From Department of Medical Biochemistry and Biophysics
Karolinska Institutet, Stockholm, Sweden

ANTIBODIES AS PATHOGENIC FACTORS AND BIOMARKERS IN RHEUMATOID ARTHRITIS

Erik Lönnblom

Karolinska Institutet

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Cover illustration: Painting by Eugène Delacroix depicting the Sack of Constantinople in 1204. *"It is tragic that the assailants, who set out to secure free access for Christians to the Holy Land, turned against their brothers in the faith."* - Pope John Paul II

Antibodies as Pathogenic Factors and Biomarkers in Rheumatoid Arthritis
THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Erik Lönnblom

The thesis will be defended in public at Gustaf Retzius Lecture hall, Berzelius väg 3, Karolinska Institutet, Solna, Friday the 11:th of June 2021, at 9:00 am

Principal Supervisor:
Professor Rikard Holmdahl, MD, PhD
Karolinska Institutet
Department of Medical Biochemistry and Biophysics
Division of Medical Inflammation Research

Co-supervisor(s):
Professor Kutty Selva Nandakumar, PhD
Southern Medical University
School of Pharmaceutical Sciences
SMU-KI United Medical Inflammation Center

Dr. Chris Kessel, PhD
WWU Medical Center (UKM)
Department of Pediatric Rheumatology & Immunology
Division of Translation Inflammation Research

Opponent:
Professor Carl Turesson, MD, PhD
Lund University
Department of Clinical Sciences
Division of Rheumatology

Examination Board:
Professor Kristina Lejon, PhD
Umeå University
Department of Microbiology
Division of Immunology/Immunochemistry

Dr. Aikaterini Chatzidionysiou, MD, PhD
Karolinska University Hospital
Rheumatology Unit

Assistant Professor Lina Marcela Diaz-Gallo, PhD
Karolinska Institutet
Department of Medicine
Division of Rheumatology

What we have done for ourselves alone dies with us; what we have done for others and the world remains and is immortal.
– Albert Pike

I hate quotations. Tell me what you know.
– Ralph Waldo Emerson

POPULAR SCIENCE SUMMARY OF THE THESIS

The largest leap in the evolution of our immune system occurred around 400 million years ago with the development of a new compartment known as the adaptive immune system. Instead of relying on inherited immune receptors passed down over generations, a new set of tools allowed the random generation of new immune receptors capable of binding almost any antigen, even those never encountered before. While the new system greatly improved our ability to quickly adapt to new pathogens, the random receptors also posed a new risk; some of the new receptors might target antigens in our own body. This new weapon to protect the body was the equivalent of a nuclear bomb in the evolutionary arms race between host and pathogen. Although instead of fearing mutual destruction from a nuclear war, we were, from this point forward, at risk of destroying our own body. Several control mechanisms developed to prevent the immune system turning against us. Despite this, investigators today have identified over 100 different types of autoimmune diseases targeting almost every imaginable tissue, from head to toe.

One of the most common autoimmune diseases is rheumatoid arthritis (RA) which affects nearly 1% of the world population, predominantly women. In RA the joints are attacked by the immune system causing joint inflammation and pain, this eventually leads to joint destruction if not treated. The most common biomarkers used to diagnose RA in the clinics are autoantibodies; the origin and function of these are however not known.

The work presented in this thesis is an attempt at discovering the origin and function of different subclasses of autoantibodies. One of the subclasses are antibodies against type II collagen (CII), the major protein in the joint cartilage. These antibodies have previously been shown to be associated with the onset of RA, and cause arthritis when injected in animals. To better understand them, we have synthesized a library of CII peptides and used them to screen the serum of more than 4000 RA patients and 1500 healthy controls, confirming that several of them are associated with RA.

If these antibodies could be identified in patients at an early stage, they may become the target for new treatment strategies. Depleting or neutralizing them could reduce symptoms such as antibody mediated pain or may even prevent RA from becoming established.

There is currently no diagnostic test available for these antibodies, although the hope is that the new knowledge generated during this PhD project may help generate new biomarkers to aid in the diagnosis and treatment of RA.

ABSTRACT

Ever since the evolution of an adaptive immune system capable of creating immune receptors that may recognize self-antigens, we have been at risk of autoimmunity. There are over 100 different types of autoimmune diseases targeting almost every available tissue from head to toe, with joints and connective tissues being a common target. In addition to autoimmune diseases, infections and degenerative joint diseases can cause joint inflammation making differential diagnosis between them difficult.

