Umeå University Medical Dissertations, New Series No 1490

Pathogenetic Factors of Importance for the Development and Progression of Rheumatoid Arthritis

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Umeå 2012

Responsible publisher under Swedish law: the Dean of the Medical Faculty

New Series No 1490
ISBN: 978-91-7459-403-4
ISSN: 0346-6612
Elektronisk version tillgänglig på http://umu.diva-portal.org/
Printed by: Print & Media, Umeå Universitet
Umeå, Sweden, 2012

To my family

“There are no shortcuts to any place worth going”

Paulo Coelho

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Abstract

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by joint inflammation eventually leading to the destruction of cartilage and bone. The aetiopathogenesis is not completely understood, but previous studies have shown that the disease is multifactorial with genetic, environmental and hormonal factors involved. Immune cells, *e.g.*, T- and B-cells, and macrophages, migrate into the joints, with increased expression of numerous soluble factors such as cytokines, chemokines and adhesion molecules functionally active both locally and systemically. Analyses of blood samples from the Medical Biobank in Umeå from individuals before the onset of symptoms of joint disease showed that anti-citrullinated protein/peptide antibodies (ACPA) preceded the development of disease by years and this finding has been confirmed by other studies.

The aim of this thesis was to identify signs of activation of the immune system analysed as up-regulation of pro- and anti-inflammatory cytokines, sero-positivity for autoantibodies, and genetic factors identified as relevant for the development and disease progression of RA. The concentrations of 30 cytokines and chemokines were measured in blood samples from individuals before the onset of symptoms, and when diagnosed with RA, together with population-based matched controls using a multiplex system. The predictive value of different isotypes (IgG, IgA, and IgM) of ACPA and rheumatoid factor (RF) before onset of symptoms and different types of ACPA (*e.g.*, mutated citrullinated vimentin, MCV) were analysed for disease development and progression in patients with early RA and controls from Northern Sweden. These factors were related to the genetic markers, HLA-shared epitope (SE) alleles and the 1858C/T polymorphism of the *protein tyrosine phosphatase non-receptor type 22* (*PTPN22*) gene.

In **paper I**, it was shown that in individuals who later developed RA (*i.e.*, pre-patients) the levels of several cytokines and related factors that represent the adaptive immune system (Th1, Th2, and T regulatory cell related factors) were significantly elevated compared with controls, whereas, after the onset of disease the involvement of the immune system was more general and widespread. In **paper II**, the presence of different isotypes (IgM, IgA and IgG) of ACPA in pre-patients, patients and controls was evaluated showing that both the IgG and IgA isotype predicted the onset of RA by years with the IgG isotype having the highest predictive value. In **paper III**, the association of the 1858T variant of *PTPN22* with RA was confirmed. Furthermore, the association was restricted to autoantibody positive disease and this variant was correlated with an earlier age for disease onset. In **paper IV**, anti-MCV antibodies were identified as being associated with a more severe disease course of RA, measured by disease activity score, erythrocyte sedimentation rate, and swollen joint count over time compared with anti-CCP2, anti-CCP3, and anti-CCP3.1 antibodies.

In conclusion, individuals who later developed RA had increased concentrations of inflammatory markers reflecting an activation of the immune system years before the clinical symptoms of the disease developed. Also, the presence of ACPA of IgG and IgA isotype prior to disease onset predicted the development of RA. The *PTPN22* 1858T variant was associated with sero-positive RA and anti-MCV antibodies were associated with a higher inflammatory activity compared with anti-CCP2, -CCP3 and -CCP3.1 antibodies. These findings together present a possibility to better predict the development and progression of RA.

Key abbreviations

ACPA	anti-citrullinated protein/peptide antibody
ACR	American College of Rheumatology
Anti-CCP	anti-cyclic citrullinated peptide
APC	antigen presenting cell
AUC	area under the curve
BCR	B cell receptor
bFGF	basic fibroblast growth factor
CI	confidence interval
CD	cluster of differentiation
CRP	C reactive protein
DAS	disease activity score
DMARD	disease modifying anti-rheumatic drug
ELISA	enzyme-linked immunosorbent assay
ESR	erythrocyte sedimentation rate
EULAR	European League Against Rheumatism
G-CSF	granulocyte-colony stimulating factor
GM-CSF	granulocyte macrophage-colony stimulating factor
HLA-SE	human leukocyte antigen-shared epitope allele
IFN	interferon
IQR	inter quartile range
Ig	immunoglobulin
IL	interleukin
IP	interferon inducible protein
Lyp	lymphoid tyrosine phosphatase
MCP-1	monocyte chemoattractant protein-1
MCV	mutated citrullinated vimentin
MHC	major histocompatibility complex
MIF	macrophage migration inhibitory factor
MIG	monokine induced by interferon-γ
MIP	macrophage inflammatory protein
MMP	matrix metalloproteinase
MONICA	Monitoring of Trends and Determinants in Cardiovascular Disease
MS	multiple sclerosis
NK	natural killer
NSHDS	Northern Sweden Health and Disease Study
OR	odds ratio
PAD	peptidyl arginine deiminase
PCR	polymerase chain reaction
PDGF	platelet derived growth factor

PTPN22	protein tyrosine phosphatase non-receptor type 22
RA	rheumatoid arthritis
RANTES	regualted activation normal T-cell expressed and secreted
RF	rheumatoid factor
ROC	receiver operating characteristics
RR	relative risk
SLE	systemic lupus erythematosus
TCR	T cell receptor
TGF	tumour growth factor
Th	T helper
TNF	tumour necrosis factor
Treg cell	regulatory T cell
UA	undifferentiated arthritis
VAS	visual analogue scale
VEGF	vascular endothelial growth factor
VIP	Västerbotten Intervention Programme
WHO	World Health Organization

List of publications

This thesis is based on the following publications, which are referred to in the text by their Roman numerals:

I. **Kokkonen H**, Söderström I, Rocklöv J, Hallmans G, Lejon K, Rantapää-Dahlqvist S. Up-regulation of cytokines and chemokines predates the onset of rheumatoid arthritis. *Arthritis Rheum* 2010; 62: 383-391.

II. **Kokkonen H,** Mullazehi M, Berglin E, Hallmans G, Wadell G, Rönnelid J, Rantapää-Dahlqvist S. Antibodies of IgG, IgA and IgM isotype against cyclic citrullinated peptide precede the development of rheumatoid arthritis. *Arthritis Res Ther* 2011; 13: R13.

III. **Kokkonen H**, Johansson M, Innala L, Jidell E, Rantapää-Dahlqvist S. The *PTPN22* 1858C/T polymorphism is associated with anti-cyclic citrullinated peptide antibody-positive early rheumatoid arthritis in Northern Sweden. *Arthritis Res Ther* 2007; 9: R56.

IV. Innala L, **Kokkonen H**, Eriksson C, Jidell E, Berglin E, Rantapää-Dahlqvist S. Antibodies against mutated citrullinated vimentin are a better predictor of disease activity at 24 months in early rheumatoid arthritis than antibodies against cyclic citrullinated peptides. *J Rheumatol* 2008; 35: 1002-1008.

Sammanfattning på svenska

Reumatoid artrit (RA), ledgångsreumatism, är en kronisk autoimmun sjukdom som drabbar knappt 1,0% av den vuxna befolkningen. Vid RA börjar kroppens immunförsvar av okänd anledning att attackera vävnaderna och organ. Det uppstår en kronisk inflammation i ledvävnaden i framförallt händer och fötter och man kan också få inflammation i andra organ så som lungsäck, hjärtsäck, ögon eller i blodkärl.

Sjukdomens svårighetsgrad varierar mycket mellan olika individer, från mild och självbegränsande till mycket aggressiv med snabb nedbrytning av brosk och ben. För att undvika dessa irreversibla vävnadsskador är det mycket viktigt att försöka identifiera och diagnostisera individer med RA så tidigt som möjligt och att sätta in rätt behandling.

Förekomst av autoantikroppar är karakteristiskt för RA och finns hos ca 70-80% av patienter med sjukdomen. De två vanligaste autoantikropparna och diagnostiskt viktigaste är reumatoid faktor (RF) och antikroppar mot citrullinerade proteiner/peptider (ACPA).

Vad som orsakar sjukdomen är till stor del okänt, men gener, hormoner och miljöfaktorer tros vara involverade.

Syftet med denna avhandling var att försöka idenfiera faktorer som kan vara av betydelse för att utveckla RA och även deras betydelse för sjukdomsutvecklingen, så som röntgenprogression.

I delarbete I, analyserades blodprover från Medicinska Biobanken i Umeå från 86 individer som donerat prov innan insjuknandet i RA och även från 69 av dem vid tipunkten för diagnosen av RA. Dessutom analyserades blodprover från 256 kontrollindivider som lämnat prov till Medicinska Biobanken. Koncentrationen av 30 olika cytokiner, cytokinrelaterade faktorer och chemokiner som fungerar som signaleringsproteiner i immunsystemet, analyserades i blodproverna. Resultaten visade att individer som senare utvecklar RA, hade signifikant förhöjda koncentrationer av flertalet faktorer jämfört med kontrollerna. Samband till flera olika delar av förvärvade immunförsvaret kunde påvisas och det fanns starka associationer till autoantikroppar för många av faktorerna.

I det andra delarbetet analyserades olika isotyper av ACPA, sk immunoglobuliner (Ig) hos individer innan insjuknandet i RA, patienter vid diagnos och även friska kontroller. De isotyper som studerades var IgA, IgM,

och IgG. Resultaten visade att både IgA och IgG isotypen av ACPA fanns i signifikant högre koncentration hos individerna innan insjuknandet i RA jämfört hos kontrollerna och att dessa isotyper även predikterade utvecklingen av RA.

I delarbete III analyserades en genetisk variation i genen *PTPN22* hos patienter från norra Sverige med RA och kontroller i relation till autoantikroppar och även i förhållande till olika variabler för sjukdomsutfall. Denna gen kodar för ett protein som är involverat i regleringen av immunceller. Denna genetiska variation, en sk single nucleotide polymorphism (SNP) på position 1858 resulterar i ett aminosyra-utbyte som gör att funktionen hos proteinet blir förändrat. Denna SNP var associerad med sero-positiv (RF eller ACPA) RA i den studerade populationen och till ett tidigare insjuknande. I studien identifierades också rökning vara en riskfaktor för utveckling av RA oberoende av autoantikroppar och den studerade genetiska variationen i *PTPN22*.

I det sista delarbetet undersöktes betydelsen av olika typer av ACPA för sjukdomsprogressionen vid RA. Resultaten visade att en viss typ av ACPA, antikroppar mot muterat citrullinerat vimentin (anti-MCV) var associerat med en aktivare sjukdom, mätt med olika faktorer associerade med inflammationen. Alla undersökta ACPA-typer var i lika stor grad betydelsefulla för röntgenprogressionen.

Slutsatserna från dessa studier är att immunsystemet är aktiverat med flertalet olika celltyper aktiverade långt innan man börjar visa kliniska symtom på sjukdomen och även att mönstret skiljer sig innan de klinska symtomen och vid tidpunkten för diagnos. Olika isotyper av ACPA förekommer innan symtomdebut och är signifikanta för utvecklingen av RA. En genetisk variation i genen *PTPN22* var associerad med ett tidigare insjuknande i sero-positiv RA jämfört med de patienter som inte hade denna. Anti-MVC ACPA var associerat med en högre inflammatorisk aktivitet hos patienter med RA.

Introduction

Rheumatoid arthritis

Autoimmune diseases such as rheumatoid arthritis (RA), type 1 diabetes, multiple sclerosis (MS), and systemic lupus erythematosus (SLE) are chronic conditions that can develop when the sophisticated protective functions of the immune systems fails to distinguish foreign pathogens from the self-tissues of the body, with the consequence that the immune system starts to attack the tissues of the individual resulting in disease. Autoimmune diseases are estimated to affect approximately 4.5% of the population (Hayter and Cook, 2012).

Rheumatoid arthritis is a chronic systemic autoimmune disease that primarily affects the synovium of joints and tendons with a massive infiltration of immune cells into the synovial tissue and fluid resulting in hyperplasia of the synovial lining cells followed by neoangiogenesis. The disease progression is associated with increased disability and, in some cases, extra-articular manifestations such as pleuritis, pericarditis and vasculitis. An increase in co-morbidity, such as cardiovascular disorders has been shown to increase mortality in patients with RA (Wallberg-Jonsson, *et al.*, 1997; Gabriel, *et al.*, 2003). Without treatment up to 30% of patients with RA would become permanently disabled during the first three years of disease (Rindfleisch and Muller, 2005).

Epidemiology

According to population-based studies approximately 0.5-1.0% of the adult population in developed countries are affected with RA (Alamanos, *et al.*, 2006; Neovius, *et al.*, 2011). The disease has a clear female dominance and is two to three times more frequent among pre-menopausal women compared with men, whilst after the menopaus the incidence is somewhat similar in females and males. The incidence is approximately 20-50 cases per 100,000 adults developing RA annually (Tobón, *et al.*, 2010). The prevalence and incidence of RA varies among different populations, *e.g.*, in American Indians and Alaskan natives a prevalence of up to 7% has been reported (Ferucci, *et al.*, 2005) whereas among some countries in Asia the calculated prevalence of RA is approximately 0.2% (Shichikawa, *et al.*, 1999; Zeng, *et al.*, 2008). It has also been observed that populations in North America and Northern Europe have a higher frequency of RA compared with Southern Europe (Alamanos and Drosos, 2005).

Diagnosis

An individual affected with RA typically presents with several symmetrical swollen and/or painful joints of the hands and feet, fatigue and sometimes slight fever. The disease is diagnosed according to the American College of Rheumatolgy (ACR) 1987 classification criteria for RA (Table 1) (Arnett, *et al*., 1988). These criteria were developed to diagnose RA in comparison with other inflammatory diseases but have poor sensitivity and specificity for diagnosing RA during the early stages of disease.

In 2010, new criteria for the classification of RA were developed that aimed at targeting features of early disease using a scoring system (Aletaha, *et al*., 2010). These features include the assessment of joint involvement, presence of rheumatoid factor (RF) and anti-citrullinated protein/peptide antibodies (ACPA), and acute phase response as measured by the C-reactive protein (CRP) and the erythrocyte sedimentation rate (ESR).

Table 1. The 1987 American College of Rheumatology (ACR) classification criteria for rheumatoid arthritis. Four out of the seven of following criteria should be fulfilled for diagnosis and criteria 1-4 must have been present ≥6 weeks.

1. Morning stiffness in and around the joints, lasting at least one hour before maximal improvement
2. Arthritis of three or more joint areas simultaneously, with soft tissue swelling or fluid
3. Arthritis of hand joints (wrist, MCP or PIP joints)
4. Symmetrical arthritis
5. Rheumatoid nodules
6. Rheumatoid factor in sera
7. Radiographic changes visible in hand or wrist radiographs

MCP=metacarpophalangeal, PIP= proximal interphalangeal

Aetiology

The aetiology leading to development of RA is not yet completely understood. During recent years several genetic, environmental, and hormonal factors have been identified as being related to the disease and

these factors are considered to interact making the aetiology of RA even more complex.

Twin studies have estimated that approximately 60% of the occurrence of RA can be explained by genetic factors (MacGregor, *et al.*, 2000). The strongest and most replicated genetic factor identified for RA is within the human leukocyte antigen (HLA) alleles of the major histocompatibility complex (MHC) located on the short arm of chromosome 6. The shared epitope (SE) hypothesis postulates that individuals carrying a conserved amino acid sequence (*i.e.*, epitope) at positions 70-74 in the third hyper variable region of the DRB1 chain in the MHC class II have an increased risk of developing RA (Gregersen, *et al.*, 1987). This association has been shown to be confined to patients that are sero-positive for autoantibodies, such as RF and ACPA, (Padyukov, *et al.*, 2004; Huizinga, *et al.*, 2005). The associations of HLA alleles with RA suggest the possible involvement of antigen presentation, peptide affinity, T-cell differentiation or molecular mimicry of the SE by microbial proteins in promoting an autoreactive immune response (Weyand and Goronzy, 1990; De Almeida, *et al.*, 2010).

