From Department of Medicine, Solna
Karolinska Institutet, Stockholm, Sweden

ON THE ORIGIN OF ACPA: EXPLORING THE ROLE OF *P. GINGIVALIS* IN THE DEVELOPMENT OF RHEUMATOID ARTHRITIS

Natalia Sherina

Karolinska Institutet

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On the origin of ACPA: exploring the role of *P.gingivalis* in the development of rheumatoid arthritis

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Natalia Sherina

Principal Supervisor:
Dr. Karin Lundberg
Karolinska Institutet
Department of Medicine, Solna
Division of Rheumatology

Co-supervisor(s):
Prof. Robert Harris
Karolinska Institutet
Department of Clinical Neuroscience
Division Neuro

Dr. Khaled Amara
Karolinska Institutet
Department of Medicine, Solna
Division of Rheumatology

Opponent:
Prof. Hani El-Gabalawy
University of Manitoba Arthritis Centre
Department of Internal Medicine

Examination Board:
Dr. Meliha Kapetanovic
Lund University
Department of Clinical Sciences

Prof. Torbjörn Bengtsson
Örebro University
Department of Medical Sciences

Prof. Marie Wahren-Herlenius
Karolinska Institutet
Department of Medicine, Solna
Division of Rheumatology

The public defense will take place on **Friday 13th of December at 9:00 am** at the Center for Molecular Medicine (CMM), Lecture hall, Visionsgatan 18, L8:00, Karolinska University Hospital, Solna

To my family

ABSTRACT

Rheumatoid Arthritis (RA) is a systemic autoimmune disease characterized by chronic synovial inflammation, joint destruction, and the presence of anti-citrullinated protein autoantibodies (ACPA) in a majority of patients. Accumulating evidence suggests that ACPA play an important role in RA pathogenesis. ACPA are often detected years before the onset of clinical symptoms, with increasing levels and epitope-spreading preceding the diagnosis of RA. The presence of ACPA is associated with a more severe disease course and recent studies imply that ACPA may directly contribute to inflammation, mediate pain, osteoclast differentiation, and fibroblast migration, and ACPA have been shown to worsen experimental arthritis. Despite progress in revealing ACPA-mediated pathology, the origin of the ACPA response remains largely unknown. The best-known genetic risk factor for RA, *HLA-DRB1* shared epitope (SE) alleles, and the most studied environmental risk factor, cigarette smoking, have been linked to ACPA-positive RA. In addition, chronic periodontitis (PD), an inflammatory disease of the tooth supporting tissue, has been epidemiologically linked to RA. Shared genetic and environmental risk factors, together with a unique ability of the periodontal pathogen *Porphyromonas gingivalis* to express an enzyme that can citrullinate proteins, led to the hypothesis that the initial brake of immune tolerance to citrullinated proteins may be triggered in the gum mucosa during chronic PD. Based on this hypothesis, the aim of my thesis was to investigate the role of *P.gingivalis* in the etiology of ACPA-positive RA.

We have examined the anti-*P.gingivalis* antibody response in a PD/non-PD cohort and in population-based RA/pre-RA case-control cohorts, and found that anti-*P.gingivalis* antibody levels are not only significantly elevated in PD patients compared to periodontally-healthy individuals, but in RA patients (in particular ACPA-positive RA) compared to non-RA controls. We identified an association between anti-*P.gingivalis* antibodies and RA, that was even stronger than the well-known association between smoking and RA, and we could show elevated anti-*P.gingivalis* antibody levels more than 10 years before clinical onset of RA.

Moreover, we have shown that not only RA patients, but a substantial proportion of the general population (likely individuals with PD), have a citrulline-specific antibody response against *P.gingivalis*. Furthermore, analysis of gingiva-derived monoclonal antibodies from patients with PD revealed the presence of citrulline-reactive B cells in inflamed gingival tissue, and B cells with cross-reactivity between a citrullinated *P.gingivalis* peptide and citrullinated human antigens were found in the gingival tissue, as well as in peripheral blood and bronchoalveolar lavage of ACPA-positive RA patients. One of the most intriguing findings was that two of these clones were positive in the gold standard clinical CCP2 test, and when one of the clones was converted back to the predicted germline sequence, autoreactivity was lost, while some reactivity against *P.gingivalis* remained. These data suggest that the initial antibody response was directed against *P.gingivalis*, and that autoimmunity developed as a result of somatic mutations during affinity maturation of the B cell response.

In summary, this thesis supports the hypothesis that in a subset of RA patients, loss of tolerance to citrullinated self-proteins may be triggered in the gum mucosa during chronic PD caused by *P.gingivalis*. However, in order to establish a causative role for *P.gingivalis* in the development of ACPA-positive RA, further research is warranted.

LIST OF SCIENTIFIC PAPERS

I. **Antibodies to *Porphyromonas gingivalis* indicate interaction between oral infection, smoking, and risk genes in rheumatoid arthritis etiology**
Kharlamova N, Jiang X, **Sherina N**, Potempa B, Israelsson L, Quirke AM, Eriksson K, Yucel-Lindberg T, Venables PJ, Potempa J, Alfredsson L, Lundberg K.
Arthritis Rheumatol. 2016 Mar; 68(3): 604-13

II. **Concentration of antibodies against *Porphyromonas gingivalis* is increased before the onset of symptoms of rheumatoid arthritis**
Johansson L*, **Sherina N***, Kharlamova N, Potempa B, Larsson B, Israelsson L, Potempa J, Rantapää-Dahlqvist S, Lundberg K.
Arthritis Res Ther. 2016 Sep 7;18:201

III. **The antibody response to a citrullinated *Porphyromonas gingivalis* PAD peptide could give clues to the aetiology of ACPA-positive rheumatoid arthritis**
Sherina N*, Kharlamova N*, de Vries C, Brynedal B, Jiang X, Hansson M, Mathsson-Alm L, Eriksson K, Saevarsdottir S, lsraelsson L, Yucel-Lindberg T, Rönnelid J, Alfredsson L, Lundberg K.
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IV. **Citrulline-reactive B cells are present in inflamed gingival tissue and display cross-reactivity between *P. gingivalis* and human antigens**
Sherina N, Sippl N, Liljefors L, Joshua V, Thyagarajan R, Israelsson L, Stålesen R, Wähämaa H, Kharlamova N, Hensvold AH, Eriksson K, Yucel-Lindberg T, Catrina AI, Lanzavecchia A, Piccoli L, Grönwall C, Malmström V, Amara K, Lundberg K.
Manuscript

*These authors contributed equally to work and share first authorship

SCIENTIFIC PAPERS NOT INCLUDED IN THE THESIS

V. **Low levels of antibodies against common viruses associate with anti-citrullinated protein antibody-positive rheumatoid arthritis; implications for disease aetiology**
Sherina N*, Hreggvidsdottir HS*, Bengtsson C, Hansson M, Israelsson L, Alfredsson L, Lundberg K
Arthritis Res Ther. 2017 Sep 30;19:219

VI. **A cross-sectional investigation into the association between Porphyromonas gingivalis and autoantibodies to citrullinated proteins in a German population**
Oluwagbemigun K, Yucel-Lindberg T, Dietrich T, Tour G, **Sherina N**, Hansson M, Bergmann M, Lundberg K, Boeing H
Therapeutic Advances in Musculoskeletal Disease 2019, 11:1759720X19883152

*These authors contributed equally to work and share first authorship

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LIST OF ABBREVIATIONS

Aa	*Aggregatibacter actinomycetemcomitans*
ACPA	Anti-citrullinated protein antibodies
ACR	American College of Rheumatology
AMPA	Anti-modified protein antibodies
AU	Arbitrary unit
BAL	Bronchoalveolar lavage
BCR	B cell reseptor
BLAST	Basic local alignment search tool
BM	Bone marrow
CarP	Carbamylated proteins
CII	Collage type II
CCP2	Cyclic citrullinated peptide, second generation
CEP1	Citrullinated alpha-enolase peptide-1
CI	Confidence interval
Cit-fib	Citrullinated fibrinogen
Cit-fil	Citrullinated filaggrin
Cit-his	Citrullinated histone
Cit-vim	Citrullinated vimentin
CPP3	Citrullinated *P.gingivalis* PAD peptide
CRP	C-reacive protein
DAS28	Disease activity score for 28 joints
EIRA	Epidemiological Investigation of RA
ELISA	Enzyme-linked immunosorbent assay
EULAR	European League Against Rheumatism
GT	Gingival tissue
GWAS	Genome-wide association study
HLA	Human leukocyte antigen
Ig	Immunoglobulin
IL	Interleukin

KS	Karolinska Sjukhuset / Karolinska University Hospital
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MBNS	Medical biobank of Northern Sweden
MHC	Major histocompatibility complex
NETs	Neutrophil extracellular traps
NSHDS	Northern Sweden Health and Disease Study
OD	Odds ratio
OR	Optical density
PAD	Peptidyl-arginine deiminase
PB	Peripheral blood
PCR	Polymerase chain reaction
PD	Periodontitis
P.gingivalis	*Porphyromonas gingivalis*
PPAD	*P.gingivalis* PAD enzyme
PTMs	Post-translational modifications
PTPN22	Protein tyrosine phosphatase, non-receptor type II
RA	Rheumatoid arthritis
RF	Rheumatoid factor
RgpB	Arginine gingipain B
ROS	Reactive oxygen species
RPP3	Arginine-containing control peptide for CPP3
RT-PCR	Reverse transcription PCR
SE	Shared epitope
SF	Synovial fluid
SNP	Single-nucleotide polymorphism
TNF	Tumor necrosis factor

1 INTRODUCTION

1.1 RHEUMATOID ARTHRITIS

1.1.1 Prevalence and diagnosis of RA

Rheumatoid arthritis (RA) is a common autoimmune disease, characterized by chronic inflammation that typically affects small and medium-sized joints in a symmetric fashion, which may be accompanied by cartilage and bone destruction, leading to disability [1]. The prevalence of RA is believed to be increasing, affecting between 0.5-1% of the world's populations, with a female to male ratio of 3:1, and a peak incidence in the fourth to fifth decades [2, 3]. There is no single diagnostic test and in order to differentiate RA from other inflammatory arthritides, the American College of Rheumatology (ACR) classification criteria (1987) have often been used [4]. The ACR criteria were developed based on manifestations of established RA, and had poor sensitivity for diagnosis of early RA. Therefore the 2010 ACR/European League Against Rheumatism (EULAR) classification criteria for RA were proposed, facilitating the diagnosis at early stages [5]. These criteria use a score-based algorithm that considers type and number of joints involved, serological parameters (low/high levels of rheumatoid factor (RF) and/or anti-citrullinated protein antibodies (ACPA)), acute-phase reactants (erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP)), and duration of symptoms.

1.1.2 Autoantibodies in RA

RA is usually subdivided in two major groups: seronegative and seropositive, depending on the absence or presence of autoantibodies. Based on the 1987 ACR classification criteria, seropositive and seronegative RA are clinically rather similar at baseline, but differ in disease course, with seropositive patients developing more erosions [6-9]. Historically RA seropositivity was referred only to the presence of RF, and only from 2010 the presence of ACPA was included in the ACR/EULAR criteria. However, with the new classification criteria, seronegative patients (i.e. RF-/ACPA-) risk delayed diagnosis, as they require more clinical symptoms to be classified as RA, and usually present with higher disease activity and older age at diagnosis, when compared to seropositive patients [10].

Rheumatoid factor

The first clue that self-reactivity plays a role in RA was the identification of "rheumatoid factor" in the blood of affected patients. Since the discovery in the 1930-40s [11, 12], detection of RF became a hallmark of RA and is still included in the ACR/EULAR classification criteria. RF is an autoantibody directed against the constant (Fc) part of immunoglobulin (Ig) G, and usually of IgM isotype, however IgA, IgG and IgE are also present in RA sera [13]. Although these antibodies are detected in around 70% of RA patients [14], they are not specific for RA. RF can also be detected in other autoimmune diseases, B-cell lymphomas, lung diseases, during bacterial and viral infections, and in systemically healthy individuals – the presence and levels of RF increase with age [15].

Anti-citrullinated protein antibodies

The discovery that a majority of RA patients have antibodies against citrullinated proteins (known as ACPA) marked an essential progress in understanding potential pathological disease mechanisms. It started with the detection of anti-perinuclear factor (APF), and anti-keratin (AKA) antibodies in sera of RA patients in 1964 and 1979, respectively [16, 17]. These antibodies were shown to be the most specific serological markers of RA [18]. However, the complicated nature of the antibody-detection techniques precluded extensive use in clinic. In subsequent studies it was found that these antibodies recognise the same antigen, human epidermal filaggrin and (pro)filaggrin-related proteins of buccal epithelial cells, so the antibodies were proposed to be re-named anti-filaggrin autoantibodies (AFA) [19]. In the late 1990s, AFA's specificity against citrullinated epitopes was described [20, 21], and the first anti-cyclic citrullinated peptide (CPP) antibody test was developed, based on cyclic citrullinated filaggrin peptides [22]. Since then a number of citrullinated proteins, including fibrinogen [23], vimentin [24], collagen type II (CII) [25], α-enolase (CEP-1) [26], and histone 4 [27] have been identified as anti-citrullinated protein antibody targets. Several tests detecting ACPA have been developed, and currently assays using the second generation of cyclic citrullinated peptide(s) as artificial antigen(s) (CCP2) are the most commonly used in clinical practice. Anti-CCP2 antibodies are found in 60-70% of RA patients, and their presence is highly specific for RA (although up to 2% of non-RA individuals are positive, and somewhat higher frequencies have occasionally been reported for patients with other rheumatic conditions) [28].

ACPA are often detected years before the first clinical symptoms develop, with increased levels and "epitope-spreading" preceding the diagnosis of RA [29-33]. The presence of ACPA is associated with higher disease activity over time, and more severe radiographic damage [34, 35]. In addition to clinical indications, recent *in vitro* and *in vivo* studies imply that ACPA may directly contribute to inflammation, and mediate pathogenic effects such as pain, osteoclast differentiation, fibroblast migration and neutrophil extracellular trap formation (NETosis), and ACPA have also been shown to worsen experimental arthritis in mice [34, 36-41].

Other autoantibodies

Recent studies indicate that around 50% of RA patients rather have a spectrum of anti-modified protein antibodies (AMPA) directed against post-translational modifications (PTMs), including citrullination, carbamylation, acetylation, and malondialdehyde modifications (MDA) [42-45]. In addition, extensive cross-reactivity between citrullinated, carbamylated, and acetylated antigens has been shown [39, 46-49]. The large number of autoantibody reactivities, together with presence of different isotypes, creates a broad heterogeneity of autoantibody reactivity patterns in individual RA patients.

