451066 and rs189866 on chromosome 14 and rs11908352 and rs62215576 on chromosome 20 were in high LD ($R^2=0.995$ and $R^2=0.998$ respectively).

Phase-2

Four SNPs did not pass quality control in phase-2, all other non-HLA variants ($n=29$) were assessed. From the 29 SNPs, four SNPs passed the threshold for significance and were associated with joint damage progression. These were rs451066 and rs189866 on chromosome 14 (both $MAF=0.20$ and $p=0.002$) and rs11908352 and rs62215576 on chromosome 20 (both $MAF=0.21$ and $p=0.002$). Rs451066 and rs189866 are in high LD ($R^2=0.995$). After conditioning for rs451066, rs189866 was no longer significant, indicating that both SNPs reflect one signal. The same was observed for rs11908352 and rs62215576 on chromosome 20 ($R^2=0.998$). Consequently, our study actually revealed two replicated risk loci for the radiographic severity on RA, one on chromosome 14 and one on chromosome 20.

Rs451066

The progression of joint destruction of the patients with different rs451066 genotypes is presented in Figure 1 (in addition the by the model predicted mean values are presented in Supplementary Figure 2). Rs451066 is located on chromosome 14, downstream of the genes *ZFP36L1* and *C14orf181*. Rs451066 was included on the ImmunoChip because rs1465788, another SNP on this locus, is associated with type-1 diabetes. A conditional analysis including both rs451066 and rs1465788 was performed to evaluate whether rs451066 actually reflected the same risk factor as rs1465788, here only rs451066 was significantly associated with progression of joint destruction ($p=6.3 \times 10^{-5}$). Fine mapping data of the locus in relation to progression of joint destruction (Figure 1B) revealed that the strongest association in this region was indeed observed for rs451066.

Rs11908352

The raw SHS-scores of patients with different rs11908352 genotypes are presented in Figure 2 and the by the model predicted mean values in Supplementary Figure 2. As depicted, carrying the minor C-allele was associated with less severe joint damage progression. Rs11908352, on chromosome 20, was included on the ImmunoChip as part of the fine-mapping of the *CD40* locus. Rs11908352 is located 92 kb downstream of *CD40*-rs4810485, a known factor for RA-susceptibility. Rs11908352 is however in low LD ($R^2=0.033$) with rs4810485 and a conditional analysis including both rs11908352 and rs4810485 showed an independent association for rs11908352 ($p=0.006$) with RA severity. Rs11908352 is located 25 kb at the 3'-end of the gene encoding for MMP-9 and in high LD with some variants located within the *MMP-9* gene (see Figure 2D for fine mapping results). Several variants within the *MMP-9* gene showed association to RA-severity, however, in a conditional analysis including these variants, only rs11908352 yielded a significant result ($p=0.035$). Altogether, we observed that rs11908352 was the strongest with progression of joint damage associating SNP in this region.

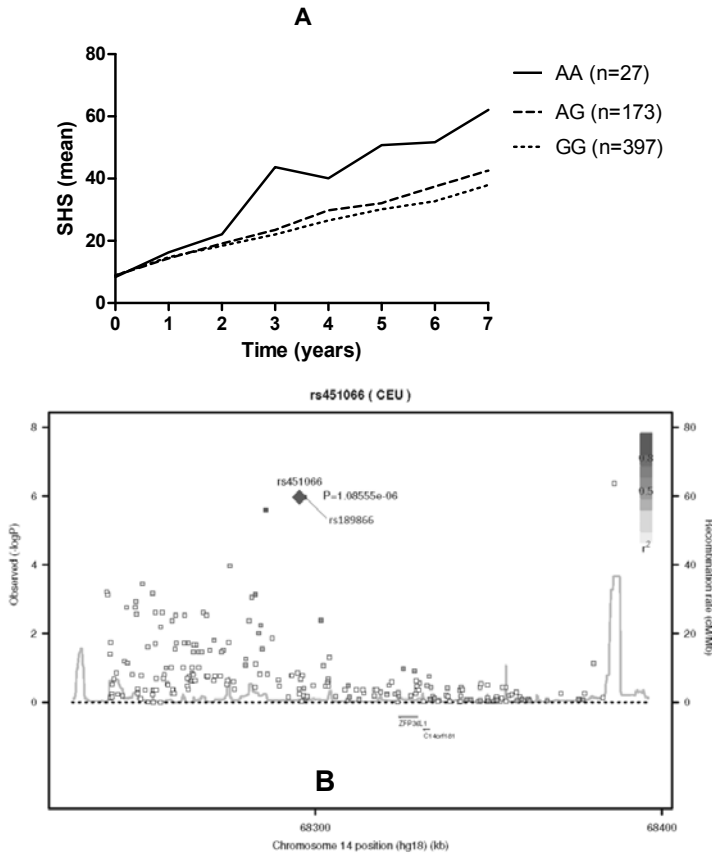


Figure 1. Joint damage (raw SHS scores) over 7 years of disease for rs451066 (A) and fine-mapping of the region of significantly associating SNPs on chromosome 14 (B). Depicted are the mean SHS values during 7 years of follow-up of Dutch RA-patients (A) and p-values of the SNPs of the region from 68238574 Kb to 68387815 Kb on chromosome 14, their linkage and the genes located in this region.

MMP-9 serum levels

Subsequently, we tested whether the different genotypes of rs11908352 were related to different serum levels of *MMP-9* using a linear regression analysis. Patients carrying the AA genotype, which was also associated with more severe joint damage, had significantly higher serum levels of *MMP-9* compared to patients carrying the CC (major) allele combination ($p=0.007$, see Figure 2C). In order to evaluate whether the effect was independent of markers of systemic inflammation, this analysis was repeated with the log-transformed CRP values at baseline as a covariate. Patients carrying the AA genotype still had significantly higher serum levels of *MMP-9* ($p=0.008$).

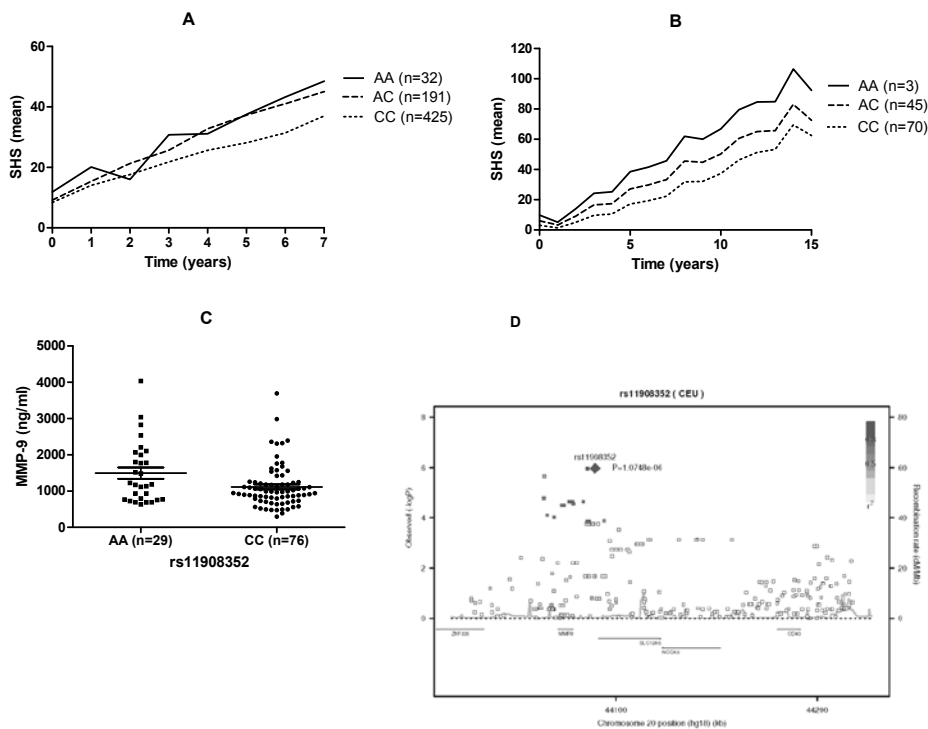


Figure 2. Joint damage in RA-patients in phase-1 and phase-2 for different rs11908352 genotypes, serum MMP-9 levels for different genotypes of rs11908352 and fine-mapping of rs11908352.

Depicted are the mean SHS values during 7 years of follow-up of Dutch RA-patients (A) and the predicted (by the multivariate normal regression model) SHS values (mean) during 15 years of follow-up of patients with repeated measurements available in phase 2 (B) for rs11908352 on chromosome 20. In Figure 2C, MMP-9 serum levels in patients of phase-1 carrying rs11908352 AA and CC genotype are depicted. The mean serum level for the patients carrying the AA alleles was 1493ng/ml, whereas the mean level for the patients carrying the CC alleles was 1112ng/ml. In Figure 2D, the p-values of the SNPs of the region from 44027635 to 44217743 Kb on chromosome 20, their linkage and the genes located in this region are depicted. Rs6073985 was, after rs11908352 and rs62215576 the next best hit in this region ($p=2.26 \times 10^{-6}$; the R^2 between rs11908352 and rs6073985 was 0.38).

Subanalyses

With regards to RA susceptibility it has been observed that some genetic variants predispose only to ACPA-positive or ACPA-negative disease. In line with this it can be questioned whether genetic factors associating with joint damage progression are also different for ACPA-positive and ACPA-negative RA.²⁰ It was observed that for rs451066 the effect sizes were almost similar in both subgroups; for rs11908352 the effect size was larger in the ACPA-positive than in the ACPA-negative subgroup. However, considering the small number of patients per subgroup, none of these analyses resulted in significant p-values. Larger studies are required for definite conclusions on the differential association of these variants in ACPA-positive and ACPA-negative RA.

DISCUSSION

The present study aimed to identify the genetic variants that associate with progression of joint destruction in RA. Associated genetic risk factors, and therefore presumably also part of the underlying pathogenesis, are shared in several auto-immune diseases. Furthermore, genetic variants that confer risk to disease development may also affect disease progression. These estimations prompted us to evaluate associations between genetic risk factors for a wide range of auto-immune diseases and the severity of the radiographic joint destruction in RA. The present study identified and provided independent replication for two new genetic risk variants for the severity of joint damage in RA and an association with the protein expression for one of these two variants.

Rs11908352 was observed to be associated with the severity of joint damage in RA. This variant is located on chromosome 20, in a region also encompassing rs4810485 (*CD40*) that is associated with susceptibility to RA. In a previous study, we reported an association between this SNP and the severity of joint destruction.⁴ Rs11908352 is however in low LD (R^2 0.062) with rs4810485 on *CD40* and the finding observed here is independent of this variant in *CD40*. Rs11908352 is located at the 3' site of *MMP-9* and in high LD with SNPs located within *MMP-9*. *MMP-9* is a member of a group of secreted zinc metalloproteinases which degrade the collagens of the extracellular matrix. *MMP-9* is, amongst others, produced by macrophages and increased serum levels of *MMP-9* have been described in RA.²¹ Expression of *MMP-9* has also been demonstrated in fibroblasts and osteoblast-like cells in the subchondral bone of destructive joints in RA.²⁰ We subsequently explored how this genetic variant could affect progression and questioned whether rs11908352 influences joint destruction by a mechanism that also influences the serum levels of *MMP-9*. We observed that patients with the AA genotype that was associated with more severe joint damage also had higher serum levels of *MMP-9*. This may suggest that rs11908352 affects *MMP-9* production.

Interestingly, the rs11908352 variant is located in the region active chromatin, as indicated by the recent findings of Encyclopedia of DNA Project (ENCODE).²² In the majority of cell types (127 out of 148) included to the ENCODE project, the locus containing rs11908352 is annotated to be in the DNase hypersensitive site (DHS). DHS sites are enriched for regulatory and promoters regions and is a sign of active genome locus. Moreover, the rs11908352 C/A variant itself alters the regulatory motifs of EVI-1 and HBP1, according to the HaploReg database.²³ Both are the transcriptional regulators that bind to DNA sequence in the promoter regions of target genes and regulate their expression. This can possibly explain the mechanism of the effect of the rs11908352 genotype on *MMP-9* transcription. Rs11908352 was not annotated as an eQTL.

The other SNP that was identified in both phases was rs451066. The region of rs451066 was previously shown to be associated with susceptibility to type-1 diabetes.²⁴ A conditional analysis revealed that the association between rs451066 and joint destruction is independent of the effect of rs1465788, the SNP linked to type-1 diabetes susceptibility. Nonetheless, the present data may imply that type-1 diabetes and progression of RA have a partly shared genetic background.

Interestingly, the variants observed here were not identified as risk factors for the development of RA in the Immunochip study of Eyre et al.¹¹ In addition, Eyre et al identified genetic risk factors for RA susceptibility that did not appear in the top list of SNPs of the present study. The p-values of the association between the known RA susceptibility factors and the severity of joint damage in phase-1 are presented in Supplementary Table 2. *IL-2RA* was identified as risk factor for RA development and joint damage progression in previous studies.^{11;13} *TNFAIP3-OLIG3* was identified but not replicated as severity factor.²⁵ The other variants that achieved nominal significance were not yet studied in independent cohorts. It should be noted that the present study contained considerably less patients than the study of Eyre et al. Nonetheless these data indicate that the genetic factors associated with susceptibility to and progression of RA are mostly different, suggesting that the processes driving the development of RA and progression of joint damage within RA are different as well.

Strengths of this study include the evaluation of RA-patients with serial X-rays; the presence of repeated radiological measurements increases the reliability with which the outcome measure can be determined, and hence increases the power. Clinical data were collected in eras when treatment was less aggressive than today and biologics were not yet common. Hence the natural course of the patients studied was less inhibited than that of patients that are currently being treated. The patients included in the Leiden EAC were treated conventionally, though with different strategies and adjustments for these differences were made. The US patients were included in even earlier time periods and no treatment effects were observed. Another strength of the study is that the identified loci were densely genotyped by the Immunochip allowing detection of the genetic variant in the region that has the strongest association.

The approach taken also encompasses several disadvantages. In contrast to conventional GWAS, the Immunochip only covers pre-selected regions on the genome. Therefore, our study-design is not suitable for detecting associations between genomic regions not included on this chip. Furthermore, the present study may include false-negative findings. This is inherent to a study design in which a lot of variants are genotyped and analyzed.

In addition, although our study included a considerable number of patients and X-rays, achieving adequate power in longitudinal cohort studies remains difficult as high-quality long-term outcome data are required. Hence the approach is focused on finding replication of the most significant signals, we can therefore not exclude that other SNPs located on the Immunochip platform, that had p-values higher than 1.1×10^{-6} in phase-1 could be associated with the rate of joint destruction in RA.

In conclusion, the current data indicate that two loci that confer risk to autoimmune disease may specifically affect the severity of joint destruction in RA, and one of these effects is mediated by a mechanism that also affects *MMP-9* serum levels. Further studies are required to unravel the functional consequences of carrying these risk factors, and the processes mediating radiographic progression in RA.

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SUPPLEMENTARY DATA

Supplementary Table 1. P-values obtained in the first phase of the study

Chromosome	SNP	MAF	P-value
1	1kg1_92917314	0.01	2.3×10^{-09}
2	rs4567998	0.26	4.0×10^{-09}
2	rs4575770	0.26	4.1×10^{-09}
2	rs6709667	0.26	4.2×10^{-09}
2	rs4952660	0.26	4.9×10^{-09}
2	rs11306948	0.25	2.2×10^{-07}
2	rs4241188	0.03	7.7×10^{-07}
3	rs6550923	0.33	9.2×10^{-07}
5	rs6872021	0.25	1.6×10^{-07}
5	rs7724098	0.24	3.4×10^{-07}
5	rs4976688	0.32	2.8×10^{-07}
5	rs4075958	0.29	1.7×10^{-07}
5	rs4976646	0.35	1.7×10^{-07}
5	rs11746443	0.30	1.1×10^{-06}
6	rs1264423	0.48	5.2×10^{-07}
6	rs1264423	0.48	5.2×10^{-07}
6	rs1264419	0.48	5.2×10^{-07}
6	rs1140809	0.48	3.1×10^{-07}
6	rs1075496	0.42	1.0×10^{-08}
6	rs2076536	0.28	3.2×10^{-10}
6	rs9268494	0.36	8.0×10^{-08}
6	rs9268497	0.36	8.0×10^{-08}
6	rs9268835	0.34	3.0×10^{-08}
6	rs6923504	0.24	1.1×10^{-11}
6	rs6903608	0.24	1.1×10^{-11}
6	rs9268838	0.34	3.0×10^{-08}
6	rs9268853	0.37	2.0×10^{-08}
6	rs9268882	0.24	1.1×10^{-11}
6	rs9268923	0.37	2.0×10^{-08}
6	rs2395185	0.37	2.0×10^{-08}
6	rs9268969	0.37	5.0×10^{-09}
6	rs9405108	0.37	2.0×10^{-08}
6	rs9269081	0.24	1.6×10^{-11}
6	rs9269110	0.24	1.1×10^{-11}
6	rs1964995	0.47	9.2×10^{-14}
6	rs477515	0.34	5.3×10^{-07}
6	rs660895	0.28	2.1×10^{-07}
6	rs521539	0.28	2.1×10^{-07}

Supplementary Table 1. (continued)

