



UMEÅ UNIVERSITY

Insights into the processes preceding the onset of rheumatoid arthritis

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To my family

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Abstract

Rheumatoid arthritis (RA) is a chronic inflammatory disease, characterized by the production of anti-citrullinated protein antibodies (ACPA) in the majority of all patients and a persistent inflammation in the synovial tissue leading to joint destruction. The aetiology of RA remains to a large extent unknown but is believed to be a complex interplay between genetic, environmental and stochastic factors. Recently, several infectious agents have been shown to have the capacity to induce citrullination of both endogenous and exogenous antigens *e.g.*, Epstein-Barr virus (EBV) and *Porphyromonas gingivalis* (*P.gingivalis*). Disease progression in patients with RA is suggested to be a longstanding process that begins several years before symptom onset of RA. This hypothesis is supported by studies showing increased antibody levels against ACPA and disease related cytokines/chemokines several years before symptom onset of RA. The presence of ACPA is highly specific for RA and is already used as an indicator of progression and prognosis of the disease. This thesis is aimed to further investigate the origin and role of ACPA and the processes preceding the development of RA. New insights into these processes are of importance in order to be able to prevent the disease onset, achieve better diagnostic methods and treatments in the future.

All of the individuals included in these papers, had attended to the Department of Rheumatology at Umeå University to receive their diagnosis of RA. The register of the patients were thereafter co-analysed with the register of the Medical Biobank of Northern Sweden. Plasma/sera samples were analysed for antibodies and receptor activator of nuclear factor kappa-B ligand (RANKL) using different ELISA techniques from individuals before symptom onset (pre-symptomatic individuals) and at disease onset (patients). Cytokines/chemokines were analysed using Meso Scale Discovery methods. Levels of marginal jawbone loss were measured using dental radiographs from premolar/molar regions. The Larsen score at disease onset was used to grade radiographs of hands and feet.

In **Paper I** antibodies against Epstein-Barr virus nuclear antigen (EBNA) 1 and 2 (VCP1 and VCP2) and histone 4 (H4) derived citrullinated peptides (HCP1 and HCP2) were found to predate symptom onset of RA. In **Paper II**, antibodies against anti-*P.gingivalis* (anti-CPP3 and -RgpB IgG) were significantly increased in pre-symptomatic individuals and were detectable several years before symptom onset of RA. In **Paper III** the concentration of RANKL was shown to be increased several years before symptom onset of RA, especially in ACPA/rheumatoid factor (RF)/anti-carbamylated (CarP) antibody positive individuals. Positivity for RANKL was found to appear later in time than both positivity for ACPA, RF and anti-CarP antibodies. The highest Larsen score at disease onset was yielded when combining positivity for RANKL and anti-CarP

antibodies. In ***Paper IV*** periodontitis, defined as marginal jawbone loss was significantly higher in pre-symptomatic individuals who never smoked, compared with matched controls. RANKL positive individuals particularly those that were also ACPA positive, had a significantly greater extent of jawbone loss in comparison to those individuals who were RANKL negative.

Antibodies against citrullinated exogenous and endogenous peptides were found to be associated with the symptom onset of RA. No hierarchy among the citrullinated epitopes could be identified. RANKL levels were particularly increased in ACPA-positive individuals, and RANKL positivity appeared later in time than the general ACPA response. Periodontitis, defined as marginal jawbone loss was significantly higher in pre-symptomatic individuals, who never smoked.

Abbreviations

Aa	Aggregatibacter actinomycetemcomitans
ACPA	Anti-citrullinated protein/peptide antibody
ACR	American College of Rheumatology
Anti-CarP antibody	Antibody against carbamylated protein
Anti-CCP antibody	Antibody against citrullinated protein
Anti-CCP-1 antibody	Antibody against filaggrin ³⁰⁷⁻³²⁴
Anti-CEP-1 antibody	Antibody against α -enolase ⁵⁻²¹
Anti-cFibβ36-52 antibody	Antibody against citrullinated fibrinogen β 36-52
ARA	American Rheumatism Association
AU	Arbitrary Units
BCR	B-cell receptor
CI	Confidence interval
CPP₃	Cyclic citrullinated peptide 3
CRP	C-reactive protein
DMARD	Disease Modifying Anti-Rheumatic Drug
EBNA	Epstein-Barr virus nuclear antigen
EBV	Epstein-Barr virus
EIRA	Swedish Epidemiological Investigation of Rheumatoid Arthritis
ELISA	Enzyme-linked immunosorbent assay
ESR	Erythrocyte sedimentation rate
EULAR	European League of Arthritis and Rheumatism
Fab	Fragment antigen binding
Fc	Fragment crystallizable
H4	Histone 4
HCP	Histone citrullinated peptide
HLA	Human leukocyte antigen-shared epitope allele
IP-10	IFN- γ -inducible protein 10
Ig	Immunoglobulin
IU	International unit
IL	Interleukin
IQR	Interquartile range
Ltx A	Leukotoxin A
MCH	Major immunohistocompatibility complex
MCP	Monocyte chemotactic protein 1
MCP	Metacarpophalangeal
MDC	Macrophage-derived chemokine
MTP	Metatarsophalangeal
MTX	Methotrexate
NETs	Neutrophil extracellular traps
NSHDS	Northern Sweden Health and Disease Study
OR	Odds ratio
OPG	Osteoprotegerin
PAD	Peptidyl arginine deiminase enzyme
PD	Periodontitis
PIP	Proximal interphalangeal

P.gingivalis	Porphyromonas gingivalis
PTPN22	Protein tyrosine phosphatase non receptor type 22
RA	Rheumatoid Arthritis
RANKL	Receptor activator of nuclear factor kappa-B ligand
RgpB	Arginine gingipainB
RF	Rheumatoid Factor
ROC	Receiver operating characteristic
RPP3	Arginine-containing control peptide
SE	Shared epitope
SEM	Standard error of mean
SD	Standard deviation
SNP	Single nucleotide polymorphism
VCP	Viral citrullinated peptide
VIP	Västerbotten Intervention Programme

List of papers

- I. **Johansson L***, Pratesi F*, Brink M, Ärlestig L, D'Amato C, Bartaloni D, Migliorini P*, Rantapää-Dahlqvist S*. Antibodies directed against endogenous and exogenous citrullinated antigens pre-date the onset of rheumatoid arthritis. *Arthritis Research & Therapy* 2016;18:127
- II. **Johansson L***, Sherina N*, Kharlamova N, Potempa B, Larsson B, Israelsson L, Potempa J, Rantapää-Dahlqvist S*, Lundberg K*. Concentration of antibodies against *Porphyromonas gingivalis* is increased before the onset of symptoms of rheumatoid arthritis. *Arthritis Research & Therapy* 2016;18:201
- III. **Johansson L**, Ärlestig L, Kokkonen H, Brink M, Rantapää-Dahlqvist S. An increased concentration of receptor activator of nuclear factor kappa-B ligand predates the onset of rheumatoid arthritis. *Rheumatology* 2017;12:2190-2196
- IV. Kindstedt E, **Johansson L**, Palmqvist P, Koskinen Holm C, Kokkonen H, Johansson I, Rantapää-Dahlqvist S*, Lundberg P*. Marginal jawbone loss is associated with onset of rheumatoid arthritis and is related to plasma level of receptor activator of nuclear factor kappa-b ligand (RANKL). *Arthritis & Rheumatology* 2017: DOI 10.1002/art.40394

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Enkel sammanfattning på svenska

Reumatoid artrit (RA), ledgångsreumatism är en kronisk inflammatorisk autoimmunsjukdom som drabbar ungefär 0.5 – 1 % av Sveriges befolkning. Sjukdomen karaktäriseras av destruktiva förändringar i främst fötter och händer, men även andra organ såsom hjärta, lungor och blodkärl kan drabbas. Idag finns ingen botande behandling mot RA, men en tidig diagnos och ett snabbt insättande av rätt behandling är viktig för prognosen. Orsaken till varför vissa människor insjuknar i RA är ännu inte känd. Man tror dock att sjukdomen beror på ett komplext samspel mellan genetiska, miljö (t.ex. rökning) och stokastiska faktorer. Under de senaste åren har även intresset för betydelsen av parodontit (tandlossning) och infektioner såsom körtelfeber för sjukdomsutvecklingen vuxit. ACPA (antikroppar mot citrullinerade peptider) är typiskt för individer med RA och återfinns hos ca 70 % av alla patienter. En av dessa antikroppar, cyklisk citrullinerade peptid (CCP2) har idag fått en nyckelroll i diagnostiken. Mekanismen bakom bildningen av ACPA och deras betydelse för sjukdomsutvecklingen är ännu inte helt klarlagd. Tidigare studier har visat ökade nivåer av ACPA flera år innan symtomdebut av RA, vilket tyder på att sjukdomen potentiellt startar flera år innan insjuknade. Syftet med denna avhandling är att öka kunskapen om de processer som kan ha en betydelse för RA sjukdomsutvecklingen och bildningen av ACPA. Genom att öka denna kunskap hoppas man i framtiden på att kunna förbättra tidig diagnos och behandling, men även förhindra uppkomst av sjukdomen genom olika preventiva åtgärder.

I denna avhandling har blodprov som donerats till den Medicinska biobanken, Norra Sverige från individer innan de insjuknat i RA ("pre-symptomatiska individer") och matchade kontroller analyserats. Även blodprov tillhörande patienter från den tidiga RA kohorten vid Umeå Universitetssjukhus har undersökts. I **delarbete I** såg man en ökad förekomst av antikroppar mot citrullinerad histon 4 och peptider från Epstein-Barr viruset, flera år innan insjuknande. Både koncentrationer och positivitet för dessa antikroppar ökade desto närmare symtomdebut av RA. I **delarbete II** analyserades antikroppar mot den orala bakterien *Porphyromonas gingivalis* (*P.gingivalis*) roll i antikroppsbildningen och utvecklandet av RA. En patogen ofta associerad med tandlossning och som potentiellt har en förmåga att citrullinera peptider och bryta immuntoleransen mot dessa. Resultatet visade att nivåer av antikroppar mot *P.gingivalis* var signifikant ökade hos pre-symptomatiska individer jämfört med kontroller och att koncentrationer av dessa antikroppar ökade desto närmare symtomdebut. I **delarbete III** analyserades sambandet mellan receptor activator of nuclear factor κ B ligand (RANKL) och ACPA, anti-karbamylerade antikroppar (anti-CarP IgG) och cytokiner/kemokiner, och deras association till bendedstruktion. Koncentrationer av RANKL var ökad flera år innan symtom

debut av RA. Positivitet för RANKL visades sig tidsmässigt komma efter de övriga analyserade antikropparna. Kombinationen av positivitet för både RANKL och anti-CarP antikroppar var associerad med den högsta Larsen score (benskada) vid symptomdebut. I **delarbete IV** undersökte vi om parodontit definierat som förlust av käkben var associerade med utvecklingen av RA. Av de inbjudna i studien hade totalt 46 stycken tandröntgen innan insjuknande av RA, 45 av dessa individer kunde matchas med kontroller utifrån kön, ålder och rökstatus. Genom denna studie kunde man se att käkbensförlust var associerade med utvecklandet av RA, hos icke-rökare. Pre-symptomatiska individer positiva för RANKL hade även ökad mängd käkbensförlust jämfört med individer negativa för RANKL, särskilt i kombination med ACPA positivitet.

Background

Rheumatoid Arthritis (RA)

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease, characterized by a persistent inflammation in the synovial tissue of the joints, leading to destruction of the cartilage and bone (Harris, 1990; Firestein, 2003). As the disease progresses the patient may experience disabilities, pain and fatigue. Involvement of other organs “extra-articular manifestations” in heart, lungs and blood vessels resulting in myocardial infarction, lung fibrosis and vasculitis may also be a part of the disease and are often associated with a shorter life expectancy (Turesson, 2013). The prevalence of RA is estimated to be approximately 0.5-1 % in the adult population and the overall incidence is 41 of 100 000 individuals. The mean age at disease onset have been reported to be between 53-60 years. Approximately two-thirds of patients with RA are women (Neovius et al., 2011; Eriksson et al., 2013).

Diagnosis and classification criteria

The clinical diagnosis of RA is based on clinical features and/or radiological findings or/and laboratory tests. In 1957, the first classification criteria for RA was defined with the purpose to “homogenize” the patient group and discrete them from patients with other rheumatic diseases (Ropes et al., 1957). In 1987, a new improved version of the RA classification criteria was defined by the American Rheumatism Association (ARA) (*Table 1*) (Arnett et al., 1988). The 1987 classification criteria, were well suited for established disease, but not for identifying individuals in the early stages of RA (Harrison et al., 1998). In 2010, an updated version of the classification criteria was defined by the American College of Rheumatology (ACR) together with the European League of Arthritis and Rheumatism (EULAR) (*Table 2*). The 2010 classification criteria was better applicable on patients with earlier stages of the disease. In the 2010 classification criteria for RA, the presence of anti-citrullinated protein antibodies (ACPA) and not only rheumatoid factor (RF) were included. Except from ACPA, markers for an ongoing inflammation and immune activation were also added (C-reactive protein (CRP) and erythrocyte sediment rate (ESR)). Furthermore, the presence of radiographic changes were excluded and new definitions of joint involvement were constructed (Aletaha et al., 2010). In comparison with the 1987 ARA classification criteria, the 2010 ACR/EULAR classification criteria have a higher sensitivity, but is poor in identifying erosive disease. Both of the criteria are in general relatively poor in identifying erosive disease in early arthritis (van der Helm-van Mil and Huizinga, 2012; Berglin and Dahlqvist, 2013).

