Chapter 8

Summary and Discussion
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The focus of this thesis was profiling of seropositive arthralgia patients to discover additional biomarkers for the prediction of arthritis development.

Rheumatoid arthritis (RA) is a chronic inflammatory joint disease with varying clinical symptoms and disease severity. Many patients have autoantibodies against citrullinated proteins (ACPA) and rheumatoid factor (RF). These autoantibodies are often detectable years before the clinical symptoms of RA arise\(^1\),\(^2\). Individuals who are autoantibody positive and have painful joints without swelling of the joints are referred to as seropositive arthralgia patients. Approximately 40\% of these seropositive arthralgia patients develop RA within 5 years\(^3\). Some RA patients have erosions at diagnosis or develop them shortly after diagnosis\(^4\),\(^5\). Early intervention helps to decrease the progression of the disease\(^6\),\(^7\). Profiling of arthralgia patients might identify patients that will develop RA and thereby reveal individuals that will benefit from early treatment to prevent joint damage or even development of RA.

Biomarkers for the prediction of arthritis development

To gain more insight in the prediction of arthritis development in arthralgia patients it is important to study biomarkers that discriminate arthralgia patients who do develop arthritis (converters) from arthralgia patients who do not develop arthritis (non-converters). A whole genome gene expression study on healthy controls (HC), arthralgia patients and established RA patients revealed five distinct gene sets: cytokine/chemokine, haematopoiesis, B cell, (immunity and defence with) type I IFN response genes and genes which did not relate to pathways\(^8\). In chapter 2, the type I IFN signature is validated for the prediction of arthritis development in seropositive arthralgia patients and blood bank donors from the Medical Biobank of Northern Sweden. Sixty percent of the arthralgia patients with a high type I IFN signature developed arthritis within two years, while only 32\% of the arthralgia patients with a low type I IFN signature developed arthritis within the same time span. This resulted in a significant association of a high type I IFN signature with the development of arthritis. Similar results were obtained in the Northern Sweden cohort where pre-symptomatic RA patients had a significantly increased type IFN score compared to the population-based controls. Cox regression analyses on the seropositive arthralgia patients revealed that IFN\(^\text{high}\) arthralgia patients have a significant risk of developing arthritis and that this risk is independent of ACPA and RF status. Receiver operating characteristics curve (ROC) analysis showed that the combination of the type I IFN signature with ACPA/RF status was better than ACPA/RF status alone in predicting arthritis development with an area under the curve (AUC) that increased from 62\% to 79\%. 
In chapter 3 the improvement of the prediction of arthritis development in arthralgia patients with the type I IFN signature by including a B cell signature is described. In the same arthralgia patient cohort as used for the validation of the type I IFN signature, B cell related genes were measured. This study revealed that IFN$^\text{high}$$B$ cell$^\text{low}$ seropositive arthralgia patients have a high risk of developing arthritis, while IFN$^\text{low}$$B$ cell$^\text{high}$ patients seem to be protected from the development of arthritis within the time frame of the study (two years). Furthermore, the B cell signature strongly correlated to the B cell count as measured by flow cytomtery. Subtyping of the B cell compartment revealed that converters have lower numbers of conventional memory B cells in their blood compared to the non-converters. This lower number of conventional memory B cells is in line with recent studies that showed that DMARD naïve early RA patients have decreased conventional memory B cells compared to HC$^{9-11}$. The decrease in circulating conventional memory B cells may be related to the migration of the B cells to the joints. Indeed, in synovial fluid of established RA patients an increased percentage of memory B cells was detected$^9$.

