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

INVESTIGATIONS OF PORPHYROMONAS GINGIVALIS AS A POSSIBLE TRIGGER OF AUTOIMMUNITY IN THE DEVELOPMENT OF RHEUMATOID ARTHRITIS

Nastya Kharlamova

Karolinska Institutet

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Investigations of *Porphyromonas gingivalis* as a Possible Trigger of Autoimmunity in the Development of Rheumatoid Arthritis

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Nastya Kharlamova

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

Co-supervisor(s):
Professor Lars Klareskog
Karolinska Institutet
Department of Medicine, Solna
Rheumatology Unit

Associate Professor Dzmitry Charnashei
Belarusian State Medical University
Department of Microbiology and Immunology

Opponent:
MD, MPH, PhD Paola de Pablo
University of Birmingham
Department of Rheumatology
Institute of Inflammation and Ageing

Examination Board:
Associate Professor Anna Fogdell-Hahn
Karolinska Institutet
Department of Clinical Neuroscience

Dr Shauna Culshaw
Oxford University
Institute of Infection Immunity and Inflammation

Associate Professor Rille Pullerits
University of Gothenburg
Department of Rheumatology and Inflammation

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Science is magic that works.
Anything can make me stop and look and wonder, and sometimes learn.

Kurt Vonnegut

To my mom, who made it all possible

And my father who is always in my heart

ABSTRACT

Rheumatoid Arthritis (RA) is a severe autoimmune disease affecting 0.5-1% worldwide. Patients suffer from pain, disability, chronic joint inflammation, comorbidities and increased mortality. Although, the aetiology of RA is largely unknown, both genes and environment have been shown to contribute. The major risk factors of RA, smoking and *HLA-DRB1* shared epitope (SE) alleles, associate primarily with the presence of autoantibodies to citrullinated proteins (ACPA). These antibodies are present in two thirds of patients, precede clinical symptoms, and predict a more destructive disease course. However, the exact trigger of ACPA production remains unclear. In addition to smoking, periodontitis (PD) has been epidemiologically linked to RA. Both smoking and PD cause local inflammation and increased protein citrullination. Moreover, *Porphyromonas gingivalis* (*P.gin*) - the keystone pathogen in chronic PD - is the only known prokaryote to express an enzyme that can citrullinate polypeptides (peptidyl arginine deiminase, denoted *P.*PAD). With this unique property, it was suggested that *P.*PAD could generate citrullinated epitopes in the inflamed periodontium, which would trigger a systemic ACPA response that eventually cause intraarticular inflammation. Based on this hypothesis, the overall aim of my PhD project was to investigate the role of *Porphyromonas gingivalis* in the aetiology of ACPA-positive RA, in terms of ACPA production, association with classical risk factors, and disease progression.

Most studies in this thesis were performed in the population-based RA case-control cohort EIRA, where serum samples and information on genes (i.e. SE and *PTPN22* polymorphism) and smoking history were available. In **Study I**, we showed that classical RA risk factors associated with specific ACPAs rather than the magnitude of the ACPA response, suggesting that the production of different ACPA fine-specificities is governed by partly different mechanisms. In **Study II**, we identified an association between anti-*P.gin* antibodies and RA (in particular ACPA-positive RA) that was even stronger than the association between smoking and RA. Moreover, we observed interactions between anti-*P.gin* antibodies and both SE and smoking in ACPA-positive RA. With **Study III**, we could demonstrate that anti-*P.gin* antibodies pre-dated clinical RA with up to 12 years. **Study IV** revealed a citrulline-specific antibody response to a *P.*PAD epitope in non-RA PD patients. Moreover, we identified a monoclonal antibody derived from RA blood, which exhibited cross-reactivity between citrullinated epitopes on bacterial (*P.*PAD) and human (vimentin) proteins.

The data presented in this PhD thesis support a role for *P.gin* in the development of ACPA-positive RA, and we propose that the pathway involves citrullination by *P.*PAD, followed by an antibody response, which cross-reacts with citrullinated human proteins, and that expansion of the autoimmune ACPA response in genetically susceptible individuals eventually triggers RA. It is my hope that the data presented herein can serve as a basis for disease-preventive strategies, as well as more detailed studies of disease mechanisms in RA aetiopathogenesis, ultimately aimed at the development of curative therapies.

LIST OF SCIENTIFIC PAPERS

I. **Genetic and environmental determinants for disease risk in subsets of rheumatoid arthritis defined by the anti-citrullinated protein/peptide antibody fine-specificity profile**
Lundberg K, Bengtsson C, **Kharlamova N**, Reed E, Jiang X, Kallberg H, Pollak-Dorocic I, Israelsson L, Kessel C, Padyukov L, Holmdahl R, Alfredsson L, Klareskog L.
Ann Rheum Dis. 2013 May; 72 (5): 652-8

II. **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

III. **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

IV. **Characterisation of the antibody response to a citrullinated peptide derived from Porphyromonas gingivalis PAD in RA**
Kharlamova N, Brynedal B, Jiang X, Sherina N, Eriksson K, Yücel-Lindberg T, Hansson M, Israelsson L, Steen J, Malmström V, Alfredsson L, Amara K, Lundberg K
Manuscript

SCIENTIFIC PAPERS NOT INCLUDED IN THE THESIS

V. **Affinity purified anti-citrullinated protein/peptide antibodies target antigens expressed in the rheumatoid joint**
Ossipova E, Cerqueira CF, Reed E, **Kharlamova N**, Israelsson L, Holmdahl R, Nandakumar KS, Engström M, Harre U, Schett G, Catrina AI, Malmström V, Sommarin Y, Klareskog L, Jakobsson PJ, Lundberg K.
Arthritis Res Ther. 2014 Aug 12;16(4):R167.

VI. **Antibodies to carbamylated α-enolase epitopes in rheumatoid arthritis also bind citrullinated epitopes and are largely indistinct from anti-citrullinated protein antibodies**
Reed E, Jiang X, **Kharlamova N**, Ytterberg AJ, Catrina AI, Israelsson L, Mathsson-Alm L, Hansson M, Alfredsson L, Rönnelid J, Lundberg K.
Arthritis Res Ther. 2016 May 4;18(1):96

VII. **Effects by periodontitis on pristane-induced arthritis in rats**
Eriksson K, Lönnblom E, Tour G, Kats A, Mydel P, Georgsson P, Hultgren C, **Kharlamova N**, Norin U, Jönsson J, Lundmark A, Hellvard A, Lundberg K, Jansson L, Holmdahl R, Yucel-Lindberg T.
J.Trans Med 2016: 14:311

CONTENTS

LIST OF ABBREVIATIONS

Aa	*Aggregatibacter actinimycetemcomitans*
ACPA	Anti-citrullinated protein antibodies
ACR	American College of Rheumatology
AMPA	Anti-modified protein antibodies
AU	Arbitary unit
CII	Collage type II
CCP	Cyclic citrullinated peptide
CCP2	Cyclic citrullinated peptide, second generation
CEP-1	Citrullinated alpha-enolase peptide-1
CI	Confidence interval
Cit-fib	Citrullinated fibrinogen
Cit-vim	Citrullinated vimentin
CMV	Cytomegalovirus
CNS	Central nervous system
CRP	C-reacive protein
CPP3	Citrullinated P.gin PAD
CXCL	Chemokine CXC motif ligand
DAS 28	Disease activity score for 28 joints
EBV	Epstein-Barr virus
EIRA	Epidemiological Investigation of RA
ELISA	Enzyme-linked immunosorbent assay
EULAR	European League Against Rheumatism
FT	Flowthrough
HLA	Human leukocyte antigen
Ig	Immunoglobulin
IL	Interleukin
LPS	Lipopolysaccharide
MHC	Major histocompatibiloty complex

MCV	Mutated citrullinated form of vimentin
OD	Optical density
OR	Odds ratio
NETs	Neutrophil extracellular traps
NSHDS	Northern Sweden Health and Disease Study
PAD	Peptidyl arginine deiminase
PD	Periodontitis
P.gin	*Porphyromonas gingivalis*
P.PAD	*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
SE	Shared epitope
SF	Synovial fluid
TNF	Tumor necrosis factor

1 INTRODUCTION

1.1 RHEUMATOID ARTHRITIS

Rheumatoid arthritis (RA) is a heterogeneous chronic inflammatory autoimmune disease, affecting 0.5-1% of the population, with a female to male ratio of 3:1. The disease is characterized by synovial inflammation, so called synovitis, and the subsequent formation of the pannus, a thick cellular layer on the joint surface. The leukocyte infiltrate includes granulocytes, monocytes/macrophages, B cells, T cells ($CD4^+$ and $CD8^{+)}$), mast cells, and NK cells, which produce large amounts of proinflammatory cytokines, chemokines, and degrading enzymes [1, 2].

Chronic joint inflammation causes cartilage and bone destruction, dysfunction of diarthrodial joints, pain and disability. Joint pain is one of the dominant symptoms of RA and often develops before joint inflammation and clinical symptoms of arthritis [3]. During disease progression, other organs may also become affected, and as a consequence, systemic cardiovascular, pulmonary, and skeletal complications frequently present [2].

1.1.1 Classification criteria of RA

Until 2010, the American College of Rheumatology (ACR) 1987 revised classification criteria of RA [4] were used for diagnosing patients, and in clinical trials and for treatment recommendations. However, this set of criteria has been criticized for the lack of sensitivity in early disease. Hence, to overcome this limitation, the ACR and the European League Against Rheumatism (EULAR) created and published new classification criteria, aimed at achieving earlier diagnosis and treatment (Table 1) [5].

Table 1. The 2010 ACR/EULAR classification criteria for RA

Criteria	Points
Joint involvement	
Large joint	0
2-10 large joints	1
1-3 small joints (with or without involvement of large joints)	2
4-10 small joints (with or without involvement of large joints)	3
More than 10 joints (at least one small joint)	4
Serology (at least one test result is required)	
RF negative and CCP negative	0
Low titer of RF and low titer of anti-CCP	2
High titer of RF and high titer of anti-CCP	3
Acute-phase reactants (at least one test result is required)	
Normal CRP and normal ESR	0
Abnormal CRP and abnormal ESR	1
Duration of symptoms	
Less than 6 weeks	0
6 (or more) weeks	1
Requirement for using the criteria: Patient should have at least one swollen joint (synovitis) that is not better explained by another disease. To classify for rheumatoid arthritis: Add all the applicable points from each subgroup. A score of 6 (or more) is required for classification as definite RA.	

Table adapted from Aletaha et al. 2010 Rheumatoid arthritis classification criteria: an American College of Rheumatology/European League Against Rheumatism collaborative initiative. Arthritis Rheum 62(9): 2569-2581.

1.2 AUTOANTIBODIES IN RA

Rheumatoid arthritis is known as an autoimmune disease due to the presence of autoantibodies. The most frequent and the most studied RA-related autoantibodies are the rheumatoid factor (RF) and the anti-citrullinated protein antibodies (ACPA). More recently, a variety of anti-modified protein antibodies (AMPA) have been described in RA, and there is data showing that more than 50% of patients have a spectrum of AMPAs directed against post-translational modifications (PTMs), including citrullination, acetylation, and carbamylation [6].

1.2.1 Rheumatoid factor

Historically, rheumatoid factor (RF) was described as the main serological marker in RA, and it was the only serological marker included in the 1987 revised classification criteria [5]. Rheumatoid factor is an antibody (mainly IgM, but also IgG and IgA are present) reactive with the Fc portion of IgG. Although RF is detected in approximately 70% of RA patients, the presence of RF is not specific for RA as these autoantibodies are also present in a variety of other diseases and in 5% of the general population. Some studies suggest a synergistic role for RF and ACPA in initiating RA-associated inflammation. The presence of both IgM RF and ACPA IgG was shown to associate with significantly increased disease activity score for 28 joints (DAS28) as well as with increased levels of inflammatory markers, suggesting that interaction between IgM RF and ACPA IgG may directly contribute to the pathogenesis of RA [7].

1.2.2 ACPA

Anti-citrullinated protein antibodies are the most disease-specific autoantibodies in RA, with a specificity of around 98% and a sensitivity of 60-70% [6, 8-10]. ACPA can be detected more than 10 years prior to RA manifestations and are strongly associated with genetic and environmental risk factors for RA [11, 12]. The presence of these autoantibodies also predicts a more severe, erosive and destructive disease process, suggesting a pathogenic involvement [13]. Moreover, recent studies show that ACPAs can induce osteoclast activation and bone resorption *in vitro* and in experimental animal models, and that this effect is mediated by IL-8 [14, 15].

There is also experimental evidence suggesting that ACPAs can induce joint pain. Mice injected with either human or murinized ACPAs developed pain-like behavior; ACPAs bound osteoclasts in the bone marrow and induced CXCL1/2 expression in the joints. CXCL1 is a nociceptive chemokine, an analog to human IL-8, which activates nociceptive nerve signaling and pain [16].

1.3 PROTEIN CITRULLINATION

Citrullination, also known as deimination, is the post-translational conversion of positively charged peptidylarginine to neutral peptidylcitrulline. This is an enzymatic process driven by a family of calcium-dependent peptidyl arginine deiminases (PADs), (Figure 1) [17]. Citrullination can affect the three-dimensional structure of a protein and its solubility, which is crucial in generating new structural proteins, but in the context of RA it could also result in the generation of neo-epitopes, and the subsequent breach of immunological tolerance.

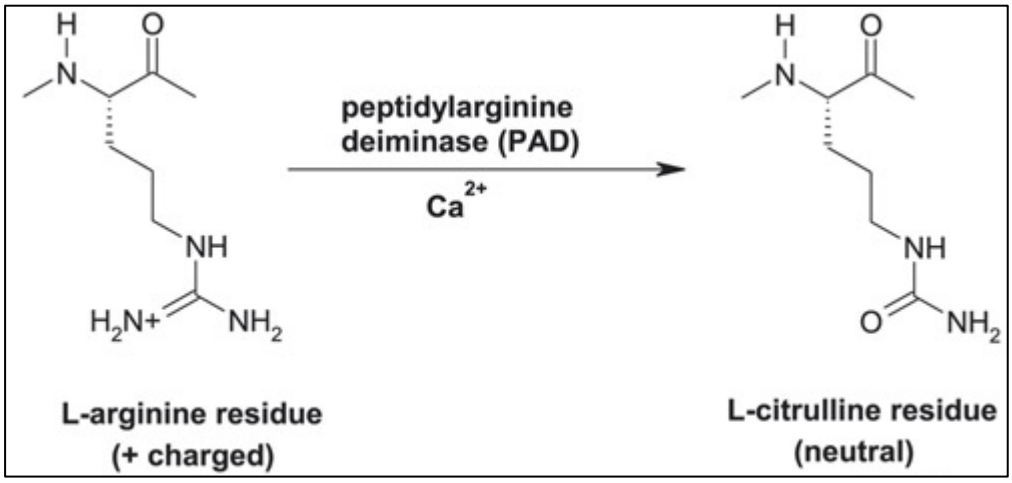

Figure 1 Schematic illustration of the peptidyl-arginine deamination process by PAD. *Figure from van Boekel et al, Arthritis Res. 2002;4(2):87-93.*

Citrullination has an important role in physiological processes and occurs naturally: [I)] in the skin, citrullination plays a role in keratinization and cornification, by deimination of pro-filagrin and keratin; [II)] in the central nervous system (CNS), citrullination is important for plasticity and insulation of neuronal axons, by citrullination of myelin basic protein; and [III)] through chromatin remodeling, citrullination participates in gene regulation. Deimination is also part of the innate immune system's response to bacterial infection, through hypercitrullination of histones, which is important for the generation of neutrophil extracellular traps (NETs) during NETosis. In addition, increased protein citrullination has been linked to chronic inflammation in various tissues. So, citrullination is not restricted to RA, and it has been suggested to play a pathogenic role in several diseases, including multiple sclerosis, Alzheimer's disease, psoriasis, glaucoma, neuropathy, and myositis [18].

1.3.1 PAD enzymes

Protein citrullination is catalysed by PAD enzymes, and since PAD enzymes require high concentrations of Ca^{2+}, deimination is more likely to occur in association with cell death (e.g. apoptosis, necrosis and terminal differentiation of cells), when the membrane integrity is lost resulting in Ca^{2+} influx, and in condition such as chemokine receptor ligation, when there is mobilization of free intracellular calcium [17, 19].

Five PAD enzymes (PAD1, 2, 3, 4 and 6) have been characterized in humans. All of them are encoded by a single gene cluster on chromosome 1p35-36, but each PAD isotype has a different tissue distribution [20]. PAD1 and PAD3 are mainly expressed in the epidermis and in hair follicles, and are primarily cytoplasmic enzymes. PAD1 citrullinates keratin K1 while PAD3 targets filaggrin and the structural protein of the hair follicles, trichohylagrin [21]. PAD2 is detected in multiple tissues including skeletal muscle, secretory glands, brain, and hematopoietic cells [22]. PAD2 is primarily known as a cytoplasmic protein, but has also been detected in the nucleus. Vimentin in skeletal muscles and macrophages, myelin basic protein in the CNS, as well as α- and β-actins, are all recognized as PAD2 substrates. Moreover, histones H3 and H4 can also be citrullinated by nuclear PAD2, suggesting a role for PAD2 in gene regulation. PAD4 is expressed mainly in hematopoietic cells under normal physiological conditions. However, overexpression of PAD4 was found in a wide range of tumors implying that PAD4 plays a role in tumorigenesis [23, 24]. PAD4 resides mainly within the nucleus and is the only PAD enzyme with a nuclear localization signal sequence. PAD4 plays a crucial role in nuclear function by targeting nuclear proteins including histones H2A, H3 and H4, ING4, p300/CBP, and nucleoplasmin [25, 26]. PAD6 in humans is mainly restricted to ovary, testis and white blood cells, and regulates oocyte cytoskeletal sheet formation and female fertility. Due to the loss of the conserved Ca^{2+} binding residues, it has been suggested that PAD6 is not an active enzyme [27, 28].

