Chapter 1

General Introduction
Introduction

Rheumatoid Arthritis
Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease that mostly affects the joints, which leads to cartilage and bone destruction. The cartilage and bone destruction increases over the years resulting in a decline in functional capacity of the patients. The decreased functional capacity in turn influences the social economic status of the patients, by a negative effect on work participation. In addition to the economic costs through loss of work participation and economic assistance, the costs of treatment and healthcare for RA increased to over 568 million euro in the Netherlands in the year 2011. If it were possible to cure RA in an early phase and prevent the disabling joint destruction it would be possible to decrease the costs of healthcare for RA significantly. New biomarkers may be able to help to identify RA patients or persons at risk for RA in an early phase before joint damage occurs, in order to enable timely treatment.

Development of Rheumatoid Arthritis
RA is considered to be a heterogeneous disease with different clinical symptoms, progression and severity of the disease. Long before the manifestation of RA, healthy individuals with certain genetic dispositions come into contact with environmental factors such as smoking, certain dietary factors, silica components or infections. This may cause an immune response, which can trigger the formation of autoantibodies. Some of these individuals will develop RA like symptoms without the clinical diagnosis of RA and are called arthralgia patients. Arthralgia is defined as having joint complaints without swelling of the joints, without visible erosions of hands and feet on x-rays and without previous disease modifying anti-rheumatic drug (DMARD) treatment. Most of the time patients with painful joints report to the general practitioner who sometimes tests blood from the patients for autoantibodies. Patients with persistent joint complaints with or without positive autoantibodies are often referred to the rheumatologist in the Netherlands. For research purposes, at Reade Amsterdam, arthralgia patients who are autoantibody positive are followed for possible development of arthritis. Arthritis is diagnosed when the arthralgia patient has one or more swollen joints and at that time point 90% of the patients meet the 2010 ACR/EULAR classification criteria for RA. Approximately 40% of the autoantibody positive patients will develop RA within 5 years and in the other 60% of the patients the symptoms may persist, diminish or even disappear. It is not clear why some patients progress and others do not. One hypothesis that awaits scientific evidence is that a so-called ‘second hit’ like trauma or infection causes progression. Currently treatment with conventional DMARDS is started when the patients is diagnosed with RA on the basis of swollen joints. Early recognition and treatment helps to prevent joint damage. Theoretically even better results could be obtained by improved prediction and preventive treatment in the at-risk phase of the disease.
Figure 1. Development of Rheumatoid Arthritis (RA). The development of RA starts with a healthy individual with a certain genetic profile that encounters environmental factors. This could lead to the formation of autoantibodies. Some individuals that are autoantibody positive develop joint complaints without swelling and are called arthralgia patients. Only 40% of the arthralgia patients develop RA.

Risk factors for the development of Rheumatoid Arthritis

Genetic risk factors contribute for approximately 50-65% to RA susceptibility. The most relevant genetic risk factor is the shared epitope (SE) accounting for 30-50% of overall genetic susceptibility. The SE is a cluster of HLA-DRB1 genes that share a conserved amino acid sequence in the DRβ1 chain. Over 40 other genetic risk factors are described for RA including PADI4, PTPN22, STAT4, TNFAIP3, and CLA4. Most of the genetic risk factors are only associated with autoantibody positive RA and not with autoantibody negative RA, which points towards two different diseases with different etiologies.

Besides genetic risk factors for RA there are also environmental risk factors such as smoking, diet, silica components and infections. Klareskog et al., described a hypothesis for the etiology of RA in which it all starts with an environmental factor like smoking in a genetically predisposed individual. This causes the citrullination of proteins, e.g. in the lungs. The citrullinated proteins induce a T and B cell response leading to autoantibody production. The memory B cells will move to the bone marrow. After a “second hit” such as an infection or a trauma in the joints, an inflammatory response is induced attracting among others immune cells such as the citrullinated protein specific memory B cells and T cells. This process will eventually lead to a vicious circle of inflammation in the joints known as RA.
Autoantibodies
There are two major types of autoantibodies in RA, rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPA). RF was already discovered in 1937 by Waaler, but only since the early 1960s it is known that it is an antibody against the Fc portion of IgG. The standardization of the test for RF made the test comparable between labs and useful for clinical practice. ACPAs were discovered in 1964 as antiperinuclear factor (APF), which later were found to be antibodies against keratin, soon followed by antibodies against filaggrin. Later it was established that these antibodies were specific for citrullinated arginine. The reaction against the citrullinated part of all those proteins starts mostly with the reaction against the citrullinated part of one protein and spreads to other proteins over time. There is no standard pattern of epitope spreading, but the larger the variation in ACPA epitopes the higher the chance of RA development. Not all RA patients have autoantibodies, but in patients that are autoantibody positive, the autoantibodies are sometimes detectable years before the disease onset. However, only 40% of the autoantibody positive individuals will develop RA within 5 years. RF as well as ACPA are part of the new 2010 ACR/EULAR classification criteria for RA and are seen as risk factors for the development of RA, with a potential involvement in the pathology of RA.

Immune cells, chemokines and cytokines in Rheumatoid Arthritis
RA is a chronic inflammatory autoimmune disease and many immune cells play a role in the pathogenesis of RA. The most obvious sign of RA is the swelling of the joints. In the joints there is an infiltration of lymphocytes (B, T and NK-cells), macrophages, mast cells, dendritic cells, neutrophils and fibroblast like synoviocytes (FLS) into the synovium. In RA synovium many chemotactants are found including CCL2 (MCP-1), CCL3 (MIP-1α), CCL4 (MIP-1β), CCL5 (Rantes), CXCL1 (GRO-α), CXCL5 (ENA-78) and CXCL8 (interleukin-8). Chemotactants involved in neutrophil recruitment are CXCL8, CXCL1 and CXCL5. Interestingly, CXCL5 is also described as a monocyte attractant if it is citrullinated. T cell activation and monocyte recruitment, are among others, stimulated by CCL2, CCL3, CCL4 and CCL5.

The most important pro-inflammatory cytokines that are involved in RA are tumor necrosis factor-α (TNF-α), interleukin (IL)-1, IL-6 and IL-17A. TNF-α and IL-17A are mostly produced by monocytes and FLS and are implicated in the destruction of cartilage and bone resorption. TNF-α and IL-17A also stimulate the production of CXCL8 and IL-6. The latter is a well-known osteoclast activator, stimulating bone absorption and thereby bone destruction. Another important group of cytokines that are involved in RA are the type I and II interferons (IFN). The type I IFN consists of 16 subtypes and the most relevant ones are IFN-α and IFN-β. Type I IFN is involved in defence against viruses and has several regulatory functions. The type I IFN binds to the IFN-α/β receptor IFNAR1/2 heterodimer and signals through the JAK-STAT pathway.
pathway to the IFN-stimulated response element (ISRE), transcribing multiple IFN response genes. This can lead to DC activation and maturation and antigen presentation, it affects cell cycle, apoptosis and chemotactic ability. The antiviral activity is mediated by stimulating, among others, cytolytic activity of CD8+ T cells and NK cells. IFN-gamma is the type II IFN, which has a direct anti-viral effect by stopping the replication of viruses. Furthermore, type II IFN stimulates NK and CD56+ T cells in the innate immune response and increases MHC class II antigen presentation.

**Treatment**

For the treatment of RA there are several options like non-steroidal anti-inflammatory drugs (NSAIDs), conventional DMARDs and biological DMARDs, which are divided among others, in anti-TNF agents, anti-IL-6, anti-CTLA4 and B cell depleting agents. The most common NSAIDs are aspirin, ibuprofen and naproxen. Some examples of conventional DMARDs are Methotrexate (MTX) and Sulfasalazine. For the biological DMARDs there are Infliximab, Adalimumab and Etanercept belonging to the anti-TNF agents, Tocilizumab is an IL6 agents, Abatacept an anti CTLA4 and for the B cell depletion Rituximab.

Most often treatment in RA is started as soon as a clinical diagnosis of RA is made. Treatment schemes differ slightly between EULAR and ACR recommendations, but both start with conventional DMARD monotherapy like MTX or with contraindication for MTX start with monotherapy of Leflunomide or Sulfasalazine. Patients with a high disease activity can also start with combination therapy of conventional DMARDS like MTX with Sulfasalazine. After a disease duration of more than 6 months the patients are considered to have established RA. Treatment of established RA is based on disease activity and poor or good prognostic factors, such as autoantibody status. If a patient is not responding to a therapy the rheumatologist can switch to a combination of conventional DMARDs or a biological DMARD, often starting with anti-TNF followed, if needed, by IL-6 blockade, costimulation blockade or B cell depletion treatment.

**Predicting Rheumatoid Arthritis development**

To be able to study treatment at an earlier stage of RA development it is important to identify which individuals will develop RA. Some steps have already been made to investigate arthralgia patients and to identify (bio)markers which can discriminate between arthralgia patients that develop arthritis (converters) and arthralgia patients that do not develop arthritis (non-converters). A prediction model built on 9 clinical parameters (family members with RA, alcohol use, duration of symptoms, morning stiffness, VAS score, history of swollen joints and autoantibody status) divides arthralgia patients into three groups with low risk, intermediate risk and high risk of developing arthritis. Although the high risk group has a significantly higher chance of developing arthritis, the uncertainty for the intermediate
Introduction

The aim of this thesis was to profile seropositive arthralgia patients to discover biomarkers to predict arthritis development. Therefore, a prospective cohort of seropositive arthralgia patients was studied for the development of arthritis.

In chapter 2 the type I IFN signature is described to be predictive for arthritis development in a seropositive arthralgia cohort as well as in a preclinical RA cohort of blood bank donors who developed RA. To further increase the predictive value of the type I IFN signature for the development of arthritis a B cell signature is added to the type I IFN signature in a seropositive arthralgia cohort as described in chapter 3. In chapter 4 the longitudinal expression of the type I IFN signature is described, beginning at the arthralgia stage into early arthritis. This is followed by reporting in chapter 5 that the type I IFN signature is mainly produced by granulocytes as measured in a cohort of early arthritis patients. Chapter 6 reports the changes in peripheral blood lymphocytes during arthritis development in arthralgia patients. In chapter 7 the biomarkers described in chapter 2 and 3 (type I IFN and B cell signatures) are combined with the presence of anti-carbamylated protein antibodies and a clinical prediction rule to describe the best prediction of arthritis development in arthralgia patients. The results of this thesis are summarized and discussed in chapter 8.
Chapter 1

References


Introduction


23. Elshazli R, Settin A. Association of PTPN22 rs2476601 and STAT4 rs7574865 polymorphisms with rheumatoid arthritis: A meta-analysis update. *Immunobiology.* 2015;


35. Van De Stadt LA, De Koning MHMT, Van De Stadt RJ, et al. Development of the anti-


The type I IFN signature as a biomarker of pre-clinical Rheumatoid Arthritis

Joyce Lübbers*
Mikael Brink*
Lotte A. van de Stadt
Saskia Vosslamber
John G. Wesseling
Dirkjan van Schaardenburg
Solbritt Rantapaa-Dahlqvist
Cornelis L. Verweij


*both authors contributed equally to this study
Abstract

Objectives:
To validate the presence and demonstrate the clinical value of the type I interferon (IFN)-signature during arthritis development.

Method:
In 115 seropositive arthralgia patients who were followed for the development of arthritis (Amsterdam Reade cohort), and 25 pre-symptomatic individuals who developed RA later and 45 population-based controls (Northern Sweden cohort) the expression levels of 7 type I IFN response genes were determined with multiplex qPCR and an IFN-score was calculated. The diagnostic performance of the IFN-score was evaluated using Cox regression and Receiver Operating Characteristics (ROC)-curve analysis.

Results:
In 44 of the 115 at risk individuals (38%) from the Amsterdam Reade cohort, arthritis developed after a median period of 8 months (IQR 5-13). Stratification of these individuals based on the IFN-score revealed that 15 out of 25 IFN^{high} individuals converted to arthritis, compared to 29 out of 90 IFN^{low} individuals (P=0.011). In the Northern Sweden cohort, the level of the IFN-score was also significantly increased in pre-symptomatic individuals who developed RA compared to population-based controls (P=0.002).

Cox regression analysis of the Amsterdam Reade cohort showed that the hazard ratio for development of arthritis was 2.38 (P=0.008) for IFN^{high} at risk individuals after correction for ACPA and RF. The ROC-curve area under the curve (AUC) for the IFN-score combined with ACPA and RF in the prediction of arthritis was 79% (P=0.0001, 95% C.I. 0.70-0.87).

Conclusion:
The results demonstrated clinical utility for the IFN-signature as a biomarker in the prediction of arthritis development.
Chapter 2

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory joint disease eventually leading to destruction of cartilage and bone. No curative treatment is currently available and prolonged treatment with disease modifying anti-rheumatic drugs (DMARDS) or biologicals is required to suppress disease activity and joint damage. Early diagnosis in combination with timely initiation of treatment was demonstrated to increase the chance of remission and to prevent irreversible joint damage. Hence, recognition of individuals at high risk of developing RA may provide a major step forward towards strategies for the very early possibly preventive treatment of RA.

Accumulating evidence suggests a role for a dysregulated immune system prior to the appearance of clinical symptoms. Accordingly, the presence of anti-citrullinated protein antibodies (ACPA) and rheumatoid factor (RF) up to 14 years before the onset of disease was demonstrated. Together with the demonstration of epitope spreading of the ACPA response, this suggests that immune tolerance has been lost many years before the disease manifests clinically. In a prospective study, it was demonstrated that 20% of ACPA positive individuals at risk and 40% of ACPA and RF positive individuals at risk developed RA within two years. These findings show that the presence of these antibodies is important but not sufficient in itself to develop RA. Therefore, efforts to identify additional biomarkers such as cytokines, chemokines and gene signatures have been made to improve the prediction of RA.

Recently, we described gene signatures relevant to the development of RA. The results suggested a major role for type I interferon (IFN) mediated immunity, as shown by the presence of an IFN-signature in blood cells in ACPA and/or RF positive individuals, who developed arthritis, independent of ACPA positivity. The type I IFN signature consists of type I IFN response genes, and was eviously shown to be also present in a subset of patients with established RA, suggesting that pathological processes in the pre-clinical phase of RA are reminiscent of those in established RA.

In the present study, we aim to study the association between type I IFN-signature and arthritis development in two independent cohorts, namely another seropositive persons at risk cohort (Amsterdam cohort) and a cohort of individuals before onset of symptoms of later diagnosed RA (Northern Sweden cohort), and demonstrate its clinical utility to predict RA development.
Materials and Methods

Study populations
The Amsterdam cohort consisted of 115 newly included seropositive arthralgia patients at risk for RA without clinical arthritis from the Jan van Bremen Research Institute | Reade, i.e. not the same patients as analysed in our previous publication. Inclusion criteria were the absence of arthritis despite joint complaints as determined by two independent investigators (LvdS and DvS), a seropositive status (ACPA and/or IgM-RF positive) and a minimal follow-up of 12 months. Exclusion criteria were: a history of arthritis ascertained by a physician, erosions on radiographs and previous use of DMARDs. Patients were followed biannually for the first year and then annually for the development of arthritis, defined as having one or more swollen joints by two independent investigators.

The Northern Sweden cohort consisted of 25 samples donated 2.9 years (IQR 2.2-5.5) before onset of symptoms of RA (referred to as pre-symptomatic individuals) and 45 population based sex and age matched controls (PBC), identified from the Medical Biobank of Northern Sweden. Twenty-three of the individuals were also sampled when they were diagnosed with early RA (1987 ACR criteria). They had a follow-up time of a median of 11 years (IQR 9-12). ACPA, RF, C-reactive protein (CRP), erythrocyte sedimentation rate (ESR) determinations were as previously described.

All patients gave written informed consent and the study was approved by the Regional Ethics Committee in both countries.