Table 1. The 1987, ARA classification criteria for RA (Arnett et al., 1988).

Criteria
1. Morning stiffness: In/around joints at least 1 hour before maximal improvement
2. Arthritis of at least 3 or more joints: Soft tissue swelling of fluid in PIP ¹ , MCP ² , wrist, elbow, knee, ankle and/or MTP ³
3. Arthritis of hand joints: At least one area swollen in wrist, PIP ¹ or MCP ²
4. Symmetric arthritis: Simultaneous involvement of the same joint areas on both sides of the body
5. Rheumatoid nodules: Subcutaneous nodules
6. Rheumatoid factor: Abnormal levels of S-RF ⁴
7. Radiographic changes: Erosions in hand/wrist and/or periarticular osteopenia in hand/wrist

¹PIP = Proximal interphalangeal, ²MCP = metacarpophalangeal, ³MTP = metatarsophalangeal, ⁴S-RF=Rheumatoid factor in serum. 4/7 criteria are needed for a classification of RA, where criteria 1-4 must have been present for ≥6 weeks.

Table 2. The 2010, ACR/EULAR classification criteria for RA (Aletaha et al., 2010).

Criteria¹	Score*
Joint involvement	
1 large joint	0
2-10 large joint	1
1-3 small joints (with or without involvement of large joints)	2
4-10 small joints (with or without involvement of large joints)	3
>10 joints (at least 1 small joint)	5
Serology	
RF ⁻² and ACPA ⁻³	0
Low RF ²⁺ or low ACPA ³⁺	2
High RF ²⁺ or high ACPA ³⁺	3
Acute-phase reactants	
Normal CRP ⁴ and normal ESR ⁵	0
Abnormal CRP ⁴ or abnormal ESR ⁵	1
Duration of symptoms	
<6 weeks	0
≥6 weeks	1

¹This criterion is applied to individuals with ≥ 1 swollen joint. ²RF=Rheumatoid factor, ³ACPA=Anti-citrullinated protein antibody, ⁴CRP= C-reactive protein, ⁵ESR= Erythrocyte sedimentation rate. *A score of ≥6/10 is needed to be fulfilled for a diagnosis of RA.

Treatment

Therapies for RA are primarily aimed to at decreasing the disease activity by dampening the inflammation, preventing joint destruction and/or progression (van der Heijde, 2012; Kavanaugh et al., 2013) and to minimize the risk of future co-morbidities (Thiele et al., 2013). Without treatment, approximately 20-30 % of the patients, would be work-disabled within two to three years after the clinical onset of RA (Rindfleisch and Muller, 2005). According to the current EULAR recommendations, DMARD (Disease Modifying Anti-Rheumatic Drug) should be started as soon as the diagnosis of RA is confirmed. DMARDs are usually divided into conventional synthetic DMARDs (*e.g.*, methotrexate (MTX), sulfasalazine, hydroxychloroquine, leflunomide), biological DMARDs (*e.g.*, TNF- α and IL-6 inhibitors) and targeted synthetic DMARDs (*e.g.*, Janus kinase inhibitor (JAK)). MTX should be considered the first choice of treatment for patients with active RA, alone or in combination with other DMARDs. Low-dose glucocorticoids are usually added to the initial treatment in combination with DMARDs. In patients whose response to the treatment is low, prescription of biological drugs *e.g.*, TNF- α blocker is recommended. The main goal of the treatment is to attain symptom relief, remission and decrease disease activity (Baecklund E., 2017; Smolen et al., 2017). The response to treatment is highly variable, several factors *e.g.*, levels of ESR and CRP, high number of swollen joints and presences of RF and ACPA, is one of many factors suggested to have an effect on the therapy response in these patients (Wijbrandts and Tak, 2017).

Radiology

Plain radiography (X-ray) of hands and feet is not only used as a routine for identifying joint destruction in patients with RA, but also to follow the disease progression and to estimate the severity of the disease. Several scoring systems for assessing radiographic changes in hands and feet have, through the years been developed. In 1971 Sharp presented a method for scoring both joint erosions and space narrowing in hand and wrists (Sharp et al., 1971). After 1985, the Sharp method was modified twice (Sharp et al., 1985; Sharp et al., 1991) and also by van der Heijde who included the evaluation of the feet in the scoring system (van der Heijde et al., 1989). The Larsen score method was also developed in the 1970's and has thereafter been modified several times during the years (Larsen et al., 1977; Larsen et al., 1984; Larsen and Thoen, 1987; Larsen, 1995). In the Larsen score, joints in the hands (proximal interphalangeal joint (PIP) and metacarpophalangeal joint (MCP)), wrist and feet (metatarsophalangeal joints (MTP)) are scored between 0 and 5 according to the degree of joint destruction, bone erosion and joint narrowing in comparison to standard reference films. The maximal score that can be received by this method is 160 (Larsen, 1995). The

Sharp/Heijde and Larsen scoring systems have, in studies, been found to be significantly correlated (Pincus et al., 1995).

Risk factors for the development of rheumatoid arthritis

The aetiology of RA remains to a large extent unknown, but is believed to be a complex interplay between genetic, environmental and stochastic factors. A large number of risk factors have been identified through the years and many of them have been suggested to interact several years prior to the onset of disease.

Genetic factors

In twin studies, monozygotic twins have been shown to have a higher probability to inherit the same disease in comparison to dizygotic twins. The heritability of RA in the general population were accounting for approximately 60 % (Aho et al., 1986; Silman et al., 1993; MacGregor et al., 2000). In a study made with first degree relatives and second-degree relatives, the familial odds ratio (OR) for RA was approximately 3 in first-degree relatives and 2 in second-degree relatives (Frisell et al., 2013). In a Swedish twin study, genetic factors were estimated to contribute to 41 % of all cases of ACPA-positive RA, 39 % of all RA and 10 % for ACPA without RA (Hensvold et al., 2015b). More than 100 risk gene loci for RA have been identified through the years (Terao et al., 2016).

The strongest and the most well established genetic risk factor for RA is the shared epitope (SE) alleles of the human leukocyte antigen (HLA), first identified in 1976 (Stastny, 1976). The HLA-SE consist of a group of alleles (*04:01, *04:04, *04:05, *04:08, *10:01, *01:01 and *01:02) in the HLA-DRB1 gene located on chromosome 6p21 encoding for the beta chain of the major immunohistocompatibility complex type II (MCH II) (Gregersen et al., 1987). MCH II is expressed on antigen-presenting cells and play a central part in displaying peptides to the immune system, for CD4+ T-lymphocytes. Carriers of HLA-SE, have been shown to have an increased risk of developing RA, especially seropositive (RF and/or ACPA positive) disease (Padyukov et al., 2004; Huizinga et al., 2005). The HLA-DRB1 alleles *04:01 and *04:04 are particular strongly associated with ACPA positive RA (Lundström et al., 2009; Mackie et al., 2012). The strong association between ACPA and RA has suggested that peptides presented by HLA-SE might be citrullinated. Hill et al., showed that the SE encoded MCH molecules had an increased affinity against the citrullinated form of a peptide in comparison to the non-citrullinated form of the protein (Hill et al., 2003). Another study found that SE alleles preferentially bound citrullinated peptides, whereas other alleles encoding for the MCH bound both the arginine and citrullinated form (Scally et al., 2013).

The protein tyrosine phosphatase 22 (*PTPN22*), located on chromosome 1p13 is the second strongest genetic risk factor for RA, especially the *PTPN22* (rs2476601) C1858T variant (Begovich et al., 2004). *PTPN22*, encodes for LYP a protein tyrosine phosphatase (PTP) mainly expressed in the lymphoid cells (Gjörloff-Wingren et al., 1999; Hasegawa et al., 2004). The LYP protein has been suggested to be a regulator of T-cell activities and the C1858T variant has been found to increase lymphocyte survival (Vang et al., 2013). Except from HLA-SE and *PTPN22*, cytotoxic T-lymfocyte antigen 4 (CTLA4) (Seidl et al., 1998), signal transducer and activator of transcription 4 (STAT4) (Remmers et al., 2007) and peptidyl arginine deiminase 4 (PADI4) (Suzuki et al., 2003) are one of many gene variants found to be associated with RA, with an estimated risk ratio between 1-2.

Smoking and air exposure

Tobacco smoke has long been considered a well-established environmental risk factor for RA, particular ACPA-positive RA, independent of adjustments for geographic location of residence, social class and age (Vessey et al., 1987; Heliovaara et al., 1993; Stolt et al., 2003). Smoking has been estimated to increase the risk of RA almost 2-fold (Sugiyama et al., 2010). One large twin study has suggested that environmental (including smoking) and stochastic factors may play a larger role than genetic factors in the development of ACPA. Whilst, genetic factors may have a large role in identifying which ACPA positive individual will develop arthritis (Hensvold et al., 2015b). Smoking was found to induce citrullination of peptides by the activation of PAD enzymes in the lungs (Makrygiannakis et al., 2008; Reynisdottir et al., 2014). In a Swedish study, smoking was estimated to be responsible for approximately 35 % of all ACPA-positive RA cases, and 55 % of all ACPA-positive RA cases carrying two sets of the HLA-DRB1 SE (Källberg, 2011). In accordance with a previous study, Klareskog et al. found that the risk of developing ACPA-positive RA for a smoker who carried two copies of the HLA-SE alleles were 21-fold higher than for non-smoking HLA-SE carrier (Klareskog et al., 2006). The interaction between HLA-SE and smoking has been found to be associated with several different citrullinated antibodies and not to be specific for antibodies against certain citrullinated antigens (Willemze et al., 2011). The association between smoking and RA remain several years after cessation of smoking, (Di Giuseppe et al., 2013) and has also been demonstrated in seronegative individuals (Bang et al., 2010). Except, from smoking, silica released during *e.g.*, stone dust and crushing were found to be associated with an increased risk for ACPA-positive RA (Khuder et al., 2002; Stolt et al., 2010).

Hormonal and dietary factors

It is well-known that RA is 2-4 times more common in women than men, although the mechanism behind this is, to a large extent unknown. Several studies have shown a protective effect of contraceptives in RA (Vandenbroucke et al., 1982; Berglin et al., 2010). Recently, in a large Swedish case-control study, oral contraceptives were associated with a lower risk of developing ACPA-positive RA, in comparison to women whom never used oral contraceptives. Furthermore, ever smokers who never used oral contraceptives had a higher risk of developing seropositive RA in comparison with ever smokers who used oral contraceptives (Orellana et al., 2017). The interest in dietary factors in the development of RA has throughout the years increased. Recently, omega-3-fatty acids were shown to decrease the risk for ACPA-positive RA and reduce the inflammatory burden in individuals already positive for ACPA (Gan et al., 2017). In one study by Sundström et al., a high intake of dietary salt in combination with smoking doubled the risk of developing RA in comparison with those with low salt intake (Sundstrom et al., 2015). Alcohol consumption is another well debated area, and a moderate alcohol consumption has been found to decrease the risk of developing RA, especially in ACPA-positive RA patients in comparison with non-drinkers (Scott et al., 2013; Jin et al., 2014).

Infections and infectious agents

Several infectious agents have been suggested to have the potential to induce citrullination of both endogenous and exogenous antigens in RA. Recent studies have suggested that dysbiosis in the oral and gut microbiome potentially could lead to chronic inflammation and trigger an ACPA response (Zhang et al., 2015; Horta-Baas, 2017).

A link between periodontitis (PD) and RA has long been suggested (Bartold et al., 2005). Both of the diseases are characterized by chronic inflammation resulting in soft-tissue inflammation and that later may result in loss of adjacent bone and a lifelong disability (Pihlstrom et al., 2005; Klareskog et al., 2009). Furthermore, both PD and RA share similar genetic and environmental risks factors *e.g.*, the HLA-DRB1 alleles (Katz et al., 1987) and smoking (Heliovaara et al., 1993; Tonetti, 1998). Several serum markers used in the diagnosis of RA, have also been found to be elevated in periodontitis, *e.g.*, ACPA, RF and CRP (The and Ebersole, 1991; Noack et al., 2001; Harvey et al., 2013). The connection between PD and RA has been suggested to be explained by the periodontal pathogen *Porphyromonas gingivalis* (*P.gingivalis*) (Rosenstein et al., 2004). *P.gingivalis* has for many years been the only bacteria known to have the capacity to citrullinate both bacterial and human proteins with help of its PAD enzyme, *P.PAD* (McGraw et al., 1999; Wegner et al., 2010; Quirke et al., 2014) The *P.gingivalis* virulence

factor arginine ginpain (Rgp) protease cleaves the proteins at arginine residues and enable the citrullination of the *P.PAD* enzyme at the C-terminal of the protein (Wegner et al., 2010; Goulas et al., 2015). A study by Lundberg et al. interestingly demonstrated a cross-reactivity between human and *P.gingivalis* citrullinated α -enolase (Lundberg et al., 2008). Several studies has recently shown an association between antibodies against *P.gingivalis* and ACPA-positive RA (Hitchon et al., 2010; Kharlamova et al., 2016) while other studies have not been able to confirm these findings (Seror et al., 2015). Studies have suggested that the action of *P.PAD* may lead to a chronic exposure of citrullinated proteins in the inflamed periodontium. Cross-reactivity with self-peptides may thereafter break the tolerance and trigger the ACPA production in susceptible individuals (Rosenstein et al., 2004; Lundberg et al., 2010; Quirke et al., 2014).