In the context of RA pathogenesis, PAD2 and PAD4 have been recognized as key players. Both enzymes are found within the inflamed rheumatoid joint, in synovial fluid as well as in the synovial membrane [22, 29, 30]. Since the concentration of Ca^{2+} in the extracellular space is at millimolar levels, PAD released from dying cells during apoptosis, necrosis or NETosis could become activated and citrullinate extracellular joint proteins [31]. This makes PAD2, and PAD4, the strongest candidates for generating the citrullinated antigens, which are targeted by the ACPA response. Furthermore, several reports have demonstrated that PAD4 itself is a target of autoantibodies (anti-PAD4) in a subgroup of RA patients, and that anti-PAD4 autoantibodies associate with erosive RA [32, 33], suggesting that anti-PAD4 autoantibodies may be useful as a severity biomarker in RA. It has also been shown that RA patients remain positive for anti-PAD4 antibodies over time and that some patients seroconvert from anti-PAD4 negative to anti-PAD4 positive during disease progression. Anti-PAD4 antibodies did not affect the enzymatic activity of PAD4 when the small substrate N-α-benzoyl-L-arginine ethyl ester was used. However, this

finding may not exclude an *in vivo* effect on protein citrullination in RA. Human genetic studies also support the involvement of PAD4 in RA [34].

Taken together, these findings support the idea to use PAD enzymes as novel targets for drug development. Indeed, it has been shown that in the context of tumorigenesis, inhibition of PAD4 by small molecules can turn on the expression of the tumor suppressor genes in cancer cells [20]. It has also been demonstrated that PAD4 inhibition alone is sufficient to block the development of murine arthritis [35], and in a PAD2 knockout model, arthritis was ameliorated [36], suggesting that PAD inhibitors might be a promising strategy in future RA therapy.

1.3.2 Citrullinated autoantigens in RA

Despite the fact that citrullination is not restricted to RA, the formation of autoantibodies to citrullinated proteins, is highly specific for RA. The main clinical test used today for the detection of ACPA is the CCP2 ELISA assay, which is based on synthetic cyclic citrullinated peptides. These peptides are patent-protected, and were originally identified from a phage display library, after screening hundreds of peptides for reactivity with RA sera and healthy control sera. The peptides with the highest sensitivity, combined with the highest specificity, were selected and included in the CCP2 test [37]. Importantly, the CCP2 peptides do not correspond to any known human protein sequence; hence give no information with regard to the driving autoantigen(s) in RA. A lot of effort has therefore been put into the identification of the "true" *in vivo* targets of the ACPA response, and a number of candidate antigens have been put forward, including fibrinogen, vimentin, α-enolase, collagen type II (CII), and more recently histones [38-41].

Alpha-enolase

Alpha-enolase is a glycolytic enzyme expressed as a homo-dimer in most tissues. It is a multifunctional protein primarily present in the cytosol, although it is also found in the nucleus where it functions as a tumor suppressor, and on the cell surface where it serves as a plasminogen-binding receptor, which is upregulated during inflammation. Alpha-enolase can bind plasminogen not only on the surface of eukaryotic cells but also on the cell surface of bacteria, fungi and parasites [42-44]. Citrullinated α-enolase was first identified as a candidate autoantigen in RA after blotting PAD-treated monocytic HL60 cells with RA sera, followed by mass spectrometry analysis of the reactive 47kD protein band [45]. Approximately 40% of patients with RA have antibodies to the immunodominant epitope citrullinated α-enolase peptide-1 (CEP-1), which corresponds to amino acids 5-21 of the full-length protein [46]. Alpha-enolase, including the citrullinated form, is abundantly expressed in synovial fluid and synovial tissue, in correlation with joint inflammation [47]. A bacterial version of enolase exists, with high sequence homology to human α-enolase, and anti-CEP-1 antibodies purified from RA sera cross-react with bacterial enolase [46]. Based on this observation, it was hypothesized that molecular mimicry could be involved in the development of ACPA-positive RA [48].

Fibrinogen

Fibrinogen is a hexameric plasma glycoprotein containing pairs of α, β and γ chains. As a precursor of fibrin, fibrinogen plays a central role in coagulation. Deposition of fibrin in the rheumatoid joints is found in patients with early arthritis, and there is evidence suggesting that this deposition may trigger pannus formation [49]. Fibrinogen, including the citrullinated form, is highly expressed in inflamed synovia and synovial fluid of patient with RA, and citrullinated fibrinogen (Cit-fib) is one of the most recognized autoantigens in RA, with anti-Cit-fib antibodies detected in approximately 50-60% of patients [50, 51]. Studies have shown that ACPA - Cit-fib immune complexes can activate macrophage cytokine production by binding to Fcγ receptor IIa and TLR4 [7, 52, 53].

Vimentin

Vimentin is an intermediate filament protein abundantly expressed in the cytoskeleton of eukaryotic cells. Vimentin plays an essential role in organelle transport, cell migration, and proliferation. Cell-surface expression and secretion of vimentin from macrophages have been shown to be enhanced in response to bacterial infection, and some bacteria use extracellular vimentin as an attachment receptor [54]. Citrullinated vimentin (Cit-vim) is present in the inflamed joint and antibodies to a mutated citrullinated form of vimentin (MCV) are found in approximately 65% of RA patients [55]. Based on these findings, a commercial ELISA test, the MCV assay, was developed, with similar sensitivity and specificity as the CCP2 ELISA [56]. Anti-Cit-vim antibodies have been shown to induce osteoclastogenesis and to play a role in apoptosis and inflammation [14].

Collagen type II

Collagen type II (CII) is a major component of articular cartilage, and immunization with CII cause arthritis in mice and rats [57], making CII an attractive autoantigen in RA. Antibodies to the native form of CII can be detected in 15-25% of RA patients. However, these antibodies are also found in other inflammatory and autoimmune diseases [58]. Citrullination of CII has been shown to enhance arthritis in an experimental rat model [59]. Moreover, an immunodominant citrullinated epitope on CII, the Cit-C1 epitope, have been described as an antibody target in approximately 40% of RA patients [60], and anti-Cit-C1 antibodies also mediate arthritis in mice [61]. In addition to Cit-C1, often Cit-CII epitopes have been described and affinity purified ACPA against them could bind RA cartilage, suggesting that the anti-Cit-CII antibody response contributes to cartilage degradation [62].

Histones

Histones are nuclear proteins, which enable package of DNA into tight bundles, and in response to posttranslational modifications, such as phosphorylation, acetylation, methylation and citrullination, histones can regulate gene transcription. Neutrophils infiltrating the joints contribute to inflammation through the release of reactive oxygen

species (ROS), as well as the release of NETs during NETosis. Core histones are the most abundant proteins in NETs [63], and several reports indicate that hypercitrullinated histones in NETs (i.e. H2A, H2B, H3 and H4) are targets of the ACPA response [38, 64]. High levels of Cit-H2B and immune complexes containing Cit-H2B have been detected in the synovial fluid of RA patients, and Cit-H2B has also been shown to have an arthritogenic potential in a mouse model of arthritis [65].

1.4 GENETIC RISK FACTORS FOR RA

1.4.1 *HLA-DRB1* SE

The connection between genetic susceptibility and RA has been studied intensively during the past 40 years. Since then many breakthroughs have been made. The major susceptibility alleles for RA are present in *HLA-DR4*, *-DR1*, and *-DR10*, principally *DRB1*0101, *0102, *0401, *0404, *0405, *0408, *1001,* and **1402*. These *HLA-DR* alleles have been described as the "Shared Epitope" (SE), as they encode a similar amino acid sequence, the shared epitope sequence, within the peptide-binding groove of the beta chain of the *HLA-DR* molecule [66, 67]. Interestingly, the association between *HLA*-SE and RA is restricted to ACPA-positive RA [11], and it has been shown that the positively charged P4 pocket in *HLA-DR* favors binding of peptides containing neutral citrulline, rather than positively charged arginine [8]. Hence, the transformation of arginine to citrulline by PAD enzymes could promote *HLA*-SE binding and antigen presentation to T cells, (Figure 2).

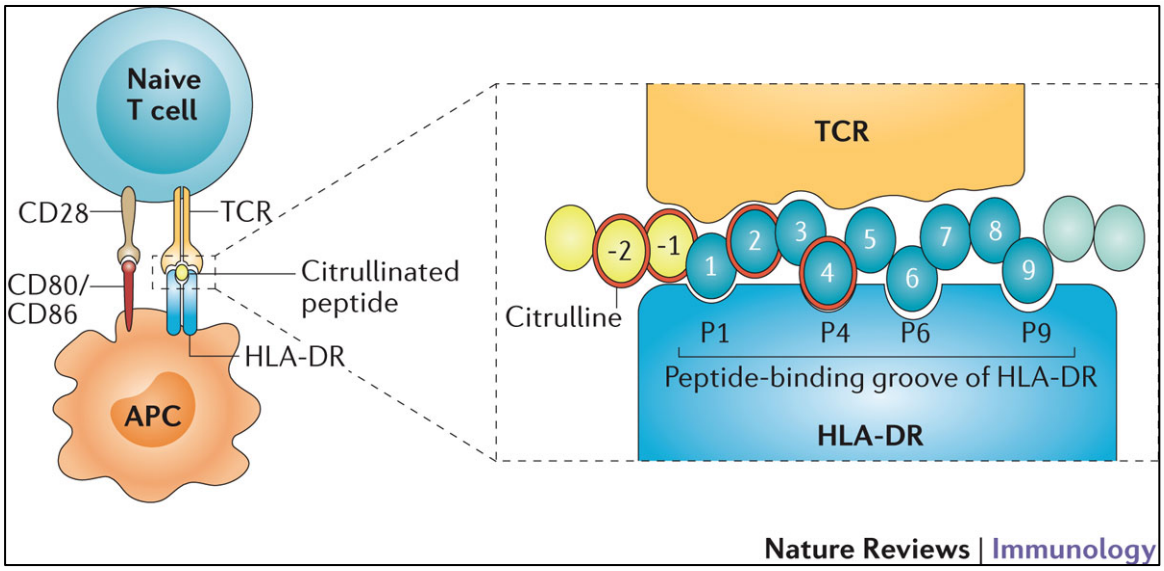

Figure 2 Schematic illustration of the interaction between the citrullinated peptide-antigen - MHC complex on an antigen-presenting cell (APC), and the T cell receptor (TCR) on a naïve T cell. Neutral citrullinated peptide residues (red rings) bind the positively charged P4 pocket of *HLA-DRB1* SE molecules, or interact directly with the TCR. *Figure from Malmström et al, Nat Rev Immunol. 2017 Jan;17(1):60-75.*

1.4.2 PTPN22 polymorphism

Another genetic risk factor described in RA is the protein tyrosine phosphatase non receptor type 22 (*PTPN22*) 1858C/T polymorphism. *PTPN22* encodes the protein tyrosine phosphatase LYP that is involved in T- and B-cell signaling. The presence of the risk allele has been shown to influence activation of autoreactive T cells and reduce negative selection of B cells. This results in a more autoimmune-prone repertoire of B- and T cells [8, 68-70]. Like *HLA*-SE, the *PTPN22* risk allele has been reported to be related mainly to ACPA-positive RA, and *PTPN22* shows a strong gene-gene interaction with *HLA*-SE in the development of ACPA-positive RA [71].

1.4.3 Other risk genes

Genome-wide association studies in several large cohorts describe additional gene loci such as TRAF1-C5, STAT4, REL, TNFAIP3, CTLA, and CD40 in RA pathogenesis [72]. Most of these have been associated specifically with ACPA-positive disease [40, 73]. Another genetic factor associated with ACPA-positive RA is polymorphism of the PADI4 gene [34]. This association seems to be stronger in Japanese and Korean population, compared to Western Europeans.

1.5 ENVIRONMENTAL RISK FACTORS FOR RA

In addition to genetic susceptibility, environmental factors and life style contribute to the development of RA. Smoking, silica- and textile dust, air pollution, viruses and other microbial exposures, as well as hormones have all been described as risk factors for RA [40].

1.5.1 Smoking

The best-known environmental risk factor for the development of RA is cigarette smoking. The association between smoking and RA was first found in 1987 [74], and later confirmed in a number of studies, including twin studies [75]. Cigarette smoking contributes more to RF-positive [76] and ACPA-positive RA [77]. Moreover, there is a strong interaction between smoking and *HLA-DRB1* SE alleles in ACPA-positive RA [11, 78, 79]. It is well known that cigarette smoke contains a lot of toxic compounds that may cause tissue damage and inflammation. Cigarette smoke increases recruitment and activation of immune cells in the lungs, and elevated serum levels of matrix metalloproteinases, CRP, IL-6, IL-1β, TNF-α, and fibrinogen, as well as increased expression of Fas on T and B cells [40]. Nicotine, the most abundant component of cigarette smoke, is probably not responsible for the association between cigarette smoking and risk of RA. In some studies, nicotine has even been demonstrated to have protective effects [77, 80]. Interestingly, increased expression of PAD2 and citrullinated proteins

have been described in the lungs of smokers compare to non-smokers [11, 81], and the presence anti-CCP IgA, as well as RF IgA, is associated with smoking in RA patients [82, 83].

1.5.2 Microbial exposure: viruses and bacteria

An attractive hypothesis for the development of RA, as well as other autoimmune diseases, is that the disease is triggered by an infection. An immune response against pathogens could cause tissue destruction and cell death, which could lead to the release and exposure of intracellular and/or nuclear proteins to the immune system. In an inflammatory environment, and in the presence of danger molecules, this may cause break of immune tolerance to self-proteins. Alternatively, an immune response against viral or bacterial antigens may target epitopes on human proteins by mechanisms of molecular mimicry. Historically, a number of pathogens, both viruses and bacteria, have been linked to RA. However, data from different groups have been contradictory.

Viruses

The association between infection by viruses and the development of RA has been a long lasting discussion. The link between RA and a virus was first described for Epstein-Barr virus (EBV). It was shown that patients with RA compared to controls have increased antibody levels against EBV [84, 85]. Later on, the involvement of other viral infections such as human parvovirus B19 (B19) [86] and cytomegalovirus (CMV) [87] was described in the pathogenesis of RA. However, studies investigating the link between viral infections and RA are contradictory. Some serological studies have demonstrated higher antibody levels against mentioned viruses in RA compared to controls, while others were not able to show the same results [88]. Moreover, some studies reported the detection of viral DNA in the synovium and bone marrow of RA patients [89]. However, others studies could not confirm these findings [90, 91]. A recent report suggests an association between low anti-EBV/anti-B19 antibody levels and ACPA-positive RA, in the context of *HLA-DRB1* SE, suggesting that high anti-viral antibody levels could potentially protect against ACPA-positive RA [92].

Bacteria

In addition to viruses, there is growing evidence suggesting that bacterial dysbiosis contributes to the initiation and progression of RA. Due to the fact that autoantibodies precede the onset of clinically classifiable RA, it has been hypothesised that the initial inflammation begins outside of the joints, in mucosal tissues such as the lungs, the gut and the periodontal regions [3]. Involvement of the lungs in RA etiology and pathogenesis is well recognized today [77]. In addition, several animal studies have shown the ability of intestinal microbes to trigger arthritis, and RA patients have an altered gut microflora compared to controls [93]. Based on the epidemiological link between chronic periodontitis and RA, the gum mucosa has also raised interest in this respect [94, 95].

1.6 AETIOLOGY OF PD

Periodontitis is one of the most common chronic inflammatory diseases in humans, affecting approximately 30% of the population worldwide, with 10-15% suffering from the most severe form [96]. Chronic PD is a complex, multifactorial disease, characterized by the destruction of the tooth-supporting tissue. A microbial shift in the gingiva - towards pathogenic microorganism - results in a local inflammation called gingivitis, and if untreated gingivitis could progress to chronic periodontitis (PD).

The surface of the oral cavity harbours around six billion bacteria, which are represented by approximately 700 species. The diverse community of oral microbiota includes also other types of the microorganisms such as fungi, mycoplasma and protozoa [96]. This community, called the "climax community", is very stable, and a shift of microbiota from gram-positive, facultative, fermentative microorganisms to predominantly gram-negative, anaerobic, proteolytic organisms triggers pathological changes in the periodontium. The consortium of periodontal pathogens detected in periodontal pockets includes: *Aggregatibacter actinimycetemcomitans (Aa), Porphyromonas. gingivalis (P.gin), Tannerella forsythia, Prevotella intermedia, Fusobacterium, Campybacter rectus, Eubacterium, Streptococcus intermedia and Treponema denticola*; the three most common species (*P.gin, T.forsythia and T. denticola)* form the so-called "red complex".

The induction and progression of PD begins with a plaque accumulation initiated by early colonizers, followed by late colonizers, i.e. the red complex pathogens, and release of bacterial substances, which cause an inflammatory response. Over time, the bacteria in the periodontal pocket become resistance to attack by neutrophils and phagocytes, ROS, bactericidal proteins and peptides, and this ultimately leads to the development of chronic inflammation [97, 98]. In other words, tissue damage of the periodontium results from a dysregulated innate immune response against the bacteria rather than by the bacteria themselves.

1.6.1 *Porphyromonas gingivalis*

Porphyromonas gingivalis is recognized as a keystone pathogen involved in the pathogenesis and progression of PD. This bacterium was found in more than 85% of samples from patients with chronic PD, and there are a number of studies showing that serum levels of antibodies against *P.gin* are higher in patients with PD, compared to periodontally healthy controls. *P. gingivalis* serves as a secondary colonizer of dental plaque and often adhere to the primary colonizers, including *P. intermedia*. It can be divided into invasive and non-invasive strains, and both *in vivo* and *in vitro* data show that the invasive form of *P.gin* is more pathogenic than the non-invasive [99].