Chromosome	SNP	MAF	P-value
6	rs3104413	0.27	5.0x10 ⁻⁰⁸
6	rs6931277	0.27	3.0x10 ⁻⁰⁸
6	rs9271488	0.36	2.0x10 ⁻⁰⁸
6	rs9271588	0.44	9.9x10 ⁻¹¹
6	rs3129769	0.27	4.0x10 ⁻⁰⁸
6	rs9273363	0.35	3.5x10 ⁻⁰⁷
6	rs9273448	0.2	8.0x10 ⁻⁰⁸
6	rs3828800	0.24	4.0x10 ⁻⁰⁸
6	rs7774434	0.43	1.1x10 ⁻⁰⁶
6	rs9275224	0.40	1.0x10 ⁻⁰⁷
6	rs2858324	0.35	3.8x10 ⁻⁰⁷
6	rs6457617	0.41	4.6x10 ⁻⁰⁷
6	rs2647012	0.35	6.2x10 ⁻⁰⁷
6	rs9275313	0.19	4.2x10 ⁻⁰⁹
6	rs9275330	0.20	4.0x10 ⁻⁰⁸
6	rs9275332	0.37	5.5x10 ⁻¹⁰
6	rs9275334	0.18	4.5x10 ⁻⁰⁹
6	rs9275338	0.20	7.0x10 ⁻⁰⁸
6	rs9275371	0.37	5.4x10 ⁻¹⁰
6	rs9275390	0.37	5.4x10 ⁻¹⁰
6	rs9275393	0.37	2.3x10 ⁻¹⁰
6	rs9275407	0.37	5.4x10 ⁻¹⁰
6	rs2856717	0.35	6.2x10 ⁻⁰⁷
6	rs9275428	0.37	5.3x10 ⁻¹⁰
6	rs9275439	0.36	2.1x10 ⁻¹⁰
6	rs9275495	0.18	2.0x10 ⁻⁰⁸
6	rs9275530	0.18	2.0x10 ⁻⁰⁸
6	rs9275532	0.18	2.0x10 ⁻⁰⁸
6	rs9275578	0.35	8.4x10 ⁻¹⁰
6	rs9275580	0.35	1.8x10 ⁻¹⁰
6	rs9275582	0.35	8.4x10 ⁻¹⁰
6	rs7764856	0.46	8.0x10 ⁻¹⁰
6	rs7454108	0.18	2.0x10 ⁻⁰⁸
6	rs3957146	0.18	2.0x10 ⁻⁰⁸
6	rs3998158	0.34	2.3x10 ⁻¹⁰
6	rs3998159	0.18	4.0x10 ⁻⁰⁸
6	rs3957148	0.18	1.0x10 ⁻⁰⁸
6	rs9275599	0.17	3.3x10 ⁻⁰⁷
6	rs17429444	0.10	2.7x10 ⁻⁰⁷

Supplementary Table 1. (continued)

Chromosome	SNP	MAF	P-value
6	rs3819717	0.43	4.7x10 ⁻⁰⁹
6	rs3819721	0.29	1.1x10 ⁻⁰⁹
6	rs6903433	0.18	9.5x10 ⁻⁰⁷
6	rs3101944	0.08	5.0x10 ⁻⁰⁸
6	rs3130595	0.08	1.8x10 ⁻⁰⁷
6	rs1431393	0.08	1.8x10 ⁻⁰⁷
6	rs1431394	0.08	1.8x10 ⁻⁰⁷
6	rs1367727	0.08	1.8x10 ⁻⁰⁷
6	rs9276935	0.08	1.8x10 ⁻⁰⁷
6	rs15912	0.08	1.8x10 ⁻⁰⁷
6	rs2071876	0.08	6.6x10 ⁻⁰⁷
6	rs3135034	0.08	1.8x10 ⁻⁰⁷
6	rs3097646	0.08	1.8x10 ⁻⁰⁷
9	rs348472	0.03	4.2x10 ⁻⁰⁷
9	rs2038621	0.26	4.7x10 ⁻⁰⁷
9	rs732726	0.25	4.4x10 ⁻⁰⁷
10	rs780849	0.20	9.0x10 ⁻⁰⁸
10	rs1938525	0.04	1.1x10 ⁻⁰⁶
11	rs10898200	0.13	1.8x10 ⁻⁰⁷
12	rs1565190	0.10	8.4x10 ⁻⁰⁷
12	rs10783780	0.04	3.0x10 ⁻⁰⁸
12	rs3809129	0.04	1.0x10 ⁻⁰⁸
12	rs12422499	0.04	3.0x10 ⁻⁰⁸
12	rs7305258	0.48	5.3x10 ⁻⁰⁷
13	rs9590793	0.17	7.9x10 ⁻⁰⁷
14	rs451066	0.20	1.1x10 ⁻⁰⁶
14	rs189866	0.20	1.1x10 ⁻⁰⁶
14	rs57418859	0.01	4.3x10 ⁻⁰⁷
18	rs206514	0.35	2.7x10 ⁻⁰⁷
19	rs8102662	0.43	2.0x10 ⁻⁰⁸
20	rs62215576	0.21	1.7x10 ⁻⁰⁷
20	rs11908352	0.21	1.7x10 ⁻⁰⁷

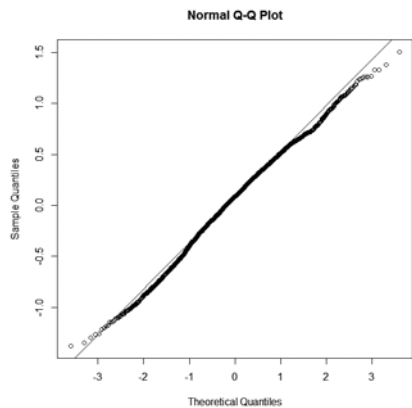
Supplementary Table 2. Presented are the associations (p-values) between recently identified non-HLA risk loci for RA-susceptibility and joint damage severity in phase-1 of the present study.

Genetic variant	Gene	Locus	Minor allele frequency	P-value
Rs2476601	<i>PTPN22</i>	1p13	0.10	0.70
Rs3087243	<i>CTLA4</i>	2q33	0.44	0.26
Rs10499194	<i>TNFAIP3-OLIG3</i>	6q23	0.27	0.0034

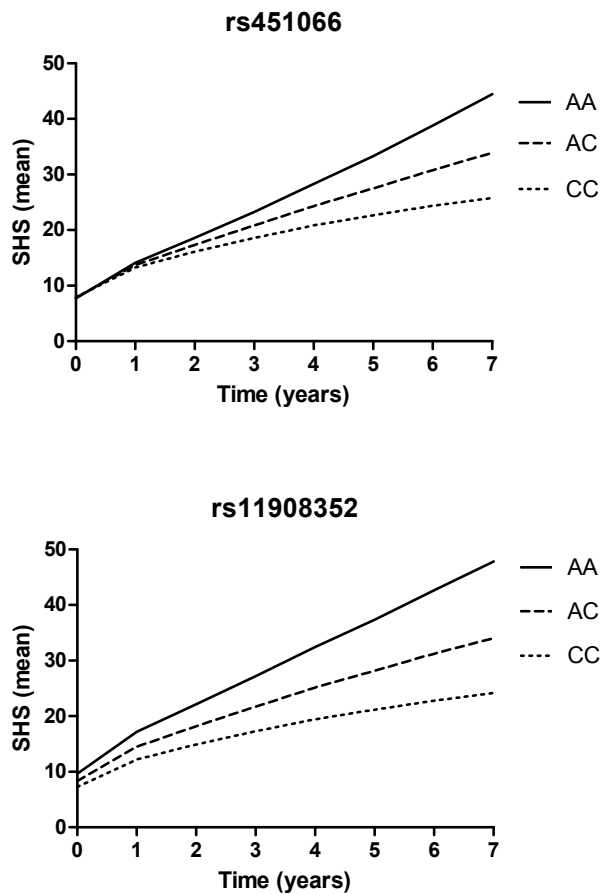
Supplementary Table 2 (continued)

Genetic variant	Gene	Locus	Minor allele frequency	P-value
Rs7574865	<i>STAT4</i>	2q32	0.22	0.09
Rs10818488	<i>C5-TRAF1</i>	9q33	0.40	0.17
Rs2104286	<i>IL-2RA</i>	10p15	0.24	0.016
Rs743777	<i>IL-2RB</i>	22q12	0.33	0.59
Rs6822844	<i>IL-21</i>	4q27	0.18	0.23
Rs4810485	<i>CD40</i>	20q13	0.25	0.16
Rs2812378	<i>CCL21</i>	9p13	0.34	0.75
Rs3890745	<i>MMEL</i>	1p36	0.33	0.43
Rs4750316	<i>PRKCQ</i>	10p15	0.19	0.82
Rs1678542	<i>KIF5A</i>	12q13	0.38	0.53
Rs42041	<i>CDK6</i>	7q21	0.24	N/A
Rs13031237	<i>REL</i>	2p16	0.37	0.23
Rs2736340	<i>BLK</i>	8p23	0.25	0.16
Rs394581	<i>TAGAP</i>	6q25	0.30	0.007
Rs1980422	<i>CD28</i>	2q33	0.24	0.58
Rs540386	<i>TRAF6</i>	11p12	0.14	0.38
Rs10919563	<i>PTPRC</i>	1q31	0.13	0.29
Rs12746613	<i>FCGR2A</i>	1q23	0.12	0.019
Rs548234	<i>PRDM1</i>	6q21	0.33	0.039
Rs11586238	<i>CD2/CD58</i>	1p13	0.24	0.12
Rs10865035	<i>AFF3</i>	2q11	0.47	0.069
Rs6859219	<i>ANKRD55/IL-6ST</i>	5q11	0.21	0.069
Rs26232	<i>C5-orf30</i>	5q21	0.32	0.044
Rs3093023	<i>CCR6</i>	6q27	0.43	0.54
Rs10488631	<i>IRF5</i>	7q32	0.11	0.54
Rs13315591	<i>PXK</i>	3p14	0.10	0.97
Rs874040	<i>RBIP</i>	4p15	0.30	0.90
Rs934734	<i>SPRED2</i>	2p14	0.49	0.59
Rs951005	<i>CCL21</i>	9p13	0.16	1.00
Rs34536443	<i>TYK2</i>	19p13	0.04	0.60
Rs13397	<i>IRAK1</i>	Xq28	0.12	NA
Rs8026898	<i>TLE3</i>	15q23	0.29	0.63
Rs8043085	<i>RASGRP1</i>	15q14	0.25	0.77
Rs2240336	<i>PADI4</i>	1p36	0.42	0.50
Rs8192284	<i>IL6R</i>	1q21	0.42	0.28
Rs13330176	<i>IRF8</i>	16q24	0.22	0.35

NA, not available



Supplementary Figure 1. Q-Q plot of the p-values obtained in phase-1



Supplementary Figure 2. Predicted values for rs451066 and rs11908352
Depicted are the by the model predicted mean values. These data are based on the RA patients studied in phase-1.



Part III



Chapter 11

Can anti-cyclic citrullinated peptide antibody negative RA be subdivided into clinical subphenotypes?

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ABSTRACT

Introduction

Studies investigating genetic risk factors for susceptibility to rheumatoid arthritis (RA) studied anti-citrullinated peptide antibody (CCP)-positive RA more frequently than anti-CCP-negative RA. One of the reasons for this is the perception that anti-CCP-negative RA may include patients that fulfilled criteria for RA but belong to a wide range of diagnoses. We aimed to evaluate the validity of this notion and explored whether clinical subphenotypes can be discerned within anti-CCP-negative RA.

Methods

The 318 patients with anti-CCP-negative RA (1987 ACR criteria), included in the Leiden Early Arthritis Clinic between 1993 and 2006, were studied for baseline characteristics and radiologic progression data during a mean follow-up of 5 years. Grouping was studied both at variable and patient levels. Principal components analysis and partial least-squares regression were applied to study for clustering of variables. A cluster analysis was performed to look for clustering of patients.

Results

The simultaneous presence of patient characteristics at disease presentation was observed for several groups; however, the three largest groups of patients' characteristics explained only 26.5% of the total variance. Plotting the contribution of each patient to these three groups did not reveal clustering of patients. Comparable observations were made when data on progression of joint destruction were studied in relation to baseline clinical data. A cluster analysis, evaluating whether patients resemble each other, revealed no grouping of patients. Altogether, no clinically distinguishable subphenotypes were observed.

Conclusions

The current data provide evidence that, for risk-factor studies, anti-CCP-negative RA patients can be studied as one group.

INTRODUCTION

Rheumatoid arthritis (RA) has been considered a relatively homogeneous clinical syndrome for more than 50 years. However, our current view of RA as a single disease may become untenable, and the disease may be subdivided into a range of disorders based on improved knowledge of its driving immunologic markers¹⁻³. During the last decade, a number of studies suggested that RA can be divided into two syndromes: anti-citrullinated peptide antibody (CCP)-positive and anti-CCP-negative RA. This subdivision was based on differences in genetic risk factors, histopathologic differences, and differences in outcome of anti-CCP-positive and anti-CCP-negative RA⁴.

Several successful genome-wide studies for genetic risk factors for anti-CCP-positive RA have been performed. Studies on genetic risk factors for anti-CCP-negative RA are thus far lacking. One of the reasons for this is the fear of phenotypic misclassification, as anti-CCP-negative RA is often considered to be a heterogeneous disease^{1,5}. For future risk-factor, translational, and outcome studies on the subgroup of anti-CCP-negative RA patients, it is essential to provide epidemiologic and clinical evidence on whether anti-CCP-negative RA can be considered one entity. In this study, we therefore aimed to determine whether the group of anti-CCP-negative RA patients can be separated into clinically distinguishable subphenotypes.

MATERIALS AND METHODS

Patients

The 704 patients who were included between 1993 and 2006 in the Leiden Early Arthritis Clinic and who were diagnosed with RA according to the 1987 ACR criteria were selected; 318 patients had anti-CCP-negative RA and were therefore selected for further analysis. The Leiden Early Arthritis Clinic previously has been described extensively⁶. In short, it is a population-based inception cohort of patients presenting with arthritis to the Department of Rheumatology of the Leiden University Medical Center. This is the only referral center in a health care region of approximately 400,000 inhabitants. Written informed consent was obtained from all patients, and the cohort was approved by the local medical ethical committee (Ethics Committee of the Leiden University Medical Center). At first visit, the rheumatologist completed a questionnaire regarding the presenting symptoms, as reported by the patient: type, localization and distribution of initial joint symptoms, duration and course of the initial symptoms, and the presence of inflammatory back pain and skin abnormalities. The patient's smoking history and family history were assessed. Patients rated morning stiffness in minutes (mean, 103; SD, 112). The Health Assessment Questionnaire (HAQ) was used to provide an index of disability. A 44-joint count for swollen joints (SJC)

was performed. Anti-CCP2 antibodies were measured in sera collected at baseline with enzyme-linked immunosorbent assay (ELISA) (Immunoscan RA Mark 2; Eurodiagnostica, Arnhem, The Netherlands). Samples with a value less than 25 units/ml were considered negative, according to the manufacturer's instructions. IgM-Rheumatoid Factor (RF) was determined with ELISA. RF titers ranged from 0 to 200 IU/ml. For the analyses, RF levels were divided into three groups: RF normal, RF moderately increased (1 to 3 times the reference value), and RF highly increased (>3 times reference value)⁷. Anti-modified citrullinated vimentin (MCV) antibodies were also measured with ELISA (Orgentec Diagnostika, Mainz, Germany); here, the cutoff level was 20 arbitrary units, according to the manufacturer's instruction. All the mentioned baseline variables were studied here, as they may, on their own or in combination with other characteristics, point to different disease subsets. Annual radiographs of the hands and feet were taken during a mean follow-up period of 5 years (minimum, 0; maximum, 14 years) and scored according to the Sharp-van der Heijde method (SHS) by an experienced reader. The intraclass observer correlation coefficient for the radiographic progression rate was 0.97. The radiographic SHS progression per year was calculated for each patient by using a linear regression analysis with the following formula: $Y = \alpha + \beta x$. All available radiographs per patient were used to estimate a patient's progression rate (the β in the equation). At a group level, the median SHS values ($\pm$ SD) at the subsequent time points were 5.0 (9.8) at baseline; 7.0 (13.3) at 1-year follow-up; 9.0 (15.6) at 2 years; 9.0 (17.2) at 3 years; and 10.5 (22.4) at 5 years of follow-up.

Statistical analysis

To investigate whether clinical subphenotypes can be discerned, two types of analyses were done. First, we studied whether groups of patient characteristics frequently occur together; such clustering at the variable level was studied by using the variable reduction techniques Principal Components Analysis (PCA) and Partial Least Squares regression (PLS). Second, we studied whether subgroups of patients can be discerned; such grouping at the patient level was studied by using a cluster analysis.

PCA

Some overlap between clinical variables is extremely common (for example, a high swollen-joint count will often be accompanied by a high number of tender joints). PCA makes use of such overlap and combines variables that frequently occur together into components. In this way, the number of variables explaining data can be reduced, which makes datasets easier to interpret. The components resulting from the PCA are based on the observed variance and not on predefined hypotheses, making this technique suitable for exploring unknown relations between variables. For each component, the loading of each variable to the component is provided. Loadings >0.4 are generally considered relevant. For each

component, an observed variance is presented, indicating the percentage of the total variance in clinical variables that is explained by this component. Here, a PCA was performed, and the contribution of each patient to the most important components was plotted to look for clustering of patients. The PCA was performed by using the following baseline variables: age at inclusion, gender, symptom duration, acuteness of the onset of symptoms (subacute, within 1 week, or insidious, over more than 1 week), morning stiffness, fatigue, fever, smoking, family history of RA, three variables on the distributions of involved joints (upper extremities, lower extremities, or both; symmetric or asymmetric; large joints, small joints, or both), C-reactive protein, RF, the presence of baseline erosions, the number of swollen joints, anti-MCV positivity, inflammatory back pain, and skin abnormalities.

PLS

A limitation of PCA is that it cannot take outcome measures into account. Because patient response obtained during the disease course is at least of equal importance to baseline characteristics for the aim of the present study, we also performed a Partial Least Squares regression. PLS does an analysis that is comparable to PCA, but that has the advantage that it makes use also of outcome measures⁸. Here, a PLS regression was applied to the same variables as included in the PCA as independent variables, but with the addition of the radiologic damage over time as a dependent variable. This analysis allowed us to assess whether variance between patients can be characterized by distinguishable subgroups. Also here, identified factors were plotted to look for clustering, which may represent clinical subphenotypes. The PCA and PLS analyses were done by using SPSS version 17.0 (SPSS Inc., Chicago, IL, USA). To perform PLS, the relevant SPSS extension packages were downloaded from the links provided by the official SPSS website.