RNA isolation and gene expression profiling
Total RNA was isolated from whole blood using the PAXgene RNA system (PreAnalytix, Hombrechtikon, Switzerland) for the Amsterdam cohort and the Trizal (Invitrogen, Bleiswijk, The Netherlands) isolation method on buffy-coat cells for the Northern Sweden cohort, according to manufacturers’ instructions. RNA was purity tested and amplified as previously described. Multiplex q-PCR was performed using the 96.96 Biomark™ Dynamic Array systems (Fluidigm Corporation, San Francisco, U.S.A) at ServiceXS (Leiden, The Netherlands), according to the manufacturers’ instructions. Expression levels of target genes were log transformed and calculated relative to GAPDH.

IFN-score calculation and statistical analyses
The IFN gene set that makes up the IFN signature, consisted of the 7 strongest correlating type I IFN response genes, i.e. IFI44L, IFI6, IFIT1, MXA, OAS3, RSAD2 and EPSTI (r=0.74), which discriminated between converting and non-converting arthralgia patients in a previous study using DNA micro-array analysis. An IFN-score was calculated by averaging the relative expression of these genes (log2 based). Patients were stratified in a IFNhigh and IFINlow group.
based on a cut-off determined by ROC-curve analysis using the IFN-score for arthritis development correlating with a specificity of 85%. Statistical analyses were done with Mann Whitney U, Chi-square, Cox Regression and ROC-curve analysis using GraphPad PRISM 5.0 or SPSS 15.0. P-values <0.05 were considered to be significant.

Results

Demographic and clinical characteristics for both cohorts are shown in table 1.

Table 1: Patient characteristics from the Amsterdam Reade cohort and the Northern Sweden cohort.

<table>
<thead>
<tr>
<th></th>
<th>Amsterdam Reade cohort</th>
<th>Northern Sweden cohort</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Non-converting</td>
<td>Converting</td>
</tr>
<tr>
<td></td>
<td>individuals at risk</td>
<td>individuals at risk</td>
</tr>
<tr>
<td>Individuals nr</td>
<td>71</td>
<td>44</td>
</tr>
<tr>
<td>Female nr, (%)</td>
<td>55 (77)</td>
<td>38 (86)</td>
</tr>
<tr>
<td>Median age at sample collection in years (IQR)</td>
<td>49 (41-56)</td>
<td>46 (39-55)</td>
</tr>
<tr>
<td>ACPA positive nr, (%)</td>
<td>44 (62)</td>
<td>40 (91)</td>
</tr>
<tr>
<td>RF positive nr, (%)</td>
<td>31 (44)</td>
<td>26 (59)</td>
</tr>
<tr>
<td>ACPA and RF Positive nr, (%)</td>
<td>12 (17)</td>
<td>23 (52)</td>
</tr>
<tr>
<td>Shared epitope nr, (%)</td>
<td>30 (46)^</td>
<td>26 (67)^</td>
</tr>
<tr>
<td>ESR (IQR)</td>
<td>13.5 (5.0-20.0)</td>
<td>9.5 (5.5-19.25)^s</td>
</tr>
<tr>
<td>CRP (IQR)</td>
<td>2.00 (0.86-4.95)^s</td>
<td>2.68 (0.97-4.75)^s</td>
</tr>
<tr>
<td>NSAID use nr, (%)</td>
<td>18 (25)</td>
<td>14 (32)</td>
</tr>
<tr>
<td>DMARD use nr, (%)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Corticosteroids (≤7.5 mg) use nr, (%)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Median follow-up time in months (IQR)</td>
<td>23 (12-35)</td>
<td>20 (12-26)</td>
</tr>
<tr>
<td>Median time to conversion in months (IQR)</td>
<td>NA</td>
<td>8 (5-13)</td>
</tr>
<tr>
<td>2010 ACR/EULAR criteria nr, (RA/UA)</td>
<td>NA</td>
<td>40/4</td>
</tr>
<tr>
<td>1987 ACR criteria nr, (RA/UA)</td>
<td>NA</td>
<td>18/26</td>
</tr>
</tbody>
</table>

ACPA = anti-citrullinated protein antibodies; RF= rheumatoid factor; ESR= erythrocyte sedimentation rate, CRP= C-reactive protein; NSAID= non-steroidal anti-inflammatory drugs; RA= rheumatoid arthritis; UA= undifferentiated arthritis; ND = not determined; NA = not applicable. ^available for 34 out of 45 patients, ^available for 65 out of 71 patients, "available for 39 out of 44 patients, "available for 38 out of 44 patients, "available for 69 out of 71 patients.
Firstly, we determined the IFN-score in seropositive arthralgia patients at risk for RA from the Amsterdam Reade cohort. During clinical follow-up (median follow-up time of 23 months (IQR 12-30)) 44 at risk individuals developed arthritis, 40 (91%) RA according to the 2010 American college of rheumatology/ European league against rheumatism (ACR/EULAR) criteria and 4 (9%) undifferentiated arthritis, after a median of 8 months (IQR 5-13). Analysis of the linear IFN-scores revealed a trend for an elevated mean IFN-score in the group of converting at risk individuals compared to the non-converting at risk individuals (P=0.066) (figure 1A). When we stratified patients for a high and low IFN-score a total of 25 patients were categorized as IFN\textsuperscript{high} of whom 15 (60%) converted to arthritis compared to 29 out of 90 (32%) IFN\textsuperscript{low} patients, revealing a clear association of IFN\textsuperscript{high}-status and arthritis conversion (Chi-square, P=0.011) (figure 1B).

Figure 1: IFN-score in association with arthritis development in the Amsterdam Reade cohort (A and B) and pre-symptomatic individuals from the Northern Sweden cohort (C and D). A) In the Amsterdam Reade cohort, a trend for an elevated mean IFN-score in the group of converting at risk individuals compared to the non-converting at risk individuals was observed (P=0.066). B) Stratification of the 115 seropositive arthralgia patients of the Amsterdam Reade cohort in an IFN\textsuperscript{high} and IFN\textsuperscript{low} group showed a higher arthritis development in the IFN\textsuperscript{high} group (15 out of 25, i.e. 60%) compared to the IFN\textsuperscript{low} group (29 out of 90, i.e. 32%) (χ\textsuperscript{2}, P=0.011). C) In the Northern Sweden cohort a significant increase in IFN-score in pre-symptomatic individuals and RA patients compared to population based controls was observed (P=0.002 and P=0.001, respectively). D) Stratification of the Northern Swedish patients in an IFN\textsuperscript{high} and IFN\textsuperscript{low} group revealed that 14 out of 23 RA patients (61%) and 12 out of 25 pre-symptomatic individuals (48%) have an IFN\textsuperscript{high} status compared to 10 out of 45 population based controls (22%) (χ\textsuperscript{2}, P=0.004).
Secondly, we studied the IFN-response gene expression in pre-symptomatic RA patients from the Northern Sweden cohort. The results revealed a significant elevated IFN-score in the RA patients compared to PBC (P=0.0012) (figure 1C). We also observed that the IFN-score was significantly increased in the pre-symptomatic individuals compared with PBC (P=0.0019). Stratifying patients in an IFN<sup>high</sup> and IFN<sup>low</sup> group, revealed that an IFN<sup>high</sup> status was present in 61% (14/23) of the RA patients and 48% (12/25) of the pre-symptomatic individuals (median time before disease onset 2.9 years (IQR 2.2-5.5)) compared to 22% (10/45) in PBC (Chi-square, P=0.004) (figure 1D). The results from the two independent validation cohorts confirm the association of an increased IFN-score with at risk or pre-clinical individuals who develop RA.

In order to study the predictive value of the IFN-score for development of arthritis, we determined the hazard ratio (HR) for the IFN<sup>high</sup> versus IFN<sup>low</sup> at risk patients in relation to arthritis development in the Amsterdam Reade cohort. This analysis revealed that IFN<sup>high</sup> patients have a significantly higher risk of developing arthritis compared to IFN<sup>low</sup> patients after correction for ACPA and RF status (HR of 2.38, P=0.008, 95% C.I. 1.26-4.49) (figure 2A). Age, shared epitope, CRP, ESR and non-steroidal anti-inflammatory drugs (NSAID) had no significant association with the conversion status.

![Figure 2: Survival curves and clinical utility of 115 seropositive at risk individuals from the Amsterdam Reade cohort. A) Cox regression analysis revealed a Hazard ratio for developing arthritis of 2.376 (P=0.008, 95% C.I. 1.26-4.49) for IFN<sup>high</sup> patients after correction for ACPA and RF status. B) ROC-curve analysis showed an AUC of 0.619 (P=0.032, 95% C.I. 0.514-0.724) for ACPA and RF (dotted line), an AUC of 0.602 (P=0.066, 95% C.I. 0.491-0.714) for the IFN-score (dashed line), and an AUC of 0.785 (P=0.0001, 95% C.I. 0.699-0.872) when ACPA and RF are combined with the IFN-score (black line).](image)

Next we used the ROC-curve area under the curve (AUC) analysis in the Amsterdam Reade cohort to determine the accuracy of ACPA/RF and/or IFN-score in separating arthritis converters from non-converters. First we calculated the AUC for ACPA and RF as a predictor for arthritis development, which resulted in an AUC of 0.62 (P=0.032, 95% C.I. 0.514-0.724)
Type I IFN as a biomarker for RA development

The IFN-score by itself gave an AUC of 0.602 (P=0.066, 95% C.I. 0.491-0.714), whereas the combination of ACPA/RF and IFN-score revealed an AUC of 0.785 (P=0.0001, 95% C.I. 0.699-0.872). This result demonstrates that the combination of ACPA/RF and IFN-score reached the highest AUC and means that this combination correctly diagnoses 78.5% of randomly drawn pairs of arthralgia patients at risk for RA. Based on these data a cut-off could be chosen to predict future arthritis with a specificity of 85%, and a sensitivity of 52.3% correlating with a positive predictive value (PPV) of 65%.

Discussion

Here we support findings from a prior study suggesting that the IFN-signature genes are elevated in the blood cells of individuals at risk for RA. To this end, we used two independent validation cohorts of different nature. In the Amsterdam Reade cohort we determined the IFN-score in seropositive individuals at risk for development of RA who were monitored for the development of arthritis. The other cohort consisted of pre-symptomatic individuals from the Medical Biobank of Northern Sweden, who subsequently developed RA. In both cohorts we demonstrated a statistically significant association between high IFN-score and the risk of arthritis. Previously we have shown that arthritis development is related to high-positive ACPA or ACPA and RF double positive status. Now we could demonstrate that the contribution of a high IFN-score to the risk of arthritis development is independent of ACPA and RF. These results reveal the utility of the IFN-signature to identify individuals at high risk for progression to arthritis.

In this study the significance for the IFN-high-score in predicting the conversion to arthritis was already observed using a relatively short follow-up period (median follow-up of 23 months (IQR 12-30)). This relatively short follow-up period leaves open the possibility that future converters may still be present in the non-converter group, which may explain the finding that we did not reach significance (trend) for an association with the linear IFN-score values. Since we have the impression that the majority of conversions in the at risk cohort takes place in the first two years after inclusion, it will be of interest to study the development of the IFN-activity in relation to the time to onset of arthritis.

The increased IFN-activity represented by the high IFN-score likely reflects various underlying processes that are associated with an activated immune status. This correlates with findings of elevated concentrations of pro-inflammatory cytokines and chemokines in the pre-clinical phase of RA. However, since cytokine and chemokine biomarkers are associated with autoantibodies the IFN-score is likely to provide novel and additional clinical value. Underlying processes that may specifically be linked to the IFN-activity include a break in
tolerance, dendritic cell (DC) differentiation, stimulation of the humoral and cellular arms of the immune system and chemokine activity\textsuperscript{16-18}. Of particular interest is the capacity of continued IFN induced maturation of DCs. This may lead to the induction of co-stimulatory activity of immature DCs, leading in turn to a break in tolerance through the activation of auto-reactive B cells\textsuperscript{8}. This process may be essential to facilitate epitope spreading of the ACPA response.

**Conclusion**

Our data demonstrated that an elevated IFN-signature represents an additional risk factor to predict RA. Since multiplex qPCR technology allows easy and accurate transcript quantification in peripheral blood cells, measurement of the IFN-signature represents an ideal methodology for biomarker assessment. Hence, the IFN-signature could be useful as biomarker for the prediction of RA in at risk individuals such as seropositive arthralgia patients and first degree relatives of RA patients.

**Acknowledgements**

The Medical Biobank of Northern Sweden (head: Professor Göran Hallmans) is gratefully acknowledged for the contribution of samples.

**Funding**

This research was performed with support from the “TRACER” consortium in the framework of the Center for Translational Molecular Medicine (CTMM) (www.ctmm.nl), and the Dutch Arthritis Foundation, (grant 041-202 and grant 0801034, respectively).
Type I IFN as a biomarker for RA development

References


Chapter 3

B cell signature contributes to the prediction of RA development in arthralgia patients

Joyce Lübbers
Saskia Vossolamber
Lotte A. van de Stadt
Marian van Beers-Tas
John G. Wesseling
B. Mary E. von Blomberg
Birgit I. Witte
Hetty J. Bontkes
Dirkjan van Schaardenburg
Cornelis L. Verweij

Published as letter in:
Annals of the rheumatic Diseases 2015;74:1786-9
Abstract

Objectives:
To validate and study the predictive value of the B cell signature during arthritis development.

Methods:
In 115 seropositive arthralgia patients, followed for arthritis development, expression levels of four B cell genes were determined resulting in a B cell score. Patients were stratified into B cell$^{\text{high}}$ and B cell$^{\text{low}}$ based on Receiver Operating Characteristics (ROC)-curve analysis. The diagnostic performance of the B cell score alone or combined with the previously described IFN-score to predict arthritis development was evaluated using Cox regression and ROC-curve analysis.

Results:
Within 24 months 38% of the patients had developed arthritis. A high B cell score was protective of arthritis, 73% of B cell$^{\text{high}}$ versus 54% of B cell$^{\text{low}}$ individuals did not develop arthritis (p=0.040). Risk for arthritis development increased from Hazard ratio (HR) of 2.4, based on the previously described IFN score to HR 5.6 (95%CI 1.9-16.1) in IFN$^{\text{high}}$B cell$^{\text{low}}$ patients compared to IFN$^{\text{low}}$B cell$^{\text{high}}$ patients. Moreover, addition of the B cell score improved the ROC area under the curve value in IFN$^{\text{high}}$ patients from 0.82 (95%CI 0.66-0.98; p=0.008) to 0.90 (95%CI 0.77-1.0; p=0.001). The B cell$^{\text{low}}$ score was associated with a low percentage CD27$^+$CD19$^+$ conventional memory B cells in converting patients.

Conclusion:
Low B cell score contributes to prediction of arthritis development within two years in seropositive IFN$^{\text{high}}$ arthralgia patients and is associated with a low percentage conventional memory B cells.
Chapter 3

Introduction

Early recognition followed by treatment of rheumatoid arthritis (RA) helps to maintain joint integrity and functional capacity, suggesting it may be beneficial to intervene in arthralgia patients before RA develops. However, to justify early intervention it is important to be able to accurately select individuals with high risk of developing RA.

Anti-citrullinated protein antibodies (ACPA) and rheumatoid factor (RF) are established predictive markers, but only 20%-40% of ACPA and/or RF positive arthralgia patients develop RA within two years. A model including clinical and serological characteristics has shown to be a promising approach to predict risk of RA in arthralgia patients. Recently, we have demonstrated that the type I interferon (IFN) signature is another promising biomarker. In the seropositive arthralgia group the IFN score correctly identifies 52% of the patients that will develop arthritis within two years, at a specificity of 85%. Additional biomarkers are needed to increase the sensitivity for the prediction of arthritis development.

Our previous study suggested that a B cell related gene signature was associated with protection against arthritis development, and could aid in the prediction of arthritis development. We therefore studied the clinical value of the B cell signature for the prediction of arthritis development in an independent cohort of ACPA and/or RF positive arthralgia patients and explored the phenotypical nature of this B cell signature.