P. gingivalis produces a broad array of virulence factors, including: lipopolysaccharide (LPS), lipoteichoic acids, haemagglutinins, gingipains, outer membrane proteins and

vesicles. Some of these virulence factors, and the associated effects, are listed in Table 2. *P. gingivalis* uses these factors to overcome the host external protective barriers, colonize the subgingival plaque, and modulate the host immune response.

Table 2. Virulence factors of *P.gin* and their effects

Virulence factor	Effects
Enzymes (e.g. hyaluronidase, chondroitin sulfatase, capsule)	Decrease phagocytosis, Inhibition of chemotaxis
Lipopolysaccharide (LPS)	Bone resorption Immunoglobulin proteases
Fimbriae, exopolysaccharide, outer membrane proteins	Adhesion or attachment to host cell outer membrane
Collagenase, trypsin-like protease	Degradation of plasma protease inhibitors Destruction of periodontal tissue
Gingipains	Uncontrolled proteolysis Activation of the kallikrein/kinin pathway Development of edema Activation of the complement system Degradation of fibrin
P.PAD	Citrullination of proteins/ peptides (e.g. bradikinin, anaphylotoxin C5a, and epidermal growth factor)
Aminopeptidase	Degradation of iron transport protein

Table adapted from How et al: Porphyromonas gingivalis: An Overview of Periodontopathic Pathogen below the Gum Line. Front Microbiol 2016, 7:53

P.gin LPS

Lipopolysaccharide is key factor in the development of PD, and detected in over 50% of patients suffering from chronic PD. *P.gin* LPS triggers release of pro-inflammatory cytokines such as IL-1β, IL-6 and IL8, and matrix metalloproteinase, which cause tissue destruction. Moreover, *P.gin* LPS inhibits alkaline phosphatase activity and production of collagen type I, and stimulates the expression of adhesion molecules in a dose dependent manner. These mechanisms help *P.gin* colonize the subgingival plaque, and to evade the immune system [96].

P.gin gingipains

Arginine- and lysine-specific cysteine proteases, called gingipains, are recognized as the main virulence factors of *P.gin.* They are expressed in cell-associated- as well as secretory forms, and they cleave peptides after arginine- (R-gingipains) or lysine residues (K-gingipains). The action of gingipains results in uncontrolled proteolysis and kallikrein/kinin pathway activation, which leads to the development of edema. Furthermore, R-gingipain activates complement and neutrophil infiltration, and

K-gingipain contributes to increased bleeding by degradation of fibrin. Moreover, degradation of antimicrobial peptides by gingipains helps other bacteria to co-aggregate with *P.gin* and thereby to persist in the gingival tissue. Gingipains also cleave and activate the proteinase-activated receptor-2 (PAR-2) on neutrophils, which helps to maintain a pro-inflammatory-signaling pathway [96, 100].

P.gin PAD enzyme

P. gingivalis is the only known microorganism to express a PAD enzyme (*P.*PAD). The *P.*PAD gene is not related to the human PADI genes, and *P.*PAD has different properties compared to human PADs. First of all, *P.*PAD does not require Ca^{2+} for activity, but needs a higher pH than human PADs for optimal function. Moreover, *P.*PAD, unlike human PADs, deiminates C-terminal arginine residues, and is also able to citrullinate free arginine[101].

Importantly, there is no citrullination detected in arginine-gingipan-null mutants of *P.gin,* meaning that citrullination depends on the activity of R-gingipains [102, 103]. Indeed, R-gingipains co-localize with *P.*PAD on the bacterial outer membrane or in secreted vesicles, and cleave host proteins/peptides after arginine, exposing C-terminal arginine, which is subsequently citrullinated by *P.*PAD [101]. *P.*PAD participates in inflammation and homeostasis in the infected periodontium by citrullinating bradikinin, anaphylotoxin C5a, and epidermal growth factor. There is also data suggesting that *P.*PAD plays a role in prostaglandin secretion from infected fibroblasts [104].

The generation of citrullinated host peptides and protein fragments by the combined actions of *P.*PAD and R-gingipains may lead to the exposure of endogenous neo-epitopes to the immune system, and there is data demonstrating that *P.*PAD can citrullinate both α-enolase and fibrinogen, two of the main candidate autoantigens in RA [103]. In addition to citrullination of host proteins, there is emerging evidence suggesting that *P.*PAD is capable of autocitrullination; analysis by mass spectrometry, reveled citrullination of 7 out of 18 arginines in the *P.*PAD polypeptide chain. Interestingly, all of them were internal arginines/citrullines [105]. A recent analysis of the *P.gin* "citrullinome" showed that up to 25 *P.gin* proteins were potentially citrullinated [106]. The same study also showed that the ability to generate citrullinated proteins was dependent on the *P.gin* strain, as only one out of two commonly used lab strains, and one out of three clinical isolates, could produce citrullinated peptides.

1.7 THE LINK BETWEEN PD AND RA

Both RA and PD are chronic inflammatory diseases ultimately resulting in soft tissue destruction and progressive bone erosion. The link between these two diseases could be non-causal, and depend on common genetic and environmental risk factors such as smoking and lifestyle, including stress, nutrition and socioeconomic status. Among the

genetic risk factors, expression of *HLA-DRB1* SE subtypes (0401, 0404, 0405, 0408) have been associated to both ACPA positive RA and progressive severe periodontitis [98]. A causal link on the other hand, where PD drives RA, was first proposed by Rosenstein et al [107], and involves citrullination of bacterial and human proteins by *P.*PAD, followed by break of immune tolerance and production of ACPA in genetically predisposed individuals [48, 98]. This hypothesis is supported by the fact that the autoimmune response in RA - in the form of ACPA - often precede clinical joint symptoms by several years [12], suggesting that RA is precipitated outside the joints, potentially in the gum mucosa, where citrullinated proteins are exposed to the immune system in an inflammatory environment [108]. In addition to *P.*PAD, human PAD2 and PAD4 are expressed and active in the gingiva during chronic periodontitis [109], and another oral pathogen, *Aa*, has been suggested to play a role in the generation of citrullinated proteins in the oral cavity by inducing hypercitrullination in neutrophils and thereby generate multiple RA autoantigens [110].

1.7.1 Epidemiological evidence

Many well-designed studies show that PD is more prevalent in patients with RA compared to non-RA controls, and *vice versa*. In one study for example, RA patients were found to be more often edentulous and suffer from PD, compared to non-RA controls [111]. In another study, the largest population-based case-control study performed to date (13 779 RA cases and 137 790 controls), an association was identified between PD and incident cases of newly diagnosed RA [94]. It should be mentioned though, that a number of other studies have not been able to find a significant association between PD and RA, including the largest prospective register-based study conducted so far (81 132 participants including 292 incident RA cases), where a history of periodontal surgery and/or tooth loss was investigated in relation to risk of RA [112]. Also a recent study, where the Epidemiological Investigation of RA (EIRA) cohort was linked to the Swedish Dental Health Register, failed to find an association between RA and PD [113]. Still, a meta-analysis recently confirmed that there is strong evidence for an epidemiological association between PD and RA [95].

In addition, another recent meta-analysis demonstrated significantly higher anti-*P.gin* antibody levels in RA, compared to healthy controls [114], and a positive correlation between anti-*P.gin* antibody levels and ACPA has been shown [115]. Moreover, elevated anti-*P.gin* antibody levels have been detected in individuals at increased risk of developing RA [116, 117]. Thus, the epidemiological association between PD and RA seems to be present even before clinical onset of arthritis [118].

1.7.2 Evidence from animal studies

The majority of animal studies of RA and/or PD describe aggravated clinical symptoms of arthritis in mice and rats after being infected with *P.gin*. A number of studies have shown massive influx of leukocytes, accumulation of osteoclasts as well as cartilage and bone

erosion in joints after *P.gin* infection [98, 119]. Moreover, the inflammatory reaction in infected mice went beyond the joint and oral cavity and resulted in a systemic Th17 response [98, 119]. Another study confirmed the role of IL-17 in linking experimental PD and arthritis. In this study, mice with adjuvant-induced arthritis and *P.gin* infection had more severe joint damage, elevated number of Th17 cells and neutrophils, as well as increased TNF and IL-17 levels, compared to non-infected mice [120]. In another experimental study, ACPAs were found in animals infected with wild type *P.gin* but not in mice infected with the *P.*PAD-null strain [121].

1.8 AN AETIOLOGICAL HYPOTESIS LINKING P.GIN TO ACPA-POSITIVE RA

Mucosal surfaces, such as gums and lungs, may be exposed to environmental agents like smoking and microorganisms, including *P.gin* and *Aa*, which could trigger innate immune reactions and local inflammation. In this setting, human and bacterial PAD enzymes could become activated, which leads to increased protein citrullination, including autocitrullination of *P.*PAD, and the generation of neo-epitopes (Figure 3.1). In the presence of "danger signals" (e.g. toxic components from the smoke, bacterial DNA and LPS), B cells may become activated and start to produce anti-*P.gin* antibodies as well as low titers of low-affinity ACPAs, which can be detect in the circulation years before clinical symptoms of RA develops (Figure 3.2). In individuals carrying risk alleles such as the *HLA-DRB1* SE and *PTPN22* polymorphism, autoreactive T cells - that have escaped negative selection - could be activated by B cells presenting citrullinated peptides, derived from endogenous proteins, on MHC class II (i.e. SE) (Figure 3.3). As a consequence, activated autoreactive T cells stimulate the citrulline-specific B cells to produce high titers of high-affinity ACPAs. Through epitope-spreading and/or cross-reactivity, these ACPAs may later target citrullinated joint proteins, for example on osteoclasts, form immune complexes and ultimately cause chronic joint inflammation (Figure 3.4).

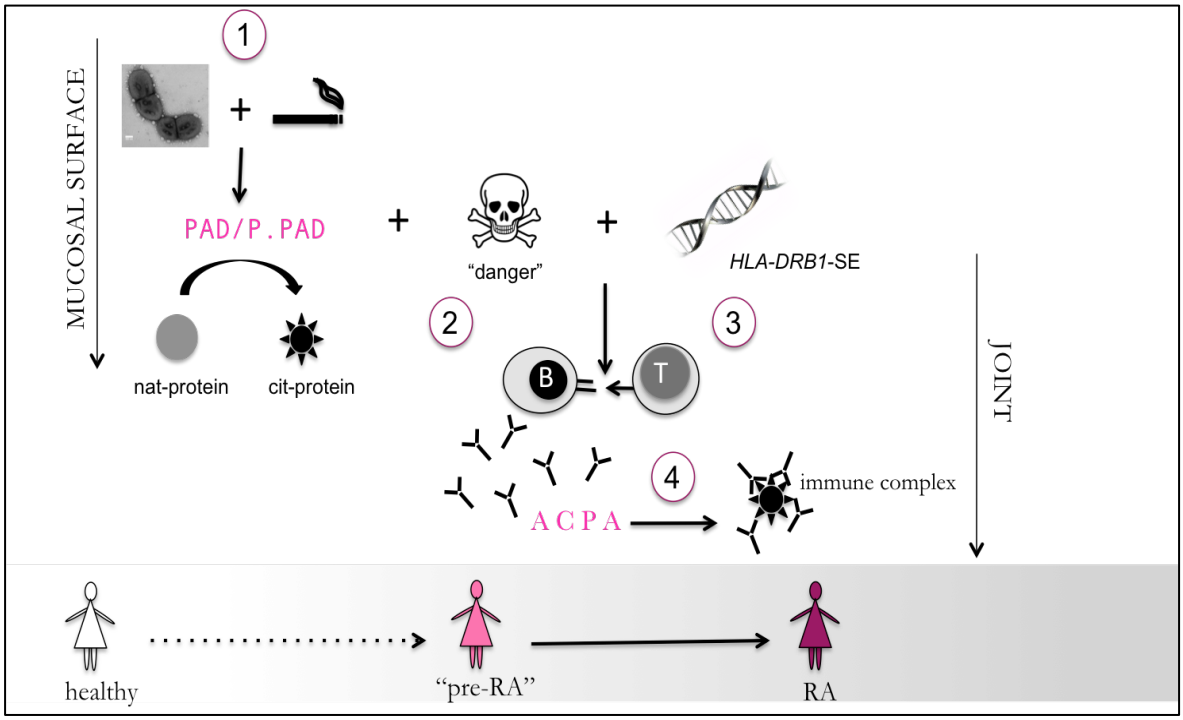

Figure 3 Schematic illustration of the etiological hypothesis linking *P.gingivalis* to the development of ACPA-positive RA.

2 AIMS OF THE THESIS

The overall aim of this PhD project was to investigate whether the oral pathogen *Porphyromonas gingivalis* is etiologically linked to the development of RA, specifically ACPA-positive RA. Specific aims included:

1. To investigated the fine-specificity of the ACPA response in relation to well-known RA risk factors (i.e. *HLA-DRB1* SE, *PTPN22* polymorphism and cigarette smoking) (**Study I**)

2. To characterise the subset of RA patients with a heightened immune response to *P.gin* R-gingipain, in terms of genetic risk factors, smoking and the ACPA response (**Study II**)

3. To analyse the antibody response to *P.gin* in blood samples collected before the onset of clinical RA, in relation to the ACPA response (**Study III**)

4. To characterize the antibody response to a citrullinated peptide derived from *P.*PAD in RA, in relation to genetic risk factors, smoking, disease activity, and the ACPA response **(Study IV)**

3 MATERIAL AND METHODS

3.1 PATIENT MATERIAL

All four studies included in this PhD thesis are based on human material, from patients with RA or PD and healthy controls. In **Study I, II** and **IV**, the serum biobank from the population-based RA case-control study EIRA (Epidemiological Investigation of RA) was used, and in **Study III**, serum samples from the population-based Biobank of the Northern Sweden Health and Disease Study (NSHDS) cohort as well as the Maternity cohort was used. In addition, a small serum cohort of patients with PD and periodontally healthy controls was used in **Study II** and **IV**, and monoclonal antibodies derived from RA blood, -synovial tissue and -synovial fluid was used in **Study IV**. All samples and information related to the study subjects were collected with informed consent and ethical approval in compliance with the Declaration of Helsinki [122].

3.1.1 EIRA

In **Study I, II and III** we analysed antibody reactivities in serum samples from the EIRA cohort. This population-based case-control study was initiated in May 1996 and is ongoing. Incident cases of RA (aged 18-70 years), diagnosed in accordance with the 1987 revised ACR criteria, were recruited within 12 months after the first symptoms of arthritis from the southern and middle parts of Sweden. Controls were randomly selected from the national population register, and matched on age, gender and residential area [76]. All EIRA participants donated blood at the time of inclusion and filled in an extensive questionnaire relating to life style and environmental exposures, including detailed smoking history. Serum samples were frozen (stored at minus 80°C) for future serological analyses; genotyping was performed on DNA from whole blood, for *HLA-DRB1* shared SE alleles (Olerup SSP kit) and *PTPN22* (rs2476601) polymorphism (TaqMan allelic discrimination PCR) [71]. Patients in EIRA are also registered in the nationwide Swedish Rheumatology Register, from where data on disease activity score for 28 joints (DAS28) and C-reactive protein (CRP) levels (mg/l) were collected for **Study IV** [123].

3.1.2 A case-control study within NSHDS and the Maternity cohort

In **Study III**, antibody reactivities were analysed in plasma/serum samples collected before the onset of clinical RA. This was done as a case-control study within the population-based Biobank of the Northern Sweden Health and Disease Study (NSHDS) cohort and the Maternity cohort of Northern Sweden**.** The study design has been described previously [12]. Briefly, the NSHDS cohort is based on health surveys, where all habitants of Västerbotten County are continuously invited to participate. Study subjects donate blood and complete a self-administered questionnaire for the collection of demographic, medical, and lifestyle information, including smoking status and diet. The Maternity cohort is based on blood samples collected from pregnant women that have been screened for immunity to rubella. In order to identify individuals in these cohorts who have donated blood before the onset of

symptoms of RA, a register linkage was performed using the register of patients with RA (fulfilling the ARA 1987 classification criteria for RA) at the Department of Rheumatology, University Hospital, Umeå established since 1995. A total of 251 RA patients were identified who had donated at least one blood sample before having symptoms of the subsequent RA disease. These individuals had together donated 422 plasma/serum samples at various time points before the onset of symptoms of RA (375 from the Biobank cohorts and 47 from the Maternity cohort): the majority had only donated one blood sample; 92 individuals (36.6%) had donated two samples; 46 individuals (18.3%) had donated three samples; 22 individuals (8.8%) had donated four samples; nine individuals (3.6%) had 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 of these 251 individuals. Controls (n=198) were selected from the same cohorts as the pre-symptomatic individuals and matched for age and gender. Serum/plasma samples were stored frozen for future serological analyses; genotyping for *HLA-DRB1* SE alleles and *PTPN22* (1858C/T) polymorphism were performed as described [124].

3.1.3 PD- and non-PD cohort

In addition to the larger population-based cohorts described above, serum samples from patients with chronic PD (n=66) and gender-matched periodontally healthy controls (n=63) were screened for antibody reactivities in **Study II** and **IV**. All study subjects were examined by dentists at the Department of Dental Medicine, Karolinska Institutet, Stockholm, Sweden. Clinical criteria for PD were: bone resorption with attachment loss ≥5mm, pocket probing depth ≥4mm, and bleeding on probing. Periodontally healthy controls had no signs of gingival inflammation, clinical attachment level ≤3.5 mm, pocket probing depth ≤3.0 mm, and no bleeding on probing.

3.2 ELISA

The main method used in the studies included in this PhD thesis is the ELISA assay. For detection of anti-CCP2 IgG (**Study I-IV**), the commercially available Immunoscan CCPlus® kit was used (Euro-Diagnostica AB, Malmö, Sweden), according to the manufacturer's instructions with a cut-off for positivity set at ≥25 AU/ml. Information on RF status (positive or negative) in **Study II** had been collected from patient journals, and was in most cases assayed by nephelometry, while analysis of RF in **Study III**, was performed using the EliA assay on the Phadia 2500-system was used (Phadia GmbH, Freiburg, Germany), in accordance with the manufacturer's instructions. For detection of antibodies against bacterial antigens and ACPA fine-specificities (**Studies 1-IV**), in-house protein- and peptide ELISAs were used (descriptions of these antigens are presented in Table 3).