Cluster analysis

Subsequently, a cluster analysis was performed, which, in contrast to PCA and PLS, evaluates not grouping of patient characteristics, but grouping of patients. In other words, given all characteristics that were available of the patients, this evaluated which patients resemble each other. Accordingly, not the variables denoting them, but the patients themselves are subject to combination into clusters. Hierarchic clustering was performed by using Gene Cluster, as described by Eisen *et al.*⁹

RESULTS

PCA

The baseline characteristics of the anti-CCP-negative RA patients are depicted in Table 1. Entering baseline variables into a PCA resulted in nine components. The first component

Table 1. Baseline characteristics of the study population and the factors found in PCA

Variable	Component									
	Baseline Frequency	1	2	3	4	5	6	7	8	9
Age at inclusion (yrs, mean $\pm$ SD)	59.2 (16.2)	0.761								
Female gender (n, %)	219 (68.9)	0.448			0.394					
Subacute onset of symptoms (versus insidious)	188 (59.1)		0.592		-0.306			0.316		
Morning stiffness (min; mean $\pm$ SD)	103.18 (112.0)				-0.496			0.484		
Fatigue (VAS; mean $\pm$ SD)	45.1 (29.9)	-0.389				0.522				
Symptom duration (days; mean $\pm$ SD)	172.1 (180.2)		0.394		-0.349			0.455		
Positive family history of RA (n, %)	62 (19.5)	-0.326		0.594	0.380					
Past or present smoking (n, %)	128 (40.3)				0.652		0.375			
Fever (n, %)	22 (6.9)						-0.319		-0.426	
Involvement of small/large joints (n, %)	*	0.604								
Symmetry of involved joints (n, %)	216 (67.9)	-0.379		0.308	0.331				0.370	
Involvement of upper/lower extremities	**	0.602			-0.348					
Inflammatory back pain (N, %)	16 (5.0)					0.466	0.531		0.321	
Skin abnormalities (N, %)**	56 (18.1)		0.374							
Swollen Joint Count (Mean $\pm$ SD)***	11.3 (8.5)	0.445	0.365							
CRP (mg/L; mean $\pm$ SD)	30.10 (34.4)	0.315	0.434						0.512	
RF (IU/ml) (mean $\pm$ SD)****	7,12 (19,5)			0.305	-0.345	0.315		-0.602		
MCV positivity (n, %)	59 (18.6)		0.470	0.376		-0.321				
Erosive disease at baseline (n, %)	202 (63.5)	0.642				0.332				
Explained variance per component (%):		10.0	8.6	8.0	7.4	6.8	6.5	5.6	5.5	5.4

Legend

VAS: Visual Analogue Scale, CRP: C-reactive protein; RF: Rheumatoid Factor; MCV: Mutated Citrullinated Vimentin; involvement of upper extremities: shoulder, elbow, wrist or hand joints; involvement of lower extremities: joints of hip, knee, ankle, feet or toe joints.

*Involvement of small/large joints: in 163 patients (51.3%), only small joints of hands and feet were involved. In 53 patients (16.7%) only large joints were involved. In 93 patients (29.2%), both small and large joints were involved.

** Involvement of upper/ lower extremities: in 156 patients (49.1%) only joints in the upper extremities were involved. In 28 patients (8.8%), only joints in the lower extremities were involved. In 96 patients (30.2%) joints in both lower and upper extremities were involved.

***Skin abnormalities: absence or presence of dermatological abnormalities such as psoriatic lesions, ulcers, rheumatoid nodules etcetera.

**** Swollen Joint Count: 44-Swollen Joint Count.

***** Analyses were performed on RF in groups (less than reference value, 1-3 times reference value or more than 3 times reference value.

explained 10.0% of the variance, the second component, 8.6%, and the third component, 8.0%. The relative importance of the variables contributing to the different components is depicted in Table 1. For example, in the first component, the variables age, gender, and the presence of baseline erosions were grouped. In the second component, the involvement of small joints versus the involvement of large joints or both SJC and CRP were grouped. The component scores for individual patients of factors 1 through 3 were plotted against each other, and no evident clustering was observed (Figure 1a-c). As the first three components explain most variance, these components were plotted. In these plots, each dot indicates

Figure 1A. PCA Component loadings of Component 1 and Component 2

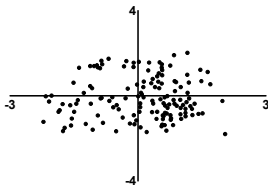


Figure 1B. PCA Component loadings of Component 1 and Component 3

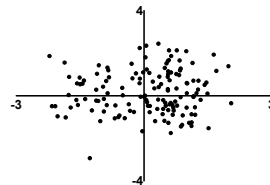


Figure 1C. PCA Component loadings of Component 2 and Component 3



Figure 1D. PLS regression Factor Loadings of Factor 1 and Factor 2

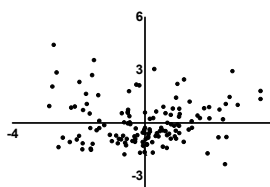


Figure 1. Plots of the most important Component Loadings from PCA and PLS on 318 anti-CCP negative RA-patients

In these plots, each dot indicates one single patient. Component scores indicate how strongly each component is represented in each patient. For example in Figure 1A, a dot indicates how much the variance in an individual patient is being described by factor 1 on the x-axis (age, gender and the presence of baseline erosions) in relation to factor 2 on the y-axis (involvement of small joints vs the involvement of large joints or both, Swollen Joint Count and CRP). If there would be concurrence of components, clustering of patients would be visible. In the PCA clinical variables at disease onset were explored. The same applies for the factors in PLS regression. In the PLS regression, the clinical variables at disease onset were explored together with radiological data on progression of joint destruction during mean of 5 years of disease.

one single patient. For example in Figure 1a, a dot indicates how much the variance in an individual patient is described by factor 1 (age, gender, and the presence of baseline erosions) in relation to factor 2 (involvement of small joints versus the involvement of large joints or both SJC and CRP).

PLS

Apart from baseline characteristics, outcome data may be informative to identify differences between patient populations, so we next performed a data-reduction method that allows assessing radiologic-outcome data in addition to baseline data. To identify subsets of patients, PLS regression was used. With PLS, two latent factors were found that together accounted for 30.1% of the observed variation. The major important variables in the first latent factor were gender, symmetry of involved joints, rheumatoid factor positivity, anti-MCV positivity, age at inclusion, symptom duration at inclusion, and the presence of baseline erosions. The major variables in the second factor were the same; this suggests that little difference exists. The individual patient scores on these factors were plotted against each other. Also here, no clustering was observed (Figure 1d).

Because of the absence of clustering, we sought a positive control, to verify that the method and data used do allow finding clusters. To this end, the PLS with baseline and radiologic progression data was repeated on the total group of 704 RA patients instead of on the subgroup of anti-CCP-negative patients. Anti-CCP status was not included in this analysis, so that the analysis was not influenced by this variable. Again, two factors were found, explaining together 10.1% of the observed variance. The main variables of these components were gender, rheumatoid factor, age at inclusion, and CRP. Clustering the first two factors revealed two clusters; one for anti-CCP-positive patients and one for anti-CCP-negative patients (see Figure 2).

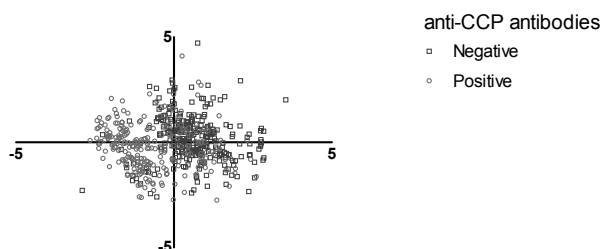


Figure 2. Plots of the two major Component Loadings from PLS on the whole Leiden Early Arthritis Clinic (n=704)

Each dot indicates one single patient. Component scores indicate how strongly each component is represented in each patient. Patients positive for anti-CCP antibodies are colored blue, whereas patients negative are colored red.

Cluster analysis

Finally, we explored whether anti-CCP-negative patients can be grouped into subgroups of patients with similar characteristics. To this end, a heat map was made in which the patients with the most similarity clustered together. Cluster analysis showed no clustering of patients in the heat map, and this finding is supported by the dendrogram (Figure 3). Therefore, also with these analyses, no distinguishable groups of patients with similar characteristics were recognized.

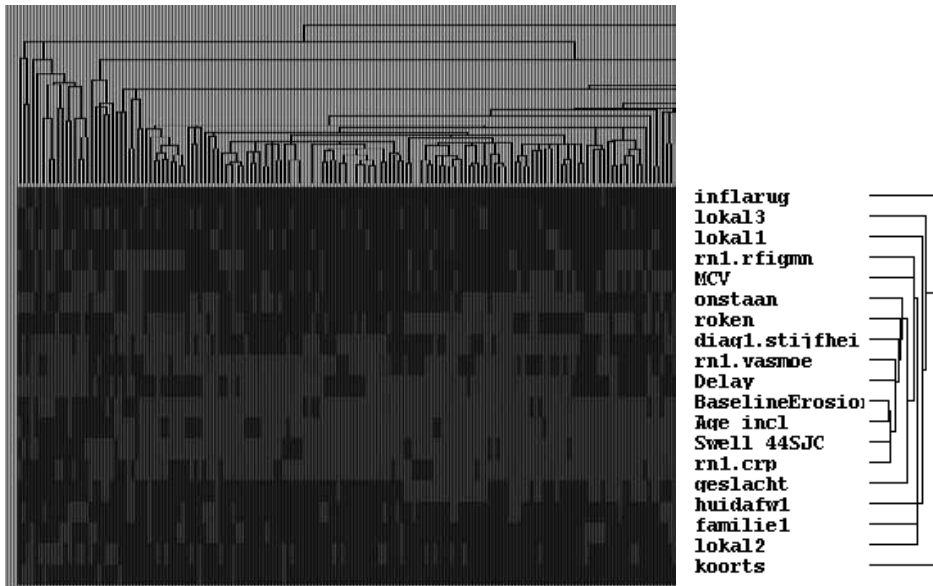


Figure 3. Heatmap of the Cluster Analysis of 318 anti-CCP negative RA-patients

Heatmap representing the presence or absence of disease characteristics in individual patients. In order to make variables comparable, all values were transformed into binary values. For those variables on a continuous scale, the following cut-offs were made: Morning Stiffness: $> \text{ or } \leq 60$ minutes; Fatigue: fatigue rated $> \text{ mean } (45.1)$ on Visual Analogue Scale; Symptom duration $> \text{ or } \leq 12$ weeks; Age at inclusion: age $> \text{ mean age } (59.2 \text{ years})$; swollen joint count > 4 swollen joints; CRP: CRP $> \text{ or } > \text{ reference value } (10 \text{ mg/L})$. The dendrograms depict the relative strength of correlations between the variables and the patients respectively.

Sensitivity analysis

Patients negative for anti-CCP antibodies can harbor other autoantibodies. Here 24.2% of the anti-CCP-negative patients were positive for RF, and 18.6% were positive for anti-MCV. It can be argued that it is more appropriate to perform the analyses on patients negative for anti-CCP, RF, and anti-MCV. Therefore, all analyses were repeated in this subgroup ($n = 171$). Similar observations were made (data not shown).

DISCUSSION

This study determined whether anti-CCP-negative patients fulfilling the 1987 ACR criteria for RA can be subdivided into clinical subphenotypes and explored extensive phenotypic characteristics at baseline, as well as data on progression in joint destruction during the disease course. In addition, several methods were applied, intending to find subgroups of either variables or patients. With any method used, no clearly distinguishable clusters were observed, although the methods used did distinguish CCP-positive and CCP-negative patients as identifiable subphenotypes. Therefore, these data do not support the hypothesis that anti-CCP-negative RA is composed of different subsets, but rather provide evidence that anti-CCP-negative RA can be regarded as one disease, and therefore, risk-factor studies in anti-CCP-negative RA are feasible.

The data evaluated concerned a wide variety of clinical characteristics, such as the acuteness of the onset of symptoms, the distribution of involved joints, the severity of fatigue, fever, skin abnormalities, inflammatory back pain, acute-phase reactants, and radiologic baseline and progression data. The variables assessed are, in our view, variables that might in combination form patterns characteristic of different disease subsets. However, despite the evaluation of a large range of characteristics at disease presentation and the evaluation of long-term radiologic follow-up data, no clear subphenotypes were discerned. We cannot exclude that when other variables are assessed, conclusions might be different.

To test whether the currently used data and methods are able to find clinical subsets, we also studied the total RA population, including 704 patients, of whom 318 were anti-CCP-negative patients. We observed that PLS regression is able to discriminate between anti-CCP-positive and anti-CCP-negative disease. This is in line with published data that anti-CCP-positive RA has more progressive joint destruction during the disease course than does anti-CCP-negative RA¹⁰

The present study did not aim to find statistically significant associations of baseline variables with the outcome. The present study also does not give any indication on whether the pathogenesis of anti-CCP-negative RA is heterogeneous or homogeneous between patients. We explored whether clinical data provide evidence that different groups of patients compose the group of anti-CCP-negative RA patients. If subclinical phenotypes had been identified, this is relevant for future pathophysiological studies. Then it could be suggested that, to prevent phenotypic misclassification, studies should be done on anti-CCP-negative subphenotypes. The present observation of a lack of phenotypic heterogeneity within anti-CCP-negative RA suggests that future studies on pathogenic mechanisms underlying anti-CCP-negative RA can be done on the total population of anti-CCP-negative patients.

CONCLUSIONS

Based on the present data, we suggest that risk factors studied on the anti-CCP-negative RA patients can be performed on the total group of 1987 ACR criteria-positive, anti-CCP-negative RA patients.

ABBREVIATIONS

CCP, citrullinated peptide antibody; CRP, C-reactive protein; HAQ, Health Assessment Questionnaire; MCV, modified citrullinated vimentin; PCA, principal components analysis; PLS, partial least-squares regression; RA, rheumatoid arthritis; RF, rheumatoid factor; SHS, Sharp-van der Heijde Score; SJC, swollen joint count; VAS, visual analogue scale.

COMPETING INTERESTS

The authors report that they have no competing interests.

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Chapter 12

Genetic factors for the severity of ACPA-negative Rheumatoid Arthritis in two cohorts of early disease: a genome-wide study

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Submitted

ABSTRACT

Objective

ACPA-negative and ACPA-positive Rheumatoid Arthritis (RA) are increasingly regarded as separate clinical entities. Although ACPA-negative patients have a less severe disease course at group level, considerable inter-individual differences in the amount of joint destruction occur. As no studies focusing on genetic risk factors underlying the differences in joint destruction in ACPA-negative patients have been performed thus far, we performed the present study.

Methods

A Genome-Wide Association Study was performed using Illumina Human CytoSNP-12v2 in relation to radiographic joint destruction in 276 ACPA-negative early RA-patients included in the Leiden Early Arthritis Clinic (EAC). According to the Bonferroni correction on the number of tested SNPs, the threshold for genome wide significance was $p < 2 \times 10^{-7}$. Subsequently, the significant SNPs were evaluated for association with the progression of radiographic joint destruction in 253 ACPA-negative early RA-patients included in the BARFOT-study. As 11 uncorrelated SNPs were tested, the Bonferroni threshold for significance was 0.0045. In all patients, joint destruction was measured by Sharp-van der Heijde Score with good reproducibility.

Results

33 SNPs associated significantly to the severity of joint damage ($p < 2 \times 10^{-7}$) in phase-1. In phase-2, two SNPs showed a trend towards a significant association with joint damage, rs2833522 ($p = 0.0049$) and rs17763915 ($p = 0.047$). A combined analysis of both the Leiden and BARFOT datasets of rs2833522 showed a highly significant association with joint destruction ($p = 3.57 \times 10^{-9}$), the presence of the minor allele associated with more severe damage.

Conclusion

Rs2833522 might be associated with the severity of joint damage in ACPA-negative RA. Larger, longitudinal, studies are needed for confirmation.

INTRODUCTION

Rheumatoid Arthritis (RA) is diagnosed by clinical characteristics and not based on pathophysiological processes. Nonetheless as recent studies have found differences in genetic susceptibility factors, histopathology and outcome of anti citrullinated peptide antibodies (ACPA)-positive and ACPA-negative RA, RA is considered to consist of two sub-entities. One subset is characterized by presence of these auto-antibodies and another subset is characterized by the absence of ACPA.¹⁻³ On the group-level ACPA-negative RA patients have a less severe disease course with less joint damage than ACPA-positive patients. On the individual level however, disease severity is variable and severe joint destruction also occurs in auto-antibody negative patients (this is illustrated in Figure 1). The number of studies addressing risk factors for joint damage progression in the total group of RA-patients is relatively small, and joint damage progression in ACPA-negative RA has less frequently been explored as the majority of cohorts included predominantly ACPA-positive RA patients.

The heritability of joint damage in the total population of RA-patients is estimated to be 45-58%.⁴ Whether this is different for ACPA-positive and ACPA-negative RA is unknown. Joint destruction in RA is considered to be the net result of the influence of inflammation on bone. Hypothetically, several processes (for instance processes that determine the sensitivity of bone and cartilage to get destroyed in response to inflammatory stimuli) may be similar in ACPA-positive and ACPA-negative RA, whereas other (immunological and/or inflammatory) pathways may be differently regulated in ACPA-positive and ACPA-negative RA and affect the severity of joint damage. Under the assumption that this notion is correct, risk factors for progression of joint destruction may in part be shared between these subsets of RA and in part be subset specific.