Recently, the periodontal gram-negative bacteria *Aggregatibacter actinomycetemcomitans* (*Aa*) has been found to have the capacity to initiate citrullination of proteins via its virulence factor, leukotoxin A (LtxA). The pore-forming protein LtxA allows extracellular calcium influx and activation of the PAD enzymes in neutrophils, leading to hypercitrullination of intracellular proteins that subsequently were released into the extracellular space (Taichman et al., 1991; Konig et al., 2016). The citrullinated proteins induced by LtxA mechanism showed a markedly overlap with the previously identified citrullinated proteins found in RA synovial fluid. Furthermore, antibodies against both LtxA and *Aa* were found to be increased in patients with RA (Konig et al., 2016).

Epstein-Barr virus (EBV) is a herpes virus infecting a majority of the general adult population. The EBV infection has been found to targeting epithelial cells and B-lymphocytes, resulting in a life-long latent infection in memory B-cells (Kalla and Hammerschmidt, 2012). Through, the years several studies have suggested a link between EBV and RA (Tosato et al., 1984; Goldstein et al., 2012; Erre et al., 2015; Westergaard et al., 2015). Studies has shown that EBV is present in a high frequency in ACPA producing plasma cells in patients with RA (Croia et al., 2013), and that patients with RA have elevated levels of EBV DNA compared to controls (Balandraud et al., 2003). Previous studies have shown an increased reactivity against the two EBV derived proteins Epstein-Barr nuclear antigen (EBNA) 1 and 2 in RA patients (Pratesi et al., 2006; Pratesi et al., 2011).

Neutrophil extracellular traps (NETs)

Neutrophils are one of the cells of the innate immune system that have received attention in the pathogenesis of RA. Cell death in different forms *e.g.*, NETosis has been suggested to be an important source of citrullinated autoantigens in RA. Neutrophil extracellular traps (NETs) are produced by neutrophils in the defence

against bacterial, fungal and parasitic infections. During NETosis, decondensed chromatin fibers containing histones, proteins and antimicrobial granules is released into the extracellular space (Brinkmann et al., 2004). The process has been demonstrated in many studies to be dependent upon activation of PAD enzymes (Li et al., 2010; Leshner et al., 2012). PAD are one of the enzymes in our body with the capacity to citrullinate proteins. Enzymatically active PAD2 and PAD4 have been suggested to be released into the extracellular environment during NETosis and citrullinate proteins in the surrounding tissue (Spengler et al., 2015). RA synovial fluid displays enhanced NETosis compared with healthy individuals and patients with RA were shown to have increased levels of antibodies against citrullinated peptides from *e.g.*, histones 4 (H4). Suggesting NETosis as a potential endogenous source of citrullinated antigens (Pratesi et al., 2014).

Peptidyl arginine deiminase (PAD)

As mentioned above, the peptidyl arginine deiminase (PAD) are an enzyme dependent upon binding of calcium ions that induces a conformational change and generates an active form of the enzyme. The enzyme is responsible for the physiological process of citrullination/deamination, during that process PAD converts the strongly basic amino acid arginine to the more natural amino acid citrulline (*Figure 1*).

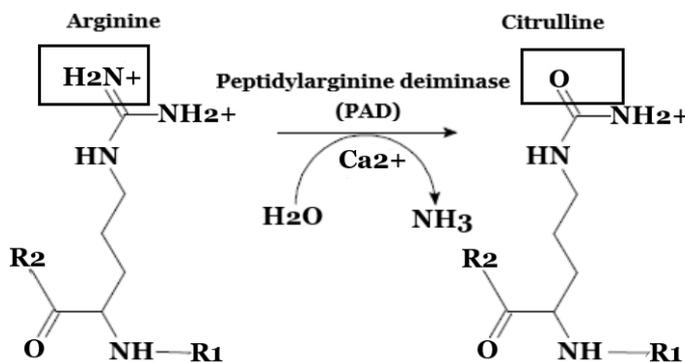


Figure 1. The citrullination process by the peptidyl arginine deiminase (PAD) enzyme.

In humans, five PAD isoenzymes have been identified so far, PAD 1-4 and PAD 6, all located at 1p36.15 (Vossenaar et al., 2003; Chavanas et al., 2004). The PAD enzymes, have as the citrullinated proteins, been identified in several tissues in the body. PAD1 in the uterus and epidermis, PAD2 in skeletal muscle, inflammatory cells and secretory glands, PAD3 in keratinocytes and hair follicles,

PAD4 in granulocytes, *e.g.*, neutrophils and PAD6 in oocytes and embryo (Vossenaar et al., 2003; Chavanas et al., 2004; Makrygiannakis et al., 2006). The PAD enzymes share a sequence similarity of approximately 50 % (Arita et al., 2004). The levels of autoantibodies directed against PAD4 has been shown to be significant higher in RA patients compared with controls and has also been found to correlate both with both disease severity and anti-CCP2 antibody positivity (Halvorsen et al., 2008; Harris et al., 2008; Kolfenbach et al., 2010).

Antibodies

The immune system is essentially divided into an innate and an adaptive immunity. The phagocytes *e.g.*, macrophages and neutrophils but also natural killer cells of the innate immune system provide a first line defense against microorganism. Pathogens that enter the circulation or the tissues by breaching the mucosal or the epithelial barrier are met immediately by these cells. The more specialized adaptive immunity develops much slower. The lymphocytes (CD4+, CD8+ T-lymphocytes and B-lymphocytes) and antibodies of the adaptive immunity recognize a much wider variety of molecules produced by microbes, but also non-infectious substances. The adaptive immune system also has a “memory function” for previous encountered pathogens and can more effective protect the host. Together the adaptive and the innate immunity cooperate to eliminate pathogens of different characteristic *e.g.*, via antibodies.

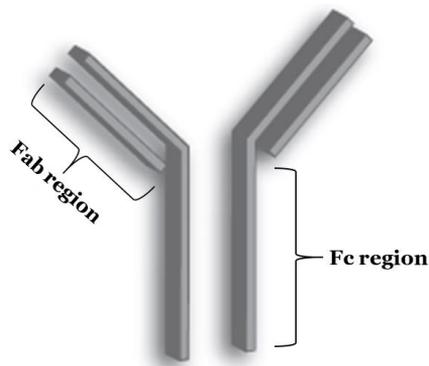


Figure 2. Overview of IgG antibody structure. Abbreviations: fragment antigen-binding (Fab) region and fragment crystallizable (Fc) region.

Antibodies consist of two light and two heavy chains, with one antigen-binding region named Fab (fragment antigen binding) and a Fc (fragment crystallizable) region (*Figure 2*). Antibodies are in general glycoproteins, with carbohydrate chains attached to both the Fc and the Fab region of the antibody. The variation of the Fc segment results in different classes of antibodies *e.g.*, IgG or IgM. Naïve

B-lymphocytes express membrane bound antibodies, also known as the B-cell receptor (BCR). The antigen binding of the BCR often result in the differentiation of the naïve B-lymphocytes into antibody-secreting plasma cells and memory B-cells. The plasma cells synthesises and secretes antibodies with many different heavy-chain isotypes (classes): immunoglobulin (Ig) G, IgA, IgE, IgM and IgD. After secretion the antibodies enter the bloodstream and reach the site of infection and act as a defence against *e.g.*, microbes. The different isotypes have different functions in the immune system: IgG is the most common antibody in plasma, eliminates microbes by *e.g.*, activating the complement system and enable opsonisation of microbes by macrophages and neutrophils. IgG can be divided into four subclasses, IgG1-4, whereas the IgG-1 is the most common subclass with the highest concentration in plasma. IgM together with IgD are expressed as a BCR on naïve B-cells and IgM with its pentameric shape is much larger than the other antibodies. Like IgG, IgM can activate the complement pathway. IgA is mainly found in the mucosal sites and can take the shape of a monomer, dimer or trimers. IgE is predominantly associated with allergic reactions.

The balance between recognition of pathogens and self-antigens is an important task for the immune system. It is hypothesized that susceptibility genes interfere with pathways of self-tolerance and can cause a persistence of self-reactive T- and B-lymphocytes. Environmental or stochastic stimuli, *e.g.*, infections and smoking can cause a cell or tissue injury leading to activation of these cells and resulting in production of autoantibodies and a generation of effector T-lymphocytes. In the bone marrow, where the B-cell maturation take place, naïve B-lymphocytes are tested against self-antigens. Only B-lymphocytes with properly BCR signaling and with one antibody specificity will continue the maturation process. The cells that bind too strongly to self-antigens, will go through further receptor editing of the light chain, anergy or apoptosis. The mechanism of early distinguish between B-lymphocytes that have the potential to be autoreactive is suggested to be impaired in patients with RA (Shlomchik, 2008).

Citrullination and ACPA

The conversion of arginine residues to citrulline residues in protein is a calcium-dependent physiological process called citrullination/deamination (*Figure 1*) and was first described in 1977 (Rogers et al., 1977; Vossenaar et al., 2003). The process was found to be catalyzed by the enzyme peptidyl arginine deiminase (PAD) and was partly purified in 1981 (Fujisaki and Sugawara, 1981). Citrullinated proteins are present in a wide range of inflammatory tissues independently of the disease (Makrygiannakis et al., 2006), while Schellekens et al. found that immunity against citrullinated proteins are specific for RA. Approximately 70 % of all patients with RA have antibodies against citrullinated

proteins (ACPA) (Schellekens et al., 1998; Nishimura et al., 2007). In the RA synovial fluid a ten-fold of differential of intra- and extracellular citrullinated proteins has been identified, but only a few of them have been identified as ACPA targets. The synovial “citrullinome” has been a term used for describing the of the whole set of citrullinated proteins in the inflamed synovium (Van Beers et al., 2012). Citrullinated vimentin (Vossenaar et al., 2004), fibrinogen (Masson-Bessiere et al., 2001), α -enolase (Kinloch et al., 2005) and collagen II (Burkhardt et al., 2005) are all well-known citrullinated protein targets for ACPA.

The anti-perinuclear factor (APF) from buccal mucosal cells was the first ACPA found with a high specificity for RA (Nienhuis and Mandema, 1964) and in 1979 the anti-keratin antibodies (AKA) was identified (Young et al., 1979). These two antibodies were later found to share the specificity for the citrullinated form of filaggrin (Simon et al., 1993; Schellekens et al., 1998; Girbal-Neuhauser et al., 1999). The first generation of enzyme-linked immunosorbent assay (ELISA), anti-CCP IgG test was later developed by the same group, Schellekens et al. By making a cyclic form of filaggrin in comparison to the linear form the sensitivity of the test increased. When comparing the anti-CCP1 test with RF, a higher specificity but a lower sensitivity were shown for the anti-CCP1 test (Schellekens et al., 2000; Nishimura et al., 2007). An improved version of the anti-CCP1 test, the anti-CCP2 test was later developed, with a higher sensitivity and a specificity of approximately 98% (van Gaalen et al., 2005; van Venrooij et al., 2011). To this present day, the identity of the anti-CCP2 test is unknown. A third-generation CCP-test, anti-CCP3 test was later developed (Santiago et al., 2008). Most studies could not show evident improved sensitivity and specificity of the third generation test, therefore the CCP2 test is predominantly used in the clinic today (Coenen et al., 2007; Santiago et al., 2008). ACPA is presented in three isotypes, IgG, IgM and IgA, whereas ACPA of IgG type is so far the most common measured (Chapuy-Regaud et al., 2005; Lundström et al., 2014).

The presence of antibodies divides patients with RA into a seronegative and a seropositive disease, groups that differ in risk factors and clinical outcomes. Patients with ACPA usually have a more aggressive disease with increased radiographic progression, disability and mortality (van Gaalen et al., 2004; van der Helm-van Mil et al., 2005; van der Linden et al., 2009; Syversen et al., 2010) and often respond better to B-cell depletion treatments (Chatzidionysiou et al., 2011). Individuals with seronegative disease need a higher inflammatory activity to fulfill the 2010 ACR/EULAR classification criteria in comparison with patients with seropositive disease (Nordberg et al., 2017). A few patients have been found to seroconvert after disease onset (Barra et al., 2011).

Rheumatoid factor (RF)

Rheumatoid Factor (RF) was the first diagnostic test for RA and being introduced in 1940 by Erik Waaler, and due to communication difficulties during the war later rediscovered in 1948 by Rose et al. The test thereafter received the name Waaler-Rose agglutination test (Waaler, 1940; Rose et al., 1948), first in 1949 the test were named rheumatoid factor because of its association with RA (Pike et al., 1949). RF of IgM type was a long time the only diagnostic test for RA and is included in both the ARA and the ACR/EULAR classification criteria for RA (Arnett et al., 1988; Aletaha et al., 2010). Although, RF is only present in approximately 60-80 % of all patients with RA and is a nonspecific marker for the disease (Steiner and Smolen, 2002). Patients with extra articular manifestations tend to have higher levels of RF (Naranjo Hernandez et al., 1997). High titers of RF have also been found in patients with other rheumatic disease, primarily Sjögren's syndrome and chronic infections (Williams, 1988; van Boekel et al., 2002).

Several isotypes of the RF immunoglobulin have been identified *e.g.*, IgG, IgM, IgA, IgE (Gioud-Paquet et al., 1987) and has been shown to be directed against the Fc region of IgG (Lawrence and Williams, 1967). IgA RF has the highest sensitivity until onset of symptoms of RA, whereas IgM RF on the other hand has the highest sensitivity after disease onset (Rantapaa-Dahlqvist et al., 2003; Jorgensen et al., 2008). The combination of both ACPA and RF was found to increase the specificity, but decreased the sensitivity for RA (Rantapaa-Dahlqvist et al., 2003; Nielen et al., 2004). Approximately, 90% of all ACPA positive patients are positive for RF and almost 50% of all individuals negative for ACPA are RF positive (Brink et al., 2016).