Materials and Methods

Study population
In total 115 newly included ACPA and/or RF positive arthralgia patients who were followed for arthritis development and analysed previously for the clinical utility of the IFN signature. All patients gave their informed consent and the study protocol was approved by the medical ethics committee.

RNA isolation and gene expression profiling
Whole RNA was isolated as previously described. Taqman gene expression assay for the B cell related genes CD79A, CD79B, CD19, CD20 which were previously found to be associated with protection from arthritis development and housekeeping gene GAPDH (Life Technologies, Bleiswijk, the Netherlands) were used for quantitative real-time PCR (qPCR) (7500 Fast-qPCR system, Applied Biosystems, Foster City, CA, USA). Relative quantities were calculated using standard curves for all genes and were log2 expressed relative to GAPDH. B cell score was calculated as average relative expression of the four B cell genes representing the B cell signature. IFN score was determined as previously described.
Flow cytometry analyses
Absolute number and percentage of peripheral B cell subsets were determined with flow cytometry (FACSCalibur) in a representative subgroup (21 converting and 42 non-converting patients; flow cytometry measurements were included at approximately the second half of cohort, but characteristics are similar to the whole cohort). Absolute number of B cells was measured using quantification beads (Trucount-tubes) in combination with CD45 fluorescein isothiocyanate (FITC) and CD19 allophycocyanin, according to the manufacturer’s instructions. Percentage naive (CD27- CD19+) and conventional memory B cells (CD27+ CD19+) within the lymphocyte pool were measured on fresh whole peripheral blood with CD27 FITC, CD19 phycoerythin and CD45 peridinin chlorophyll protein (all from BD Biosciences, San Jose, California, USA). Flow cytometry data were analyzed using FACSDIVA software version 6.1.3. Forward, sideward scatter and CD45<sup>bright</sup> were used to select lymphocytes.

Statistical analyses
Statistical analyses were performed in GraphPad PRISM 5.0 or IBM SPSS statistics V20 using χ² or Mann Whitney-U tests for discriminating between patient groups, cox-regression analysis to model time to arthritis development since study inclusion and receiver operating characteristics (ROC) curves to predict arthritis development within two years as described previously. Spearman’s rho correlation coefficient between CD19<sup>+</sup> B cell counts and B cell score was computed. P-values <0.05 were considered to be significant.

Results
Patient characteristics are shown in table one.
In total, 44 patients (38%) developed arthritis (defined as one or more swollen joints) within two years. Of these, four patients had undifferentiated arthritis and 40 patients fulfilled the 2010 American College of rheumatology/ European League against Rheumatism (ACR/EULAR) criteria for RA. Patients were stratified into B cell<sub>high</sub> and B cell<sub>low</sub> based on ROC-curve analysis using a cut-off that resulted in 73% specificity and 47% sensitivity (a high B cell score is associated with protection against arthritis). Seventy-three percent of B cell<sub>high</sub> versus 54% of B cell<sub>low</sub> individuals did not develop arthritis (χ², p=0.040). In a Cox-regression analysis a B cell<sub>high</sub> signature revealed protection compared to a B cell<sub>low</sub> signature (HR 0.50, 95%CI 0.26-0.98, p=0.042). Significance was lost after correction for RF and ACPA status (HR 0.63, 95%CI 0.32-1.3, p=0.19).

Combining the previously described IFN with the B cell signature correctly classified 82% (9 out of 11) of patients developing arthritis based on their IFN<sup>high</sup>B cell<sup>low</sup> profile and 81% (25 out of 31) of the patients not developing arthritis based on the IFN<sup>low</sup>B cell<sup>high</sup> profile (χ²,
p<0.001). Patients with an IFN<sup>high</sup>B cell<sup>low</sup> profile have an increased risk compared to patients with an IFN<sup>low</sup>B cell<sup>high</sup> profile (HR 7.4, 95%CI 2.6-21.1, p<0.001). This remained significant after including ACPA and RF status (HR 5.6; Figure 1 A) and was considerably higher compared to the original IFN<sup>high</sup> profile alone (HR 2.4, p=0.008). No significant association was found with age, C reactive protein (CRP) and erythrocyte sedimentation rate (ESR) in any of the analyses.

### Table 1: Patient characteristics of 115 arthralgia patients from the Amsterdam arthralgia cohort.

<table>
<thead>
<tr>
<th></th>
<th>Arthralgia patients that develop arthritis within 2 years</th>
<th>Arthralgia patients that do not develop arthritis within 2 years</th>
</tr>
</thead>
<tbody>
<tr>
<td>Individuals, nr (%)</td>
<td>44 (38)</td>
<td>71 (62)</td>
</tr>
<tr>
<td>Female, nr (%)</td>
<td>36 (86)</td>
<td>55 (77)</td>
</tr>
<tr>
<td>Median age in years (IQR)</td>
<td>46 (39-55)</td>
<td>49 (41-56)</td>
</tr>
<tr>
<td>ACPA positive, nr (%)</td>
<td>40 (91)</td>
<td>44 (61)</td>
</tr>
<tr>
<td>RF positive, nr (%)</td>
<td>26 (59)</td>
<td>31 (44)</td>
</tr>
<tr>
<td>ACPA and RF Positive, nr (%)</td>
<td>23 (52)</td>
<td>12 (17)</td>
</tr>
<tr>
<td>Shared epitope, nr (%)</td>
<td>26 (59)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>30 (42)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>CRP median (IQR)</td>
<td>2.7 (0.97-4.8)</td>
<td>2.0 (0.86-5.0)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Median follow-up in months (IQR)</td>
<td>23 (12-35)</td>
<td>20 (12-26)</td>
</tr>
<tr>
<td>Median time to conversion in months (IQR)</td>
<td>8 (5-13)</td>
<td>NA</td>
</tr>
<tr>
<td>2010 ACR/EULAR criteria, nr (RA/UA)</td>
<td>40/4</td>
<td>NA</td>
</tr>
</tbody>
</table>

ACPA = anti-citrullinated protein antibodies; RF= rheumatoid factor; CRP= C-reactive protein; RA= rheumatoid arthritis; UA= undifferentiated arthritis; ND = not determined; NA = not applicable. <sup>a</sup>available for 39 out of 44 patients, <sup>b</sup>available for 65 out of 71 patients, <sup>c</sup>available for 69 out of 71 patients. No significant difference in baseline characteristics was found between the converting and non-converting arthralgia patients.

Clinical relevance of the biomarkers was established by ROC curve analysis. For ACPA and RF the area under the curve (AUC) was 0.62, combined with the IFN score this increased to 0.79. Adding the B cell score did not improve the AUC (0.79; Figure 1B). However, a two-step analysis, in which RF and/or ACPA positive patients were first stratified in an IFN<sup>high</sup> (to predict arthritis development) and an IFN<sup>low</sup> groups (to predict not developing arthritis), revealed an AUC for ACPA and RF of 0.82 and 0.60, respectively, which increased to 0.90 and 0.73 after adding the B cell score (Figure 1C and 1D).
Figure 1: Combination of the type I IFN and B cell signatures at inclusion in arthralgia patients in relation to arthritis development. A) Cox-regression analysis revealed that patients with the IFN<sup>high</sup>B cell<sup>low</sup> profile had a significantly higher risk of developing arthritis (HR 5.6) compared to the IFN<sup>low</sup>B cell<sup>high</sup> profile, corrected for ACPA and RF status. B) Receiver Operating Characteristics (ROC) curve analysis of ACPA, RF, IFN and B cell score for 115 patients within a time-window of two years. The area under the curve (AUC) for ACPA and RF of 0.62 increased to a good AUC of 0.79 if only the IFN score is included or if both the IFN and the B cell scores are included. C) ROC curve in 25 seropositive IFN<sup>high</sup> patients revealed an AUC of 0.82 for ACPA and RF, which increased to a very good AUC of 0.90 after including the B cell score. D) ROC curve in 90 seropositive IFN<sup>low</sup> patients revealed an AUC of 0.60 for ACPA and RF and this increased to a good AUC of 0.73 after including the B cell score.

Flow cytometry analysis, in a representative subgroup (n=63) showed a positive correlation between the B cell score and the absolute B cell number (ρ=0.73, p<0.001). While the proportion of CD27<sup>−</sup>CD19<sup>+</sup> naive B cells did not differ between the groups (Figure 2A), the proportion of CD27<sup>+</sup>CD19<sup>+</sup> conventional memory B cells was significantly higher in the arthralgia patients that did not develop arthritis (p=0.014; Figure 2B).
Figure 2: B cell subsets measured by flow cytometry in 63 arthralgia patients. A) Scatter plot of the CD27−CD19+ naive B cells within the lymphocyte pool revealed no differences between arthralgia patients that do not develop arthritis and arthralgia patients that do develop arthritis. B) Scatter plot of CD27+CD19+ conventional memory B cells within the lymphocyte pool revealed that arthralgia patients that do not develop arthritis had a significantly higher percentage of conventional memory B cells than arthralgia patients that do develop arthritis.

Discussion

In the present study, we validated and extended previous findings that suggest that a B cell signature in a subset of ACPA and/or RF positive individuals at risk for RA is associated with arthritis development. We have shown that a patient with a high B cell signature had a lower risk of developing arthritis and that in combination with the previously described type I IFN signature the HR for developing arthritis increased, corrected for ACPA and RF. Furthermore, adding the B cell score to the IFNhigh profile increased the predictive value of arthritis development compared to the type I IFN signature alone, corrected for ACPA and RF. Additionally, we demonstrated that this B cell signature reflects increased presence of conventional memory B cells in whole blood of arthralgia patients that do not develop arthritis.

Our data reveals that low percentage of CD19+CD27+ conventional memory B cells, and not naive B cells, is associated with conversion towards RA. This observation is in line with recent studies that showed that within DMARD naive (very) early RA patients with less than 6 months disease duration, conventional memory B cells are decreased compared to healthy controls.

The decreased percentage of conventional memory B cells suggests that these cells play an important role in the development of arthritis. The reduced number of conventional memory B cells in the periphery may reflect extravasation of these cells to tissues such as lymphoid tissue, the lungs and/or the joints, before clinical arthritis may occur. The type I IFN system could add to this process by its chemotactic properties. Moreover, type I IFNs may play...
a role in the break of tolerance leading to expansion of auto reactive T cells that may provide help to RF and ACPA producing B cells\textsuperscript{11,12}. Hence our findings corroborate results of Sellam et al. who reported that a reduction of conventional memory B cells is inversely correlated with CCL19, CXCL12 and CXCL13 in established RA patients, pointing to redistribution of conventional memory B cells into different anatomic compartments\textsuperscript{13}.

Alternatively, the reduction of CD19$^+$CD27$^+$ conventional memory B cells seen in arthralgia patients that develop arthritis could point towards a reduction of a subset of the memory B cell, i.e. the regulatory B cells that are CD19$^+$CD27$^+$CD24$^+$ and produce IL10\textsuperscript{14,15}. A decrease of the immune suppressive cells may facilitate early disease initiation. Reduced numbers of regulatory B cells are indeed negatively correlated with disease activity in patients with (new onset) RA\textsuperscript{16,17}.

The B cell score, together with ACPA and RF, reached an excellent predictive value in the IFN\textsuperscript{high} group (AUC 0.90) corresponding with a sensitivity of 80\% and 100\% specificity. The ultimate specificity is highly important when these biomarkers will be used in decision making for early treatment of arthralgia patients who are likely to develop RA. One of the limitations is the small number of patients that is actually characterized by an IFN\textsuperscript{high}B cell\textsuperscript{low} profile (n=11) and thus a low percentage of converters will be detected with 100\% specificity in the preclinical phase of arthritis using these biomarkers. However, these biomarkers also identify a substantial number (31) IFN\textsuperscript{low}B cell\textsuperscript{high} patients, who will not develop arthritis in the next two years. In this study, subjects had a relative short follow-up period (median 23 months, IQR 12-30). Therefore, we cannot exclude the presence of arthralgia patients that develop arthritis at a later time point. Therefore, longitudinal studies are needed that focus on evaluating the stability and time-window of the IFN and B cell signatures to predict arthritis development in arthralgia patients.

**Conclusions**

In conclusion, the combination of the B cell and previously described type I IFN signature represents a clinically useful biomarker to predict arthritis development in ACPA and/or RF positive arthralgia patients. The B cell score corresponds to conventional memory B cell numbers in the periphery which might indicate that this B cell subset plays a critical role in the pre-clinical phase of arthritis.

**Acknowledgements**

S. de Ridder of the inflammatory disease profiling unit, department of Pathology, is gratefully acknowledged for contributing to flow cytometry data conversion.

The technicians of the Unit of Medical Immunology, department of Pathology, are gratefully acknowledged for their help with flow cytometry data collection.
Chapter 3

Funding
This research was performed with support from the ‘TRACER’ consortium in the framework of the Center for Translational Molecular Medicine (CTMM) (http://www.ctmm.nl)
References


Chapter 4

Longitudinal expression of type I interferon response genes in arthralgia patients

Joyce Lübbers
Marian H. van Beers-Tas
Saskia Vossalamber
Tamara de Jong
Samina Turk
Sander de Ridder
Elise Mantel
John G. Wesseling
Johannes W. Bijlsma
Dirkjan van Schaardenburg
Hetty J. Bontkes
Cornelis L. Verweij

Manuscript in preparation
Introduction

Auto-antibodies against citrullinated proteins (ACPA) and/or rheumatoid factor (RF) are often present years before the clinical onset of rheumatoid arthritis (RA)\(^1,2\). Arthralgia patients harbouring such autoantibodies are at an increased risk of developing RA\(^3\). The type I IFN signature was shown to predict the development of arthritis in seropositive arthralgia patients, where IFN\(_{\text{high}}\) arthralgia patients have an increased risk of developing arthritis within 2 years compared to IFN\(_{\text{low}}\) patients with a hazard ratio of 2.38 (95%CI 1.26-4.49)\(^4\). From a pathogenic and diagnostic view it is of interest to know whether the type I IFN response gene expression is stable over time within a patient in the early stage of the disease. We therefore studied the longitudinal expression of these genes in arthralgia patients during clinical follow-up.

Materials and Methods

The type I IFN score (average expression of IFI44L, IFI6, IFIT1, MXA, OAS3, RSAD2 and EPSTI1) was measured with multiplex qPCR as described before\(^4\) in 55 seropositive arthralgia patients during follow-up (two to four time points). Patients were designated IFN\(_{\text{high}}\) or IFN\(_{\text{low}}\) based on the cut-off that was previously determined\(^4\). All patients gave written informed consent and the regional medical ethical committee approved this study. Statistical analyses were performed with IBM SPSS statistics version 20 and GraphPad PRISM 6.