1.1.3 Protein citrullination

Citrullination (or deimination) is a post-translational enzymatic conversion of positively charged peptidylarginine to neutral peptidylcitrulline, catalysed by calcium-dependent peptidyl arginine deiminases (PADs) (Figure 1). This normal physiological process occurs in different

tissues and is involved in hair growth, terminal differentiation of the epidermis, myelin formation, the regulation of gene expression, NETosis, and several other biological processes. However, it has also been suggested to play a role in a variety of diseases, including cancer, psoriasis, multiple sclerosis, inflammatory bowel disease and RA [50, 51].

Citrullination of the protein can occur extracellularly, in the cytoplasm, and in the nucleus, and results in a small increase in molecular mass (approximately 1 Da) for each modified arginine residue, influencing protein's ionic and hydrogen bond-forming capacity, that in turn may influence the structure and function of a protein and its antigenicity.

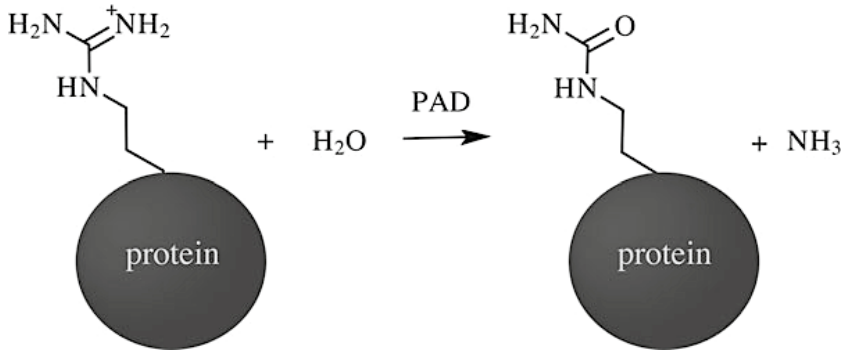


Figure 1. *The PAD enzymes catalyse citrullination (deimination) of protein substrates leading to formation of peptidylcitrulline and the release of ammonia. From Rohrbach et al. [52].*

In mammals, five PAD enzymes were identified (PAD1-3, PAD4/5, PAD6), which exhibit high amino acid sequence homology (~41-55%), but differ in terms of their tissue and cellular distributions. PAD2 is expressed in many tissues, including secretory glands, brain, spleen, pancreas, uterus, and skeletal muscles, that makes it the most widely expressed PAD, while expression of other members is more tissue-restricted: PAD1 (epidermis, uterus, hair follicles), PAD3 (epidermis and hair follicles), PAD4 (spleen, bone marrow and lymphoid tissue) and PAD6 (embryos, ovaries, oocytes, testis, small intestine, spleen, lung, liver) [52-54]. *In vitro* studies also indicate that under favourable conditions (i.e. high calcium concentration, long incubation time and high temperature) PADs can citrullinate all sorts of arginine-containing proteins. Although being structurally similar, each PAD enzyme has been shown to have distinct substrate preferences. Interestingly, PAD1, 2, 3 and 4 can also be auto-citrullinated, which could impair their activity [52-54].

PAD enzymes are activated during inflammation, and citrullinated proteins have been detected in various inflamed tissues, during various inflammatory conditions, including for example RA, polymyositis and inflammatory bowel disease, but generally not in healthy (non-inflamed) tissues, supporting a role of PAD enzymes during inflammation. The only PAD enzymes identified so far in the RA synovium are PAD2 and PAD4, and their expression correlates with the degree of inflammation, suggesting that these enzymes could play a role in RA pathogenesis [53-55].

1.1.4 Risk factors

Despite decades of investigation, the etiology of RA is still largely unknown. Like most autoimmune diseases, more women than men are affected, and both long term use of oral contraceptives and breastfeeding has been shown to reduce the risk for developing RA, in particular ACPA-positive RA, pinpointing hormones as etiological factors [56]. RA is considered to be a complex multifactorial disease, which may occur as a result from interaction of specific genes and environmental factors [57]. Epidemiological and genetic studies of RA have revealed that specific *HLA-DRB1* alleles termed the shared epitope (SE), give the strongest genetic association [58], while cigarette smoking has been identified as the most important environmental risk factor for RA [59]. Additional candidate genes (e.g. *PTPN22, PADI4, TRAF1*) and environmental risk factors (mineral oil, silica dust, and certain infections) have also been suggested to link to RA.

HLA genes

The strongest genetic risk factor for RA, i.e. genes located on the human leukocyte antigen (HLA) region, has been known since the 1970s [58]. The HLA system encodes membrane proteins on antigen presenting cells (APC) that display antigen peptides to T lymphocytes, initiating the adaptive immune response. The HLA genes can be divided into three classes (I-III), where class I and II are involved in cell-mediated immune responses, while class III genes encode proteins regulating immune responses, such as complement proteins (C2, C4), tumor necrosis factor (TNF) and heat shock proteins [60].

In the context of autoimmunity and RA, the HLA class II alleles are best studied and constitute the most important risk factor for RA. In 1987, the so-called shared epitope (SE) hypothesis was postulated [58], and suggested that certain HLA alleles (including *HLA-DRB1**0401, *0404, *0101 and *1001, collectively called *HLA-DR* SE alleles) encode a highly conserved region in the amino acid residues 70-74 (QKRAA, QRRAA and RRRAA) of the third hypervariable region of the DRß-chain. A more recent study defined five amino acids in the peptide-binding groove of three HLA proteins (*HLA-DRB1, HLA-B, HLA-DPB1*) that could explain the HLA association to RA risk [61]. Three amino acids (valine at position 11, lysine at 71 and alanine at 74) in *HLA-DRB1* and two additional amino acids at position 9 in *HLA-DP* and *HLA-B* (belongs to class I) showed the strongest association with ACPA-positive RA and were estimated to account for 12.7% of the genetic risk in RA development. The SE hypothesis implicates a functional impact of these amino acid positions on antigen presentation to T cells, either during early thymic development or during peripheral immune responses, and supports an important role for both CD8+ and CD4+ T cells in RA pathogenesis.

HLA-DRB1 SE alleles have been confirmed as a risk factor only for ACPA-positive RA, suggesting a role for SE in the presentation of citrullinated peptides to T cells [57, 62, 63]. SE alleles have also been associated with radiographic erosions [64], and this association is dose-dependent - patients with two SE alleles have more radiographic erosions and more joint replacements than patients with non-disease associated *HLA-DRB1* alleles [65]. Recent crystallographic and structural analysis demonstrated that the positively charged P4 pocket of DRB1 formed by SE variants favours binding of citrulline-containing peptides, rather than arginine variants of the same peptides (Figure 2) [66]. Such an arginine to citrulline conversion

by PADs may enhance HLA binding and therefore the presentation of peptides that would not be bound in their native form [67].

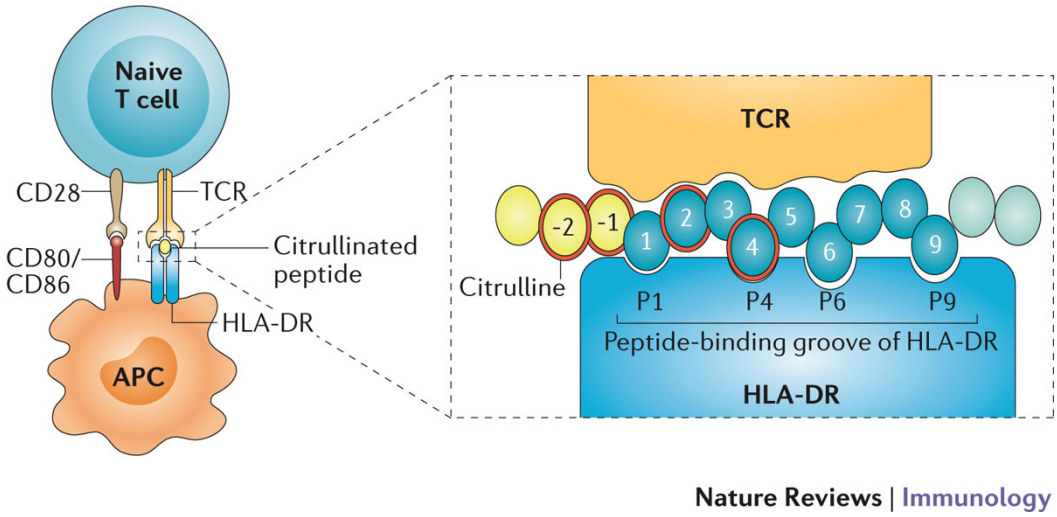


Figure 2. *An interaction between the peptide–MHC complex on an antigen-presenting cell (APC) and the T cell receptor (TCR) on a T cell. Neutral citrullinated peptides (as indicated by red rings) bind the P4 pocket of the peptide-binding groove of the HLA-DR SE molecules associated with rheumatoid arthritis (RA), whereas the positively charged amino acid arginine is unlikely to fit into this structure. From Malmstöm et al. [67]*

PTPN22

Polymorphism in the protein tyrosine phosphatase non-receptor type 22 (*PTPN22*) gene is the second most important genetic risk factor for RA, and the first described genetic variance outside the HLA region [68-70]. *PTPN22* polymorphism associates with both seropositive and seronegative RA, although the association with seropositive disease is stronger [71-74], and in addition, *PTPN22* associates with a number of other autoimmune diseases [70]. The *PTPN22* gene encodes a tyrosine phosphatase that is primarily expressed in lymphoid tissue, and was suggested to play a critical role in regulating early T-cell signalling events [75]. However, a recent study proposed other mechanism of action for this risk allele (at least, in the context of RA) through the generation of CD4+ T cells with cytotoxic characteristics [76]. In addition, the *PTPN22* risk allele has been linked to higher numbers of autoreactive circulating B cells in healthy individuals [77]. A significant gene-gene interaction has been identified between the two major genetic risk factors for developing ACPA-positive RA, i.e. *HLA-DRB1*-SE and polymorphism in *PTPN22*, with a 10-fold increased risk for developing RA in individuals carrying at least one copy of the susceptible variants, compared to those carrying none [73].

Other non-HLA genes

Recent genome-wide association studies (GWAS) have identified more than 100 loci associated with RA, including single-nucleotide polymorphisms (SNPs) in *PADI4, STAT4, TNFAIP3, ANKRD55, CTLA4* and *CD40* [78-82]. Many of them are associated with ACPA-positive RA, while only few were found to be exclusively associated with ACPA-negative RA

(polymorphisms in the *CLEC4A* and *IRF-5* genes as well as in the *HLA DRB1**03 alleles) [83-85]. Some of them also show population-specific associations. For instance, SNP haplotypes in the *PADI4* gene, encoding for the PAD4 enzyme involved in citrullination, were shown to be associated with increased ACPA levels in RA patients mainly in Asian populations [86, 87]. Only approximately 20% of RA risk variants have been identified in coding regions (for instance, *PTPN22, IL6R, TNFAIP3*), while other RA-associated variants occur in non-coding regions, therefore the majority of RA risk genes and variants remain to be identified [82].

Environmental risk factors

Since RA cannot be explained only by genetics, environmental factors clearly contribute to the development of the disease. Cigarette smoking constitutes the best-established environmental risk factor for RA, mainly seropositive RA [57, 59, 88]. Several studies have shown that the duration and intensity of smoking correlates with the risk of developing RA, and that the risk remains elevated for several years even after smoking secession [89]. Gene-environment interaction analysis has revealed interactions between SE and smoking, in seropositive RA [90, 91], suggesting that smoking may trigger the development of RA specifically in genetically predisposed individuals [92]. Specifically, it has been suggested that smoking triggers protein citrullination in the lungs, since smokers have been shown to have increased presence of citrullinated proteins and expression of PAD enzymes in the lungs, compared to non-smokers [91-94].

Occupational exposure to silica dust, textile dust, asbestos and mineral oils have also been shown to associate with RA [95-99], while alcohol consumption – one of the most studied lifestyle factors – was shown to be protective against RA [100, 101]. One explanation could be the downregulating effect alcohol has on pro-inflammatory cytokines involved in progression of RA [102, 103]. Several studies have also examined diet and dietary supplements (red meat, fish, fruits, vegetables, D-vitamin, Omega-3 etc.) in relation to the development of RA, although with inconsistent results.

Infectious agents, like common viruses, have for long been discussed in the context of autoimmune diseases, including RA. A number of studies have addressed the role of Epstein-Barr virus (EBV), human parvovirus B19 and cytomegalovirus (CMV) in RA development. However, data show conflicting results, and it has been difficult to link a specific virus to RA [104-108]. More recently, alterations in the gut microflora has been highlighted in the context of autoimmune diseases like RA [109]. In addition, periodontal pathogens, including *Porphyromonas gingivalis,* has been suggested to be causatively linked to RA [110].

1.2 PERIODONTITIS AND RA

1.2.1 Etiology of PD

Periodontal diseases (gingivitis and periodontitis) are probably the most common diseases of mankind (Guinness World Records 2001) [111]. These chronic inflammatory conditions affect the tissues surrounding and supporting the teeth, and are highly prevalent in adult populations all over the world, with prevalence rates of 47-60% [112, 113]. Initially, periodontal disease presents as gingivitis, a reversible inflammation of the gingival mucosa, resulting in gingival bleeding and swelling. In susceptible individuals, untreated gingivitis might progress to chronic periodontitis, resulting in destruction of the periodontal tissue support and surrounding alveolar bone, and can ultimately lead to tooth loss [114].

The severity of periodontal disease is largely driven by an interplay between the subgingival biofilm and the host immune system, modulated by various genetic and environmental factors. Risk factors for periodontal disease include male sex, cigarette smoking, diabetes mellitus, obesity, and genetic factors [115]. The main cause of periodontal disease is poor oral hygiene leading to an accumulation of plaque biofilm at the gingiva–tooth interface, that eventually extends below the gingival margin. The primary PD-associated pathogens include Gram-negative anaerobic bacteria: *Porphyromonas gingivalis (P.gingivalis), Tannerella forsythia, Treponema denticola* and *Aggregatibacter actinomycetemcomitans (Aa)* [116]. Of these, *P.gingivalis* is recognized as a keystone pathogen in the pathogenesis of PD, causing a disturbed interplay between the subgingival biofilm and the host response [117]. Periodontal disease is associated with several other chronic diseases, including cardiovascular diseases, chronic respiratory and kidney diseases, and rheumatoid arthritis, and this may be related to shared common inflammatory mediators and pathways [118].