The most common autoimmune disease to afflict the joints is rheumatoid arthritis (RA), affecting nearly 1% of the world population, predominantly women. The etiology of RA is not known, although it involves interaction between multiple genes and environmental risk factors. It is characterized by chronic inflammation of the joints, which without successful treatment can lead to joint destruction. One of the hallmarks of RA is the presence of autoantibodies, often observed in serum several years before any symptoms of disease. The two classes of autoantibodies focused on today are rheumatoid factors (RF) and anti-citrullinated protein antibodies (ACPA), the latter being a highly specific biomarker for a large subset of RA-patients. The ACPA have greatly aided in diagnosing RA in many patients. Yet their function and origin are still not known. Nevertheless, a subset of patients still lacks a specific biomarker.

All studies in this thesis have autoantibodies in arthritis as a common theme, and four of them use a bead-based multiplex platform established during the PhD-project. In **Study I**, we explored the hypothesized link between periodontitis induced by the oral pathogen *P.gingivalis*, and its effect on arthritis progression and the production of ACPA. This study revealed a citrulline specific antibody response against *P.gingivalis* peptidyl arginine deiminase derived peptide, although the link to the arthritis development could not be confirmed. In **Study II**, we synthesized a library of triple helical peptides (THP) as a tool to characterize antibodies against type II collagen (CII). The peptides were tested in two cohorts of RA patients, as well as on monoclonal antibodies (mAb), and in collagen induced arthritis. The THPs were subsequently used in **Study III** to elucidate the specificity and function of antibodies against type XI collagen (CXI), revealing a shared epitope between CXI and CII in mice, rats and humans with arthritis. In addition, the THPs were also used in **Study IV** to explore the cross-reactivity of a joint-reactive mouse ACPA, demonstrating a molecular mechanism of how an ACPA can trigger arthritis. For **Study V**, the specificity of several human ACPA were dissected with a bead based multiplex assay and compared to polyclonal responses in two RA cohorts. Crystal structures of the ACPA revealed for the first time the structural basis of how human ACPA bind citrulline residues on different peptides.

The data presented in this thesis provide further evidence that the major determinant of the arthritogenicity of antibodies lies in their ability to cross-react to joint proteins. Dissecting these specificities may lead to the establishment of new clinical biomarkers.

LIST OF SCIENTIFIC PAPERS

I. **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, L Lundmark A, Hellvard A, Lundberg K, Jansson L, Holmdahl R, Yucel-Lindberg T.
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* These authors contributed equally.

II. **Synthesis of an Array of Triple-Helical Peptides from Type II Collagen for Multiplex Analysis of Autoantibodies in Rheumatoid Arthritis**
Viljanen J, **Lönnblom E**, Ge C, Yang J, Cheng L, Aldi S, Cai W, Kastbom A, Sjöwall C, Gjertsson I, Holmdahl R, Kihlberg J.
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III. A Shared Epitope of Collagen Type XI and Type II Is Recognized by Pathogenic Antibodies in Mice and Humans with Arthritis
Tong D, **Lönnblom E**, Yau ACY, Nandakumar KS, Liang B, Ge C, Viljanen J, Li L, Bãlan M, Klareskog L, Chagin AS, Gjertsson I, Kihlberg J, Zhao M, Holmdahl R.
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VI. **Natural polymorphism of Ym1 regulates pneumonitis through alternative activation of macrophages**
Zhu W, **Lönnblom E**, Förster M, Johannesson M, Tao P, Meng L, Lu S, Holmdahl R.
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VII. **Cartilage-binding antibodies initiate joint inflammation and promote chronic erosive arthritis**
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VIII. **The autoantibody response to cyclic citrullinated collagen type II peptides in rheumatoid arthritis**
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X. **Influence of hydrocarbon oil structure on adjuvanticity and autoimmunity**
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CONTENTS

LIST OF ABBREVIATIONS

ACPA	Anti-citrullinated protein antibodies
ACR	American College of Rheumatology
AMPA	Anti-modified protein antibodies
Anti-CarP	Anti-carbamylated protein antibodies
AKA	Antikeratin antibodies
APK	Antiperinuclear Factor
BCR	B cell receptor
CAIA	Collagen antibody induced arthritis
CCP	Cyclic citrullinated peptides
CCP2	Cyclic citrullinated peptides, version 2
CD	Circular Dichroism
CIA	Collagen induced arthritis
CII	Type II collagen
CEP-1	Citrullinated human alpha-enolase peptide 1
CFA	Complete Freund’s adjuvant
CLR	C-type lectin receptors
COMP	Cartilage oligomeric matrix protein
CPP3	Citrullinated *P*.PAD peptide 3
CTLA4	Cytotoxic T-lymphocyte-associated protein 4
CXI	Type XI collagen
DAMP	Damage-associated molecular patterns
DIP	Distal interphalangeal
EULAR	The European League Against Rheumatism
GPI	Glucose-6-phospho-isomerase
IgG	Immunoglobulin G
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MCP	Metacarpophalangeal
MHC	Major histocompatibility complex
NET	Neutrophil extracellular trap