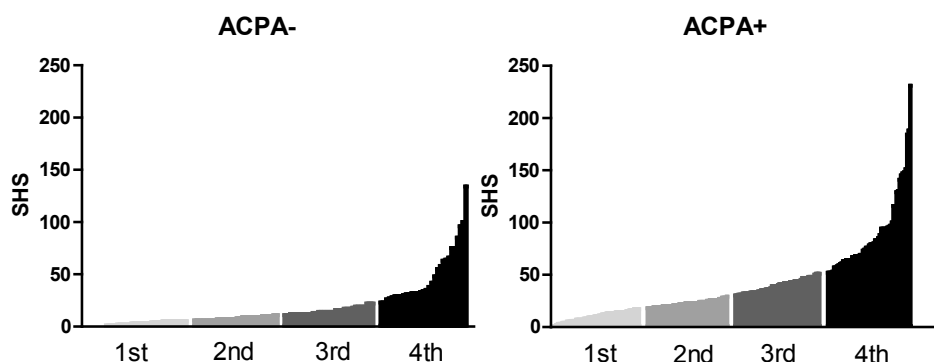


Figure 1. SHS scores after 5 years of follow-up in ACPA- and ACPA+ patients included in Leiden EAC
Depicted are the first, second, third and fourth quartiles of patients and their SHS score after 5 years of follow-up.

We aimed to identify genetic risk factors for the severity of joint damage in ACPA-negative RA using a genome wide approach. Two cohorts of ACPA-negative early RA patients who had serial radiographs over time and were recruited in eras when treatment strategies were milder than today and when biological therapy was uncommon were evaluated.

PATIENTS AND METHODS

Patients

A two-staged study was performed. In both phases RA was defined according to the 1987-American College of Rheumatology (ACR) criteria for RA. In all patients, written informed consent had been obtained and the medical ethical committee's of the participating centers had approved the study.

In the first phase, 276 early ACPA-negative RA-patients who were included in the Leiden-EAC between 1993 and 2006 were studied.⁵ Anti-CCP2 antibodies were measured in stored sera that were collected at the first visit using enzyme-linked immunosorbent assay (ELISA) (Immunoscan RA Mark 2; Eurodiagnostica, Arnhem, The Netherlands). Samples with a value below 25 units/ml were considered negative according to the manufacturer's instructions. Radiographs of the hands and feet were taken at baseline and yearly thereafter during 7 years of follow-up. (in total 1,266 sets of hands and feet radiographs). Radiographs were chronologically scored by one reader using the Sharp-van der Heijde Score (SHS) with good ICC (Intra-class observer Correlation Coefficient).⁶ Treatment strategies were different in three treatment-periods (1993-1995, initial treatment with non-steroid anti-inflammatory drugs (NSAIDs), 1996-1998, initial treatment with chloroquine or sulfasalazine and 1999-2006, prompt treated with methotrexate or sulfasalazine) as described in more detail previously.⁵

The second phase concerned 253 ACPA-negative RA-patients included in the Better Anti-Rheumatic Farmaco Therapy (BARFOT)-cohort, a Swedish multicentre observational study of patients with early (disease duration ≤ 1 year) RA.⁷ Clinical, laboratory and radiological assessments were performed at inclusion and after 1, 2 and 5 years of follow-up. Hands and feet radiographs (total number 842) were scored according to the SHS score by 2 readers with good ICCs. Average scores of the readers have been used. During follow-up 50 patients participated in a 2-year randomized study on low dose prednisolone as an addition to Disease Modifying Anti-Rheumatic Drug (DMARD)-therapy.⁷

Baseline characteristics of the ACPA-negative study populations are shown in Table 1.

Table 1. Baseline characteristics of the ACPA-negative study population

	Cohort	
	Leiden-EAC (n=257)	BARFOT (n=253)
Year of diagnosis	1993-2006	1993-1999
Total number of x-ray sets	1,266	842
Follow-up years (maximum)	7 years	5 years
Method of scoring	SHS ¹	SHS ¹
Female, n (%)	175 (68.1%)	166 (68.0)
Age at diagnosis (years), mean±SD	58.6 (16.4)	56.6 (16.9)
ACPA-negative ²	100%	100%

1. SHS –Sharp van der Heijde method.

Genotyping

In phase 1, genotyping was done using Illumina Human CytoSNP-12v2. Results of 244.655 SNPs were obtained. Of these all call rates were >97% and no SNPs were excluded due to a low call rate. 31 SNPs were excluded from the analyses because they were not in Hardy-Weinberg equilibrium (threshold 10^{-4}), leaving 244.624 SNPs to be analyzed for an association with the progression of joint destruction. DNA on 276 patients was used for genotyping; 2 patients were excluded because of failed genotyping, 1 patient due to high homozygosity and 11 patients were excluded as relatives (based on Identity By State (IBS) analysis). Thus, in total 244.624 SNPs were studied in 262 ACPA-negative patients.

In phase 2, SNPs that showed a significant association in the first phase and with MAF>0.1 (n=18) were genotyped using Sequenom iPLEX. Apart for rs4926674, for which genotyping failed, success rates were all >98.5% and error rates 0% (based on the samples typed in duplo). No patients were excluded from the analyses.

Statistical methods

In phase 1, associations with the SNPs with MAF >0.05 and radiographic joint destruction were studied based on a linear mixed effects model for the longitudinal log-transformed SHS data.⁸ Such a model can accommodate the within patient correlations induced by the repeated radiological measurements and allows studying effects on disease progression. To model the evolution of joint destruction in time we have used natural cubic splines with 3 knots located at the sample quantiles of the visiting times. This method was used to model the non-linear progression of joint damage and the correlation between the X-ray scores adequately. Adjustments have been made for gender, age and inclusion period (as a proxy for differences in treatment) as described previously⁵ and SNP under an additive genetic model. It has been shown that in this cohort, time is an adequate proxy for different treatment regimes.⁹ For the random effects component, we have assumed random-intercepts and random-slopes modeled again using the splines effects. The effect of each

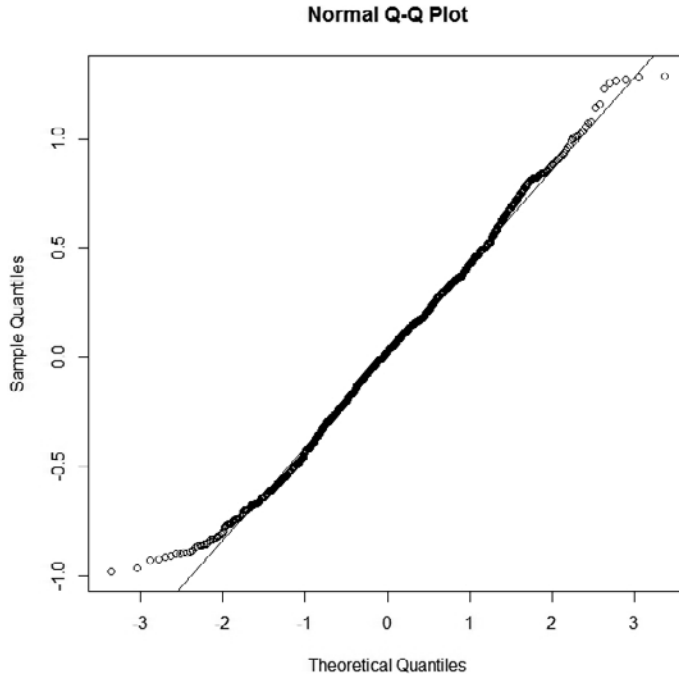


Figure 2. Normal Quantile-quantile plot of the marginal residuals based on the fitted linear mixed effects model

The normal Quantile-quantile plot for the marginal residuals obtained by the linear mixed effects model on the, log-transformed radiographic joint destruction does not show any severe deviations from the normal distribution. This is based on the data of phase-1.

SNP on the disease progression has been tested using the likelihood ratio test, which is assumed to follow under the null hypothesis the chi-squared distribution with 4 degrees of freedom. Thereby we can investigate whether the patients' profiles for patients carrying different genotypes were different. The normality assumption for the linear mixed effects model on the log-transformed SHS scores is validated using the normal quantile-quantile plot of the marginal residuals, which does not show any serious deviation from the normal distribution (Figure 2). Since analyses were done on 244.624 SNPs, the threshold for significance was set at $p < 2 \times 10^{-7}$.

In phase 2, a similar model as described in phase 1 was used to study an association with joint destruction. Also here, analyses were corrected for age, gender and treatment differences (participation in the corticosteroids trial). The Bonferroni correction for the number of uncorrelated SNPs ($R^2=0.8$, $n=11$) was used; the threshold for significance was 0.0045.

The results obtained in the two cohorts for rs2833522, the strongest associating SNP, were combined into one analysis. This combined analysis was performed by combining the

data of the two cohorts and correcting for participating in either the Swedish or the Dutch cohort in a statistical analysis similar to the one described in phase-1 and 2, so a linear mixed effects model was applied on all data. This combined analysis bears similarities with the fixed effects meta-analysis in the sense that both studies are estimating the same effect size, and the contribution of each study on the estimation of the combined effect is determined by the amount of information available by each one.

All analyses were done using the R statistical software package.¹⁰

RESULTS

Phase-1

In total 244.624 SNPs were studied in 262 ACPA-negative patients in relation to the radiological severity of joint destruction over 7 years of follow-up. A Q-Q plot for observed versus expected values of the likelihood ratio test statistic under a chi-squared distribution with 4 degrees of freedom is shown in Figure 2. The lambda score for genomic control was 0.994. The results are depicted in the Manhattan plot (Figure 3). 33 SNPs related significantly ($p < 2 \times 10^{-7}$) to the progression of joint destruction (Table 2).

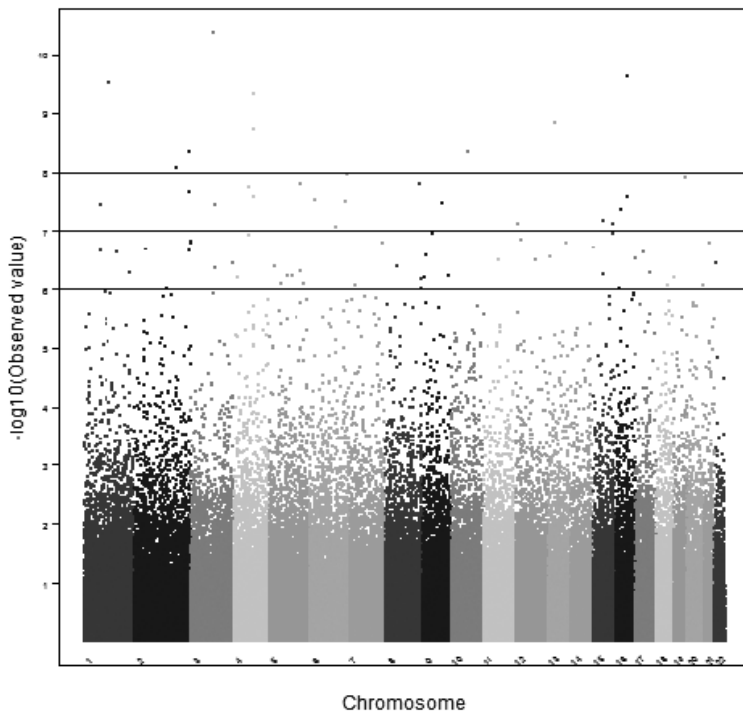


Figure 3. Manhattan plot of all p-values obtained in phase-1

Table 2. SNPs that passed the significance threshold of 2×10^{-7} in phase-1 and the p-value obtained in phase-2

Chrom	SNP	MAF	p phase-1	Gene	p phase-2
1	rs4926674	0.35	3.7×10^{-8}	<i>USP24</i>	na
2	rs7605224	0.15	8.7×10^{-9}	<i>ITGA6</i>	0.17
2	rs7574103	0.48	4.6×10^{-9}	<i>AF279775</i>	0.26
2	rs11681769	0.16	2.2×10^{-8}	<i>AF279775</i>	0.48
2	rs1316929	0.06	1.6×10^{-7}	<i>GPC1</i>	-
2	rs9973786	0.12	1.5×10^{-7}	<i>GPC1</i>	0.74
3	rs7632765	0.10	1.0×10^{-11}	<i>CBLB</i>	-
3	rs11919628	0.084	3.5×10^{-8}	<i>PVRL3</i>	-
4	rs17763915	0.14	1.8×10^{-8}	<i>SLC4A4</i>	0.05
4	rs316440	0.16	1.2×10^{-7}	<i>ADAMTS3</i>	0.49
4	rs2726486	0.073	1.9×10^{-8}	<i>PPA2</i>	-
4	rs9307305	0.10	2.6×10^{-7}	<i>PPA2</i>	-
4	rs1388040	0.10	5.0×10^{-10}	<i>FLJ2084</i>	-
5	rs161034	0.30	1.6×10^{-8}	<i>PPP2R2B</i>	0.49
6	rs17654008	0.05	3.1×10^{-8}	<i>AK098665</i>	-
6	rs6931103	0.17	8.7×10^{-8}	<i>RFPL4B</i>	0.56
6	rs9480379	0.23	3.1×10^{-8}	<i>ARID1B</i>	0.25
6	rs1967290	0.29	1.1×10^{-8}	<i>PDE10A</i>	0.88
7	rs7778273	0.06	1.6×10^{-7}	<i>Cullin 1</i>	-
7	rs11979066	0.06	1.6×10^{-7}	<i>Cullin 1</i>	-
8	rs16906415	0.10	1.6×10^{-8}	?	-
9	rs1418247	0.44	3.4×10^{-8}	<i>GRIN3A</i>	0.24
10	rs2305210	0.29	4.6×10^{-9}	<i>CDH23</i>	0.53
12	rs11044895	0.10	1.4×10^{-7}	<i>AEBP2</i>	-
13	rs2028809	0.20	1.5×10^{-9}	<i>C13orf31</i>	0.51
14	rs28840384	0.15	2.0×10^{-7}	<i>AX746996</i>	na
15	rs4646644	0.09	6.6×10^{-8}	<i>ALDH1A2</i>	-
15	rs1834210	0.09	1.1×10^{-7}	<i>AK057337</i>	-
15	rs8041896	0.10	7.8×10^{-8}	<i>AK057337</i>	-
16	rs7184684	0.45	4.4×10^{-8}	<i>ERCC4</i>	0.45
16	rs8061387	0.10	2.0×10^{-10}	<i>KIFC3</i>	-
16	rs16960143	0.09	2.6×10^{-8}	<i>GINS3</i>	-
19	rs268909	0.12	1.2×10^{-8}	<i>KLK5</i>	0.84
21	rs2833522	0.37	1.7×10^{-7}	<i>SFRS15 – HUNK</i>	0.01

In phase 2, data for rs4926674 and rs28840384 were not available (na) because the assay for this SNP failed. SNPs with MAF<0.1 were not tested in phase 2, therefore, no p-value is reported for these SNPs.

Phase-2

From the 33 variants identified in phase 1, only the SNPs that had a MAF of >0.1 ($n=18$) were studied for an association with joint destruction in ACPA-negative patients included in the BARFOT-cohort. Here, two SNPs, rs2833522 ($p=0.0049$) and rs17763915

($p=0.047$) associated with the progression of joint destruction. However, none of these passed the Bonferroni threshold for multiple testing, though the p -value of rs2833522 was just above this threshold. The joint destruction scores of the ACPA-negative Leiden and BARFOT-patients having a minor, heterozygous or major genotype of this SNP are depicted in Figure 4A and 4C respectively. The fitted profiles by the model of both cohorts are presented as well (Figure 4B and 4D). As shown, in both cohorts presence of the minor allele was associated with more severe damage progression.

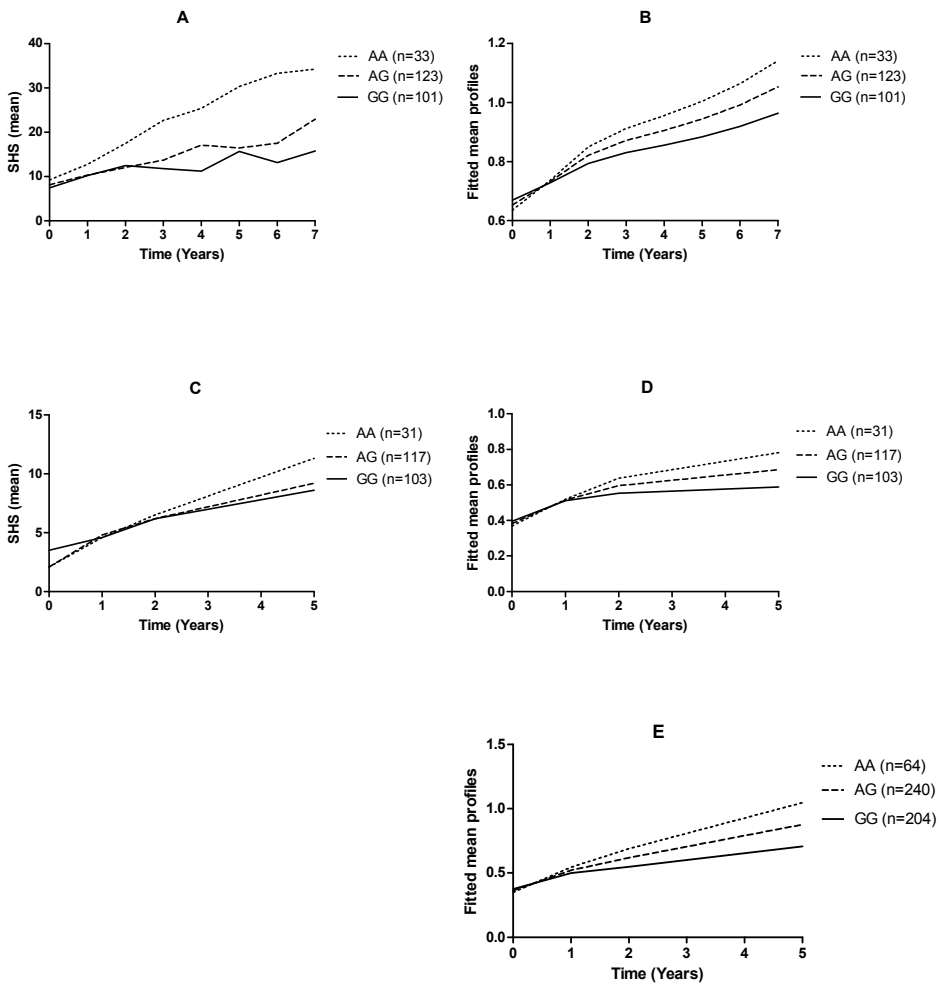


Figure 4. Progression of joint damage for the ACPA-negative RA patients carrying the homozygous major, heterozygous and homozygous minor genotype of rs2833522

Portrayed are the sample mean SHS-scores, thus the raw SHS data on the normal scale (A, Leiden EAC C, BARFOT), the mean log-transformed SHS as modeled by the linear mixed model (B, Leiden EAC, D, BARFOT) and the mean log-transformed SHS as modeled by combined analysis of both cohorts (E).

