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

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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 IFNhigh individuals converted to arthritis, compared to 29 out of 90 IFNlow 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 IFNhigh at risk individuals after correction for ACPA and RF. The ROC–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 IFN–signature is found to be consistently and significantly associated with presymptomatic arthritis and demonstrated clinical utility. These results indicate that the IFNsignature may be used as a biomarker for the prediction of RA development in the presymptomatic phase.
INTRODUCTION
Rheumatoid arthritis (RA) is a chronic inflammatory joint disease eventually leading to destruction of cartilage and bone.\footnote{1} 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.\footnote{2} 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 deregulated 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.\footnote{3,4} Together with the demonstration of epitope spreading of the ACPA response,\footnote{5,6} 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.\footnote{7} 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.\footnote{8-11}

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/RF seropositive individuals at risk, who developed arthritis within 2–5 years, independent of ACPA positivity.\footnote{9} The type I IFN signature consists of type I IFN response genes, and was previously shown to be also present in a subset of patients with established RA,\footnote{12} suggesting that pathological processes in the pre–symptomatic 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 and a cohort of individuals before onset of symptoms of a later diagnosed RA, 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. Inclusion criteria were the absence of arthritis despite joint complaints as determined by two independent investigators, a seropositive status (ACPA and/or IgM-RF positive) and a minimal follow-up of 12 months. Exclusion criteria were: (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.

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, Nederland) 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 log² 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 an IFN$^{\text{high}}$ and IFN$^{\text{low}}$ 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

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>
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<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</td>
<td>49 (41-56)</td>
<td>46 (39-55)</td>
</tr>
<tr>
<td>collection in years</td>
<td></td>
<td>40 (91)</td>
</tr>
<tr>
<td>(IQR)</td>
<td></td>
<td>26 (59)</td>
</tr>
<tr>
<td>ACPA positive nr, (%)</td>
<td>12 (17)</td>
<td>23 (52)</td>
</tr>
<tr>
<td>RF positive nr, (%)</td>
<td>30 (46)$^\dagger$</td>
<td>26 (67)$^\ddagger$</td>
</tr>
<tr>
<td>ACPA and RF Positive</td>
<td>14 (5.0-20)</td>
<td>9.5 (5.5-19)$^\S$</td>
</tr>
<tr>
<td>nr, (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ESR (IQR)</td>
<td>2.0 (0.86-5.0)$^\S$</td>
<td>2.7 (1.0-4.8)</td>
</tr>
<tr>
<td>CRP (IQR)</td>
<td>18 (25)</td>
<td>14 (32)</td>
</tr>
<tr>
<td>NSAID use nr, (%)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>DMARD use nr, (%)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Corticosteroids ($\leq$7.5 mg) use nr, (%)</td>
<td>23 (12-35)</td>
<td>20 (12-26)</td>
</tr>
<tr>
<td>Median follow-up time</td>
<td>2010 ACR/EULAR</td>
<td>8 (5-13)</td>
</tr>
<tr>
<td>in months (IQR)</td>
<td>criteria nr, (RA/UA)</td>
<td></td>
</tr>
<tr>
<td>DMARD use nr, (%)</td>
<td>1987 ACR criteria nr,</td>
<td>40/4</td>
</tr>
<tr>
<td>Corticosteroids (≤7.5 mg) use nr, (%)</td>
<td>18/26</td>
<td>18/26</td>
</tr>
</tbody>
</table>

ACPA = anti-citrullinated peptide 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.
Demographic and clinical characteristics for both cohorts are shown in table 1. 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 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-high of whom 15 (60%) converted to arthritis compared to 29 out of
90 (32%) IFN$^{\text{low}}$ patients, revealing a clear association of IFN$^{\text{high}}$-status and arthritis conversion (Chi-square, $P=0.011$) (figure 1B).

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$^{\text{high}}$ and IFN$^{\text{low}}$ group, revealed that an IFN$^{\text{high}}$ 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$^{\text{high}}$ versus IFN$^{\text{low}}$ at risk patients in relation to arthritis development in the Amsterdam Reade cohort. This analysis revealed that IFN$^{\text{high}}$ patients have a significantly higher risk of developing arthritis compared to IFN$^{\text{low}}$ 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.

Next we used the ROC-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) (figure 2B). 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 PPV of 65%.
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 IFNhigh 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).

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 IFNhigh–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.\textsuperscript{8,10} However, since cytokine and chemokine biomarkers are associated with autoantibodies the IFN-score is likely to provide novel and additional clinical value.\textsuperscript{15} 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 T cells.\textsuperscript{18} 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.
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