Other antibodies in RA

ACPA is not the only antibody against posttranslational modified proteins associated with RA. Anti-carbamylated antibodies (anti-CarP IgG) (Shi et al., 2011), anti-acetylated vimentin antibodies (Juarez et al., 2016) and anti-malondialdehyde-acetaldehyde antibodies (MAA) (Thiele et al., 2015) have been associated with the disease.

Autoantibodies in the pre-symptomatic period

ACPA can exist several years without causing any clinical symptoms of RA (Rantapaa-Dahlqvist et al., 2003; Nielen et al., 2004) and their presences does not necessarily lead to an autoimmune disease in all individuals (Ioan-Facsinay et al., 2008). Several explanatory models for the production of ACPA and the development of RA have through the years been suggested. The initial trigger that

breaks the immune tolerance against citrullinated proteins and the production of ACPA has been called the “first hit”. A “second or multiple hits” are suggested to be necessary for tip the balance over from a pre-symptomatic stage against a clinical disease. The second hit is suggested to involve the HLA-SE on the antigen presenting cells (Scherer et al., 2018). Several autoantibodies against different citrullinated antigens *e.g.*, α -enolase, fibrinogen, vimentin and filaggrin (Brink et al., 2013) but also RF (Rantapaa-Dahlqvist et al., 2003; Brink et al., 2016) and anti-CarP antibodies (Brink et al., 2015) are present prior to symptom onset of RA. ACPA of IgG type have been found to appear first in time followed by IgA and thereafter ACPA of IgM type (Kokkonen et al., 2011; Bos et al., 2014). Studies have shown that the concentration and antibody positivity increases closer symptom onset of RA, and that the profile of antigens recognized by autoantibodies expand closer to disease onset (van der Woude et al., 2010; Brink et al., 2013). Furthermore, in a study based on arthralgia patients, the ACPA Fc glycosylation pattern was found to change after disease onset. Fucosylation increased whilst galactosylation decreased closer to disease onset leading to a more pro-inflammatory phenotype in these antibodies (Rombouts et al., 2015). Except from autoantibodies, several cytokines/chemokines representing the adaptive immune system *e.g.*, IL-6, IL-2, IL-4, IL-10 have been found to be up-regulated years before symptom onset of RA (Kokkonen et al., 2010).

Joint inflammation and bone damage

The main characteristic of RA are a chronic inflammation in the joint, leading to both inflammation and bone destruction. Joint inflammation in RA is characterized by hyperplasia of the synovial membrane with a local accumulation of inflammatory cells (CD4+ and CD8+ T-lymphocytes, B-lymphocytes, plasma cells and macrophages). Several pro-inflammatory cytokines *e.g.*, IL-6 and TNF- α are produced and are of importance in driving the inflammation. As the disease progresses, the synovium and the activated cells subsequently invade and destroy the cartilage and underlying bone of the joint (Tak and Bresnihan, 2000; Choy and Panayi, 2001). Bone erosion is a common outcome in patients with RA and a predictive marker for a more severe disease, with both a higher degree of disability and mortality (Scott et al., 2000; Odegard et al., 2006). Bone damage in RA has been shown to be present in approximately half of all untreated patients after only 6 months following disease onset (van der Heijde, 1995), and even before the presentation of the first clinical symptom of RA (Kleyer et al., 2014). ACPA has been suggested as the strongest risk factor for bone damage in RA (van Gaalen et al., 2004; van der Linden et al., 2009; Syversen et al., 2010). Antibodies against citrullinated vimentin have in many studies been associated with bone damage in RA (Mathsson et al., 2008; Syversen et al., 2010). A study by Harre et al. demonstrated that autoantibodies against mutated citrullinated vimentin (MCV) have the capacity to bind in to the osteoclast surfaces and increase both

osteoclastogenesis and bone resorption, both in vivo and in vitro (Harre et al., 2012). Furthermore, the presence of anti-carbamylated antibodies has also been shown to be associated with radiological bone damage in patients with RA (Shi et al., 2011; Brink et al., 2015)

An osteoblast and osteoclast imbalance driven by inflammatory process, leading to an increased resorption by osteoclasts is suggested to be a central part in the bone pathogenesis in RA. The receptor activator of nuclear factor kappa-B (RANK) is mainly expressed by osteoblast, osteocytes, T-lymphocytes, stromal cells and synovial fibroblast. The protein is a part of the TNF superfamily and is presented either in a membrane-bound (most common) or secreted form. RANK ligand/osteoprogenin (RANKL/OPG) has been shown to play a central part in osteoclastogenesis. The binding of RANKL into RANK on osteoclast precursors have been shown to trigger osteoclast maturation, whilst binding to a mature osteoclast mediates activation and survival of the cell (Khosla, 2001). Cytokines *e.g.*, IL-6 and IL-8 have previously been found to induce RANKL expression in osteoblast and stimulate osteoclastogenesis (Bendre et al., 2003; Krishnamurthy et al., 2016; Sims, 2016). Whilst, OPG is a protein with the ability to block the RANK-RANKL interaction and attenuate osteoclast differential (Khosla, 2001). Furthermore, RANKL has, in several studies, been found to be a potential bone marker in RA (van Tuyl et al., 2010; Hensvold et al., 2015a) In a recent study, RANKL was found to be associated with ACPA, especially antibodies against citrullinated vimentin, suggesting a direct link between ACPA, RANKL and bone destruction in RA (Hensvold et al., 2015a; Boman et al., 2017).

Study population

The individuals included in this thesis have been identified from the cohorts of The Medical Biobank of Northern Sweden, Umeå and the early RA cohort, Department of Rheumatology, Umeå University Hospital.

The Medical Biobank of Northern Sweden

The cohort of The Medical Biobank of Northern Sweden is composed of several different cohorts.

- **The Northern Sweden Health and Disease Study (NSHDS)** includes three sub-cohorts:
 - The Västerbotten Intervention Programme (VIP)*
 - The Northern Swedish MONICA project (MO)*
 - The Mammography Screening project (MA)

*All of the samples within NSHDS have been sampled after overnight fasting or a minimum of 4 hour fasting. The samples were stored within one hour after sampling at -80°C .

- **The Northern Sweden Maternity cohort**

Västerbotten Intervention Programme (VIP)

The VIP cohort was first developed in 1985 in Norsjö as a cardiovascular and diabetes survey intervention programme, and has thereafter been successively implemented across the whole county of Västerbotten. In 1991, the entire county was included (Weinehall et al., 2001). All habitants living in the county of Västerbotten, aged 30 (included before 1996), 40, 50 and 60 years have regularly been invited to participate in the programme. After attending to the local health care center, the participant's answered a lifestyle and health questionnaire, anthropometric (*e.g.*, blood pressure, height, weight) and blood glucose measurements was performed by a nurse and they donated blood samples. In September 2017, the VIP included 105 700 individuals, 40 700 of these individuals have donated several samples. The participation rate ranged between 48-67 % during the years (Norberg et al., 2010).

The Northern Swedish MONICA project (MO)

In 1985 the Monitoring of Trends and Determinants in Cardiovascular Disease (MONICA) project was initiated in Västerbotten and Norrbotten County, Sweden by the WHO as a part of a multi-national project. The main task of the programme

was to monitoring the trends of cardiovascular diseases in the population. Seven population surveys has so far been performed with a total of 11 800 randomly selected individuals during 1986, 1990, 1994, 1999, 2004, 2009 and 2014. For each screening occasion, 2500 individuals aged 25-74 years was invited to participate. As a part of the programme, the participants answered health and lifestyle questioners, anthropometric measurements (*e.g.*, blood pressure, length, and weight) and donate blood samples. The participation rate ranged between 63-81 % during the different occasions. In Mars 2015, the MONICA study included 11 800 individuals, 3500 of these individuals have donated several samples.

The Mammography Screening project (MA)

Blood samples and data are collected in connection to mammography screening during 1995-2006 in Västerbotten County. The cohort consisted of women aged 18-82 years, whereas 95 % of the women were between 48-70 years old. The women were invited to participate every second year. The cohort consist of 28 800 individuals of whom 14 600 individuals have donated several samples.

The Northern Sweden Maternity cohort

The Maternity cohort was established in 1975 at the University Hospital of Umeå, Sweden (NUS). The cohort include serum samples from pregnant women screened for immunity against the rubella virus (*i.e.*, German measles) from the four northern counties of Sweden Västerbotten, Norrbotten, Jämtland and Västernorrland.

The serum samples were collected in a maternity healthcare clinic located in one of the four counties and thereafter shipped and stored at -20 °C at Umeå University Hospital. Until 1987, the samples were heat-treated to inactivate potential contagious agents. In august 2013, the cohort contained more than 126,000 serum samples donated from 91,000 women.

Pre-symptomatic individuals and patients with early RA

Pre-symptomatic individuals

A pre-symptomatic individual is defined as a blood donor to one of the Medical Biobank of Northern Sweden cohorts, before the first symptoms of RA. The pre-symptomatic individuals were identified by co-analysing the register of patients with RA (early and established) fulfilling the ARA 1987 classification criteria for RA at the Department of Rheumatology with the cohorts of The Medical Biobank of Northern Sweden (*Figure 3*).

The pre-symptomatic individuals included in *Paper II* and *IV* were identified by the first co-analysing process, whilst individuals included in *Paper I* and *III* were identified by a second co-analysing process. All individuals from *Paper II* and *IV* are the same individuals as in *Paper I* and *III* who are similar individuals. The slight deviation from exactly being the same individuals is due to lack of sample and for *Paper IV* the individual must be alive (to answer the questionnaire). Individuals included in each paper is presented below.

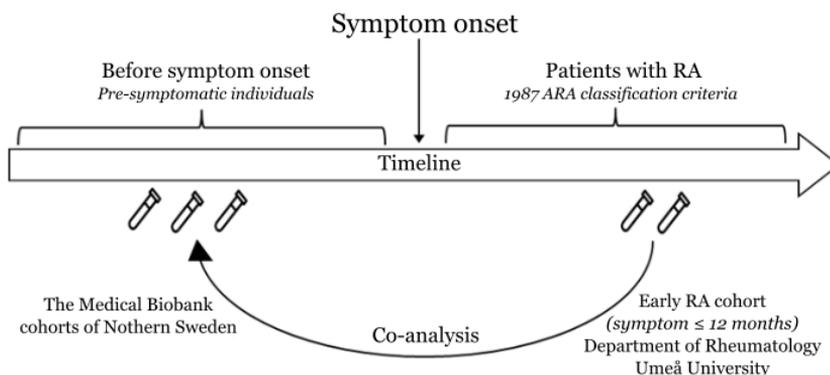


Figure 3. Illustration of the strategy used to perform the co-analysis of the register of pre-symptomatic individuals with the register of patients with early and established RA. The pre-symptomatic individuals donated blood samples to the Medical Biobank cohorts of Northern Sweden before onset symptoms onset of RA and later attended to the Department of Rheumatology at Umeå University Hospital to receive their diagnosis of RA. At time of diagnosis all pre-symptomatic individuals fulfilled the 1987 ARA classifications criteria of RA and had a symptom duration of ≤ 12 months.

Patients with early RA

Since December 1995, all of the patients attending at the Department of Rheumatology at the University Hospital of Umeå, Sweden to receive their diagnosis of RA with disease duration of ≤ 12 months, were included into the early RA cohort. The patients fulfilled the 1987 ARA classifications criteria for RA at the time of diagnosis (Arnett et al., 1988). Blood samples were collected into a Biobank at the Department of Rheumatology, Umeå University Hospital and data has been stored in the Swedish Rheumatology Quality Register (SRQ). The blood samples were divided into aliquots and stored at -80 °C.

All of the patients with early RA who are resident in one of the four northern counties of Sweden; Västerbotten, Norrbotten, Jämtland and Västernorrland constitute the early RA cohort of northern Sweden.

The population based controls

After identification of the pre-symptomatic individuals, population based controls were randomly selected and matched for sex, age at the time of sampling and area of residual from the cohorts of The Medical Biobank of Northern Sweden.

Paper I

Paper I, included first 531 pre-symptomatic individuals and 277 matched controls from the cohorts of the Medical Biobank. Only 521 pre-symptomatic individuals and 272 controls were available for plasma analysis. Samples from all 241 patients from the early RA cohort were also analysed of whom all having donated one sample before they were diagnosed with RA. These samples were included among the 521 pre-symptomatic individuals (*Table 3*).

Paper II

Paper II, included 251 pre-symptomatic individuals who had donated a total of 422 plasma/sera samples. Of the 422 pre-symptomatic individuals, 375 individuals were identified in the registers of the Medical Biobank cohort and 47 in the Maternity cohort. One sample was identified for each of the 251 pre-symptomatic individuals; two samples were identified for 92 individuals (36.6 %), three samples for 46 individuals (18.3 %), four samples for 22 individuals (8.8 %), five samples for nine individuals (3.6 %) and six samples for two individuals (0.8 %) The study also included blood samples from 192 patients with early RA of whom 153 had donated blood before onset of symptoms and were included among the 422 pre-symptomatic individuals. A total of 198 population-based controls (173 plasma samples from the Medical Biobank and 25 sera samples from the Maternity cohort) were also included in the study (*Table 3*).

Paper III

Paper III, included 470 pre-symptomatic individuals and 96 matched controls, all from the Medical Biobank cohort. All of the 470 individuals had one sample before symptom onset of RA (*Table 3*).

Table 3. Descriptive data on the pre-symptomatic individuals, patients with RA and controls included in *Paper I-III*.