Combined analysis

Finally the data of rs2833522 of both cohorts were analyzed in a combined analysis. This showed a significant association ($p=3.57 \times 10^{-9}$); the fitted profiles are depicted in Figure 4E.

DISCUSSION

Several successful Genome-Wide Association studies (GWAs) on genetic risk factors for RA-development have been performed.^{11,12} The majority of these studies included ACPA-positive RA-patients. Hence the majority of identified genetic factors predispose to both ACPA-positive and negative RA or ACPA-positive RA only. A recent study using the ImmunoChip custom SNP array on almost 12,000 RA patients and 16,000 controls performed stratified analyses in ACPA-positive and ACPA-negative patients separately. Two variants predisposed to ACPA-negative RA with a genome-wide significant p-value: rs414332 in the HLA region and rs71624119 in *ANKRD55*.¹³ No GWAs has thus far been published on ACPA-negative RA and disease severity. One possible reason for a lack of genetic studies in ACPA-negative RA patients is the fear of phenotypic misclassification, as ACPA-negative RA is sometimes thought to be heterogeneous and containing patients with unrecognized diseases other than RA.^{14,15} A recent study attempted to address this issue and evaluated a broad range of characteristics (clinical, serological and radiological), both at first presentation and during the course of the disease, using different variable reduction techniques. Based on the characteristics studied no subgroups of patients could be discerned¹⁶ and it was concluded that, for risk-factor studies, ACPA-negative RA patients can be studied as one group. Although no phenotypic subgroups could be discerned, not all ACPA-negative RA patients are alike; some patients develop no or mild erosions, whereas others have a severe destructive disease course, which is illustrated by Figure 1. Studying genetic factors underlying these differences in joint destruction in ACPA-negative RA is important as it may lead to new insights into the pathophysiology of the progression of radiographic joint destruction in this disease subset. We therefore performed the first GWAs on the severity of joint destruction over time in ACPA-negative RA.

The heritability of the severity of joint destruction in the total RA population is estimated at 58%.⁴ Although the heritability in ACPA-negative RA is not known, we assumed that also here genetic factors play a role. In our two-staged study, 33 SNPs passed the threshold of genome-wide significance in phase-1 and two of these were associated with the severity of joint damage in phase-2 at 5% significance level. After the rather conservative Bonferroni correction for multiple testing in phase-2, rs2833522 showed a trend towards statistical significance ($p=0.0049$, whereas Bonferroni threshold for significance is $p=0.0045$).

In both cohorts presence of the minor allele of rs2833522 was associated with a higher rate of joint damage progression. Rs2833522 is located on chromosome 21, between *SFRS15* and *HUNK*. As thus far little is known about the functions of these genes, it is difficult to speculate about the potential pathophysiologic effect of this SNP. In addition, since we did not perform fine-mapping of the region, it is likely that another SNP in this locus is the actual genetic variant associated with the progression of joint damage in ACPA-negative RA.

A proportion of ACPA-negative patients was rheumatoid factor-positive (24%). A stratified analysis of rs2833522 in ACPA-negative RA-patients positive and negative for rheumatoid factor yielded significant results in both groups ($p=0.015$ and $p=0.011$ respectively), indicating that the association observed was not driven by the presence of rheumatoid factor in part of the patients.

In phase-2 a significant association was observed also for rs17763915, which is located 257821 kb from the *SLC4A4* gene, encoding for a sodium bicarbonate cotransporter. To our knowledge, this gene has thus far not been related to RA or other, auto-immune or anti-inflammatory disorders. Since this association disappeared after correction for multiple testing, we cannot make a definite conclusion on the value of this SNP for joint damage severity.

A genome wide approach is focused on identifying true positive risk factors and false negative results are inherent to the study design. In our case, the risk of false negatives is of special importance. The Bonferroni correction for multiple testing applied in phase-1 is rather conservative, especially because we corrected for the total number of SNPs and not the number of uncorrelated SNPs; this choice may have introduced false negatives. Furthermore, the array that was used in phase-1 covered relatively few variants, making it likely that also for this reason not all genetic variants associating with joint damage severity in ACPA-negative RA were found. Nonetheless, the choice of the array does not affect the validity of the positive results obtained in this study.

Furthermore several issues may have affected the power of the present study to find statistically significant associations. The numbers of patients and radiographs studied were relatively small. Additionally, the severity of joint damage was relatively mild in a large number of ACPA-negative RA-patients. The differences in progression rates between genotype groups are therefore lower than those observed in total population of RA-patients or the ACPA-positive RA patients, making it more difficult to find significant associations. A limited power may be an explanation for the fact that we could not achieve independent replication of our results.

Our study also has several strengths. The two observational cohorts studied had many similarities in design and populations, they consisted both of adult, western European early RA-patients and in both cohorts treatment was much less intense than today. Although the number of ACPA-negative patients was relatively small, the patients in both cohorts were radiographed serially in time. As shown recently, the presence of repeated measurements yields more precise estimations of the radiographic progression rate and a considerable increased power.⁹ Our study did not include a third phase as we did not identify a longitudinal observational cohort with ACPA-negative RA patients including an equal or larger amount of radiographs compared to the number of radiographs studied in phases-1 and -2. Cohorts of ACPA-negative RA-patients with repeated radiological measurements available are extremely rare.

The statistical model used in this study took advantage of the within patient correlations present in repeated radiological measurements and combined all radiological data in one test. This model was chosen because, especially in ACPA-negative patients, the rate of joint destruction is not homogenous through the whole follow-up period and differences between the patients' joint damage progression profiles are present. We decided to adopt the splines functions into the linear mixed effects model used for our analyses. This allows more precise modeling the progression of joint damage over the total follow-up period. Despite the advantage of being able to flexibly model the non-linear profiles in time, a disadvantage of the chosen model is that we cannot quantify the SNP effect over the whole follow-up period using an overall effect size. Therefore, to visualize the SNP effects on the progression of joint destruction, we presented the raw SHS data and the fitted marginal profiles in time per genotype category (see Figure 4).

In phase-1 of the study, an interesting association was found for rs7778273 and rs11979066, which are both located on the *Cullin 1* gene. Cullin 1 has been thought to influence RA by altering lymphocyte signal transduction.^{17;18} However, due to the chosen selection criterion for genotyping in stage-2 of a MAF >0.1, this SNP (MAF 0.06) was not proceeded to phase-2. Whether this variant comprised a false negative result remains undetermined.

Eyre et al. recently described that rs414332 in the HLA region and rs71624119 in *ANKRD55* are associated with the development of ACPA-negative RA.¹³ These variants were not in the list of variants identified in phase 1 of this study. This might indicate that the genetic variants, and hence the underlying pathophysiological processes, of developing ACPA-negative RA and progression of ACPA-negative RA are different.

In conclusion, we found that rs2833522 might be associated with the severity of joint damage in ACPA-negative RA. Larger, longitudinal studies are needed for confirmation

and subsequent functional studies are required to elucidate the processes relevant for joint destruction in RA that are influenced by this genetic variant.

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Part IV



Chapter 13

Does the season at symptom onset influence the severity of radiographic joint destruction in Rheumatoid Arthritis?

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ABSTRACT

Objective

Several season-associated environmental factors have been related to the onset of rheumatoid arthritis (RA) and the severity of joint complaints. However, whether seasonality affects the long-term outcome of RA is unknown. We aimed to evaluate the influence of the season at symptom onset on long-term radiographic progression in two large cohorts.

Methods

688 RA-patients included in the Dutch Leiden Early-Arthritis-Clinic (EAC) and 830 RA-patients included in the Swedish BARFOT-study were studied. Repeated radiological measurements during 7 and 5 years of follow-up, respectively, were scored according to the Sharp-van-der-Heijde method. The majority of patients were recruited in an era when early aggressive treatment was not yet the standard. Seasons were defined meteorologically.

Results

In both cohorts, the proportion of RA-patients who had their symptom onset in winter was larger than those for other seasons; though significance was noted only in the Swedish cohort ($p=0.005$). The radiological progression rates over time, in contrast, were not different in the four seasonal groups, neither in the Dutch ($p=0.29$) nor in the Swedish cohort ($p=0.46$).

Conclusion

Meteorologically defined seasons at symptom onset were not associated with the severity of joint damage in RA, making it unlikely that season-related environmental factors influence long-term outcome.

INTRODUCTION

The severity of the joint destruction in Rheumatoid Arthritis (RA) is highly variable between patients. Genetic factors explain only part of the variance in joint destruction¹, hence environmental factors likely play a role as well. Several environmental factors such as infections or Vitamin D levels portray seasonal clustering, and seasonal factors are thought to influence RA²⁻⁴. Some studies observed that RA starts more frequently in winter than in other seasons^{5,6} and that both the onset and severity of symptoms are season-related.^{7,8} A seasonal effect for the onset of RA was not observed by others.⁹

It has not been established whether the season of onset of first arthritis symptoms is related to the severity of joint destruction over time. A recent French study found that early arthritis patients whose symptoms started between January 1st and June 30th had more often erosive disease after one year than patients whose symptoms started in the second half of the year.¹⁰ However, long term effects of the season of symptom onset on progression of radiographic joint damage are unknown.

A finding of seasonal influences on the severity of RA would indicate that season-related pathogenic factors play a role in joint destruction. Therefore, we studied the possible effects of the season of onset of first symptoms on long-term progression of joint destruction in early RA-patients from two observational cohorts.

MATERIALS AND METHODS

Patients

In both cohorts RA was defined according the 1987-ACR criteria, written informed consent was obtained from all patients and approval by the local medical ethical committees was obtained.

Cohort 1 consists of 688 Dutch RA-patients included between 1993 and 2006 in the Leiden Early Arthritis Clinic, a population-based inception cohort.¹ In all patients the self-reported date of symptom-onset was recorded. Hands and feet X-rays taken at baseline and yearly intervals till 7 years of follow-up were studied (total number of X-rays 4030, mean follow-up duration 4.9 years). All X-rays were chronologically scored by one experienced reader who was unaware of clinical data using the Sharp-van der Heijde score (SHS).¹¹ The within reader ICC was 0.91.¹ The treatment of these patients could be divided into three treatment periods. Patients included in 1993-1995 were initially treated with NSAIDs, patients included in 1996-1998 were initially treated with chloroquine or salazopyrine and patients included after 1999 were promptly treated with methotrexate or salazopyrine.

Cohort 2 contains 830 RA-patients from the BARFOT project, a Swedish multicentre observational study of patients with early (disease duration ≤ 1 year) RA.¹² In all patients the self-reported date of symptom-onset was recorded. Clinical, laboratory and radiological assessments were performed at inclusion and after 1, 2 and 5 years of follow-up. These hands and feet X-rays (total number 2960, mean follow-up duration 4.3 years) were scored according to the Sharp-van der Heijde score by 2 readers. The between reader ICCs for total Sharp score at baseline and after two years were 0.93 and 0.94, respectively. At inclusion, no patient had got prior treatment with DMARDs or glucocorticoids. During follow-up 213 patients participated in a 2-year randomized study on low dose prednisolone as an addition to DMARD-therapy.

Defining the seasons

Seasons were defined according to meteorologists in the Netherlands and Sweden as spring starting on March 1st, summer on June 1st, autumn on September 1st and winter on December 1st.

Statistical analyses

Chi-Square tests were used to compare the proportions of patients. Radiographic scores were log-transformed to approximate a normal distribution. A multivariate normal regression analysis was used with the radiographic score as response variable; see ref¹³ In the Dutch cohort, adjustments were made for age, gender and treatment strategy.¹³ In the Swedish cohort, adjustments were made for age and participating in a corticosteroid study. Analyses were done using SPSS, versions 17.0 and 18.0 (SPSS Inc., Chicago, IL, USA), p values < 0.05 were considered significant.

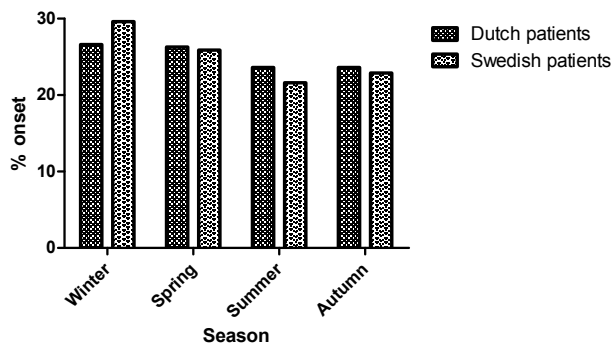
RESULTS

The patient characteristics are depicted in Table 1. 26.6% of the Dutch RA-patients had their onset of symptoms during the winter (Figure 1). Although this percentage was higher than that of the other seasons, the difference was not significant ($p=0.56$). In the Swedish RA-patients, 29.6% of the patients had symptom onset during the winter (Figure 1), which was significantly higher than the other seasons ($p=0.005$).

Subsequently we analyzed the association between the four meteorologically defined seasons and the progression of joint destruction. In the Dutch RA-patients, we did not observe that patients with a symptom onset in one of the four seasons had a different rate of progression of joint destruction compared to symptom onset in the other seasons ($p=0.29$) (see Figure 2A). Also in the Swedish RA-patients, no significant association between the

Table 1. Baseline characteristics of the Leiden EAC and the BARFOT patients

	Leiden (n=688)	Barfot (n=830)
Women, n (%)	462 (67.2)	538 (64.1)
Age (years), mean (SD)	56.7 (15.7)	56.7 (15.4)
Symptom duration (weeks), mean (SD)	26.2 (22.4)	26.9 (13.8)
Swollen Joint Count (28 joints), mean (SD)	9.1 (6.9)	10.6 (5.6)
Anti-CCP2 positive, n (%)	349 (50.7)	418 (55)
CRP, mean (SD)	30.0 (34.4)	33.8 (37.7)

**Figure 1. Percentage of RA-patients with symptom onset in each of the seasons in Dutch RA-patients and Swedish RA-patients**

season of symptom onset and the severity of joint destruction over time was observed ($p=0.46$) (see Figure 2B).

Likewise, when comparing only two seasons namely the patients with symptom onset in summer or winter, no significant differences in progression of joint destruction were observed in both cohorts.

In a recent paper, Mouterde et al. reported a significant effect of a first symptom onset in winter and spring on erosions development during the first 12 months of follow-up.¹⁰ In their study, seasons were defined differently, namely winter = January 1-March 31, spring = April 1-June 30, summer = July 1-September 30 and autumn = October 1-December 31 (personal communication with professor B. Combe). Having learned this, we recoded our seasons in the same way in order to replicate the French findings. Performing the analyses over 7 years in the Dutch dataset, we obtained an association for disease onset in winter and radiographic joint destruction ($p=0.02$) Analyzing the 5-year follow-up data in the Swedish dataset revealed no significant association ($p=0.89$).

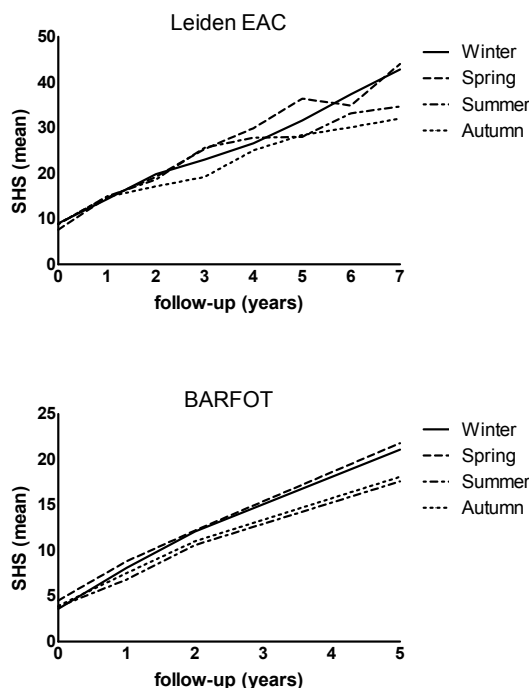


Figure 2. Effect of meteorologically defined seasons on radiographic damage progression over 7 years of follow-up in Dutch RA-patients (A) and Swedish RA-patients (B)

DISCUSSION

Several studies reported that season-related factors influence the onset of RA, though other studies challenged this.^{3;8;10} The interpretation of observed seasonal influences is often that RA may be triggered by for instance viral infections or different Vitamin D levels. A recent study observed that the season of symptom onset is also associated with erosive disease after 12 months and concluded that season related environmental factors are relevant for disease outcome.¹⁰ This is the only study evaluating the association of seasons with structural damage. Since erosiveness early in the disease is the most potent predictor for a progressive destructive disease course, we investigated seasonality in relation to long-term progression of joint damage, taking advantage of two large longitudinal datasets (total number of X-rays 6990, total number of patients 1473). Despite our finding that there was a tendency towards an effect of seasons on onset of RA symptoms, this was not accompanied by an effect on long-term outcome of RA.

In both cohorts, patients were included in a period when early and aggressive treatment strategies were not standard, and treatment adjustments were not target-driven. Hence the effect of treatment on the progression of joint destruction was supposed to be limited. In

addition, analyses were adjusted for differences in treatment strategies between patients. Furthermore, the effect of treatment on the progression of joint damage would not be different between patients with symptom-onset in different seasons. Altogether it seems unlikely that our negative findings are due to treatment effects.