Results

Patient characteristics are shown in Table 1. Development of arthritis (≥1 swollen joint) was seen in 20 (36.4%) arthralgia patients after a median follow-up of 8.4 months (IQR 4.3-12.8). An IFN\(_{\text{high}}\) score was observed at inclusion in 11 patients (55%) who developed arthritis and in 7 patients (20%) who did not develop arthritis (Fishers exact, p=0.016). In the 18 IFN\(_{\text{high}}\) patients some fluctuation was observed. The IFN score remained high in 10 patients (56%), whereas it shifted from IFN\(_{\text{high}}\) to IFN\(_{\text{low}}\) in 7 patients (39%). One patient (5%) revealed a shift from IFN\(_{\text{high}}\) to IFN\(_{\text{low}}\) and back to IFN\(_{\text{high}}\) before conversion. The IFN\(_{\text{low}}\) score is much more stable, as 33 out of 37 patients (89%) remained IFN\(_{\text{low}}\) during follow-up. All observations were irrespective of arthritis development (Figure 1).
### Table 1: Patient characteristics

<table>
<thead>
<tr>
<th></th>
<th>Arthralgia patients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Arthritis development within 5 years</td>
</tr>
<tr>
<td>Individuals, n</td>
<td>20</td>
</tr>
<tr>
<td>Females, n (%)</td>
<td>17 (85)</td>
</tr>
<tr>
<td>Age at inclusion in years, median (IQR)</td>
<td>46.5 (41.3-54.0)</td>
</tr>
<tr>
<td>ACPA positive, n (%)</td>
<td>17 (85)</td>
</tr>
<tr>
<td>RF positive, n (%)</td>
<td>11 (55)</td>
</tr>
<tr>
<td>RF and ACPA positive, n (%)</td>
<td>8 (40)</td>
</tr>
<tr>
<td>Follow-up time in months, median (IQR)</td>
<td>8.5 (4.3-12.8)</td>
</tr>
<tr>
<td>2010 ACR/EULAR criteria for RA (at arthritis development), n (%)</td>
<td>17 (85)</td>
</tr>
</tbody>
</table>


---

**Figure 1: Longitudinal expression of type I IFN score in arthralgia patients.** The type I IFN score in 20 arthralgia patients who did develop arthritis and 35 arthralgia patients who did not develop arthritis within five years. The arthralgia patients have ≥ 2 and ≤ 4 measuring time points (depicted in months) of the type I IFN score. The dotted line indicates the cut-off (based on ROC curve analysis as previously described) to designate IFN\textsuperscript{high} and IFN\textsuperscript{low} patients.

---

**Discussion and Conclusion**

The present study is the first to describe the longitudinal expression of the type I IFN response genes in arthralgia patients at-risk for RA. The stability of the IFN signature, or the absence thereof, is important for successful incorporation in RA prediction models, as at-risk patients will be screened at varying time points before onset of clinical disease. In arthralgia patients...
the IFN$^{\text{low}}$ score remained stable over time irrespective of arthritis development. Therefore, in arthralgia patients with an IFN$^{\text{low}}$ score at inclusion, there would be no need to repeat IFN response gene expression analysis during follow-up. In arthralgia patients with an IFN$^{\text{high}}$ score (“IFN signature”) at inclusion, more fluctuation is observed during follow-up where in 40% of the cases the signature decreased to an IFN$^{\text{low}}$ score. Follow-up measurements are necessary to determine whether these patients remain IFN$^{\text{high}}$ and how this is related to their risk for arthritis development. To uncover pathological implications of the type I IFN signature in (the development of) RA, studies in larger cohorts with more analysis time points during follow-up of patients, from the at-risk phase towards established RA, are needed.

**Funding**
This research was performed with support from the consortium in the framework for Translational Molecular Medicine (CTMM) (http://www.ctmm.nl) and the Dutch Arthritis Foundation (grant LLP-20)
Chapter 4

References


Longitudinal expression of Type I IFN
Chapter 5

The type I interferon signature in leukocyte subsets from peripheral blood of early arthritis patients; a major contribution by granulocytes

Tamarah D. de Jong
Joyce Lübbers
Samina Turk
Saskia Vosslamber
Elise Mantel
Hetty J. Bontkes
Conny J. van der Laken
Johannes W. Bijlsma
Dirkjan van Schaardenburg
Cornelis L. Verweij

Arthritis Research & Therapy 2016;18:165
Abstract

Background:
The type I interferon (IFN) signature in rheumatoid arthritis (RA) has shown clinical relevance in relation to disease onset and therapy response. Identification of the cell type(s) contributing to this IFN signature could provide insight into its functional consequences. This study aimed to investigate the contribution of peripheral leukocyte subsets to the IFN signature in early arthritis.

Methods:
Blood was collected from 26 early arthritis patients and lysed directly or separated into peripheral blood mononuclear cells (PBMCs) and polymorphonuclear granulocytes (PMNs). PBMCs were sorted into CD4⁺ T cells, CD8⁺ T cells, CD19⁺ B cells and CD14⁺ monocytes by flow cytometry. mRNA expression of three IFN response genes (IRGs; RSAD2, IFI44L and MX1) and type I interferon receptors (IFNAR1 and IFNAR2) was determined in whole blood and blood cell subsets by qPCR. IRG expression was averaged to calculate an IFN score for each sample.

Results:
Patients were designated “IFN⁹h” (n=8) and “IFN⁹l” (n=18) based on the IFN score cutoff in whole peripheral blood from healthy controls. The difference in IFN score between IFN⁹h and IFN⁹l patients was remarkably large for the PMN fraction (mean 25-fold) compared to the other subsets (mean 6-9-fold), indicating that PMNs are the main inducers of IRGs. Moreover, the relative contribution of the PMN fraction to the whole blood IFN score was 3-fold higher than expected from its abundance in blood (p=0.008), whereas this was 3-6-fold lower for the other subsets (p≤0.063), implying that the PMNs are most sensitive to IFN signaling. Concordantly, IFNAR1 and IFNAR2 were upregulated compared to healthy controls selectively in patient PMNs (p≤0.0077) but not in the PBMCs.

Conclusions:
Polymorphonuclear granulocytes are the main contributors to the whole blood type I IFN signature in early arthritis patients, which seems due to increased sensitivity of these cells to type I IFN signaling. Considering the well-established role of neutrophils in the pathology of arthritis, this suggests a role of type I IFN activity in the disease as well.
Introduction

Rheumatoid arthritis (RA) manifests itself as a heterogeneous disease with a clinical spectrum ranging from mild to severe disease. This heterogeneity most likely has its origin in the multifactorial nature of the disease, whereby specific combinations of genetic risk factors together with an appropriate environmental trigger influence not only susceptibility, but also the severity, pathogenesis and therapy outcome.

Heterogeneity of RA is partly reflected at the level of gene expression. Genome-wide gene expression analysis revealed evidence for molecular differences between RA patients, in particular in the type I interferon (IFN) response gene program. Part of the RA patients display a so-called “IFN signature”, which is characterized by relatively high expression of type I IFN response genes (IRGs). Induction of these IRGs is triggered via the activation of the type I IFN receptor, IFNAR1 and IFNAR2, which dimerize and subsequently activate the JAK-STAT signalling pathway, more specifically JAK1, TYK2, STAT1 and STAT2, eventually resulting in recruitment of IRF9 and formation of the ISGF3 transcription factor complex. Although the presence of the IFN signature in RA is not found to be associated with disease parameters such as disease activity or presence of rheumatoid factor and/or anti-citrullinated protein antibodies, several studies have demonstrated that the IFN signature in RA does have potential clinical relevance.

Presence of the IFN signature was shown to be a risk factor for arthritis development in preclinical disease. In later phases of the disease, presence of an IFN signature was found to be associated with clinical response to different treatment regimens, such as rituximab and tocilizumab. Furthermore, type I IRG expression appears to be differentially regulated between responders and non-responders during treatment with rituximab and anti-TNF therapy.

Peripheral blood is an easily accessible source for biomarker identification and the studies mentioned above demonstrate that the peripheral blood from RA patients reflects pathogenic processes related to the disease. However, the peripheral blood consists of several cell types and consequently the transcriptomic profile is an accumulation of all gene expression programs that are induced in these cell types. Identification of the cell type(s) contributing to the IFN signature could provide insight into its functional consequences and potentially into personalized treatment strategies. The present study aimed to investigate the contribution of the major leukocyte subsets to the IFN signature in whole blood from early arthritis patients. Using this patient group allowed us to study the IFN signature without interference of treatment with immune-modulatory drugs that are known to affect type I IFN signaling, such as glucocorticoids or hydroxychloroquine.
Methods

Patient recruitment and blood collection
Patients (n=26) were consecutively recruited from the early arthritis cohort (EAC) within the Amsterdam Rheumatology and Immunology Center, location Reade, Amsterdam, the Netherlands. Inclusion criteria were presence of ≥ 1 arthritic joint, disease duration <6 months and no previous use of DMARDS or biologicals. The majority of patients (81%) fulfilled the 2010 American College of Rheumatology criteria for the classification of RA16. The remaining 5 patients were diagnosed with seronegative rheumatoid arthritis (n=4) and monoarthritis (n=1), according to the rheumatologist’s assessment. Healthy controls (HC, n=25) were recruited at the VU University medical center, Amsterdam. From each donor, approximately 20ml blood was collected by venipuncture into heparin tubes and a PAXgene tube (PreAnalytix, GmbH, Germany). The PAXgene tube was stored at -20°C until further processing. The heparinized blood was processed on the same day it was drawn. This study was approved by the medical ethics committee of VU university medical center and Reade, Amsterdam, the Netherlands and informed consent was obtained from all donors.

PBMC isolation and PMN isolation
PBMCs were isolated from heparinized blood by density gradient centrifugation using Lymphoprep (Axis Shield, Oslo, Norway), according to the manufacturers protocol. PBMCs were washed and a minimum of 1x10^6 PBMCs was directly lysed in 350μl RLT buffer (Qiagen Benelux BV, Venlo, The Netherlands). A minimum of 7x10^6 PBMCs was resuspended in PBS containing 1% BSA for subsequent flow cytometric cell sorting. Polymorphonuclear (PMN) leukocytes (granulocytes) were isolated from the remaining erythrocyte/PMN pellet by lysing the erythrocytes with EL buffer (Qiagen Benelux BV, Venlo, The Netherlands) as described before17,18, according to the manufacturer’s protocol. The remaining PMN-enriched pellet was washed with PBS and lysed in 350μl RLT buffer. RLT lysates were stored at -20°C until RNA isolation.

Flow cytometry and cell sorting
Absolute number and percentage of monocytes and lymphocyte subsets were determined using flow cytometry (FACS Calibur) on whole heparinized blood. Quantification beads (Trucount, BD, San Jose, USA) in combination with fluorescein isothiocyanate (FITC)-conjugated anti-CD45, phycoerythrin (PE)-conjugated anti-CD14, peridinin chlorophyll (PerCP)-conjugated anti-CD3 and allophycocyanin (APC)-conjugated anti-CD19 were used to measure absolute number of lymphocytes, monocytes, T cells and B cells, according to the manufacturer’s instructions (all from BD, San Jose, USA). For the T cell subsets anti-CD45 and anti-CD3 were taken along combined with PE-conjugated anti-CD8 and APC-conjugated anti-CD4 (all from BD, San Jose, USA).
The following antibodies were used for the cell sorting procedure (all from BD, San Jose, USA): Pacific blue-conjugated or Horizon™ V450-conjugated anti-CD3, PE-conjugated anti-CD4, FITC-conjugated anti-CD8, APC-conjugated anti-CD19, and PerCP-conjugated anti-CD14. Labeled cells were analyzed and separated using FACS Aria and FACS DIVA software 6.1.3 (Becton Dickinson, San Jose, USA). The nozzle size was 70 μm and sorting speed of 3000-5000 cells/s. For sorting purposes, a gate was set around lymphocytes and subsequent gates were set for CD3’CD4+ T helper Cells, CD3’CD8+ cytotoxic T cells and CD19+ B cells, based on positivity of the markers. Monocytes were gated based on forward and side scatter properties as well as CD14 positivity. From each population, a minimum of 3x10^5 cells was sorted and subsequently spun down at 400g for 5 minutes, lysed in 350μl RLT and stored at -20°C until RNA isolation. Sorting purity was >90% for 95/104 sorted samples. Three sorted samples, two CD19-enriched fractions and one CD14-enriched fraction were excluded due to purities below 80%.

RNA isolation and cDNA synthesis
RNA was isolated from the cell lysates and PAXgene tubes using the RNeasy Micro or Mini kit (Qiagen Benelux BV, Venlo, The Netherlands) or PAXgene RNA isolation kit (PreAnalytix, GmbH, Germany), respectively, according to the manufacturers’ protocols. In both procedures, a DNAse (Qiagen Benelux BV, Venlo, The Netherlands) step was included to remove any genomic DNA. RNA quantity and purity were determined using the Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, USA). Either 50ng (cell fractions) or 250ng (PAXgene whole blood) RNA was used for cDNA synthesis, which was performed using the Revertaid H-minus cDNA synthesis kit (Thermo Scientific, Waltham, USA), according to the manufacturer’s protocol. Two CD19-enriched samples were excluded because of low RNA yield.

qPCR and calculation of the IFN score
We determined the mRNA expression of three IFN response genes (IRGs) (IFI44L, MX1 and RSAD2) that are described to be components of the IFN signature in RA1,7,10, and thus believed to reflect the type I IFN response in peripheral blood. IRG mRNA expression was measured on cDNA by quantitative PCR (qPCR). qPCR was performed using SYBR Green (Applied Biosystems, Foster City, USA) and an ABI Prism7500HT Sequence detection system (Applied Biosystems, Foster City, USA), according to the manufacturer’s protocols. Primers were designed using Primer Express software and guidelines (Applied Biosystems, Foster City, USA) and are listed in Table S1. To calculate arbitrary values of mRNA levels and to correct for differences in primer efficiencies a standard curve was constructed. Expression levels of target genes were calculated relative to housekeeping gene GAPDH. Expression levels of the IRGs were highly correlative for all studied cell fractions (r≥0.708, p<0.0001), therefore an IFN score was calculated by averaging the expression levels of all 3 genes for each sample.
Presence of a type I IFN signature (referred to as IFN\textsuperscript{high}) was defined as an IFN score above mean + 2*SD in healthy controls. Each IRG was also analyzed individually, which yielded comparable results as described below (data not shown).

**Statistical analysis and calculation of expected and observed contributions**

All analyses were performed using Mann Whitney U tests in Graphpad Prism 5 Software. In order to study the relative contribution of each cell type to the whole blood IFN signature, we calculated an “expected” and “observed” IFN score contribution. The “expected” contribution is based only on the distribution of the cell types in the blood and assumes that each cell type would contribute equally to the whole blood IFN signature. E.g. for a whole blood sample with an IFN score of 2.5 which contained 3.3% monocytes, the expected contribution of the monocytes would be 2.5 * 0.033 = 0.0825. The “observed” contribution is the IFN score as it was measured in a sorted cell subset, corrected for the abundance of this subset in whole blood. E.g. if the sample described above had an IFN score of 3.5 in the CD14-enriched fraction, the observed IFN score contribution of the monocytes is 3.5 * 0.033 = 0.1155.

**Results**

**Patients characteristics and selection of IFN\textsuperscript{high} and IFN\textsuperscript{low} patients**

First, patients were separated into an IFN\textsuperscript{high} and IFN\textsuperscript{low} group based on their IFN score in whole blood. As displayed in Figure 1, the IFN signature was present in 8/26 patients, these will be referred to as “IFN\textsuperscript{high}”, and the remaining 18 patients were designated “IFN\textsuperscript{low}”. Patient characteristics are shown in Table 1. The IFN\textsuperscript{high} group displayed slightly shorter duration of symptoms and a higher percentage of ACPA positive patients, but this did not reach statistical significance (Symptom duration p=0.137, ACPA positivity p=0.084 (Fisher’s exact)).