1.2.2 Epidemiological associations between PD and RA

Despite differences in initiating etiological mechanisms, numerous clinical and epidemiological studies reported a positive association between PD and RA, with PD being more prevalent in individuals with RA and *vice versa*, when compared to the general population [119-123]. The largest case-control study to date, that included 13 779 newly diagnosed RA patients and 137 790 matched non-RA controls within the Taiwanese National Health Insurance Research Database (NHIRD), reports a significant and independent association between RA and history of PD [124]. However, not all studies could confirm this association [125, 126]. These conflicting reports may result from differences in disease classification criteria for PD and differences between RA cohorts, where such factors as treatment use, disease duration, age and sex of study subjects could affect the outcome. Another important factor introducing discordance could be self-reported PD. For instance, Eriksson *et al.* examined the periodontal status of RA patients included in the Swedish Epidemiological Investigation of RA (EIRA) case-control study [126]. This study included 2740 RA cases and 3942 matched controls and the prevalence of PD amongst participants was investigated using

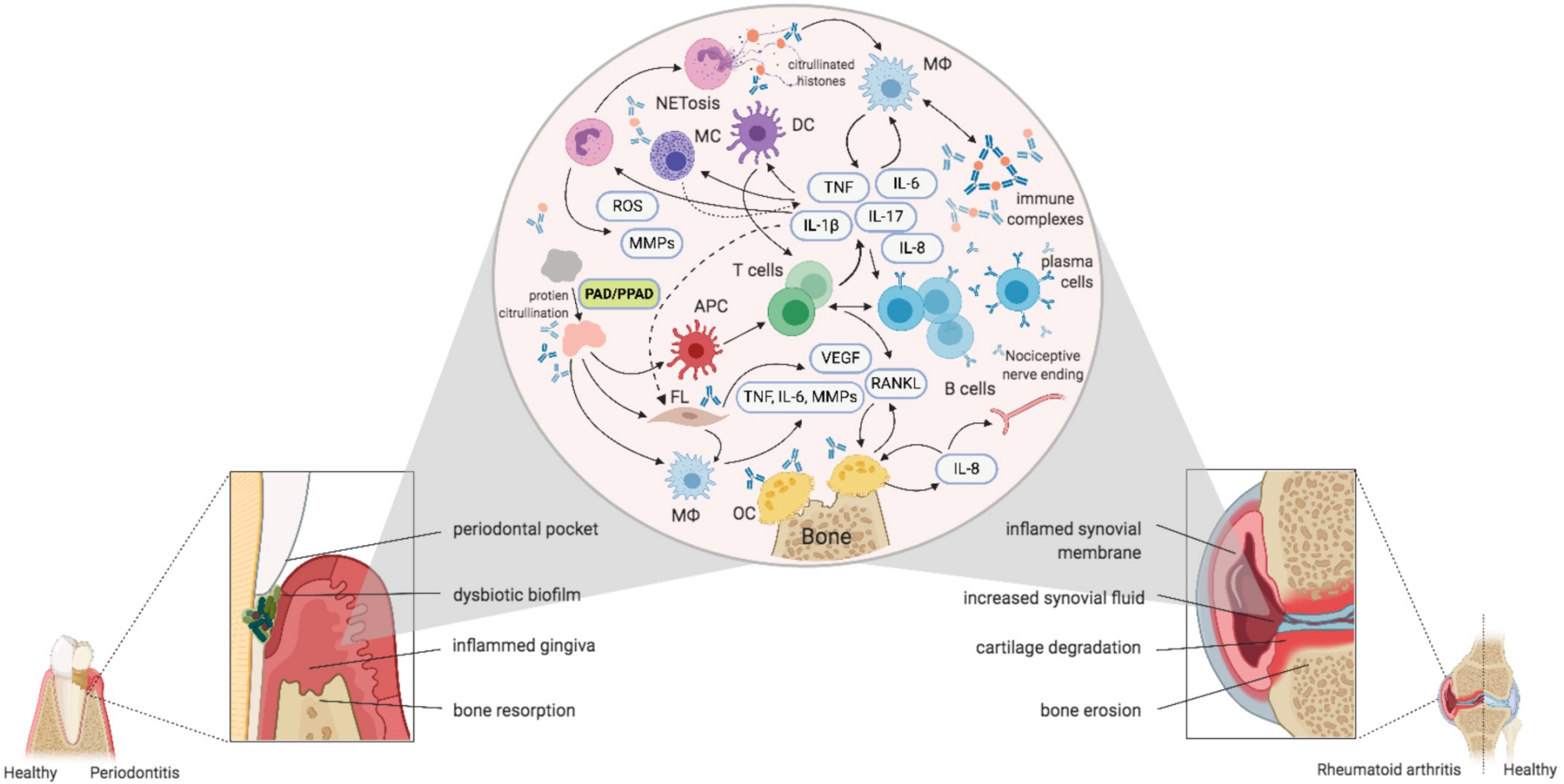
a self-administered questionnaire, and showed no significant differences in prevalence of (self-reported) PD between RA cases and controls. However, when self-reported PD from 48 RA cases and 49 controls was validated via linking of the EIRA to the National Dental Health Registry (DHR) to confirm PD diagnosis, only 29 RA cases (60%) and 34 controls (69%) appeared to have PD according to the diagnostic codes in DHR, indicating that self-reported PD could not be validated. Thus, only data from dental examination should be used in order to avoid potentially misleading conclusions.

The two most recent meta-analyses confirm a significant association between RA and PD [127, 128], and one of them showed that RA patients had a 13% greater risk of PD compared to healthy controls [128]. Analysis of periodontal status in first-degree relatives of patients with rheumatoid arthritis (FDR-RA) revealed higher prevalence and severity of PD in ACPA-positive compared to ACPA-negative FDR-RA individuals [129]. Moreover, a recent case-control study found that PD (characterized as marginal jawbone loss) preceded the clinical onset of RA symptoms, further supporting a possible role of PD in the development of RA [130]. In addition, higher RA disease activity (measured as higher disease activity score for 28 joints (DAS28)) was observed in RA patients with severe PD, when compared to RA patients with no or moderate PD [122, 131].

1.2.3 The biological link between PD and RA

The reported association between PD and RA can be non-causal, as both conditions share genetic and environmental risk factors, such as *HLA-DRB1* SE and cigarette smoking [126, 132, 133]. Furthermore, pathogenic pathways are similar in RA and PD, where inflammatory cells and pro-inflammatory cytokines drive degradation of collagen-rich tissues: gingiva, periodontal ligament, and alveolar bone in PD; and bone, cartilage, and other periarticular tissues in RA [134]. Both diseases are characterized by the presence of activated osteoclasts and fibroblasts, high levels of pro-inflammatory cytokines (IL-1β, IL-6, IL-23, TNF-α) and degradation enzymes (MMP1, MMP8, MMP9 and MMP13), decreased levels of anti-inflammatory cytokines (such as IL-10), and recruitment of a variety of cells, including neutrophils, monocytes, lymphocytes, plasma cells, and mast cells (Figure 3) [116, 135].

Neutrophils are among the first cells to be recruited to the site of inflammation, and when activated by multiple inflammatory mediators they can release enzymatic content to the exterior of the cell, forming a web-like structure – so-called NETs, composed of DNA and attached histones, and other proteins (such as lactoferrin, cathepsins, neutrophil elastase), with a purpose to kill and immobilize pathogens [136]. In chronic inflammatory conditions, such as RA and PD, the release of NETs along with presence of reactive oxygen species (ROS) provokes tissue damage, exacerbates the inflammatory response, and exposes possible autoantigens, including citrullinated histones [137]. Notably, elevated NET formation was reported in both PD and RA [138, 139].


citrullinated histones
MΦ
NETosis
MC
DC
TNF
IL-6
ROS
IL-17
immune complexes
IL-1β
MMPs
IL-8
T cells
plasma cells
protien citrullination
PAD/PPAD
APC
VEGF
Nociceptive nerve ending
RANKL
B cells
FL
TNF, IL-6, MMPs
IL-8
MΦ
OC
Bone
periodontal pocket
dysbiotic biofilm
inflammed gingiva
bone resorption
Healthy
Periodontitis
inflamed synovial membrane
increased synovial fluid
cartilage degradation
bone erosion
Rheumatoid arthritis
Healthy

◄ ***Figure 3*** *Pathogenic mechanisms involved in progression of periodontitis and rheumatoid arthritis, are driven by activated osteoclasts and fibroblasts, high levels of pro-inflammatory cytokines (IL-1β, IL-6, IL-23, TNF-α) and degradation enzymes (MMPs), and presence of a variety of immune cells, including neutrophils, monocytes/macrophages, lymphocytes (B cells, Th1, Th17), plasma cells, dendritic cells, and mast cells. Local inflammation in both conditions is accompanied with increased cell death and activation of human and (in case of PD) bacterial PAD enzymes, and NETosis, leading to increased local citrullination and generation of neo-epitopes, that are presented by antigen-presenting cells and activate an adaptive immune response with (auto)antibody formation. The antibodies contribute to local inflammation via immune-complex formation, compliment activation, stimulation of neutrophils and macrophages, and activation of osteoclast and fibroblast migration (for instance, in case of ACPA). B and T cells also increase the expression of RANKL, a pivotal player in inflammatory bone resorption, that stimulates the differentiation of monocyte-MΦ-precursor cells into osteoclasts and their maturation and survival, leading to bone loss. TNF, tumour necrosis factor; IL, interleukin; RANKL, receptor activator of nuclear factor κB ligand; NET, neutrophil extracellular traps; MMPs, matrix metalloproteinases; FL, fibroblasts; OC, osteoclasts; MΦ, macrophages; DC, dendritic cells; MC, mast cells; APCs, antigen-presenting cells; ACPA, antibodies against citrullinated proteins. Based on [67, 116, 135, 140-142]*

Interestingly, presence of autoantibodies has also been reported in PD patients, with collagen type I being one of the best-evaluated autoantigens. Higher levels of anti-collagen antibodies were detected in PD sera compared to healthy controls, and in PD gingival tissue, as well as PD gingival crevicular fluids (GCF), compared to autologous sera [143-145]. Analysis of dissociated cells from gingivae of PD patients revealed numerous cells producing antibodies to collagen type I and also CD4+ T cells reactive with collagen type I [146-148]. In addition to collagen, other periodontal autoimmune targets include fibrinogen, vimentin and α-enolase, which are well-known RA autoantigens when citrullinated [149, 150]. Indeed, in a study by de Pablo *et al.* the antibody response in PD patients was predominantly directed to arginine-containing peptides of the RA autoantigens examined [151]. However, increased citrullination in gingival tissue of PD patients has also been reported [152-154]. Moreover, ACPA has been detected in GCF of individuals with PD [155-157], indicating that ACPA production could be triggered in the gum mucosa, and that such an antibody response may prime an individual for a subsequent systemic ACPA response. Hence, these data may suggest a causal relationship between PD and RA.

1.2.4 Citrullination caused by bacteria and the link to RA

In 1995, McGraw and colleagues reported that the periodontal pathogen *P.gingivalis* expresses a PAD enzyme, denoted PPAD [158]. Together with the protozoa *Giardia lamblia*, *P.gingivalis* is the only known microorganism with the ability to citrullinate proteins [159]. Based on this knowledge, Rosenstein *et al.* proposed that individuals predisposed to *P.gingivalis* infection may be exposed to citrullinated antigens in the gum mucosa, that subsequently become systemic immunogens and lead to systemic ACPA production and intraarticular inflammation [110]. Unlike human PAD, PPAD does not require calcium for activity, is active at higher pH, and citrullinates C-terminal arginine residues and free arginine.

PPAD has been shown to citrullinate both bacterial and human proteins, and studies suggest that PPAD can also be auto-citrullinated [160-162].

Since then, many studies have addressed a potential role for *P.gingivalis* in triggering RA, yet with inconclusive results. Several studies have shown that RA patients, especially the ACPA-positive subset, have elevated anti-*P.gingivalis* antibody levels, compared to population controls, including antibodies targeting citrullinated PPAD, and in one of the studies presented in this thesis, we have shown that these antibodies precede onset of clinical symptoms of RA by up to 10 years [162-166]. In contrast, other studies were not able to replicate these findings [122, 167-169]. These conflicting results may result from absence of a standard assay to measure the antibody response to *P.gingivalis*. All studies are based on in-house ELISAs with different types of *P.gingivalis* antigen (bacterial lysates, purified recombinant proteins, *P.gingivalis*-specific LPS or outer membrane antigens). Differences between RA cohorts could also explain discrepant results, and geographical differences in the profiles of PD microbiota, including different *P.gingivalis* strains, have been reported [170, 171].

In addition to *P.gingivalis*, another PD pathogen, *A.actinomycetemcomitans (Aa),* has been associated with RA [172]. Konig *et al.* demonstrated that *Aa* induces hypercitrullination in neutrophils by the actions of leukotoxin A (LtxA), a major virulence factor of *Aa* with pore-forming capacity. Moreover, the anti-LtxA antibody response in RA patients associated with ACPA and RF positivity. Although these findings are intriguing, they could not be confirmed by Volkov *et al.*, using the same LtxA ELISA protocol in a Dutch early arthritis cohort [173].

1.2.5 *P. gingivalis* in experimental models of arthritis

In addition to epidemiological data, a number of experimental studies using animal models have been performed to investigate the relationship between *P.gingivalis* and RA. Oral exposure of mice and rats to this periodontal pathogen have been shown to affect the development and severity of arthritis. In several studies infection with *P.gingivalis* exacerbated experimental arthritis in mouse and rat models, measured as earlier onset of arthritis, accelerated progression and enhanced severity, including significantly increased bone and cartilage destruction [174-178]. Moreover, in these studies ACPA were found in sera of mice infected with wild-type *P.gingivalis*, but not with a *P.gingivalis* PPAD-null mutant strain (dPAD) [174, 177]. Arthritis and anti-CEP1 antibodies were induced by immunizing DR4-IE-transgenic mice with *P.gingivalis* enolase in study by Kinloch *et al.* [179]. Furthermore, intravascular dissemination of *P.ginigvalis* to the synovial joints was demonstrated in orally-infected collagen-induced arthritis (CIA)-prone B10.RIII mice, that was associated with arthritis exacerbation, increased inflammatory cell infiltration, destruction of articular cartilage, erosions, and pannus formation [180]. In another study, orally administered *P.gingivalis* not only aggravated arthritis with increased interleukin-17 levels in sera, but also changed the gut microbiome, that resulted in impairment of the gut barrier function [181].

Other studies showed that the aggravation of arthritis by *P.gingivalis* was accompanied by a systemic Th17 response, increased TNF and IL-17 production and articular neutrophil infiltration, leading to higher joint damage in exposed mice [178, 182, 183]. In a rat model of pristane-induced arthritis (PIA), the pre-existence of periodontitis induced by *P. gingivalis* resulted in antibody response against a citrullinated peptide derived from PPAD, but did not alter the development or severity of PIA [184]. However, in a recent study by Courbon *et al.* oral exposure of Lewis rats to *P.gingivalis* for one month was sufficient to induce anti-CCP2-positive arthritis, with systemic and joint-specific inflammation and bone erosions [185].