In order to look for the discrepancies between our findings and those published by Mouterde et al., we asked the French group for their definition of seasons. In their analyses, winter started at January 1, which is dissimilar from a meteorological or astronomical definition of seasons. By this definition an association between winter and joint damage was observed in the Dutch but not in the Swedish RA-patients. Considering the overall negative comparisons and the number of tests performed, the result obtained in the Dutch patients could be a consequence of multiple testing.

It may be both a limitation and strength of the present study to analyze populations from two countries, although about 700 km apart, since one cohort might validate the results of the other. However, despite the fact that both the Netherlands and Sweden have a temperate climate, some seasonal differences between the two countries, e.g. in the amount of daylight, temperature and moisture, exist.

Seasonality should not be mixed up with weather effects. Irrespective of the degree of latitude, seasons vary from year to year in temperature, hours of sunshine or wetness. The present data do not allow drawing conclusions on occasional weather effects.

Studying seasonality and RA onset was not our main aim. Nonetheless, symptoms of Swedish RA-patients started slightly more often in winter.^{5,6} This may be by chance or a true finding. Vitamin-D levels are the lowest at the end of winter⁴ and are presumably lower in the Swedish than the Dutch population. Given the observation that the effect of seasons on RA onset was less pronounced in the Dutch patients, it is tempting to speculate about the role of vitamin D in RA onset. Nevertheless, other explanations are also possible. Importantly, if winter-related pathogenic factors affect the onset of RA, they do not seem to inflict on the severity of the disease course.

ACKNOWLEDGEMENTS

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Chapter 14

Smoking as a risk factor for the radiological severity of Rheumatoid Arthritis: a study on six cohorts

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Under review

ABSTRACT

Background

Smoking is a risk factor for the development of ACPA-positive Rheumatoid Arthritis (RA). Whether smoking predisposes to severe joint damage progression is not known, since deleterious, protective and neutral observations have been made. We aimed to determine the effect of smoking on joint damage progression.

Patients and methods

Smoking status was assessed in 3,158 RA-patients included in 6 cohorts (Leiden-EAC, BARFOT, Lund, Iceland, NDB and Wichita). In total 9,412 radiographs were assessed. Multivariate normal regression and linear regression analyses were performed. Data were summarized in a random effects inverse variance meta-analysis.

Results

When comparing radiological progression for RA-patients that were never, past and current smokers, smoking was significantly associated with more severe joint damage in Leiden-EAC ($p=0.042$) and BARFOT ($p=0.015$) RA-patients. No significant associations were found in the other cohorts, though a meta-analysis on the six cohorts showed significantly more severe joint damage progression in smokers ($p=0.01$). Since smoking predisposes to ACPA, analyses were repeated with ACPA as additional adjustment factor. Then the association was lost (meta-analysis $p=0.29$).

Conclusion

This multi-cohort study indicated that the effect of smoking on joint damage is mediated via ACPA and that smoking is not an independent risk factor for radiological progression in RA.

INTRODUCTION

The severity of joint damage in Rheumatoid Arthritis (RA) is highly variable between patients. Genetic factors are estimated to explain half of this variance¹; environmental factors likely play a role as well. No clear environmental risk factors for joint damage progression have been identified.

Smoking has been implicated as one of the most important environmental risk factors for the onset of Rheumatoid Arthritis (RA), especially for the ACPA-positive subgroup.²⁻⁵ It has been hypothesized that smoking contributes to the development of RA-related auto-antibodies.⁴ Whether smoking influences the severity of RA as well, is less clear. Smoking was associated with more severe radiographic progression in a Swedish study⁶, but this relationship has not been established in other cohorts.⁷⁻⁹ Intriguingly, in a North-American cohort, smoking was shown to protect against joint replacement surgery¹⁰, and a Swiss study showed a trend towards less progression of radiographic joint damage in heavy smokers.⁹ The presence of ACPA is associated with more joint damage. It is unclear whether smoking as such affects progression of joint damage or whether smoking induces ACPA-production and thereby affects joint damage progression.

This study therefore aimed to determine the association between smoking and joint damage in RA and whether this association is mediated through ACPA. In total 9,412 radiographs of 3,158 RA-patients, from six cohorts, were studied and the results were summarized in meta-analyses.

PATIENTS AND METHODS

Patients

Cohort 1 consisted of 703 Dutch RA-patients included between 1993-2006 in the Leiden Early Arthritis Clinic (Leiden-EAC), a population-based inception cohort that is described more extensively elsewhere.¹¹ Hands and feet radiographs were taken at baseline and yearly over 7-years (total number 3,656, mean follow-up 4.9 years) and chronologically scored by one reader unaware of clinical data using the Sharp-van der Heijde score (SHS).¹² The within-reader ICC was 0.91.¹¹ Treatment strategies differed for different inclusion periods, as described in¹¹. Smoking status (present/past/current) was assessed by questionnaires.

Cohort 2 contained 839 RA-patients included between 1992-1999 from the BARFOT-study, a Swedish multicentre observational study of patients with early (disease duration

≤ 1 year) RA.¹³ Clinical, laboratory and radiological assessments were performed at inclusion and after 1, 2 and 5-years. Hands and feet radiographs (total number 2,870, mean follow-up 4.3 years) were SHS-scored by 2 readers. The between-reader ICCs at baseline and 2-years were 0.93 and 0.94 respectively. At inclusion, no patient had got prior treatment with DMARDs or glucocorticoids. During follow-up, 213 patients participated in a 2-year randomized study on low dose prednisolone as an addition to DMARD-therapy. Smoking status (present/past/current) was assessed by the rheumatologist at inclusion.

Cohort 3 consisted of 339 RA-patients that were recruited from a practice in Wichita, Kansas.¹⁴ Serial hands radiographs (total 1,062) were made during 15 years of follow-up. Cohort 4 consists of 885 RA-patients included in the National Databank of Rheumatic Diseases (NDB).¹⁵ Hands radiographs were made at a single time-point. The radiographs of cohorts 3 and 4 were SHS-scored by one experienced reader (ICC=0.98). All patients in these two cohorts developed RA between 1963-1999, thus in eras when early treatment and use of biologics were uncommon.¹⁶ Smoking status was assessed by questionnaires as a binary variable indicating ever or never smoking.

Cohort 5 consisted of 265 patients from Iceland, referred to Landspítali- The National University Hospital of Iceland or the private clinic of Reykjavik between 1970-2008. Radiographs were made at a single time-point. Joint damage was determined using SHS by two trained readers. The ICCs between and within readers were all >0.95 . Smoking status (present/past/current) was assessed by questionnaires.

Cohort 6 consisted of 127 early RA-patients from Lund, Sweden that were prospectively followed during 5-years.[17, 18] Patients were recruited during 1985-1989. Radiographs of hands and feet were taken at baseline and annually for 5-years. Radiographs were scored chronologically according to the Larsen score by one of two readers (ICC between readers 0.94).¹⁹ Smoking status (present/past/current) was assessed by the rheumatologist.

All cohorts had been approved by the local medical ethical committee and all patients had given informed consent. RA was defined according to the 1987-ACR-criteria except for the Lund-cohort, where the 1958-criteria were used.

Statistical methods

Radiographic scores were log10-transformed to approximate a normal distribution. In the Leiden-EAC, BARFOT, Wichita and Lund cohorts, repeated radiographs were available. For these cohorts multivariate normal regression analyses were used with the log10-transformed radiographic score as response variable, as described previously.²⁰ In the NDB and Icelandic patients, one radiograph per patient had been made. Here

the estimated yearly progression rate was calculated (total SHS divided by disease years since diagnosis at time of radiograph) and linear regression analyses performed. The statistical methods used is extensively described in reference 20. In all cohorts the radiological progression scores were compared between smoking groups, resulting in a relative difference in radiological progression. This effect estimate had no units and could be compared between data-sets. All analyses were adjusted for age and gender. In the Leiden-EAC adjustments were made for the inclusion period (1993-1995, 1996-1999, 1999-2006) as a proxy for treatment strategy as described previously.¹¹ Also in the Iceland data, adjustments were made for inclusion before or after 2000, to correct for different treatment regimes. In the BARFOT-cohort, adjustments were made for participating in a corticosteroid study. No treatment adjustments were made in the cohorts where all patients were included ≤ 1999 and treatment effects were not observed. Subsequently, all analyses were repeated with ACPA as additional adjustment factor in the regression analyses.

When possible the effect of smoking was first assessed by comparing three categories (present/past/never smokers); for the two North-American data-sets only binary variables were available. The results of the individual cohorts were summarized in an inverse variance meta-analysis testing for random effects. In these meta-analyses, for all cohorts, present and past smokers (combined as smokers) were compared with never smokers. Regression analyses were done using SPSS versions 20.0 (SPSS Inc., Chicago, IL, USA), meta-analyses were performed using STATA. Two-sided p-values < 0.05 were considered significant.

RESULTS

Table 1 presents characteristics of the patients from the different cohorts. Overall 52-69% of the RA-patients smoked or had smoked.

Table 1. Baseline characteristics of the study-population

	Leiden EAC	BARFOT	NDB	Wichita	Iceland	Lund
No of patients	703	839	885	339	265	127
No of radiographs	3,656	2,870	885	1,062	265	674
No female (%)	475 (67.6)	538 (69.1)	703 (79.4)	256 (75.5)	214 (80.8)	87 (68.5)
Age at baseline (mean, SD)	56.5 (15.7)	56.7 (15.4)	49.5 (12.8)	47.8 (14.2)	47.6 (13.7)	50.2 (10.9)
No. past or present smoker at baseline (%)	317 (50.5)	475 (56.7)	529 (60.3)	193 (56.9)	197 (69.4)	66 (52.0)
No ACPA+ (%)	368 (52.3)	418 (49.8)	539 (60.9)	275 (81.1)	130 (49.1)	100 (78.7)
Years of inclusion	1993-2006	1993-1999	1980-1999	1963-1999	1970-2008	1985-1990

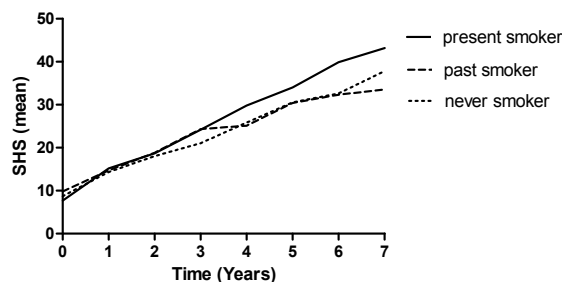


Figure 1. Joint destruction over 7 years of disease in 703 Leiden-EAC RA-patients according to their smoking status (being present, past or never smoker).

In this analyses, three groups of patients were defined: active smokers, former smokers and never smokers. Active smokers had a 1.01 (1.00-1.02) times higher radiological progression rate per year compared to former smokers, who had a 1.01 (1.00-1.02) times higher progression rate per year follow-up compared to never smokers.

In the Leiden-EAC, smoking was significantly associated with the progression of radiographic joint damage (p -value=0.042, Figure 1). When smoking was categorized in two groups, smokers had a 1.02-fold (1.00-1.04) times higher progression rate per year (p =0.07).

In the BARFOT-patients, when comparing present, past and never smokers, smoking was associated with more severe joint damage, an effect that was constantly present over time (p -value=0.015). When 'ever smokers' were compared with 'never smokers', significance disappeared (p =0.12).

When the progression rates were compared between present, past and never smokers in the Icelandic and Lund cohorts, no significant results were obtained (p =0.75 and p =0.53 respectively). When smokers were compared with non-smokers also no significant results were obtained (p =0.45 for and p =0.38 respectively).

For the North-American cohorts only binary data were available. In both cohorts smokers had no significant difference in joint damage progression compared to non-smokers (p =0.16 for the Wichita cohort, p =0.77 for NDB).

However importantly, in all mentioned cohorts, except for the Icelandic data-set, the directionality of the effect was similar with smokers having more severe joint damage progression. Subsequently the results on the analyses comparing past and present smokers with never smokers were summarized in a meta-analysis, showing that smoking was significantly associated with more radiologic progression (p =0.01, Figure 2A).

As smoking predisposes to ACPA-formation and that ACPA is associated with more severe joint destruction, we repeated all analyses with ACPA-status as additional covariate. No significant results were obtained in any of the cohorts. The directionality of the effects was diverse. Also in the meta-analysis, smoking was no longer associated with joint damage progression (p =0.29, Figure 2B), indicating that the observed effect of smoking was mediated through ACPA-formation.

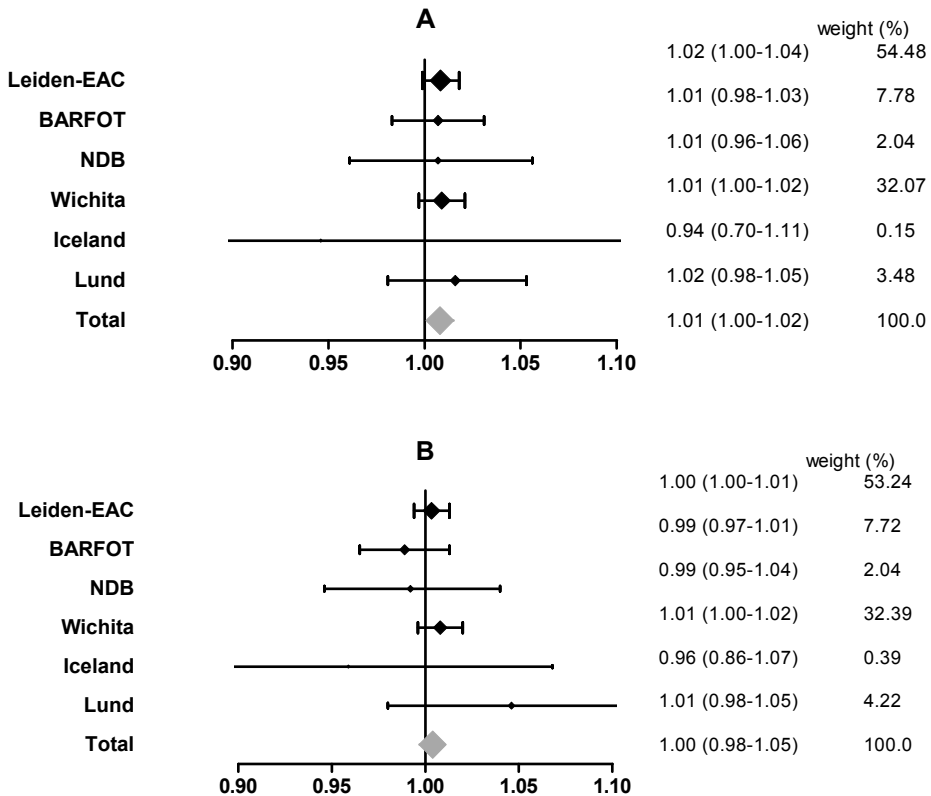


Figure 2. Meta-analysis on the effect of smoking (assessed as past and present smokers versus never smokers) on joint damage progression in six cohorts

Depicted are the results of the individual cohorts and of the meta-analysis. The meta-analysis is based on a random-effects model. The I-square was 0.0%, indicating little heterogeneity between the cohorts. In A, the meta-analysis without adjustment for ACPA-status is depicted; in B the analyses on all cohorts were also adjusted for ACPA. The effect-sizes indicate the increase in progression rate of smokers compared to non-smokers. Analyses were done on log10 transformed data, the effect estimated were backtransformed to the normal scale and presented here. For example, for Leiden-EAC patients depicted in the figure, smokers had a 1.02-fold (1.00-1.04) times higher progression rate per year ($p=0.07$) than never smokers; this indicates 2% more severe joint damage per year of follow-up. In all data-sets the relative difference in radiological progression between smokers and non-smokers was assessed, yielding per data-set an effect estimate without units and that could be compared between data-sets, independent on whether the radiographs were initially scored according to the SHS or Larsen method. The cohorts with repeated radiological measurements yielded more precise estimations of the progression rate, resulting in smaller standard errors and a higher weight in the meta-analysis.

DISCUSSION

In this study we aimed to determine whether smoking is associated with the severity of the course of RA, reflected by the severity of joint damage. In a meta-analysis combining the data of six cohorts, it was observed that smokers had more severe joint damage. However since smoking predisposes to ACPA-development, the analyses were repeated with adjust-

ments for ACPA. Then the association was lost, indicating that the effect of smoking on joint destruction is mediated via the development of ACPA.

Advantages of this study are the large number of radiographs and patients, predominantly recruited in eras when early and aggressive treatment was less common. Hence the disease course of many of these patients may be more reflective of the natural disease course compared to many currently treated RA-patients. Treatment differences occurred in part of the cohorts studied, adjustment were made where appropriate. Some cohorts had serial radiographs and others single radiographs per patient. The former results in more precise estimations of the radiological progression rate, which is reflected by smaller confidence intervals of the effect estimates (see Figure 2).

Part of the patients in the BARFOT, Iceland and Wichita cohorts were also assessed in earlier studies on smoking and joint damage.[6, 8, 10] Given that some previous studies had contradictory results, an advantage of the present study is that we could combine data from these and other cohorts.

Our study has some limitations. Smoking was mainly assessed by questionnaires at disease onset. It is possible that patients may have failed to recall their former smoking habits. We had no information on the number of pack-years or on smoking habits during the disease course. Finally we could not differentiate between past and present smokers in some cohorts, hence in the meta-analysis on all cohorts smokers were compared with non-smokers.

Studies on environmental risk factors for joint damage are relevant as such factors are potentially modifiable. Given that the effect of smoking was mediated via ACPA and that ACPA-development occurs in the preclinical phase of RA, the current data may imply that preclinical environmental factors influence the long-term disease outcome.

ACKNOWLEDGEMENTS

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Chapter 15

Discussion

No arthritis patient is similar. A patient suffering from an arthritic joint can recover spontaneously, or can develop a full-blown Rheumatoid Arthritis (RA). Also within the group of RA patients, whether they have been diagnosed by the 1958, 1987 or 2010 criteria, large differences in the severity of the disease exist. The main aim of this thesis was to improve the understanding of the differences in the severity of arthritic disease. Next to the outcome measures such as remission of the disease or progression of Undifferentiated Arthritis (UA) into RA, the majority of the research that has been reported in this thesis was on understanding the differences in the radiologic severity of the disease. The research was focused mainly on identifying individual risk factors for more severe disease, but also towards finding differences in (possible) subgroups of patients.