Table 3 Coating antigens used in the in-house ELISAs

Antigen	Peptide sequence	Origin
RgpB	Full length protein	Arginine gingipain B, purified from *P.gin*
CPP3	C-AKTDSYWT-CIT-DYTGWFAMYD-C	*P.gin* PAD, amino acids: 121-139
CEP-1	C-KIHA-CIT-EIFDS-CIT-GNPTVE-C	Human α-enolase, amino acids: 5-21
Cit-Vim$_{60-75}$	VYAT-CIT-SSAV-CIT-L-CIT-SSVP	Human vimentin, amino acids: 60-75
Cit-Fib$_{36-52}$	NEEGFFSA-CIT-GHRPLDKK	Human fibrinogen β-chain, amino acids: 36-52
Cit-C1	(GPP*)$_5$-GA-CIT-GLTG-CIT-P*-GDA-(GPP*)$_2$-GKKYG	Human CII, C1 epitope, amino acids: 355-378

CIT = citrulline; P* = hydroxyproline

3.2.1 ACPA fine-specificity ELISA

In **Study I**, we measured antibodies against four synthetic citrullinated peptides, described as candidate autoantigens in RA: CEP-1, Cit-vim$_{60-75}$, Cit-fib$_{36-52}$, and Cit-C1, corresponding to epitopes on citrullinated α-enolase, vimentin, fibrinogen and CII (Table 3). Reactivity against the corresponding arginine-containing control peptides was analysed in parallel. In brief, 96-well plates were coated with peptide antigens diluted in 50mM carbonate buffer: CEP-1/REP-1 (5µg/ml) and Cit-C1/Arg-C1 (10µg/ml) were coated on MaxiSorp (Nunc) plates at 4°C over night; biotinylated Cit-vim$_{60-75}$/Arg-vim$_{60-75}$ and Cit-fib$_{36-52}$/Arg-fib$_{36-52}$ (1µg/ml) were coated on streptavidine (Pierce, Thermo Scientific) plates at room temperature (RT) for 1h (note, streptavidine plates were washed in PBS, 0.5% Tween, prior addition of antigen). Following antigen incubation, plates were washed (PBS, 0.5% Tween) and CEP-1/Cit-C1 plates were also blocked (PBS, 1% BSA) for 1h at RT, before adding serum samples, diluted 1:100 in RIA buffer (10mM Tris, 1% BSA, 350mM NaCl, 1% Triton-X, 0.5% sodium deoxycholate, 0.1% SDS). The serum samples were subsequently incubated for 1h at RT, before plates were washed again (PBS, 0.5% Tween) and incubated for 1h in RT with horseradish peroxidase (HRP)-conjugated goat anti-human IgG (Jacksson), diluted 1:10,000 in RIA buffer. After a final wash, TMB substrate (Sigma) was added, and the colour reaction stopped by addition of 1M H_2SO_4. Absorbance was measured at 450nm, and optical density (OD) was expressed as arbitrary units (AU/ml) for the ACPA responses, based on standard curves. The standard curves were derived from antibody-positive serum pools added to the plates in serial dilutions. Serum samples were analysed in duplicates, and blank wells (only RIA buffer), as well as positive and negative control serum, were included on all plates. Cut-off values for positivity were based on the 98th percentile among 150 EIRA controls. For comparison of ACPA fine-specificity responses, each cut-off value was converted to 10AU/ml.

3.2.2 CPP3/RPP3 IgG ELISA

In **Study III** and in **Study IV**, we analysed reactivity against the citrullinated *P.*PAD-derived peptide CPP3 and the arginine-containing control version RPP3. The same protocol as described above was used, with some modifications: Coating concentration for CPP3 and RPP3 was 10µg/ml. In **Study III**, cut-off for CPP3-positivity was calculated using receiver operating characteristic (ROC) curves, based on reactivity among 192 RA cases and 198 population-based controls from the NSHDS and the Maternity cohorts**,** giving a specificity of 96%. In **Study IV**, a cut-off based on the 100th percentile among 63 periodontally healthy controls was used when analyzing the anti-CPP3 antibody response in patients with chronic PD. In addition, 218 human monoclonal antibodies were screened at 10µg/ml for CPP3/RPP3 reactivity using the CPP3/RPP3 ELISA; positive clones were re-analysed in serial dilution (1:2 steps, starting at 20µg/ml), and unspecific binding was evaluated by including uncoated wells (i.e. 2% BSA).

3.2.3 RgpB IgG ELISA

In **Study II** and **Study III** we used a protein-based ELISA to analyse the presence of antibodies against R-gingipain B (RgpB). The coating antigen, C-terminal hexahistidine-tagged RgpB protein, was purified from the growth medium of genetically modified *P.gin* strain W83, by affinity chromatography on Ni-Sepharose, as previously described [125]. The same protocol as outlined above was used, with some modifications: Coating concentration was 2.5µg/ml, and serum was diluted 1:800. In **Study II**, cut-off for positivity was set at the 95th percentile, based on reactivity among 59 periodontally healthy controls, while in **Study III**, no specific cut-off was set for the anti-RgpB antibody response. Anti-RgpB IgG levels were presented as arbitrary units (AU/ml), based on a standard curve made from a serially diluted highly positive serum pool.

3.3 PEPTIDE ABSORPTION ASSAY

In **Study I,** cross-reactivity between different ACPA fine-specificities (CEP-1, Cit-vim$_{60-75}$, Cit-fib$_{36-52}$, and Cit-C1) was examined by peptide absorption experiments. Briefly, serum samples with high anti-CCP IgG levels (>800 AU/ml) and multiple ACPA reactivities were diluted 1:100 in RIA buffer and incubated with each of the four peptides (CEP-1, Cit-vim, Cit-fib or Cit-C1) at 10 µg/ml, or in the presence of buffer alone, for 2h hours at RT, during constant agitation. Afterwards, samples were centrifuged at 1000g for 15 minutes, and supernatants transferred to peptide-coated ELISA plates, and assayed as described above. Evaluation of cross-reactivity was made by comparing ACPA responses in peptide pre-absorbed serum samples and serum samples incubated without any peptide.

3.4 MULTIPLEX PEPTIDE MICROARRAY

In **Study IV**, we used a custom-made multiplex peptide microarray, based on the ImmunoCAP® ISAC system (Phadia AB, Uppsala, Sweden), for the detection of antibody responses, in addition to the ELISA method. This assay allows the simultaneous detection of multiple antibody reactivities by high-throughput screening of large numbers of serum samples [126]. In brief, glass slides were spotted with peptide antigens before incubated with serum (or monoclonal antibodies). Unbound antibodies were then removed by washing, and bound antibodies detected using Cy3-conjugated goat anti-human IgG (Jackson ImmunoResearch Laboratories, Newmarket, UK) and visualized by laser scanner. Fluorescence intensity was converted to normalized arbitrary units, by comparison to calibrator samples on each assay run. Cut-off values for each antibody response were set at the 98th percentile among 370 EIRA controls. Peptide antigens printed on the glass slides included: CPP3 and RPP3, as well as eight citrullinated peptides (and the arginine-containing equivalents) derived from human proteins: filaggrin (cfc1-cyc), fibrinogen (Cit-fib$\alpha_{563-583}$, Cit-fib$\alpha_{580-600}$, Cit-fib$ß_{36-52}$), vimentin (Cit-vim$_{2-17}$ and Cit-vim$_{60-75}$), α-enolase (CEP-1) and collagen type II (Cit-C1).

Study III also used data from the multiplex peptide array, for ACPA fine-specificities: CEP-1, Cit-fib$_{36-52}$ and cfc1-cyc. Cut-off values for these antibody responses were set based on 198 population-based controls from the NSHDS and the Maternity cohorts.

3.5 GENERATION OF HUMAN MONOCLONAL ANTIBODIES

In **Study IV**, we analysed 218 human monoclonal antibodies for reactivity against CPP3/RPP3. These antibodies had been generated previously in the lab by *in vitro* cloning [127-130]. Briefly, memory or plasma B cells were isolated from RA blood, synovial tissue or synovial membrane, and subsequently single cell sorted into PCR plates. Immunoglobulin cDNA was synthesized and amplified for individual Ig heavy (H) and light (L) (κ or λ) chain genes by reverse transcriptone-PCR, using primers previously described [131]. Matching IgH (γ) and Igκ/Igλ amplicons were sequenced (Eurofins MWG Operon) and compared against germline sequences using IgBLAST and IMGT/V-quest, in order to determine Ig gene usage, complementary determining region 3 (CDR3) features, and number of variable (V) gene usage. Immunoglobulin genes were subsequently cloned into human Igγ1, Igκ, or Igλ IgG expression vectors, and the vectors were transformed into DH5α bacteria (Gibco Invitrogen) before isolated by NucleoSpin plasmid DNA purification kits (Macherey-Nagel). Recombinant monoclonal antibodies (IgG1) were produced in the Expi293 system (Thermo Fisher Scientific) with transient transfection using PEI-max [132], and purified on Protein G Fast Flow Sepharose, (GE Healthcare Life Sciences).

3.6 STATISTICAL METHODS

Continuous data were compared using the non-parametric Mann–Whitney U-test for independent groups (**Study I-IV**), or Wilcoxon signed rank test including two groups, or Kruskal–Wallis test including several groups (**Study III**). For categorical data, the chi-square test or Fisher's exact test was used (**Study III**). Correlation analyses were performed using Spearman's rank correlation coefficient (**Study II** and **III**), or Pearson correlation (using R v. 3.3.3) (**Study IV**). Associations between RA risk factors (i.e. smoking, *HLA DRB1* SE and *PTPN22*) and presence of different antibodies in different 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 (CI). Analyses were adjusted for age, gender and residential area (**Study I, II** and **IV**), or age and gender (**Study III**). Interaction was defined as departure from additivity of effects [133], and was evaluated between different RA risk factors in different subsets of RA (**Study I-III**). The attributable proportion due to interaction (AP) was calculated together with 95% CI, as previously described [134]. The AP value between two interacting factors reflects the joint effect beyond the sum of the independent effects [135]. All statistical analyses were performed using GraphPad Prism6 or SAS (version 9.1 or higher) (**Study I, II** and **IV**), or SPSS 23.0 software (Chicago, IL, USA) (**Study III**). P-values ≤ 0.05 were considered statistically significant.

3.7 METHODS NOT INCLUDED IN SUDY I-IV

3.7.1 ACPA purification

For use in both *in vitro* and *in vivo* experiments, polyclonal ACPA IgG was purified from highly CCP2-positive plasma samples of patients with RA. Purification was done through affinity chromatography using columns with the CCP2 peptides (donated by Euro-Diognostica AB) coupled to Sepharose beads. In brief, plasma samples were first centrifuged and diluted in PBS, before the IgG fraction was isolated on Protein G columns. The IgG fraction was then put on the CCP2 column, and the anti-CCP2 IgG was eluted and concentrated, followed by buffer exchanging to PBS. The CCP2-column IgG flow-through (FT) fraction was also collected, and used as a control in various *in vitro* and *in vivo* experiments where the purified ACPAs were further investigated. The ACPA purification method and related data was presented in **Study V**: "*Affinity purified anti-citrullinated protein/peptide antibodies target antigens expressed in the rheumatoid joint*" (Study V is not included in the thesis) [136].

3.7.2 Western blot

The ACPA and FT pools described above, were used in Western blot experiments: initially, to investigate their reactivity against citrullinated RA candidate autoantigens (α-enolase, vimentin and fibrinogen) and non-citrullinated counterparts (**Study V**), and subsequently to

study cross-reactivity with carbamylated proteins (α-enolase and fibrinogen) in **Study VI**: "*Antibodies to carbamylated α-enolase epitopes in rheumatoid arthritis also bind citrullinated epitopes and are largely indistinct from anti-citrullinated protein antibodies*" (Study VI is not included in the thesis) [137]. In addition, in an ongoing study, the ACPA/FT pools as well as the monoclonal ACPAs described above are being used for blotting *P.gin* lysates as well as lysates of arthritic mouse joints, in order to detect relevant ACPA targets. Briefly, modified and non-modified proteins (or *P.gin*/mouse joint lysates) were separated on NuPAGE Bis-Tris 4-20% gels by electrophoresis, and transferred to nitrocellulose membranes. After blocking (5% milk in TBS/0.05% Tween), membranes were incubated over night at 4°C with purified ACPA/FT, or monoclonal antibodies (all at 1 or 2μg/ml), or the secondary antibody alone. As a secondary detecting antibody, HRP-conjugated goat-anti human IgG was used (diluted 1:10,000) for 1h at RT. Detection of bound antibodies was visualized using the ECL chemiluminescence method.

3.7.3 *In vitro* generation of citrullinated proteins

Citrullination of the proteins used in the immunoblotting experiments described above was performed *in vitro*, by incubating proteins (α-enolase, vimentin and fibrinogen) with the rabbit skeletal PAD2 enzyme (Sigma, St. Louis, MO, USA) at a protein concentration of 1mg/ml, and an enzyme concentration of 2U/mg protein, in PAD buffer (100 mM Tris, 10 mM $CaCl_2$, 5 mM dithiothreitol (DTT), pH 7.6) for 2h at 37° C. Citrullination was stopped by adding 20mM EDTA, followed by dialysis to calcium-free PBS. Citrullination was confirmed by mass spectrometry.

3.7.4 Animal experiments

In an ongoing study, aimed at investigating the effect of *P.gin* and ACPA on arthritis and pain development, we have used adult male BALB/c mice. In a pilot experiment, mice were injected intraarticularly with *P.gin* LPS at three different doses (0.5 μg, 1.5 μg and 3 μg). Acute arthritis (paw swelling/oedema) was scored by measuring paw thickness using a digital caliper for up to 6 days post injection (p.i.). Mice were sacrificed at the peak of inflammation (around day 2 p.i.), or when inflammation had subsided (day 6 p.i.). Mice were bled, serum was collected, and paws/ankles were frozen. In order to determine the optimal time point for subsequent ACPA transfer, i.e. when joint inflammation - and more importantly joint citrullination - was at its peak, presence of citrullinated proteins was examined by Western blot using the ACPA/FT pool or monoclonal antibodies, followed by mass spectrometry. As a positive control we used paws from severely arthritic mice, and as negative control, we used contra lateral paws, or paws from naïve mice.

To assess pain during the experiment, we used the mechanical sensitivity test. Briefly, after acclimatization on the top of a mesh-like surface, optiHair filaments were applied under each paw until buckling of the hair filament was observed. Spontaneous withdrawal within three seconds was considered a positive response; 50% withdrawal was calculated using Dixon's up-down method [16].

3.7.5 Protein extraction

Proteins from the mouse joints (bone and soft tissue) were extracted from *P.gin* LPS-injected paws and the contralateral paws, as well as from naïve mice and severely arthritic mouse paws. For this, we used a lysis buffer containing 150mM NaCl, 1mM EDTA, 50mM Tris and 1% SDS, followed by sonication and centrifugation. Supernatants were collected and the protein concentration was determined using the Micro BCA assay. Samples were stored at -80°C until further analysis.

3.8 ETHICAL CONSIDERATIONS

This PhD project has been aimed at increasing our understanding for the aetiology of RA, in order to develop better treatments for patients, and potentially a cure in the future, as well as to identify preventive strategies. To achieve this, we have used patient material, and in the ongoing study, also experimental animals.

We have ethical permits for all studies described in this PhD thesis: from the regional ethics review board at Karolinska Institutet, Stockholm (**Study I, II** and **IV**), as well as the regional ethics review board in Umeå, Department of Medical Research (**Study III**), and for the ongoing animal study, from the local ethical committee for animal experiments in Stockholm. Biological samples were collected from patients and controls with informed consent. Ethical considerations included the protection of privacy and handling of personal data, which was done in accordance with the Swedish Law: Personuppgiftslagen (PUL). Information was stored behind the Karolinska University Hospital's network firewall. All studies were conducted in compliance with the Declaration of Helsinki [122].

4 RESULTS AND DISCUSSION

4.1 STUDY I

In **Study I**, we have investigated the fine-specificity of the ACPA response in relation to known RA risk factors: *HLA-DRB1* SE, *PTPN22* polymorphism and cigarette smoking. When this study was initiated, it was known that SE mainly associated with CCP2-positive RA, and that *PTPN22* and cigarette smoking each interacted with SE to strengthen this association [11, 71]. It had also recently been shown that anti-CCP2 antibodies are a collection of ACPAs containing overlapping and non-overlapping reactivities [138]. Moreover, that the fine-specificity of the ACPA response is influenced by SE [139]. Our study was performed to further define which ACPA fine-specificities were associated with SE, *PTPN22* and smoking.

The study population comprised 1985 early RA cases and 2252 matched controls from the Swedish population-based case-control cohort EIRA. ACPAs specific for epitopes on citrullinated candidate autoantigens: α-enolase (CEP-1), vimentin (Cit-vim$_{60\text{-}75}$), fibrinogen (Cit-fib$_{36\text{-}52}$), and collagen type II (Cit-C1) were measured by in-house peptide ELISAs. Cross-reactivity between antibodies was investigated by peptide absorption experiments. Data on CCP2 status, genotype and smoking habits had been generated/collected previously, and were retrieved from the EIRA database. Associations between risk factors and specific ACPAs were calculated by logistic regression analysis, and presented as OR, with 95% CI.