Based on animal studies, various mechanisms have been proposed by which *P.gingivalis* may promote autoimmune arthritis in experimental animals and humans, including: 1) induction of autoantibodies [174, 175, 185]; 2) cross-reactivity between host and bacterial antigens [179]; 3) induction of Th17 responses [178, 182, 183]; 4) through direct dissemination of *P.gingivalis* to the joints [180]; and 5) by alteration of gut microbiota caused by swallowing the bacteria [181]. Still, generation of citrullinated antigens by PPAD remains the most credible scenario mechanistically linking PD and RA [186].

1.2.6 Effect of PD treatment on RA, and *vice versa*

The treatment of RA is complex and includes pharmacological therapies (primarily aimed to decrease disease activity, and inhibit bone and cartilage damage), and non-pharmacological therapies (psychological support, education, physical activity and, in late disease, surgery). The current EULAR recommendations for RA treatment advice that: 1) the therapy with conventional synthetic (cs) disease-modifying antirheumatic drugs (DMARDs) should be started as soon as the diagnosis of RA is made; 2) methotrexate (MTH) should be part of the first treatment strategy; 3) short-term glucocorticoids (GC) should be considered when initiating or changing csDMARDs (MTH, leflunomide, sulfasalazine); 4) in the presence of unfavorable prognostic markers (e.g. autoantibodies, high disease activity, early erosions, or failure of second csDMARD), any biological DMARD (TNF-inhibitors (adalimumab, certolizumab pegol, etanercept, golimumab, infliximab), IL-6 inhibitors (tocilizumab, clazakizumab, sarilumab and sirukumab), T/B cell targeting therapies (abatacept, rituximab), and biosimilar DMARDs) or targeted synthetic DMARD (Janus kinase (Jak)-inhibitor tofacitinib, baricitinib) should be added to the csDMARD [187].

Despite the availability of many efficacious agents, current treatment of RA is based on targeting established pathological processes. As the understanding on how RA develops increase, along with emerging needs of assessing patients earlier in their disease course and individuals at risk, a need for preventive treatments becomes apparent. In this instance treatment of PD could offer a relatively inexpensive and safe non-surgical intervention with potential benefit for RA patients and individuals at risk for developing RA.

Effect of PD treatment on RA

To date, twelve clinical studies have investigated the effect of periodontal therapy on RA [188-199]. In these studies, different parameters, such as ESR, CRP, health assessment questionnaire (HAQ), DAS28, RF, anti-CCP and anti-*P.gingivalis* IgG were evaluated after non-surgical treatment of PD. Although, these studies are small (with a range of 12 – 75 RA patients), have a varied criteria for PD diagnosis, have short follow-up times after periodontal treatment (8 weeks – 6 months), include RA patients with various disease duration and undergoing different treatment, and investigate distinct outcome measures, all of them generally agree on a positive effect of periodontal treatment on RA severity (measured as reduced ESR, DAS28 or CRP). The recent systematic review and meta-analysis [200], that included four of these clinical studies [189-192], showed a significant reduction in DAS28 score following non-surgical periodontal treatment in RA patients with PD. However, no significant difference in blood CRP and ESR levels were observed, suggesting that disease activity is better evaluated by a composite score (such as DAS28) than by individual clinical and inflammatory markers. Further controlled clinical trials including a larger number of patients and longer follow-up periods are necessary to prove efficacious effect of periodontal treatment on RA clinical outcome.

Effect of RA treatment on PD

A number of studies have addressed the effect of RA treatment on the course of PD, and a majority of them were focused on biological DMARDs and suggested a beneficial effect on PD. Anti-TNF therapy has been shown to significantly decrease clinical parameters of PD severity (determined by gingival index, bleeding on probing, and probing depth) in RA patients without additional periodontal treatment [201-204]. Similar changes in periodontal parameters were observed after IL-6 receptor (IL-6R) inhibition therapy [202]. Anti-TNF therapy also resulted in significant decrease in gingival crevicular fluid (GCF) volume and levels of pro-inflammatory cytokines (IL-1β, IL-8, MCP-1) in GCF and saliva [205], and in significantly decreased attachment lost [203]. However, several studies reported absence of significant effects of anti-TNF therapy on the periodontal condition [190, 206]. Beneficial effects on PD was also observed after anti-B cell therapy (i.e. rituximab) [207].

1.3 ETIOLOGICAL HYPOTHESIS

Autoimmunity in RA may be triggered in genetically predisposed individuals, as a result of exposure at mucosal surfaces (such as the lungs, the gums, and the gut) to environmental stimuli (e.g. cigarette smoke, textile dust, silica, or microorganisms like the periodontal pathogens *P.gingivalis* and/or *Aa*) (Figure 4.1). This could cause a local inflammation, increased cell death and activation of human and bacterial PAD enzymes, leading to increased local citrullination and generation of neo-epitopes. Already at this point, B cells may be activated and start producing low levels of low-affinity ACPA, that can be detected several years before clinical symptoms of RA develop [160, 208].

The autoimmune ACPA response will gradually mature with epitope-spreading and increasing levels of high-affinity ACPA, as a result of interactions between autoreactive T cells and citrulline-specific B cells in individuals carrying risk alleles associated with RA (such as the *HLA-DRB1* SE) (Figure 4.2). An individual at this stage of disease progression would be characterized as "at risk" for the development of RA, and may experience arthralgia and even bone loss as a result of ACPA binding to and activating osteoclasts, which release IL-8, that in turn stimulates nociceptors [209].

IL-8 is also a strong chemokine for recruiting neutrophils, and has been shown to induce PAD activity in fibroblasts [39]. Hence, through increased epitope-spreading and/or cross-reactivity, ACPA may later target citrullinated proteins on fibroblast-like synoviocytes, mediating increased fibroblast migration, and citrullinated histones in NETs, forming soluble immune complexes. Such ACPA-immune complexes could then stimulate monocytes/macrophages, with increased production of pro-inflammatory cytokines (e.g. TNF and IL-6) [210, 211], ultimately causing chronic joint inflammation (Figure 4.3).

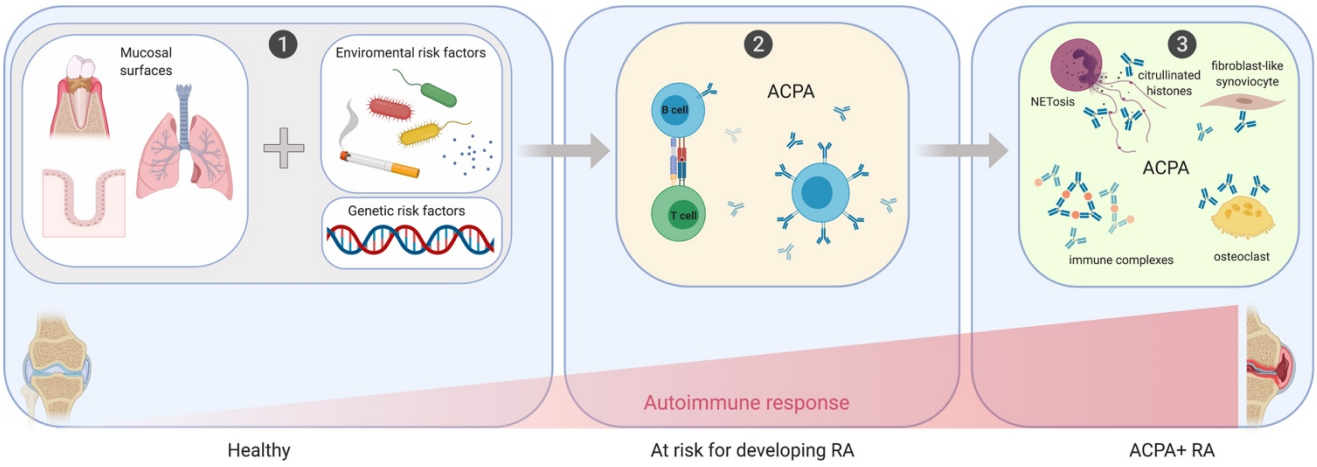


Figure 4. *Etiological hypothesis of the longitudinal development of ACPA-positive RA.*

2 AIMS OF THE THESIS

The overall aim of this PhD project was to investigate the role of the periodontal pathogen *Porphyromonas gingivalis* in the etiology of Rheumatoid Arthritis (RA), particularly in the ACPA-positive subset of RA. Specific aims included:

- To characterise the subset of RA patients with a heightened immune response to *P.gingivalis,* in terms of genetic risk factors, smoking and the ACPA response (**Study I**)
- To investigate the antibody response to *P.gingivalis* in blood samples of pre-symptomatic individuals and controls, in order to determine whether anti-*P.gingivalis* antibodies preceede RA onset (**Study II**)
- To analyse the antibody response to a citrullinated peptide derived from the *P.gingivalis* PAD enzyme in RA, PD, and controls; and to characterise the antibody-positive subset of RA regarding genetics, smoking, clinical phenotype, and ACPA (**Study III**)
- To isolate and clone gingiva-derived B cells, and to characterise recombinantly expressed monoclonal antibodies from these cells in order to investigate whether citrulline-reactive B cells reside in inflamed gingival tissue (**Study IV)**

3 MATERIALS AND METHODS

3.1 Ethical considerations

The purpose of this thesis has been to gain a better understanding of triggers and drivers of disease processes in ACPA-positive (ACPA+) RA, with an aim to ultimately benefit patients. The project has involved ethical considerations regarding the collection of biological samples, clinical data, and personal information from patients with RA and/or PD, as well as healthy donors.

All biological samples were collected with informed consent and ethical approval granted from the regional ethics review board at Karolinska Institutet, Stockholm (**Studies I, III** and **IV**), and the regional ethics review board in Umeå, Department of Medical Research (**Study II**).

Protection of privacy for the study participants was ensured by handling personal information according to PUL (personuppgiftslagen), and keeping information within the network security system of the Karolinska University Hospital (KS) (**Studies I, III** and **IV**), or Umeå University (**Study II**). Stored data does not include personal identifiers. All scientists and clinicians involved in the project have adhered to Good Clinical Practice / Good Laboratory Practice guidelines, and all studies were conducted in compliance with the Declaration of Helsinki [212].

3.2 Patient material

All studies included in this PhD thesis are based on human material from RA patients, individuals with PD, and healthy controls. Serum samples from the Swedish population-based EIRA case-control study, and from a small cohort of patients with PD and periodontally healthy controls (non-PD) were used in **Studies I** and **III**. **Study II** was performed using the serum samples from the Medical Biobank of Northern Sweden, Umeå. **Study IV** is based on recombinantly expressed monoclonal antibodies generated from B cells isolated from RA peripheral blood, bone marrow, bronchoalveolar lavage, synovial tissue and synovial fluid, as well as gingival tissue of PD patients with and without RA.

EIRA

In **Studies I** and **III,** serum samples from the Epidemiological Investigation of Rheumatoid Arthritis (EIRA) cohort were used. EIRA is an ongoing Swedish population-based case-control study of RA, initiated in 1996. Newly diagnosed RA patients, aged 18-70 years, are included in the study and donate blood at inclusion. Controls are randomly selected individuals from the Swedish population register, matched to cases on age, sex and residential area. All participants donate blood at inclusion, and answer a questionnaire, that includes information on smoking, occupational history, alcohol consumption, dietary intake, and disease history. Blood samples taken from EIRA participants are used for serological analysis, and for genotyping for RA risk

genes (e.g. *HLA-DRB1* SE and *PTPN22* 1858C/T polymorphism). Details of the study have been described previously [89]. In **Study I,** we analysed 1974 patients with RA and 377 controls from EIRA, and in **Study III**, we analysed 2859 RA and 370 control samples, for presence of anti-*P.gingivalis* antibodies and ACPA fine-specificities. Information on CCP2 and RF status, risk genes, smoking status, C-reactive protein (CRP) levels, and disease activity score for 28 joints (DAS28) were retrieved from the EIRA database. Verified data on periodontal status were not available for EIRA participants.

The PD and non-PD cohort

In addition to the EIRA cohort, serum samples from chronic PD patients (n=65) and gender-matched periodontally healthy controls (n=63) were screened in **Studies I** and **III**. All study subjects were examined by dentists at the Department of Dental Medicine, Karolinska Institutet, Stockholm, Sweden. Clinical criteria for PD included bone resorption with attachment loss ≥5mm, pocket probing depth ≥4mm, and bleeding on probing. Periodontally healthy controls had no signs of any periodontal disease (no gingival inflammation, clinical attachment level ≤3.5 mm, pocket probing depth ≤3.0 mm, and no bleeding on probing).

Medical Biobank of Northern Sweden

In **Study II**, a case-control study was performed within the Medical Biobank of Northern Sweden (MBNS), which is composed of the Northern Sweden Health and Disease Study and the Maternity cohort. Information regarding recruitment, sample collection and storage conditions has been described previously [213]. To identify pre-symptomatic individuals, i.e. individuals who donated blood samples prior to onset of RA symptoms, the Medical Biobank has been linked to the clinical register of patients fulfilling the 1987 classification criteria for RA [4]. In total, 251 pre-symptomatic individuals, who had donated (one or more) blood samples at different time points pre-dating the RA diagnosis, were identified. A blood sample taken at the time of diagnosis was available for 192 individuals (RA patients), 153 of which were identified within the group of pre-symptomatic individuals. From the same cohorts, 198 population-based controls, matched for age and sex, were included. A total of 422 pre-RA, 192 RA, and 198 control serum/plasma samples were analysed for the presence of anti-*P.gingivalis* antibodies. Anti-CCP2 antibody status and ACPA fine-specificities, risk genes (i.e. *HLA-DRB1* SE alleles and *PTPN22* 1858C/T polymorphism), and smoking status had been analysed previously [214, 215], and data were retrieved from the MBNS database. Information regarding the periodontal status or treatment was not available.

Single sorted B cells from RA and PD patients

In **Study IV,** we screened recombinant monoclonal antibodies (mAbs) for reactivity against a citrullinated peptide derived from *P.gingivalis* and citrullinated peptides derived from human proteins. These mAbs were generated from single-cell sorted CD19+ cells isolated from various anatomical sites, such as peripheral blood (PB), synovial fluid (SF), bone marrow (BM), bronchoalveolar lavage (BAL), and gingival tissue (GT). PB samples were obtained

from ACPA+ RA patients (n=4), recruited at KS and at the University of Minnesota, USA. Synovial fluid (n=6) samples were obtained from ACPA+ RA patients undergoing joint-replacement surgery, and were collected at KS and at the University of Birmingham, UK. Bone marrow samples were collected from proximal or distal femur of ACPA+ RA patients (n=4) undergoing hip replacement surgery due to secondary osteoarthritis at KS. Bronchoalveolar lavage samples were obtained from early untreated ACPA+ RA patients (n=2) undergoing bronchoscopy at KS. Gingival tissue biopsies were collected during the surgical treatment of severe PD in individuals with (n=3) or without (n=4) RA, and one GT biopsy originated from an individual with gingivitis. The dental surgeries took place at the Dental Department of KS and at Folktandvården, Mörby Centrum, Stockholm. In total, nine GT biopsies were processed for single-cell sorting, four of them were sorted directly after the surgery (i.e. "fresh" samples), and five GT biopsies were snap-frozen in liquid nitrogen and stored at -80°C between 12 and 184 days prior to B cell isolation.