Although highly relevant, this last research question was not entirely new, as in 1923, Archibald Garrod, the son of Alfred Garrod wrote: *‘...perhaps several maladies had been confused together under the name of rheumatoid arthritis. For this name, which was introduced by my father, I have naturally a pious respect, but I am fully alive to its shortcomings... Another question upon which it is to be hoped that light will be thrown in the course of this discussion, is whether there be any one specific disease to which the name rheumatoid arthritis may be applied, or whether the condition so called is rather a syndrome...’*¹

Since 1923, our knowledge of Rheumatoid Arthritis (RA) has increased dramatically, but despite all research that has been done, the question made by Archibald Garrod still has not been finally answered, although in the last decade, considerable progress has been made. While Garrod was convinced that RA was an infectious disease, it is nowadays widely accepted that RA is an auto-immune disease with a multifactorious origin.² Whether or not RA can and to what extent should be subdivided into subgroups remains an issue and the research described in **chapter 11** and **chapter 12** provides some new insights on this.

After the discovery of anti-citrullinated peptide antibody antibodies (ACPA), a number of studies suggested that RA can be divided into two subgroups: (ACPA)-positive and ACPA-negative RA.³⁻⁵ This subdivision was based on differences in genetic risk factors, histopathologic differences, and differences in outcome of ACPA-positive and ACPA-negative RA.⁶ In general, patients with ACPA-positive disease have higher rates of joint destruction and are less likely to achieve remission. Many studies focus on ACPA-positive patients. One of the reasons for this is the fear of phenotypic misclassification, as ACPA-negative RA is often considered to be a heterogeneous disease³⁻⁷.

Before we performed the Genome Wide Association Study GWAs described in **chapter 12**, we wanted to be sure that we could analyze all ACPA-negative patients as one group,

and that no clinically distinguishable subgroups existed. We therefore performed the study described in **chapter 11**. Using Principal Components Analysis, Partial Least Squares Regression and a Cluster Analysis, we could not identify any subgroups.

In **chapter 12**, the GWAs that we performed in the ACPA-negative patients, is described. We found that rs2833522 might be associated with the progression of joint destruction, but that larger, longitudinal studies are needed for confirmation.

In ACPA-negative RA, although large interindividual variance in the amount of joint damage exists, on average the amount of joint damage is less compared to ACPA-positive RA. In our ACPA-negative study, the differences in progression rates between genotype groups were lower compared to ACPA-positive patients, making it more difficult to find significant associations.

The importance of this study is that it shows that it seems that the severity of ACPA-negative RA is caused by multiple factors.

Another subdivision that is currently widely used is the division between patients with an Undifferentiated Arthritis (UA), those patients with arthritis in one or more joints, and those patients fulfilling one or more of the RA-criteria sets (**chapter 2**). This subdivision is not based on distinct clinical entities, but is a division based on cutoffs in a spectre from mild to severe arthritic conditions.⁸ One cannot say that all UA is pathofysiologically different from RA. The distinction is still clinically relevant though, as the patients fulfilling either the 1987 or 2010 criteria tend to have a worse prognosis and outcome.⁹ For that reason, it is important to find those patients that (will) fulfill the criteria timely. A prediction model has been derived to this end.¹⁰

In **chapter 2**, the Leiden Early Arthritis Clinic is described. This is a population-based prospective cohort that was started in 1993 in order to detect and treat inflammatory disorders early in the disease state, especially early RA. Patients are seen within two weeks. Inclusion took place when arthritis was confirmed at physical examination and symptom duration was <2 years. Radiographs of the hands and feet were made at baseline and yearly thereafter. Patients included between 1993 and 1995 were initially treated with analgesics and were subsequently treated with HCQ or SSZ if they had persistent active disease. Between 1996 and 1998, patients who were included were promptly treated with chloroquine or SSZ, whereas after 1998, the initial treatment strategy consisted of either MTX or SSZ. Treatment of UA patients was not protocolized. **Chapter 2** shows data obtained in this cohort on the progression from UA to RA, the development of persistent disease and the prediction of the outcome in RA in terms of the rate of joint damage. Risk factors that were observed to associate with RA development and disease persistency were largely the same, and mainly inflammatory markers and autoantibodies.

In **chapter 3**, we evaluated the predictive ability of measuring Bone Mineral Density loss by Digital X-ray Radiogrammetry (DXR) in early UA. BMD loss may be a relevant predictor for the development of RA. It may be especially useful for those patients that cannot be adequately predicted by current prediction models.

When assessing the radiographic severity of the disease, **chapter 2** shows that the presence of autoantibodies has the largest influence on this severity measure. When we combined individually associating risk factors, the overall explained variance for the severity of joint destruction was 32%. Importantly, only clinical risk factors were analyzed here and not for example genetic or environmental ones.

One of the main aims of this thesis was to increase our knowledge of the factors responsible for the severity of joint destruction. Genetics are a good candidate to study, as the genotype of a patient is stable over time and, as far as we know now, not influenced by treatment or other factors. This is at the same time a disadvantage of studying genetics: a patient cannot change his or her DNA. Only his physician can apply a more aggressive treatment regimen. Knowledge of genetic factors is useful for the researcher, because this knowledge can be used for the development of new drugs.

In **chapter 6**, we described a candidate-gene study on four genes that are involved in the Wnt/ β -catenin signaling pathway in relation to the severity of progression of joint destruction in RA. Our major finding was that RA-patients carrying the risk alleles of several genetic variants in *Dkk-1* have higher serum levels of functional Dkk-1 and more progressive joint destruction over time. These findings are in line with recent observations that Dkk-1 serum levels are associated with joint destruction.¹¹⁻¹³ Based on these data, it may also be hypothesized that blocking the binding of Dkk-1 to LRP-5 or LRP-6 may lead to new therapeutic strategies to protect patients against progression of joint damage. Two genetic variants in *Sost* were suggestively associated with progression of joint destruction, though after correction for multiple testing, only a trend towards statistical significance remained. We studied interactions between the *Dkk-1* and *Sost* SNPs that were significant in phase-2 and observed significant multiplicative interactions.

In **chapter 7**, we show that SNPs on *TRAF1-C5* and *TNFAIP3-OLIG3* that were reported to associate with the progression of joint destruction in RA some years ago^{14,15}, could not be replicated in more cohorts and with more radiographic data available.

Chapter 8 shows that in a meta-analysis of several cohorts, we found that RA-patients with the minor-allele of *OPG*-rs1485305 expressed higher rates of joint destruction compared to patients without the risk variants ($p=2.35 \times 10^{-4}$).

In the last candidate gene-study in this thesis (**chapter 9**), we report that RA-patients carrying the minor alleles of several SNPs in *IL2RA* have less severe progression of joint destruction. This genetic association is further substantiated by fine-mapping data as well

as functional data. Patients carrying the minor alleles also have lower levels of soluble IL-2RA and hence may have less activation of CD4+ T cells, preventing severe inflammation and reducing long term joint destruction.

The Immunochip allowed us to study 186 loci associated with 12 auto-immune diseases in the Leiden EAC patients and to subsequently replicate our findings in 686 North-American patients.¹⁶ In **chapter 10**, we describe how we analyzed these data and how we discovered two new genetic risk loci for the radiographic severity of RA. In most studies on SNPs influencing the radiographic severity of RA, it is not possible to obtain significant replication in entirely independent cohorts, because replication cohorts usually have less patients and less radiologic data, decreasing the power. In this study however, we did find truly independent replication of two genetic variants. For the first variant found, on chromosome 14, it is difficult to speculate how this variant could influence radiographic disease progression in RA. For the variant on chromosome 20, this is different. The region of rs11908352 was on the Immunochip because of rs4810485 (*CD40*) that is associated with susceptibility to RA. However, rs11908252 is in low LD with this SNP, and rs11908352 lies close to *MMP-9*, and is in high LD with several SNPs in *MMP-9*. *MMP-9* is a member of a group of secreted zinc metalloproteinases which degrade the collagen of the extracellular matrix. Increased serum levels of *MMP-9* have been described in RA.¹⁷ We observed that at their first visit, patients with the AA genotype that was associated with more severe joint damage, had significantly higher serum levels of *MMP-9*.

When looking at the results of the various genetics studies that are part of this thesis, it is interesting to notice that the hits found by the candidate-gene approach used in **chapters 6-10** are different from the top-hits in found in **chapter 12** (describing the GWAS on the radiologic severity of ACPA-negative RA and in **chapter 10**, describing the SNPs tested by Immunochip in relation to RA severity). Here, the variants found to be risk factors for a more severe disease were different from the variants identified in the Immunochip study by Eyre et al on the susceptibility to RA.¹⁸ This latter difference may be due to the fact that our study contained considerably less patients than the study of Eyre et al. Also, the genetic factors associated with the susceptibility to and progression of RA may very well be different.

An important difference between the Immunochip and the GWAS in ACPA-negative RA on the one site, and the candidate-gene studies on the other, is that in the first two studies, hundred thousands of SNPs were tested, making a strong correction for multiple testing mandatory. Consequently, the threshold for significance was much lower compared to the threshold of significance in the candidate-gene studies where relatively few SNPs were tested. However, the GWAS-like studies focus on finding positive associations, and

false negative results are inherent to this study-design. A candidate-gene approach allows studying those SNPs that are unlikely to be found by GWAS-like studies. This does not mean that the results obtained in the candidate-gene studies are less-important compared to the results reported in **chapters 10 and 12**. On the contrary, as for the SNPs studied in candidate gene studies, we already had some ideas about their (possible) relation to RA severity, it was extremely important to gain more insight into their possible association with joint destruction. One may argue that the p-values obtained in the candidate-gene studies are generally much higher compared to the p-values obtained in **chapters 10 and 12**, and, that therefore there is more chance that the results reported in the candidate gene studies are due to coincidence. Such argumentation is flawed however, firstly, because the study sizes of the GWAS-like and the candidate-gene studies are different, making a comparison of p-values impossible, and also, because for the candidate-gene SNPs, a lot more evidence of a possible relationship to RA-severity exists. The weight of this evidence cannot be easily transformed to a numerical value, but should be taken into account when comparing the results of candidate-gene studies to GWAS-like studies. A good example of this is the SNP found on chromosome 14 in the Immunochip (rs451066) (**chapter 10**). This SNP was included because it was near to a SNP associated with the risk on type-1 diabetes. Apart from a p-value in the replication phase of 0.002, we do not have any evidence on how this SNP could influence the radiologic severity of RA. Of course, future studies may provide insights into this.

Thanks to these studies, we gained more insight into the genetic factors determining the severity of RA. But how useful is this knowledge? A patient can stop smoking or drinking alcohol, or make an attempt to loose weight or get more exercise. A patient cannot change his or her DNA though. Still, when the severity of RA can be predicted on an individual level, physicians can apply a more aggressive treatment regimen in those patients where a more severe disease course is expected. In this way, genetic studies could be useful to individual patient care. However, when van der Helm et al added the presence of genetic risk factors to their prediction model on RA-development, the model did not improve.¹⁹ Whether this will be different for predicting the severity of the disease course is subject for further studies. Nonetheless, it is not to be expected that genetic markers alone will enable adequate prediction making.

Does that mean that a large part of the research presented in this thesis has no clinical value? Absolutely not! First of all, knowledge of genetic factors may reveal new pathophysiologic pathways that enhance our understanding of the disease. A good example is our finding of *MMP-9* as a risk factor for the severity of RA. The candidate gene studies presented in this thesis (**chapters 6-9**) show that some genes of pathways influencing bone metabolism or auto-immunity have a strong effect on the severity of RA (e.g. *Dkk-1* and *IL2-RA*, whereas other have a less pronounced effect (e.g. *Sost*) or seem to have no effect at all, improving our understanding of the pathophysiology of RA joint destruction.

Secondly, now that we have found several risk factors for the severity of RA, we can use this information as a starting point for new research to additional factors determining RA severity. Last but not least, for studying the genetics of the severity of RA, a lot of the methodology used had to be ‘invented’, e.g. the statistical models used, and the optimal ways of combining data into meta-analyses.²⁰⁻²² This knowledge is extremely useful research to other RA-severity risk factors.

For future studies, two strategies are most promising. From conception till death, our lives are influenced by environmental risk factors varying from maternal smoking to the intake of healthy nutrients to air pollution. Exposure to most environmental factors can be diminished if necessary. So research into the environmental factors responsible for RA-severity may have direct benefits for patients. In **chapter 13**, we investigated whether the season of RA-symptom onset causes differences in the progression of joint destruction. Although many patients and doctors have thoughts on seasonal influences on RA, research has not yet provided us with very clear answers.²³⁻²⁶ Seasonal differences could be explained by different weather conditions, but also by different levels of vitamin D. Vitamin D has long been thought to influence the immune system, and several studies on possible associations between vitamin D and RA have been published.²⁷⁻²⁹ In contrast to a French study, we did not find clear seasonal differences.

A clear association between smoking and RA susceptibility has been described.³⁰⁻³² The effects of smoking on joint destruction were less clear though.³³⁻³⁷ In **chapter 14**, we report a study to the influence of smoking on the progression of joint destruction in RA. We found that smoking influences the long-term outcome of RA, but that this influence is not independent of ACPA-status.

A lot more of this kind of research should be done, but it should be realized that although we have excellent tools to analyze the radiographic progression of RA, research into environmental factors is much more complicated than research into genetic factors. A genotype is stable over time, and although expensive, it is well possible to genotype patients reliably. Exposure to environmental factors can change over time and is often susceptible to recall bias. Furthermore, information on exposure to environmental factors is often scarcely available in medical records. Still, during the last years, several reports have been published on the environmental factors in relation to RA-susceptibility and, to a lesser extent, RA-severity.^{28;30;38-40} Moreover, most patients have some ideas about the causes of their illness, and often they think environmental factors are important.⁴¹ Even if it shows up that environmental factors have no influence on RA-severity, this information will be useful in the communication to our patients.

Another promising research strategy is to investigate epigenetic factors. The last years, a large amount of research has become available suggesting that epigenetic factors such as DNA-methylation play an important role in the processes driving RA.^{42;43} Our current view of genetic variances as a risk factor for RA-development and RA-severity is almost certainly too simplistic, and it is likely that the influence of genetic variants on RA-severity can only be fully understood in combination with epigenetic factors. However, when studying epigenetic factors, one should first and foremost have knowledge about the relevant genetic factors on RA-severity. Studying these genetic factors was the aim of this thesis.

Of course, the research reported in this thesis also provided us with some more specific research questions. To give some examples: how useful is using DXR-online in daily clinical practice? And is it cost-effective? (**chapter 3**) What is the function of rs451066 on chromosome 14? (**chapter 10**) Can rs2833522 be replicated as a risk factor for joint destruction in other ACPA-negative cohorts? How does MMP-9 influence bone destruction, and how can we block this process? (**chapter 10**) It would be interesting to learn the answers to these questions in the near future.

In conclusion, although in the last decades, the prognosis of RA has improved dramatically, much is unknown about the processes driving joint destruction. At this moment, we do not understand these processes completely, and predicting the disease course in individual patients is still far away. The research in this thesis expanded our knowledge of the pathophysiology of joint destruction in RA both by increasing our knowledge on factors suspected to influence joint damage and by showing new factors responsible for joint destruction in RA. The main goal of the research was to better understand the processes behind joint destruction. In this respect, the research described in this thesis was successful, although we still have some way to go.

We should not forget the ultimate goal of medical research however, which is to reduce the suffering of patients. Although the research in this thesis was still some distance away from the bedside and will not directly change clinical practice, it is easy to describe its clinical usefulness. For example, we showed that DXR-online has good diagnostic abilities, especially for those patients that cannot be predicted by current models. We showed the importance of several pathways for joint destruction. These pathways could be novel therapeutic targets. Many physicians think of ACPA-negative RA as a heterogeneous disease and it is often feared that within ACPA-negative RA, specific subgroups exist. Using three sophisticated statistical methods, we could not identify any subgroups within ACPA-negative RA. Both patients and physicians often have some beliefs on seasonal influences on RA severity. We showed though that the season of first symptom onset seems not to relate to the radiologic severity of RA. All this research may help in achieving better

patient outcomes or at least be helpful in the communicating with and understanding our patients. Also for future research, we should always critically think whether it will be helpful to our patients. As mentioned before, although technically difficult, if well conducted research on environmental factors is especially promising.

In the end however, it is all about the well-being of our patients. No matter how much, how large and how excellent our research projects are they have only value when we address our patients as good physicians. Even though it is not easy to say what a good physician should be like and perceptions on this tend to change over time, we should always keep in mind though that no matter how much knowledge we may gather, medical knowledge is only useful when at the same time we respect patient autonomy, think and debate on ethical issues and always place the patient at the center of our medical universe.

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Nederlandse samenvatting

RHEUMATOÏDE ARTRITIS

Reumatoïde Artritis (RA) is een auto-immuunziekte waarbij ontsteking van de gewrichten op de voorgrond staat. Ongeveer 0,5 tot 1% van de bevolking heeft RA, de ziekte treft voornamelijk vrouwen van middelbare leeftijd. Wanneer geen behandeling plaatsvindt, kan de ziekte tot functieverlies in de gewrichten, invaliditeit, en uiteindelijk zelfs vroegtijdige sterfte leiden. Alhoewel RA tegenwoordig voor de meeste patiënten nog steeds een ongeneeslijke ziekte is, is in de afgelopen 30 jaar enorme vooruitgang geboekt in het onderzoek naar en de behandeling van RA. Bij een meerderheid van de patiënten kunnen de ziekteverschijnselen effectief worden onderdrukt. Bij sommige patiënten is het zelfs mogelijk dat de ziekte helemaal in remissie komt, wat betekent dat patiënten, zonder dat zij medicijnen gebruiken, klachtenvrij zijn. Desalniettemin gaat RA bij de meeste patiënten nog steeds gepaard met klachten en functieverlies. De ziekte heeft daardoor een grote invloed op het dagelijks leven van veel patiënten. RA gaat gepaard met hoge kosten voor de maatschappij, zowel vanwege de directe kosten van gezondheidszorg (medicijnen tegen RA behoren tot de duurste die er zijn), als vanwege toegenomen ziekteverzuim.