Presence of different ACPA fine-specificities (35% CEP-1-positive, 37% Cit-vim-positive, 28% Cit-fib-positive and 37% Cit-C1-positive) overlapped to a large extent. Still 17 distinct RA subsets could be identified based on their different ACPA fine-specificity profiles. Data from the peptide-absorption studies suggested only limited cross-reactivity between these different ACPA fine-specificities. However, it should be mentioned that more recent studies, performed using monoclonal ACPAs, suggest a much broader cross-reactivity pattern for ACPAs (personal communication, Professor Vivianne Malmström). Our study could confirm the well-established association between SE and CCP2-positive RA, and when dividing the CCP2-positive subset further, based on the different ACPA fine-specificities, we found substantial differences. The strongest SE-association was identified for the subset of RA with antibodies targeting CEP-1 or Cit-vim, while no association was observed for Cit-fib-positive RA, and only a weak association was found for the Cit-C1-positive subset. Highest OR (49.6) was identified for the CEP-1/Cit-vim double positive subset, which could be compared to an OR of only 2.5 for the Cit-fib/Cit-C1 double positive subset. Notably, anti-CCP IgG levels were significantly higher in Cit-fib+/Cit-C1+ RA, compared to CEP-1+/Cit-vim+ RA, suggesting that SE mainly influences the fine-specificity of the ACPA response, rather than total anti-CCP IgG levels. Also the combined effect of SE, *PTPN22* and smoking associated primarily with the subset of RA characterised by anti-CEP-1 antibodies, and to a lesser extent also anti-Cit-vim antibodies, while these risk factors had no specific association with antibodies against Cit-fib or Cit-C1 (beyond the effect conveyed by CCP2) (Figure 4).

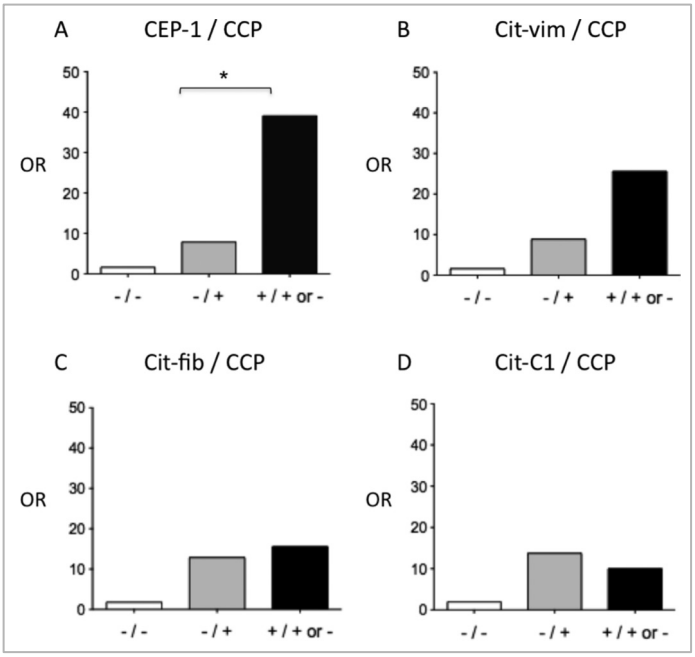

Figure 4 Combined effects of SE, *PTPN22* and smoking on disease risk in different RA subsets. RA subsets were defined based on presence/absence of one ACPA fine-specificity without taking into account which other ACPA fine-specificities may be present simultaneously. White bars = subsets negative for the ACPA fine-specificity and negative for CCP2 (-/-); grey bars = subsets negative for the ACPA fine-specificity but positive for CCP2 (-/+); black bars = subsets positive for the ACPA fine-specificity but where the CCP2 status was not considered (+/ + or -). The asterisk indicate $p < 0.05$.

This study still provides the most comprehensive picture to date of how SE, *PTPN22* and smoking are associated with the presence of specific ACPAs rather than anti-CCP2 antibody levels, suggesting that their production may be governed by partly different mechanisms.

Important to note is also that the CCP2 assay, commonly used in clinics, does not capture all ACPA fine-specificities, as 18% of the CCP2-negative patients were in fact ACPA-positive, suggesting that extended ACPA screening (in addition to the CCP2 test), should be considered. The antigens investigated in our study are likely to represent physiological targets of the ACPA response, as they are expressed in the rheumatoid joint [47, 55, 60, 140]. However, they should not be considered the only "true" autoantigens in RA. Approximately 14% of the CCP2-positive patients in our study were negative for the four ACPA fine-specificities investigated, indicating the presence of yet other ACPA fine-specificities, not investigated here. Since our study was conducted, a number of other candidate autoantigens have indeed been described, including citrullinated histones [38, 64] and citrullinated tenascin C [39], as well as citrullinated viral and bacterial antigens [46, 64, 105].

4.2 STUDY II

Study II was aimed at investigating the role of the oral pathogen *Porphyromonas gingivalis* in RA etiology, by measuring antibodies to arginine gingipain B (RgpB), the most potent virulence factor of *P.gin*, and to analyse this antibody response in relation to the presence of autoantibodies (in particular ACPAs) and RA risk factors (i.e. SE, *PTPN22* and smoking). The basis for this study was the reported epidemiological association between PD and RA,

now confirmed in a meta-analysis [95], and more specifically, the hypothesis first put forward by Rosenstein and collagues, that ACPA-positive RA is precipitated by the oral pathogen *P.gin* [107].

As in Study I, we used the EIRA cohort for these analyses. The RgpB protein was purified from *P.gin*, and anti-RgpB IgG was measured by in-house protein ELISA in 1975 RA cases and 377 controls, as well as in 65 patients with PD and 59 periodontally healthy controls. Data on genetics, smoking habits and autoantibodies were retrieved from the EIRA database, and associations with anti-RgpB IgG were calculated by unconditional logistic regression analyses, and presented as OR, with 95% CI.

We identified a significant association between elevated anti-RgpB antibodies and RA, in particular ACPA-positive RA (Table 4). Interestingly, this association was even stronger than the well-known association between smoking and RA.

Table 4 Association between elevated anti-RgpB IgG levels and RA in subgroups of patients, divided according to the presence/absence of ACPAs

Subgroup	Anti-RgpB IgG negative, no. (%)	Anti-RgpB IgG positive, no. (%)	OR (95% CI)†
Controls	341 (90.45)	36 (9.55)	1.0 (referent)
All RA patients	1,518 (76.90)	456 (23.10)	2.96 (2.00, 4.37)
ACPA-positive RA patients	1,041 (75.38)	340 (24.62)	3.24 (2.18, 4.81)
ACPA-negative RA patients	477 (80.44)	116 (19.56)	2.35 (1.51, 3.65)

* Anti-RgpB = anti–arginine gingipain type B; RA = rheumatoid arthritis; ACPAs = anti–citrullinated protein antibodies; OR = odds ratio; 95% CI = 95% confidence interval.
† Adjusted for age, sex, and residential area.

Moreover, we found that anti-RgpB antibody levels were significantly elevated in PD patients compared to non-PD controls ($p<0.0001$); in RA compared to non-RA controls ($p<0.0001$); and in ACPA-positive RA compared to ACPA-negative RA ($p<0.003$). Our data are thus in line with a number of previous reports, as well as a recent meta-analysis (which also includes our own study) [114]. Based on ACPA fine-specificity data from Study I, we also investigated whether the anti-RgpB IgG response was specifically associated with antibodies to CEP-1, Cit-vim$_{60\text{-}75}$, Cit-fib$_{36\text{-}52}$ or Cit-C1, as well as antibodies to carbamylated fibrinogen and RF. However, we could not detect any specific association with any of these antibodies, beyond the association with CCP2-positive RA. Importantly, there was no association between smoking and increased anti-RgpB IgG levels, despite smoking being a risk factor also for PD. Instead, smoking and elevated anti-RgpB IgG levels were independently associated with ACPA-positive RA. The association between anti-RgpB antibodies and RA was also independent of SE and *PTPN22*, but interestingly, statistically

significant interactions between elevated anti-RgpB IgG levels and both SE and smoking was identified for ACPA-positive RA (not for ACPA-negative RA).

In a recent study from our lab, it was shown that 33% of RA patients and controls in EIRA suffer from PD [113], while in the present study we show that 23% of RA patients, but only 9.4% of controls have elevated anti-RgpB IgG levels. This apparent contradiction could be related to the investigation of early RA in our study, *versus* established RA in the other study (as investigations of PD status was performed years after inclusion in EIRA). Alternatively, presence of anti-RgpB antibodies should not be considered as surrogate marker for PD, as anti-RgpB IgG could reflect a historical *P.gin* infection, and PD could also be caused by other pathogens than *P.gin,* and *P.gin* infection may not always cause PD. Moreover, different *P.gin* strains show differences in expression of virulence factors [141, 142] and a recent study could demonstrate that only some *P.gin* strains express a *P.*PAD enzyme capable of peptidyl-citrullination [106]. Taken together, Study II supports a causative role for *P.gin* in ACPA-positive RA etiology, and we propose that *P.gin* could account for the previously reported link between chronic PD and RA.

4.3 STUDY III

Building on data from Study II, where we identified an association between elevated anti-*P.gin* antibody levels and RA, **Study III** examines whether anti-*P.gin* antibodies pre-date RA symptom onset and ACPA production. In order to do this, we analysed the presence of anti-*P.gin* antibodies in plasma/serum samples collected before (and after) clinical onset of RA.

This study included 251 pre-RA/RA cases - who had donated in total 422 blood samples before symptom onset (median: 5.2 years; interquartile range 6.2) - and 198 population-based non-RA controls, from the Biobank of Northern Sweden Health and Disease Study (NSHDS) as well as the Maternity cohort. Antibodies to two *P.gin*-specific antigens were analysed by ELISA: RgpB and CPP3 (a citrullinated peptide derived from *P.*PAD, that has been identified as an immunodominant epitope in autocitrullinated *P.*PAD) [105] . Data on genetics, smoking habits and autoantibodies were retrieved from a database. Continuous data were compared using Mann–Whitney U-test/Wilcoxon signed rank test (including two groups), and Kruskal–Wallis (including several groups). Chi-square test or Fisher's exact test was used for categorical data, Spearman's rank correlation was used for correlation analysis, and logistic regression was used to calculate associations between antibodies and risk factors.

This study showed significantly elevated anti-RgpB antibody levels in pre-symptomatic individuals and in RA patients, compared to controls ($p < 0.001$); mean concentration of anti-RgpB IgG in pre-symptomatic individuals exceeded that of controls 12 years before symptom onset. Antibodies against CPP3 were detected in 6.8% of pre-symptomatic individuals and in 7.8% of RA patients, with significantly increased levels compared to controls ($p < 0.001$); mean concentration of anti-CPP3 IgG in pre-symptomatic individuals exceeded that of

controls eight years before symptom onset. Anti-RgpB IgG levels were stable in pre-symptomatic individuals with a significant increase over time when analysing individuals with at least four consecutive pre-dating samples ($p < 0.05$), and a non-significant decline ($p < 0.088$) after RA diagnosis, while anti-CPP3 IgG levels followed the classical ACPA response [143], with increasing concentrations over time (Figure 5).

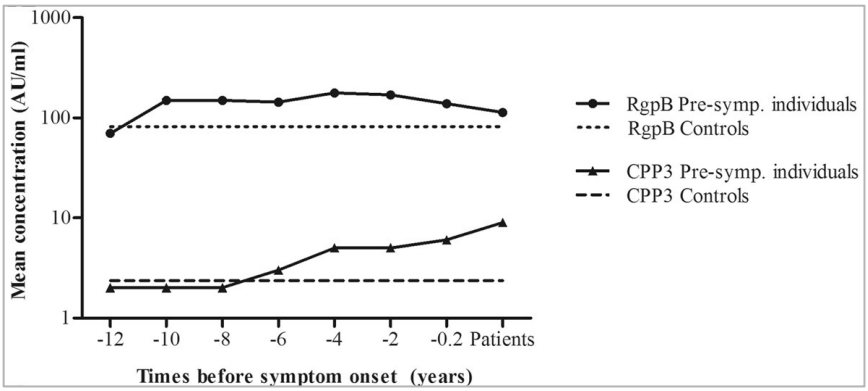

Figure 5 Anti-*P.gin* antibody responses during the pre-dating time until the onset of symptoms of RA. Logarithmic mean antibody concentrations during 2-year periods of anti-RgpB and anti-CPP3 antibodies are shown for pre-symptomatic individuals (n=422), patients with RA (n=192), and controls ($n = 198$).

Increased anti-RgpB antibody levels appeared to precede the classical ACPA response, and a (weak) correlation between anti-RgpB and the ACPA response (measured as anti-CEP-1 antibody concentrations) was observed. The median pre-dating time for anti-CPP3 antibody positivity was closer to RA onset (−3.42 years), when compared to the classical ACPA response (CCP2: −4.56 years). Notably though, we treated anti-CPP3 IgG as a classical ACPA, and therefore set cut-off for positivity based on reactivity among 198 non-RA controls, of unknown periodontal status. However, CPP3 is not an autoantigen, it is a bacterial peptide, with no sequence homology to human proteins. Hence, it is possible that the cut-off for positivity was set too low, as we did not have access to periodontally healthy controls. No cut-off for positivity was set for the anti-RgpB antibody response for this reason.

Elevated anti-RgpB IgG levels (>75th percentile) were associated with being pre-symptomatic, while anti-CPP3 IgG was associated with having RA (after adjustment for age, gender and SE, or *PTPN22)*. No associations were identified between anti-*P.gin* antibodies and classical RA risk factors, in line with data from our Study I. However, SE in combination with anti-CPP3 IgG revealed a stronger association with both pre-RA and RA, than SE alone, and the same was observed for smoking and anti-CPP3 IgG in RA.

It should be mentioned that a previous study failed to identify associations between anti-RgpB or anti-CPP3 IgG and pre-RA [144], although other studies have shown elevated anti-*P.gin* antibody levels in individuals at increased risk of developing RA [116, 117], and taken

together, we conclude that our data from Study III further supports an etiological role for *P. gingivalis* in the development of RA.

4.4 STUDY IV

Study IV focuses on the antibody response to the *P.*PAD derived citrullinated peptide CPP3, which we found elevated in pre-symptomatic RA as well as in RA patients, when compared to population-based controls in Study III. In a similar manner as with the antibody response to RgpB (Study II), we set out the characterise the anti-CPP3 antibody response in relation to RA risk factors and the classical ACPA response in the EIRA cohort, in order to address the hypothesis that *P.gin* may be driving ACPA-production in a subset of RA.

Presence of anti-CPP3 antibodies was analysed by ELISA, in 65 PD patients and 63 periodontally healthy controls, and in 2859 RA cases and 370 non-RA controls using the ISAC multiplex array, also containing citrullinated peptides from five human proteins. In addition, 218 human monoclonal antibodies (generated in-house by recombinant expression of immunoglobulins from RA B cells) were screened for CPP3 reactivity. Data on CCP2 status, genetics and smoking habits were retrieved from the EIRA database. Co-occurrence of anti-CPP3 antibodies with classical ACPA fine-specificities was investigated by pairwise Pearson's correlation; associations with RA risk factors were calculated by logistic regression.

Increased anti-CPP3 IgG levels were detected in PD compared to non-PD controls ($p<0.0001$), and this antibody response seemed to be citrulline-specific, since reactivity with the arginine-containing control peptide RPP3 was significantly lower. By using non-RA EIRA controls with unknown periodontal status to set cut-off for positivity, 11% of EIRA RA patients were anti-CPP3 antibody positive, and the majority was found within the CCP2-positive subset. Still, while the classical ACPAs showed high co-occurrence, anti-CPP3 IgG showed only weak correlations to other ACPAs (with strongest correlation identified for anti-Cit-vim$_{60\text{-}75}$ IgG), which may suggest partly different mechanisms for the production of anti-CPP3 IgG as compared to the classical ACPA response. Some support for this came from analyses of genetic risk factors, where anti-CPP3 IgG (unlike antibodies to CEP-1 and Cit-vim$_{60\text{-}75}$) showed no specific association with SE or *PTPN22*. However, a significant association was identified between smoking and anti-CPP3 IgG.

A total of four monoclonal antibodies derived from RA B cells, showed reactivity against CPP3, with low or no cross-reactivity against RPP3. Strikingly, one of these monoclonal antibodies (BVCA1:01A01) also bound CCP2, and Cit-vim$_{60\text{-}75}$, demonstrating cross-reactivity between bacterial and human citrullinated epitopes (Figure 6).

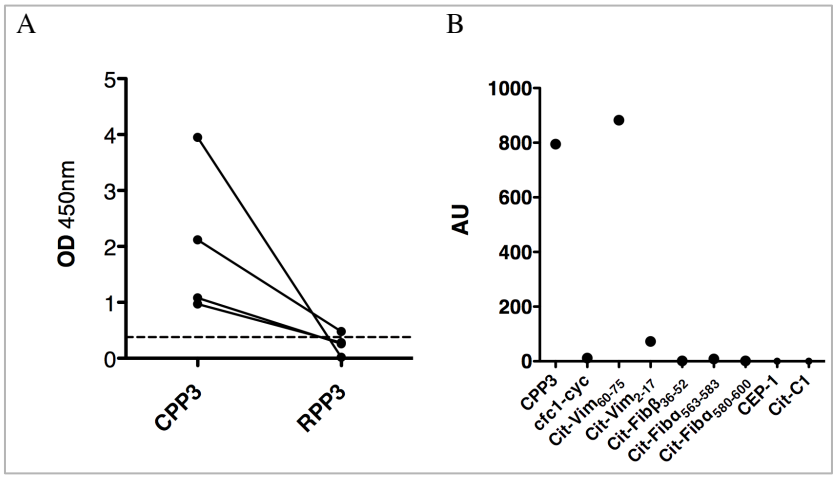

Figure 6 Reactivity of monoclonal antibodies derived from RA B cells. Four (out of 218) monoclonal antibodies showed reactivity against CPP3 in ELISA, with low or no reactivity against RPP3 (A). The monoclonal antibody with the strongest CPP3-reactivity (BVCA:01A01) also showed reactivity against citrullinated peptides derived from human proteins (in particular Cit-Vim$_{60\text{-}75}$), when analysed on the peptide multiplex array (B). OD = optical density; AU = arbitrary units.