In 2004, the association of another immune regulatory gene, *the protein tyrosine phosphatase non-receptor type 22* (*PTPN22*), with RA was discovered (Begovich, *et al.*, 2004). This association has been replicated in several studies (Hinks, *et al.*, 2005; Viken, *et al.*, 2005; Simkins, *et al.*, 2005; Seldin, *et al.*, 2005) including genome wide association scans (Wellcome Trust Case Control Consortium, 2007; Plenge, *et al.*, 2007) and is today the strongest genetic association with RA outside the HLA region. This gene encodes for the lymphoid tyrosine phosphatase (Lyp) protein, which is involved in negative regulation of T-cell activation (Hasegawa, *et al.*, 2004). The substitution of the nucleotide cytosine (C) with tyrosine (T) at position 1858 (rs2476601) resulting in an amino acid substitution from arginine to tryptophan (R620W) leads to a gain of function variant of Lyp resulting in a stronger suppression of T-cell activation (Vang, *et al.*, 2005). The *PTPN22* 1858T variant has also been associated with other autoimmune diseases, *e.g.*, SLE (Kyogoku, *et al.*, 2004; Reddy, *et al.*, 2005), type I diabetes (Bottini, *et al.*, 2004) and systemic sclerosis (Dieudé, *et al.*, 2008).

Other genetic loci that have been implicated in the pathogenesis of autoantibody positive RA include *TRAF1-C5* (Plenge, *et al.*, 2007; Kurreeman, *et al.*, 2007), *CTLA4* (Plenge, *et al.*, 2005), the 6p23 locus near *TNFAIP3* (Plenge, *et al.*, 2007; Thomson, *et al.*, 2007), *STAT4* (Remmers, *et al.*, 2007), *PADI4* (Suzuki, *et al.*, 2003), *CD40* (Raychaudhuri, *et al.*, 2008), *CCL21* (Raychaudhuri, *et al.*, 2008) and *KIF5A* (Raychaudhuri, *et al.*, 2008; Plant, *et al.*, 2010).

The genetic risk factors are less well established for autoantibody negative disease. Associations have been found with HLA-DRB1*03 alleles (Irigoyen, *et al*., 2005), *IRF5* (Sigurdsson, *et al*., 2007; Wang, *et al*., 2011), and a gene complex encoding for C-type lectin-like receptors (Lorentzen, *et al*., 2007).

The strongest environmental risk factor that has repeatedly been associated with RA is smoking (Vessey, *et al*., 1987; Heliövaara, *et al*., 1993; Stolt, *et al*., 2003). It has also been shown that smoking mainly predispose to ACPA positive disease. In 2006, Klareskog and colleagues suggested that in individuals carrying the HLA-SE alleles, smoking could trigger citrullination of proteins based on their observation of a strong gene-environment interaction between smoking and HLA-SE genes in ACPA positive patients and that smokers had more citrullinated proteins in bronchoalveolar lavage cells compared with non-smokers. Although in another study only a modest gene-environment interaction was observed of HLA-SE and smoking for the development of ACPA positive disease (Lee, *et al*., 2007). Furthermore, it was subsequently shown that the combination of HLA-SE alleles and smoking was associated with both ACPA positive and ACPA negative disease but with a stronger effect among ACPA positive patients (Bang, *et al*., 2010). Additionally, it was recently reported that smoking in the context of HLA-SE could promote citrullination of proteins that was non-specific, involving several different antigens, instead of citrullination of only specific antigens (Willemze, *et al*., 2011).

Moreover, the bacterium *Porphyromonas* (*P*.) *gingivalis* that plays a role in the progression of chronic periodontitis has been suggested to be involved in the development of RA (Lundberg, *et al*., 2008) due to its expression of bacterial peptidyl arginine deiminase (PAD) enzymes (McGraw, *et al*., 1999) that potentially could generate citrullinated epitopes. For example, it has been shown that PAD from *P. gingivalis* is able to citrullinate human fibrinogen as well as α-enolase (Wegner, *et al*., 2010). In a study by Mikuls and colleagues (2009) they demonstrated that concentrations of antibodies against *P. gingivalis* were increased in sera from patients with RA compared with controls and among the patients, high concentrations of antibodies against *P. gingivalis* were significantly associated with CRP, and ACPA of the IgM, IgG-2, and IgG-4 isotypes, further strengthening the hypothesis of its role in the pathogenesis of RA.

Other infectious agents, such as Epstein-Barr virus (Alspaugh, *et al*., 1981) or parvovirus B19 (Stahl, *et al*., 2000) and bacteria including *Streptococcus, Mycoplasma, Proteus* and *Echerichia coli* (Reviewed by Carty, *et al*., 2004) have also been suggested to be involved in the disease process.

Female hormonal factors are believed to be involved in disease susceptibility due to the female predominance in the incidence of RA prior to the menopaus. Oral contraceptive use has, in several studies, been suggested to be protective against RA (Vandenbroucke, *et al*., 1982; Jorgensen, *et al*., 1996; Berglin, *et al*., 2010). The influence of breast-feeding on the risk of developing RA has yielded conflicting results with some studies reporting an increased risk (Brennan and Silman, 1994; Berglin, *et al*., 2010), and others reporting a protective effect (Karlson, *et al*., 2004; Pikwer, *et al*., 2009).

Disease onset and synovial inflammation

The initiation of a disease such as RA involves a long process. It is hypothesized that in certain genetically predisposed individuals specific pathogenic immune processes (*e.g.*, antibody formation) can be activated by an environmental factor such as smoking that can cause inflammation of the airways generating local production of autoantigens followed by production of autoantibodies like ACPA and RF, which then can diffuse from the lungs and be spread throughout the vascular circulation (Demoruelle, *et al*., 2011).

The initiation of joint inflammation could either originate from a primary inflammation of the synovial membrane, which recruits immune cells that subsequently invade adjacent tissue as well as the bone marrow. Alternatively, the inflammation could start in the bone marrow and later migrate to the synovial membrane (Schett and Firestein, 2010).

The synovial inflammation characteristic of RA involves a variety of cell types, *e.g.*, macrophages, neutrophils, T-cells, B-cells, dendritic cells, mast cells, natural killer (NK) cells and synovial fibroblasts that act together to promote the observed synovial hyperplasia and degradation of cartilage and bone (Feldmann, *et al*., 1996; Thomas, *et al*., 1999; Kim and Berek, 2000; Weyand, 2000; Tak and Bresnihan, 2000). It is proposed that an autoantigen that is yet to be identified is presented to T-cells by an antigen presenting cell (APC) in the synovial membrane resulting in an immune response recruiting macrophages and alterations in the production of cytokines. Subsequently, synovial fibroblasts and other cell types like osteoclasts, chondrocytes and mast cells are activated by the cytokines secreted from the activated macrophages (McInnes and Schett, 2007). The synovial fibroblasts promoted by cytokines such as tumour necrosis factor (TNF)α and interleukin (IL)-1 (Karouzakis, *et al*., 2006) produce the matrix metalloproteinases (MMPs), proteins that are part of the cartilage degradation process, for example by degrading type II collagen and aggrecan (Konttinen, *et al*., 1999). The synovial fibroblasts are also believed to be

involved in the activation of osteoclasts at the bone surface leading to bone erosions (Pap, *et al.*, 2000). The B-cells are activated by T-cells or other APC that present the correct autoantigen and consequently differentiate into autoantibody producing plasma cells, thereby contributing to the inflammatory process associated with RA. The autoantibodies secreted are speculated to be involved in the formation of immune complexes that cause tissue damage (Kim and Berek, 2000).

All these processes lead to synovial hypertrophy and formation of a tumour-like pannus that ultimately results in joint damage with the joint space narrowing and production of marginal erosions. Subsequently, the joint loses its shape and position, which in turn leads to pain and disability for the patient.

Disease activity and radiological scoring

To assess the activity of RA both clinical and laboratory markers of inflammation are utilized. An often used clinical measure is the disease activity score (DAS) for 28 joints (Prevoo, *et al.*, 1995), which is a simplified version of the original European League Against Rheumatism (EULAR) DAS, that was validated for patients with early RA by van der Heijde (van der Heijde, *et al.*, 1992). DAS28 includes the patients' global health visual analogue scale (VAS), number of swollen and tender joints (hands, wrists, elbows, shoulders, and knees) and ESR. DAS28 scores ranges from 1 to 9, with the high scores indicating a more active disease. A DAS28 score >5.1 is regarded as high disease activity, <3.2 as low disease activity, and <2.6 as remission (Fransen, *et al.*, 2004). Recently, a new definition of remission in patients with RA was proposed following collaboration between ACR and EULAR with remission being defined as either a maximum value of 1 for each of the following: tender joint count, swollen joint count, CRP, and patient's global assessment (0-10 scale) or a simplified disease activity index of ≤3.3 (sum of tender- and swollen joint count, CRP, patient's and physician's global assessment) (Felson, *et al.*, 2011).

A patients response to treatment is defined by the ACR whereby ACR20 corresponds to an improvement of at least 20% in swollen and tender joints and 20% improvement in 3 out of 5 of the following: ESR, patient's and physician's global assessment, pain and disability. Likewise, the ACR50 and ACR70 responses correspond to a 50% and 70% improvement, respectively (Felson, *et al.*, 1995). Therapeutic response can also be assessed according to the EULAR response criteria based on the DAS28 score where "good", "moderate" or "no response" is based on the change in DAS from baseline,

i.e., when the therapy/drug is introduced to a given time point (van Gestel, *et al.*, 1996).

Determining the degree of joint destruction is useful for evaluating disease outcome in RA. This is usually performed with conventional radiography although magnetic resonance imaging (MRI) has been shown to be more sensitive for the detection of erosions and can directly visualize the bone and soft tissue in three dimensions (Backhaus, *et al.*, 2002; Østergaard, *et al.*, 2003). Other techniques for assessing RA include ultrasonography (Backhaus, *et al.*, 1999), and positron emission tomography (PET) (Beckers, *et al.*, 2004).

The scoring systems that are most widely used for evaluating radiographs are based on studies by Sharp and colleagues (Sharp, *et al.*, 1971) and have been modified several times, with the final modification done by van der Heijde (Sharp, *et al.*, 1985; Fries, *et al.*, 1986; van der Heijde, *et al.*, 1989) and Larsen (Larsen, *et al.*, 1977) which also have been modified over the years (Larsen, *et al.*, 1984; Larsen and Thoen, 1987; Larsen, 1995). The Larsen scoring method is based on a global score for both erosions and joint space narrowing for each joint, whilst the Sharp method includes separate scores for erosions and joint space narrowing. The Larsen scoring is performed by comparison with standard reference films. The two scoring methods are significantly correlated (Pincus, *et al.*, 1997) but the Larsen method is less time-consuming and easier to perform compared with the Sharp method (Sokka, 2008).

Treatment

Treatment strategies for RA have changed greatly over the years. The earlier approach was to start treatment with a non-steroid anti-inflammatory drug (NSAID) and subsequently add a disease modifying anti-rheumatic drug (DMARD) *e.g.*, methotrexate or sulfasalazine, if the patient continued to suffer a high disease activity. Proof over the years has established that the best approach to reduce disease activity and the inflammatory process that leads to joint destruction is to start an immediate aggressive treatment with DMARDs and perhaps to include glucocorticoids as early as possible (Möttönen, *et al.*, 1999; Svensson, *et al.*, 2005; Nell, *et al.*, 2004). If the patient's response to treatment is not adequate then a biological drug (*e.g.*, a TNF-inhibitor) should be added (Smolen, *et al.*, 2010). The anti-TNF treatments available have been shown to be very effective not only in lowering disease activity but also in slowing down the destruction of joints (Bathon, *et al.*, 2000; Klareskog, *et al.*, 2004). The goal of any treatment is

to achieve a low disease activity or optimally remission, meaning ideally a state of no symptoms for the disease. For the treatment of patients with RA both national (www.svenskreumatologi.se) and European guidelines have been formulated (Gaujoux-Viala, *et al.*, 2010; Nam, *et al.*, 2010; Knevel, *et al.*, 2010; Gorter, *et al.*, 2010).

Rheumatoid arthritis does not appear to be a single disease, rather it is the result of several related but different pathological processes and therefore it is unlikely that one universal treatment for all patients will be found, thus individual-based therapies are needed. Identifying patients that are at a greater risk for severe disease and those that will respond to certain therapies is essential to enable introduction of the optimal therapy before any irreversible joint damage has occurred. Hence, finding markers predictive of susceptibility for disease prediction is necessary.

The immune system

In order to protect us against disease the human body comprises a highly sophisticated defence system including a variety of effector cells and processes, *i.e.*, the immune system. This system protects us by identifying and destroying harmful pathogens (*e.g.*, bacteria, viruses, and parasites) and tumour cells. The immune system can be divided roughly into **innate** and **adaptive** immunity. Although these are described as two different systems there is substantial cross-talk between these in order to combat all of the infectious agents encountered during a life time.

Innate immunity

The innate immune system, the first line of defense, is congenital and fast working. It is a non-specific system that lacks immunological memory and consists of physical barriers (*e.g.*, skin and mucus membranes), chemical barriers (*e.g.*, anti-microbial substances secreted by epithelia), phagocytic cells, NK cells, blood proteins and cytokines. If pathogens succeed in crossing these barriers, macrophages that can ingest and kill these pathogens are attracted to the site of infection. Macrophages also secrete cytokines to induce an inflammatory response at the site of infection.

Adaptive immunity

If the innate system fails to clear the invading pathogen the adaptive or acquired immune system is activated. The adaptive immune system matures during the lifetime of an individual, takes longer time to activate but is more specific and entails an immunological memory which ensures a rapid and powerful response to a previously encountered infection. Another feature of adaptive immunity is the capability of self/non-self recognition, meaning that, under normal circumstances the adaptive system only responds to foreign antigens.

The effector cells of adaptive immunity are T-lymphocytes and B-lymphocytes (T-cells and B-cells, respectively). T-cells are involved in cell-mediated immune responses and B-cells in humoral (antibody) mediated responses.

B-cells

B-cells are developed and matured in the bone marrow. The antigen receptor on B-cells, *i*.e., the B-cell receptor (BCR), is a cell surface immunoglobulin (Ig). When B-cells are activated by an antigen that binds to its receptor the naïve B-cells differentiate into antibody producing cells, referred to as plasma cells and memory B-cells. The antibodies produced and secreted by these cells have the same specificity for antigens as the B-cells own receptor. The secreted antibodies then bind pathogens, which is the main effector function of B-cells.

There are five major Ig isotypes: IgM, IgG, IgA, IgE, and IgD. In a humoral immune response the first antibody isotype to be produced and secreted is IgM that can form large pentamers. The primary function of the IgM isotype is activation of the complement system, a series of proteins that functions to targeting extracellular pathogens. Antibodies of the IgG, IgA, and IgE isotypes are smaller and more easily diffused into different tissues. The IgG isotype, of which there are different sub-classes, G1-G4, is the most common antibody isotype in blood and extra-cellular fluids. The primary functions of IgG antibodies are opsonization of pathogens as well as activation of the complement system. Antibodies of the IgA isotype are produced from mucosal B-cells through isotype class switching and are most commonly found within the secretions in the gastro-intestinal and respiratory tracts and function mainly as neutralizing antibodies. In healthy individuals, a specific increase in IgA together with transforming growth factor (TGF)-β was proposed to play a role in the mucosal immune response to allergens

(Taylor, *et al*., 2006). The IgE isotype has effector functions in allergic reactions, such as release of histamine from mast-cells. Finally, the IgD isotype is mostly present as a surface Ig on mature B-cells where it is co-expressed with IgM (Pernis and Forni, 1976) but the functions of this isotype is still not fully understood.

It has recently been demonstrated that B-cells with a regulatory capacity and production of IL-10 is present in the peripheral blood of healthy individuals, whereas in patients with SLE these cells had defects in their suppressive capacity (Blair, *et al*., 2010). Similar observations of these regulatory B cells has been reported in patients with RA (Iwata, *et al*., 2011).

T-cells

T-cells are like B-cells developed in the bone marrow but with the difference that they are matured in the thymus. T-cells are responsible for cell mediated immune responses. The binding of an autoantigen to the T-cell receptor (TCR) leads to the activation of the cell resulting in subsequent production and secretion of cytokines which in turn activates various cells, such as NK cells, macrophages, B-cells, cytotoxic T-cells, and regulatory T-cells (Treg cells).