**Table 1: Patient characteristics**

<table>
<thead>
<tr>
<th></th>
<th>Healthy controls</th>
<th>All patients</th>
<th>IFN\textsuperscript{low}</th>
<th>IFN\textsuperscript{high}</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>25</td>
<td>26</td>
<td>18</td>
<td>8</td>
</tr>
<tr>
<td>Female, n (%)</td>
<td>16 (64)</td>
<td>20 (77)</td>
<td>13 (72)</td>
<td>7 (88)</td>
</tr>
<tr>
<td>Age, mean (SD)</td>
<td>35 (10)</td>
<td>47 (14)</td>
<td>48 (16)</td>
<td>44 (9)</td>
</tr>
<tr>
<td>DAS28, mean (SD)</td>
<td>n/a</td>
<td>4.6 (1.2)</td>
<td>4.7 (1.3)</td>
<td>4.4 (1.0)</td>
</tr>
<tr>
<td>Duration of symptoms in weeks, mean (SD)*</td>
<td>n/a</td>
<td>16 (25)</td>
<td>20 (29)</td>
<td>8 (8)</td>
</tr>
<tr>
<td>IgM RF positivity, n (%)</td>
<td>n/a</td>
<td>19 (73)</td>
<td>13 (72)</td>
<td>6 (75)</td>
</tr>
<tr>
<td>ACPA positivity, n (%)</td>
<td>n/a</td>
<td>15 (58)</td>
<td>8 (44)</td>
<td>7 (88)</td>
</tr>
</tbody>
</table>

*Data missing for 1 patient
SD, standard deviation; IgM RF, IgM rheumatoid factor; ACPA, anti-citrullinated protein antibodies
Figure 1: Whole blood IFN scores in all 26 early arthritis patients. Eight patients were designated IFN\textsuperscript{high} based on the mean+2SD cutoff in healthy controls. Patients within the 95\% limits of healthy controls (indicated between the dashed lines) were considered IFN\textsuperscript{low}.

General abundance of cell subsets in relation to the whole blood type I IFN profile
In order to gain insight into the cell subset composition of the peripheral blood in relation to the presence of the IFN signature, we compared the number of total CD3\(^+\) T cells, CD4\(^+\) T helper cells, CD8\(^+\) cytotoxic T cells, CD19\(^+\) B cells, CD14\(^+\) monocytes, and granulocytes (PMNs) between IFN\textsuperscript{high} and IFN\textsuperscript{low} patients. As shown in Table 2, we observed a tendency towards lower numbers of all lymphocyte subsets in IFN\textsuperscript{high} patients compared to IFN\textsuperscript{low} patients, but this did not reach statistical significance (p≥0.07). Remarkably, only the number of PMNs was significantly higher in IFN\textsuperscript{high} patients compared to IFN\textsuperscript{low} patients, (1.6-fold, p=0.031). The cell percentages also displayed a slightly higher PMN percentage and lower lymphocyte percentage in IFN\textsuperscript{high} patients compared to IFN\textsuperscript{low} patients, although this was not significant (Table 2). The fold difference we observed in whole blood IFN score between IFN\textsuperscript{low} patients and IFN\textsuperscript{high} patients (12-fold) greatly exceeded the fold difference observed in PMN abundance (1.6-fold), indicating that the presence of the IFN signature in these patients is not primarily caused by predominance of a particular cell subset.
Table 2: Abundance of leukocytes and subsets in patients whole blood.

<table>
<thead>
<tr>
<th></th>
<th>Based on</th>
<th>All patients</th>
<th>IFN&lt;sup&gt;low&lt;/sup&gt;</th>
<th>IFN&lt;sup&gt;high&lt;/sup&gt;</th>
<th>Fold difference between means</th>
<th>Comparison of means, p value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total leukocytes</strong></td>
<td>CD45+</td>
<td>7191 ± 2626</td>
<td>7271 ± 2981</td>
<td>7020 ± 1814</td>
<td>1.04 (low &gt; high)</td>
<td>0.798</td>
</tr>
<tr>
<td><strong>Lymphocytes</strong></td>
<td>CD45+, FSC/SSC</td>
<td>1856 ± 481 (27.8% ± 9.7)</td>
<td>1955 ± 478 (29.7% ± 10.6)</td>
<td>1646 ± 442 (24.0% ± 6.1)</td>
<td>1.19 (low &gt; high)</td>
<td>0.194</td>
</tr>
<tr>
<td><strong>T cells</strong></td>
<td>CD3+</td>
<td>1349 ± 365 (20.2% ± 7.2)</td>
<td>1441 ± 363 (21.8% ± 7.8)</td>
<td>1153 ± 304 (16.9% ± 4.6)</td>
<td>1.25 (low &gt; high)</td>
<td>0.066</td>
</tr>
<tr>
<td><strong>Helper T cells</strong></td>
<td>CD3+, CD4+</td>
<td>850 ± 228 (13.1% ± 5.0)</td>
<td>899 ± 214 (13.8% ± 5.3)</td>
<td>729 ± 229 (11.6% ± 4.1)</td>
<td>1.23 (low &gt; high)</td>
<td>0.130</td>
</tr>
<tr>
<td><strong>Cytotoxic T cells</strong></td>
<td>CD3+, CD8+</td>
<td>465 ± 232 (6.9% ± 3.4)</td>
<td>514 ± 245 (7.6% ± 3.7)</td>
<td>345 ± 148 (5.2% ± 1.9)</td>
<td>1.49 (low &gt; high)</td>
<td>0.187</td>
</tr>
<tr>
<td><strong>B cells</strong></td>
<td>CD19+</td>
<td>268 ± 125 (4.0% ± 1.9)</td>
<td>284 ± 140 (4.2% ± 2.1)</td>
<td>234 ± 81 (3.5% ± 1.3)</td>
<td>1.21 (low &gt; high)</td>
<td>0.549</td>
</tr>
<tr>
<td><strong>Monocytes</strong></td>
<td>CD14+</td>
<td>336 ± 127 (4.8% ± 1.5)</td>
<td>353 ± 140 (5.0% ± 1.4)</td>
<td>299 ± 92 (4.4% ± 1.5)</td>
<td>1.18 (low &gt; high)</td>
<td>0.406</td>
</tr>
<tr>
<td><strong>PMNs</strong></td>
<td>FSC/SSC</td>
<td>3757 ± 2715 (67.4% ± 10.1)</td>
<td>3137 ± 2945 (65.3% ± 11.0)</td>
<td>5075 ± 1598 (71.6% ± 6.7)</td>
<td>1.61 (low &lt; high)</td>
<td>0.031</td>
</tr>
</tbody>
</table>

FSC, forward scatter; SSC, side scatter. Cell amounts are indicated in numbers per μl, mean ± standard deviation. Percentages of total leukocytes are indicated between brackets, mean ± standard deviation.

Contribution of sorted cell subsets to the IFN score

Next, we compared the contribution of individual leukocyte subsets to the IFN signature. As shown in Figure 2, IFN scores were significantly different between IFN<sup>high</sup> and IFN<sup>low</sup> patients for all cell subsets, which is to be expected as all cell types presumably possess type I IFN signaling ability. The difference between IFN<sup>high</sup> and IFN<sup>low</sup> patients was most prominent for the PMN fraction, which displayed a 25-fold higher mean IFN score in IFN<sup>high</sup> patients compared to IFN<sup>low</sup> patients (p<0.0001, Figure 2). These measurements are normalized on RNA input and the expression levels are relative to the housekeeping gene GAPDH, hence these data are irrespective of cell abundance. In order to investigate the relative contribution of the leukocyte subsets in relation to their distribution in peripheral blood, we used the expression data in whole blood from IFN<sup>high</sup> patients and the relative abundance of each subset to estimate an “expected” cell subset contributions, assuming that each subset would contribute equally to the IFN score. Subsequently, we compared the estimated cell subset contributions to the actual contributions as measured in the sorted cell subsets, corrected for its abundance (“observed” contribution). Details about the calculation of the expected and observed contributions are described in the methods section.

As shown in Table 3, all cell types showed a difference between the observed contributions and the expected contributions to the IFN score. The observed contributions of CD4<sup>+</sup> Helper
T cells, CD8⁺ cytotoxic T cells, CD19⁺ B cells and CD14⁺ monocytes were 2.8-6.3-fold lower than the expected contributions, which was significant for most subsets (p≤0.0625). Remarkably, the observed contribution of the PMNs was 3.4-fold higher than its expected contribution (p=0.0078). This tendency remained present after correction for any differences in RNA yield between subsets (data not shown). The sum of the RNA-corrected observed contributions of all cell subsets per patient was somewhat higher than the total IFN score as measured in whole blood (mean difference 1.2-fold, not significantly different). This could be explained by slight impurities in each isolated subset, and implies that there is no other cell population substantially contributing to the whole blood IFN score, as this would have resulted in a lower sum compared to the whole blood IFN score. Altogether, these data indicate that PMNs are the main contributors to the whole blood IFN score, not only because of its high abundance in whole blood, but also because of an increased potency to induce IRGs.

Figure 2: IFN scores per leukocyte subset of IFN<sub>low</sub> and IFN<sub>high</sub> patients. Fold differences between the two groups, as well as p values of the statistical comparisons, are indicated below the graph.
Table 3: Expected and observed contributions of leukocyte subsets to the IFN score in whole blood of IFN<sup>high</sup> patients.

<table>
<thead>
<tr>
<th>Subset</th>
<th>Expected</th>
<th>Observed</th>
<th>Direction</th>
<th>Mean fold difference</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4 0.184 ± 1.715</td>
<td>0.521 ± 0.507</td>
<td>Exp &gt; Obs</td>
<td>3.94 ± 1.71</td>
<td>0.0156</td>
<td></td>
</tr>
<tr>
<td>CD8 0.341 ± 0.504</td>
<td>0.156 ± 0.127</td>
<td>Exp &gt; Obs</td>
<td>5.36 ± 2.90</td>
<td>0.0223</td>
<td></td>
</tr>
<tr>
<td>CD19 0.530 ± 0.499</td>
<td>0.298 ± 0.263</td>
<td>Exp &gt; Obs</td>
<td>2.78 ± 0.68</td>
<td>0.0625</td>
<td></td>
</tr>
<tr>
<td>CD14 0.679 ± 0.594</td>
<td>0.126 ± 0.110</td>
<td>Exp &gt; Obs</td>
<td>6.25 ± 2.53</td>
<td>0.0156</td>
<td></td>
</tr>
<tr>
<td>PMNs 11.71 ± 11.53</td>
<td>48.33 ± 65.89</td>
<td>Exp &lt; Obs</td>
<td>3.35 ± 1.29</td>
<td>0.0078</td>
<td></td>
</tr>
</tbody>
</table>

Relation between IFN score and type I IFN receptor expression in subsets and whole blood

The data described above suggests an increased sensitivity of IFN<sup>high</sup> PMNs to type I IFNs. To gain more insight into the mechanism behind this increased sensitivity, we measured the mRNA expression of the upstream receptors of type I IFN signaling, i.e. IFNAR1 and IFNAR2. Although we observed a correlation between the subset’s IFN score and IFNAR1 expression for all subsets, the correlation between the subset’s IFN score and IFNAR2 expression was only significant for the PMN fraction (Spearman r: 0.461, p=0.020, Table 4). Furthermore, both IFNAR1 and IFNAR2 expression was highest in the PMN fraction compared to the other fractions, indicating that PMNs could be more sensitive to type I IFN binding.

Table 4: IFNAR1 and IFNAR2 expression and the relation to the subset IFN scores

<table>
<thead>
<tr>
<th>IFNAR1</th>
<th>Average expression</th>
<th>Correlation with subset's IFN score</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4 2.473 ± 1.157</td>
<td>3.440 ± 1.309</td>
<td>Spearman r p value</td>
</tr>
<tr>
<td>CD8 1.723 ± 0.701</td>
<td>1.297 ± 0.730</td>
<td>0.363 0.069</td>
</tr>
<tr>
<td>CD19 3.687 ± 1.756</td>
<td>3.844 ± 1.487</td>
<td>0.525 0.012</td>
</tr>
<tr>
<td>CD14 0.687 ± 0.294</td>
<td>0.908 ± 0.354</td>
<td>0.371 0.068</td>
</tr>
<tr>
<td>PMN 5.089 ± 2.243</td>
<td>6.529 ± 1.649</td>
<td>0.461 0.020</td>
</tr>
<tr>
<td>IFNAR2</td>
<td>Average expression</td>
<td>Correlation with subset's IFN score</td>
</tr>
<tr>
<td>CD4 4.544 ± 3.247</td>
<td>3.531 ± 1.234</td>
<td>Spearman r p value</td>
</tr>
<tr>
<td>CD8 3.952 ± 2.736</td>
<td>2.969 ± 0.994</td>
<td>0.179 0.382</td>
</tr>
<tr>
<td>CD19 4.213 ± 2.389</td>
<td>2.978 ± 0.908</td>
<td>-0.03 0.857</td>
</tr>
<tr>
<td>CD14 0.946 ± 0.562</td>
<td>0.748 ± 0.304</td>
<td>0.042 0.842</td>
</tr>
<tr>
<td>PMN 5.749 ± 5.039</td>
<td>6.231 ± 3.340</td>
<td>0.507 0.010</td>
</tr>
</tbody>
</table>

Specific upregulation of type I IFN receptors in early arthritis PMNs

Since the PMN fraction showed high activation of the IFN response that appeared to be related to expression of the type I IFN receptors, we compared IFNAR1 and IFNAR2 mRNA expression in isolated PBMCs and PMNs of patients to those of healthy controls. As shown in Figure 3, we observed no differences in expression of IFNAR1 or IFNAR2 in the PBMC fractions of patients compared to healthy control PBMCs (p=0.387 and p=0.902, respectively).
However, both IFNAR1 and IFNAR2 expression were considerably increased in the PMN fraction of patients compared to healthy control PMNs (IFNAR1 3.0-fold, p<0.001, IFNAR2 2.5-fold, p=0.008). Only IFNAR1 expression was significantly different between IFN$^{\text{low}}$ and IFN$^{\text{high}}$ patients (p=0.021, See Figure 3A), implying that the extent of the IFN signature might not solely depend on IFNAR expression.

**Figure 3:** Selective upregulation of IFNAR expression in PMNs from early arthritis patients. IFNAR1 and IFNAR2 expression in PBMCs and PMNs from healthy controls and early arthritis patients. ** p 0.01 - 0.001; *** p≤0.001.
Discussion

The type I IFN signature in peripheral blood from RA patients was first described in 2007 [1] and since then, it has been extensively studied in relation to disease onset and therapy response. Occasionally, IFN response gene (IRG) expression in RA was assessed in isolated cell subsets instead of whole blood, such as PBMCs [6,19], monocytes [20] or neutrophils [21]. The present study is the first to demonstrate that there is diversity in the contribution of whole blood cell subsets to the extent of the type I IFN response, with a major contribution by PMNs.

Patients with an IFN signature (IFNhigh) did not appear clinically different from patients without this signature (IFNlow). Although our cohort is rather small, this corroborates previous studies [1,3]. We observed slightly lower lymphocyte counts and slightly higher neutrophil counts in IFNhigh patients compared to IFNlow patients, but these differences were too small to fully explain the difference in whole blood IFN score between the two groups. Concordantly, our data suggest that the whole blood IFN signature is facilitated by a selective change in PMN sensitivity to type I IFN signaling rather than by a great difference in cell abundance.

The PMN fraction primarily consists of neutrophils, which have been shown to play a role in RA. They are the first cells to enter the joint when the disease starts and are the most abundant cell type present in the joint [22,23]. Neutrophils in the RA joint display a “primed” phenotype compared to control neutrophils, resulting in increased cytokine and chemokine production, decreased apoptosis rates [24], the gained ability to present antigens [25] and upregulation of chemokine receptors to induce migration of other immune cells [26].

We observed that patient PMNs, but not the PBMCs, displayed type I IFN receptor (IFNAR1 and IFNAR2) upregulation compared to healthy controls, which was not completely dependent on the presence of the IFN signature. It has been suggested that RA neutrophils would mainly become primed and activated within the inflamed joint due to the large amount of cytokines present. However, the IFNAR1 and IFNAR2 upregulation in the circulating PMNs suggests that these cells could also have gained a primed phenotype. Wright and colleagues have described the gene profiles that are induced upon neutrophil priming with TNFα or GM-CSF, which did not involve upregulation of IFNAR1, and even seemed to cause downregulation of IFNAR2 [27]. Broader gene expression and protein expression studies on RA PMNs, possibly paired with synovial PMNs are required to gain more insight into the exact gene profile and source of the priming.