Comparison of somatic mutations in the IgG V genes, revealed a higher number of mutations in BVCA1:01A01 than in the other CPP3-reactive monoclonal antibodies, suggesting extensive/repetitive autoantigen-driven activation of this B cell response. Based on our data, we speculate that an initial immune response against the CPP3 epitope of *P.gin* at some point cross-reacts with citrullinated human vimentin by mechanisms of molecular mimicry, and in SE-positive/*PTPN22*-positive individuals this may trigger activation of autoreactive T cells, clonal selection, affinity maturation and expansion of the ACPA response, in line with our etiological model presented in the Introduction (Figure 3).

In light of the data from Study IV, we would like to re-phrase our discussion on the CPP3 response in Study III, where we suggested that the anti-CPP3 antibody response: "*rather than being P.gin specific, simply belongs to the generic ACPA response, or rather represents cross-reactivity with another citrullinated antigen*". That conclusion was based on not finding a correlation between the anti-RgpB antibody response and the anti-CPP3 antibody response, and not finding an increased frequency of CPP3-positivity in pre-symptomatic individuals compared to controls. We now believe that this could (at least partly) be explained by the very high cut-off for CPP3-positivity used in the pre-RA study. That cut-off, as well as the cut-off used for the EIRA cohort in Study IV, was based on non-RA controls with unknown periodontal status. In study IV, we also show elevated anti-CPP3 IgG levels in approximately half of the PD patients, when compared to periodontally healthy controls, and moreover, that this antibody response is citrulline-specific. Hence, with a lower cut-off for positivity, more pre-symptomatic individuals would have been positive in Study III, but at the same time more population-based controls would have been positive. In an additional experiment, we

have now compared the anti-CPP3/RPP3 antibody response in PD (n=65) and non-PD (n=63), with CCP2-positive (n=101) and CCP2-negative RA (n=99), as well as non-RA controls (n=120), (Figure 7).

A B

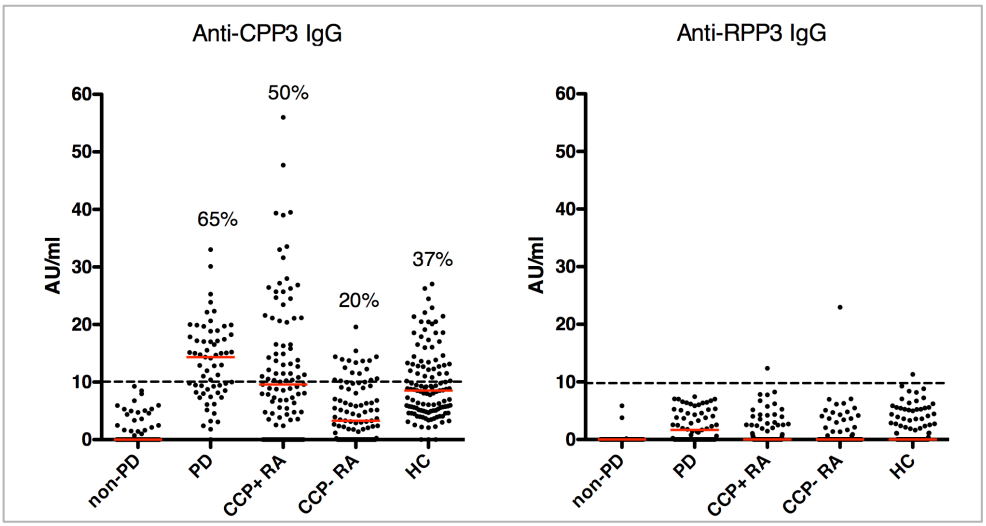

Figure 7 The antibody response to CPP3 (A) and RPP3 (B) in PD, CCP2-positive RA, CCP2-negative RA, and non-RA controls, in comparison to non-PD controls. The dotted line indicates cut-off for anti-CPP3/RPP3 IgG positivity. The percentages in graph A indicate the frequency of anti-CPP3 IgG positive individuals in each subset. AU = arbitrary units; HC = healthy controls (i.e. non-RA controls).

Setting a cut-off value at 10AU, based on CPP3-reactivity among non-PD controls, this analysis shows that 37% of non-RA controls have elevated anti-CPP3 IgG levels, which should be compared to 50% of CCP2-positive RA, and 65% of PD patients. Importantly, anti-RPP3 IgG levels were significantly lower in all subsets investigated, demonstrating that the antibody response to this *P.gin* epitope is citrulline-specific. To our knowledge, this is the first substantial ACPA response described in non-RA individuals. Note that high anti-CPP3 IgG levels (>25AU) were almost exclusively detected in CCP2-positive RA, and correspond to approximately 10% of all RA patients, which is similar as in Study III where 7.8% were anti-CPP3 antibody positive, and Study IV where 11% were anti-CPP3 antibody positive.

5 CONCLUSIONS AND FUTURE PERSPECTIVES

In **Study I** we provided novel data concerning the relationships between specific autoimmune reactions and genetic and environmental determinants for the risk of developing RA, with the overall conclusion that *HLA-DRB1* SE alleles, *PTPN22* polymorphism and cigarette smoking are associated with the presence of specific ACPA-reactivities rather than the magnitude of the ACPA response measured as total levels of anti-CCP2 antibodies. Our data thus emphasize the complexity of the gene–environment influence on ACPA-positive RA, and suggest that different ACPA fine-specificities may develop through partly different mechanisms.

Today, multiplex assays, like the one used by us in **Study IV** [126], and protein/tissue arrays - rather than single-antigen based ELISAs - are being used for studies of the broad ACPA response, where hundreds or even thousands of citrullinated epitopes can be analysed simultaneously. These types of studies will better pinpoint which proteins and specific epitopes are targeted *in vivo*, and at what time point during the disease process. Ongoing studies also focus on the hypothesis that different citrullinated peptides are presented by different SE-alleles. Recently after our study was published, another study was published, demonstrating that specific amino acids in specific positions in the peptide binding groove of the MHC class II molecule determine the association with ACPA-positive RA [145]. Extended studies of the ACPA fine-specificity response in relation to different SE alleles/different amino acids in the peptide-binding groove, as well as investigations of T cell responses to citrullinated peptides, will help uncover disease pathways in ACPA-positive RA, and form the basis for the development of novel therapies.

Study II, III and **IV** all focused on the etiological model that the periodontal bacteria *P. gingivalis* triggers ACPA-positive RA. The basis for this hypothesis is the unique feature of *P.gin* to express its own PAD enzyme, which is capable of the formation of new citrullinated antigens in the gum mucosa, and potentially also in the joint, since *P.gin* DNA has been detected in rheumatic joints [146]. Our studies clearly show that the antibody response to *P.gin* is associated with RA, in particular ACPA-positive RA. Since EIRA comprise patients with DMARD-naïve early RA, and since anti-*P.gin* antibody levels were found elevated also in blood samples collected before the onset of clinical RA, we do not think that this antibody response is a consequence of RA. Both antigens used in these studies, RgpB and CPP3, represents *P.gin*-specific antigens, hence we propose that anti-RgpB and anti-CPP3 antibodies could be used as surrogate markers for *P.gin* infection, past or present, and we conclude that our data support a causative role for *P.gingivalis* in the development of ACPA-positive RA. Moreover, that *P.gin* could be an etiological factor explaining the epidemiological link between PD and RA [95].

The most intriguing findings presented in this thesis were: [I)] the citrulline-specific antibody response against an epitope on *P.*PAD in non-RA individuals, and [II)] the cross-reactivity

between *P.gin* CPP3 and human Cit-vim$_{60\text{-}75}$ on a monoclonal antibody level. These data fit very well into the etiological model presented in figure 3 in the Introduction: antibodies against *P.gin* develop in response to *P.gin* infection, and if the *P.gin* strain expresses a *P.*PAD enzyme capable of autocitrullination, the CPP3 epitope may be exposed and trigger a CPP3-specific antibody response, which could cross-react with citrullinated epitopes on human proteins by mechanisms of molecular mimicry. *HLA-DRB1* SE and *PTPN22* were not specifically associated with the anti-CPP3 antibody response, but with the anti-Cit-vim$_{60\text{-}75}$ antibody response, suggesting that SE and *PTPN22* are not required for the anti-CPP3 antibody response, but increase the risk for the autoimmune ACPA response, and subsequently RA, to develop. The etiological model then propose that the expanded ACPA response eventually target citrullinated proteins exposed in the joint, form immune complexes, activate osteoclasts, and recruit neutrophils which undergo NETosis and release more PADs and citrullinated proteins, and this vicious circle ultimately cause chronic RA [3, 8].

In order to further investigate this hypothesis, and to more directly study the involvement of ACPAs in arthritis induction, we aim to develop an animal model based on ACPA transfer, following intraarticular *P.gin* injection. This model is bypassing the first step in the aetiological hypothesis (i.e. the production of ACPA); rather it is focusing on the second stage (i.e. the ability of ACPA to mediate chronic arthritis). To this date, we have injected mice intraarticularly with *P.gin* LPS. This local insult induces a transient joint inflammation that lasts for approximately a week. However, the induction of local citrullination (evaluated by Western blot using human ACPAs and mass spectrometry) seems to be rather poor. Hence, we will use *P.gin* lysates in future experiments, as *P.gin* lysates will contain additional virulence factors, including *P.*PAD/autocitrullinated *P.*PAD. After *P.gin* intraarticular injection, we will transfer human ACPAs, starting with the CPP3/Cit-vim$_{60\text{-}75}$ cross-reactive monoclonal BVCA:01A01 antibody, which has the ability to bind both autocitrullinated *P.*PAD and citrullinated vimentin, which was one of the endogenously citrullinated proteins detected by mass spectrometry in the mouse joints. This type of experiment could provide more direct evidence for ACPA's arthritogenic potential.

As a concluding remark, all our studies clearly points towards an involvement of both smoking and oral infection in the development of RA, especially in genetically predisposed individuals. Hence, in future studies we will evaluate anti-CPP3 IgG as a potential serological marker for the identification of PD patients at increased risk of developing RA. Moreover, we should encourage individuals at risk to never start smoking and to go for regular dental/oral check-ups, in order to try to prevent the development of RA.

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7 REFERENCES

1. Feldmann M, Brennan FM, Maini RN: **Rheumatoid arthritis**. *Cell* 1996, **85**(3):307-310.

2. McInnes IB, Schett G: **The pathogenesis of rheumatoid arthritis**. *N Engl J Med* 2011, **365**(23):2205-2219.

3. Catrina AI, Svensson CI, Malmstrom V, Schett G, Klareskog L: **Mechanisms leading from systemic autoimmunity to joint-specific disease in rheumatoid arthritis**. *Nat Rev Rheumatol* 2017, **13**(2):79-86.

4. Aletaha D, Neogi T, Silman AJ, Funovits J, Felson DT, Bingham CO, 3rd, Birnbaum NS, Burmester GR, Bykerk VP, Cohen MD *et al*: **2010 Rheumatoid arthritis classification criteria: an American College of Rheumatology/European League Against Rheumatism collaborative initiative**. *Arthritis Rheum* 2010, **62**(9):2569-2581.

5. Arnett FC, Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS, Healey LA, Kaplan SR, Liang MH, Luthra HS *et al*: **The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis**. *Arthritis Rheum* 1988, **31**(3):315-324.

6. Dekkers JS, Verheul MK, Stoop JN, Liu B, Ioan-Facsinay A, van Veelen PA, de Ru AH, Janssen GMC, Hegen M, Rapecki S *et al*: **Breach of autoreactive B cell tolerance by post-translationally modified proteins**. *Ann Rheum Dis* 2017, **76**(8):1449-1457.

7. Sokolove J, Johnson DS, Lahey LJ, Wagner CA, Cheng D, Thiele GM, Michaud K, Sayles H, Reimold AM, Caplan L *et al*: **Rheumatoid factor as a potentiator of anti-citrullinated protein antibody-mediated inflammation in rheumatoid arthritis**. *Arthritis Rheumatol* 2014, **66**(4):813-821.

8. Malmstrom V, Catrina AI, Klareskog L: **The immunopathogenesis of seropositive rheumatoid arthritis: from triggering to targeting**. *Nat Rev Immunol* 2017, **17**(1):60-75.

9. Schellekens GA, Visser H, de Jong BA, van den Hoogen FH, Hazes JM, Breedveld FC, van Venrooij WJ: **The diagnostic properties of rheumatoid arthritis antibodies recognizing a cyclic citrullinated peptide**. *Arthritis Rheum* 2000, **43**(1):155-163.

10. Schellekens GA, de Jong BA, van den Hoogen FH, van de Putte LB, van Venrooij WJ: **Citrulline is an essential constituent of antigenic determinants recognized by rheumatoid arthritis-specific autoantibodies**. *J Clin Invest* 1998, **101**(1):273-281.

11. Klareskog L, Stolt P, Lundberg K, Kallberg H, Bengtsson C, Grunewald J, Ronnelid J, Harris HE, Ulfgren AK, Rantapaa-Dahlqvist S *et al*: **A new model for an etiology of rheumatoid arthritis: smoking may trigger HLA-DR (shared epitope)-restricted immune reactions to autoantigens modified by citrullination**. *Arthritis Rheum* 2006, **54**(1):38-46.

12. Rantapaa-Dahlqvist S, de Jong BA, Berglin E, Hallmans G, Wadell G, Stenlund H, Sundin U, van Venrooij WJ: **Antibodies against cyclic citrullinated peptide and IgA rheumatoid factor predict the development of rheumatoid arthritis**. *Arthritis Rheum* 2003, **48**(10):2741-2749.

13. Ronnelid J, Wick MC, Lampa J, Lindblad S, Nordmark B, Klareskog L, van Vollenhoven RF: **Longitudinal analysis of citrullinated protein/peptide antibodies (anti-CP) during 5 year follow up in early rheumatoid arthritis: anti-CP status predicts worse disease activity and greater radiological progression**. *Ann Rheum Dis* 2005, **64**(12):1744-1749.

14. Harre U, Georgess D, Bang H, Bozec A, Axmann R, Ossipova E, Jakobsson PJ, Baum W, Nimmerjahn F, Szarka E *et al*: **Induction of osteoclastogenesis and bone loss by human autoantibodies against citrullinated vimentin**. *J Clin Invest* 2012, **122**(5):1791-1802.

15. Krishnamurthy A, Joshua V, Haj Hensvold A, Jin T, Sun M, Vivar N, Ytterberg AJ, Engstrom M, Fernandes-Cerqueira C, Amara K *et al*: **Identification of a novel chemokine-dependent molecular mechanism underlying rheumatoid arthritis-associated autoantibody-mediated bone loss**. *Ann Rheum Dis* 2016, **75**(4):721-729.

16. Wigerblad G, Bas DB, Fernades-Cerqueira C, Krishnamurthy A, Nandakumar KS, Rogoz K, Kato J, Sandor K, Su J, Jimenez-Andrade JM *et al*: **Autoantibodies to citrullinated proteins induce joint pain independent of inflammation via a chemokine-dependent mechanism**. *Ann Rheum Dis* 2016, **75**(4):730-738.

17. Witalison EE, Thompson PR, Hofseth LJ: **Protein Arginine Deiminases and Associated Citrullination: Physiological Functions and Diseases Associated with Dysregulation**. *Curr Drug Targets* 2015, **16**(7):700-710.

18. Baka Z, Gyorgy B, Geher P, Buzas EI, Falus A, Nagy G: **Citrullination under physiological and pathological conditions**. *Joint Bone Spine* 2012, **79**(5):431-436.

19. Knipp M, Vasak M: **A colorimetric 96-well microtiter plate assay for the determination of enzymatically formed citrulline**. *Anal Biochem* 2000, **286**(2):257-264.

20. Wang S, Wang Y: **Peptidylarginine deiminases in citrullination, gene regulation, health and pathogenesis**. *Biochim Biophys Acta* 2013, **1829**(10):1126-1135.

21. Ishida-Yamamoto A, Senshu T, Eady RA, Takahashi H, Shimizu H, Akiyama M, Iizuka H: **Sequential reorganization of cornified cell keratin filaments involving filaggrin-mediated compaction and keratin 1 deimination**. *J Invest Dermatol* 2002, **118**(2):282-287.

22. Vossenaar ER, Radstake TR, van der Heijden A, van Mansum MA, Dieteren C, de Rooij DJ, Barrera P, Zendman AJ, van Venrooij WJ: **Expression and activity of citrullinating peptidylarginine deiminase enzymes in monocytes and macrophages**. *Ann Rheum Dis* 2004, **63**(4):373-381.

23. Chang X, Han J: **Expression of peptidylarginine deiminase type 4 (PAD4) in various tumors**. *Mol Carcinog* 2006, **45**(3):183-196.

24. Chang X, Han J, Pang L, Zhao Y, Yang Y, Shen Z: **Increased PADI4 expression in blood and tissues of patients with malignant tumors**. *BMC Cancer* 2009, **9**:40.

25. Guo Q, Fast W: **Citrullination of inhibitor of growth 4 (ING4) by peptidylarginine deminase 4 (PAD4) disrupts the interaction between ING4 and p53**. *J Biol Chem* 2011, **286**(19):17069-17078.

26. Nakashima K, Hagiwara T, Ishigami A, Nagata S, Asaga H, Kuramoto M, Senshu T, Yamada M: **Molecular characterization of peptidylarginine deiminase in HL-60 cells induced by retinoic acid and 1alpha,25-dihydroxyvitamin D(3)**. *J Biol Chem* 1999, **274**(39):27786-27792.

27. Arita K, Hashimoto H, Shimizu T, Nakashima K, Yamada M, Sato M: **Structural basis for Ca(2+)-induced activation of human PAD4**. *Nat Struct Mol Biol* 2004, **11**(8):777-783.

28. Chavanas S, Mechin MC, Takahara H, Kawada A, Nachat R, Serre G, Simon M: **Comparative analysis of the mouse and human peptidylarginine deiminase gene clusters reveals highly conserved non-coding segments and a new human gene, PADI6**. *Gene* 2004, **330**:19-27.