For a proper activation of T-cells the antigen has to be presented by another cell on a specialized cell surface display molecule, the MHC. The antigen presenting cell, APC, are for T-cells most commonly B-cells, macrophages and dendritic cells. The T-cell also requires an interaction of its co-receptor CD28 with co-stimulatory molecules (*e.g.*, CD80 and CD86) on the APC for a correct activation (Janeway, *et al*., 2005).

Sub-division of T-cells

T-cells are sub-divided into $CD4^+$ and $CD8^+$ T-cells. The $CD8^+$ T-cells carry the co-receptor CD8 that binds to the antigen presenting MHC class I molecule on APC's. This binding leads to activation of the T-cells which causes differentiation to $CD8^+$ cytotoxic T-cells.

The T-cells carrying the co-receptor CD4, *i.e.*, the $CD4^+$ T-cells, have a receptor which recognize antigens associated with the MHC class II molecules on the APC. On activation the $CD4^+$ T-cells are differentiated to T helper 1 (Th1), T helper 2 (Th2), and T helper 17 (Th17) cells, named

according to the type of cytokine they secrete and their transcription factor expression (Mosmann, *et al*., 1986).

Th1 cells are involved in cell-mediated immunity by activation of macrophages, complement fixation and opsonization. Activated Th1 cells mainly secrete the cytokines interferon-gamma (IFN-γ) and IL -2. Th2 cells, by secreting cytokines such as IL-4, IL-5 and IL-6 promotes the activation of B-cells into antibody producing plasma cells. Both cell types produce the cytokines TNF-α and granulocyte macrophage-colony stimulating factor (GM-CSF), though Th1 cells produce higher amounts than Th2 cells (Lafaille, 1998).

The Th17 cell lineage is a subtype of T-cell that secretes cytokines such as IL-17A, IL-17F, IL-21, and IL-22 (Harrington, *et al*., 2005). The activation of Th17 cells is dependent on IL-6 secretion from other cells like macrophages (Miossec, *et al*., 2009). Th17 cells have been proposed to play an important role in several human diseases, *e.g.*, cancer (Kryczek, *et al*., 2009), psoriasis (Kryczek, *et al*., 2008), MS (Matusevicius, *et al*., 1999), as well as RA (Chaubaud, *et al*., 1998).

Regulatory T-cells are considered to be suppressor cells that are important for immune tolerance by functioning as natural controllers of self-reactive T-cells and their deficiency can produce autoimmune disease (Takahashi and Sakaguchi, 2003). The Treg cells are characterized as being CD4+ T-cells that express the IL-2 receptor α (CD25) (Sakaguchi, *et al*., 1995) and the transcription factor FoxP3 that is critical for the development of Treg cells (Fontenot, *et al*., 2003). The role of these regulatory cells in the pathogenesis of RA and other chronic rheumatic diseases has been demonstrated in studies that show a higher number of Treg cells accumulated in the synovial fluid compared with peripheral blood in patients with RA (van Amelsfort *et al*., 2004; Cao, *et al*., 2004; Möttönen, *et al*., 2005). One study demonstrated that these regulatory cells were impaired in patients with RA and displayed a reduced ability to suppress the expression of pro-inflammatory cytokines (Ehrenstein, *et al*., 2004).

Central and peripheral tolerance

The development of lymphocytes must remain under tight control by different surveillance mechanisms in order to maintain those cells that respond to foreign antigens in an appropriate manner, while deleting the ones that respond to self antigens. Central tolerance is performed within the thymus where the TCR of T-cell progenitors binds to self-peptide presented

on an MHC molcule and depending on the affinity of the binding, T-cells are either deleted or maintained. T-cells with little or no affinity are neglected and will, therefore, die whilst cells with intermediate affinity for the self-antigen-MHC will undergo a process named **positive selection** and differentiate to mature T-cells that will enter the periphery.

Thymocytes that bind with high affinity to self-peptides are deleted by apoptosis in a process called **negative selection** (Rammensee and Bevan, 1984; Sebzda, *et al*., 1999). A number of self-reacting T-cells do overcome the negative selection in the thymus and enter the periphery and consequently it is of importance to also have mechanisms in the periphery to control these autoreactive cells. One of the peripheral tolerance mechanisms is performed by Treg cells as shown by experiments were mice depleted of these $CD25^+$ regulatory cells were prone to autoimmunity (Asano, *et al*., 1996).

Cytokines and chemokines

Cytokines are small proteins responsible for cell to cell communication, *i.e*., they are produced and secreted by one cell and affect the behaviour of other cells by binding to specific receptors. The binding of a cytokine to its receptor results in intracellular signaling cascades that may have diverse effects including up- or down-regulation of genes and transcription factors, production and secretion of other cytokines, as well as feedback inhibition of the cytokine itself. Cytokines are involved in processes such as cell growth, cell differentiation, tissue repair and regulation of immune responses.

Chemokines (an abbreviation of **chemo**-attractive cyto**kine**) are small proteins with chemoattractant properties that stimulate the migration and activation of cells, especially lymphocytes. Chemokines are systematically sub-divided according to the arrangement of cysteine residues, C, CC, CXC, or CX3C. They utilize their function by binding to different specific chemokine receptors on immune cells (Sallusto, *et al*., 2000).

Cytokines and chemokines in rheumatoid arthritis

Cytokines are involved in the pathogenesis of several autoimmune diseases. In RA, multiple cytokines are both expressed and active in synovial tissue and the fact that cytokine based therapies such as blockade of TNF-α show a significant effect in patients with RA proves the contribution to pathogenesis of such cytokines (Feldmann, *et al*., 1996; Feldmann and Maini, 2008). It is

not clear how or to what extent certain cytokines contribute to the different disease processes, such as, the induction of autoimmunity, the chronic inflammation, and the destruction of joints observed in patients with RA, although an imbalance of pro-inflammatory and anti-inflammatory cytokines is involved.

Some of the cytokines that have been suggested to have an involvement in RA are: TNF-α, IL-1β, -1α, -6, -7, -15, -17A, -17F, -18, -21, -23, -32, and -33 (Dinarello and Moldawer, 2002). Several of these have therapies directed towards them or involving their receptor, either as an established and approved drug (*e.g.*, TNF-α, IL-1Ra, and IL-6R) or are currently in clinical trial (*e.g.*, IL-17).

Rheumatoid arthritis has conventionally been considered to be a Th1 cell type mediated disease but lately the Th17 lineage has been implicated to be involved with the production of IL-17A, -17F, -21, -22, and TNF-α (Chabaud, *et al.*, 1998; Miossec, *et al.*, 2009). IL-17A together with TNF-α promotes the activation of fibroblasts and chondrocytes (McInnes and Schett, 2011). The secretion of IL-17 enhances secretion of other cytokines, increases the production of cartilage destructive enzymes, such as the MMPs and the receptor activator of NFκB ligand (RANKL) that is involved in osteoclast activation and bone destruction (Sato, *et al.*, 2006). In a two-year prospective study Kirkham and colleagues (2006) showed that the expression of TNFα, IL-1, and IL-17 from the synovial membrane was predictive for joint destruction.

In 2005, Raza *et al.*, found that samples of synovial fluid from patients with early RA (<12 weeks after the onset of symptoms) were characterized by a transient cytokine profile with high concentrations of IL-1, -2, -4, -13, -15, -17, basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF) compared with other arthritides, this profile was no longer present in established disease (Raza, *et al.*, 2005). Also, an extensive analysis of cytokines and cytokine-related markers in serum samples from individuals before the onset of RA showed increased concentrations of IL-1α, -1β, -1 receptor antagonist (Ra), -4, -10, TNF-α, and soluble TNF-receptor I up to five years before the diagnosis of RA; after disease onset there was an up-regulation of several more cytokines (Jørgensen, *et al.*, 2007).

The role of chemokines in RA is believed to be the trafficking of immune cells to the joints, utilizing their expression of the specific chemokine receptors required for migration (Vergunst, *et al.*, 2005). Therapies directed at blocking the chemokine receptor CCR1 induced a clinical improvement in a study of patients with RA, thus supporting the role of chemokines in RA

(Haringman, *et al*., 2003). Conversely, in another study no effect of blocking the CCR1 receptor was observed (Vergunst, *et al*., 2009).

Interleukin-8 (CXCL8), which is a potent chemoattractant for neutrophils and T-cells, is one of the chemokines that has been suggested to be involved in the pathogenesis of RA and is abundantly present in both synovial tissue and synovial fluid from patients (Endo, *et al*., 1991; Deleuran, *et al*., 1994). Other chemokines implicated to be involved in this disease include monocyte chemoattractant protein (MCP) -1/CCL2 (Koch, *et al*., 1992), which was increased in ACPA positive individuals years before the diagnosis of RA (Rantapää-Dahlqvist, *et al*., 2007). Additionally, the chemokines: regulated activation normal T-cell expressed and secreted (RANTES/CCL5) (Volin, *et al*., 1998), macrophage inflammatory protein (MIP)-1α/CCL3 (Koch, *et al*., 1994), monokine induced by interferon-γ (MIG/CXCL9), and fractalkine/CXC3CL1 (Iwamoto, *et al*., 2008) among others, have also shown to be enhanced in RA.

Autoantibodies

A characteristic of RA as well as other autoimmune diseases is the presence of various antibodies specific for self-antigens, *i*.e., the so-called autoantibodies.

Autoantibodies that have been found in sera or synovium in patients with RA *ex vivo* include rheumatoid factor (RF), various anti-citrullinated protein/peptide antibodies (ACPA), antibodies against the immunoglobulin binding protein (BiP/p68), anti-calpastatin, anti-RA33, anti-neutrophil cytoplasmic antibody (ANCA), and anti-nuclear antibody (ANA) (reviewed by Rantapää-Dahlqvist, 2005). However, many of these can also be present in other autoimmune diseases and have lower sensitivities and specificities when compared with RF and ACPA.

Rheumatoid factor

The most well-known autoantibody for RA is RF, which is found in approximately 70% of patients with established RA. This autoantibody is routinely measured by agglutination of human- or sheep erythrocytes using Waaler-Rose, latex test or nephelometry, methods that do not discriminate between the various isotype sub-classes of RF. Using enzyme-linked immunosorbent assays, (ELISA) different isotypes of RF can be differentiated (Jonsson, *et al*., 1986). RF, which is generally of the IgM

isotype, is directed against the Fc portion of the IgG antibody. In the inflamed joint, RF can form immune complexes capable of complement fixation and induction of the release of chemotactic factors that attract immune cells to the site of inflammation. The drawback of detecting RF is that even though the sensitivity is relatively high the specificity is low as it is also present in healthy individuals at a frequency of approximately 5%, which increases in an age-dependent manner (Husby, *et al.*, 1988; Palosuo, *et al.*, 2003). Furthermore, in patients with other inflammatory conditions such as Sjögren's syndrome and systemic infections, RF can be detected at higher concentrations when compared with healthy individuals (Newkirk, 2002).

Individuals who are positive for RF at diagnosis of RA often have a disease course that has been shown to be less favorable compared with RF negative patients (van der Heijde, *et al.*, 1992; Combe, *et al.*, 1995; Sihvonen, *et al.*, 2005). The presence of the IgM isotype of RF has been shown to precede the onset of RA by several years (Aho, *et al.*, 1985) as does the IgA isotype that was demonstrated to predict the development of RA (Rantapää-Dahlqvist, *et al.*, 2003). Moreover, having IgA RF at onset of disease was associated with a worse radiological outcome later in disease (Berglin, *et al.*, 2005).

Anti-citrullinated protein/peptide antibodies

In 1964 and 1979, antibodies against perinuclear factor (APF) and anti-keratin antibodies (AKA), respectively, were described as being more specific for RA compared with RF (Nienhuis and Mandema, 1964; Hoet, *et al.*, 1991, Young, *et al.*, 1979). Since, these autoantibodies were shown to be directed at the autoantigen filaggrin they were termed anti-filaggrin antibodies (AFA) (Simon, *et al.*, 1993; Sebbag, *et al.*, 1995). In 1998, Schellekens *et al.*, found that the antigen that was recognized by AFA contained citrulline residues that had been post-translationally modified from arginine by the enzyme PAD (Vossenar, *et al.*, 2003). Schellekens *et al.*, (2000) constructed an antibody test directed against cyclic citrullinated peptides referred to as anti-cyclic citrullinated peptide (anti-CCP2) antibody test. All antibodies directed against citrullinated peptides or proteins (*e.g.*, fibrinogen and vimentin) are denoted ACPA (anti-citrullinated protein/peptide antibodies).

Citrulline is a non-standard amino acid resulting from the post-translational modification of arginine by PAD in the presence of high concentrations of calcium ions (Ca^{2+}). Citrullination or deimination is a naturally occurring process that is described as being involved in cell differentiation, programmed cell death (apoptosis), and also has a role in the generation of structural tissues like skin, hair follicles, and the myelin sheats of nerve

fibres (Baka, *et al*., 2012). The citrullination of proteins is not specific for RA as it also occurs in other inflammatory conditions, such as MS (Moscarello, *et al.*, 1994) and Alzheimer's disease (Ishigami, *et al*., 2005). In psoriasis the levels of citrullinated proteins have been shown to be down-regulated (Ishida-Yamamoto, *et al*., 2000). Additionally, in nephritis tissues from patients with SLE, the expression of inducible nitric oxide synthase (iNOS) that is associated with inflammation correlated with the presence of citrulline (Bollain-y-Goytia, *et al*., 2009). Instead, it is the presence of autoantibodies against the particular citrullinated proteins that appears to be specific for RA, with an abnormal humoral response to the citrullinated proteins (Vossenaar, *et al*., 2004a).

It has been hypothesized that, when inflammatory cells migrate to the joints in patients with RA, the activation of PAD enzymes stimulates apoptosis of the cells of the joints by marking them for degradation utilizing citrullination. In certain genetically predisposed individuals the cleareance of the citrullinated proteins is abnormal, thus citrullinated proteins can remain and result in the production of autoantibodies against these proteins, thus initiating an inflammatory response (Van Venrooij and Pruijn, 2008).

To date, five different sub-types of PAD have been identified in man (PAD1-4, and -6) with different distributions in the tissues as well as different localization in cells. The sub-types of PAD enzymes involved in the citrullination of peptides in the synovium in RA are believed to be PAD2 expressed from macrophages and PAD4 from granulocytes (Vossenar, *et al*., 2003).

Several citrullinated proteins have been identified in the inflamed synovial tissue or sera from patients with RA. These include vimentin, a protein found in mesenchymal cells and macrophages (Vossenar, *et al*., 2004b), α-enolase (Kinloch, *et al*., 2008), collagen type II (Burkhardt, *et al*., 2005), and the α- and β-chains of fibrinogen (Masson-Bessiére, *et al*., 2001). However, the role in pathogenesis of RA played by these citrullinated proteins is as yet not fully understood.

It has been suggsted that the citrullinated form of vimentin is involved in the recruitment of T-cells for the response of ACPA producing B-cells. In 2010, Feitsma and co-workers identified for the first time CD4+ T-cells that were reactive against epitopes on citrullinated vimentin in HLA-DRB1*0401 positive patients with RA. Recently, reactivity against citrullinated vimentin was observed both in patients and healthy individuals carrying the HLA-DRB1*0401 allele, but with a unique pattern for a pro-inflammatory cytokine

response restricted to the CD4+ T-cells derived from patients with RA (Snir, *et al.*, 2011).

The first available ELISA that had been developed to detect ACPA used a synthetic cyclic citrullinated peptide derived from the sequence of human filaggrin; this test is referred to as anti-CCP1 and has a sensitivity of 68% with a specificity of 98% (Schellekens, *et al.*, 2000). To increase the sensitivity a second generation test (anti-CCP2) was developed by screening a pool of sera from patients with RA for a library of citrullinated peptides and utilized those that showed the highest reactivity; this approach increased the sensitivity to 75-80% (van Venrooij, *et al.*, 2002). Anti-CCP2 tests are used more and more frequently in the diagnosis of RA as well as in clinical studies. A third generation test, anti-CCP3, has also been developed. The anti-CCP2 and –CCP3 tests have the same specificity as the original CCP1 test but with increased sensitivities (dos Anjos, *et al.*, 2009). A number of tests that detect the presence of autoantibodies recognizing specific citrullinated proteins, like vimentin (anti-MCV) and α-enolase, have also been developed (Dejaco, *et al.*, 2006; Wagner, *et al.*, 2009; Lundberg *et al.*, 2008).