NLR	NOD-like receptors
PAD	Peptidyl Arginase Deiminase
PAMP	Pathogen-associated molecular patterns
P.gingivalis	*Porphyromonas gingivalis*
P.PAD	*P.gingivalis* PAD enzyme
PRRs	Pattern recognition receptors
PTM	Posttranslational modification
PTPN22	Protein tyrosine phosphatase, non-receptor type 22
RA	Rheumatoid arthritis
RF	Rheumatoid factor
RgpB	Arg-gingipain B
PIA	Pristane induced arthritis
PIP	Proximal interphalangeal
ROS	Reactive oxygen species
RLR	RIG-I-like receptors
SE	Shared epitope
SLE	Systemic lupus erythematosus
TCR	T cell receptors
THP	Triple helical peptide
TLR	Toll-like receptors
TNF-α	Tumor necrosis factor-alpha

1 INTRODUCTION

1.1 THE IMMUNE SYSTEM

The role of the immune system is to protect the organism from diseases caused by foreign organisms and materials such as bacteria, viruses, fungi, and toxins. Even though we lack records from the earliest forms of life, it can be argued that the evolution of the immune system started with the origin of life. At a first glance the complexity of the human immune system involving a multitude of organs, cells and small molecules could be mistaken for an example of intelligent design. Yet as we examine our evolutionary ancestors, we can follow the gradual evolution of the immune system along the phylogenetic tree of life[1].

The degree of complexity of the immune system varies between organisms of different kingdoms and species, although it can be divided into different layers of protection. Surface barriers are the first line of defence to keep pathogens outside. They can be either mechanical, chemical, or biological. In humans they can be exemplified by the skin, low-pH environment in the stomach and commensal flora.

1.2 INNATE IMMUNITY

If the surface barriers are breached, the immediate response to the threat is orchestrated by the innate immune system, which is found in nearly all forms of life. Even bacteria have simple heritable defense mechanisms[2], while all multicellular organisms appear to have a complex innate immune system[3,4]. The first function of the innate immune system is to identify the pathogens. This is done through pattern recognition receptors (PRRs)[5], which are mainly expressed on cells of the innate immune system, such as macrophages, monocytes, dendritic cells, neutrophils, and mast cells. PRRs recognize molecules of two different classes: pathogen-associated molecular patterns (PAMP), which are conserved molecular motifs expressed on pathogens and damage-associated molecular patterns (DAMP) released by host cells as a result of cell damage or cell death. Examples of PRRs include Toll-like receptors (TLR) capable of binding lipopolysaccharide (LPS) of Gram-negative bacteria[6], and C-type lectin receptors (CLR) binding carbohydrate structures such as mannan on fungi cell walls[7]. The two other families of PRRs are Nod-like receptor (NLR) and RIG-I-like receptor (RLR)[8]. Once the PRRs of the immune system have been activated, cells release inflammatory mediators to recruit neutrophils and macrophages to the site of inflammation. Some of the effector mechanisms to kill the pathogens include phagocytosis, complement system and release of reactive oxygen species (ROS)[9].

1.3 ADAPTIVE IMMUNITY

The final line of defense is the adaptive immune system. The principal components of our adaptive immune system are T and B lymphocytes, which through somatic recombination can assemble T and B cell receptors (TCR and BCR) specific for an immense range of antigens. In contrast to the innate immunity, an adaptive immunity response is not immediate. However, it is able to form immunological memory. These components are found in all jawed vertebrates

and marks a clear distinction in the evolution of the immune system, although jawless vertebrates carry a precursor to our adaptive immune system with other recombinatorial genes[10]. To put this into perspective, the earliest fossil records of jawed vertebrates are more than 400 million years old[11]. The random generation of TCRs and BCRs does however have the potential to generate receptors capable of recognizing self-antigens. To address this problem the development of T and B cells undergo a stringent process of selection to ensure self-tolerance; any cell not following the protocol may soon find itself on a path to autoimmunity[12].

1.4 RHEUMATOID ARTHRITIS

Rheumatoid arthritis (RA) is an autoimmune disease characterized by chronic inflammation of peripheral joints causing cartilage destruction and bone erosion. The disease occurs in up to 1% of the population, with women more often being affected than men. While the peripheral joints of the hands, feet and wrists are frequently affected, it is considered a systemic disease since it can also affect cardiovascular, skeletal, and respiratory systems. The cause of RA is not known, although the current dogma is that for a subset of patients, autoimmunity starts in a mucosal tissue in response to a chronic inflammation. This subset of RA patients is characterized by anti-citrullinated protein antibodies (ACPA) present in around 70% of patients[13], measured in clinics using the commercial CCP2-test (cyclic citrullinated peptides kit, version 2). ACPA are a heterogenous subset of antibodies that can be detected in 1/5 of the patients already 10 years before onset of arthritis[14]. The other prominent autoantibody is rheumatoid factor (RF), often present together with ACPA[15]. The strongest genetic association to RA is with the HLA-DRB1 allele which together with environmental risk factors such as cigarette smoking show an epistatic interaction in the ACPA positive subset of RA patients[16]. There is currently no cure for RA, although the introduction of biological drugs targeting pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) has significantly reduced the burden of disease[17].