	Paper I	Paper II	Paper III
Pre-symptomatic individuals (n¹)	521	251	470
Samples (n ¹)	521	422	470
Women (%)	71.2	76.9	71.1
Age at sampling, mean (SD ²), years	52.4 (9.4)	50.5 (11.9)	52.3 (9.4)
Predating time before onset of symptoms, median (IQR ³), years	5.3 (6.0)	5.2 (6.2)	5.0 (5.1)
RA patients (n¹)	241	192	-
Women (%)	69.3	75.0	-
Age at sampling, mean (SD ²), years	60.1 (9.7)	56.5 (11.3)	-
Symptom duration before diagnosis, median (IQR ³), years	0.6 (0.63)	0.6 (0.48)	-
Controls (n¹)	272	198	96
Women (%)	61.4	84.3	62.5
Age at sampling, mean (SD ²), years	51.8 (9.5)	49.3 (14.8)	53.2 (9.7)

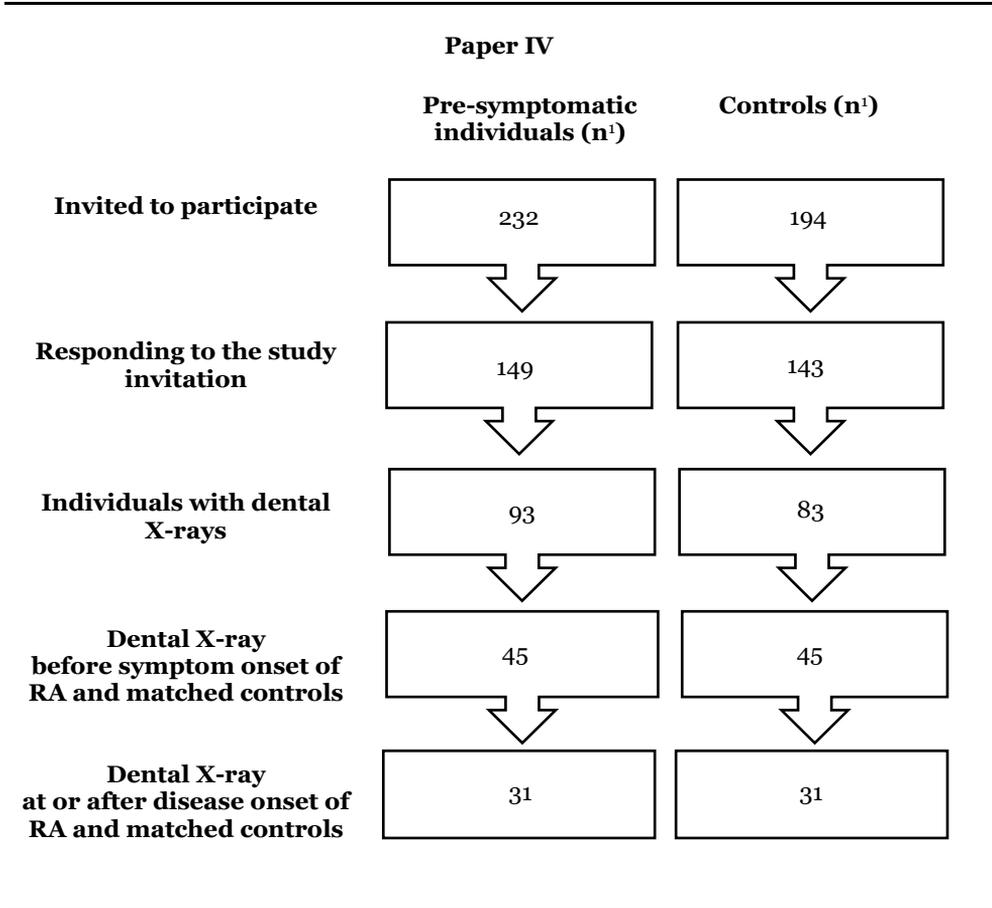
¹n=Number of individuals ²Standard deviation ³Interquartile range

Paper IV

In *Paper IV*, 232 pre-symptomatic individuals and 194 matched controls were identified from the register of the Medical Biobank in Northern Sweden. These individuals were invited to participate in the study via mail. A total of 149 (64.2 %) of the pre-symptomatic individuals and 145 (74.7 %) of the controls gave their informed consent to participate. All participants were asked to fill in a questionnaire on self-assessed dental status, smoking habits and information about their dental care provider during over the years.

A total of 93 pre-symptomatic individuals and 83 controls had dental X-rays (n=688.2 vs n=655.7, respectively). Of the pre-symptomatic individuals, 46 had an X-ray before onset of symptoms for onset of RA. Only 45 of these could be matched with one control based upon sex, age and smoking status, due to the low frequency of smokers among the controls. 31 of the matched pairs had also dental X-ray after symptom onset of RA (Table 4).

Table 4. Descriptive data of the selection of the pre-symptomatic individuals and controls into the different analysis included in Paper IV.



¹n=Number of individuals

Methods

Enzyme linked immunosorbent assay (ELISA)

Anti-CCP2 antibodies/ACPA

Anti-CCP2 antibodies was measured using a commercially available ELISA kit (Euro-Diagnostica, Malmö, Sweden), with the cut-off for positivity set at >25 arbitrary units (AU)/mL. The measurements and cut-off for positivity were made according to manufacturer's protocol.

Multiplex analysis of antibodies against citrullinated peptides

In *Papers I-III*, the results from the most frequently appearing antibodies from Brink et al (Brink et al., 2013), against citrullinated proteins were used. Four different ACPA fine-specificities were analysed using a microarray based on the ImmunoCAP ISAC system (Phadia AB, Uppsala, Sweden). The specificity for each of the antibodies was set according to receiver operating characteristic (ROC) curves, giving the specificity of $\geq 95\%$ as previously described (Brink et al., 2013). The description of the method and validation in comparison to a regular ELISA method are described previously (Hansson et al., 2012; Brink et al., 2013).

- *Anti-citrullinated fibrinogen β 36-52 (cFib β 36-52) IgG*: Citrullinated at position: 44. Amino acid sequence: NEEGFFSA (cit) GHRPLDKK (linear)
- *Anti-citrullinated α -enolase5-21 (CEP-1) IgG*: Citrullinated at position: 9 and 15. Amino acid sequence: CKIHA (cit) EIFDS (cit) GHPTVEC (cyclic)
- *Anti-citrullinated filaggrin307-324 (CCP-1) IgG*: Citrullinated at position 13. Amino acid sequence: SHQEST (cit) GRSRGRSGRSGS (cyclic)
- *Anti-citrullinated vimentin60-75 (cVim60-75) IgG*: Citrullinated at position 64, 69 and 71. Amino acid sequence: VYAT (cit) SSAV (cit) L (cit) SSVP (linear)

Anti-VCP and -HCP IgG ELISA

In *Paper I*, ELISA polystyrene plates (Nunc MaxiSorp F96; Nunc Roskilde, Denmark) were coated with viral citrullinated peptide (VCP)₁ or VCP₂ (5 μ g/ml in PBS) or with histone citrullinate peptide (HCP)₁ or HCP₂ (10 μ g/ml in 50 mM sodium carbonate/bicarbonate buffer pH 9.6) as previously described (Pratesi et al., 2011; Pratesi et al., 2014). The plates were incubated overnight at +4°C.

Saturation was carried out with (PBS, BSA 3% for anti-VCP assays) and with (PBS, Porcine gelatin 1% for anti-HCP assays) for 45 minutes in room temperature (RT). Plasma samples, diluted 1:200 in (PBS, BSA 1%, Tween-20 0.05% for anti-VCP) or (PBS, Gelatin 0.5%, Tween 0.05% for anti-HCP) were incubated on the plates for 2 hours at RT. After washing, alkaline phosphatase-conjugated anti-human IgG was diluted and added to the wells and the plates incubated for 2 hours at RT. Alkaline phosphatase activity was revealed with p-nitrophenylphosphate in sodium carbonate/bicarbonate buffer. ROC curves were used to define the cut-off values for antibody positivity. The cut-off for anti-VCP1 IgG positivity was set at 32.89 AU/mL, anti-VCP2 IgG at 9.09 AU/mL, anti-HCP1 IgG at 19.51 AU/mL and anti-HCP2 IgG at 11.64 AU/mL, corresponding to specificity of 98.2 %.

Anti-RgpB and -CPP3 IgG ELISA

In *Paper II*, plasma/serum samples from patients, pre-symptomatic individuals and controls were analysed for presence of anti-RgpB IgG using an in-house ELISA, modified from Quirke et al (Quirke et al., 2014; Kharlamova et al., 2016). The coating antigen, C-terminal hexahistidine-tagged RgpB protein was purified from growth medium of a genetically modified *P.gingivalis* strain (W83) by using an affinity chromatography on Ni-Sepharose as previous described by Potempa and Nguyen (Potempa and Nguyen, 2007).

The synthetic cyclic citrullinated peptide 3 (CPP3) derived from the *P.gingivalis* PAD enzyme (*P.PAD*), (C-AKTDSYWT-Cit-DYTGWFAMYD-C), or the arginine-containing analogue RPP3 (Innovagen AB, Lund, Sweden) were used as coating antigens in an in-house ELISA. A cut-off value for anti-CCP3 IgG positivity was set at >29.19 AU/mL, giving a specificity of 96 % using ROC curves. The ROC curve were based on the plasma/serum anti-CPP3 IgG concentration in RA patients and controls included in the study.

Anti-CarP IgG ELISA

In-house ELISA methods were used to measure antibodies against carbamylated peptides, as previously described in Brink et al (Brink et al., 2015). Carbamylated foetal calf serum (FCS) or non-modified FCS were used as coating antigens. The cut-off for positivity was set at 256.07 AU/mL, by using ROC curve based on patients with RA as described by Brink et al (Brink et al., 2015).

Rheumatoid Factor (RF)

The rheumatoid factor (RF) against IgM isotypes were analysed using a commercially available ELISA (Orgentec Diagnostica GmbH), with the cut-off for

positivity set at ≥ 20 international units (IU)/mL. The measurements and cut-off were made according to manufacturer's protocol. Some of the analysed RF IgM were analysed using the commercially available EliA assay (Phadia GmbH, Freiburg, Germany) as described by Brink et al (Brink et al., 2016).

Receptor activator of nuclear factor kappa-B ligand (RANKL)

In *Paper III* and *IV*, a commercial sandwich ELISA method was used to measure quantitatively total (bound and unbound) RANKL (BioVendor, Karasek, Czech Republic) in plasma, in accordance to manufacturer's protocol. A cut-off value for RANKL positivity was defined using ROC curve analysis based on RA patients and controls, yielding a cut off value for positivity at >0.927 nmol/L, with a specificity of 97.9 % (Boman et al., 2017). All of the samples were re-analysed with a commercial RF blocker (HeteroBlock; Omega Biologicals Inc., Mozeman, MT, USA). The results with and without RF blocker correlated significantly for all of the analyses ($p < 0.001$). The results without RF blocker are presented in the *papers*.

Meso Scale Discovery V-plex analyses of cytokines/chemokines

In *Paper III*, eight cytokines/chemokine were analyzed using Meso Scale Discovery V-plex methods (Rockville, MD, USA). Interleukin (IL)-6, IL-10, IL-2, IL-4, and IL-8 were determined using the Chemokine Panel 1 (human) Kits (K15047D). Eotaxin, monocyte chemotactic protein 1 (MCP-1), macrophage-derived chemokine (MDC) and IFN- γ -inducible protein 10 (IP-10) were analyzed with the chemokine Panel 1 (human) Kits (K15047D). The MSD cytokine/chemokine assays are sandwich immunoassays coated with capture antibodies on well-defined spots on the plate (*Figure 4*). Plasma samples are thereafter subsequently added together with detection antibodies conjugated with electrochemiluminescent labeled (MSD-SULFO-TAG). The detection antibody binds into the analyte (chemokine/cytokine) and emit light (proportional to the amount of analyte present in the added sample). The emitted light can be measured using Meso Scale Discovery (MSD) Model 1250 Sector Imager 2400 plate reading instrument.

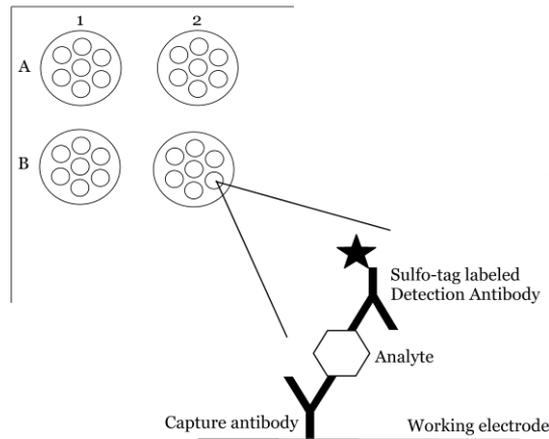


Figure 4. Illustrative figure of the Meso Scale Discovery V-plex methods.

Genetic Analyses

Genotyping of HLA-DRB1, shared epitope (SE) defined as HLA-DRB1*0401/0404/0405/0408/0101, were performed using polymerase chain reaction sequence-specific primers from a DR low-resolution kit and DRB1*04 and *01 subtyping kit (Dynal, Oslo, Norway) as previously described by Johansson et al (Johansson et al., 2006). Data on gene polymorphisms were extracted from ImmunoChip analysis (SNP&SEQ Technology Platform Uppsala, Sweden) covering 314 single nucleotide polymorphisms (SNPs) for PADI gene loci. Information about the protein tyrosine phosphatase, non-receptor type 22 (*PTPN22*) C1858T was also extracted from the ImmunoChip analysis (Eyre et al., 2012).

Radiographs of hands and feet

Radiographs of both hands and feet from patients at the disease onset of RA (baseline) were graded according to Larsen Score (Larsen, 1995). The radiographs were graded by two trained clinicians in consensus, at the Department of Rheumatology, Umeå University Hospital. The clinicians were blinded from the rest of the data.