Anti-citrullinated protein/peptide antibodies have been shown to be present at all stages of RA: pre-disease, early disease and established disease. In samples from pre-diseased individuals, ACPA could be detected up to 14 years before the onset of RA (Rantapää-Dahlqvist, *et al.*, 2003; Nielen, *et al.*, 2004) and an age-relationship has been suggested with appearance of pre-clinical ACPA in older individuals long before the development of clinical symptoms whilst in younger individuals ACPA appear closer to the clinical onset of RA (Majka, *et al.*, 2008).

ACPA in combination with the genetic risk variants HLA-SE or *PTPN22* 1858T are strongly associated with future onset of RA, the latter with a 100% specificity for the disease (Berglin, *et al.*, 2004; Johansson, *et al.*, 2006)

Several studies have shown that the presence of ACPA is a powerful predictor of disease course and having ACPA is strongly associated with joint damage with higher concentrations of these autoantibodies correlating with erosive RA (Forslind, *et al.*, 2004; van Gaalen, *et al.*, 2005; Berglin, *et al.*, 2006). Also, a higher level of ACPA at diagnosis predicts greater disease activity for the following years of disease (Kastbom, *et al.*, 2004). Additionally, the presence of ACPAs in individuals with the genetic risk factor HLA-SE either in one or two copies had a significantly higher risk for a severe disease (van Gaalen, *et al.*, 2004).

Generally, the amount of ACPA that is measured is the total level of the IgG isotype. In 2006, Verpoort *et al.* showed that in individuals with undifferentiated arthritis (UA) who developed RA compared with those who did not had a complete usage of IgM-, IgA- and sub-classes of IgG (G1-G4) ACPA early in the disease course which was not observed in UA. Also, having more than four different isotypes (including the different sub-classes of IgG) yielded a higher risk of developing RA compared with having less than three (Verpoort, *et al.*, 2006). The number of different isotypes has also been related to long-term radiographic progression in ACPA positive RA patients with more isotypes yielding a higher risk for radiographic destruction (van der Woude, *et al.*, 2010). Furthermore, the occurrence of IgA ACPA has been reported to be associated with smoking in patients with RA (Svärd, *et al.*, 2008).

To understand the pathogenesis leading to disease development in RA the presence of citrullinated proteins functioning as autoantigens and the distribution of these in the inflamed tissues such as the joints, is of fundamental importance.

Identified biomarkers prior to onset of symptomatic RA

To summarize, results from multiple studies on individuals sampled before the onset of symptoms of RA increased frequencies and levels of several different biomarkers have been demonstrated. These biomarkers are RF, ACPA, CRP, secretory phospholipase A^2, a broad range of different cytokines and chemokines involved in several different aspects of the immune system, as well as changes of the lipid profile, together revealing possible pathways in the development of a disease such as RA (Aho, *et al.*, 1991; Rantapää-Dahlqvist, *et al.*, 2003, Nielen, *et al.*, 2004a; Nielen, *et al.*, 2004b; Rantapää-Dahlqvist, *et al.*, 2007; Jørgensen, *et al.*, 2008; Karlson, *et al.*, 2009; Deane, *et al.*, 2010; Nielen, *et al.*, 2006). The biomarkers clearly indicate that the development of RA is a gradual process over many years.

Aims

The overall aim of this thesis was to identify potential predictors for the development of and disease progression of RA.

The specific aims were the following:

– To analyze whether there is an activation of the immune system, as measured by inflammatory markers, prior to the onset of symptoms of RA.

– To evaluate the prevalence and predictive values of anti-citrullinated protein/peptide antibodies (ACPA) of IgG, IgA and IgM isotype for the development of RA.

– To investigate the association of the genetic factors, *protein tyrosine phosphatase non- receptor type 22* (*PTPN22*) 1858T and HLA- shared epitope (SE), in relation to autoantibodies and smoking habits, for the risk and severity of RA.

– To evaluate the predictive value for disease progression of RA for different ACPA and their relation to the genetic factors, *PTPN22* 1858T and HLA-SE.

Study populations and methods

Study populations

In these studies two different cohorts were included: firstly, a case-control cohort from the Medical Biobank of Northern Sweden comprising individuals before the onset of any symptoms of joint disease (referred to as "pre-patients") and controls matched to the pre-patients according to sex, time point of blood sampling, age at the time of blood sampling, and area of residence (urban or rural).

The second case-control cohort utilized in the study included individuals from the four northern-most counties of Sweden with early diagnosed RA (symptom duration ≤12 months), and population-based controls from the Medical Biobank of Northern Sweden.

The Medical Biobank of Northern Sweden consists of The Northern Sweden Health and Disease Study (NSHDS) cohort, which includes the following three sub-cohorts:

- The *Västerbotten Intervention Programme* (VIP), a long term project among the population of Västerbotten intended for health promotion. Since 1985, all individuals 40, 50, and 60 years of age are invited to participate in the project. The participants are asked to complete a questionnaire and donate a blood sample to the Medical Biobank for future research. Additional samples are collected at intervals of ten years.

- The Northern Sweden part of the *World Health Organization* (WHO) study for *Monitoring of Trends and Determinants in Cardiovascular Disease* (MONICA), which includes individuals from the counties of Norrbotten and Västerbotten (approximately 400,000 inhabitants) that are invited for cardiovascular screening (screening performed 1986, 1990, 1994, and 1999).

- The Mammary Screening Project comprising women from Västerbotten in the age range 50 to 69 years. The cohort has an annual recruitment of approximately 7,000 women included from mammographic screening since 1995. In this cohort sampling is performed every second year.

The NSHDS cohort currently includes 210,500 blood samples from 131,000 individuals in the age range of 25 to 74 years. Individuals included in the

NSHDS cohort completed a questionnaire regarding demographic, medical and lifestyle information such as smoking.

The Maternity cohort started in 1975 is composed of samples from pregnant women screened for rubella (*i.e.*, German measles) within the general healthcare from the four northern-most counties of Sweden and includes approximately 70,000 serum samples.

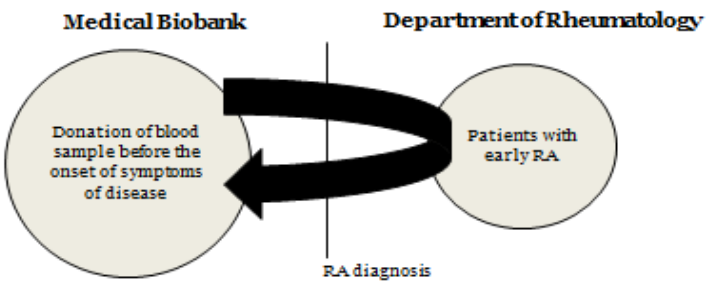
Identification of individuals sampled before any symptoms of joint disease

To identify individuals who had donated blood samples to the Medical Biobank prior to the onset of symptoms of joint disease, the registers from the NSHDS and the Maternity cohort were co-analyzed with the registers of patients with early RA at the Department of Rheumatology at the University Hospital in Umeå (illustrated in Figure 1). All of the patients who fulfilled the 1987 ACR classification criteria for RA (Arnett *et al*., 1988) and had a known date for disease onset were included in the co-analysis.

The co-analysis within the NSHDS cohort was restricted to patients with disease onset after 1985 (*i*.e., when the NSHDS cohort was started) and the co-analysis within the Maternity cohort was restricted to female patients of fertile age (≤ 45 years) and disease onset after 1975 (*i.e.*, when the Maternity cohort was started).

In total, 92 individuals that had donated blood samples before the onset of any symptoms of joint disease were identified. The median sampling time before disease onset was 2.4 years (inter quartile range (IQR) 1.2–4.9 years) and the median time to diagnosis of RA after symptom onset was 7.8 months (IQR 5.0–10.0 months). For every pre-patient, four controls were randomly selected from within the same registers of the Medical Biobank of Umeå and matched for sex, age at the time of blood sampling, time point of blood sampling, and for rural or urban residence. The total number of control subjects was 368. The number of individuals included in the separate studies is shown in Table 2. To date, this cohort includes 406 individuals, who have donated blood samples to the Medical Biobank prior to the onset of symptoms of RA.

Figure 1. *Illustration of the strategy used for the co-analysis performed to identify individuals who had donated blood samples to the Medical Biobank of Northern Sweden before the onset of symptoms of RA and later attended the Department of Rheumatology at the University Hospital.*



Patients with early RA

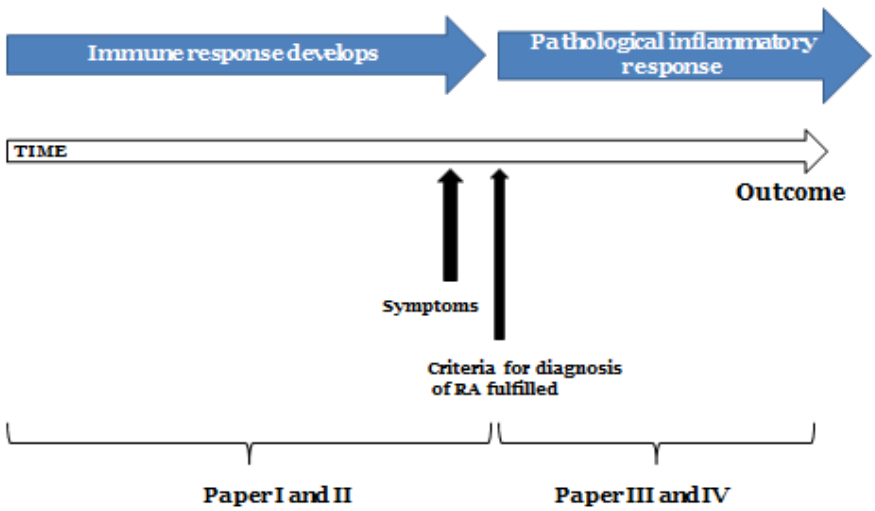
The cohort of patients with early RA (*i.e.*, having a disease duration of ≤ 12 months) consists of patients fulfilling the ACR classification criteria for RA from the four northern-most counties of Sweden.

Patients are consecutively included to this early RA project and currently includes approximately 1,000 individuals. All patients are included in the nationwide Swedish Rheumatoid Arthritis Registry.

Pre-patients, patients and controls paper I-IV

In Figure 2 the course of the development of RA is illustrated with a overview on the time point in the pathogenesis that the different cohorts of individuals in the various papers were included.

Figure 2. *Overview on the course of development of disease and the time point that the individuals in different papers were included.*



Data on the distribution of pre-patients, patients and controls for the different papers is presented in Table 2.

All individuals included in this thesis gave their written informed consent and the Regional Ethics Committee at the University Hospital, Umeå, approved these studies.

Table 2. *Demographic data on the subjects included in the different papers.*

	Paper I	Paper II	Paper III	Paper IV
Pre-patients (n)	86	71	85	-
Female %	76	80	75	-
Age at sampling (mean, years; range)	52 (30-69)	49 (20-67)	53 (30-69)	-
RA patients (n)	69	60	505	210
Female %	75	78	68	69
Age at disease onset (mean, years; range)	56 (34-72)	54 (28-68)	55 (18-87)	54 (18-84)
Symptom duration prior to diagnosis (months, mean)	7.7	7.0	7.8	6.4
Controls (n)	256	276	970	102
Female %	76	80	73	69
Age at sampling (mean, years; range)	52 (30-69)	50 (19-69)	57 (25-79)	59 (30-88)

Methods

Measurement of cytokines and chemokines

Thirty cytokines and chemokines were measured in blood samples using multiplex detection kits from Bio-Rad Laboratories Inc (Hercules, CA). The factors measured were: IL-1β, IL-1Ra, IL-2, IL-2Rα, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, IL-15, IL-17, eotaxin, bFGF, granulocyte-colony stimulating factor (G-CSF), GM-CSF, IFN-γ, interferon inducible protein (IP)-10, MCP-1, MIP-1α, MIP-1β, platelet derived growth factor (PDGF)-BB, RANTES, TNF-α, vascular endothelial growth factor (VEGF), MIG, and macrophage migration inhibitory factor (MIF). All samples were assayed in duplicate and analyzed using a Bio-Plex Array Reader (Luminex^{200} Labmap™ system). Data analyses were performed with the Bio-Plex Manager software version 4.1.1 (Bio-Rad). The cytokines were grouped

according to their main function in the immune system or their origin (Table 3).

Table 3. *Grouping of the different cytokines, cytokine related factors, and chemokines analyzed according to their main function.*

General activation	Th1	Th2	Th17	Treg	Bone marrow derived	Stromal cells and angiogenic factors	Chemokines
IL-1β	IL-12	IL-4	IL-17	IL-10	IL-7	b-FGF	MIF
IL-1Ra	IFNγ	IL-5			GM-CSF	PDGF-BB	MIG
IL-2Rα		IL-9			G-CSF	VEGF	IL-8
TNFα		IL-13					IP-10
IL-6		Eotaxin					MCP-1
IL-2							MIP-1α
IL-15							MIP-1β

As RF of the IgM isotype is suggested to cause false-positive results in immunoassays (de Jager, *et al.*, 2005; Todd *et al.*, 2011) several different strategies for blocking non-specific binding by RF was tested. A combination of 40% mouse serum, 20% goat serum, and 20% rabbit serum was tested, as well as Heteroblock (Omega Biologicals, Bozeman, MT), and/or ProteinL (Pierce, Rockford, IL), but none of the different approchaes produced reliable or reproducible results. Furthermore, the effect of RF cross-binding activity was tested by using different specificities of capture and detection antibodies (anti-IL-2-coated beads with anti-eotaxin antibodies) with plasma from patients known to display high concentrations of RF, but no signals were detected. Consequently, no blocking agent was added to the samples prior to analyzes.

Genotyping of PTPN22 1858C/T and HLA-DRB1

Genomic DNA was purified from EDTA-treated blood from pre-patients, patients, and controls using the standard salting out procedure (Miller, *et al.*, 1988).

HLA-DRB1 genotyping for shared epitope (SE) alleles was performed using polymerase chain reaction (PCR) sequence specific primers from a DR low resolution kit and DRB1*04 subtyping kit from Dynal (Oslo, Norway), and Olerup SSP AB (Saltsjöbaden, Sweden). The SE alleles were defined as DRB1*0401 and DRB1*0404.

The *PTPN22* 1858C/T polymorphism (rs2476601) was determined using the 5´nuclease assay as previously described (Johansson, *et al*., 2006). All PCR's were performed according to the manufacturer's instructions (Applied Biosystems, Foster City, CA, USA). The different genotypes were determined using an ABI PRISM 7900HT Sequence Detector System and the data was processed with the SDS 2.1 software (Applied Biosystems, Foster City, CA, USA).

Autoantibody analyses

In paper I and II, ACPA were measured in samples using the anti-CCP2 enzyme immunoassay from EuroDiagnostica (Arnhem, the Netherlands) the cut-off value for positivity being 25 units/mL (U/mL). ACPA were analyzed using the Diastat kit (anti-CCP2) from Axis-Shield Diagnostics (Dundee, UK) with a cut-off value for positivity of 5 U/mL as recommended by the manufacturer in paper III and IV. The two methods have previosuly been tested on the same samples (n=100) and showed 100% concordance for positivity according to the separate cut-off (personal communication from S. Rantapää-Dahlqvist).

Additionally, in paper II ACPA (analyzed as anti-CCP2) of IgG, IgA and IgM isotype were determined using EliA anti-CCP assay on ImmunoCAP 250 (Phadia Diagnostic AB, Uppsala, Sweden), according to the manufacturer's instruction. Within-study reference ranges were defined by receiver operating characteristic (ROC) curves based on the studied population in paper II and the cut-off for positivity were based on the concentrations giving the most optimal sum of sensitivity and specificity, and accordingly were: 15.0 U/mL for IgG, 2.5 U/mL for IgA and 90.0 U/mL for IgM.

In paper IV, QuantaLite™ anti-CCP3 using an ELISA for IgG (3rd generation antigen; Inova Diagnostics, San Diego, CA, USA), and for anti.CCP3.1 (QuantaLite™, Inova) detecting both IgG and Ig A was used. Anti-MCV antibodies were measured using the Orgentec 548 Anti-MCV ELISA (Orgentec Diagnostics, GmbH, Mainz, Germany). A cut-off of 20 U/mL for was used for all three tests.

Rheumatoid factors of the IgM, IgA, and IgG isotype were determined in serum or plasma samples in duplicate using an in-house ELISA (Department of Immunology, Karolinska University Hospital, Huddinge, Sweden). In paper III and IV the IgM isotype was determined using the agglutination test as originally described according to Waaler-Rose.