**Type I IFN signature**

To investigate the underlying mechanisms of the type I IFN biomarker for the development of arthritis, we examined the biomarker in seropositive arthralgia patients from inclusion until development of arthritis. The type I IFN signature has been described in a broad spectrum of auto-immune diseases such as systemic lupus erythematosus (SLE), Multiple Sclerosis (MS), RA and Myositis$^{13-15}$. In established RA no link between clinical parameters and the type I IFN signature was observed (unpublished data). However, a high type I IFN signature in established RA patients has predictive value for non-response to rituximab treatment$^{16,17}$. The good responders on rituximab revealed an increase of the type I IFN signature after 3 months of treatment compared to baseline and this increase was not detected in the non-responders on rituximab$^{18}$. Thus, an IFN$^\text{high}$ signature in seropositive arthralgia patients indicates the development of RA and at a later stage a similar type I IFN signature predicts non-response to rituximab treatment. In chapter 4, the longitudinal expression of the type I IFN response genes is described from the inclusion time point of the arthralgia patients towards the early RA stage. This revealed that an IFN$^\text{low}$ expression is overall stable and that there is some fluctuation in arthralgia patients with an IFN$^\text{high}$ expression, i.e. an IFN signature, where some patients remain IFN signature positive and other patients lose the signature during follow-up. Further research on the stability of the IFN signature is necessary to uncover the relevance of the fluctuation to the prediction of arthritis development in arthralgia patients.

In chapter 5 we investigate which cell type is the major source of the type I IFN signature in early arthritis patients. Comparison of whole blood, peripheral blood mononuclear cells (PBMCs), polymorhonuclear cells (PMNs), CD14$^+$ monocytes, CD19$^+$ B cells, CD4$^+$ and CD8$^+$ T cells revealed that PMNs are the major contributors to the type I IFN signature in early arthritis.
patients. Furthermore, an increase in type I IFN receptor (IFNAR1 and IFNAR2) was detected in PMNs of early arthritis patients, but not in healthy controls or PBMCs from early arthritis patients. This could reflect a more mature status of the neutrophils, since mature neutrophils display an upregulation of the IFNAR1 and IFNAR2, as well as type I IFN response genes compared to immature neutrophils\textsuperscript{19}. In established RA patients an increase in neutrophil extracellular trap (NET) formation has been observed and that ACPAs are important inducers of NET formation in RA patients\textsuperscript{20}. This could indicate that ACPAs are inducing NET formation by neutrophils in arthralgia patients, possibly before joint symptoms arise, and thereby upregulating the type I IFN response genes and receptors.

**Peripheral blood lymphocyte subsets during arthritis development**

To gain more insight in the leucocyte composition of peripheral blood of arthralgia patients, lymphocyte subsets and monocytes were determined in seropositive arthralgia patients at inclusion and close to the time of arthritis development. In inflamed joints of RA patients an infiltration of lymphocytes (B, T and NK cells), monocytes, macrophages, mast cells, dendritic cells, neutrophils etc. is detected\textsuperscript{21–25}. In peripheral blood of established RA patients a decrease in (pre-switched) memory B cells was detected compared to HC\textsuperscript{9,10}. No differences between T and NK cell populations were found, however, there was a shift within the naïve and memory T cell subsets toward more CD8\textsuperscript{+} terminal differentiated effector memory T cells\textsuperscript{26}. In chapter 3 a decrease of memory B cells in converting arthralgia patients is described. Further evaluation of the lymphocyte subsets in seropositive arthralgia patients is described in chapter 6. In converting arthralgia patients a significant decrease of CD8\textsuperscript{+} T cells and memory B cells was seen compared to non-converters. Furthermore, this decrease of CD8\textsuperscript{+} T cells is mainly seen in the late converters (conversion >12 months after inclusion). In the early converters a decrease in number of memory B cells was detected and a similar trend was visible for the activation (CD80) and migration (CXCR3) marker positive B cells. In converters the ratio of conversion time point and 12 or 24 months before conversion revealed a relative increase of activation (CD80) marker positive B cells towards conversion. In a recent article a decrease of NK cells, especially CD56\textsuperscript{dim} NK cells was described in seropositive arthralgia patients compared to HC\textsuperscript{27}. In our comparison of converting and non-converting seropositive arthralgia patients no decrease of overall NK cells was visible, however, no sub classification of NK cells on CD56 dim or bright was made. Overall the decrease in CD8\textsuperscript{+} T cells and memory B cells in converting seropositive arthralgia patients could indicate that in the very early stages, possibly years before RA development, cytotoxic T cells and memory B cells migrate towards the lymph nodes or joints.
Chapter 8