Dental radiographs

The marginal jawbone loss was evaluated using X-ray of the bitewings, the premolar/molar section of the jaws. X-ray from the pre-symptomatic individuals were taken mean 3.8 (95% CI 3.2, 4.4) years before symptom onset of RA. For the

controls, the X-ray from the matching age were used. Jawbone loss were scored if the reduction of the marginal bone level corresponding to more than $\frac{2}{3}$ (3.7 mm) of the height of the crown of the same tooth (Page and Eke, 2007; Eke et al., 2012). Total bone loss were defined as the sum of the number of lost teeth and teeth with scored bone loss, in proportion of the number of teeth assessable for scoring.

The following characteristics were assessed for each tooth:

- **Intact tooth:** The tooth could be evaluated in its entirety and showed no sign of bone loss exceeding $\frac{2}{3}$ of the height of the tooth crown.
- **Bone loss:** The tooth with bone loss exceeding $\frac{2}{3}$ of the height of the crown. If the bitewing radiographs could not capture the marginal bone levels due to too severe bone loss it was also scored as bone loss.
- **Missing:** The tooth was missing or a retained root tip was present.
- **Not assessable:** The tooth could not be evaluated.

Statistics

Papers I – IV

The statistical analyses were performed using SPSS software for Windows version 23 (SPSS, Chicago, IL, USA). Nonparametric test, Kruskal-Wallis for several groups, Mann-Whitney for two groups and the Friedman's test for matched pairs were used to analyse continues data e.g., comparison of antibody concentration between study groups/matched pairs (controls, patients and pre-symptomatic individuals) or sexes (women vs. men). While, Student's T-test was used for normal distributed values. In *Paper IV*, RANKL concentrations were logarithmically transformed to become normally distributed. Categorical data for calculation of antibody positivity between study groups were analysed using chi²-test or Fischer's exact. Correlation analysis for antibody concentrations were made using Spearman's rank correlation test (r_s). In *paper I*, the frequency distribution among the antibodies were compared using Kendall's tau-test. Logistic regression and/or univariate analysis were performed to identify associations between antibody/RANKL positivity/concentrations in the development of RA or in relation to Larsen score at baseline. The same analyses were also made for adjustment for potential confounders e.g., age, sex, smoking, RF and anti-CCP2 antibody positivity. In *Paper IV*, Cox (proportional hazard) regression using a time dependent covariate (age), were used to compare the marginal jawbone loss between the matched pairs and to adjust for potential confounders (dental care giver and year when X-ray was taken). A cut-off value for positivity for each of the antibodies (anti-CPP3, -VCP1, -VCP2, -HCP1, -HCP2, -cfibrinogen β 36-52, - α -enolase5-21, -cfilaggrin307-324 and -cvimentin60-75 IgG) and RANKL was made using ROC-curve analysis. The p-values $p \leq 0.05$ considered as significant and were presented as two-sided. Specificities and sensitivities with confidence interval (CI) were calculated using the "XLSTAT-Life" add-on software (Addinsoft).

Paper I

PLINK (1.07) (Purcell et al., 2007) with Bonferroni correction and Haploview (4.2) were used for genetic analyses of SNPs in relation to concentration. Haploview (4.2), is a program used for haplotype analysis (BroadInstitute, 2018). Information about the *PTPN22* C1858T polymorphism was also extracted from the Immunochip analysis.

Paper II

In Paper II, we were unable to establish a cut-off value for the anti-RgpB antibody

response, due to a lack of data regarding the periodontitis status. Stratification of the anti-RgpB antibody concentration, above or below the 75th percentile were therefor used in the analyses. Interactions between anti-CPP3 antibody and HLA-DRB1 SE or smoking were measured according to standard methods (Zou, 2008).

Aim

General aim

The overall aim of this thesis was to gain knowledge about the pathogenic processes and factors preceding the development of RA and the ACPA response in individuals before the first symptom of RA.

Specific aims

- To study the evolution of the ACPA response and the development of RA by analysing antibodies against citrullinated endogenous antigens (citrullinated histones 4) and exogenous antigens (Epstein-Barr viral peptides).
- To investigate the role of the periodontal pathogen, *Porphyromonas gingivalis* in the development of RA and the ACPA response.
- To explore the relationship between RANKL and ACPA, RF, anti-CarP IgG and inflammatory markers, and their relationship to bone destruction in individuals before symptom onset of RA.
- To investigate whether periodontitis (defined as marginal jawbone loss) preceded the onset of symptoms of RA, and if ACPA and RANKL was associated with the jawbone loss.

Results and Discussion

Paper I

In *paper I*, the evolution of the ACPA response were studied in individuals before symptom onset of RA by analysing antibodies against citrullinated endogenous antigens HCP1 and HCP2 (citrullinated histones 4) and exogenous antigens VCP1 and VCP2 (Epstein-Barr viral peptides).

In this present study an increased concentration of anti-VCP1, -VCP2, -HCP1 and -HCP2 IgG in individuals before and after symptom onset of RA compared with controls ($p < 0.001$) were found, consistent with previous studies in RA (Pratesi et al., 2006; Pratesi et al., 2011; Pratesi et al., 2014) The concentration and the accumulated frequency of positivity were found to increase gradually until symptom onset, for all of the four antibodies (*Figure 5 & 6*).

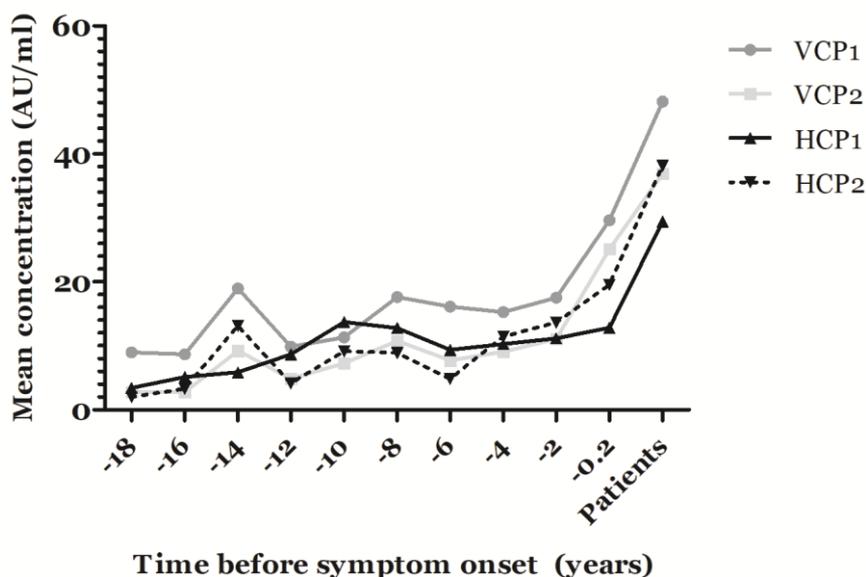


Figure 5. The mean concentration of the four antibodies (anti-VCP1, -VCP2, -HCP1 and -HCP2 IgG) during the pre-dating time until symptom onset of RA.

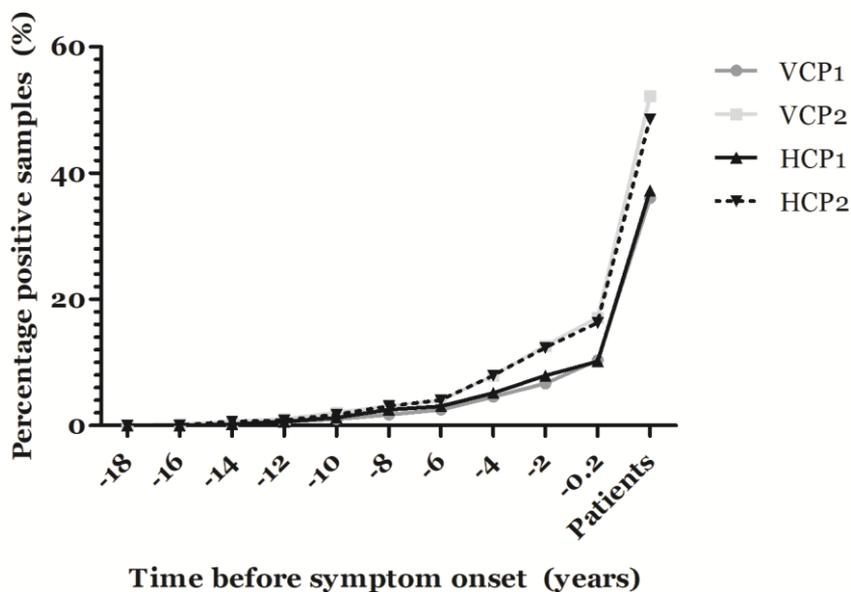


Figure 6. Accumulated percentage of positivity of the four antibodies (anti-VCP1, -VCP2, -HCP1 and -HCP2 IgG) during the pre-dating time until symptom onset of RA.

The anti-VCP2 and -HCP2 IgG achieved the highest frequency of positivity before (17.1% and 16.3 %) and after symptom onset of RA (52.3% and 48.5%), consistent with previous findings (Pratesi et al., 2006; Pratesi et al., 2014). These studies reported a slightly higher frequency of these antibodies in RA patients, probably due to the inclusion of individuals with established disease compared to our study on individuals with early RA (symptom duration ≤ 12 months). Positivity for any of the four antibodies could be observed in 28.2 % of the pre-symptomatic individuals and the majority of the individuals positive for these antibodies were also anti-CCP2 antibody positive. The major overlap between anti-CCP2 antibodies and ACPA positivity, has previously been observed in other studies (Brink et al., 2013). Only a minor overlap was shown between (anti-VCP1, -VCP2, -HCP1 and -HCP2 antibodies) and (anti-cFib β 36-52, -CEP-1, -cFilaggrin antibodies), suggesting a minor cross-reactivity or an independent production of these antibodies. In pre-symptomatic individuals, the combinations of triple negativity of anti-CCP2 and/or -VCP1 and/or -VCP2 and/or HCP1 and/or HCP2 antibodies became triple positive after disease onset of RA. This trend supports the hypothesis of both anti-VCP and -HCP antibodies participating in the development of RA and the ACPA response.

The number of positive antibodies increased closer to the onset of symptoms, as the regular ACPA response, suggesting an epitope spreading (van der Woude et al., 2010; Brink et al., 2013). In this present study we were unable to detect any differences in time of first appearance of antibody positivity among the anti-HCP and -VCP antibodies in relationship to each other and other studied ACPA. As a consequence of this we were unable to identify a single inciting antigen in the RA development.

When investigating the role of anti-HCP and -VCP antibodies in the development of RA, the risk for being a pre-symptomatic individual increased with the number of positive antibodies (*Table 5*). The combination of anti-VCP1 and -HCP2 IgG positivity yielded the highest risk for RA development (OR= 18.9). The risk increased even more when combining positivity for anti-VCP1, or -VCP2 or -HCP1 antibody with anti-CCP2 antibody positivity (OR=24.5–25.8). These combinations yielded higher risk of being a pre-symptomatic individuals, than positivity for anti-CCP2 IgG alone (OR=21.9).

Table 5. Association between the development of RA and number of positive antibodies of anti-VCP1, -VCP2, -HCP1 or -HCP2.

Number of antibody positive	Pre-symptomatic individuals, n=521 /Controls, n=272 (n/n)	OR¹	95% CI²	P-value
None	375/259		Ref.	
One Antibody	63/7	6.23	2.81, 13.83	< 0.001
Two Antibodies	41/5	5.68	2.21, 14.56	< 0.001
Three Antibodies	35/1	24.93	3.40, 182.98	0.002
Four Antibodies	7/0	-	-	-

¹OR= Odds ratio, ²CI=confidence interval

Furthermore, the relationship between genes and the anti-VCP and -HCP antibodies were investigated. Only the anti-VCP2 antibody was significantly associated with HLA-SE before symptom onset, whilst all four antibodies were related with HLA-SE after symptom onset as observed previously for other citrullinated antibodies (Kokkonen et al., 2015). In accordance with previous studies on a Caucasian population, the *0401 allele was associated with both anti-VCP2 and -HCP2 IgG positivity (Saal et al., 1999; Pratesi et al., 2012). No

association could be found between the *PTPN22* T-variant and these four antibodies. Interestingly, three single nucleotide polymorphisms (SNPs) (rs3003444, rs7542629 and rs11800688) in the PADI3/PADI4 region were associated with anti-HCP1 antibodies, also after Bonferroni correction and adjustments for anti-CCP2 antibodies and carriage of HLA-SE. The PADI enzyme has in many studies been suggested to be important for histone citrullination and for the formation of NETs and the release of citrullinated antigens (Li et al., 2010; Leshner et al., 2012). Interestingly, the SNP (rs3003444, A-allele) has in a previous study been associated with RA patients (Too et al., 2012).

All together this present study suggest that exogenous anti-VCP antibodies and endogenous anti-HCP antibodies act as the regular ACPA response and have a potential role in the development of RA. A role that could not be fully explained by the other ACPA included in this study. In this present study, also certain ACPA combinations were identified that increased the risk for being a pre-symptomatic individual more than *e.g.*, anti-CCP2 antibody positivity alone. Although, no temporal relationship between first positivity for anti-VCP and -HCP antibodies and the other ACPA included in the study could be found.

Paper II

In *Paper II*, the role of the periodontal pathogen *Porphyromonas gingivalis* in the development of RA and the ACPA response was analysed. The presence of antibodies against *P.gingivalis* virulence factor arginine gingipain B (RgpB) and a cyclic citrullinated peptide 3 (CPP3) derived from the *P.gingivalis* PAD enzyme (P.PAD) were analysed in individuals before and after symptom onset of RA.