Standard serological analyses

C reactive protein (CRP; mg/L) and ESR (mm/h) were measured in patients with RA at baseline and at a regular basis of every six months up to 24 months.

Measures of disease activity and outcome

The disease activity score for 28 joints (DAS28) was calculated at baseline, and every six months up to 24 months. The score includes the number of tender and swollen joints, the patient's global health visual analogue scale (VAS), and ESR. The area under the curve (AUC) at 24 months was calculated utilizing the DAS28 values for the different time points, baseline, 6, 12, 18, and 24 months (Matthews, *et al.*, 1990).

Radiographs were evaluated according to the Larsen score (Larsen, 1995) by comparison of anterior-posterior radiographs of the hands, wrists and fore-feet in frontal projection with standard reference radiographs by two rheumatologists conversant with this protocol. The assessment included 32 joint areas: metacarpophalangeal (MCP) II-V, proximal interphalangeal (PIP) II-V, four areas in each wrist, and metatarsophalangeal (MTP) II-V. Each area was graded from 0-5 based on the degree of destruction, yielding a maximum score of 160.

Statistics

Statistical calculations were performed using SPSS for Windows versions 14.0-17.0 (SPSS, Chicago, IL, USA) and the EPI Info Software version 5 (EPI info, Center of Disease Control, Atlanta, GA, USA).

The Chi-square test was used for testing categorical data between groups or Fisher's exact test when appropriate. Continuous data were analyzed using the Student's t-test for independent samples and paired t-test for related samples when applicable. For differences in continuous data for unrelated samples not normally distributed the non-parametric Mann-Whitney U test was used. Related samples were analyzed with the Wilcoxon's signed rank test for matched pairs (pre-patients versus patients) and conditional logistic regression analyses (pre-patients versus matched control subjects). Stratified data were compared with simple logistic regression adjusted for age and sex. Variations of continuous data over time within and between groups were analyzed with the Kruskal-Wallis test (for three groups or more). In paper I,

calculations were performed using the logarithm of the measured concentrations since the observed concentrations of the different factors were not normally distributed. Odds ratios (OR) were calculated with a 95% confidence interval (95% CI). All *P* values are two-sided, and *P* values less than or equal to 0.05 were considered significant.

In paper I, Random Forest modeling was used for multi-dimensional scaling to demonstrate clustering of groups (randomforest 4.5-25, R-project).

Calculations of sensitivity and specificity for the different cytokines and chemokines in paper I were based on cut-off values for positivity if concentrations were above the 95th percentile of the concentrations in control subjects as previously described (Jørgensen, *et al*., 2008).

In paper II and IV, ROC-curves were constructed for each ACPA isotype and the different types of ACPA, respectively.

In paper IV, variations over time between groups were assessed by analysis of variance for repeated measurements using StatView v 4.51 (Abascus Concepts, Berkely, CA, USA). Backward logistic regression analyses were used to estimate the OR's for radiological progression at 24 months.

In paper IV, positive likelihood ratios (LR) were calculated using the constructed ROC curves and at a given specificty of 98% for all tests.

Results and Discussion

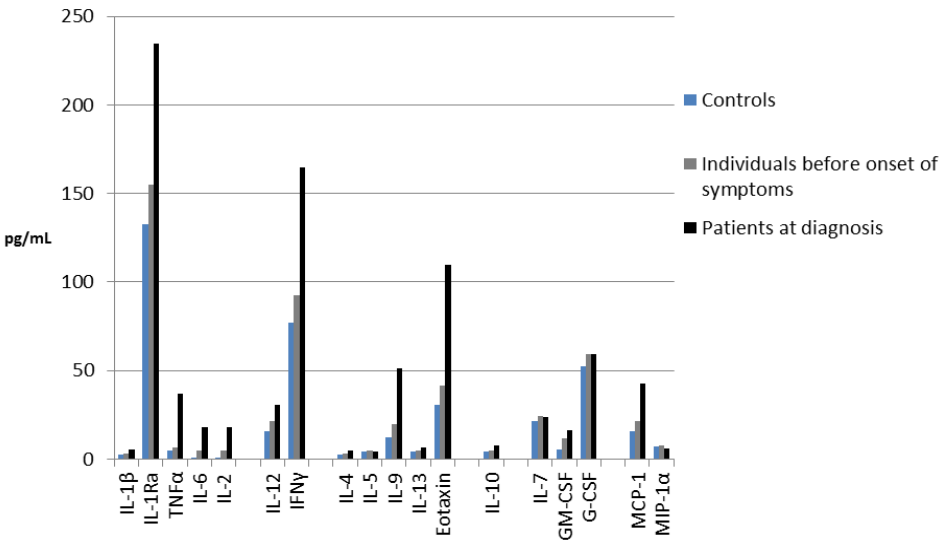
Paper I

Up-regulation of cytokines and chemokines predates the onset of rheumatoid arthritis

In order to identify wheter there were any signs of an activation of the immune system besides presence of ACPA in individuals before they presented with any symptoms of joint disease, *i.e.,* in the pre-disease state, the concentrations of a number of cytokines, cytokine-related markers and chemokines related to different aspects of the immune system were analyzed. These factors were measured in blood samples from 86 individuals before the onset of symptoms of RA (*i.e.*, pre-patients), 69 of these individuals when diagnosed with RA, and 256 control subjects matched to the pre-patients, using a multiplex bead-based system.

Conditional logistic regression analysis performed on the logarithm of the measured concentrations showed that in individuals who later developed RA there was a statistically significant increase in several of the analyzed factors. These included markers of general activation, factors related to Th1 cells, Th2 cells, Treg cells, bone marrow derived, and chemokines. Most of the factors identified were further increased when these individuals were diagnosed with RA (Figure 3). These cytokines represent several aspects of the immune system, and both anti-inflammatory and pro-inflammatory immunological responses.

Figure 3. *Cytokines, cytokine related factors, and chemokines significantly increased in individuals prior to the onset of symptoms of RA compared with controls, the levels (median concentration) of the factors at diagnosis of RA are also shown.*



The sensitivitiy and specificitiy for the development of RA in individuals before the onset of symptoms of RA compared with controls for the different cytokine factors (defined as being positive if the mean concentration was above the 95th percentile of the value in control subjects) as well as for the autoantibodies, IgM RF and ACPA, were calculated (Table 4). The highest sensitivity among the cytokines for predicting RA was for eotaxin, with a sensitivity of 22.4%, followed by IL-1Ra, IL-2, GM-CSF, and IFNγ, all with a sensitivity of 18.8 % (Table 4).

The most prominent cytokine-group that was significantly associated with ACPA positivity among pre-patients was that related to Th2 cells (χ^2=14.6, P<0.0001), followed by factors associated with general immune activation (χ^2=7.1, P<0.01), Th1 cell related (χ^2=5.6, P<0.05), and Th17 cell related (χ^2=4.06, P<0.05) cytokines.

Table 4. *Sensitivity and specificity of the various factors for the development of RA in the pre-patients compared with matched controls.*

Factor	Sensitivity	Specificity	OR	95% CI	Group
ACPA	38.7	98.6	41.4	12.2-140.6	Autoantibody
RF	27.4	94.0	5.9	2.66-13.16	Autoantibody
Eotaxin	22.4	95.3	5.8	2.70-12.62	Th2 cell related
IL-1Ra	18.8	95.3	4.7	2.12-10.40	General activation
IL-2	18.8	95.3	4.7	2.12-10.40	General activation
GM-CSF	18.8	95.3	4.7	2.12-10.40	Bone marrow derived
IFNγ	18.8	95.3	4.7	2.12-10.40	Th1 cell related
IL-4	17.6	95.3	4.3	1.94-9.70	Th2 cell related
IL-9	17.6	95.3	4.7	2.08-10.80	Th2 cell related
TNFα	16.5	95.3	4.0	1.77-9.02	Th2 cell related
IL-12	16.5	95.3	4.0	1.77-9.02	Th1 cell related
IL-10	16.5	95.3	4.0	1.77-9.02	Treg cell related
IL-1β	16.5	95.3	4.0	1.77-9.02	General activation
IL-6	15.3	95.3	3.6	1.60-8.36	General activation
MCP-1	14.3	95.3	3.8	1.47-9.56	Chemokine
IL-15	10.6	95.3	2.6	1.05-6.55	General activation
IP-10	10.6	95.7	2.6	1.05-6.55	Chemokine

In samples obtained from patients at the time of their RA diagnosis, the concentrations of all analytes except of IL-17, MIF, MIP-1α, and bFGF were significantly increased compared with control subjects. Comparison of matched pairs, *i.e.*, the same individual before onset of symptoms of joint disease and at the time of a diagnosis of RA (n=69) the concentrations of most of the analyzed variables further increased with the exception of TNFα, IL-5, IL-7, IL-13, GM-CSF, G-CSF, bFGF, MIP-1α, MIF, and IL-17. Interestingly, there was actually a significant decrease in the concentration of IL-17 in sera after disease onset compared with the samples obtained prior to onset of any symptoms of disease.

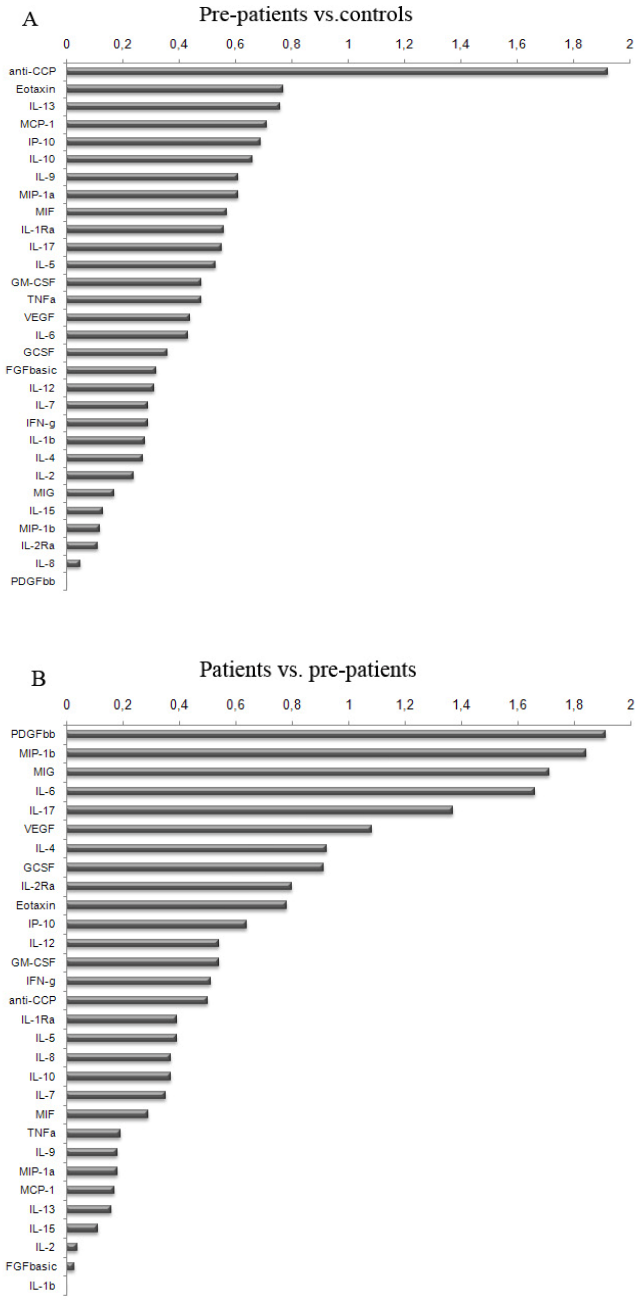
Stratifications for ACPA and RF status (positive or negative according to established cut-off values) in pre-patients compared with control subjects with relation to cytokine concentrations showed that in ACPA positive individuals (n=32) IL-2Rα, was in addition to the previous associations shown in Figure 3, significantly increased ($P<0.01$). In ACPA positive patients with RA (n=47), almost all factors except for G-CSF, bFGF, IL-8, MIP-1α, and MIP-1β were significantly up-regulated compared with those of control subjects and many of these remained significant in ACPA negative patients, the exceptions being IL-2, IL-15, IL-5, IL-9, IL-13, IL-10, IL-7, GM-CSF, and MIG ($P>0.05$).

In RF positive pre-patients (n=23) the levels of IL-2Rα, IL-5, IL-9, IL-13, IL-17, and MIG were additionally significantly increased compared with controls whereas the significance for TNFα and IL-7 was lost. In pre-patients negative for RF (n=61) almost no factor was significantly increased compared with control subjects whereas in samples from patients after the onset of disease who were negative for RF (n=12) the concentrations of IL-6, eotaxin, MIG, IP-10, GM-CSF, PDGF-BB, and TNFα were significantly increased. All markers except IL-7, IL-8, G-CSF, IP-10, bFGF, MIP-1α, MIP-1β, and MIF were significantly increased in patients with RF (n=57) compared with controls.

When stratifying the samples obtained prior to disease on the basis of being collected either more or less than three years before the onset of RA it observed that several factors were significantly increased if collected closer to disease onset. The factors included: IL-1β, IL-2Rα, IL-9, eotaxin, IL-10, GM-CSF, bFGF, IP-10, and MIG.

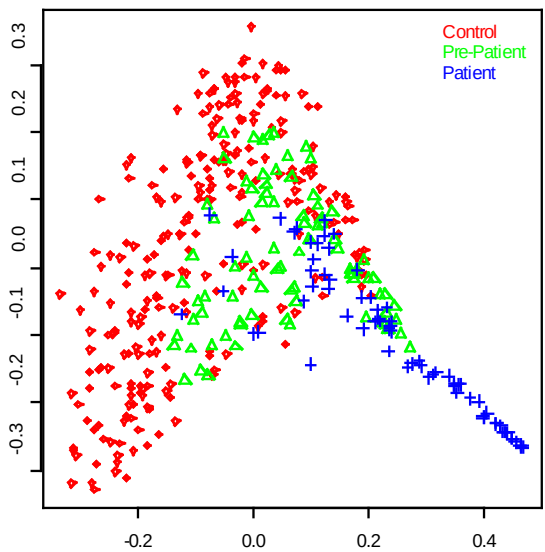
Random Forest modeling was used to determine the relative importance for each factor analyzed in classifying between the different groups: pre-patients, patients and control subjects (Figure 4A and 4B). The importance of the factors in discriminating pre-patients from control subjects as shown in Figure 4A indicates that following ACPA the most important factors were eotaxin, IL-13, MCP-1, IP-10, and IL-10. Pre-patients were distinghuished from patients mainly by PDGF-BB, MIP-1β, MIG, IL-6, and IL-17 (Figure 4B). The factors that best distinguished patients after disease onset from control subjects using this modeling were ACPA, PDGF-BB, IL-6, MIP-1β, MIG, eotaxin, IL-10, and G-CSF (data not shown).

Figure 4. *The order of analyzed factors according to their ability to discriminate pre-patients from control subjects (Panel A) and patients from pre-patients (Panel B).*



Multidimensional scaling using a summary of the Random Forest modeling including all factors analyzed demonstrated clustering of the different groups: control subjects, pre-patients, and patients (Figure 5). Performing Random Forest modeling with only ACPA in the model gave a high specificity of 98.8% and a sensitivity of 36.9% for predicting the development of RA, whilst inclusion of all analyzed factors in the model increased the sensitivity to 51.2% with a specificity of 91.9%.

Figure 5. *Visualization of the multi-dimensional scaling demonstrating clustering of control subjects, pre-patients, and patients. The axes represent the dominant clustering directions between the groups scaled as proximities.*



Discussion of paper I

This study demonstrated that several cytokines, cytokine related factors and chemokines are up-regulated in individuals several years prior to the onset of RA, which is further increased and immunologically more widespread after the onset of RA when compared with control subjects. The most prominent up-regulated markers before the start of symptoms of disease are factors related to Th2 cells (eotaxin, IL-4, IL-9), Th1 (IL-12, IFNγ), and also the Treg cell related IL-10; all factors that are mainly representive of the adaptive immune system. Also, many of these factors were among those that best distinguished pre-patients from control subjects in Random Forest modeling. The increases in the concentration of IL-4, IL-10 and IFNγ are consistent with a Norwegian study performed using serum collected from

blood donors prior to the diagnosis of RA (Jørgensen, *et al.*, 2008). Recently another study, reported by Deane and co-workers, also demonstrated an up-regulation of IL-10 in sero-positive samples from individuals before the onset of clinical RA (Deane, *et al.*, 2010). A role for IL-4 in the early phase of RA has also been suggested following analysis of synovial fluid samples from individuals with early arthritis (Raza, *et al.*, 2005).