1.5 CLASSIFICATION OF RA

> *In a disease such as rheumatoid arthritis in which the etiology is unknown and in which there is no available proof of the diagnosis, a broad description of the disease usually suffices for teaching and for diagnosis in individual cases. When such a method is used for classifying patients for study, however, there is little uniformity in the cases included in any series labelled rheumatoid arthritis.* (Ropes MW et al, 1957)[18]

One of the first attempts to classify RA was made by the American Rheumatism Association (today known as ACR, American College of Rheumatology) in 1957, in which patients needed to fulfill 5 out of 11 criteria to be classified as "definite" RA[18]. There were gradual modifications to the criteria over time, although it was not until a major overhaul leading to the 1987 ACR revised classification criteria for RA[19], that this classification became widespread. The revised 1987 guidelines were designed with the intention to be more specific creating a stricter definition of RA. In addition, it also removed 3 earlier criteria which required invasive procedures.

Table 1. The 1987 ACR revised classification criteria for RA.

Criterion	Description
Morning stiffness	Morning stiffness in and around the joints, lasting at least one hour before maximal improvement.
Arthritis of three or more joint areas	At least three joint areas (out of 14 possible areas; right or left PIP, MCP, wrist, elbow, knee, ankle, MTP joints) simultaneously have had soft tissue swelling or fluid (not bony overgrowth alone) as observed by a physician.
Arthritis of hand joints	At least one area swollen (as defined above) in a wrist, MCP, or PIP joint.
Symmetric arthritis	Simultaneous involvement of the same joint areas (as defined above) on both sides of the body (bilateral involvement of PIPs, MCPs, or MTPs, without absolute symmetry is acceptable).
Rheumatoid nodules	Subcutaneous nodules over bony prominences or extensor surfaces, or in juxta-articular regions as observed by a physician.
Serum rheumatoid factor	Demonstration of abnormal amounts of serum rheumatoid factor by any method for which the result has been positive in less than 5 percent of normal control subjects.
Radiographic changes	Radiographic changes typical of rheumatoid arthritis on posteroanterior hand or wrist radiographs, which must include erosions or unequivocal bony decalcification localized in, or most marked adjacent to, the involved joints (osteoarthritis changes alone do not qualify).
Note: For classification purposes, a patient has RA if at least four of these criteria are satisfied (the first four must have been present for at least six weeks).	

Table adapted from Arnett et. al, The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis, Arthritis & Rheumatism (1988) 31(3) 315-324

Table 2. 2010 ACR/EULAR RA classification criteria

Domain	Category	Point score
A	**Joint involvement (0–5 points)**	
	1 large joint	**0**
	2–10 large joints	**1**
	1–3 small joints (large joints not counted)	**2**
	4–10 small joints (large joints not counted)	**3**
	>10 joints including at least one small joint	**5**
B	**Serology (0–3 points)**	
	Negative RF and negative ACPA	**0**
	Low positive RF or low positive ACPA	**2**
	High positive RF or high positive ACPA	**3**
C	**Acute-phase reactants (0–1 point)**	
	Normal CRP and normal ESR	**0**
	Abnormal CRP or abnormal ESR	**1**
D	**Duration of symptoms**	
	<6 weeks	**0**
	≥6 weeks	**1**
The points from each of domains A through D are added and the sum is considered to be the total score. A total score of ≥6 is needed to classify a patient as having definite RA.		

Table adapted from Aletaha D, Neogi T, Silman AJ, et al. 2010 Rheumatoid arthritis classification criteria: an American College of Rheumatology/European League Against Rheumatism collaborative initiative, Arthritis Rheum, 2010, vol. 62 (pg. 2569-81)