In this study, the concentration of anti-RgpB IgG was shown to be significantly increased in pre-symptomatic individuals compared with control subjects ($p < 0.001$). These findings were consistent with a previous study showing elevated antibody levels in patients with RA, especially in ACPA-positive RA (Kharlamova et al., 2016). The anti-RgpB antibody concentration increased significantly during the predating time ($p < 0.05$) and exceed the mean concentration of the controls almost 12 years before symptom onset. A trend against lower antibody levels was observed after disease onset ($p = 0.088$) (*Figure 7*). This trend could potentially be due to a relative higher percentage of ever smoking among the RA patients (67.2 %) in comparison to the pre-symptomatic individuals (64 %), because not all pre-symptomatic individuals had corresponding samples after disease onset. Several studies have previously shown lower levels of anti-*P.gingivalis* antibodies in smokers compared with non-smokers (Vlachojannis et al., 2010; Seror et al., 2015; Kharlamova et al., 2016). In this study, an association between smoking and lower concentrations of anti-RgpB antibodies in patients ($p < 0.012$), but not in pre-symptomatic individuals was found. Anti-RgpB

antibodies were found to be associated with being a pre-symptomatic individual (OR=2.31, 95% CI 1.41, 3.78, $p < 0.001$), independent of smoking, HLA-SE or the *PTPN22* T-variant, in line with a previous study (Kharlamova et al., 2016). No association between anti-RgpB antibodies and being a patient could be found, which could be due to the low number of patients included in the study.

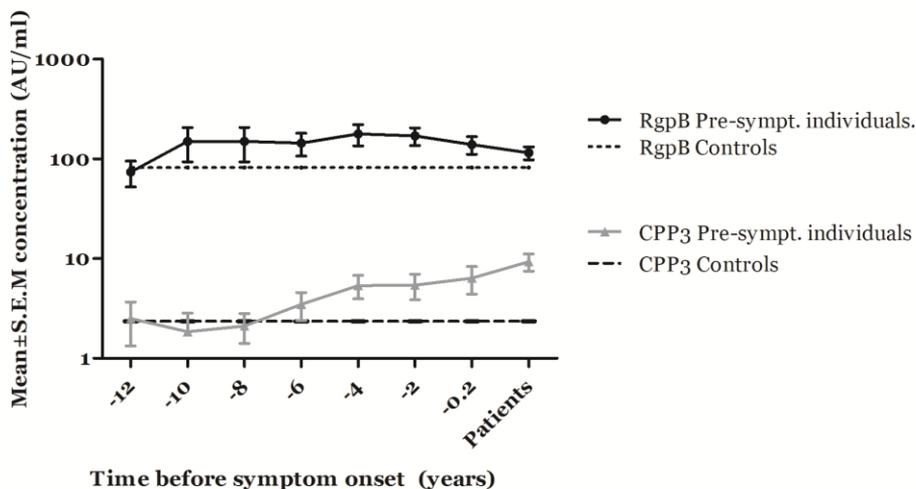


Figure 7. The logarithmic mean \pm S.E.M concentration of anti-RgpB and -CPP3 antibodies during the predated time until symptom onset of RA ($n=251$) and the mean concentration in all of the controls ($n=198$).

The concentration of the second antibody analysed in this study, anti-CPP3 IgG was found to be significantly increased in pre-symptomatic individuals compared with controls, in agreement with a previous study in RA patients (Quirke et al., 2014). The antibody concentration was found to exceed the controls almost 8 years before symptom onset (Figure 7). The anti-CPP3 antibody levels and the accumulated percentage of positive samples increased gradually the closer to onset of RA and was acting as the regular ACPA response. Although, the percentage of positivity was lower in comparison to the general ACPA response, defined as the anti-CCP2, -CEP-1, -cFib β 36-52 and -cFilaggrin antibodies (Brink et al., 2013). Furthermore, no reactivity against the arginine-control peptide was found and the majority of the anti-CPP3 IgG positive individuals were also anti-CCP2 IgG positive. No association could be found between anti-CPP3 and -RgpB IgG. All together, these findings suggest that anti-CPP3 IgG rather belongs to the generic ACPA response or is cross-reactive with other citrullinated antigens than being *P.ginivalis* specific. No association between anti-CPP3 IgG positivity and being a pre-symptomatic individual could be found. Although, anti-CPP3 IgG positivity in combination with HLA-SE revealed a stronger association of being a

pre-symptomatic individual, (OR=6.74, 95% CI 1.43, 31.81) or a patient, (OR=8.80, 95% CI 1.80, 43.03), than being negative for both of the factors as a reference. Similar results could be shown when combining anti-CCP3 antibody positivity with smoking compared with being negative for both factors as a reference, (OR=3.61, 95% CI 1.05, 12.44) in patients.

Several studies have investigated the role of *P.gingivalis* in RA, although only a few of them have analysed the role of *P.gingivalis* in the development of RA and the ACPA response. One study by Fisher et al, was unable to show any associations between anti-CCP3 and -RgpB IgG, and pre-RA cases (Fisher et al., 2015). Although, our studies differ in several aspects, their study was based on a smaller study population (n=103) derived from different southern countries in Europe. Our study included 251 pre-symptomatic individuals from northern Sweden, with a total of 422 samples. Not all individuals in the study by Fisher et al. were confirmed to develop RA, whereas all of our individuals fulfilled the 1987 ARA classifications criteria for RA after disease onset. Studies have also shown that the periodontal microbiota differ between countries (Haffajee et al., 2004) and also bacterial strain diversity has been reported, with associated variation in expression of virulence factors (Sundqvist et al., 1991). Together these differences can contribute to the inconsistency between the results. In line with our study, a study by de Smit et al. showed increased antibody levels against *P.gingivalis* in individuals positive for ACPA, but no higher levels were observed for individuals with arthralgia whom subsequently developed RA. Although, we were unable to investigate these findings further in our study (de Smit et al., 2014). Mikuls et al. could also, in accordance with our data, report increased concentrations of anti-*P.gingivalis* antibody levels in individuals at high-risk of developing RA (Mikuls et al., 2012). In this present study, purified RgpB protein from *P.gingivalis* was used as a coating antigen in the ELISA assay, while many other studies instead used outer membrane or whole bacterial lysates. By using purified RgpB protein in our analysis, the presence of citrullinated epitopes could be avoided and thereby potential false positive result due to cross-reactive ACPA.

This study is the largest population-based study, investigating antibodies against *P.gingivalis* in individuals before onset of symptoms of RA. Although, our study has limitations, e.g., the individuals included are from different cohorts and information regarding PD status or *P.gingivalis* infection (past or present) was lacking. Although, anti-RgpB antibody levels has in several studies been found to be clearly elevated in individuals with PD compared with healthy individuals and has also been found to be associated with DNA from *P.gingivalis*. These findings, suggest anti-RgpB IgG to be good marker for *P.gingivalis* infection (Socransky et al., 1998; Hajishengallis, 2015; Schwenzer et al., 2017). Although, anti-RgpB IgG should not be used as a marker for PD, since *P.gingivalis* does not have to be present for PD development and many individuals are asymptomatic carrier of

P.gingivalis (Pussinen et al., 2011). Due to missing information concerning PD status, we were unable to set any cut off value for the anti-RgpB antibodies.

Paper III

In *Paper III*, the relationships between RANKL and ACPA, RF, anti-CarP IgG and inflammatory markers and their relationship with bone destruction in individuals before symptom onset of RA were investigated.

The RANKL concentration ($p < 0.001$) and percentage of positivity for RANKL were found to increase constantly during the pre-dating time. The frequency and the concentration of RANKL were higher in pre-symptomatic individuals compared with control subjects, (13.4 % vs. 2.1 %, $p < 0.001$) and (mean 0.5 vs. 0.22 nmol/l, $p < 0.001$), especially in ACPA positive individuals (*Figure 8*).

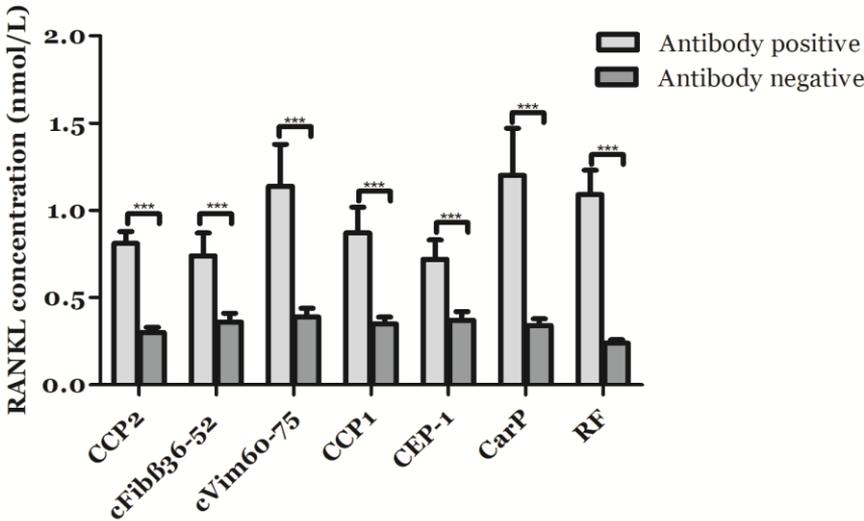


Figure 8. RANKL concentrations in antibody positive/negative pre-symptomatic individuals. *** $p < 0.001$ between antibody positive/negative individuals.

These findings were in line with previous studies, showing increased levels of RANKL in ACPA positive patients (Hensvold et al., 2015a; Boman et al., 2017). In this study, we were unable to separate the relationship between RANKL and ACPA from the association with RF due to the low frequency of these factors. In

a previous study on RA patients the relationship between RANKL and ACPA was independent of RF status, supporting the importance of ACPA (Boman et al., 2017). In contradiction with our study, van Schaardenburg et al. were unable to show any association between RANKL and pre-RA cases. The discrepancy between these studies could potentially be explained by the small study group in their study (n=79), furthermore information as to whether free and/or bound RANKL were measured were also lacking thus making comparison difficult (van Schaardenburg et al., 2011).

The first positivity for RANKL appeared 10.5 years before symptom onset. A time difference in the first appearance of positivity among RANKL and the other antibodies were observed, suggesting a much later appearance of RANKL positivity than that of anti-CCP2, -CarP antibodies, RF and the general ACPA response (defined as anti-cVim60-75, -cFib β 36-52, -CEP-1, -cFilaggrin) (Brink et al., 2013). The first appearance of antibody positivity was 11 years for anti-CarP IgG and 13 years before symptom onset for the other ACPA (*Figure 9*).

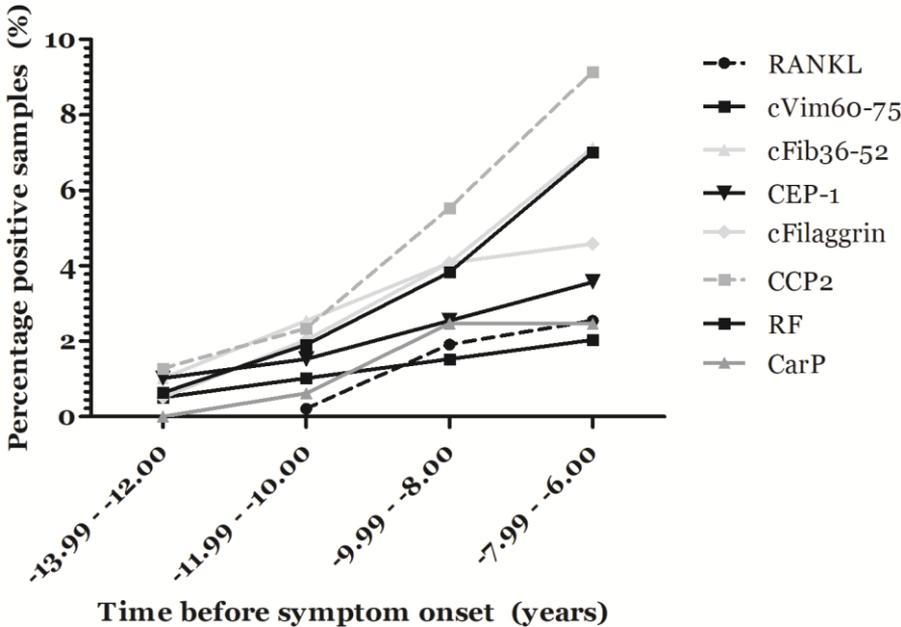


Figure 9. Accumulated percentage of positive samples for RANKL, anti-cVimentin60-75 (cVim60-75), anti-cfibrinogen β 36-52 (cFib β 36-52), anti-CEP-1 (CEP-1), anti-cfilaggrin (cfilaggrin), anti-CCP2 (CCP2), anti-carbamylated antibodies (CarP) antibodies and rheumatoid factor (RF), with focus on the first appearing time points.