Samples from the individuals at the time of diagnosis with RA were discriminated from their corresponding pre-patient samples mainly by chemokines (*e.g.*, MIG and IL-8) and stromal cell and angiogenic related factors (PDGF-BB and VEGF). Additionally, confirmed patients had significantly increased levels of almost all analytes compared with controls reflecting a more general and widespread activation of the immune system after the onset of disease.

In this study, IL-17, which has been suggested to be involved in the pathogenesis of RA via several different processes (reviewed by McInnes and Schett, 2011) was present in its highest concentration in the sera from pre-patients and was significantly decreased already during the first year following the onset of symptoms of RA implicating its role in the beginning of the pathogenesis.

In the report by Dean *et al.*, they demonstrated that the number of cytokine factors present at elevated concentrations was predictive for time to diagnosis of RA in relation to age (Dean, *et al.*, 2010). In the present study no association with age was observed but there was a clear trend for the concentrations of the cytokines to increase the closer to the onset of disease that the sample was collected, although statistical significance was only reached for some of them and the increase in concentration was mostly related to the presence of sero-positivity for ACPA.

The cytokine-groups that were significantly associated with ACPA were related to: Th2 cells, Th1 cells, Treg cells, along with Th17. This strengthens the opinion that the immune system, especially the adaptive division, was up-regulated in the direction of RA. Stratification according to ACPA and RF status showed that most of the significant associations with increased levels of markers were in the autoantibody positive groups which could indicate an initiation of the disease process and thereby elevated concentrations of cytokine markers.

The previous finding of increased levels of MCP-1 before disease onset (Rantapää-Dahlqvist, *et al.*, 2007) was also confirmed as this was the most

prominently increased chemokine in pre-patients compared with control subjects.

Due to the relatively limited number of samples analyzed there are statistical limitations to this study although the use of samples from the same individual before and after disease onset is considered a strength as well as the inclusion of controls matched for sex, age, area of residence, and time of blood sampling (*i.e.*, the storage conditions were the same for samples from the pre-patient and their matched controls). Also, the possible effect of false positive results due to heterophilic autoantibodies such as IgM-RF should be taken into consideration (Todd, *et al.*, 2011).

Based on the results obtained in this study we can hypothesize on which parts of the immune system that are activated during the initiation of a disease such as RA. However, due to a lack of samples from several different time points from the same individuals it is not possible to conclude the order in which the factors become involved in the pathogenic processes leading to the initiation of disease. The increase in the concentrations of G-CSF, GM-CSF, and IL-7, typical bone marrow derived factors, could indicate the enhanced production of neutrophils, monocytes, and lymphocytes and also be indicative of a start of pathogenic processes from the bone marrow that later is spread to other compartments. Th1, Th2, Th17, and Treg cell types are all involved in the initiation of the disease and chemokines, such as MCP-1 and MIP-1α are secreted, thus promoting activation of Th1 and Th2 cells and enhancing the migration of monocytes. Macrophages activated by the increased levels of IFNγ and IL-10 secrete several factors that further enhance the immune response. These processes could occur in peripheral lymphoid organ, or in some other organ or tissue, *i.e.*, the bone marrow, the lungs, or the oral cavity, and later when the disease symptoms start to appear and the joints also are involved, the pattern changes. Now, factors involved in tissue remodeling, such as PDGF-BB and VEGF are also up-regulated, thereby facilitating processes like the growth of synovial tissue. Most of the cytokines and related factors have a further increased expression reflecting a more elevated response that also now is more general and widespread. In line with this, it has recently been suggested that there exists a systemic autoimmunity prior to the development of the joint inflammation seen in patients with RA, since it was observed that in individuals positive for autoantibodies (RF and/or ACPA) before the disease had developed, no inflammation induced synovial changes were present (van de Sande, *et al.*, 2011).

Paper II

Antibodies of IgG, IgA, and IgM isotypes against cyclic citrullinated peptide precede the development of rheumatoid arthritis

In this paper, the predictive value of three different ACPA and RF isotypes, IgG, IgA, and IgM was assessed in individuals prior to the onset of symptoms of RA (n=71), in relation to cytokine factors, genetic factors, and smoking habits. Also, the effect on the radiological progression from baseline up to 24 months of these ACPA isotypes that were present prior to symptom onset was evaluated.

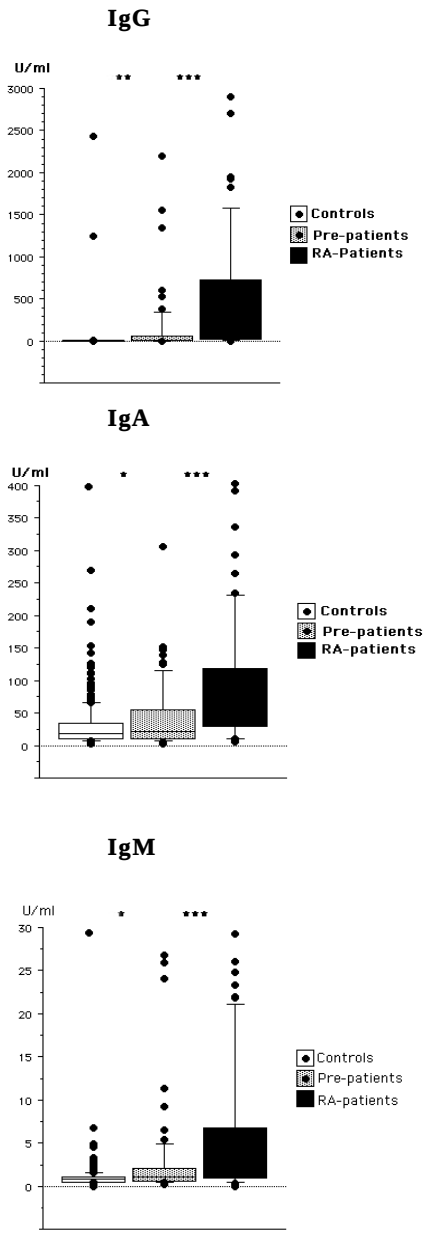
There were significantly increased levels of all three ACPA isotypes in the blood samples from the pre-patients compared with controls and these increased further after the onset of disease (Figure 6). Kruskal-Wallis analyses comparing all three groups showed that the increase in concentration of all isotypes was highly significant ($P<0.001$). Both the IgA and IgG isotype were present in significant frequencies in samples from pre-patients compared with controls, whereas all isotypes were statistically significant in patients after the onset of RA compared with controls (Table 5).

Table 5*. Sensitivities, and specificities of ACPA isotypes in individuals before onset of RA (pre-patients, n=71), and at the time of diagnosis of RA (patients, n=53) defined using ROC curves calculated on the data from patients and controls.*

	Sensitivity	**Sensitivity**	**Specificity**
ACPA isotype	pre-patients	patients	%
IgG	33.8***	71.7***	98.9
IgA	23.9***	43.3***	97.1
IgM	11.8	32.8***	93.9

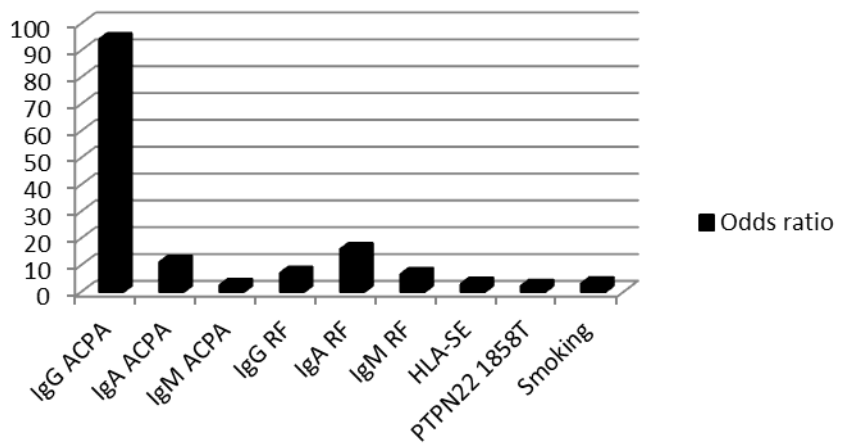
***$P<0.001$

Figure 6. *Concentrations (median, U/mL) of ACPA isotypes in individuals before onset of symptoms of RA (pre-patients), matched controls, and the same individuals as the pre-patients when diagnosed with RA.* ***$P<0.001$, **$P<0.01$, *$P<0.05$



When calculating the predictive value for the development of RA for the various factors using simple conditional logistic regression analyses it was found that the IgG ACPA had the highest OR for predicting RA followed by IgA RF and IgA ACPA (Figure 7). All factors were significantly associated with the development of RA except for IgM ACPA, which was only borderline significant (P=0.054).

Figure 7. *Simple logistic regression analyses of different isotypes of ACPA, RF, genetic factors and smoking in individuals prior to onset of symptoms of RA and matched controls.*



The accumulated percentage of positive samples of ACPA and RF of different isotypes (illustrated in Figure 8A and 8B) over time before the onset of symptoms of RA and until the diagnosis of RA showed a pattern in which both IgG and IgA ACPA appeared first, with the highest percentage positivity being shown for IgG ACPA (Figure 8A). For RF, a different appearance pattern was observed with the first isotype to be detectable being IgA followed by IgG and IgM (Figure 8B).

In multiple logistic regression models including only IgM RF and IgG ACPA, the IgG ACPA was significant for development of RA (OR=32.4 (95% CI 9.0-116.9) but not the IgM RF alone, whereas the combination of these autoantibodies strengthened the OR for development of the disease (OR=71.2, 95% CI 9.9-566.7) suggesting an interaction between these autoantibodies. The same pattern was observed in analyses for IgM RF and IgA ACPA, where the individual antibodies had an OR of 3.8 (95% CI 4.6-321.6) and 6.9 (95% CI 2.6-18.4), respectively, whilst the combination gave an OR of 38.7 (95% CI 4.6-321.6).

Figure 8A. *Accumulated percentage of positive samples of IgA, IgG, and IgM isotypes of ACPA in individuals before the onset of symptoms of RA and at diagnosis of RA.*

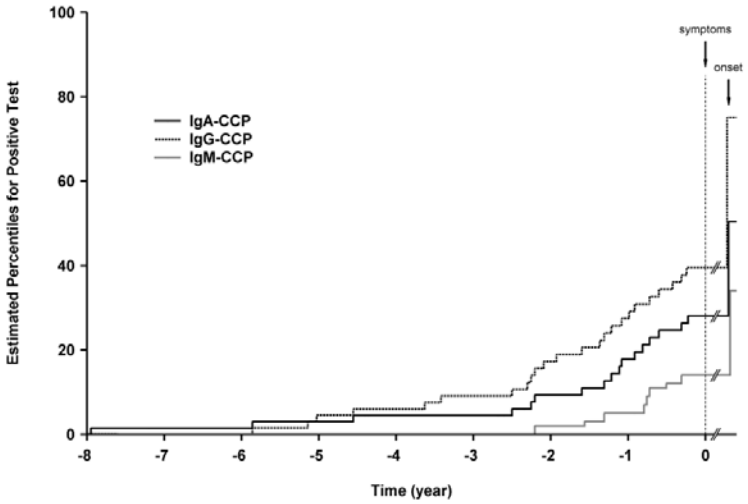
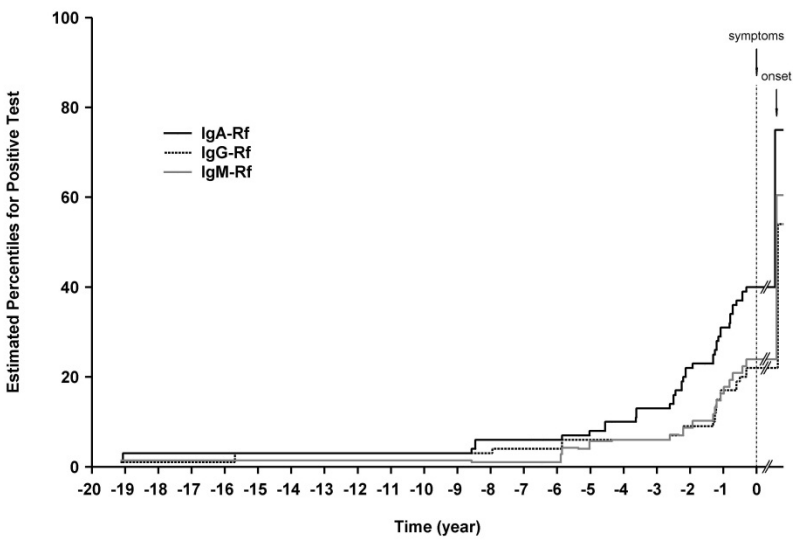
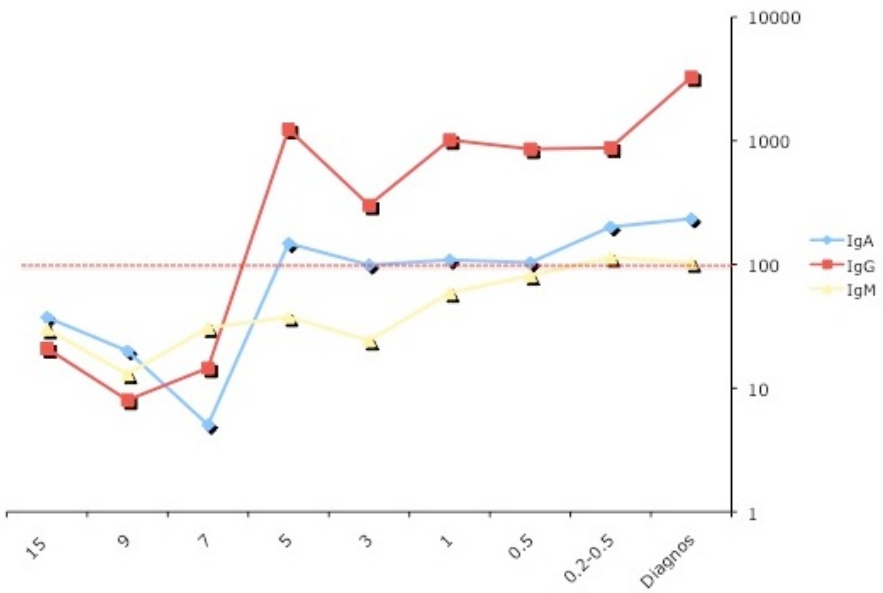


Figure 8B. *Accumulated percentage of positive samples of IgA, IgG, and IgM isotypes of RF in individuals before the onset of symptoms of RA and at diagnosis of RA.*



The concentration of the IgG isotype of ACPA increased significantly over time until the onset of RA (P<0.0001); the increase having started up to five years before the onset of RA and remained rather constant until just before the onset of symptoms (0.25 years before) when there was a significant increase to the time of diagnosis (P<0.05) (Figure 9). Also, a gradual increase of the IgA isotype could be observed until just before the onset of symptoms (P<0.05) as well as to the time of diagnosis (P<0.01) (Figure 9). For the IgM isotype the increase in concentrations started later than for the other isotypes, *i.e.*, about three years before the onset of disease, but the increase was significant until 0.25 years before the onset of symptoms (P<0.02) and at the time of diagnosis (P<0.001) (Figure 9).

Figure 9. *Increase in concentrations, in percentage of cut-off value (indicated with a dotted line), of the different ACPA isotypes analyzed before the onset of symptoms of RA and at diagnosis.*



Stratification of the samples according to positivity for IgA, IgG, and IgM ACPA in relation to the concentrations of the different cytokines and other soluble markers showed a fairly similar pattern in IgG ACPA and IgA ACPA positive individuals with higher concentrations of cytokines related to general activation (*e.g.*, IL-2Rα, and IL-6), Th1 cells (IL-12), Th2 cells (*e.g.*, IL-9, and eotaxin), and the angiogenesis associated factor VEGF, compared with autoantibody negative individuals (Table 6). Although, there were dissimilarities in the associations with chemokines, which were more pronounced in IgA ACPA positive individuals with significantly elevated levels of IP-10, MCP-1, and MIP-1β, besides MIG that also was up-regulated in IgG ACPA positive individuals. No significant associations whatsoever were observed in individuals with IgM ACPA compared with those lacking this isotype (data not shown).