A strength with the work presented in this thesis has been that all studies were based on human (patients and controls) material. It has been an advantage to examine the antibody response to *P.gingivalis* in such large and well-characterized cohorts as EIRA and the Medical Biobank of Northern Sweden, with properly matched population controls. A weakness, however, has been that we did not have access to periodontal status in these cohorts. Questions regarding periodontitis was included in the EIRA questionnaire, but self-reported PD could not be verified in a study comparing the questionnaire to diagnostic codes retrieved from the Swedish Dental Health Registry [126], in line with previous reports [216].

Another weakness is the small sample size of the PD/non-PD cohort, and that the two groups were not matched on age. Also, we had no information regarding RA diagnosis in the PD/non-PD cohort, although all study participants reported that they had no chronic diseases (other than PD).

A shortcoming with the pre-RA cohort is the different number of blood samples donated before diagnosis from each patient, and the variation in time points before diagnosis for those blood samples. The antibody status (positive or negative; high-levels or low-levels) of patients that donated a high number of samples, from an early time point, may skew the dataset.

3.3 ELISA AND MULTIPLEX

Anti-RgpB IgG ELISA

All serum samples in **Studies I** and **II** were analysed for the presence of anti-RgpB IgG by in-house ELISA. The protocol was modified from Quirke *et al.* [162] and included adjustments in coating concentration (2.5µg/ml), and dilution of sera (1:800). The coating antigen, RgpB protein, was purified by affinity chromatography on Ni-Sepharose from the culture medium of genetically modified *P.gingivalis* W83 secreting RgpB with the C-terminal hexahistidine-tag, as previously described by Potempa and Nguyen [217]. Serial dilutions of a highly positive

serum pools were included on all ELISA plates and used as a standard curve and anti-RgpB IgG levels were calculated and presented as arbitrary units (AU/ml). In **Study I,** a cut-off for positivity was set using the 95th percentile among non-PD controls.

Anti-CPP3/RPP3 IgG ELISA

In **Studies II, III** and **IV** we analysed reactivity against the synthetic citrullinated PPAD-derived peptide CPP3 and the arginine-containing control version RPP3 (Innovagen AB, Lund, Sweden). The same protocol as described above was used [162], with modification of the coating concentration (10 μg/ml), and serum dilution (1:100). In **Study II,** a cut-off for anti-CCP3 IgG positivity was set to give a specificity of 96% using receiver operating characteristic (ROC) curves based on reactivity among RA patients and controls. In **Study III,** a cut-off for positivity was set at the 100th percentile among non-PD controls. In **Study IV**, recombinant mAbs were tested at a concentration of 5 μg/ml, and positive clones were re-analysed in serial dilutions. See also the section on **4.4 Supplementary Information to Studies III and IV**, page 41, for a detailed discussion on the stability of the synthetic CPP3 peptide, and how this may affect antibody binding.

Anti-CCP2 IgG ELISA

Anti-CCP2 IgG data for all serum samples (in **Studies I**, **II** and **III**) were generated using the commercial Immunoscan CCPlus® ELISA (Euro-Diagnostica AB, Malmö, Sweden) in accordance with the kit instructions, with a cut-off for positivity set at ≥25 U/mL. The same kit was used in **Study IV,** where mAbs were analysed at a concentration of 5 μg/ml. For EIRA and the Medical Biobank of Northern Sweden, anti-CCP2 antibody results were published previously [218, 219], and data retrieved from databases.

ACPA fine-specificities and anti-CarP ELISAs

In **Study IV,** mAbs were tested for reactivity against different citrullinated peptides, and their arginine-containing equivalents, using previously described in-house peptide ELISAs [48, 161, 218]. Peptide antigens were derived from PPAD (denoted CPP3/RPP3) or human proteins (α-enolase, fibrinogen, vimentin, filaggrin and histone 4). All mAbs were tested at a concentration of 5 μg/ml, and reactive clones were analysed in at least two independent experiments. Unspecific binding was evaluated by including uncoated wells blocked with 2% BSA, and poly-reactivity was assayed by in-house ELISAs (i.e. LPS, insulin, dsDNA, and soluble membrane protein (SMP) fraction from Hek293 cells), following the protocols described previously [220, 221].

For **Study I**, we also analysed ACPA fine-specificities (α-enolase, fibrinogen, vimentin and collagen type II), and anti-CarP antibodies. These data had been generated previously, using the same in-house ELISAs [48, 161, 218], and a previously described in-house carbamylated fibrinogen ELISA [222], and data was retrieved from the EIRA database.

Multiplex peptide microarray

The RA, pre-RA and non-RA control serum samples included in **Studies II** and **III** have been previously analysed for the presence of different ACPA fine-specificities, using a multiplex peptide microarray, based on the ImmunoCAP ISAC system (Phadia AB, Uppsala, Sweden) [223], and data was retrieved from the EIRA and the MBNS databases [224]. Citrullinated peptide antigens (and their arginine-containing equivalents) included: citrullinated α-enolase peptide 1 (CEP-1_{5-21}), citrullinated fibrinogen (cit-fibα$_{563-583}$, cit-fibα$_{580-600}$, and cit-fibß$_{36-52}$), citrullinated vimentin (cit-vim_{2-17} and cit-vim_{60-75}), citrullinated collagen type II (cit-$C1_{359-369}$), and citrullinated filaggrin (CCP1/cit-$fil_{307-324}$).

In the present studies, both commercial (CCP2) and in-house methods, single-peptide ELISAs and multiplex assays, were used for the detection of ACPA / citrulline-reactivity. For all assays, arginine-containing control peptides were used to ensure citrulline-specificity. All Cit-peptide antigens have been previously published, evaluated, and described as autoantigens in RA. Still, the antigens are linear peptides, some coupled to biotin and some with C- and N-terminal cysteine to enhance the formation of conformational epitopes, but binding to the corresponding citrullinated proteins have not been confirmed in our studies. Although, several reports suggest that unlike classical antibodies, which target conformational epitopes, ACPA target short motifs, containing citrulline and just a few flanking amino acids, where glycine next to citrulline has been highlighted [48, 225]. Two *P.gingivalis* antigens, the purified RgpB protein and the PPAD-derived CPP3 peptide, were used in our studies. However, direct binding to *P.gingivalis* lysates and autocitrullinated PPAD protein is yet to be confirmed by immunoblotting and/or ELISA in consecutive studies.

3.4 Cloning and production of recombinant monoclonal antibodies

Recombinant monoclonal antibodies in **Study IV** were generated, following the protocol by Amara *et al.* [220]. In brief, B cells were isolated from GT and single-cell sorted into 96-well plates (Figure 5). Paired Ig heavy (IgH) and light chains (IgL) were PCR amplified, sequenced and analysed by IgBLAST [226] and IMGT/V-QUEST [227]. Ig gene-specific PCR was run to introduce restriction sites for expression vector cloning. Purified digested PCR products from each single cell was ligated into expression vectors containing human Igγ1, Igλ or Igκ constant regions, and subsequently transformed into DH5α bacteria. Purified IgH and IgL plasmids were sequenced to confirm identity with original PCR products. Monoclonal antibodies were produced by transient co-transfection (IgH and corresponding IgL plasmids) in high-density suspension cultures of Expi293 cells using the polyethyleneimine (PEI)-precipitation method. Supernatants were collected after 5 days of culture, and mAbs were purified on protein G-sepharose 4 Fast flow beads and eluted with 0.1M glycine buffer, into neutralization buffer (1M Tris-HCl, pH 8.0), with following buffer exchange to PBS. Purified hIgG1 concentrations were determined using an anti-human IgG ELISA. Monoclonal antibodies derived from RA bronchoalveolar lavage (BAL) B cells (n=44), bone marrow (BM)

plasma cells (n=39), synovial fluid (SF) plasma cells (n=142), and peripheral blood (PB) memory B cells (n=36), including 19 previously reported anti-CCP2 positive ACPA clones, were also produced following the same protocol [48, 220, 228-230].

To further evaluate the contribution of somatic mutations to the citrulline reactivity of one ACPA mAb (BVCA1), the sequence was converted into the germline (GL), using the closest predicted germline sequence in the IMGT database, as described previously [48]. Germline VH and VL nucleotide sequences were codon-optimized and synthesized by Genscript, and their accuracies were confirmed by sequencing

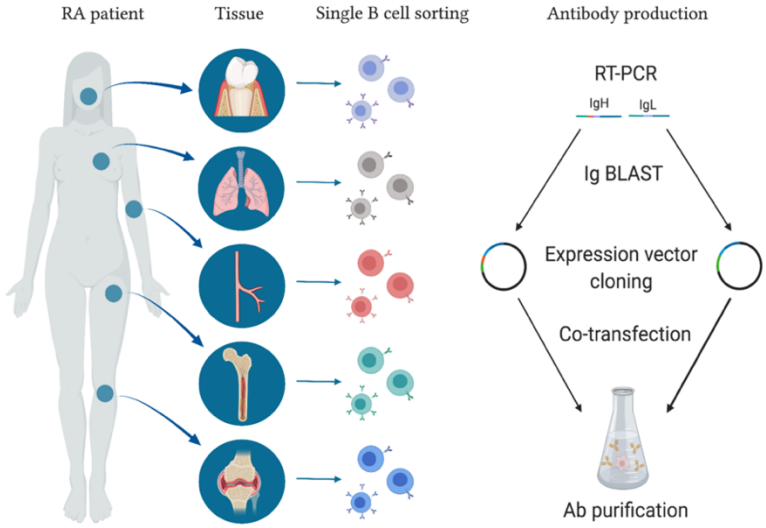


Figure 5 *Schematic illustartion of the generation of recombinant monoclonal antibodies. Single B cells were isolated and sorted from biopsy material of RA patients (gingival tissue, bronchoalveolar lavage, peripheral blood, bone marrow, and synovial fluid). Antibodies from sorted B cells were subsequently produced following the protocol by Amara et al. [220].*

3.5 Statistical methods

Continuous data (e.g., comparison of antibody concentration between study groups/matched pairs (controls, patients and pre-symptomatic individuals)) were compared using the non-parametric Mann–Whitney U-test for independent groups (**Studies I-IV**), or Kruskal-Wallis test for several groups, or the Friedman's test for matched pairs (**Study II**). Categorical data (e.g., calculation of antibody positivity between study groups) were analysed using the chi-square test or Fisher's exact test (**Studies II and IV**). Correlation analyses (e.g., for antibody concentrations, or ACPA co-occurrence) were performed using Spearman's rank correlation coefficient (**Studies I** and **II**), or Pearson correlation (**Study III**). Associations between RA risk factors (smoking, *HLA-DRB1* SE and *PTPN22* polymorphism) and presence of different antibodies in RA subsets were calculated using unconditional logistic regression models with unexposed cases and/or controls as reference group, and presented as odds ratios (OR) with 95% confidence intervals (95% CI). Analyses were adjusted for age, sex and residential area

(**Studies I** and **III**), or age and sex (**Study II**). Interaction, defined as departure from additivity of effects [231], was evaluated between RA risk factors and antibody response (**Studies I and II**). The attributable proportion due to interaction (AP) and 95% CI were calculated, as previously described [232]. The AP value between two interacting factors reflects the joint effect beyond the sum of the independent effects [233]. All statistical analyses were performed using GraphPad Prism6 or SAS (version 9.1) (**Studies I, III and IV**), or SPSS 23.0 software (Chicago, IL, USA) (**Study II**). P values ≤ 0.05 were considered statistically significant.

4 RESULTS AND DISCUSSION

4.1 Study I: The anti-RgpB IgG response in RA

A number of studies has shown clinical and epidemiological association between chronic PD and RA, that was confirmed in a large meta-analysis [128]. A non-causal relationship that could explain the epidemiological link has been suggested, based on shared environmental and genetic risk factors (e.g. smoking and *HLA-DRB1* SE), and shared inflammatory pathways, recently reviewed by de Molon *et al.* [234]. The fact that ACPA are often detected years before the onset of clinical symptoms of RA [29, 30], suggests that RA may arise outside joints, potentially at mucosal sites, such as lungs or gums. Thus, a causal link between RA and PD, where PD triggers and/or drives RA, has also been proposed [110]. Specifically, the oral pathogen *Porphyromonas gingivalis* has been pinpointed as a possible causative agent linking PD to ACPA+ RA, based on its unique feature of expressing a citrullinating enzyme, PPAD. In order to investigate the hypothesis that development of RA could be triggered by *P.gingivalis*, we have in **Study I** analysed the antibody response to *P.gingivalis* virulence factor arginine gingipain B (RgpB), which co-localize with PPAD in the outer membrane and in secreted vesicles. The anti-RgpB IgG response was investigated in relation to RA-associated autoantibodies and risk factors in patients with PD and RA, and in controls.

We measured anti-RgpB IgG by in-house ELISA in EIRA serum samples (1975 RA cases and 377 controls), and in PD and non-PD sera (65 and 59 samples, respectively). Data on smoking habits, genetics, anti-CCP2 status, ACPA fine-specificities and anti-CarP antibodies were retrieved from the EIRA database for the analysis of associations between elevated anti-RgpB IgG levels and RA-risk factors or RA-related autoantibodies.

We found that anti-RgpB antibody levels were significantly elevated in PD patients compared to periodontally healthy controls ($p<0.0001$); in RA compared to non-RA controls ($p<0.0001$); and in ACPA-positive RA compared to ACPA-negative RA ($p<0.003$) (Figure 6). We also identified a significant association between elevated anti-RgpB IgG levels and RA (OR=2.96), that was stronger in the ACPA-positive subgroup (OR=3.24). Interestingly, this association was even stronger than the well-known association between smoking and RA (OR=1.37).

These results are consistent with previous reports, where a heightened antibody response against *P.gingivalis* was shown in RA patients [165, 235-238], but yet contradictory to others [122, 168, 169], where no difference in anti-*P.gingivalis* antibody levels in RA cases compared to controls was observed. These conflicting findings may result from absence of a standard assay to measure antibodies to *P.gingivalis*. All reported studies are based on using of in-house ELISAs with different types of *P.gingivalis* antigen (bacterial lysates, purified recombinant proteins, *P.gingivalis*-specific LPS or outer membrane antigens). The discrepant results between studies could also be explained by differences between RA cohorts, where such factors as treatment use, disease duration, age and sex of study subjects could affect the outcome.