1.6 GENETICS OF RA

The heritability of RA is estimated to be around 60% based on twin studies, with a concordance rate among monozygotic twins at 15-30%[20]. The major histocompatibility complex (MHC) region contributes to more than half of the identified genetic heritability, with a set of HLA-DRB1 alleles known as "shared epitope" having the largest contribution[21]. Shared epitope is a five amino acid motif (70-74) in the DRβ1 chain with either QKRAA, QRRAA or RRRAA shared between many DRB1 alleles. The most strongly associated allele in Caucasians is the DRB1*0401 which in addition also encodes risk associated amino acids in position 11, 71 and 74[22]. Over 100 risk loci have been identified in RA[23], although the disease association among the individual non-MHC genes is much weaker. Many of these are involved in regulation of the adaptive immune system (e.g., loci containing PTPN22, CD28 and CTLA4). Importantly, the MHC class II region and several non-MHC genes are associated with a specific autoantibody response to citrullinated proteins (anti-citrullinated protein antibodies, ACPA)[24]. While the heritability is the same for ACPA positive and negative RA, there is no predominant contribution of specific HLA-alleles in ACPA negative RA[12]. Another locus found in genetic studies of Japanese and Korean cohorts contains PADI4 which encodes the enzyme Peptidyl Arginine Deiminase 4 (PAD4) expressed in myeloid cells. PAD4 is proposed to be the PAD enzyme responsible for generating citrullinated autoantigens during chronic inflammation in mucosal tissues[25]. Surprisingly, this locus is associated to both ACPA positive and negative RA[16,26]. The total contribution of the risk genes identified so far cannot explain the estimated heritability in RA[16], this gap is known as "the problem of missing heritability", is seen in many complex diseases and is hypothesized to be caused by rare genetic variants, gene-gene interactions or gene-environment interactions not yet discovered[27]. Another explanation could be that the heritability from early twin studies is overestimated. However, a recent study on monozygotic twins shows that 15% have a substantial number of early developmental mutations specific to only one of them, implicating we may have underestimated the contribution of heritability in twin studies.[28]

1.7 ENVIRONMENTAL RISK FACTORS

1.7.1 Cigarette smoking

The strongest environmental risk factor to date is cigarette smoking that has been demonstrated in a multitude of cohorts with RA patients[29–33]. The association with smoking also shows a possible epistatic effect together with several genetic risk factors highlighting the complex interactions between genes and environment in RA[16,34,35]. Adding to the complexity, cigarette smoke is a complex mixture of more than 7000 chemicals. One major component of cigarette smoke is nicotine which induces an anti-inflammatory effect through the cholinergic pathway and is shown to be protective in several autoimmune diseases and animal models of RA[36,37]. Nicotine is however also reported to induce formation of neutrophil extracellular traps (NETs) which release intracellular components that may act as autoantigens[38]. There are in addition other components of cigarette smoke shown to correlate with RA[39,40].

1.7.2 Aerosols

Cigarette smoking is only one of the many aerosols that have been implicated in the etiology of RA as well as in several other autoimmune diseases; other examples are air pollution, silica dust, textile dust, pesticides, wood dust and mineral oils. Although they may act through different molecular pathways for the breach of self-tolerance, the common denominator is that these compounds may induce a chronic inflammation in the lung and in addition many of them show an epistatic effect together with cigarette smoking[16,41].

1.7.3 Periodontitis

Periodontitis is an inflammation of the periodontium, which in its severe chronic form affects 10% of the world population[42]. The association between oral health and inflammatory arthritis was described already by Hippocrates in ancient Greece who suggested pulling out teeth could cure arthritis. In addition to sharing pathophysiological similarities, they also have genetic (HLA-DRB1) and environmental (cigarette smoking) risk factors in common.

1.7.4 Porphyromonas gingivalis

One of the bacteria causing periodontitis (PD) is *Porphyromonas gingivalis (P.gingivalis)* found in around 80% of patients with periodontal disease[43]. It expresses a PAD enzyme (*P*.PAD) capable of citrullination[44] not found in any other prokaryote to date. The proposed mechanism is that *P*.PAD will generate citrullinated epitopes of bacterial origin, which through a process of molecular mimicry will lead to cross-reactivity to citrullinated self-antigens (Figure 1)[45]. Ever since a hypothesis that citrullination by *P*.PAD could be a causative link between PD and RA[46] was presented, the scientific community has put considerable effort into investigating this with mixed results[45,47,48]. While most reported experimental models show an increased severity in the group treated with *P.gingivalis*, one major problem in characterizing the ACPA response is the lack of a non-citrullinated control; the CCP2-kit used in most studies does not include one. Moreover, other virulence factors in *P.gingivalis* such as LPS, collagenase and arginine gingipain B (RgpB) may also have a contribution to the link between PD and RA[49]. In summary, although epidemiological and experimental data support the role of *P. gingivalis* in the etiology of RA, there is yet no conclusive experimental evidence proving that citrullination by *P*.PAD is the causative link[47].