Analysis of the effect of smoking on the presence of the different isotypes of RF and ACPA showed that smoking increased the risk for developing IgG ACPA and IgA RF ($P<0.05$) and that smokers developed IgA ACPA significantly earlier compared with non-smokers ($P<0.05$), *i.e.*, 2.4 years before the onset of RA compared with 0.6 years for non-smokers.

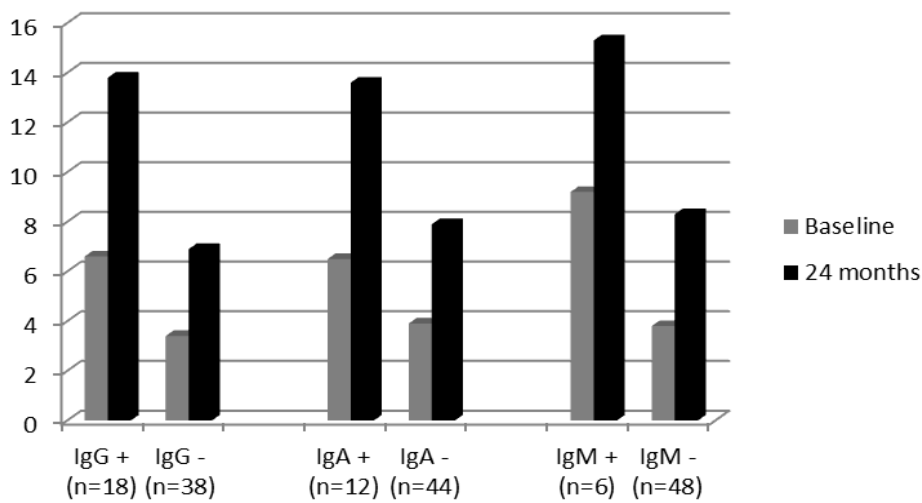
Table 6. *Concentration of cytokines, cytokine related factors, and chemokines (median pg/mL, (IQR)) significantly increased in individuals before the onset of symptoms of RA stratified for IgA and IgG isotypes of ACPA.*

	IgA positive (n=14)	**IgA negative (n=38)**	**IgG positive (n=20)**	**IgG negative (n=32)**
		General activation		
IL-1β	7.2* (2.9-36.4)	3.4 (2.3-5.1)	5.9 (2.3-25.5)	3.3 (2.3-4.9)
IL-2Rα	66.9** (38.6-110.0)	33.4 (22.3-51.3)	60.9*** (35.9-88.7)	32.2 (20.8-47.6)
IL-6	19.8** (5.9-147.7)	4.3 (1.1-11.5)	16.2* (3.6-111.6)	4.8 (1.1-8.2)
IL-2	74.7* (2.8-141.5)	3.8 (1.1-18.3)	49.1*** (10.5-106.8)	1.4 (1.1-11.7)
IL-15	2.4 (0.2-9.6)	0.5 (0.2-4.2)	4.2* (0.8-4.8)	0.5 (0.2-4.2)
		Th1 cell related		
IL-12	54.4* (21.0-135.2)	21.2 (13.7-32.3)	53.5* (21.4-161.3)	20.5 (13.5-29.3)
IFNγ	113.4 (84.5-1711.0)	92.9 (50.5-150.1)	152.2* (97.1-1167.6)	78.5 (50.4-130.8)
		Th2 cell related		
IL-4	4.4* (3.2-26.4)	3.0 (2.2-4.2)	4.4* (3.3-17.4)	2.9 (2.3-4.0)
IL-9	128.3* (22.7-283.6)	19.6 (5.7-85.9)	121.1* (24.7-255.9)	17.6 (5.6-71.7)
Eotaxin	80.7** (43.2-429.8)	42.0 (26.1-53.1)	80.7** (44.0-363.8)	36.7 (26.0-47.6)
		Th17 cell related		
IL-17	31.3 (17.7-46.5)	22.3 (9.9-38.1)	31.5* (20.3-42.0)	21.2 (9.6-36.9)
		Bone marrow derived		
GM-CSF	23.0** (12.9-103.4)	5.0 (2.5-23.9)	20.2 (5.5-62.0)	4.6 (2.5-15.7)
		Stromal cells and angiogenic factors		
VEGF	24.6* (10.0-63.7)	13.1 (5.7-23.9)	28.6* (12.0-53.1)	11.7 (4.9-15.0)
		Chemokines		
MIG	488.5** (319.0-1167.5)	272.9 (190.4-371.2)	425.6* (279.1-741.3)	272.9 (201.6-364.0)
IP-10	962.1* (696.2-2214.2)	634.3 (425.0-1062.0)	957.0 (644.2-1855.8)	624.4 (420.0-1071.5)
MCP-1	23.0* (19.0-62.4)	18.5 (12.8-28.4)	25.8 (17.6-52.7)	17.3 (12.9-26.3)
MIP-1β	44.2* (37.6-49.7)	35.7 (26.5-42.2)	42.9 (31.4-48.7)	36.5 (27.4-41.1)

* $P<0.05$, ** $P<0.01$, ***$P<0.001$, positive vs negative for IgA ACPA and IgG ACPA, respectively

The predictive effect of the different ACPA isotypes present before development of symptoms of disease on the radiological outcome at disease onset (baseline) and after 24 months was evaluated (Figure 10). The Larsen score at baseline was significantly higher in patients with IgG ACPA and IgM ACPA before disease onset compared with those negative for these autoantibodies. After 24 months the Larsen score was significantly higher in individuals who were positive for IgA ACPA and IgG ACPA before the onset versus sero-negative subjects. Also, there were significant differences in the Larsen score, both at baseline and after 24 months, for those with ≥2 isotypes compared with having none or 1 ($P<0.05$). Overall, there was a significant increase in the Larsen score for all subgroups from baseline to 24 months.

Figure 10. *Larsen score at baseline and after 24 months in patients with RA who were positive or negative for ACPA of IgG, IgA, or IgM isotypes before onset of symptoms of joint disease.*



Discussion paper II

Anti-citrullinated protein/peptide antibodies of the IgG, IgA, and IgM isotypes were all present before the onset of symptoms of RA and both IgG ACPA and IgA ACPA predicted the development of RA, with IgG ACPA showing the highest OR. Here, the most diagnostically relevant isotype was IgG followed by IgA and IgM. This pattern is entirely different when comparing with RF isotypes as previously published by Rantapää-Dahlqvist, *et al.*, (2003), in individuals before disease onset, where the most important isotype for predicting RA was IgA, followed by IgM and finally IgG. There are

no comparable data to the present study on individuals prior to the onset of RA regarding the isotype of ACPA, although, in a recently published study first-degree relatives were shown to have increased concentrations of IgA ACPA and IgM ACPA compared with healthy controls, which could either indicate a pattern that is apparent prior to disease or a form of autoimmunity that does not lead to a class switch to the IgG isotype being related to RA (Ärlestig, *et al*., 2011).

The results of this study are consistent with a previous study performed on patients with established RA in which the authors observed the highest frequency for IgG ACPA, followed by the IgA and IgM isotypes (Lakos, *et al*., 2008), as well as a study on antibodies against viral citrullinated peptides showing a high specificity for IgG and IgA isotypes for discriminating RA from other chronic arthritides and connective tissue disorders (Anzilotti, *et al*., 2007). Moreover, Verpoort, *et al*., (2006) showed that the frequency of IgA ACPA was higher among RA patients compared with those suffering from undifferentiated arthritis.

In clinical use IgM RF and IgG ACPA have the major diagnostic power and it can be hypothesized that the IgM RF represents a non-isotype switch T-cell independent immune response whilst the IgG ACPA could reflect a mature T-cell dependent immune response, although the pattern seems rather complex.

Stratifications according to positivity for the different isotypes of ACPA were performed and analyzed in relation to the concentrations of the different cytokines studied in paper I. Interestingly, there were no associations for the different cytokine factors with the IgM isotype, which could be due to the low number of samples positive for this isotype or it could be speculated that this isotype is not involved at this stage in the immune response seen in these individuals before the onset of the symptoms of RA. The IgA and IgG isotypes showed a rather similar pattern with increased levels of those cytokines related to general immune activation, Th1 cells, Th2 cells, and VEGF. The difference between these isotypes was in the up-regulation of several chemokines that was only observed for the IgA isotype positive group. It has been demonstrated that individuals who were unlikely to associate with ACPA (of the IgG isotype) had high expression of MCP-1 and MIP-1β but a low expression of IFNγ and TNFα assessed using micro-array analysis (Hitchon, *et al*., 2004), which are in line with our finding of IgA ACPA.

Smoking has been associated with higher frequencies of ACPA of the IgA (Svärd, *et al*., 2008; Verpoort, *et al*., 2007) and IgM isotype (Verpoort, *et al*.,

2007) compared with the concentrations measured in non-smokers. These results together with the finding that IgA ACPA appeared significantly earlier in smokers compared with non-smokers at the pre-patient stage could indicate a possible involvement of smoking as an environmental trigger in the appearance of this isotype.

Radiological progression has, in several studies, been shown to be predicted by the presence of ACPA (Berglin, *et al*., 2006; Syversen, *et al*., 2008). The combination of the IgG ACPA and IgA ACPA at baseline identified a group of patients with RA that had a more severe disease course during the following three years that these individuals were studied compared with those without this combination (Svärd, *et al*., 2008). Furthermore, another study showed that having several ACPA isotypes at baseline was predictive for disease severity as an increasing number of isotypes was associated with higher radiographic score (van der Woude, *et al*., 2010). Consistent with these publications the present study extends this to individuals who before the onset of RA, already were sero-positive for several ACPA isotype having a significantly higher risk of radiological damage both at baseline (*i.e.,* at diagnosis of RA) and after 24 months.

This study was performed on a relatively small number of samples and when stratifying the groups into sub-groups the results have to be interpreted with caution and should be replicated in larger cohorts of individuals sampled before the onset of symptoms of clinical RA. Also, the effects of storage time and small alquits should be considered, however, this was compensated for by selecting controls that were matched for time at sampling and storage conditions.

Paper III

The *PTPN22* 1858C/T polymorphism is associated with anti-cyclic citrullinated peptide antibody-positive early rheumatoid arthritis in Northern Sweden

The association of the *PTPN22* 1858T allele with RA in the population of Northern Sweden was investigated since it had previously been shown to be related with several different autoimmune diseases including RA, in other populations (Bottini, *et al*., 2004; Criswell, *et al*., 2005; Begovich, *et al*., 2004) and in combination with IgG ACPA the 1858T variant of *PTPN22* had a 100% specificity for predicting RA in samples collected prior to the onset of RA compared with controls in Northern Sweden (Johansson, *et al*., 2006).

The 1858T allele was significantly higher in patients (n=505) compared with matched controls (n=970) (χ^2=23.84, P<10^{-5}, OR=1.69 95%CI 1.36-2.11). Stratification according to ACPA and RF status confined the association of this gene variant to sero-positive individuals (Table 7).

Table 7. *Comparison of PTPN22 1858 genotypes in patients with early RA stratified according to ACPA and RF compared with all controls.*

	CT + TT genotype n (%)	**CC genotype n (%)**	**MAF**	**χ^2**	***P value***	**OR (95% CI)**
ACPA +	113 (35.5)	205 (64.5)	0.196	24.99	$6x10^{-7}$	2.01 (1.51-2.67)
ACPA -	40 (26.7)	110 (73.3)	0.145	1.97	0.16046	1.32 (0.88-2.00)
RF+	96 (36.4)	168 (63.6)	0.199	24.49	$7x10^{-7}$	2.08 (1.53-2.82)
RF-	28 (27.5)	74 (72.5)	0.142	1.87	0.17164	1.38 (0.85-2.23)
Controls	209 (21.5)	761 (78.5)	0.114	-	-	-

MAF= minor allele frequency

The influence of this gene variant on disease activity measured by DAS28 from baseline, *i.e.*, at diagnosis of RA, up to 24 months was assessed by calculating AUC where no difference could be seen over this period of time. Only a modest difference between those with the 1858T variant compared with 1858C variant was detected at 6 months, with 1858T carriers having a significantly higher DAS28 value (P<0.05*)*, which could not be associated with differences in the medications prescribed (*i.e.*, DMARD and glucocorticoids).

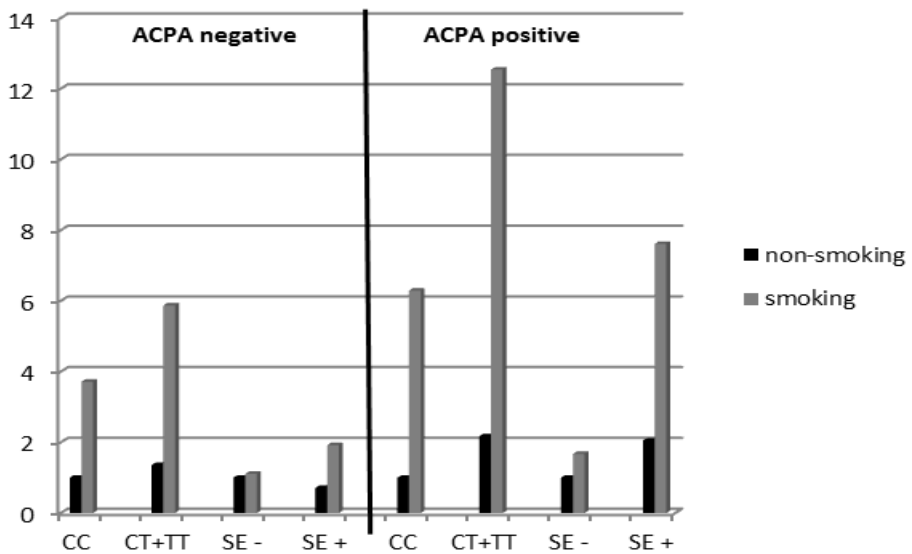
Both the 1858T variant and being sero-positive for ACPA was associated with being younger at the onset of RA compared with those patients without these parameters (P<0.05, and P<0.01, respectively). Also, the combination of having the 1858T variant and being ACPA positive contributed to an even earlier age at onset of RA (P<0.05).

In simple logistic regression analyses ACPA were the strongest risk factor for RA (OR 145.0, 95% CI 58.7-359.0), followed by smoking (OR=5.1, 95% CI 4.0-6.5), carriage of the HLA-SE (OR=2.0, 95% CI 1.4-2.9), and *PTPN22* 1858T carriage (OR=1.8 95% CI 1.4-2.3).

In analyses of different combinations of the *PTPN22* 1858T variant, HLA-SE, and smoking stratified according to ACPA for the risk of RA it could be observed that smoking was a risk factor for RA independent of ACPA and the 1858T variant (Figure 11). Although, the highest risk for RA was observed in the group of individuals who were ACPA positive, carried the 1858T variant and were smokers (OR=12.5, 95% CI 8.1-19.4).

HLA-SE among the patients was strongly associated with the presence of ACPA ($P<0.0001$) whilst the 1858T variant was not ($P>0.05$).

Figure 11. *Relative risk (RR) for developing RA in patients with different combination of either HLA-SE or PTPN22 1858 in smokers or non-smokers, stratified for ACPA.*



In a sub-cohort of patients (n=85), who had also donated blood samples to the Medical Biobank prior to the onset of RA, the frequency of ACPA was 32%. Among those who were initially negative for ACPA but were positive for these autoantibodies at the onset of disease, 63% were recruited from those being HLA-SE positive, although this did not reach statistical significance. There were no differences in individuals who had the 1858T variant compared with those lacking this allele (50% in both groups).

Discussion of paper III

This study confirmed the formerly demonstrated association of the *PTPN22* 1858T with RA and this was confined to patients who were positive for the autoantibodies ACPA and RF, as had previously been described (Begovich, *et al.*, 2004; Lee, *et al.*, 2005; Plenge, *et al.*, 2005).

In earlier studies of the population from Northern Sweden it was shown that both the 1858T variant and HLA-SE predicted RA, with an increase in the

relative risk when in combination with ACPA in individuals before the onset of symptoms of RA (Berglin, *et al*., 2004; Johansson, *et al*., 2006*)*, which was also found in this study.