De belangrijkste vraag in dit proefschrift is: wat maakt dat sommige RA-patiënten een ernstig ziektebeloop hebben en anderen een mild? Daar gaat echter een andere vraag aan vooraf: wat is ernstig RA? Hoe meet je de ernst van RA? Patiënten zullen doorgaans vooral klagen over pijn en functieverlies, klachten die moeilijk in een maat en getal zijn uit te drukken. Voor onderzoeksdoeleinden wil je het liefste een eenheid gebruiken die objectief kan worden vastgesteld. Om die reden is er in dit proefschrift voor gekozen de ernst van RA te meten door middel van de schade aan de gewrichten zoals vastgesteld op röntgenfoto's. Om deze schade te kwantificeren zijn gevalideerde scoringsmethoden ontwikkeld. Voor dit proefschrift is voornamelijk gebruik gemaakt van de Sharp-van der Heijde Score (SHS). Deze scoringsmethode telt erosies ("gaatjes" in het bot die het gevolg zijn van de ontstekingsprocessen in RA) en gewrichtsspleetvernaauwing (een gevolg van kraakbeenverlies). In de studies in dit proefschrift worden doorgaans niet de ruwe scores geanalyseerd, maar de relatieve toenames. Op die manier kunnen patiënten bij wie verschillende scoringsmethoden zijn gebruikt, toch gezamenlijk geanalyseerd worden.

Niet iedere patiënt met een ontsteking van een gewricht (een artritis) heeft ook RA. Wanneer een patiënt één of meer ontstoken gewrichten heeft zonder te voldoen aan de criteria voor RA, spreken we van een ongedifferentieerde artritis (Undifferentiated Arthritis, UA). In de praktijk blijkt dat veel UA patiënten in de loop van de tijd RA ontwikkelen. Het liefst wil je die patiënten in een zo vroeg mogelijk stadium identificeren, om met behandeling gewrichtsschade (en dus functieverlies) te kunnen voorkomen. Dit laatste geldt overigens ook voor de RA-patiënten: je wilt die patiënten, die veel gewrichtsschade zullen gaan ontwikkelen, in een zo vroeg mogelijk stadium identificeren en behandelen.

In 1993 was dit een belangrijke reden om met de Leidse *Early Arthritis Clinic* (EAC) te starten, een follow-up studie van inmiddels meer dan 3000 patiënten. Dit proefschrift is voor het grootste deel gebaseerd op gegevens die uit de EAC verkregen zijn.

Het eerste deel van dit proefschrift gaat over de EAC, over het ontstaan van RA bij UA patiënten en over het voorspellen van het ontstaan van radiologische gewrichtsschade bij RA.

Na een algemene inleiding in hoofdstuk 1, volgt in **hoofdstuk 2** van dit proefschrift allereerst een uitgebreide beschrijving van de EAC. De EAC is een prospectief onderzoekscohort dat bestaat sinds 1993. Dit cohort had als doel auto-immuunziekten in een vroeger stadium op te kunnen sporen en behandelen. Patiënten, die op de polikliniek Reumatologie van het Leids Universitair Medisch Centrum kwamen en die een artritis

(gewrichtsontsteking) hadden die minder dan twee jaar bestond, werden geïncludeerd. Jaarlijks werden röntgenfoto's van handen en voeten gemaakt. In de loop der jaren veranderden de behandelstrategieën. In de periode 1993-1995 werd voor de behandeling van RA gestart met pijnstilling, en pas als dat onvoldoende hielp, chloroquine of sulfasalzine. Van 1996 tot 1998 werd direct gestart met chloroquine of sulfasalzine. Vanaf 1998 werd direct behandeld met methotrexaat of sulfasalzine. Om ondanks deze behandelingsverschillen toch alle patiënten te kunnen vergelijken, wordt een statistische correctie uitgevoerd voor de periode waarin een patiënt in behandeling kwam. In hoofdstuk 2 wordt ook aandacht besteed aan klinische risicofactoren die gerelateerd zijn aan de progressie van UA in RA en meer radiologische gewrichtsschade bij RA-patiënten. De risicofactoren voor de ontwikkeling van RA vanuit UA en de radiologische schade in RA waren grotendeels hetzelfde, de belangrijkste risicofactoren zijn verhoogde ontstekingsparameters en de aanwezigheid van autoantistoffen.

In **hoofdstuk 3** wordt een nieuwe methode beschreven om bij bepaalde groepen UA-patiënten beter te kunnen voorspellen of ze wel of geen RA zullen ontwikkelen. Op conventionele röntgenfoto's van de handen werd met de DXR-Online techniek botdichtheidsverlies gemeten. Dit botdichtheidsverlies bleek inderdaad voorspellend voor het ontstaan van RA. Echter, de meerwaarde van deze techniek, bovenop de al bekende risicofactoren, is beperkt. Enkele jaren geleden hebben Van der Helm ea een predictiemodel ontwikkeld om het ontstaan van RA in UA patiënten te kunnen voorspellen. Voor die patiënten, waarbij met het predictiemodel van Van der Helm ea géén goede voorspelling mogelijk is, lijkt DXR-Online een nuttige optie.

In **hoofdstuk 4** werd onderzocht in hoeverre het aantal erosieve gewrichten dat een patiënt bij zijn eerste bezoek had, voorspellend was voor een snelle toename in gewrichtsschade, en of het mogelijk was een afkapwaarde van het aantal erosieve gewrichten te definiëren, waarboven meer kans was op de snelle ontwikkeling van gewrichtsschade. Alhoewel een groter aantal erosieve gewrichten met een slechtere prognose gepaard ging, was het niet mogelijk een duidelijke afkapwaarde te definiëren.

Met onderzoek beschreven in **hoofdstuk 5** van dit proefschrift wordt geprobeerd andere onderzoekers een handje te helpen. Onderzocht werd of er een correlatie is tussen SHS van de linker- en rechterhand en -voet. Deze correlaties waren zeer zwak, en voor onderzoeksdoeleinden kan men het beste röntgenfoto's van zowel de linker- als de rechterhand en -voet van de patiënt maken.

GENETICA EN DE ERNST VAN RA

In de afgelopen jaren zijn verscheidende genetische risicofactoren voor het ontstaan van RA beschreven. In wezen kan men, tweelingonderzoek daargelaten, de genetische contributie aan de (ernst van) een ziekte op twee manieren bestuderen. Een eerste manier is het zonder hypothese testen van grote aantallen genetische varianten (Single Nucleotide Polymorphisms, SNPs) in relatie tot de ziekte. Een alternatief is het eerst vormen van een hypothese dat een bepaald gen gerelateerd is aan de ziekte, en het vervolgens uitsluitend onderzoeken van SNPs op zo'n gen.

In **hoofdstuk 6** wordt een studie beschreven naar een aantal genen die betrokken zijn bij het reguleren van de Wnt-cascade (een cascade van eiwitreacties die onder meer verantwoordelijk is voor kraakbeenopbouw- en afbraak). Het bleek dat de SNP rs1898368, op het gen dat codeert voor het eiwit Dkk-1, gerelateerd was aan meer radiologische schade. Patiënten met de genetische variant die gerelateerd was aan meer radiologische schade, hadden ook hogere spiegels van het eiwit Dkk-1, dat een rem van de Wnt-cascade is.

Enkele jaren geleden werd beschreven dat SNPs op de genen *TRAF1-C5* en *TNFAIP3-OLIG3* geassocieerd waren met radiologische schade. In **hoofdstuk 7** wordt geprobeerd deze bevinding te repliceren (opnieuw aan te tonen in andere cohorten). Er werden echter geen statistisch significante verbanden gevonden.

In **hoofdstuk 8** worden de resultaten beschreven van een studie naar SNPs die coderen voor eiwitten van de OPG/RANK/RANKL/TRAF6 cascade. Patiënten met de minor variant (de minst voorkomende variant) van *OPG*-rs1485305 hadden meer gewrichtsdestructie dan patiënten met een andere variant.

In **hoofdstuk 9** wordt aangetoond dat patiënten met een minor variant van *IL2RA*-rs2104286 minder gewrichtsschade hadden dan patiënten met een andere variant. Ook hadden ze lagere spiegels van het eiwit IL2RA. Mogelijk werden de CD4+ T-cellen van deze patiënten minder sterk geactiveerd, waardoor ontsteking en gewrichtsdestructie minder waren.

Hoofdstuk 10 beschrijft eveneens onderzoek naar de genetische risicofactoren voor meer radiologische schade bij RA. Hier was de insteek echter anders. Het is bekend dat er een overlap bestaat tussen de genetische risicofactoren voor verschillende auto-immuunziekten. Om deze reden is er een Immunochip-consortium opgericht dat een Immunochip

ontwikkelde waarmee 186 genetische risicogebieden voor 12 auto-immuunziekten (waaronder de ziekte van Crohn, coeliakie, colitis ulcerosa, type-1 diabetes, multipele sclerose (MS), RA, systemische lupus erythematosus (SLE) en arthritis psoriatica) gedetailleerd in kaart werden gebracht. Uit onderzoek kwam naar voren dat een genetische variant op chromosoom 14, die eerder was beschreven in relatie met type-1 diabetes, ook van invloed was op de ontwikkeling van radiologische schade bij RA. Tevens bleek dat de SNP rs11908352, die dicht bij het gen ligt dat codeert voor het eiwit MMP-9, gerelateerd was aan meer radiologische schade. Patiënten die een “slechte” variant van deze SNP hadden, hadden bovendien significant hogere spiegels van MMP-9. MMP-9 is een enzym dat zorgt voor kraakbeenafbraak. Heel bijzonder aan deze studie is dat de resultaten gerepliceerd konden worden in een volledig onafhankelijke patiëntenpopulatie. Dit is voor het onderzoek naar de radiologische ernst van RA tamelijk uniek.

SUBGROEPEN RA-PATIËNTEN

Gedurende lange tijd werd RA als één homogene ziekte gezien, maar in de afgelopen jaren kwam er steeds meer bewijs naar voren dat er binnen RA verschillende subgroepen van patiënten onderscheiden kunnen worden. Op dit moment lijkt het erop dat de groep van RA-patiënten in twee subgroepen kan worden onderverdeeld: patiënten met antilichamen tegen gecitrullineerde eiwitten (de ACPA-positieve patiënten), en patiënten zonder deze antilichamen (de ACPA-negatieve patiënten). Over het algemeen hebben de ACPA-positieve patiënten meer gewrichtsschade en minder kans om ziekte-remissie te bereiken. In veel wetenschappelijk onderzoek worden uitsluitend ACPA-positieve patiënten onderzocht. Een reden hiervoor is dat het klinisch beeld bij ACPA-positieve RA patiënten over het algemeen duidelijker is, terwijl de ACPA-negatieve RA-patiënten een meer wisselende groep vormen. Toch zijn er ook veel ACPA-negatieve patiënten die veel gewrichtsschade oplopen en een zware ziektelast ervaren, en daarom is wetenschappelijk onderzoek naar de oorzaken van gewrichtsschade in deze patiëntengroep van groot belang.

In **hoofdstuk 11** wordt onderzocht of er binnen de groep van ACPA-negatieve patiënten klinisch verschillende subgroepen onderscheiden kunnen worden. Met een drietal geavanceerde statistische methoden werd onderzocht of op basis van klinische variabelen subgroepen onderscheiden konden worden. Dit bleek niet het geval, wat betekent dat de ACPA-negatieve RA patiënten in wetenschappelijk onderzoek als een homogene groep beschouwd kunnen worden.

In **hoofdstuk 12** worden de resultaten gepresenteerd van een Genome Wide Association Study (GWAS) naar genetische factoren die van invloed zijn op de radiologische ernst van RA. Een zeer groot aantal SNPs over het hele genoom werd geanalyseerd voor een

associatie met gewrichtsdestructie. Uit dit onderzoek bleek dat Rs2833522 mogelijk geassocieerd is met de radiologische ernst van RA. Maar om deze associatie definitief te bevestigen zijn grotere studies nodig.

OMGEVINGSFACTOREN EN DE ERNST VAN RA

Het laatste gedeelte van dit proefschrift gaat over de invloed van omgevingsfactoren op de radiologische gewrichtsdestructie bij RA. Onderzoek naar de invloed van omgevingsfactoren op de ernst van RA staat nog in de kinderschoenen. Een Franse onderzoeksgroep liet recentelijk zien dat het seizoen waarin een RA-patiënt zijn eerste klachten heeft, van invloed lijkt op de prognose. In **hoofdstuk 13** wordt geprobeerd de Franse resultaten te repliceren, er werden echter geen duidelijke seizoensinvloeden gevonden in de EAC-patiënten en in een patiënten cohort uit Zweden.

Dat roken slecht is voor de gezondheid, is alom bekend, en dat roken ook een verhoogde kans geeft op het ontstaan van RA is in meerdere studies onomstotelijk aangetoond. Wat de invloed van roken is op de ontwikkeling van radiologische schade in RA is echter minder duidelijk. In **hoofdstuk 14** worden de resultaten van een studie naar de effecten van roken op de ontwikkeling van gewrichtsschade op lange termijn onderzocht. Roken geeft meer gewrichtsschade, dit effect is echter afhankelijk van de aanwezigheid van ACPA. Dit betekent dat roken niet direct gewrichtsschade tot gevolg heeft, maar wel de vorming van ACPA stimuleert, waardoor rokende patiënten toch meer gewrichtsschade ontwikkelen.

BESCHOUWING EN VOORTUITBLIK

Een van de belangrijkste onderzoeksdoelen was het beter begrijpen van de processen die verantwoordelijk zijn voor meer radiologische gewrichtsschade bij RA-patiënten. Het onderzoek was vooral gericht op het identificeren van genetische risicofactoren. Nieuwe genetische risicofactoren werden ontdekt, zoals *Dkk-1* en *MMP-9*. Maar is dit soort onderzoek ook zinvol? Bij de huidige stand van wetenschap is het immers nog niet mogelijk om het DNA van patiënten te veranderen. Toch is dit onderzoek wel degelijk nuttig. Door het identificeren van de onderliggende genetische risicofactoren, kunnen nieuwe *pathways* van ziekteprogressie ontrafeld worden, waardoor ons begrip van de ziekte (en vervolgens ook de behandelmogelijkheden), beter worden. Daarnaast kan een deel van de kennis in dit proefschrift als startpunt dienen voor nieuw onderzoek. Ook moest voor het onderzoek in dit proefschrift een (deel van) de gebruikte (vooral statistische) methodologie speciaal ontwikkeld worden. De hiermee opgedane kennis en ervaring kan ook weer gebruikt worden in andere onderzoeken. Voor de toekomst van het onderzoek naar genetische

risicofactoren lijkt onderzoek naar epigenetische factoren, de factoren die bepalen waarom en hoe stukken DNA wel of niet worden afgelezen, het meest vruchtbaar. Voor het onderzoek naar de oorzaken van gewrichtsschade bij RA-patiënten in het algemeen is onderzoek naar omgevingsfactoren veelbelovend en belangrijk, maar ook lastig om uit te voeren. Hiervoor zijn immers grote onderzoekspopulaties met nauwkeurig vastgelegde data nodig, en de ervaring leert dat die schaars zijn. Zoals bij al het medische onderzoek is het ook hier van groot belang dat de patiënt centraal blijft staan, hetgeen betekent dat bij al het onderzoek ook de vraag moet worden gesteld hoe de patiënt, al is het via vele tussenstappen en nog weinig concreet, van het onderzoek zal kunnen profiteren.

List of Publications

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Curriculum Vitae

Diederik Pieter Constantijn de Rooy werd op 6 september 1985 geboren te Naarden. In 2003 slaagde hij voor het eindexamen Gymnasium aan het Willem de Zwijger College te Bussum. In datzelfde jaar startte hij met de studie Geneeskunde te Leiden. In 2005 startte hij daarnaast met de studie Rechtsgeleerdheid. Zijn co-schappen liep hij in ziekenhuizen in de regio Leiden. Hij deed een gecombineerde wetenschapsstage en semi-artsstage over het Hemofagocytair Syndroom bij de afdelingen Algemene Interne Geneeskunde en Hematologie van het Leids Universitair Medisch Centrum (LUMC) (begeleiders Dr. P.J.M. von dem Borne en dr. A.J. Fogteloo). In 2010 behaalde hij zowel zijn Artsexamen als zijn Bachelor Rechtsgeleerdheid. Aansluitend startte hij met promotieonderzoek op de afdeling Reumatologie van het LUMC (begeleiders prof. dr. T.W.J. Huizinga en dr. A.H.M. van der Helm-van Mil). Voor dit onderzoek verkreeg hij een beurs in het kader van het excellente studenten traject van het LUMC. Tijdens zijn promotieperiode ontving hij diverse reisbeurzen en in 2013 won hij een Abstract Award van de European League Against Rheumatism.

In de avonduren volgde hij de Master Civiel Recht. In 2012 schreef hij zijn afstudeerscriptie onder begeleiding van prof.mr. P.M.J. Mendes de Leon, met als titel "A passenger has become sick- is there a lawyer on board", over de juridische aspecten van ziekte van vliegtuigpassagiers. In 2012 studeerde hij af als Master in Laws (LL.M.). In oktober 2013 publiceerde hij een juridisch-wetenschappelijk artikel over de juridische aspecten van ziekte van vliegtuigpassagiers in het Tijdschrift Vervoer & Recht.

Thans is hij werkzaam als arts, en start hij in 2014 bij het LUMC met de specialisatie tot psychiater.