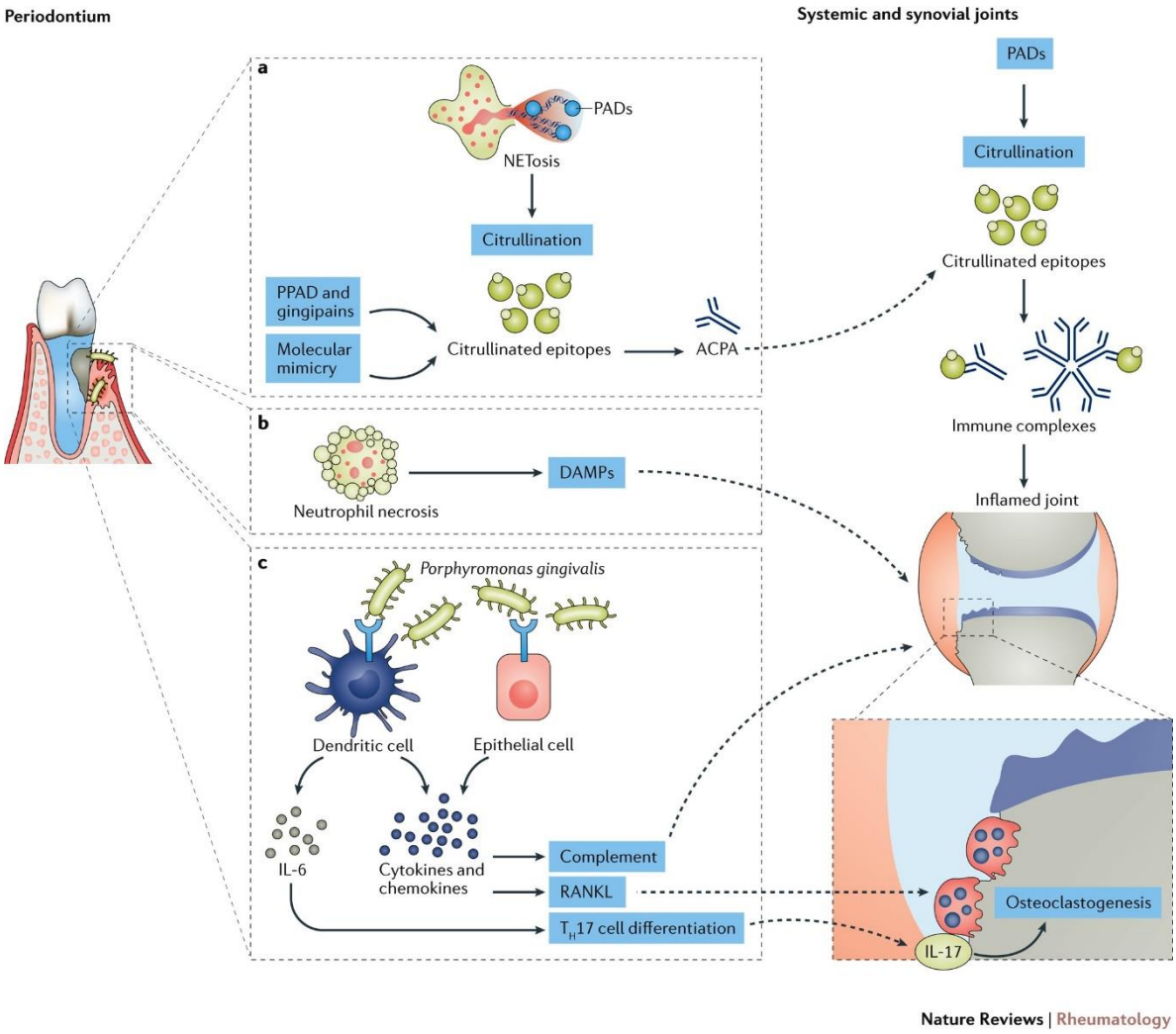

Figure 1. Proposed mechanisms underlying the links between periodontal disease and the pathogenesis of rheumatoid arthritis. *Figure from Potempa, et. al. Nat Rev Rheumatol 13, 606–620 (2017). Reprinted with permission from Nature.*

1.7.5 Aggregatibacter actinomycetemcomitans (Aa)

While the scientific community studying the possible link between PD and RA has almost exclusively focused on *P.gingivalis*, a study from 2016 by Konig *et. al.*[50] proposed the periodontal pathogen *Aggregatibacter actinomycetemcomitans* (*Aa*) as a candidate microbe responsible for the break of tolerance in RA. The proposed mechanism is that a pore-forming leukotoxin-A (LtxA) produced by *Aa* induces hypercitrullination in neutrophils, which through a process similar to NET formation exposes citrullinated self-antigens in the gingival tissue. Reports on experimental animal models exploring the link are so far scarce[51], most certainly due to earlier reports that LtxA is specific for leukocytes of human or Old World primate origin[52], although this narrow specificity is disputed[53]. The role of *Aa* in the development of RA has so far yielded contradictory data[47,54–57].

1.7.6 Other infectious agents

The idea that a pathogen may cause RA is not new, early theories suggested that the disease originated in pre-Columbian America and was spread to the Old World by the Europeans[58]. One of the most well studied pathogens is Epstein-Barr virus[59–61] which has been detected in the synovial fluid in RA patients[62]. Subsequent theories include an unknown microbe spreading through blood transfusions[63] and subclinical urinary tract infections by *Proteus mirablis*[64].