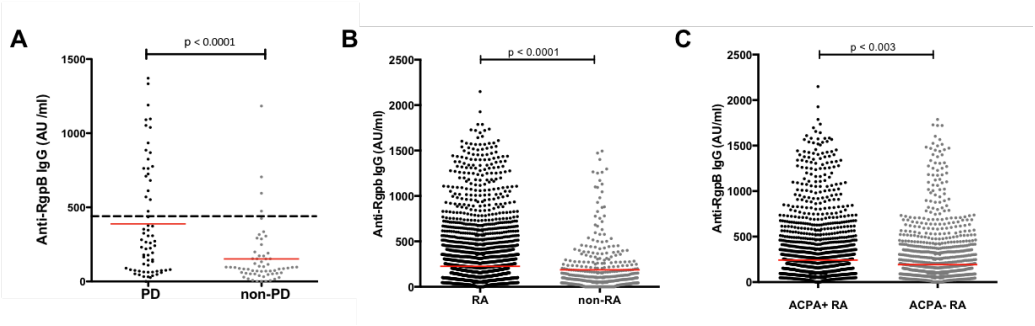


Figure 6 *Levels of anti-RgpB IgG in patients with chronic periodontitis (PD) and periodontally-healthy controls (non-PD) (**A**), in patients with rheumatoid arthritis (RA) and non-RA controls (**B**), and in ACPA-positive RA patients (ACPA+RA) and in ACPA-negative RA patients (ACPA-RA) (**C**). The dashed line in **A** indicates the cut-off value for elevated anti-RgpB IgG levels, based on the 95th percentile among controls without PD. Horizontal red lines indicate the mean.*

Since smoking is a risk factor for PD, the possible association between anti-RgpB IgG and RA seen in our study, could result from cofounding effects of smoking. However, we could not detect any association between smoking and increased anti-RgpB IgG levels. Instead, smoking and elevated anti-RgpB IgG levels were independently associated with ACPA-positive RA (Table 1). The association between elevated anti-RgpB antibodies and RA was also independent of SE and *PTPN22* polymorphism. Interestingly, statistically significant interactions between elevated anti-RgpB IgG levels and both SE and smoking were identified for ACPA-positive RA, but not for ACPA-negative RA, supporting the hypothesis of *P.gingivalis* as an etiological agent in the development of ACPA-positive RA.

Table 1. *Additive interaction (AP) between smoking and elevated anti-RgpB IgG levels in subgroups of RA, divided according to the presence of ACPA.*

Smoking	Anti-RgpB IgG	Cases (%)	Controls (%)	OR (95%CI)
ACPA-positive RA				
-	-	289 (70.83)	119 (29.17)	1.0 (ref)
+	-	751 (77.42)	219 (22.58)	1.36 (1.01, 1.83)
-	+	99 (84.62)	18 (15.38)	2.40 (1.29, 4.46)
+	+	241 (93.05)	18 (6.95)	5.35 (3.07, 9.33)
				AP 0.48 (0.12, 0.85)
ACPA-negative RA				
+	-	299 (57.72)	219 (42.28)	1.0 (ref)
-	-	176 (59.66)	119 (40.34)	1.09 (0.78, 1.52)
-	+	39 (68.42)	18 (31.58)	1.67 (0.86, 3.24)
+	+	76 (80.85)	18 (19.15)	3.01 (1.69, 5.35)
				AP 0.42 (-0.07, 0.91)

In conclusion, **Study I** demonstrates an epidemiological association between elevated anti-RgpB IgG levels and RA diagnosis, in particular ACPA+ RA, supporting the hypothesis of a causative role for *P.gingivalis* in triggering and/or driving autoimmunity and RA in a subset of patients.

4.2 Study II: The anti-RgpB and anti-CPP3 IgG responses in pre-RA

In **Study I**, we could show increased anti-*P.gingivalis* antibody levels in RA compared to controls at the time of clinical RA onset. To better understand the contribution of *P.gingivalis* to the development of RA and the ACPA response, we next analysed the presence of antibodies against *P.gingivalis* in individuals before (and after) clinical onset of RA.

Study II was based on a set of samples from the Medical Biobank of Northern Sweden cohort and included 251 pre-RA individuals, that had together donated 422 plasma/serum samples at various time points before the onset of symptoms of RA: at least one blood sample was donated by all 251 pre-symptomatic individuals; 92 individuals (36.6%) donated two samples; 46 individuals (18.3%) donated three samples; 22 individuals (8.8%) donated four samples; nine individuals (3.6%) donated five samples; and two individuals (0.8%) had donated six samples. A blood sample taken at the time of RA diagnosis (≤12 months of symptoms) was available for 192 individuals (RA patients), 153 of which were identified within the group of pre-symptomatic individuals. From the same cohort, 198 controls, matched for age and sex, were included. The samples were analysed by in-house ELISA for the presence of antibodies against virulence factor arginine gingipain B (RgpB) and a cyclic citrullinated peptide 3 (CPP3) derived from PPAD.

The levels of anti-RgpB IgG were significantly higher in pre-symptomatic individuals, as well as in RA patients, compared to controls ($p<0.001$) (Figure 7 A). These findings agree with data from **Study I**, showing increased anti-RgpB antibody levels in EIRA RA cases compared to EIRA controls. The anti-CPP3 IgG levels were significantly higher in RA patients compared to pre-symptomatic individuals ($p<0.001$), and were also significantly higher in RA patients and in pre-symptomatic individuals, when compared to controls ($p<0.001$) (Figure 7 B), in agreement with a previously reported study in RA patients [162].

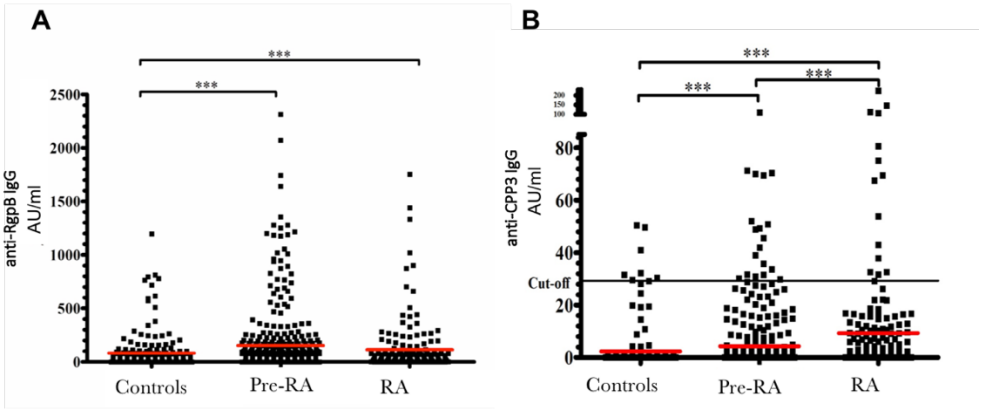


Figure 7 *Levels of anti-RgpB (**A**) and anti-CPP3 (**B**) antibodies in controls, in pre-symptomatic individuals (pre-RA), and in RA patients. Red line indicates mean. *** p<0.001*

Anti-RgpB IgG levels increased significantly during the pre-dating time ($p<0.05$) and exceeded the mean concentration of the controls more than ten years before symptom onset (Figure 8). Interestingly, a trend for lower antibody levels was observed after disease onset ($p<0.088$), that

potentially could be explained by a higher frequency of smokers in RA patients (67.2%) compared with pre-symptomatic individuals (64%), because not all pre-symptomatic individuals had corresponding samples after disease onset. An association between smoking and lower levels of anti-RgpB antibodies in RA patients ($p<0.012$), but not in pre-symptomatic individuals, was found. This finding is in line with results from **Study I**, where we observed a negative association between smoking and elevated anti-RgpB levels, and with previous reports [239, 240].

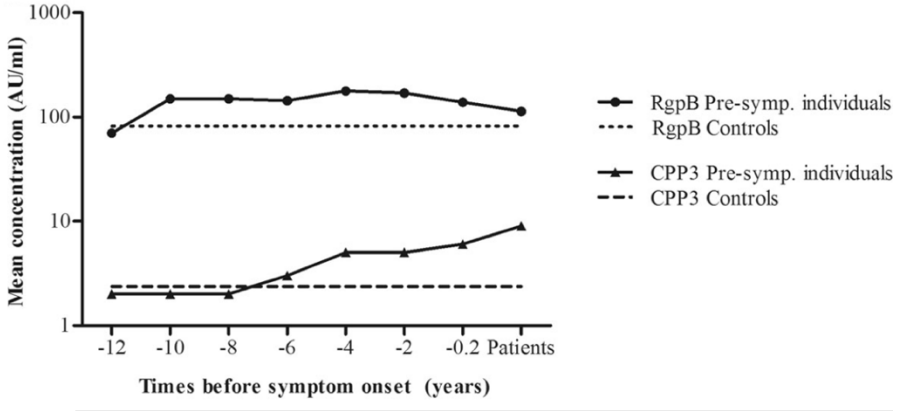


Figure 8 *The anti-P.gingivalis antibody responses during the pre-dating time until the onset of symptoms of RA. Logarithmic mean ± SEM antibody concentration of anti-RgpB and anti-CPP3 antibodies in pre-symptomatic individuals, patients with RA, and controls. SEM: standard error of the mean.*

The levels of anti-CPP3 IgG were found to increase constantly over the pre-dating time, with the mean concentration exceeding that of the controls around eight years before symptom onset (Figure 8). This pattern of increasing antibody levels over time follows the classical ACPA response, with increasing anti-CPP3 IgG concentrations over time. The majority of anti-CPP3 IgG-positive pre-symptomatic individuals and RA patients were also anti-CCP2 positive. In pre-symptomatic individuals anti-CPP3 positivity was associated with positivity for anti-cit-Fib$\beta_{36\text{-}52}$ antibodies, and anti-CPP3 IgG levels correlated with the concentration of both anti-CCP2 and anti-CEP1 antibodies. There was also a significant correlation between anti-RgpB IgG and anti-CEP1 antibodies ($p<0.05$). These findings are particularly interesting since *P.gingivalis* has been shown to efficiently citrullinate host proteins, particularly fibrinogen and α-enolase [161]. Moreover, purified anti-CEP1 antibodies have been shown to cross-react with citrullinated *P.gingivalis* enolase, which formed the etiological hypothesis of molecular mimicry in the development of ACPA+ RA [241].

Anti-RgpB antibodies were found to be associated with being pre-symptomatic ($p<0.001$), independently of smoking, SE or *PTPN22* polymorphism, in line with previous data from **Study I**. Although, no such association between anti-RgpB antibodies and RA could be shown. Anti-CPP3 antibodies were not significantly associated with being pre-symptomatic. However, anti-CPP3 IgG was associated with RA, when adjusted for age, sex, and SE or *PTPN22*. The SE in combination with anti-CPP3 IgG also revealed a stronger association with both pre-RA

and RA, than SE alone, and the same was observed for smoking and anti-CPP3 antibodies in RA.

Of note, in this study, we treated the anti-CPP3 IgG response as a classical ACPA response, in the sense that we set a cut-off based on reactivity among controls. Notably, this was not done for the anti-RgpB response, with the argument that periodontal status was unknown for both RA patients and controls. In retrospect, setting a cut-off for anti-CPP3 positivity based on non-RA controls may have been rather conservative, since we later in **Study III** found increased anti-CPP3 IgG levels in PD patients compared to non-PD controls, and importantly, in non-RA controls compared to non-PD controls. Hence, the proportion of CPP3-positive pre-symptomatic individuals (RA patients, and population controls), may be much higher than suggested in this study. In our discussion (**Study II**), we speculated that the anti-CPP3 antibody response was perhaps not *P.gingivalis*-specific, but simply a cross-reactive ACPA. Based on our data presented in **Study IV**, we now know that there is indeed cross-reactivity between *P.gingivalis* CPP3 and citrullinated human epitopes, but we also know that the unmutated common ancestor of one such cross-reactive clone was not autoreactive, but still reactive with *P.gingivalis* CPP3. Based on these data, we are more confident to say that the anti-CPP3 IgG response originates as an antibody response against *P.gingivalis.* Importantly, the CPP3 peptide sequence does not correspond to any known human protein sequence.

Taken together, data from **Study II** clearly show elevated anti-*P.gingivalis* antibody levels more than 10 years before clinical onset of RA. Hence further supporting an etiological role for this specific oral bacterium in development of RA in genetically susceptible individuals

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4.3 Study III: The anti-CPP3 IgG response in RA

To extend our findings from **Study II** showing elevated antibody levels against a citrullinated *P.gingivalis* PAD-derived peptide (CPP3) in pre-symptomatic RA and clinically established RA compared to population controls, we have in **Study III**, further investigated this antibody response in RA and non-RA controls, as well as in PD and non-PD controls**.** Moreover, in EIRA, we have characterized the CPP3-positive subset of RA in relation to RA risk factors, clinical phenotype and the classical ACPA response.

The presence of anti-CPP3 antibodies was analysed by in-house ELISA in 65 PD patients, 63 periodontally healthy controls (non-PD), in 200 RA patients (101 ACPA+ and 99 ACPA-), and 120 non-RA controls. In addition, anti-CPP3 IgG was analysed in 2859 EIRA RA cases and 370 EIRA non-RA controls using a multiplex peptide array, also containing eight citrullinated peptides from five human proteins.

Significantly elevated anti-CPP3 IgG levels were found in PD patients compared to non-PD controls ($p<0.0001$) (Figure 9A), and this antibody response seemed to be citrulline-specific, since reactivity against the arginine-containing control peptide RPP3 was significantly lower in all subsets and below cut-off for positivity for most study subjects (Figure 9B). With a cut-off set at the highest detectable reactivity among non-PD controls, 65% of PD patients were found to be anti-CPP3 IgG positive, which is not surprising, since CPP3 is a peptide derived from PPAD – a virulence factor of *P.gingivalis* capable of auto-citrullination [162]. Of note, the CPP3 epitope has been shown to be generated in PPAD during *in vitro* auto-citrullination [242], and when PD was induced experimentally in rats, by ligature placement and *P.gingivalis* infection, the animals developed anti-*P.gingivalis* antibodies, including anti-CPP3 IgG [184].