It was demonstrated that healthy mature neutrophils already display increased expression of IFNAR1 and IFNAR2 as well as type I IFN response genes compared to immature neutrophils [28]. Of interest, these mature neutrophils were more prone to IFNa-mediated induction of neutrophil extracellular trap (NET) formation than immature neutrophils. NETs are extracellular structures that consist of chromatin and neutrophil-related proteins and are released by neutrophils under (auto-)inflammatory conditions. Neutrophils from RA blood
and synovial fluid are shown to exhibit increased spontaneous NET formation compared to neutrophils from healthy controls or osteoarthritis patients. A study in SLE patients demonstrated that NETs contain a considerable source of type I IFN-inducing agents. Altogether, the upregulation of IFNAR1 and IFNAR2 we observed in RA PMNs, together with the increased spontaneous NET formation, could contribute to a positive feedback loop of subsequent NET-mediated type I IFN production, type I IFN binding and simultaneous IRG induction and more NET formation.

It has recently been demonstrated that the baseline IFN signature in RA PMNs is associated with a good response to anti-TNF therapy. Notably, earlier studies using gene expression profiling in whole blood only described a relation between IFN response regulation and therapy response during anti-TNF treatment and not between the extent of the IFN response and anti-TNF response prior to the start of therapy. Although the studies describe different types of anti-TNF treatment and the PMN findings need validation in independent studies, one could speculate that the PMN fraction is a more homogeneous source than whole blood to study the IFN signature in relation to anti-TNF response. Moreover, neutrophils are known to both bind and secrete TNFα, and multiple studies have demonstrated that TNFα and type I IFNs might influence each other’s signaling activities. Consequently, the IFN signature in PMNs might be a direct reflection of high TNFα activity and therefore indicate increased sensitivity to TNFα inhibition, ultimately resulting in a good response to therapy.

Presence of a baseline IFN signature is also shown to be associated with a poor response to rituximab treatment. The exact mechanism behind this association remains to be elucidated, but it could indicate that patients with an IFN signature have a neutrophil-dominated pathology; hence B cell depletion would have less effect on the disease activity than in IFNlow patients. Recently, it has been shown that rituximab treatment could lead to late-onset neutropenia in a small proportion of patients. It would be interesting to study this in relation to the previously reported association of rituximab-related pharmacodynamics of type I IFN response gene expression and clinical response to rituximab.

Considering these previously described findings regarding the IFN signature in relation to therapy response, we hypothesize that patients with an IFN signature in the neutrophils might benefit from therapies that target the activity of neutrophil-derived cytokines, such as anti-TNF therapy or tocilizumab therapy, whereas patients without an IFN signature might benefit from rituximab therapy instead. However, more studies on the exact role of the IFN signature in neutrophil-related RA pathology are required to support this hypothesis.

Since the PMNs are considered one of the first cell types to enter the joint and the presence of an IFN signature has been associated with an increased risk to develop arthritis, this could indicate that the neutrophils have been primed and activated to migrate towards the joint in order to inflict the first damage. Moreover, it could suggest that patients without an IFN signature that develop arthritis might have another mechanism behind the disease
onset, e.g. mediated by B cell migration\textsuperscript{4,36–38}. Extending the present study to the preclinical phase of arthritis could give more insight into the role of the IFN signature and neutrophils in disease onset.

Conclusions
Conclusively, we have demonstrated that PMNs are the main contributors to the whole blood IFN signature in early arthritis patients. Considering the well-established role of neutrophils in the pathology of arthritis, this suggests a role of type I IFN activity in the disease as well.

Funding
This research was performed with support from the Dutch Arthritis Foundation (project numbers 13-2-307 and LLP-20).
### Supplementary material

Table S1: Primers used for quantitative PCR.

<table>
<thead>
<tr>
<th>Gene of interest</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>GCCAGCCCCAGCCACATC</td>
<td>TGACCAGGCGCCCAATAC</td>
</tr>
<tr>
<td>RSAD2</td>
<td>GTGGTTCCAGAATTATGGTGATATT</td>
<td>CCACGCGCAATAAGGACATT</td>
</tr>
<tr>
<td>IFI44L</td>
<td>CCGACCGGTAGATATATTGT</td>
<td>TGCTCCCTCTGCCCATCTA</td>
</tr>
<tr>
<td>MX1</td>
<td>TTCAGCACTGTGGGCTATC</td>
<td>GTACGTCTGGAGCATAAGAAGCTG</td>
</tr>
<tr>
<td>IFNAR1</td>
<td>AAAATTGTCTGGGTGCAGAATATTACTAG</td>
<td>ACCAATCTGACCTTGAGAAAA</td>
</tr>
<tr>
<td>IFNAR2</td>
<td>TGTATACATCATGAGTAACCAGAGATT</td>
<td>TGTGTCTCCGCTGAATCCT</td>
</tr>
</tbody>
</table>
References


Chapter 6

Changes in peripheral blood lymphocyte subsets during arthritis development in arthralgia patients

Joyce Lübbers
Marian H. van Beers-Tas
Saskia Vosslander
Samina A. Turk
Sander de Ridder
Elise Mantel
John G. Wesseling
Martine Reijm
Ingrid M. van Hoogstraten
Johannes W. Bijlsma
Dirkjan van Schaardenburg
Hetty J. Bontkes
Cornelis L. Verweij

Arthritis Research & Therapy 2016;18:205
Abstract

Introduction:
Multiple lymphocyte subsets like T and B cells have been connected to joint infiltration and inflammation in rheumatoid arthritis (RA). Identification of leucocyte subsets that are dysregulated in arthritis development could provide insight in the aetiology of RA. This study aimed to investigate the composition of the peripheral blood components, i.e. CD14+ monocytes, CD4+ and CD8+ T lymphocytes (CD3+), CD80+, C-X-C chemokine receptor 3 (CXCR3)+ and CD27+ B lymphocytes (CD19+), CD16+CD56+CD3- NK cells and activated CD56+CD3+ T cells for their association with arthritis development in arthralgia patients.

Materials & Methods:
Peripheral blood was collected from 89 early RA patients (disease duration <6 months), 37 healthy controls (HC) and 113 arthralgia patients (22 developed arthritis ≤ 1 year, 18 developed arthritis >1 year and 73 did not develop arthritis). Absolute numbers of monocytes and lymphocyte subsets in whole heparinized blood was determined with flow cytometry using quantification beads in combination with fluorescent labelled antibodies for T cells, B cells, monocytes, NK cells and activated T cells.

Results:
In early RA patients a significant decrease in the number of (activated) T cells, CD80+ and memory B cells and a trend towards a lower numbers of CD8+ T cells was observed compared to HC. Similar differences were seen in arthralgia patients that developed arthritis compared to arthralgia patients that did not develop arthritis (non-converters), with significantly decreased CD8+ T cells and memory B cells. Arthralgia patients who developed arthritis were split in a group developing arthritis within 1 year (early converters) and after 1 year (late converters). The late converters showed a significant decreased number of CD8+ T cells compared to non-converters. Whereas in the early converters a decreased number of memory B cells was observed. Longitudinal analysis of converting patients showed a significant relative increase of CD80+ B cells towards conversion time point compared to 24 months prior to conversion.

Conclusion:
This study revealed that arthralgia patients that develop arthritis demonstrate a change in cellular immune parameters apparent in the periphery, starting with a decrease of cytotoxic T cells 24 months prior to arthritis development, followed by a decrease in the number of memory B cells 12 months prior to disease onset.
Introduction

Rheumatoid Arthritis (RA) is an autoimmune disease affecting the joints. Inflammation of the joints leads to destruction of bone and cartilage in the joint. Although the aetiology of the disease is still unknown it is likely that inadvertent immune activation is the basis for the development of RA. Understanding the immune mechanism and components that lead to chronic joint inflammation is of utmost importance to allow timely identification and treatment in order to prevent arthritis development and concomitant joint destruction.

Among these immune components are autoantibodies against citrullinated peptides (APCA) and rheumatoid factor (RF), which are seen years before the clinical manifestation of RA. More recently we have identified the presence of the type I interferon (IFN) signature in peripheral blood cells of 52% of patients with arthralgia who developed arthritis within 2 years. Moreover, we provided evidence that a decreased B cell count, corresponding to lower memory B cell numbers, in the periphery is associated with arthritis development.

In this study we analysed the peripheral blood components, i.e. CD14+ monocytes, CD4+, CD8+ and CD56+ T lymphocytes (CD3+), CD80+, C-X-C chemokine receptor 3 (CXCR3)+, CD27+ B lymphocytes (CD19+) and CD16+CD56−CD3− NK cells, for their association with arthritis development. We therefore, studied individuals that were seropositive and had joint complaints without swelling of the joints; so called arthralgia patients. These patients were followed until the development of arthritis, defined as having at least one swollen joint. Furthermore, we investigated the variability of the frequency of the different cell subsets over the course of arthritis development in these patients.

Materials and Methods

Study population

The Amsterdam Reade cohort used for this study consisted of 113 arthralgia patients of whom 22 developed arthritis within 1 year (converters ≤12 months) and 18 develop arthritis after 1 year (converters >12 months). Inclusion criteria for the arthralgia patients were positivity for ACPA and/or RF, joint complaints without clinical arthritis determined by two independent rheumatologists and a minimum follow-up period of 24 months. Exclusion criteria for the arthralgia patients were the (previous) use of disease modifying anti-rheumatic drugs (DMARDs), a history of arthritis and erosions on radiographs. Arthritis was defined as having one or more swollen joints as assessed by two independent rheumatologists. Furthermore, 37 healthy controls (HC) and 89 early RA patients were included at Reade. Inclusion criteria for the early RA patients were: disease duration <6 months and no previous use of DMARDs or biologicals. See Table 1 for demographic and clinical characteristics of all study groups.
Flow cytometry analysis
Absolute numbers and percentage of monocytes and lymphocyte subsets were determined with flow cytometry (FACS Calibur) on whole heparinized blood. Quantification beads (Trucount, BD) in combination with CD45 fluorescein isothiocyanate (FITC), CD14 phycoerythrin (PE), CD3 peridinin chlorophyll protein (PerCP) and CD19 allophycocyanin (APC) were used to measure absolute numbers of lymphocytes, monocytes, B cells and T cells according to the manufacturer’s instructions. Absolute numbers of cell subsets were calculated using the percentage of cells within a main cell type that was measured with Quantification beads. For the subsets CD45 and CD3 or CD19 were always taken along in multiple tubes combined with the following markers: CD8 PE, CD4 APC, CD16/56 PE, CXCR3 APC, CD80 PE, CD27 FITC (all products from BD Biosciences, San Jose, California, USA). Flow cytometry data were analysed using FACSDIVA software version 6.1.3. Forward, sideward scatter and CD45^bright were used to select lymphocytes. CD16^+CD56^+CD3^- cells were defined as NK cells and CD16/CD56^+CD3^+ cells are expected to be predominantly CD56^+CD16-. Since CD56^+CD3^- T cells, also described as NKT-like cells, have been described as activated effector cells, we define them here as activated T cells. Gating strategy is depicted in supplementary figure 1.

Statistical analyses
GraphPad Prism 5.0 was used for statistical analysis. Mann-Whitney U or Kruskal-Wallis test followed by Dunn’s multiple comparison test were used to compare the different parameters between patient groups. For the ratios, showing the timeframe from conversion time point and 12 or 24 months prior to conversion, log2 values were used. One sample T-test or Wilcoxon Signed Rank test was used to test if the median was significantly different from 0, which would indicate an increase or decrease from conversion time point. P-values <0.05 were considered to be significant.


Table 1: Demographic characteristics of healthy controls, arthralgia patients and early RA patients.

<table>
<thead>
<tr>
<th></th>
<th>Healthy controls (HC)</th>
<th>Arthralgia patients</th>
<th>Early RA patients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>All arthralgia patients</td>
<td>Arthritis development &lt;1 year (early converters)</td>
<td>Arthritis development &gt;1 year (late converters)</td>
</tr>
<tr>
<td>Individuals (n)</td>
<td>37</td>
<td>113</td>
<td>22</td>
</tr>
<tr>
<td>Median age at inclusion in years (IQR)</td>
<td>30 (27-41)</td>
<td>50 (41-57)</td>
<td>55 (32-65)</td>
</tr>
<tr>
<td>Female (n), (%)</td>
<td>16 (64)</td>
<td>86 (76)</td>
<td>17 (77)</td>
</tr>
<tr>
<td>ACPA positive (n), (%)</td>
<td>N.D.</td>
<td>69 (61)</td>
<td>18 (82)</td>
</tr>
<tr>
<td>RF positive (n), (%)</td>
<td>N.D.</td>
<td>50 (44)</td>
<td>12 (55)</td>
</tr>
<tr>
<td>ACPA and RF positive (n), (%)</td>
<td>N.D.</td>
<td>29 (26)</td>
<td>11 (50)</td>
</tr>
<tr>
<td>Median follow-up time in months (IQR)</td>
<td>N.A.</td>
<td>26.9 (19.1-42.4)</td>
<td>8.1 (1.3-12.3)</td>
</tr>
<tr>
<td>Median time to arthritis development in months (IQR)</td>
<td>N.A.</td>
<td>12.48 (7.4-22.1)</td>
<td>8.1 (1.3-12.3)</td>
</tr>
</tbody>
</table>

N.A. = not applicable, N.D. = not determined, *= available for 87 out of 89 patients
ACPA: anti citrullinated protein antibodies, RF: rheumatoid factor, IQR: interquartile range

Results

In order to gain insight in the immune differences between HC and early RA patients, the number of circulating monocytes, lymphocytes, NK cells, activated T cells, B cells, T cells and B and T cell subsets were assessed by flow cytometry. This revealed that early RA patients had a significantly overall lower number of circulating CD3+ T cells, CD3+CD56+CD16+ activated T cells, conventional memory (CD27+) B cells, and activated (CD80+) B cells compared to HC (figure 1). A similar trend was visible for the number of CD8+ cytotoxic T cells. The number of monocytes (data not shown), overall lymphocytes (data not shown), CD4+ Thelper cells, B cells and migration marker (CXCR3)+ B cells showed no differences between HC and early RA patients. This indicates that memory B cells may have left the circulation in early RA patients.

Next, we compared the same lymphocyte subsets at the inclusion time point in arthralgia patients who developed arthritis within 5 years (converters) with arthralgia patients that did not develop arthritis (non-converters). This revealed that converting arthralgia patients had
a significant lower number of CD8+ cytotoxic T cells and CD27+ memory B cells compared to non-converting arthralgia patients (Figure 2). There was no significant decrease in migration marker (CXCR3)+ B cells between non-converters and early arthritis patients ($p=0.18$). A significant decrease or a trend thereof was observed for CD27+ memory B cells in early RA patients compared to non-converters and HC ($p=0.013$ and $p=0.059$, respectively). Differences between HC, non-converters, converters and early arthritis patients are depicted in supplementary figure 2. This indicates that some of the difference seen in immunological status of early RA patients is already occurring in converting arthralgia patients at their first visit to the rheumatologist.

In the Amsterdam Reade cohort, the arthralgia patients were followed for research purposes, by a rheumatologist for a maximum of five years to determine if arthritis developed. Therefore, in this cohort, arthritis can occur between the time point of inclusion and at maximum follow-up of five years. To investigate if there is a difference in composition of circulating mononuclear cells at the inclusion time point between arthralgia patients that develop arthritis within a year and between 1 and 5 years, the converting arthralgia patients were divided in an early and late converting group. This revealed that the late converters had a significantly lower number of CD8+ Cytotoxic T cells compared to non-converters (Figure 3). No differences between late converters and non-converters were observed for B cells, memory B cells and activation and migration markers. In the early converting arthralgia patients there was a significant decrease in number of memory B cells (Figure 3). The same trend was visible for the overall number of B cells and the activation and migration marker positive B cells.