In the study by Johansson *et al*., it was observed that in individuals prior to the onset of RA there was a significant association of the 1858T variant with ACPA whereas the HLA-SE was not associated with the presence of ACPA (Johansson, *et al*., 2005). In the present study, in patients with early RA, the associations were the opposite with ACPA and HLA-SE being strongly associated whereas no association was found for the 1858T variant with ACPA. A greater number of patients were recruited from the HLA-SE positive group compared with HLA-SE negatives amongst the sub-group of pre-patients that were ACPA negative prior to disease onset but later was found to be ACPA positive when diagnosed with RA, although the sub-groups of this analysis were small and did not reach statistical significance. However, according to these results it could be hypothesized that the *PTPN22* 1858T variant is important before the disease has developed to a clinical stage and together with the notion that this gene variant is also associated with other autoimmune disease it could interfere with an earlier pathway in the pathogenesis of RA as compared with HLA-SE that seems more important later in disease development and is confined to RA. It has been possible to confirm these findings in a larger cohort of pre-patients, in that, 122 individuals with ACPA negative samples prior to the onset of disease, 62% had converted to ACPA positive when sampled again at diagnosis of RA; 65% of these were HLA-SE positive and 35% HLA-SE negative, this difference being statistically significant. Among the now ACPA positive patients only 32% carried the 1858T variant of *PTPN22* (personal communication from S. Rantapää-Dahlqvist).

Smoking was shown to be a major environmental risk factor in this study, and was independent of ACPA status and carriage of the 1858T variant. Additionally, as it has previously been demonstrated, HLA-SE was identified as a risk factor for ACPA positive RA and this risk was strengthened by a factor of approximately three when combined with smoking (Klareskog, *et al*., 2006).

Also, consistent with the reported increasing south to north gradient among white European populations, the population from Northern Sweden studied had a higher genotype and allele frequency of the *PTPN22* 1858T variant when compared with other study cohorts (Gregersen, *et al*., 2006).

Finally, it was possible to confirm the previous findings of a contribution by the 1858T variant to an earlier age at onset of RA (Plenge, *et al*., 2005;

Pierer, *et al*., 2006). Also, ACPA in combination with the 1858T allele contributed to an even earlier age at onset of RA.

Paper IV

Antibodies against mutated citrullinated vimentin are a better predictor of disease activity at 24 months in early rheumatoid arthritis than antibodies against cyclic citrullinated peptide

This study aimed at evaluating the predictive value of the following different ACPA: anti-mutated citrullinated vimentin (anti-MCV), anti-CCP2, anti-CCP3, and a combined test for the IgG and IgA isotype together, anti-CCP3.1, for disease activity and progression in early RA. These parameters were also analyzed in relation to the genetic factors, HLA-SE and *PTPN22* 1858C/T.

The highest sensitivities yielding the best specificities for the various ACPA for RA, based on ROC curves, are shown in Table 8. The highest sensitivities were for anti-CCP2 and anti-CCP3.1 and were rather similar, 80.4% and

80.5%, with specificities of 98% and 95.1%, respectively. The 95% CI for all ACPA were overlapping.

Table 8. *Sensitivity, specificity, and positive likelihood ratios (LR) calculated using ROC curves at a given specificity of 98% for all tests.*

ACPA	Sensitivity (95% CI)	Specificity (95% CI)	Sensitivity at 98% specificity (95% CI)	LR*
CCP2[#]	80.4 (74.3-85.6)	98.0 (93.7-99.7)	80.4 (74.3-85.6)	41.0 (10.3-162.1)
MCV	74.0 (67.5-79.8)	96.1 (90.8-98.7)	69.0 (62.3-75.1)	35.2 (8.9-139.2)
CCP3	79.0 (72.9-84.2)	98.0 (93.6-99.7)	79.0 (72.9-84.2)	40.3 (10.2-159.2)
CCP3.1	80.5 (74.5-85.6)	95.1 (84.4-98.2)	78.5 (72.4-83.8)	40.0 (10.1-158.2)

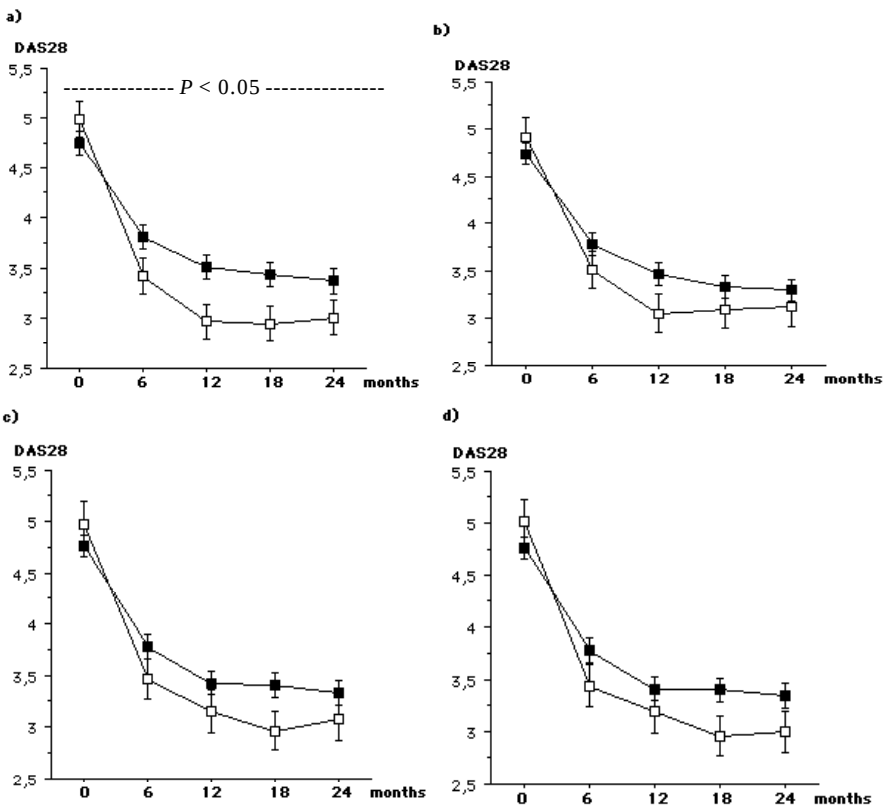
[#] n=192, *calculated with a specificity of 98 %

Overall, there was a significant reduction in DAS28 over time from baseline, *i.e*., at diagnosis of RA, to 24 months ($P<0.0001$) from a mean ± SEM of 4.8 ± 0.9 to 3.3 ± 1.0. Patients with anti-MCV ACPA had significantly less reduction in DAS28 compared with those without these (ANOVA; F-value=3.4, $P<0.01$) and also the AUC for DAS28 was significant for anti-MCV ACPA compared with the sero-negative patients ($P<0.05$) (Figure 12). No significant differences were found for the other ACPA regarding DAS28 (Figure 12). Also, anti-MCV ACPA positive individuals had significantly less reduction in the number of swollen joints during the 24 months compared with those negative for these ACPA ($P<0.05$). In anti-MCV ACPA positive

individuals the AUC for ESR and CRP was also significantly higher compared with sero-negative individuals, which was not detected for the other ACPA.

The anti-CCP2, anti-MCV and anti-CCP3 ACPA were all significantly associated with the HLA-SE ($P<0.05$) but not the anti-CCP3.1. No relationship between the 1858T variant of *PTPN22* and the various ACPA could be detected.

Figure 12. *DAS28 in patients positive compared with negative for the different ACPA, anti-MCV (a), anti-CCP3 (b), anti-CCP2 (c), and anti-CCP3.1 (d), from baseline to 24 months. Closed squares representing ACPA positive patients and open squares representing ACPA negative patients.*



Patients stratified according to positivity for the different ACPA showed that being positive for any of these compared with being negative was associated with a greater radiological damage, evaluated using the Larsen score after 24 months, this outcome was not observed for RF. Factors at baseline that were significant for radiological progression at 24 months in multiple regression

analyses were: baseline values for the Larsen score, swollen joint count, positivity for all four ACPA, and RF. Patients with radiological progression at 24 months at baseline already had significantly higher levels of anti-MCV, anti-CCP3, and anti-CCP3.1 compared with patients without progression (median levels: 205 and 66 U/ml, 305 and 222 U/ml, and 326 and 219 U/ml, respectively). Patients positive for any of the four ACPA frequently had significantly more radiological progression when compared with ACPA negative individuals ($P<0.001$).

Backward stepwise logistic regression analyses utilized to identify predictors of radiological progression at 24 months included the baseline values of the following variables: the different ACPA, RF, swollen joint count, ESR, Larsen score, HLA-SE, the *PTPN22* 1858T variant, and therapeutic response at 6,12, and 24 months, respectively. Positivity for ACPA, RF, and ESR at baseline was predictive for radiological progression ($P<0.05$) and therapeutic response at all time points was predictive for less radiographic progression ($P<0.05$).

Discussion Paper IV

This study was aimed at evaluating the predictive value of the different ACPA at the onset of RA, *i.e.*, at baseline, for disease progression and outcome up to 24 months after diagnosis. In line with the study by Bang, *et al.*, the presence of anti-MCV antibodies at disease onset was associated with a more severe disease course, such as smaller reduction of DAS28 over time as well as a larger swollen joint count compared with the other ACPA types (Bang *et al.*, 2007).

In this study all ACPA types were equally predictive for a worse radiological progression and outcome after 24 months. In one published study it had been shown that anti-Sa antibodies were associated with more severe joint damage compared with RF or ACPA (Boire, *et al.*, 2005), which we could not observe in this study.

The highest LR for diagnosing RA was for the anti-CCP2 ACPA, followed by anti-CCP3 but all of the different ACPA analyzed had overlapping confidence intervals.

In relation to the genetic risk factors *PTPN22* 1858T and HLA-SE, no significant differences were detected for the various ACPA in terms of the risk for radiological progression or enhanced inflammatory activity but this could also be due to the small sample sizes when stratifying into appropriate

sub-groups. The findings are, however, consistent with studies in larger cohorts with patients showing that neither the HLA-SE nor *PTPN22* 1858T is associated with disease severity (van der Helm-van Mil, *et al*., 2006; Harrison, *et al*., 2006). All ACPA other than the anti-CCP3.1 was associated with HLA-SE, which could suggest that the IgA isotype is not associated with HLA-SE in contrast to the IgG isotype previously shown to be associated with HLA-SE (van der Helm-van Mil, *et al*., 2006) as also was presented in the results of paper II and III, when the IgA ACPA was not associated with HLA-SE whilst the IgG isotype was.

One limitation with this study is that the number of individuals was relatively small, that could result in difficulties in terms of revealing significant differences when comparing sub-gropus stratified for different parameters.

Conclusions

The main conclusions from this thesis comprising individuals from Northern Sweden who had donated samples prior to the onset of RA and followed up to 24 months after disease diagnosis can be summarized as follows:

- Blood samples obtained from individuals before the onset of symptoms of RA have increased concentrations of pro-inflammatory cytokines, cytokine related factors as well as chemokines indicative of an already activated immune system.

- The pattern of the increased cytokines and cytokine related factors after disease onset demonstrated a more general and widespread activation of the immune system.

- ACPA of both the IgG and IgA isotypes predated the onset of RA by years and also predicted the development of RA, with the highest predictive value for IgG ACPA.

- There was a difference in the pattern of upregulated chemokines in IgA and IgG ACPA positive individuals indicating a different role for these in RA pathogenesis.

- The *PTPN22* 1858T variant was associated with seropositive RA in our population and also contributed to an earlier age at onset, especially when in combination with ACPA.

- Smoking was a risk factor for RA idependent of the *PTPN22* 1858T variant as well as ACPA.

- Anti-MCV antibodies were associated with a more severe RA as measured by DAS28, ESR, and swollen joint count as compared with anti-CCP2, anti-CCP3, and anti-CCP3.1.

- Radiological progression was predicted equally by the various ACPA, anti-MCV, anti-CCP2, anti-CCP3, anti-CCP3.1, and RF.

Future perspectives

Based on the results from this thesis as well as from other publications it would be of interest to try to replicate some of the major findings of the studies in larger cohorts of individuals before the onset of symptoms of clinical RA.

Analyzing selected cytokines and other soluble markers in blood samples from individuals in serial measurments over time until the onset of clinical RA could provide us with more knowledge regarding which parts of the immune system and which cell-types that are involved in the development of RA.

Analyzing these markers in different sub-groups according to the presence of various ACPA, RF, genetic factors, age, sex, smoking *etc*., is also important due to the complexity of this disease.

Acknowledgements

Först och främst vill jag tacka min handledare, professor **Solbritt Rantapää-Dahlqvist** som gav mig den här möjligheten. Tack för din entusiasm och ditt kunnande, du har alltid funnits till hands när jag behövt hjälp!

Stort tack också till min bihandledare **Ingegerd Söderström** för alla givande diskussioner om både forskning och annat här i livet. Tack för alla de gånger du kom med kaffe och kaka när jag var fast på labbet!

Alla de härliga personerna i våran 'lilla' grupp. **Martin** för att du handledde mig under min tid som förvirrad ex-jobbare, vi saknar ditt sällskap här! **Lisbeth Ä**, för all hjälp och trevliga diskussioner om det mesta under dessa år. **Solveig L**, du är verkligen en stor del till att den här avhandlingen blev till, tack för all hjälp på labbet! **Mikael B**, tack för all datahjälp och trevligt prat och lycka till med ditt eget projekt och studierna.

Alla på reumatologkliniken här i Umeå, speciellt till er som varit med på våra trevliga forskarluncher, **Solveig W-J, Ewa B, Lotta L, Lena I, Anna S, Gerd-Marie A, Kristina J, Sonja O, AnnCathrin K, Björn S** och **Gun-Britt J.**

Alla på det bästa fika-bordet ever! Tack till nuvarande och föredettingar på 6M, 3tr. **Maria L, Maria H, Åsa L, Mia S, Marie L, Sofia M, Linda K, Thomas J, Urban H, Susann H, Malin O, Dan H, Monica H, Anna Karin N, Ulrika NN, Lisbet L, Anna N, Stefan N** och **Mikael L**.

Våra 'grannar' på pediatriken och fysiologisk kemi.

Kristina L, tack för alla givande diskussioner och idéer i samband med 'cytokin-projektet'.

Medförfattare på de olika projekten, **Johan Rönnelid, Mohammed Mullazehi, Catharina Eriksson, Joacim Rocklöv, Göran Hallmans, Göran Wadell** och **Erik Jidell**.

Hans Stenlund och **Marie Eriksson** för statistikhjälp.

Maria B och **Kristina S**, som den där sommaren 2004 visade mig hur kul forskning kan vara.

P-A, tack för all hjälp med att skicka iväg alla prover.

Nina G, Elin L, Angelica N och **Nina N**, det har varit kul att lära känna er även utanför labbet!

Emma, för att du helt enkelt är snyggast och bäst ;) Nu kan vi börja satsa på nästa Vasaloppscykling!

Min kära vän **Veronica**, mår alltid så bra efter att jag pratat med dig. Så tråkigt att vi bor så långt ifrån varandra.

Sofia G, saknar ditt sällskap och hoppas vi hinner ses framöver. Snart får jag väl ta och samla ihop hela familjen och hälsa på er i Ö-vik.

Jennie, tänk att det snart gått 25 år sedan vi blev 'bästisar'. Nu får vi se till att umgås mer.

Tack även alla mina andra vänner utanför jobbet, ingen nämnd ingen glömd!

Stort tack till min 'andra' familj, **Carina, Staffan, Helena, Nicklas, Maria** och **Thomas** för att ni är så varma och enkla människor som gör det lätt att trivas i erat sällskap. Extra tack till Carina och Staffan för de gånger ni tagit hand om barnen!

Älskade **mamma** och **pappa**, tack för att ni alltid har trott på mig och stöttat mig i både vått och torrt. Mina systrar med familj, **Anne** och **Joel**, **Eija** och **Erik**. Tack för att ni alltid finns där.

Till sist, de viktigaste: min fantastiska, fina familj. **Peter**, tack för att du ställt upp och tagit hand om all 'markservice', utan dig hade det här aldrig gått. Finns ingen som förstår mig lika bra som du. Våra underbara barn, **Elina** och **Adam**, ni är det mest värdefulla som finns, jag är så stolt över att ha er. Älskar er!

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