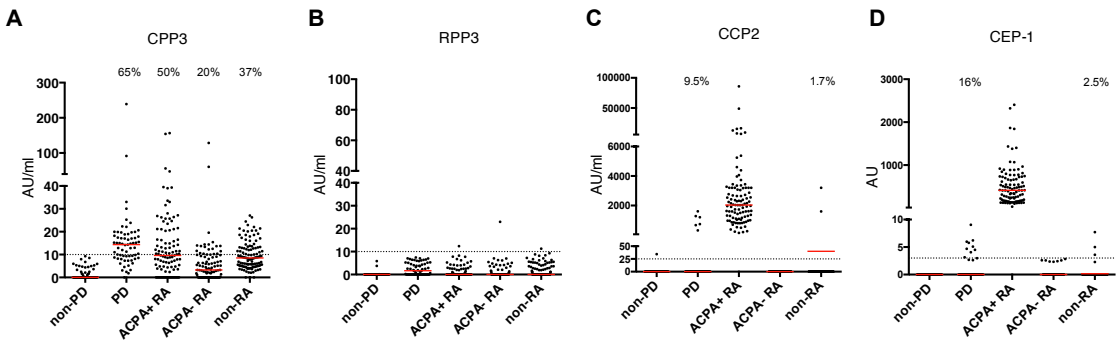

Figure 9 *The antibody response to CPP3 (**A**), RPP3 (**B**), CCP2 (**C**), and CEP-1 (**D**) in non-PD controls, PD, ACPA-positive RA, ACPA-negative RA, and non-RA controls. The dotted lines indicate cut-off for positivity. The red lines indicate mean. The percentages indicate the frequency of positive individuals in each subset. AU = arbitrary units.*

Increased anti-CPP3 antibody levels were also detected in ACPA-positive RA (50%), and to a lesser extent in ACPA-negative RA (20%) when compared to non-PD controls ($p<0.0001$ for both) (Figure 9A). Although, significantly higher anti-CPP3 antibody levels were found in ACPA-positive RA compared to ACPA-negative RA ($p<0.0001$), we could not detect a statistically significant difference in anti-CPP3 IgG levels between RA patients and non-RA controls, in this small cohort contrary to **Study II**. This could be explained by the high percentage of non-RA individuals having elevated anti-CPP3 IgG levels (37%) when compared to non-PD controls (0%), $p<0.001$. In a recent study, linking EIRA to the Swedish Dental

Health Registry it was estimated that 32% of EIRA non-RA controls have PD [126]. Even though we have no information with regard to periodontal status of the non-RA controls included in the present study, we hypothesize that the subset with increased anti-CPP3 IgG levels have (or have had) PD / *P.gingivalis* infection.

To investigate whether non-RA controls also had increased ACPA levels with regard to the classical ACPA response, we tested the two control populations, as well as RA- and PD patients, for reactivity against CCP2 and the CEP-1 peptide derived from human citrullinated α-enolase. Contrary to the anti-CPP3 antibody response, the frequency of anti-CCP2-positive, or anti-CEP-1-positive, non-RA controls was low (1.7% and 2.5%, respectively), and not significantly increased compared to non-PD controls (Figure 9C and D). On the other hand, the frequencies of anti-CCP2-positivity (9.5%) and anti-CEP-1-positivity (16%) were significantly higher in the PD group when compared to non-RA controls ($p=0.014$, and $p<0.001$, respectively). Our data thus support previous reports on elevated ACPA levels in PD compared to non-PD controls [155, 157]. A weakness in this study, however, is that we have no reliable data on RA diagnosis among the PD patients, only that they have reported no chronic diseases (except PD) at the time of donating the blood sample.

To further evaluate the anti-CPP3 antibody response in relation to the classical ACPA response, and RA risk factors, and clinical phenotype, we screened EIRA (2859 RA and 370 non-RA controls) on the multiplex peptide array, and we applied the same cut-off rule for anti-CPP3 positivity as for the classical ACPA fine-specificity response, i.e. the 98th percentile reactivity among non-RA controls. With this type of cut-off, 11% of RA patients were anti-CPP3 antibody positive (i.e. having "high-level" anti-CPP3 IgG), in agreement with our previous data reported in **Study II** and a report by Quirke *et al.* [162], both showing elevated anti-CPP3 antibody levels in RA patients compared to non-RA controls with unknown PD status.

Similar to the classical ACPA response directed against citrullinated epitopes on human α-enolase (CEP-1), vimentin (Cit-Vim$_{60-75}$) and fibrinogen (Cit-Fib$_{36-52}$), high-level anti-CPP3 antibodies were mainly detected in the anti-CCP2-positive RA subset, but only weakly correlated to other ACPA. Unlike most of the classical ACPA fine-specificities, high-level anti-CPP3 IgG showed no specific association with SE or *PTPN22* polymorphism (beyond the well-known association between these risk factors and anti-CCP2-positive RA). In a recent study, it was shown that high-level anti-CPP3 (as well as some other autoimmune ACPA, which also displayed independent expression patterns) instead showed an association with having aspartic acid at amino acid position 9 in *HLA-B* [243]. Moreover, presence of high-level anti-CPP3 IgG, but not anti-CCP2 IgG or the classical ACPA fine-specificities, associated with more pain and higher patient global assessment at disease onset. Collectively, these data suggest different mechanisms for the production of anti-CPP3 IgG as compared to the autoimmune ACPA response. We conclude that the anti-bacterial anti-CPP3 antibody response is not dependent on *HLA-DRB1* SE or *PTPN22*, which are linked to ACPA+ RA and autoimmunity, respectively [244, 245]. Furthermore, we suggest that the CPP3+ subset of newly diagnosed RA constitutes patients with PD, a diagnosis which may have influenced baseline pain and global assessment scores.

A significant association was identified between high-level anti-CPP3 IgG and smoking, a major risk factor not only for the development of RA [89, 246], but also for the development

of PD [133]. Smoking has been shown to correlate with increased protein citrullination in the lungs [93] and may have the same effect in the gingival tissue, by activating human PAD enzymes, thereby contributing to the recently reported increased citrullination seen in gingival tissue of PD patients [153].

Taken together, data from **Study III** suggest that a substantial proportion of the general population has a citrulline-specific antibody response against *P.gingivalis*. We hypothesise that such ACPA could cross-react with citrullinated self-proteins by mechanisms of molecular mimicry, and develop into an autoimmune ACPA response in genetically susceptible individuals.

In relation to the data presented in **Study III**, please see section **4.4 Supplementary Information to Studies III and IV**, page 41, for a detailed discussion on the stability of the synthetic CPP3 peptide, and how this may affect antibody detection.

4.4 Study IV: ACPA cross-reactivity between *P.gingivalis* and human antigens

To further address the hypothesis that *P.gingivalis* could be linked to break of immune tolerance to citrullinated proteins and ACPA production, we investigated whether citrulline-reactive B cells reside in inflamed gingival tissue (GT), and examined ACPA cross-reactivity between a citrullinated PPAD peptide and human epitopes on a monoclonal level in **Study IV.**

With an aim to generate recombinant monoclonal antibodies (mAbs) from inflamed GT, we first performed single-cell flow cytometry sorting of Live/CD3-/CD14-/CD19+ cells, isolated from four fresh GT biopsies. Flow cytometry analysis showed presence of memory and plasma cells in inflamed GT, in line with earlier reports on dense infiltrations of B and T lymphocytes within the gingival connective tissue [247, 248]. Since the direct processing of fresh biopsies was not always possible due to unplanned dental surgeries, we tested whether it would be feasible to isolate live B cells also from stored frozen material. Hence, we processed five GT biopsies that were snap-frozen in liquid nitrogen within a minute from tissue extraction, and stored at -80°C between 12 and 184 days. To our surprise, we were able to recover cells and isolate CD19+ cells from all frozen samples, although we could not see a clear lymphocyte population and the leukocyte yields were more variable and consistently lower than in fresh GT samples.

After performing single cell isolation of GT B cells, we analysed sequences of the variable regions of the B-cell receptor of one PD+ ACPA+RA patient (GT01, fresh biopsy) and one PD+ non-RA individual (GT06, frozen biopsy). In total, 148 variable heavy (VH) and corresponding variable light (VL) region sequences were generated from GT01 (n=94) and GT06 (n=54) samples, and B cell repertoire analysis revealed similar variable region characteristics, in terms of closest germline gene usage, number of somatic hypermutations (SHM), and the frequency of SHM-introduced N-glycosylation sites.

Next, we generated 64 mAbs from these two biopsies, i.e. one from the PD+ ACPA+RA patient (GT01, n=53 mAbs) and one from the PD+ non-RA individual (GT06, n=11 mAbs). In addition, 44 mAbs from bronchoalveolar lavage (BAL) CD19+ B cells (n=2 ACPA+ RA patients); 29 mAbs from bone marrow (BM) plasma cells (n=4 ACPA+ RA patients); 142 mAbs from synovial fluid (SF) plasma cells (n=6 ACPA+ RA patients); and 36 mAbs from peripheral blood (PB) memory cells (n=4 ACPA+ RA patients) were also analysed. Citrulline-reactivity was determined using the anti-CCP2 ELISA assay. All mAbs were also screened by ELISA for reactivity against a citrullinated peptide derived from PPAD (CPP3), and citrullinated peptides derived from human RA candidate autoantigens α-enolase, fibrinogen, histone 4, filaggrin and vimentin.

Eleven clones (out of the 64) were positive for CPP3, and four of them showed cross-reactivity with the arginine-containing control peptide RPP3 (Figure 10). The majority of GT-derived CPP3-positive clones showed cross-reactivity with one or more citrullinated peptides derived from human proteins, with a citrullinated (tetramer) peptide derived from human histone 4 as

the most common cross-reactivity. Notably, activated neutrophils are common in inflamed GT, and Cit-histone 4, which is thought to be released during NETosis, is targeted by the ACPA response [27].

A majority of the *P.gingivalis*-human cross-reactive clones also showed reactivity against the corresponding arginine-containing peptides, and several GT-mAbs derived from the non-RA sample displayed reactivity against filaggrin in both citrullinated- and arginine-versions. Interestingly, the first ACPA test, the so-called anti-perinuclear factor, was described as a substance in RA sera that bind human buccal mucosa, later identified as citrullinated filaggrin [16, 19]. Two clones showed reactivity against the CEP-1 peptide, derived from citrullinated α-enolase, and several of the GT-mAbs also exhibited low-level reactivity against native human fibrinogen and vimentin peptides. Our identification of citrulline-reactive B cells in GT follow the reports of increased citrullination in inflamed GT and an antibody response to citrullinated proteins in PD patients [152-154].

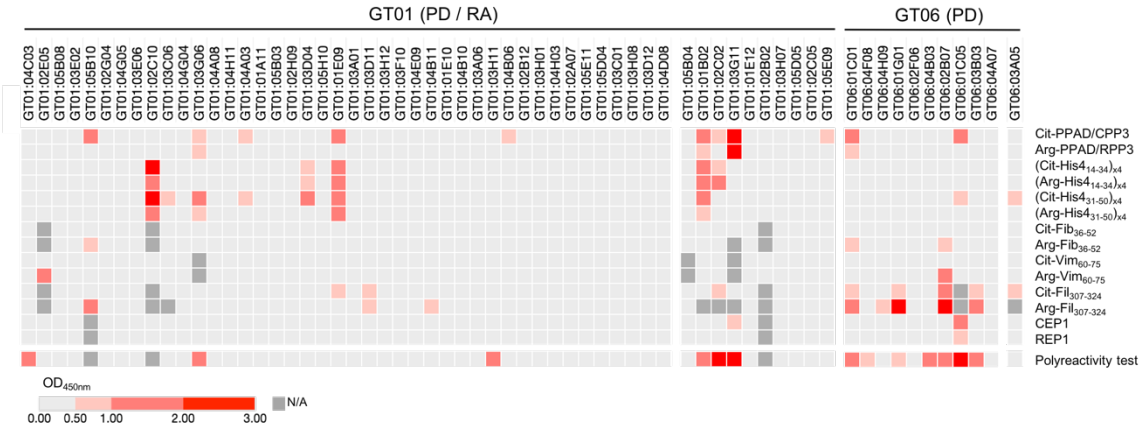

Figure 10 *Recombinant mAbs generated from GT of one PD patient with ACPA+ RA (GT01, 53 clones) and one PD patient without RA (GT06, 11 clones) exhibit distinct multi-reactive binding to citrullinated peptides. A summary of peptide ELISA results is shown as a heat map. Peptide antigens include a citrullinated peptide derived from PPAD (Cit-PPAD/CPP3) and citrullinated peptides derived from human proteins (α-enolase, fibrinogen, vimentin, filaggrin and histone 4), as well as the arginine-containing equivalents. N/A = not analyzed.*

None of the GT-derived CPP3-positive clones were polyreactive (i.e. unspecific) as measured by anti-LPS, -insulin and -dsDNA ELISAs. However, when tested in a polyreactivity ELISA based on soluble HEK293 cell membrane proteins, 13 of the GT mAbs were positive, i.e. polyreactive: 6 of the CPP3-reactive clones, and 7 of the other clones. Leaving four CPP3-reactive clones with no detected polyreactivity, of which two showed cross-reactivity with human citrullinated peptides.

Interestingly, the GT-derived polyreactive mAbs in our study were of IgG and IgA isotypes and had a high number of mutations (>15 in either Ig chain), while the more historically described polyreactive antibodies are unmutated IgM. In the spontaneously expressed natural antibody repertoire, soluble autoreactive/polyreactive IgM serve double roles in the immune system: by recognition of pathogens as a first line of defence against infections, and by recognition of autoantigens for clearance of apoptotic cells, senescent erythrocytes, and

harmful modified biomolecules to maintain homeostasis [249]. However, highly mutated low-affinity polyreactive IgG produced by memory B cells are also frequently found in normal healthy human serum, and might have different properties compared to IgM [250]. Moreover, polyreactive antibodies that are highly specific for pathogens have also been described, such as broadly neutralizing anti-HIV antibodies [251-253]. Future studies should address the role of the polyreactive B cells in PD and their potential contribution to the autoimmune ACPA response.

In addition to presence of *P.gingivalis* CPP3-reactive B cells in GT, we found B cells reactive with the Cit-PPAD peptide in blood and BAL of RA patients, as some of BAL- (n=8) and PB- (n=1) derived mAbs also bound this bacterial peptide, with some of them having weak cross-reactivity to RPP3 (n=3), in the absence of polyreactivity. Similar to the GT-derived mAbs, six of these CPP3-positive clones also cross-react with human citrullinated peptides, with histone 4 being the most common citrulline-reactivity.

Among the 13 CPP3-positive/non-polyreactive clones, we identified two clones that were also positive in the CCP2 ELISA. These clones were generated from an RA peripheral blood B cell (BVCA1) and an RA BAL B cell (L204:01A01). The two CCP2/CPP3 double positive clones reacted against several citrullinated peptides derived from human proteins, but with slightly different reactivity patterns (Figure 11A), demonstrating cross-reactivity on the monoclonal level, between a bacterial epitope, human epitopes, and the CCP2 peptide(s), used in the gold standard clinical test for RA. Peptide alignment of CPP3 with citrullinated peptides derived from human proteins (fibrinogen, vimentin, α-enolase, filaggrin and histone 4) revealed two identical amino acids adjacent to citrulline in vimentin, and one identical amino acid adjacent to citrulline in histone 4 (Figure 11B and C). Comparison of somatic mutations in the IgH and IgL variable genes of the CCP2/CPP3 double positive mAbs show substantially more mutations in the CCP2-positive clones as compared to the other CPP3-reactive monoclonal antibodies, and these double positive mAbs also displayed a higher r/s ratio as compared to the single positive mAbs, suggesting extensive autoantigen-driven activation of these B cell responses.