The data described above suggests that cytotoxic T cells and memory B cells leave the periphery before the clinical diagnosis of arthritis. To gain more insight into the time frames of action for these cell types, we investigated a small group of 17 converting arthralgia patients with flow cytometry measurement on the time point of conversion and 12 and/or 24 months prior to conversion. Supplementary figure 3 depicts the overtime changes in both converting and 68 non-converting arthralgia patients. To more clearly depict the changes in the converting arthralgia patients at 12 or 24 months prior to conversion, log2 ratios were calculated to show the relative increase or decrease compared to the conversion time point. This revealed that there was a broad distribution within the cell types, where one patient showed a relative increase towards conversion and the other revealed a relative decrease towards conversion time point (Figure 4). The most outstanding cell types were the activation marker positive B cells, which showed a significant relative increase towards conversion time point compared to 24 months prior to conversion. This may indicate that in the very early stages, possibly years before arthritis development occurs, cytotoxic T cells and conventional memory B cells migrate towards the lymph nodes or the joints.
Figure 1: Dot plots of the absolute number of cells in the lymphocyte subsets in 89 early RA patients (EAC) and 37 healthy controls (HC). The black line represents Median of the whole group. Differences between groups was tested with a Mann Whitney-U test. N.S. is not significant.
Changes in peripheral blood lymphocyte subsets

Figure 2: Dot plots of the absolute number of cells per lymphocyte subset in 40 arthralgia patients who develop arthritis within 5 years (converter) and 73 arthralgia patients who did not develop arthritis (non-converter). The black line represents Median of the whole group. Differences between groups was tested with a Mann Whitney-U test. N.S. is not significant.
Figure 3: Dot plots of the absolute number of cells per lymphocyte subset in 73 arthralgia patients who do not develop arthritis (non-converters), 22 who develop arthritis within a year (early converters) and 18 who develop arthritis between 1 and 5 years (late converters). The black line represents Median of the whole group. Differences between groups was tested with a Kruskal-Wallis test followed by Dunn’s multiple comparison test. N.S. is not significant.
Changes in peripheral blood lymphocyte subsets

Figure 4: Dot plots of the negative log₂ ratios of the absolute number of cells per lymphocyte subset at time point of arthritis development (conversion) divided by the absolute number of cells per lymphocyte subset 12 or 24 months prior to arthritis development, in 17 arthralgia patients that develop arthritis. A ratio >0 indicates increased cell number at 12 or 24 months before arthritis development compared to conversion time point. The black line represents Median of the whole group. Differences between conversion time point and 12 or 24 months prior to arthritis development (e.g. difference from 0) was tested with a one sample T-test or Wilcoxon Signed Rank test and N.S. is not significant.
Discussion

Changes in immune parameters and B cells in particular, have been extensively studied in the peripheral blood of RA patients in relation to therapy response, pathology of RA and clinical parameters. In early RA patients a decrease of total memory B cells and an increase of serum markers for the activation of B cells such as beta2 microglobulin have been described\(^8,10\). We have recently described lower numbers of memory B cells as a potential biomarker to predict arthritis development in arthralgia patients\(^5\). In the present study, we demonstrate that changes in peripheral blood mononuclear subsets, especially a lower number of CD8\(^+\) T cells were seen 24 months prior to developing arthritis. Whereas, 12 months prior to arthritis development, the number of memory B cells are lower compared to arthralgia patients that do not develop arthritis.

Our data demonstrates that there is a clear difference between early RA patients and HC with a decrease in CD8\(^+\) cytotoxic T cells, CD3\(^+\)CD56\(^+\)CD16\(^+\) activated T cells, memory B cells and activated B cells in early RA patients. This could be partly due to the age differences between the HC and the early RA patients as it was described that established RA patients have an pre-aged immune system that is about 20 years older than the healthy controls\(^11\). However, our data suggests that these changes start early in the development of RA, as converting arthralgia patients display similar changes. Late converters show lower numbers of CD8\(^+\) cytotoxic T cells, whereas the early converters have a decrease in memory B cells. The lower number of memory B cells found in peripheral blood of early converters and early RA patients is similar to what is described by McComish et al. for early DMARD naïve RA patients\(^12\). This reduced number could be due to migration of memory B cells to the synovium of the affected joints.

We observed trends towards decreased activated B cells and CXCR3\(^+\) B cells, which suggests increased migration of activated B cells to the joints. These populations and CD27\(^+\) memory B cells may be partly overlapping\(^13\). Increased migration of these subsets is in line with a study of Nanki et al. describing more memory B cells and CXCR3 positive B cells in the synovium compared to the peripheral blood in early RA patients\(^14\). Alternatively, B cells may have migrated towards the lymph nodes or bone marrow. In early RA patients significantly more B cells were observed in the draining lymph nodes of the inflamed joints than in similar lymph nodes of HC\(^9\). In established RA patients the bone marrow adjacent to the affected joints reveals a cell rich inflammatory surrounding instead of a fat rich non-inflammatory surrounding as seen in HC\(^6\). Both the increase of cells in the lymph nodes of arthralgia patients and a more inflammatory surrounding in the bone marrow adjacent to the affected joints in RA patients point towards a systemic immune activation in arthralgia patients were B cells may migrate towards the secondary lymphoid organs, bone marrow or joints approximately a year before clinical signs arise.
Reduced numbers of CD8+ cytotoxic T cells are associated with early arthritis as there was a trend towards lower numbers in the early arthritis patients and a significant reduction in (late) converters compared to non-converters. It has previously been described that in established RA patients no significant differences were found in T cell subsets compared to HC, however there was a shift seen towards more CD8+ terminally differentiated effector memory T cells. This might point towards an early activation of cytotoxic T cells in arthralgia and early RA patients which may later differentiate into the terminally differentiated effector memory T cells found in established RA patients. There are indications that there is an increased migration of CD8+ cytotoxic T cells to lymph nodes and joints in RA patients. In lymph nodes of early RA patients and arthralgia patients that did not develop RA, no differences in frequency of total CD8+ cytotoxic T cells was observed. However, there was a significant increase in activated CD69+ CD8+ T cells in the lymph nodes of early RA patients as well as a tendency in arthralgia patients. de Hair et al., described that synovial fluid of arthralgia patients had a subtle infiltration of CD8+ cytotoxic T cells. These data suggests that cytotoxic T cells in arthralgia patients become more activated in the lymph nodes before migrating towards the affected joints which might accelerate the inflammation in the joint by producing more pro-inflammatory cytokines. No differences were observed in CD4+ T helper cells between HC, non-converters, converters and early RA patients. This is in concordance with a recent publication on CD4+ T cells, where no differences were observed between HC and seropositive arthralgia patients in absolute number of CD4+ T cells, nor in CD4+ (terminally differentiated) effector memory cells. An increase in CD4+CD69+ T cells expressing a TH1 or TH1/TH17 phenotype was observed in seropositive arthralgia patients compared to HC, suggesting a role for TH17 cells in RA development. Our data suggest that increased migration of B cell subsets and CD8+ cytotoxic T cells is reflected by decreased numbers of these subsets in peripheral blood; however, larger studies are necessary to confirm this.

Recently it was described that seropositive arthralgia patients had a decrease in percentage of NK cells, especially CD56dim NK cells compared to HC, however no differences in absolute numbers of NK cells within the CD45+ pool were found. In our comparison of converting and non-converting seropositive arthralgia patients no decrease of absolute numbers and percentage (data not shown) of overall NK cells was observed, however, no sub classification of NK cells on CD56 dim or bright was made. Furthermore, no differences in NK cells between HC, non-converting and converting arthralgia patients and early RA patients were observed. This could be due to differences in patient population and inclusion criteria of the arthralgia patients.

Longitudinal data of the arthralgia patients revealed for both non-converters and converters that there is a fluctuation in percentage of all immune cells that we measured. This could be a reflection of the normal fluctuation observed as well in HC or due to seasonal infections like the influenza virus, although, patients with symptoms of the flu were asked
to return at a later time point for blood collection. What emerged from the longitudinal data, although it is a small cohort, is the significant increase in number of activated B cells within patients between 12 and 24 months before conversion. Our study provides indications of which immune cell subsets and changes therein are involved in the phase preceding development of clinical RA. Larger studies are needed to better understand the time points of these changes, as well as the transitions between the blood, bone marrow, lymph node and synovial compartments. These studies will enable more precise prediction for clinical use as well as the definition of time points and targets for specific interventions.

Conclusions
The main conclusion from this study is that arthralgia patients that develop arthritis show changes in circulating lymphocyte subsets, starting with a decrease of CD8$^+$ cytotoxic T cells 24 months prior to arthritis development, followed by a decrease in the number of activated memory B cells 12 months prior to disease onset.

Funding
This research was performed with support from the consortium in the framework of the Center for Translational Molecular Medicine (CTMM) (Http://www.ctmm.nl) and the Dutch Arthritis Foundation (grant 11-1-411 and LLP-20)

Acknowledgements
The authors thank J. Hollander, P. Bonnet and the other technicians of the Unit of Medical Immunology, department of Pathology, VU University Medical Center, the Netherlands for their help with flow cytometry data collection. We would also like to thank S. Snel of the department of Haematology, VU University Medical Center, the Netherlands for his help with the FACS DIVA software.
Changes in peripheral blood lymphocyte subsets

Supplementary data

Supplementary figure 1: Gating strategy of the flow cytometry analysis. A) Gating strategy with quantification beads. Gate 1 for living cells, which are depicted in the second dot plot. Gate 2 for lymphocytes, gate 3 for monocytes and gate 4 for the quantification beads. CD3 and CD19 positivity was determined in the lymphocyte gate and depicted in plot 5. B) Gating strategy for the immunological subsets. Lymphocyte selection based on on FSC and SSC properties (plot 1) followed by selection on CD45 properties (plot 2). All the subsets were determined in the lymphocyte gate.
Supplementary figure 2: Dot plots of the absolute number of cells in the lymphocyte subsets in 37 healthy controls (HC), 40 arthralgia patients who develop arthritis within 5 years (converter), 73 arthralgia patients who did not develop arthritis (non-converter) and 89 early RA patients (EAC). The black line represents Median of the whole group. Differences between groups were tested with a Kruskal Wallis test followed by a Dunn’s multiple comparison test. N.S. is not significant.
Supplementary Figure 3: Line plot of the absolute number of cells per lymphocyte subset in 68 non-converting and 17 converting arthralgia patients. The non-converting arthralgia patients are depicted from time point of inclusion. The converting arthralgia patients are depicted from the time point of conversion and the time before conversion.
References


Chapter 7

Can new biomarkers improve prediction of arthritis development in seropositive arthralgia patients?

Joyce Lübbers
Marian H. van Beers-Tas
Birgit I. Witte
Lotte A. van de Stadt
Leendert A. Trouw
Elise Mantel
Hetty J. Bontkes
Cornelis L. Verweij
Dirkjan van Schaardenburg

Manuscript in preparation
Abstract

Objectives:
The objective of this study was to investigate the potential added value of the biomarkers: type I Interferon (IFN) response signature, B cell signature and anti-carbamylated protein (anti-CarP) antibodies to an existing model for the prediction of arthritis in rheumatoid factor and/or anti-citrullinated protein antibody positive (seropositive) arthralgia patients. The previous model was based on clinical characteristics, symptoms and classical auto-antibody status.

Materials and Methods:
Of 350 seropositive arthralgia patients, 127 (36%) developed arthritis within a median of 13 months (IQR 6-26) and 223 did not develop arthritis within a median follow-up period of 58 months (IQR 36-64). Cox regression analysis was used to analyse biomarkers for their ability to predict arthritis development. Harrell’s C and Receiver Operating Characteristics (ROC) curve analysis with Area under the curve (AUC) in combination with internal validation through cross validation was used to describe the performance of the different models.

Results:
Univariate analysis to predict arthritis development of the type I IFN and B cell signatures and the anti-CarP antibodies revealed an increased risk of developing arthritis with an IFNhighB celllow signature (Hazard ratio (HR) 1.96, 95% CI 1.06-3.63) compared to an IFNlowB cellhigh signature, and positivity for anti-CarP antibodies (HR 2.28, 95% CI 1.61-3.25) compared to not having anti-CarP antibodies, respectively. However, including these biomarkers in the previous clinico-serological model revealed no added value to predict arthritis development, as is apparent by Harrell’s C which remained 0.74 and the AUC which remained below the AUC of the original model (0.81 after five years, 95% CI 0.64-0.94). When computing a new model, most variables from the original model were included in the model except for ‘alcohol consumption’ and ‘intermittent symptoms present’. With the new model the AUC increased slightly to 0.82 after five years (95% CI 0.64-0.93).

Conclusions:
The original clinico-serological model to predict arthritis development within five years in seropositive arthralgia patients did not improve by adding the new biomarkers type I IFN, B cell signatures and anti-CarP.
Introduction

In 2010 new American college of rheumatology (ACR)/ European league against rheumatism (EULAR) criteria for rheumatoid arthritis (RA) were developed in order to promote early recognition and treatment. A prominent role was reserved in these criteria for autoantibodies against rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPA) which are often present years before the onset of RA. ACPA positivity predicts RA development in the general population with a positive predictive value of 5-16%. In clinical practice autoantibodies are mostly measured for the first time in patients with arthralgia. Arthralgia patients that are positive for APCA and RF have a 40% risk of developing RA within 24 months. In a cohort of seropositive arthralgia patients, we have previously developed a model to predict arthritis development, using clinical characteristics, symptoms and serology. The prediction model consisted of 9 variables: RA in first degree family members, no alcohol consumption, duration of symptoms shorter than 12 months, presence of intermittent symptoms, arthralgia in upper and lower extremities, a visual analogue scale (VAS) over 50, presence of morning stiffness, swollen joints reported by the patient and antibody status. Scoring of these variables with points for presence of a symptom, divided arthralgia patients in three groups. The high-risk group (7-13 points) had 81% chance of developing arthritis within five years, whereas the intermediate-risk group (5-6 points) and low-risk group (0-4 points) had only a 43% and 12% chance of developing arthritis, respectively.

Other biomarkers that were additionally studied for the prediction of arthritis in a different subset of this cohort were the type I interferon (IFN) signature, the B cell signature and anti-carbamylated protein (anti-CarP) antibodies. The type I IFN signature was predictive for the development of arthritis within two years, while the B cell signature was found to be protective for the development of arthritis. The combination of both signatures revealed that arthralgia patients with an IFNhighB celllow signature had an increased risk of developing arthritis with a hazard ratio (HR) of 5.6 (95% CI: 1.9-16.1) compared to IFNlowB cellhigh patients. The anti-CarP antibodies have been detected in sera of asymptomatic blood bank donors years before the clinical manifestation of RA, and therefore may enhance the prediction of RA in antibody positive arthralgia patients. Indeed, in seropositive arthralgia patients anti-CarP antibodies were associated with the development of arthritis (HR 2.53; 95% CI: 1.76-3.63) compared to arthralgia patients without anti-CarP antibodies, which remained significant after correction for ACPA and RF status (HR 1.56; 95% CI: 1.06-2.29).

The type I IFN signature, B cell signature and anti-CarP antibodies were only studied for their predictive ability in comparison to ACPA and RF positivity. In the present study, we aimed to investigate whether the type I IFN and B cell signatures and/or the presence of anti-CarP antibodies have an added value to the full original clinico-serological model for predicting arthritis development.
Materials and Methods

Study population
In the Amsterdam Reade cohort 624 arthralgia patients were included for this study who were auto-antibody positive, not using DMARDs and had absence of a (history of) arthritis and erosions despite joint complaints as assessed by a rheumatologist. At second measurement 50 patients were seronegative and 4 did not have a second measurement and were therefore excluded. Another 77 patients were excluded due to loss to follow-up or had a shorter follow-up than 12 months and in 143 patients no additional biomarkers could be tested due to no or not enough material present to perform the measurements. In total 350 seropositive arthralgia patients were selected for the analysis (Figure 1), 296 (84.6%) patients were also included in the development of the original clinico-serological model. All patients were followed for arthritis development at six months and then yearly, with extra visits if the patients had increasing symptoms or suspected arthritis development. All patients gave written informed consent and this study was approved by the local medical ethical committee.