Prediction Model

To select the best (bio)markers for prediction of arthritis development in arthralgia patients, a combination of clinical, serological and gene expression biomarkers was assessed. Prediction of arthritis development in arthralgia patients may help to diagnose and treat RA earlier. In recent years a number of biomarkers and a prediction model have been presented to predict the development of arthritis in seropositive arthralgia patients. The shared epitope (SE) consists of a cluster of HLA-DRB1 genes and is associated with the development of RA28–30. In more recent research there was an association between the SE and certain ACPA epitopes as well as smoking for the development of RA31. Another source of biomarkers are the cytokines and chemokines in peripheral blood and/or serum. In established RA patients an increase in interleukin (IL)-6 is detected in serum and correlates with disease activity and C reactive protein (CRP) levels32. Upregulation of, among others, IL-1β, IL-6, IL17A and tumor necrosis factor-α have been detected in plasma of patients before the clinical onset of RA33. A third source of biomarkers are lipids. Decreased levels of lipids such as high-density cholesterol (HDL), apolipoprotein A1 (APO A1) and APO B were found in RA patients before the development of clinical symptoms and APO A1 levels and HDL presence were found to predict arthritis development in arthralgia patients34–36. In chapter 2 and 3 we describe type I IFN and B cell signature as predictive for arthritis development in arthralgia patients. In chapter 7 type I IFN and B cell signatures are tested along with the presence of anti-CarP antibodies, APCA, RF, SE and clinical characteristics of arthralgia patients to determine the best markers for prediction of arthritis development. For this, 9 clinical parameters from a sero-clinico prediction model are used, among which are morning stiffness, VAS score, alcohol use and duration of symptoms37. Type I IFN signature, B cell signature and the presence of anti-CarP antibodies were added to this sero-clinico prediction model. This revealed that the biomarkers did not add any value to the prediction of arthritis development within five years in arthralgia patients. One explanation could be that the new biomarkers are only predictive for a shorter time window or only have a pathological role before the arthralgia stage. Type I IFN signature and B cell signatures were only tested at 24 months follow-up as described in chapter 2 and 3. For the Anti-CarP antibodies this was tested up to 36 months follow up38. The model for arthritis development within 24 months includes the anti-CarP antibodies, although this did not improve prediction. Clinical markers are presently the best markers to predict arthritis development in arthralgia patients; however, further validation of the prediction rule in other cohorts is necessary.
Concluding remarks and future perspectives

In this thesis we have described new biomarkers for the prediction of arthritis revealed by profiling seropositive arthralgia patients, and although these biomarkers are not as strong as the clinical prediction rule, they could help explain possible pathological mechanisms in RA development.

The cellular composition in peripheral blood of arthralgia patients described in this thesis revealed a decrease of CD8+ T cells and memory B cells in converters compared to non-converters. It would be interesting to expand this study to more cell subsets such as dendritic cells, neutrophils and regulatory T and B cells to elucidate their contribution to the pathogenesis of RA. Dendritic cells have possible implications in RA by presenting citrullinated peptides and activating T and B cells\(^\text{39}\), however, differences in DC subsets in the development of RA remain to be elucidated. Regulatory B and T cells are reduced in early RA patients and have a negative correlation with disease activity in early RA patients\(^\text{40,41}\). It will be interesting to see if a decrease in regulatory B and T cells is also visible in converting arthralgia patients. They could be implicated in the regulation of the autoimmune response, by not reducing B and T cell activation against ACPAs. Further studies on the migration status of B and T cell subsets, especially memory B cells and CD8+ T cells, could reveal if they are migrating out of the periphery into e.g. the joints, lymph nodes or bone marrow. In lymph nodes of early arthritis patients an increase in B and T cells was detected compared to HC and a similar trend was seen in arthralgia patients\(^\text{42}\). It would be interesting to investigate whether there are differences in the cellular composition of the lymph node versus peripheral blood between non-converters and converters, which could point to events triggering RA development.