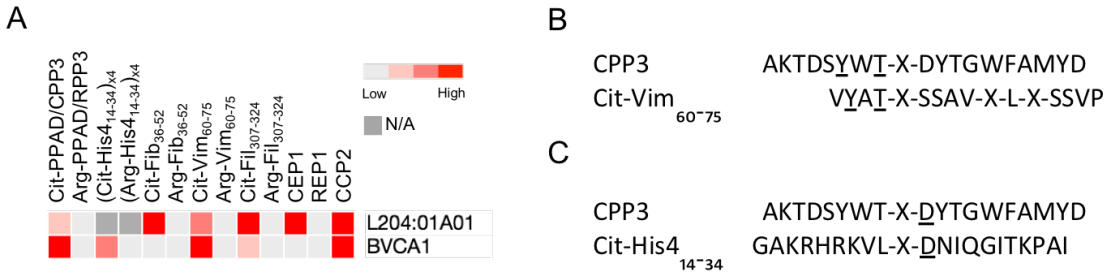

Figure 11 *Cross-reactivity of ACPA recombinant monoclonal antibodies between a citrullinated bacterial peptide, citrullinated human peptides, and CCP2.* ***A*** *A heat-map of peptide ELISA results for two CCP2+/CPP3+ clones is shown. The strength of binding is illustrated in colors; high reactivity in red, intermediate in light red and no reactivity in grey; N/A = not analyzed.* ***B*** *Peptide alignment of the citrullinated PPAD peptide (CPP3) and a citrullinated human vimentin peptide (Cit-Vim$_{60-75}$), and a citrullinated histone 4 peptide (Cit-His4$_{14-34}$)* ***(C)****. Identical amino acids are underlined. X = citrulline.*

Importantly, when converting one CPP3/CCP2 double-positive clone (BVCA1) back to the predicted germline sequence, it was no longer autoreactive (i.e. CCP2-negative), but remained *P.ginigivalis* CPP3-positive, albeit to a lower extent, suggesting that the original B cell response was not autoimmune, but directed against *P.gingivalis.* These data are supported by a separate study, where CCP2+ mAbs generated from circulating plasmablasts of ACPA+ RA patients cross-reacted with *P.gingivalis* outer membrane proteins, while when reverted back to the predicted germline sequence, reactivity in the CCP2 ELISA was lost but some reactivity to *P.gingivalis* remained [254].

In conclusion, our data from **Study IV** demonstrate the presence of citrulline-reactive B cells in gingival tissue of individuals with periodontitis. Some of these cells produce antibodies with cross-reactivity between a citrullinated *P.gingivalis* peptide (CPP3) and human citrullinated peptides, further supporting the hypothesis that a loss of tolerance to citrullinated self-proteins may be triggered in the gum mucosa during periodontitis caused by *P.gingivalis*.

4.5 SUPPLEMENTARY INFORMATION TO STUDIES III AND IV

After **Study III** was finalized, and **Study IV** was well in progress, we received a new batch of the CPP3 peptide from the company Innovagen AB, Malmö, Sweden. The purity of the peptide was stated to be >95%, like nearly all previous peptide batches we have ordered from Innovagen AB.

Our standard CPP3+ serum pool, as well as one of the CPP3-reactive monoclonal antibodies (BVCA1 from **Study IV**), showed similar to identical reactivity in ELISA, irrespective of coating with the new or the old CPP3 peptide batches (Figure 12A and B). However, when re-screening the previously CPP3-reactive monoclonal antibodies from gingival tissue and bronchoalveolar lavage (**Study IV**), they appeared negative when using the new batch of CPP3 peptide, while still positive when using the old peptide (Figure 12C).

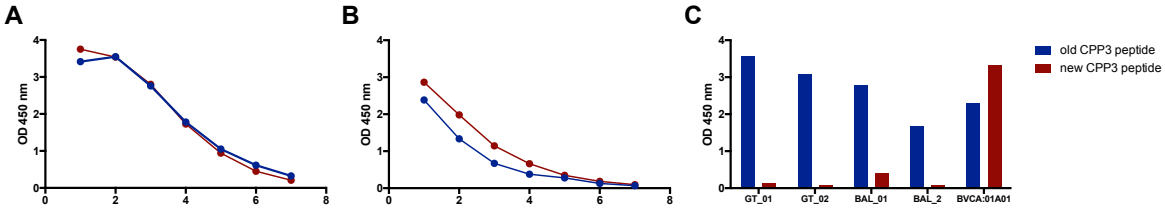

Figure 12 *The comparison of performance of two batches of CPP3 peptide (i.e "old" and "new") on ELISA. An example of standard curves based on serial dilutions of a highly CPP3-positive serum pool (A) and a CPP3-reactive monoclonal antibody (BVCA1) (B), and reactivity of five monoclonal antibodies (C). Binding to the old CPP3 peptide batch is shown in dark blue, and binding to the new CPP3 peptide batch is shown in dark orange.*

We then undertook a thorough investigation into this discrepancy. First, we re-screened the PD/non-PD serum cohort, and the ACPA+ RA sera (from **Study III**) using the CPP3 peptide from new batch, and we could no longer see elevated anti-CPP3 IgG levels in PD patients (as reported in **Study III**), but in ACPA+ RA sera, we could still see a strong anti-CPP3 antibody response (Figure 13).

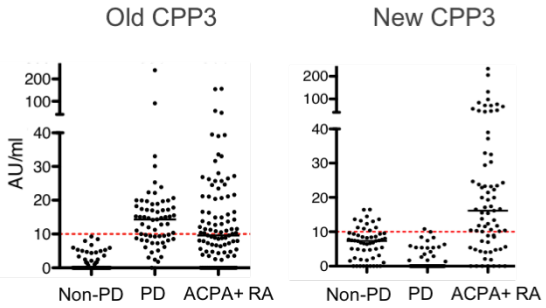

Figure 13 *CPP3 peptide ELISA results. The old and the new CPP3 peptide batches were assayed on different ELISA plates, at different time points. Non-PD = periodontally healthy; PD = periodontitis; ACPA+ RA = anti-citrullinated protein antibody positive rheumatoid arthritis; AU = arbitrary units (calculated from OD values, based on a standard curve).*

Based on these contradictory data we then analysed the old and the new peptide batches by mass spectrometry and HPLC (at Karolinska Institutet and at Innovagen AB, respectively), and these analyses found that, while the full-length peptide were detected in both batches, the old peptide batch contained more shorter fragments (Figure 14). Moreover, the new full-length peptide was primarily in a cyclic form (data not shown).

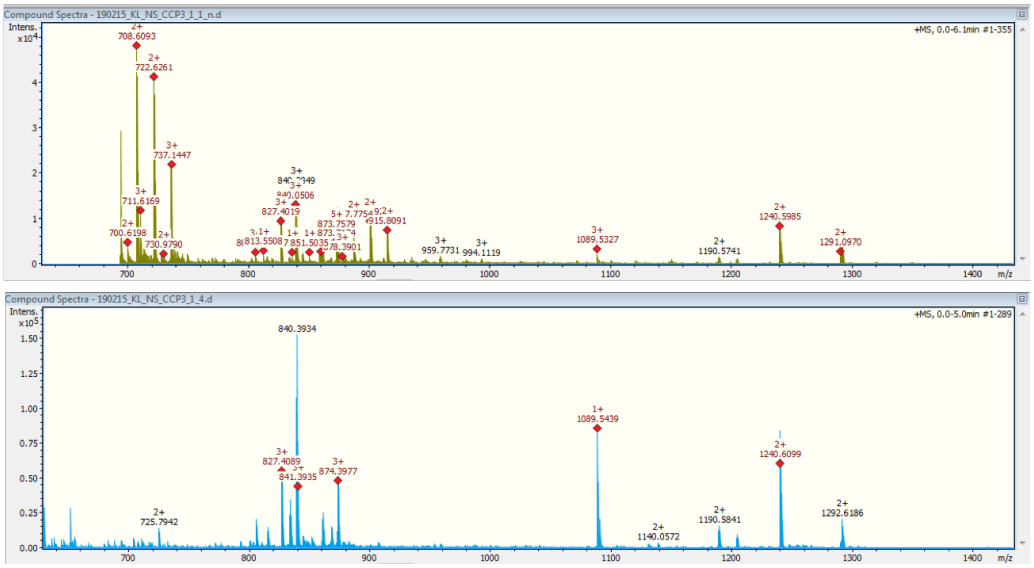

Figure 14 *Mass spectrometry analysis (analysis made by direct infusion with a positive mode of peptide solution on Bruker Q-TOF) of the old (top) and the new (bottom) CPP3 peptide batches. While both peptide batches contain the full-length sequence, the old batch contains more peptide fragments.*

Hence, we hypothesise that a cyclic peptide efficiently exposes a "citrulline motif", while the "fragmented" peptide is likely to form aggregates when coated in the ELISA plate, potentially exposing another larger/conformational, i.e. more classical, antibody epitope. Alternatively, a (primarily) cyclic peptide *versus* a fragmented (aggregated) peptide may affect the coating concentration, and/or how well the same epitope is exposed when coated in the ELISA plate.

We speculate that the monoclonal antibody with retained CPP3-reactivity (i.e. BVCA1 from **Study IV**), as well as the ACPA+ RA sera (from **Study III**), bind - with high affinity - to a short citrulline-containing motif (i.e. citrulline and a few flanking amino acids), which should be well exposed in the cyclic full-length peptide. The other CPP3-reactive monoclonal antibodies from **Study IV** and the PD sera (from **Study III**), on the other hand, either bind the same epitope but with lower affinity (and thus appear negative when coating with the new peptide, which may not coat well), or bind another – larger/conformational – epitope (which may or may not contain citrulline), which is better exposed when coating with the fragmented (aggregated) peptide, but not when coating with the (mainly) cyclic peptide.

In an attempt to break up the cyclic form of the new full-length peptide, and instead form aggregates, we have now lowered the pH of the new peptide before coating. In addition, we run the ELISA at 37°C, in order to facilitate interactions between antibody and antigen. With these modifications to the ELISA protocol, we do see increased binding also to the new CPP3

peptide, and approximately half of the (previously CPP3-positive) GT- and BAL-derived mAbs becomes CPP3-positive again.

All CPP3-data presented in **Study IV** are based on the new CPP3 and RPP3 peptide using the modified ELISA protocol. However, for **Study III** we have not been able to re-run the PD non-PD samples using the new CPP3 peptide batch and the modified ELISA protocol. Hence, at present we do not know whether the anti-CPP3 antibody response seen in PD patients (**Study III**) is true. We also do not know whether this antibody response is citrulline-specific in PD patients, since we have not yet investigated the arginine-containing control peptide RPP3 by mass spectrometry, so we do not know if this peptide is cyclic or fragmented, which seem to affect antibody binding.

We now plan to identify and screen another PD/non-PD cohort, using the new CPP3 peptide, along with the new RPP3 peptide, using the old and modified ELISA protocol, to confirm the serological data (concerning PD/non-PD sera) presented in **Study III**. We also consider ordering a new batch of CPP3/RPP3 peptides from another company.

5 CONCLUSIONS AND FUTURE PERSPECTIVES

Rheumatoid arthritis is a multifactorial disease with an incomplete understanding of etiological triggers and pathways. Both genes and environment are known to play a role, with susceptibility factors such as *HLA-DRB1* SE alleles, *PTPN22* polymorphism and smoking identified primarily for ACPA-positive RA. Many different cell types and inflammatory mediators are thought to be involved in the initiation and progression of RA. However, we do not fully understand the mechanisms. Based on an epidemiological link between chronic periodontitis and RA, and the unique ability of the periodontitis-associated oral pathogen *Porphyromonas gingivalis* to express a citrullinating enzyme, this PhD thesis has addressed the potential causative role of *Porphyromonas gingivalis* in the development of ACPA-positive RA. In summary, my studies reveal that:

- Anti-*P.gingivalis* antibody levels are not only significantly elevated in PD patients compared to periodontally-healthy individuals, but in RA patients (in particular ACPA+ RA) compared to non-RA controls.
- Anti-*P.gingivalis* antibodies antedate clinical onset of RA by more than 10 years.
- Associations between anti-*P.gingivalis* antibodies and RA are independent of *HLA-DRB1* SE, *PTPN22* polymorphism, and smoking. Still, interactions between anti-RgpB antibodies and both *HLA-DRB1* SE alleles and smoking was observed in ACPA+ RA, and a significantly stronger association was found between smoking and CCP3+/CCP2+ RA, compared to CPP3-/CCP2+ RA.
- Patients with periodontitis, i.e. a substantial proportion of the general population, seem to have a citrulline-specific antibody response against *P.gingivalis*.
- Citrulline-reactive B cells are present in inflamed gingival tissue, and produce antibodies with cross-reactivity between a citrullinated PPAD peptide (CPP3) and human epitopes.

Taken together, these studies support the hypothesis that a loss of tolerance to citrullinated self-proteins may be triggered in the gum mucosa during chronic periodontitis caused by *P.gingivalis.* However, in order to establish a causative role for *P.gingivalis* in the development of ACPA-positive RA, further research is warranted, and may include molecular, clinical and epidemiological studies, such as:

- Mechanistic studies of gingival tissue-derived citrulline-reactive monoclonal antibodies, focused on their potential pathogenic effect on monocytes/macrophages, neutrophils, osteoclasts and fibroblasts, and their ability to induce experimental arthritis.
- Further characterization of the germline versions of ACPA monoclonal antibodies generated from RA blood and tissues, in terms of reactivity against *P.gingivalis* and citrullinated epitopes; and studies confirming binding of CPP3-reactive mAbs to autocitrullinated PPAD protein.
- Detailed investigations of the oral health and the oral microbiota, with special focus on *P.gingivalis*, in newly diagnosed treatment-naïve RA patients and in individuals at risk of developing RA.

- Linkage of patient registers, where periodontal status (prior to the RA diagnosis) will be identified in the Swedish Dental Health Registry, and RA diagnosis will be captured in EIRA and/or the Swedish Rheumatology Quality Registry.
- Longitudial analysis of the effects of periodontal treatment on RA disease activity and on development of RA in at risk individuals.
- Qualitative studies to obtain a deeper understanding of how RA patients and individuals at risk perceive their oral health, in order to identify and implement strategies for preventive care and smoking cessation.
- Investigations of the anti-CPP3 IgG response as a potential marker for the identification of individuals with PD at an increased risk of developing ACPA+ RA.

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