The highest RANKL levels were found in pre-symptomatic individuals positive for anti-CarP or anti-cVim60-75 antibodies (*Figure 8*). These findings are interesting since antibodies against citrullinated mutated vimentin (MCV) have been found to induce osteoclast differentiation and bone resorption both in vivo and in vitro (Harre et al., 2012). A study by Hensvold et al, found that anti-cVim60-75 antibody positive patients had increased levels of RANKL and bone destruction (Hensvold et al., 2015a). Furthermore, a study on pre-symptomatic individuals, have showed an association between anti-CarP antibodies and radiological bone damage (Brink et al., 2015). From our study, we cannot conclude if there is a direct effect of ACPA on the RANKL production (from one or several different cell types) or if the response might be mediated via an increased chemokine/cytokine production due to inflammatory processes. Interestingly, we found increased levels of both IL-6, IL-10 and IP-10 (Kokkonen et al., 2010). IL-6 has been shown to increase RANKL expression and promote bone formation and trigger osteoclastogenesis (Sims, 2016). While, the chemokine IP-10 was found to increase RANKL expression in osteoblastic stromal cells (Wang et al., 2010). In contrast to IL-6 and IP-10, IL-10 has anti-inflammatory functions and has been shown to inhibit both osteoclast bone resorption and regulate osteoblast bone formation (Zhang et al., 2014). Together these result suggest an imbalance between bone remodeling and destructive pathways in these individuals. We were unable to show a time-dependent development between RANKL and these cytokines/chemokines. Furthermore, no association between IL-8 and RANKL could be found. These results were unexpected since IL-8 has previously been found to increase osteoclastogenesis and regulate RANKL expression (Bendre et al., 2003).

Increased concentrations of RANKL were found to be associated with a higher Larsen score at baseline in men ($p < 0.05$), but not in women. Since the number of men with radiographic examination (47/158) were few, interpretation of these data should be undertaken with care. These findings were in line with previous studies identifying RANKL or the ratio of RANKL and osteoprotegerin (OPG) as a valuable predictor of radiological progression (Geusens et al., 2006; van Tuyl et al., 2010; Hensvold et al., 2015a). The role of RANKL as a marker for radiological findings was also investigated in combination with the other antibodies. The highest association with Larsen score at baseline in pre-symptomatic individuals were received when combining positivity for both anti-CarP IgG and RANKL, mean (S.E.M.) 13.0 (3.6) in pre-symptomatic individuals, compared with being negative for both as reference 6.8 (0.6), (OR=6.18 95% CI (0.93, 11.43), $p = 0.022$) (*Figure 10*). These findings were independent of adjustments for sex, anti-CCP2 antibody or RF positivity. All together these findings suggesting a novel link between anti-CarP IgG and RANKL positivity in the development of bone damage in individuals that will subsequently develop RA.

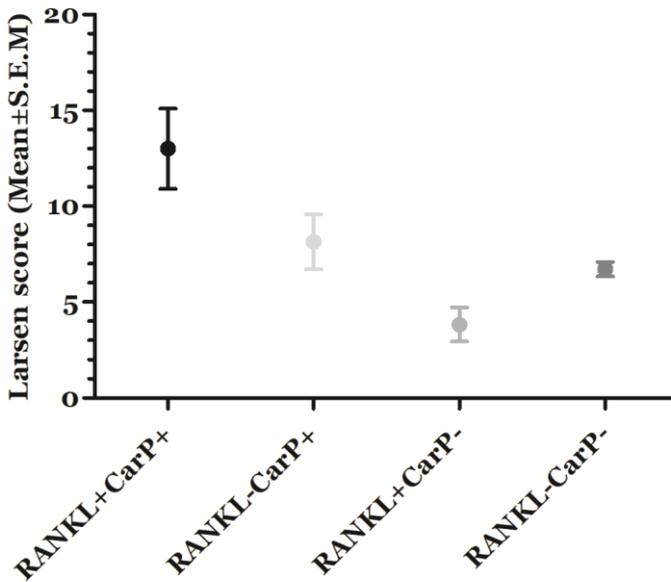


Figure 10. The mean Larsen score in different combinations of RANKL and anti-carbamylated (CarP) antibody positivity in pre-symptomatic individuals.

Our study has limitations, for example the samples were not collected on a regular basis and were from different population surveys. Our study lacks radiological examinations for several of the included in the study and information about RF and HLA-SE for the controls. All together, these limitations reduced the ability to subdivide the study group into different sub-groups of interest.

Paper IV

In *paper IV*, we wanted to investigate whether periodontitis (defined as marginal jawbones loss) preceded the onset of symptoms of RA and the impact of ACPA and RANKL for jawbone loss.

A total of 93 pre-symptomatic individuals (46 individuals with dental X-ray before symptom onset and 47 individuals with dental X-ray within the same year or after disease onset of RA) and 83 controls were included. The pre-symptomatic individuals and controls were proportionate with respect to sex, age and smoking habits. A total of 45 of the 46 pre-symptomatic individuals with dental X-ray before disease onset could be matched with a control subject based upon sex, age when dental X-ray was taken and smoking status, due to too few current smokers among the controls.

Among the matched pairs (n=45), the degree of marginal jawbone loss was significantly higher in pre-symptomatic individuals compared with controls, (p=0.017). When stratifying for smoking habits the association was only found among the non-smokers, in accordance with a previous study (Potikuri et al., 2012). When further analysing the association between the development of RA and the degree of jawbone loss in non-smokers, the association was found to be independent of adjustments for year when dental X-ray was performed and dental care provider (p=0.05) (Table 6).

Table 6. Hazard ratio (HR 95%CI) for being a pre-symptomatic for RA by increasing the levels of total jawbone loss.

	All matched subjects		Ever smokers (past+present)		Never smokers	
	HR (95 %CI)	p-value	HR (95%CI)	p-value	HR (95%CI)	p-value
Unadjusted model ¹	1.006 (0.996, 1.016)	0.246	0.998 (0.985, 1.011)	0.689	1.026 (1.005, 1.047)	0.017
Adjusted model ²	1.006 (0.997, 1.015)	0.209	1.004 (0.981, 1.018)	0.525	1.025 (1.000, 1.050)	0.050

¹Unadjusted model including total boneloss and time dependent covariate (age)

²Adjusted model including total bone loss, time dependent covariate (age), dental care provider and year x-ray was taken.

Among the 45 matched pairs with dental X-ray available before symptom onset, 31 of the pairs also had X-ray at or after symptom onset of RA. In these individuals the jawbone loss could be tracked from four years before symptom onset (jawbone loss standardized to zero) until six years after symptom onset. In the longitudinal analyses, the pre-symptomatic individuals showed a higher extent of jawbone loss than controls.

Several studies have shown that patients with RA have a higher prevalence of PD compared with the general population (Kasser et al., 1997; Mercado et al., 2001; Pischon et al., 2008; Garib and Qaradaxi, 2011; Potikuri et al., 2012; Smit et al., 2012; Joseph et al., 2013), whilst other studies could not confirm these findings (Arkema et al., 2010; Demmer et al., 2011; Eriksson et al., 2016). These studies should not be compared with this study, since we were unable to obtain information regarding periodontitis classification and diagnosis for these individuals and instead used jawbone loss as a marker for PD. The diagnosis of PD is usually based on outcomes from intraoral and radiographic examination, including *e.g.*, probing pocket depth, probing bleeding, tooth mobility and

clinical attachment level (Page and Eke, 2007). Data of *e.g.*, gingiva bleeding and pocket depth were not available in our study. Since, PD is a major cause of tooth loss in the adult population and one of the world’s most prevalent chronic oral inflammatory diseases, it is reasonable to believe that the jawbone loss is caused by past or present PD in these individuals (Hugoson et al., 2008; Dye, 2012). Furthermore, diversity in PD classification criteria, treatments, cohort size, study population and self-reported information about oral health can contribute to the diversity found among these studies.

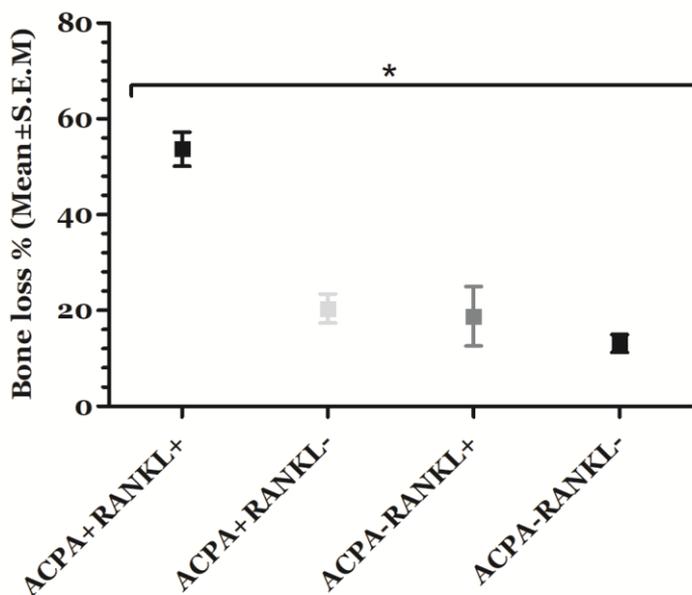


Figure 11. The mean percentage of jawbone loss in different combinations of RANKL and ACPA positivity in pre-symptomatic individuals. * $p < 0.05$

Furthermore, RANKL positive pre-symptomatic individuals have a significantly higher extent of jawbone loss compared with RANKL negative individuals ($p = 0.031$). Also, a modest correlation between RANKL concentration and jawbone loss was found in these individuals. Interestingly, the finding of a more severe jawbone loss in RANKL-positive individuals, could be a result of an imbalance in bone remodeling shared between a subgroup of pre-symptomatic individuals and individuals with severe periodontitis. In this study, no further investigations, if this was an associative or causative observation could be made. No association between RF and jawbone loss could be found, although ACPA (anti-CCP2 IgG) positive pre-symptomatic individuals had higher levels of jawbone loss compared with ACPA negative individuals (27.3 % vs 13.9 %

p<0.05). The highest extent of jawbone loss was found when combining ACPA and RANKL positivity in pre-symptomatic individuals (p<0.05) (*Figure 11*). Several oral bacteria as previously mentioned in this thesis, *P.gingivalis* and *Aa* have been shown to have the capacity to citrullinate antigens and generate an ACPA response and have therefore been suggested as a link between the two diseases, PD and RA (Rosenstein et al., 2004; Konig et al., 2016). ACPA have previously been shown to have the capacity to induce osteoclast differentiation and bone resorption (Harre et al., 2012; Krishnamurthy et al., 2016).

Our study has limitations, the blood samples and the dental X-ray were not collected at the same time point. This limitation reduced the number of individuals in every subgroup and decreased the statistical power in our analysis. Interpretation should be made by care for this study due to the limitations declared. However, our findings support the hypothesized link between PD and RA. Although, further and larger studies are required to determine if there is a causative link between the two disease or if the two disease only share the same outcome of an inflammatory burden.

Conclusions

The main findings from this thesis in pre-symptomatic individuals, early RA patients and controls are:

- Antibodies against citrullinated proteins derived from both endogenous and exogenous sources are present several years before symptom onset and are associated with the disease development.
- The concentration of antibodies and the number of antibody positive increased gradually the closer to symptom onset of RA, for all of the analysed antibodies.
- Anti-*P.gingivalis* antibodies precede the clinical onset of RA by several years, supporting an etiological role of the periodontal bacteria in the development of RA.
- Periodontitis defined as marginal jawbone loss precede the onset of RA, in non-smokers.
- Plasma RANKL increased with time approaching the symptom onset of RA and was associated with higher extent of bone damage (Larsen score) and marginal jawbone loss in pre-symptomatic individuals.
- RANKL levels were particularly increased in ACPA-positive pre-symptomatic individuals, and RANKL positivity appeared later in time than the general ACPA response, RF and anti-CarP antibodies.
- Individuals positive for both RANKL and anti-CarP antibodies yielded the highest bone damage (Larsen score) at disease onset.

Future perspectives

There is a need to increase the knowledge about the factors and processes underlying the aetiopathogenesis of RA. New insights are important in order to be able to prevent the disease onset, achieve better diagnostic methods and treatments in the future. Through this thesis the knowledge about the origin of ACPA and the processes preceding the development of RA have increased, but there are still many important aspects that remain unclear. It is now evident that the production of ACPA and the destructive processes starts several years before symptom onset of RA. Although, it is still unknown how tolerance against citrullinated proteins are broken, to which extent ACPA contribute to the disease pathogenesis and what trigger the transition of a pre-symptomatic state into the fulminant disease. Several studies have suggested that “first hits” *e.g.*, microbial agents and smoking are needed to break the immunity against citrullinated proteins. Since there are individuals positive for ACPA that never will develop RA, ACPA have been shown to not always be pathogenic or that “second or multiple hits” *e.g.*, HLA-SE are essential for the disease development.

To receive a better knowledge about the nature of the ACPA response and thus the pathogenesis of RA, the factors and processes included in this thesis need to be studied into a broader context. In the future it would be interesting to study them together, into a temporal relationship and in context to new risk factors of interest, *e.g.*, the oral bacteria *Aa*. It is also of importance to study these factors and processes in both ACPA positive/negative individuals and ACPA positive/negative RA by taking advantage of analyses of first degree relatives, twins, and individuals with arthralgia and larger cohorts with pre-symptomatic individuals/controls. Through these studies, the individual contributions from genetic factors, environmental factors and the importance of ACPA in the development of RA could be more easily distinguished and hence better understood. It is of relevance to not only focus on ACPA, but also antibodies against other post transitional modified proteins and differences in glycosylation patterns between these study groups. Furthermore, studies on the ability of RA related antibodies to directly or indirectly activate different cell types, that later could lead to inflammation, pain and bone erosions, could potentially bring us closer to the importance of ACPA in the disease development. Therefore not only observational studies are needed, but also experimental.

Also of interest would be to analyse the antibody reactivity in pre-symptomatic individuals, patients with RA and controls more thoroughly by using protein microarrays including several hundreds of proteins derived from plasma and/or synovial fluid. Finally, it would also be relevant to investigate the microbiota profile between patients and healthy individuals to find new potential agents of

interest that could be of importance for the production of ACPA and the RA pathogenesis.

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