29. Mechin MC, Coudane F, Adoue V, Arnaud J, Duplan H, Charveron M, Schmitt AM, Takahara H, Serre G, Simon M: **Deimination is regulated at multiple levels including auto-deimination of peptidylarginine deiminases**. *Cell Mol Life Sci* 2010, **67**(9):1491-1503.

30. Foulquier C, Sebbag M, Clavel C, Chapuy-Regaud S, Al Badine R, Mechin MC, Vincent C, Nachat R, Yamada M, Takahara H *et al*: **Peptidyl arginine deiminase type 2 (PAD-2) and PAD-4 but not PAD-1, PAD-3, and PAD-6 are expressed in rheumatoid arthritis synovium in close association with tissue inflammation**. *Arthritis Rheum* 2007, **56**(11):3541-3553.

31. Damgaard D, Senolt L, Nielsen MF, Pruijn GJ, Nielsen CH: **Demonstration of extracellular peptidylarginine deiminase (PAD) activity in synovial fluid of patients with rheumatoid arthritis using a novel assay for citrullination of fibrinogen**. *Arthritis Res Ther* 2014, **16**(6):498.

32. Reyes-Castillo Z, Munoz-Valle JF, Llamas-Covarrubias MA: **Clinical and immunological aspects of anti-peptidylarginine deiminase type 4 (anti-PAD4) autoantibodies in rheumatoid arthritis**. *Autoimmun Rev* 2018, **17**(2):94-102.

33. Halvorsen EH, Pollmann S, Gilboe IM, van der Heijde D, Landewe R, Odegard S, Kvien TK, Molberg O: **Serum IgG antibodies to peptidylarginine deiminase 4 in rheumatoid arthritis and associations with disease severity**. *Ann Rheum Dis* 2008, **67**(3):414-417.

34. Iwamoto T, Ikari K, Nakamura T, Kuwahara M, Toyama Y, Tomatsu T, Momohara S, Kamatani N: **Association between PADI4 and rheumatoid arthritis: a meta-analysis**. *Rheumatology (Oxford)* 2006, **45**(7):804-807.

35. Willis VC, Banda NK, Cordova KN, Chandra PE, Robinson WH, Cooper DC, Lugo D, Mehta G, Taylor S, Tak PP *et al*: **Protein arginine deiminase 4 inhibition is sufficient for the amelioration of collagen-induced arthritis**. *Clin Exp Immunol* 2017, **188**(2):263-274.

36. Bawadekar M, Shim D, Johnson CJ, Warner TF, Rebernick R, Damgaard D, Nielsen CH, Pruijn GJM, Nett JE, Shelef MA: **Peptidylarginine deiminase 2 is required for tumor necrosis factor alpha-induced citrullination and arthritis, but not neutrophil extracellular trap formation**. *J Autoimmun* 2017, **80**:39-47.

37. van Venrooij WJ, van Beers JJ, Pruijn GJ: **Anti-CCP Antibody, a Marker for the Early Detection of Rheumatoid Arthritis**. *Ann N Y Acad Sci* 2008, **1143**:268-285.

38. Khandpur R, Carmona-Rivera C, Vivekanandan-Giri A, Gizinski A, Yalavarthi S, Knight JS, Friday S, Li S, Patel RM, Subramanian V *et al*: **NETs are a source of citrullinated autoantigens and stimulate inflammatory responses in rheumatoid arthritis**. *Sci Transl Med* 2013, **5**(178):178ra140.

39. Schwenzer A, Jiang X, Mikuls TR, Payne JB, Sayles HR, Quirke AM, Kessler BM, Fischer R, Venables PJ, Lundberg K *et al*: **Identification of an immunodominant peptide from citrullinated tenascin-C as a major target for autoantibodies in rheumatoid arthritis**. *Ann Rheum Dis* 2016, **75**(10):1876-1883.

40. Wegner N, Lundberg K, Kinloch A, Fisher B, Malmstrom V, Feldmann M, Venables PJ: **Autoimmunity to specific citrullinated proteins gives the first clues to the etiology of rheumatoid arthritis**. *Immunol Rev* 2010, **233**(1):34-54.

41. Martinez G, Gomez JA, Bang H, Martinez-Gamboa L, Roggenbuck D, Burmester GR, Torres B, Prada D, Feist E: **Carbamylated vimentin represents a relevant autoantigen in Latin American (Cuban) rheumatoid arthritis patients**. *Rheumatol Int* 2016, **36**(6):781-791.

42. Lopez-Alemany R, Longstaff C, Hawley S, Mirshahi M, Fabregas P, Jardi M, Merton E, Miles LA, Felez J: **Inhibition of cell surface mediated plasminogen activation by a monoclonal antibody against alpha-Enolase**. *Am J Hematol* 2003, **72**(4):234-242.

43. Pancholi V: **Multifunctional alpha-enolase: its role in diseases**. *Cell Mol Life Sci* 2001, **58**(7):902-920.

44. Terrier B, Degand N, Guilpain P, Servettaz A, Guillevin L, Mouthon L: **Alpha-enolase: a target of antibodies in infectious and autoimmune diseases**. *Autoimmun Rev* 2007, **6**(3):176-182.

45. Kinloch A, Tatzer V, Wait R, Peston D, Lundberg K, Donatien P, Moyes D, Taylor PC, Venables PJ: **Identification of citrullinated alpha-enolase as a candidate autoantigen in rheumatoid arthritis**. *Arthritis Res Ther* 2005, **7**(6):R1421-1429.

46. Lundberg K, Kinloch A, Fisher BA, Wegner N, Wait R, Charles P, Mikuls TR, Venables PJ: **Antibodies to citrullinated alpha-enolase peptide 1 are specific for rheumatoid arthritis and cross-react with bacterial enolase**. *Arthritis Rheum* 2008, **58**(10):3009-3019.

47. Kinloch A, Lundberg K, Wait R, Wegner N, Lim NH, Zendman AJ, Saxne T, Malmstrom V, Venables PJ: **Synovial fluid is a site of citrullination of autoantigens in inflammatory arthritis**. *Arthritis Rheum* 2008, **58**(8):2287-2295.

48. Lundberg K, Wegner N, Yucel-Lindberg T, Venables PJ: **Periodontitis in RA-the citrullinated enolase connection**. *Nat Rev Rheumatol* 2010, **6**(12):727-730.

49. Sanchez-Pernaute O, Largo R, Calvo E, Alvarez-Soria MA, Egido J, Herrero-Beaumont G: **A fibrin based model for rheumatoid synovitis**. *Ann Rheum Dis* 2003, **62**(12):1135-1138.

50. Nielen MM, van der Horst AR, van Schaardenburg D, van der Horst-Bruinsma IE, van de Stadt RJ, Aarden L, Dijkmans BA, Hamann D: **Antibodies to citrullinated human fibrinogen (ACF) have diagnostic and prognostic value in early arthritis**. *Ann Rheum Dis* 2005, **64**(8):1199-1204.

51. Masson-Bessiere C, Sebbag M, Girbal-Neuhauser E, Nogueira L, Vincent C, Senshu T, Serre G: **The major synovial targets of the rheumatoid arthritis-specific antifilaggrin autoantibodies are deiminated forms of the alpha- and beta-chains of fibrin**. *J Immunol* 2001, **166**(6):4177-4184.

52. Clavel C, Nogueira L, Laurent L, Iobagiu C, Vincent C, Sebbag M, Serre G: **Induction of macrophage secretion of tumor necrosis factor alpha through Fcgamma receptor IIa engagement by rheumatoid arthritis-specific autoantibodies to citrullinated proteins complexed with fibrinogen**. *Arthritis Rheum* 2008, **58**(3):678-688.

53. Sokolove J, Zhao X, Chandra PE, Robinson WH: **Immune complexes containing citrullinated fibrinogen costimulate macrophages via Toll-like receptor 4 and Fcgamma receptor**. *Arthritis Rheum* 2011, **63**(1):53-62.

54. Mor-Vaknin N, Punturieri A, Sitwala K, Markovitz DM: **Vimentin is secreted by activated macrophages**. *Nat Cell Biol* 2003, **5**(1):59-63.

55. Bang H, Egerer K, Gauliard A, Luthke K, Rudolph PE, Fredenhagen G, Berg W, Feist E, Burmester GR: **Mutation and citrullination modifies vimentin to a novel autoantigen for rheumatoid arthritis**. *Arthritis Rheum* 2007, **56**(8):2503-2511.

56. Innala L, Kokkonen H, Eriksson C, Jidell E, Berglin E, Dahlqvst SR: **Antibodies against mutated citrullinated vimentin are a better predictor of disease activity at 24 months in early rheumatoid arthritis than antibodies against cyclic citrullinated peptides**. *J Rheumatol* 2008, **35**(6):1002-1008.

57. Courtenay JS, Dallman MJ, Dayan AD, Martin A, Mosedale B: **Immunisation against heterologous type II collagen induces arthritis in mice**. *Nature* 1980, **283**(5748):666-668.

58. Choi EK, Gatenby PA, McGill NW, Bateman JF, Cole WG, York JR: **Autoantibodies to type II collagen: occurrence in rheumatoid arthritis, other arthritides, autoimmune connective tissue diseases, and chronic inflammatory syndromes**. *Ann Rheum Dis* 1988, **47**(4):313-322.

59. Lundberg K, Nijenhuis S, Vossenaar ER, Palmblad K, van Venrooij WJ, Klareskog L, Zendman AJ, Harris HE: **Citrullinated proteins have increased immunogenicity and arthritogenicity and their presence in arthritic joints correlates with disease severity**. *Arthritis Res Ther* 2005, **7**(3):R458-467.

60. Burkhardt H, Sehnert B, Bockermann R, Engstrom A, Kalden JR, Holmdahl R: **Humoral immune response to citrullinated collagen type II determinants in early rheumatoid arthritis**. *Eur J Immunol* 2005, **35**(5):1643-1652.

61. Uysal H, Bockermann R, Nandakumar KS, Sehnert B, Bajtner E, Engstrom A, Serre G, Burkhardt H, Thunnissen MM, Holmdahl R: **Structure and pathogenicity of antibodies specific for citrullinated collagen type II in experimental arthritis**. *J Exp Med* 2009, **206**(2):449-462.

62. Haag S, Schneider N, Mason DE, Tuncel J, Andersson IE, Peters EC, Burkhardt H, Holmdahl R: **Identification of new citrulline-specific autoantibodies, which bind to human arthritic cartilage, by mass spectrometric analysis of citrullinated type II collagen**. *Arthritis Rheumatol* 2014, **66**(6):1440-1449.

63. Kaplan MJ, Radic M: **Neutrophil extracellular traps: double-edged swords of innate immunity**. *J Immunol* 2012, **189**(6):2689-2695.

64. Pratesi F, Tommasi C, Anzilotti C, Chimenti D, Migliorini P: **Deiminated Epstein-Barr virus nuclear antigen 1 is a target of anti-citrullinated protein antibodies in rheumatoid arthritis**. *Arthritis Rheum* 2006, **54**(3):733-741.

65. Sohn DH, Rhodes C, Onuma K, Zhao X, Sharpe O, Gazitt T, Shiao R, Fert-Bober J, Cheng D, Lahey LJ *et al*: **Local Joint inflammation and histone citrullination in a murine model of the transition from preclinical autoimmunity to inflammatory arthritis**. *Arthritis Rheumatol* 2015, **67**(11):2877-2887.

66. Kampstra ASB, Toes REM: **HLA class II and rheumatoid arthritis: the bumpy road of revelation**. *Immunogenetics* 2017, **69**(8-9):597-603.

67. Gregersen PK, Silver J, Winchester RJ: **The shared epitope hypothesis. An approach to understanding the molecular genetics of susceptibility to rheumatoid arthritis**. *Arthritis Rheum* 1987, **30**(11):1205-1213.

68. Begovich AB, Carlton VE, Honigberg LA, Schrodi SJ, Chokkalingam AP, Alexander HC, Ardlie KG, Huang Q, Smith AM, Spoerke JM *et al*: **A missense single-nucleotide polymorphism in a gene encoding a protein tyrosine phosphatase (PTPN22) is associated with rheumatoid arthritis**. *Am J Hum Genet* 2004, **75**(2):330-337.

69. Stanford SM, Bottini N: **PTPN22: the archetypal non-HLA autoimmunity gene**. *Nat Rev Rheumatol* 2014, **10**(10):602-611.

70. Vang T, Congia M, Macis MD, Musumeci L, Orru V, Zavattari P, Nika K, Tautz L, Tasken K, Cucca F *et al*: **Autoimmune-associated lymphoid tyrosine phosphatase is a gain-of-function variant**. *Nat Genet* 2005, **37**(12):1317-1319.

71. Kallberg H, Padyukov L, Plenge RM, Ronnelid J, Gregersen PK, van der Helm-van Mil AH, Toes RE, Huizinga TW, Klareskog L, Alfredsson L *et al*: **Gene-gene and gene-environment interactions involving HLA-DRB1, PTPN22, and smoking in two subsets of rheumatoid arthritis**. *Am J Hum Genet* 2007, **80**(5):867-875.

72. Kim K, Bang SY, Lee HS, Bae SC: **Update on the genetic architecture of rheumatoid arthritis**. *Nat Rev Rheumatol* 2017, **13**(1):13-24.

73. Plenge RM, Seielstad M, Padyukov L, Lee AT, Remmers EF, Ding B, Liew A, Khalili H, Chandrasekaran A, Davies LR *et al*: **TRAF1-C5 as a risk locus for rheumatoid arthritis--a genomewide study**. *N Engl J Med* 2007, **357**(12):1199-1209.

74. Vessey MP, Villard-Mackintosh L, Yeates D: **Oral contraceptives, cigarette smoking and other factors in relation to arthritis**. *Contraception* 1987, **35**(5):457-464.

75. Silman AJ, Newman J, MacGregor AJ: **Cigarette smoking increases the risk of rheumatoid arthritis. Results from a nationwide study of disease-discordant twins**. *Arthritis Rheum* 1996, **39**(5):732-735.

76. Stolt P, Bengtsson C, Nordmark B, Lindblad S, Lundberg I, Klareskog L, Alfredsson L, group Es: **Quantification of the influence of cigarette smoking on rheumatoid arthritis: results from a population based case-control study, using incident cases**. *Ann Rheum Dis* 2003, **62**(9):835-841.

77. Chang K, Yang SM, Kim SH, Han KH, Park SJ, Shin JI: **Smoking and rheumatoid arthritis**. *Int J Mol Sci* 2014, **15**(12):22279-22295.

78. Linn-Rasker SP, van der Helm-van Mil AH, van Gaalen FA, Kloppenburg M, de Vries RR, le Cessie S, Breedveld FC, Toes RE, Huizinga TW: **Smoking is a risk factor for anti-CCP antibodies only in rheumatoid arthritis patients who carry HLA-DRB1 shared epitope alleles**. *Ann Rheum Dis* 2006, **65**(3):366-371.

79. Lundstrom E, Kallberg H, Alfredsson L, Klareskog L, Padyukov L: **Gene-environment interaction between the DRB1 shared epitope and smoking in the risk of anti-citrullinated protein antibody-positive rheumatoid arthritis: all alleles are important**. *Arthritis Rheum* 2009, **60**(6):1597-1603.

80. Jiang X, Alfredsson L, Klareskog L, Bengtsson C: **Smokeless tobacco (moist snuff) use and the risk of developing rheumatoid arthritis: results from a case-control study**. *Arthritis Care Res (Hoboken)* 2014, **66**(10):1582-1586.

81. Makrygiannakis D, Hermansson M, Ulfgren AK, Nicholas AP, Zendman AJ, Eklund A, Grunewald J, Skold CM, Klareskog L, Catrina AI: **Smoking increases peptidylarginine deiminase 2 enzyme expression in human lungs and increases citrullination in BAL cells**. *Ann Rheum Dis* 2008, **67**(10):1488-1492.

82. Svard A, Skogh T, Alfredsson L, Ilar A, Klareskog L, Bengtsson C, Kastbom A: **Associations with smoking and shared epitope differ between IgA- and IgG-class antibodies to cyclic citrullinated peptides in early rheumatoid arthritis**. *Arthritis Rheumatol* 2015, **67**(8):2032-2037.

83. Mikuls TR, Hughes LB, Westfall AO, Holers VM, Parrish L, van der Heijde D, van Everdingen M, Alarcon GS, Conn DL, Jonas B *et al*: **Cigarette smoking, disease severity and autoantibody expression in African Americans with recent-onset rheumatoid arthritis**. *Ann Rheum Dis* 2008, **67**(11):1529-1534.

84. Alspaugh MA, Henle G, Lennette ET, Henle W: **Elevated levels of antibodies to Epstein-Barr virus antigens in sera and synovial fluids of patients with rheumatoid arthritis**. *J Clin Invest* 1981, **67**(4):1134-1140.

85. Ferrell PB, Aitcheson CT, Pearson GR, Tan EM: **Seroepidemiological study of relationships between Epstein-Barr virus and rheumatoid arthritis**. *J Clin Invest* 1981, **67**(3):681-687.

86. Takahashi Y, Murai C, Shibata S, Munakata Y, Ishii T, Ishii K, Saitoh T, Sawai T, Sugamura K, Sasaki T: **Human parvovirus B19 as a causative agent for rheumatoid arthritis**. *Proc Natl Acad Sci U S A* 1998, **95**(14):8227-8232.

87. Soderberg-Naucler C: **Does cytomegalovirus play a causative role in the development of various inflammatory diseases and cancer?** *J Intern Med* 2006, **259**(3):219-246.

88. Costenbader KH, Karlson EW: **Epstein-Barr virus and rheumatoid arthritis: is there a link?** *Arthritis Res Ther* 2006, **8**(1):204.