The hypothesis for the development of seropositive RA is that it starts with an environmental trigger, e.g. smoking in a genetic predisposed individual. Some individuals will become autoantibody positive, by the induction of T and B cell responses. This could be enhanced by a high type I IFN expression, due to break of tolerance by stimulating DCs, T and B cells\(^\text{43}\). After the first immune response, memory B cells will probably move to the bone marrow or secondary lymphoid tissue which may explain the decrease in memory B cells at inclusion of the later converting arthralgia patients. After a so called “second hit” such as an infection or trauma, an inflammatory response is induced attracting immune cells such as memory B cells\(^\text{44}\) to the joints. This will lead to a vicious circle of inflammation in the joints as depicted in Figure 1.

It is important to discriminate converting arthralgia patients from non-converting arthralgia patients at an as early as possible time point to be able to start possible preventive treatment. Therefore it is important to predict arthritis development in arthralgia patients. This thesis revealed that clinical parameters are more powerful than the type I IFN and B cell...
signatures for prediction of arthritis development in seropositive arthralgia patients. In the high risk group 81% developed arthritis within 5 years. The intermediate group of arthralgia patients have a reduced risk of arthritis development, but still 43% develops arthritis within 5 years. Therefore, more biomarkers should be tested to enhance the prediction of arthritis development in this intermediate group. It would be interesting to include other described biomarkers such as lipids (APO A1 and HDL)\textsuperscript{34,35}, susceptibility genes such as STAT4, TRAF1, PTPN22 or TNAIP3\textsuperscript{36,46-50}, macrophage PET imaging for synovitis in hands and feet\textsuperscript{51,52} and cytokine/chemokine levels in serum such as IL-6, IL-1β and TNFα\textsuperscript{33}.

In conclusion, this thesis presented a type I IFN signature mostly originating from granulocytes, and a B cell signature reflecting a decrease in memory B cells, as biomarkers for the prediction of arthritis development within two years. An already reasonably accurate clinical prediction rule for the prediction of arthritis development within five years could not be improved with these type I IFN and B cell signatures. Therefore, further biomarker studies for improved prediction of arthritis in at-risk individuals are needed.

Figure 1: Illustration of a hypothesis for the development of seropositive rheumatoid arthritis (RA). 1) An environmental trigger, such as smoking, induces citrullination of proteins in the lungs. 2) Break in tolerance to self-proteins and activation of antigen presenting cells through higher amounts of interferon (IFN) alpha and beta. This increases the uptake of citrullinated proteins by antigen presenting cells which migrate to the lymph nodes. 3) Under influence of high levels of IFN alpha and beta, T and B cell activation and proliferation is stimulated in the lymph nodes. 4) Formation of anti-citrullinated protein antibodies (ACPA) specific memory B cells (MB in picture) and (long-lived) plasma cells (PC in the picture). 5) Memory B cells and plasma cells move to the bone marrow or stay in the secondary lymphoid tissue. 6) Second hit, e.g. a virus infection, of the joint causes more citrullination of proteins. 7) Inflammatory response is induced in the joint and immune cells i.e. memory B cells, T cells and plasmablasts are recruited to the joints. 8) Production of cytokines by activated cells, which recruit and activate more cells leading to a vicious circle of inflammation in the joints. Picture adapted from Klareskog et al\textsuperscript{45} and created with Servier medical art.
References


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