Clinical and laboratory measurements
At inclusion of the arthralgia patients, the medical history as well as joint complaints, tender joints and medical examinations were recorded as described previously. Measurements of anti-CCP levels and IgM-RF were determined as described earlier. Cut-off levels for anti-CCP were 50 arbitrary units/ml (AU/ml) or 10 AU/ml, depending on type of test over the years. For RF the cut-off levels were 30 IU/ml or 5 IU/ml. Type I IFN and B cell signatures were determined in whole blood collected in PaxGene tubes. Gene expression measurements on mRNA were performed with intron exon spanning Sybr green primers and Sybr green fast mastermix (Thermo Fisher Scientific, Massachusetts, USA) for the genes described previously. The cut-off for both signatures was determined as described previously; with a Receiver operating characteristics (ROC) curve with a specificity of 85% and 73% for IFN and B cell, respectively. Anti-CarP measurements were performed in serum with an Anti-CarP ELISA at the LUMC as described before. The cut-off for positive anti-CarP antibodies was 202 AU/ml.
Figure 1: Patient selection in the Amsterdam Reade arthralgia cohort. Patients were selected for being seropositive at first and second measurement, having a minimal follow-up of 12 months and availability of stored serum/RNA for the measurement of additional biomarkers. Out of the 350 seropositive patients thus selected, 223 did not develop arthritis within five years and 127 developed arthritis within five years (93 within two years and 34 after two years).
Statistical analysis
Statistical analyses were performed with IBM SPSS statistics version 20 and the R software environment for statistical computing (R-development Core Team) with the packages: survival and pROC. Clinical and serological prediction marker selection was as previously described5. IFN and B cell signatures as well as anti-CarP antibodies were selected for their strong individual prediction of arthritis development in parts of the Amsterdam Reade cohort. Categorisation of the variables was performed as described previously for the clinical variables as well as the IFN/B cell signature and anti-CarP antibodies5,6,12. Missing data of the variables alcohol use (5.7%), symptom duration (2%), intermittent symptoms present (2.9%), shared epitope (SE) (14.9%) and symmetric arthralgia (2.6%) were imputed with the median of nearby points, creating five imputation sets. Univariate and multivariate cox regression analysis was performed on all imputation sets and pooled analyses were reported. Multivariate analysis for a new model was performed with a backward step selection procedure (P removal 0.1). Variables added to the model were tested for changing the model with a likelihood ratio test.

Internal validation of the multivariate models was performed with cross validation. Therefore, 300 cases were randomly assigned to the test set and the remaining 50 cases were assigned to the validation set to calculate the HR, the area under the curve (AUC) for developing arthritis within 1, 2, 3, 4 and 5 years and the Harrell’s C performance index. Patients with less than 1, 2, 3, 4 and 5 years of follow-up, respectively, were treated as missing in the ROC analyses. Pooled results over all imputation sets for HR, AUC and Harrell’s C were calculated, and average scores from 1000 repetitions were presented.

Results
Arthralgia patients
Of the 350 selected arthralgia patients 127 developed arthritis with a median time to arthritis development of 12.6 months (IQR 5.9-25.8), of whom 93 developed arthritis within 2 years and 34 between two and five years. Patients who did not develop arthritis had a median follow-up of 58.2 months (IQR 36.1-63.9). The patient characteristics are shown in Table 1.

Univariate analysis of new biomarkers
To analyse if the new biomarkers IFN and B cell signatures and Anti-CarP antibodies can predict arthritis development in this large group of arthralgia patients a univariate Cox regression analysis was performed. This analysis revealed that patients with an IFN<sup>high</sup>B cell<sup>low</sup> signature or positivity for Anti-CarP antibodies have a higher risk of developing arthritis than arthralgia patients with an IFN<sup>low</sup>B cell<sup>low</sup> signature or without anti-CarP antibodies, as depicted in Table 2 (HR of 1.96 and 2.28, respectively).
Table 1: Characteristics of the selected arthralgia patients

<table>
<thead>
<tr>
<th></th>
<th>Arthritis development within 5 years</th>
<th>No arthritis development within 5 years</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>127</td>
<td>223</td>
</tr>
<tr>
<td>Age at inclusion in years, median (IQR)</td>
<td>48.3 (42.1-55.8)</td>
<td>49.5 (41.4-58.8)</td>
</tr>
<tr>
<td>Female, n (%)</td>
<td>92 (72.4)</td>
<td>174 (78.0)</td>
</tr>
<tr>
<td>Time to arthritis development in months, median (IQR)</td>
<td>12.6 (5.9-25.8)</td>
<td>N.A.</td>
</tr>
<tr>
<td>2010 ACR/EULAR criteria RA, n (%)</td>
<td>112 (88.2)</td>
<td>N.A.</td>
</tr>
<tr>
<td>1987 ACR criteria RA, n (%)</td>
<td>53 (41.7)</td>
<td>N.A.</td>
</tr>
</tbody>
</table>

Table 2: Univariate analysis of new biomarkers

<table>
<thead>
<tr>
<th>Marker</th>
<th>HR</th>
<th>95% CI</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFNlowB cellhigh (reference)</td>
<td>1.00</td>
<td>1.00-1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>IFNlowB celllow</td>
<td>1.39</td>
<td>0.91-2.11</td>
<td>0.129</td>
</tr>
<tr>
<td>IFNhighB cellhigh</td>
<td>1.29</td>
<td>0.61-2.71</td>
<td>0.503</td>
</tr>
<tr>
<td>IFNhighB celllow</td>
<td>1.96</td>
<td>1.06-3.63</td>
<td>0.033</td>
</tr>
<tr>
<td>Anti-CarP</td>
<td>2.28</td>
<td>1.61-3.25</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

IFN: interferon, Anti-CarP: anti-carbamylated proteins, HR: Hazard ratio, CI: confidence Interval

**Prediction models including the new biomarkers**

To study if the biomarkers have added value to the original clinico-serological model a Cox regression analysis was performed with all original clinical and serological markers included and IFN and B cell signatures or the anti-CarP antibodies were subsequently added to the model. In both cases the biomarkers were not significant in the model and did not change the model significantly according to the likelihood ratio test (data not shown).

To investigate whether the new biomarkers would be stronger than some clinical and serological variables already in the original clinico-serological model a new model was created with a backward step selection process (p removal 0.1). This revealed that the same clinical variables were included as in the original clinico-serological model (Table 3), except for the variables no alcohol and intermittent symptoms present. In the original clinico-serological model the ‘no alcohol’ variable was also removed if a removal P value of 0.05 was used and this did not change the performance of the model. Internal validation of the new model with 1000 repetitions revealed a comparable HR with even smaller 95% confidence intervals for all the variables (Table 3).
Table 3: Multivariate model to predict arthritis development in arthralgia patients

<table>
<thead>
<tr>
<th>Variable</th>
<th>HR</th>
<th>95% CI</th>
<th>p-value</th>
<th>HR CV</th>
<th>95% CI CV</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Clinical variables</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Swollen joint(s) reported(^a)</td>
<td>1.81</td>
<td>1.26-2.59</td>
<td>0.001</td>
<td>1.81</td>
<td>1.57-2.09</td>
</tr>
<tr>
<td>VAS pain &gt;50</td>
<td>1.44</td>
<td>1.00-2.08</td>
<td>0.051</td>
<td>1.44</td>
<td>1.21-1.70</td>
</tr>
<tr>
<td>Morning stiffness &gt; 1 hour present</td>
<td>1.90</td>
<td>1.26-2.85</td>
<td>0.002</td>
<td>1.90</td>
<td>1.61-2.28</td>
</tr>
<tr>
<td>FDR with RA</td>
<td>1.87</td>
<td>1.28-2.75</td>
<td>0.001</td>
<td>1.87</td>
<td>1.61-2.22</td>
</tr>
<tr>
<td>Location in upper and lower extremities</td>
<td>1.42</td>
<td>0.99-2.03</td>
<td>0.059</td>
<td>1.41</td>
<td>1.22-1.66</td>
</tr>
<tr>
<td><strong>Antibody status</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgM-RF positive, aCCP negative (reference)</td>
<td>1.00</td>
<td>0.00</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgM-RF negative, anti-CCP low positive(^a)</td>
<td>2.36</td>
<td>1.17-4.78</td>
<td>0.017</td>
<td>2.35</td>
<td>1.79-3.26</td>
</tr>
<tr>
<td>IgM-RF negative, anti-CCP high positive(^a)</td>
<td>4.50</td>
<td>2.40-8.43</td>
<td>&lt;0.0001</td>
<td>4.45</td>
<td>3.50-5.86</td>
</tr>
<tr>
<td>IgM-RF and anti-CCP positive</td>
<td>6.62</td>
<td>3.67-11.96</td>
<td>&lt;0.0001</td>
<td>6.57</td>
<td>5.43-8.80</td>
</tr>
</tbody>
</table>

\(^a\) Presence of a swollen joint as noticed by the patient, but not verified by a rheumatologist at physical examination. \(^a\)aCCP low positive ≥ 1x cut-off < 3x cut-off. \(^a\)aCCP high positive ≥ 3x cut-off.

Performance measurement of the different models

Next, we determined the performance of the new models via Harrell’s C and AUC in the ROC curve analysis. The original clinico-serological model revealed a Harrell’s C index of 0.74 in this patient group. Harrell’s C of the other models including IFN and B cell signatures, anti-CarP antibodies or the new clinico-serological model were lower, revealing no difference in performance between all the models (Table 4). The ROC analysis predicting development of arthritis within one to five years revealed an AUC for the original model of 74% (year one) up to 81% (year five). In the new clinico-serological model this increased only slightly to 82% in year five (Table 4).

Table 4: Performance measures of the different models

<table>
<thead>
<tr>
<th>Model</th>
<th>Harrell’s C</th>
<th>AUC at 1 year</th>
<th>AUC at 2 years</th>
<th>AUC at 3 years</th>
<th>AUC at 4 years</th>
<th>AUC at 5 years</th>
</tr>
</thead>
<tbody>
<tr>
<td>Original clinico-serological model (95% CI)</td>
<td>0.74</td>
<td>0.74</td>
<td>0.75</td>
<td>0.78</td>
<td>0.80</td>
<td>0.81</td>
</tr>
<tr>
<td>(0.61-0.84)</td>
<td></td>
<td></td>
<td>(0.60-0.89)</td>
<td>(0.63-0.91)</td>
<td>(0.64-0.92)</td>
<td>(0.64-0.94)</td>
</tr>
<tr>
<td>Original clinico-serological model + IFN &amp; B cell signatures (95% CI)</td>
<td>0.73</td>
<td>0.74</td>
<td>0.75</td>
<td>0.78</td>
<td>0.80</td>
<td>0.80</td>
</tr>
<tr>
<td>(0.62-0.84)</td>
<td></td>
<td></td>
<td>(0.59-0.89)</td>
<td>(0.62-0.91)</td>
<td>(0.63-0.92)</td>
<td>(0.63-0.93)</td>
</tr>
<tr>
<td>Original clinico-serological model + Anti-CarP (95% CI)</td>
<td>0.74</td>
<td>0.74</td>
<td>0.76</td>
<td>0.78</td>
<td>0.80</td>
<td>0.81</td>
</tr>
<tr>
<td>(0.62-0.85)</td>
<td></td>
<td></td>
<td>(0.59-0.89)</td>
<td>(0.63-0.91)</td>
<td>(0.64-0.92)</td>
<td>(0.64-0.93)</td>
</tr>
<tr>
<td>New clinico-serological model (95% CI)</td>
<td>0.74</td>
<td>0.74</td>
<td>0.75</td>
<td>0.78</td>
<td>0.80</td>
<td>0.82</td>
</tr>
<tr>
<td>(0.62-0.84)</td>
<td></td>
<td></td>
<td>(0.60-0.89)</td>
<td>(0.64-0.91)</td>
<td>(0.65-0.93)</td>
<td>(0.64-0.93)</td>
</tr>
</tbody>
</table>

IFN: interferon, AUC: area under the curve, CI: confidence interval
Chapter 7

Discussion

The original published prediction rule consisting of easily assessable clinical parameters in combination with ACPA and RF status had a good AUC of 82% within five years. To assess whether this model could be further improved, the recently identified biomarkers type I IFN signature, B cell signature and anti-CarP antibodies were included in the prediction model. Unfortunately, these biomarkers were not of added value to the previously described prediction model.

In this study an overlap of 296 patients from the original clinico-serological model were included. No significant differences in clinical parameters were found between the patients in the original clinico-serological model and the 54 new patients used in this model. The validity of the new model for the prediction of arthritis development is increased, due to an increase in median follow-up from 32.0 months (IQR 13.0-48.0) in the original clinico-serological model to 36.0 months (IQR 14.4-60.1) currently and for the patients that do not develop arthritis even to 58.2 months (IQR 36.1-63.9). The newly included biomarkers type I IFN signature, B cell signature and anti-CarP antibodies were not of added value to predicting arthritis development. The HR of the combined type I IFN and B cell signature is lower in this large cohort of seropositive arthralgia patients compared to the earlier published subset of seropositive arthralgia patients. This could be due to the time window used for this model, with prediction of arthritis development within five years. In the published subset for the prediction of arthritis development with the type I IFN and B cell signatures the median follow-up time of the seropositive arthralgia patients was 23 months (IQR 12-30). This could mean that there were still arthralgia patients that developed arthritis after the 23 months. This may decrease the HR in the analysis over five years follow-up. Furthermore, we showed that the B cell gene signature is correlating to the number of (conventional memory) B cells in whole blood, and that these conventional memory B cells decreased 12 months prior to disease onset (unpublished observation). The anti-CarP antibodies are seen years before the clinical symptoms of RA, however, the anti-CarP antibodies were tested for the prediction of arthritis development in arthralgia patients with a median follow-up of 36 months. This is a decent follow-up period, however, the new model is build on a five year follow-up which includes 34 arthralgia patients that developed arthritis after two years and some even just within five years. Therefore, it was tested if the biomarkers had an additive value if the time span for the model would be 24 months. This revealed that only anti-CarP was included in the model, although it did not increase the performance of the model as described by Harrell’s C or AUC values (data not shown).
The intermediate-risk group of the clinico-serological model still has 40% chance of developing arthritis within 5 years. This suggests it may be possible to further enhance the prediction of the model by adding variables that offer information that is distinct from the present variables. A good candidate may be serum cytokine levels, since multiplex cytokine analysis revealed that cytokines related to the T helper 1, T helper 2 and T helper 17 pathways and regulatory T cell function, such as interleukin (IL)-4, IFNγ, IL-10 and IL-17 were up regulated in pre-onset patients before clinical symptoms arose. Another good candidate would be the measurement of lipid profiles such as cholesterol levels through HDL. High serum cholesterol is associated with RA development in women and differences in lipid profiles was seen years before the development of RA. Furthermore, lipid levels were associated with SE, STAT4 and TRAF1/C5 which are susceptibility genes in RA. Combination of the original clinico-serological model with genetic risk factors such as polymorphisms in SE, PTPN22, PAD14 and CTLA4 could also identify more arthralgia patients that have a high risk of developing arthritis. Furthermore, other candidate markers could be imaging parameters. Promising results have been obtained with both MRI and PET scanning. Finally, it may be possible to improve the clinico-serological model by improving the clinical parameter measurements by further standardizing the measurements and to add more measurements of symptoms that occur both in arthralgia patients and early arthritis patients.

In conclusion, we could not improve the prediction of arthritis development in seropositive arthralgia patients by adding the type I IFN, B cell signature and anti-CarP antibodies to the original published clinico-serological model.

**Funding**

This research was performed with support from the consortium in the framework of the Center for Translational Molecular Medicine (CTMM) (http://www.ctmm.nl) and the Dutch Arthritis Foundation (grant 11-1-411 and LLP-20)


References