1.8 STRUCTURE OF COLLAGEN

Collagens are the most abundant proteins; it is present in all animals with cells differentiated into tissues and makes up approximately 30% of the protein of mammals[65]. A total of 28 distinct collagen proteins have been described, with the main fibril-forming collagens (type I, II, III, V and XI) accounting for 80-90% of all collagens in humans. The structural motif that defines the collagen proteins is their ability to form a triple helix made up by three polypeptide chains (Figure 2). The trimers are made up of either three identical polypeptide chains to form homotrimers (type II), or different chains to form heterotrimer (type I, XI)[66].

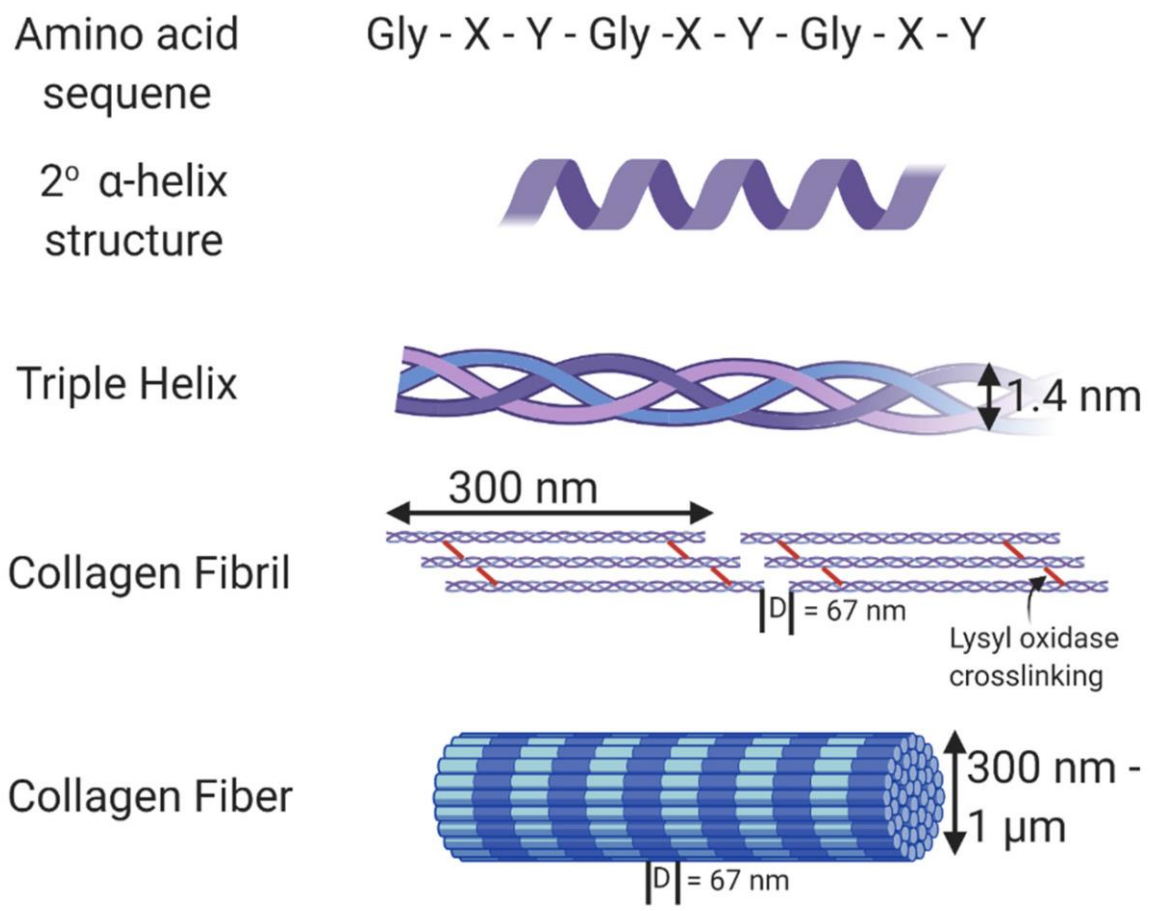

Figure 2. Structure of collagen. The amino acid sequence of collagen consists of Gly-Xaa-Yaa repeats, with Xaa and Yaa commonly occupied by proline and hydroxyproline. This unique sequence allows collagen to form an α helix secondary structure. Fibrillar collagen is a triple helix containing crosslinks formed through the action of lysyl oxidase. In vivo, these collagen fibrils form fibers with varying thickness and a D-banding pattern of 67 nm. *Figure from Walimbe et.al., Best of Both Hydrogel Worlds: Harnessing Bioactivity and Tunability by Incorporating Glycosaminoglycans in Collagen Hydrogels.Bioengineering. 2020; 7(4):156.*

1.8.1 Type II collagen (CII)

CII is the major protein of the articular cartilage covering the joint surfaces and is made up of three identical α1(II) chains translated from the *Col2a1* gene. In addition, it is also found in the cartilage of the nose, larynx, trachea and the vitreous. In similarity to other collagens, the sequence is highly conserved between species and the major B-cell epitopes are shared between humans, primates and rodents[67].