89. Mehraein Y, Lennerz C, Ehlhardt S, Remberger K, Ojak A, Zang KD: **Latent Epstein-Barr virus (EBV) infection and cytomegalovirus (CMV) infection in synovial tissue of autoimmune chronic arthritis determined by RNA- and DNA-in situ hybridization**. *Mod Pathol* 2004, **17**(7):781-789.

90. Niedobitek G, Lisner R, Swoboda B, Rooney N, Fassbender HG, Kirchner T, Aigner T, Herbst H: **Lack of evidence for an involvement of Epstein-Barr virus infection of synovial membranes in the pathogenesis of rheumatoid arthritis**. *Arthritis Rheum* 2000, **43**(1):151-154.

91. Peterlana D, Puccetti A, Beri R, Ricci M, Simeoni S, Borgato L, Scilanga L, Ceru S, Corrocher R, Lunardi C: **The presence of parvovirus B19 VP and NS1 genes in the synovium is not correlated with rheumatoid arthritis**. *J Rheumatol* 2003, **30**(9):1907-1910.

92. Sherina N, Hreggvidsdottir HS, Bengtsson C, Hansson M, Israelsson L, Alfredsson L, Lundberg K: **Low levels of antibodies against common viruses associate with anti-citrullinated protein antibody-positive rheumatoid arthritis; implications for disease aetiology**. *Arthritis Res Ther* 2017, **19**(1):219.

93. Chen J, Wright K, Davis JM, Jeraldo P, Marietta EV, Murray J, Nelson H, Matteson EL, Taneja V: **An expansion of rare lineage intestinal microbes characterizes rheumatoid arthritis**. *Genome Med* 2016, **8**(1):43.

94. Chen HH, Huang N, Chen YM, Chen TJ, Chou P, Lee YL, Chou YJ, Lan JL, Lai KL, Lin CH *et al*: **Association between a history of periodontitis and the risk of rheumatoid arthritis: a nationwide, population-based, case-control study**. *Ann Rheum Dis* 2013, **72**(7):1206-1211.

95. Fuggle NR, Smith TO, Kaul A, Sofat N: **Hand to Mouth: A Systematic Review and Meta-Analysis of the Association between Rheumatoid Arthritis and Periodontitis**. *Front Immunol* 2016, **7**:80.

96. How KY, Song KP, Chan KG: **Porphyromonas gingivalis: An Overview of Periodontopathic Pathogen below the Gum Line**. *Front Microbiol* 2016, **7**:53.

97. Hajishengallis G, Lamont RJ: **Beyond the red complex and into more complexity: the polymicrobial synergy and dysbiosis (PSD) model of periodontal disease etiology**. *Mol Oral Microbiol* 2012, **27**(6):409-419.

98. Potempa J, Mydel P, Koziel J: **The case for periodontitis in the pathogenesis of rheumatoid arthritis**. *Nat Rev Rheumatol* 2017, **13**(10):606-620.

99. Baek KJ, Ji S, Kim YC, Choi Y: **Association of the invasion ability of Porphyromonas gingivalis with the severity of periodontitis**. *Virulence* 2015, **6**(3):274-281.

100. Potempa J, Sroka A, Imamura T, Travis J: **Gingipains, the major cysteine proteinases and virulence factors of Porphyromonas gingivalis: structure, function and assembly of multidomain protein complexes**. *Curr Protein Pept Sci* 2003, **4**(6):397-407.

101. McGraw WT, Potempa J, Farley D, Travis J: **Purification, characterization, and sequence analysis of a potential virulence factor from Porphyromonas gingivalis, peptidylarginine deiminase**. *Infect Immun* 1999, **67**(7):3248-3256.

102. Koziel J, Mydel P, Potempa J: **The link between periodontal disease and rheumatoid arthritis: an updated review**. *Curr Rheumatol Rep* 2014, **16**(3):408.

103. Wegner N, Wait R, Sroka A, Eick S, Nguyen KA, Lundberg K, Kinloch A, Culshaw S, Potempa J, Venables PJ: **Peptidylarginine deiminase from Porphyromonas gingivalis citrullinates human fibrinogen and alpha-enolase: implications for autoimmunity in rheumatoid arthritis**. *Arthritis Rheum* 2010, **62**(9):2662-2672.

104. Gawron K, Bereta G, Nowakowska Z, Lazarz-Bartyzel K, Lazarz M, Szmigielski B, Mizgalska D, Buda A, Koziel J, Oruba Z *et al*: **Peptidylarginine deiminase from Porphyromonas gingivalis contributes to infection of gingival fibroblasts and**

induction of prostaglandin E2 -signaling pathway. *Mol Oral Microbiol* 2014, **29**(6):321-332.

105. Quirke AM, Lugli EB, Wegner N, Hamilton BC, Charles P, Chowdhury M, Ytterberg AJ, Zubarev RA, Potempa J, Culshaw S *et al*: **Heightened immune response to autocitrullinated Porphyromonas gingivalis peptidylarginine deiminase: a potential mechanism for breaching immunologic tolerance in rheumatoid arthritis**. *Ann Rheum Dis* 2014, **73**(1):263-269.

106. Stobernack T, Glasner C, Junker S, Gabarrini G, de Smit M, de Jong A, Otto A, Becher D, van Winkelhoff AJ, van Dijl JM: **Extracellular Proteome and Citrullinome of the Oral Pathogen Porphyromonas gingivalis**. *J Proteome Res* 2016, **15**(12):4532-4543.

107. Rosenstein ED, Greenwald RA, Kushner LJ, Weissmann G: **Hypothesis: the humoral immune response to oral bacteria provides a stimulus for the development of rheumatoid arthritis**. *Inflammation* 2004, **28**(6):311-318.

108. Nesse W, Westra J, van der Wal JE, Abbas F, Nicholas AP, Vissink A, Brouwer E: **The periodontium of periodontitis patients contains citrullinated proteins which may play a role in ACPA (anti-citrullinated protein antibody) formation**. *J Clin Periodontol* 2012, **39**(7):599-607.

109. Harvey GP, Fitzsimmons TR, Dhamarpatni AA, Marchant C, Haynes DR, Bartold PM: **Expression of peptidylarginine deiminase-2 and -4, citrullinated proteins and anti-citrullinated protein antibodies in human gingiva**. *J Periodontal Res* 2013, **48**(2):252-261.

110. Konig MF, Abusleme L, Reinholdt J, Palmer RJ, Teles RP, Sampson K, Rosen A, Nigrovic PA, Sokolove J, Giles JT *et al*: **Aggregatibacter actinomycetemcomitans-induced hypercitrullination links periodontal infection to autoimmunity in rheumatoid arthritis**. *Sci Transl Med* 2016, **8**(369):369ra176.

111. de Pablo P, Dietrich T, McAlindon TE: **Association of periodontal disease and tooth loss with rheumatoid arthritis in the US population**. *J Rheumatol* 2008, **35**(1):70-76.

112. Arkema EV, Karlson EW, Costenbader KH: **A prospective study of periodontal disease and risk of rheumatoid arthritis**. *J Rheumatol* 2010, **37**(9):1800-1804.

113. Eriksson K, Nise L, Kats A, Luttropp E, Catrina AI, Askling J, Jansson L, Alfredsson L, Klareskog L, Lundberg K *et al*: **Prevalence of Periodontitis in Patients with Established Rheumatoid Arthritis: A Swedish Population Based Case-Control Study**. *PLoS One* 2016, **11**(5):e0155956.

114. Bender P, Burgin WB, Sculean A, Eick S: **Serum antibody levels against Porphyromonas gingivalis in patients with and without rheumatoid arthritis - a systematic review and meta-analysis**. *Clin Oral Investig* 2017, **21**(1):33-42.

115. Bae SC, Lee YH: **Association between anti-Porphyromonas gingivalis antibody, anti-citrullinated protein antibodies, and rheumatoid arthritis : A meta-analysis**. *Z Rheumatol* 2017.

116. Hitchon CA, Chandad F, Ferucci ED, Willemze A, Ioan-Facsinay A, van der Woude D, Markland J, Robinson D, Elias B, Newkirk M *et al*: **Antibodies to porphyromonas gingivalis are associated with anticitrullinated protein**

antibodies in patients with rheumatoid arthritis and their relatives. *J Rheumatol* 2010, **37**(6):1105-1112.

117. Mikuls TR, Thiele GM, Deane KD, Payne JB, O'Dell JR, Yu F, Sayles H, Weisman MH, Gregersen PK, Buckner JH *et al*: **Porphyromonas gingivalis and disease-related autoantibodies in individuals at increased risk of rheumatoid arthritis**. *Arthritis Rheum* 2012, **64**(11):3522-3530.

118. Bello-Gualtero JM, Lafaurie GI, Hoyos LX, Castillo DM, De-Avila J, Munevar JC, Unriza S, Londono J, Valle-Onate R, Romero-Sanchez C: **Periodontal Disease in Individuals With a Genetic Risk of Developing Arthritis and Early Rheumatoid Arthritis: A Cross-Sectional Study**. *J Periodontol* 2016, **87**(4):346-356.

119. Marchesan JT, Gerow EA, Schaff R, Taut AD, Shin SY, Sugai J, Brand D, Burberry A, Jorns J, Lundy SK *et al*: **Porphyromonas gingivalis oral infection exacerbates the development and severity of collagen-induced arthritis**. *Arthritis Res Ther* 2013, **15**(6):R186.

120. de Aquino SG, Abdollahi-Roodsaz S, Koenders MI, van de Loo FA, Pruijn GJ, Marijnissen RJ, Walgreen B, Helsen MM, van den Bersselaar LA, de Molon RS *et al*: **Periodontal pathogens directly promote autoimmune experimental arthritis by inducing a TLR2- and IL-1-driven Th17 response**. *J Immunol* 2014, **192**(9):4103-4111.

121. Maresz KJ, Hellvard A, Sroka A, Adamowicz K, Bielecka E, Koziel J, Gawron K, Mizgalska D, Marcinska KA, Benedyk M *et al*: **Porphyromonas gingivalis facilitates the development and progression of destructive arthritis through its unique bacterial peptidylarginine deiminase (PAD)**. *PLoS Pathog* 2013, **9**(9):e1003627.

122. World Medical A: **World Medical Association Declaration of Helsinki: ethical principles for medical research involving human subjects**. *JAMA* 2013, **310**(20):2191-2194.

123. Saevarsdottir S, Wedren S, Seddighzadeh M, Bengtsson C, Wesley A, Lindblad S, Askling J, Alfredsson L, Klareskog L: **Patients with early rheumatoid arthritis who smoke are less likely to respond to treatment with methotrexate and tumor necrosis factor inhibitors: observations from the Epidemiological Investigation of Rheumatoid Arthritis and the Swedish Rheumatology Register cohorts**. *Arthritis Rheum* 2011, **63**(1):26-36.

124. Berglin E, Padyukov L, Sundin U, Hallmans G, Stenlund H, Van Venrooij WJ, Klareskog L, Dahlqvist SR: **A combination of autoantibodies to cyclic citrullinated peptide (CCP) and HLA-DRB1 locus antigens is strongly associated with future onset of rheumatoid arthritis**. *Arthritis Res Ther* 2004, **6**(4):R303-308.

125. Potempa J, Nguyen KA: **Purification and characterization of gingipains**. *Curr Protoc Protein Sci* 2007, **Chapter 21**:Unit 21 20.

126. Hansson M, Mathsson L, Schlederer T, Israelsson L, Matsson P, Nogueira L, Jakobsson PJ, Lundberg K, Malmstrom V, Serre G *et al*: **Validation of a multiplex chip-based assay for the detection of autoantibodies against citrullinated peptides**. *Arthritis Res Ther* 2012, **14**(5):R201.

127. Amara K, Steen J, Murray F, Morbach H, Fernandez-Rodriguez BM, Joshua V, Engstrom M, Snir O, Israelsson L, Catrina AI *et al*: **Monoclonal IgG antibodies**

generated from joint-derived B cells of RA patients have a strong bias toward citrullinated autoantigen recognition. *J Exp Med* 2013, **210**(3):445-455.

128. Amara K, Clay E, Yeo L, Ramsköld D, al e: **B cells expressing the IgA receptor FcRL4 participate in the autoimmune response in patients with rheumatoid arthritis**. *Journal of autoimmunity* 2017, **81**:34-43.

129. Steen J, Sahlström P, Ndlovu W, Odowd V, al. e: **Plasma cell derived monoclonal anti-citrulline antibodies from ra synovial fluid are multireactive**. *Ann Rheum Dis* 2016, **75**:A2.24.

130. Titcombe PJ, Amara K, Barsness LO, Zhang N, Krishnamurthy A, Shmagel A, Hansson M, Israelsson L, Sahlstrom P, Giacobbe L *et al*: **Citrullinated Self Antigen-Specific Blood B Cells Carry Cross-Reactive Immunoglobulins with Effector Potential**. *Annals of the Rheumatic Diseases* 2016, **75**:A28-+.

131. Tiller T, Meffre E, Yurasov S, Tsuiji M, Nussenzweig MC, Wardemann H: **Efficient generation of monoclonal antibodies from single human B cells by single cell RT-PCR and expression vector cloning**. *J Immunol Methods* 2008, **329**(1-2):112-124.

132. Mouquet H, Klein F, Scheid JF, Warncke M, Pietzsch J, Oliveira TY, Velinzon K, Seaman MS, Nussenzweig MC: **Memory B cell antibodies to HIV-1 gp140 cloned from individuals infected with clade A and B viruses**. *PLoS One* 2011, **6**(9):e24078.

133. Rothman KJ, Greenland S, Walker AM: **Concepts of interaction**. *Am J Epidemiol* 1980, **112**(4):467-470.

134. Mahdi H, Fisher BA, Kallberg H, Plant D, Malmstrom V, Ronnelid J, Charles P, Ding B, Alfredsson L, Padyukov L *et al*: **Specific interaction between genotype, smoking and autoimmunity to citrullinated alpha-enolase in the etiology of rheumatoid arthritis**. *Nat Genet* 2009, **41**(12):1319-1324.

135. Hosmer DW, Lemeshow S: **Confidence interval estimation of interaction**. *Epidemiology* 1992, **3**(5):452-456.

136. Ossipova E, Cerqueira CF, Reed E, Kharlamova N, Israelsson L, Holmdahl R, Nandakumar KS, Engstrom M, Harre U, Schett G *et al*: **Affinity purified anti-citrullinated protein/peptide antibodies target antigens expressed in the rheumatoid joint**. *Arthritis Res Ther* 2014, **16**(4):R167.

137. Reed E, Jiang X, Kharlamova N, Ytterberg AJ, Catrina AI, Israelsson L, Mathsson-Alm L, Hansson M, Alfredsson L, Ronnelid J *et al*: **Antibodies to carbamylated alpha-enolase epitopes in rheumatoid arthritis also bind citrullinated epitopes and are largely indistinct from anti-citrullinated protein antibodies**. *Arthritis Res Ther* 2016, **18**(1):96.

138. Ioan-Facsinay A, el-Bannoudi H, Scherer HU, van der Woude D, Menard HA, Lora M, Trouw LA, Huizinga TW, Toes RE: **Anti-cyclic citrullinated peptide antibodies are a collection of anti-citrullinated protein antibodies and contain overlapping and non-overlapping reactivities**. *Ann Rheum Dis* 2011, **70**(1):188-193.

139. Verpoort KN, Cheung K, Ioan-Facsinay A, van der Helm-van Mil AH, de Vries-Bouwstra JK, Allaart CF, Drijfhout JW, de Vries RR, Breedveld FC, Huizinga TW *et al*: **Fine specificity of the anti-citrullinated protein antibody response is influenced by the shared epitope alleles**. *Arthritis Rheum* 2007, **56**(12):3949-3952.

140. Takizawa Y, Suzuki A, Sawada T, Ohsaka M, Inoue T, Yamada R, Yamamoto K: **Citrullinated fibrinogen detected as a soluble citrullinated autoantigen in rheumatoid arthritis synovial fluids**. *Ann Rheum Dis* 2006, **65**(8):1013-1020.

141. Tachibana-Ono M, Yoshida A, Kataoka S, Ansai T, Shintani Y, Takahashi Y, Toyoshima K, Takehara T: **Identification of the genes associated with a virulent strain of Porphyromonas gingivalis using the subtractive hybridization technique**. *Oral Microbiol Immunol* 2008, **23**(1):84-87.

142. Sundqvist G, Figdor D, Hanstrom L, Sorlin S, Sandstrom G: **Phagocytosis and virulence of different strains of Porphyromonas gingivalis**. *Scand J Dent Res* 1991, **99**(2):117-129.

143. Brink M, Hansson M, Mathsson L, Jakobsson PJ, Holmdahl R, Hallmans G, Stenlund H, Ronnelid J, Klareskog L, Rantapaa-Dahlqvist S: **Multiplex analyses of antibodies against citrullinated peptides in individuals prior to development of rheumatoid arthritis**. *Arthritis Rheum* 2013, **65**(4):899-910.

144. Fisher BA, Cartwright AJ, Quirke AM, de Pablo P, Romaguera D, Panico S, Mattiello A, Gavrila D, Navarro C, Sacerdote C *et al*: **Smoking, Porphyromonas gingivalis and the immune response to citrullinated autoantigens before the clinical onset of rheumatoid arthritis in a Southern European nested case-control study**. *BMC Musculoskelet Disord* 2015, **16**:331.

145. Raychaudhuri S, Sandor C, Stahl EA, Freudenberg J, Lee HS, Jia X, Alfredsson L, Padyukov L, Klareskog L, Worthington J *et al*: **Five amino acids in three HLA proteins explain most of the association between MHC and seropositive rheumatoid arthritis**. *Nat Genet* 2012, **44**(3):291-296.

146. Reichert S, Haffner M, Keysser G, Schafer C, Stein JM, Schaller HG, Wienke A, Strauss H, Heide S, Schulz S: **Detection of oral bacterial DNA in synovial fluid**. *J Clin Periodontol* 2013, **40**(6):591-598.