1.8.2 Type XI collagen (CXI)

CXI is a heterotrimer composed of three distinct chains (α1(XI), α2(XI), α3(XI)). CXI forms heterotypic fibrils together with CII and type IX collagen (CIX) and is often colocalized with CII. The exact structure of the heterotypic fibrils is not determined, but it is hypothesized that CXI constitutes the inner core of the fibrils and therefore is less exposed (Figure 3). The α3(XI) is expressed by the *Col2a1*. As a result, it is identical to the α1(II) chain in CII apart from a higher degree of hydroxylation and glycosylation[68].

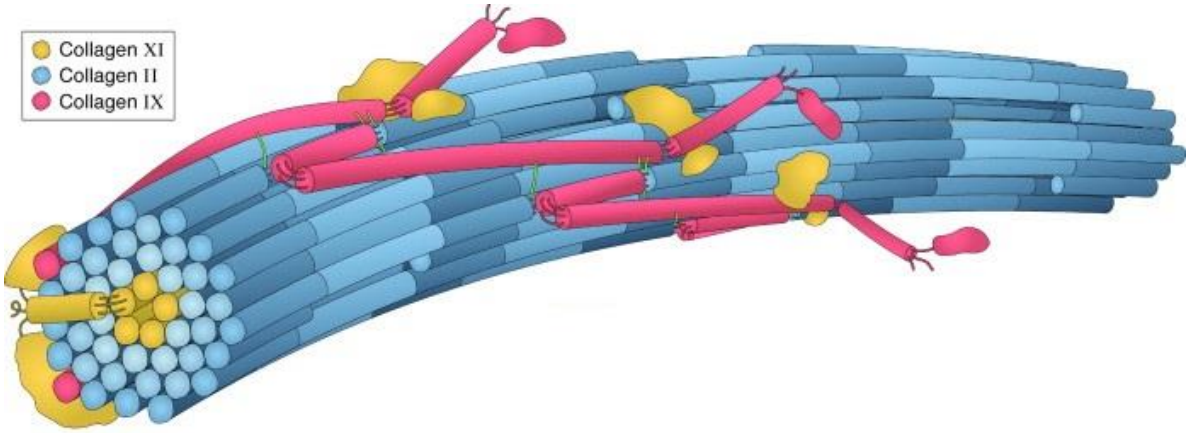

Figure 3. Three-dimensional model of the type II/IX/XI heterotypic fibril of developing cartilage matrix. *Reprinted from Methods, 45/1, Eyre et. al. Advances in collagen cross-link analysis, 65-74., Copyright (2008), with permission from Elsevier*

1.9 ANIMAL MODELS

The most commonly used animal for medical research is the mouse, and advances in recent decades to create genetically modified mice has made it into the model organism of choice for immunological studies[69]. Our latest common ancestor with the mouse is estimated at around 65 million years ago and our immune systems have since evolved not only to different ecological niches, but also different body size and life span. Some of the major differences that have emerged between the humans and mice immune system include the balance between neutrophils and lymphocytes in peripheral blood, Toll receptors, Immunoglobulin subsets, Fc receptors and regulation and development of different B- and T-cell subsets[70]. Despite the lengthy period on diverging evolutionary paths using the mouse and other animals as a model organism for the immune system has been instrumental for our current knowledge[71] and alternatives to animal models to study complex diseases are limited[72].

1.9.1 Collagen induced arthritis (CIA)

The current gold standard for preclinical experiments in arthritis is collagen induced arthritis (CIA) where CII is emulsified in complete Freund's adjuvant[73]. Similar to RA, a major genetic risk factor for CIA is the MHC haplotype requiring a CII permissive MHC such as H-2^r or H-2^q[74]. CIA can also induce arthritis in genetically susceptible rats and several species of monkeys, revealing that the major B-cell epitopes are conserved between species[75]. The disease development is dependent on both T and B-cells, leading to the production of anti-CII antibodies which are the major effector mechanism for disease induction. Finally, it was an important tool in development and testing of several biologically based therapeutics, such as IL-1[76], IL-6[77] anti-TNF-α[78].

1.9.2 Collagen type XI induced arthritis ($C^{XI}IA$)

A variant of the traditional CIA model is to substitute CII with CXI in the experimental protocol[79]. CXI forms a heterotrimer from three different chains (α1(XI), α2(XI), α3(XI)), one of which (α3) is shared with CII. As a result, a subset of anti-CXI antibodies can cross-react to CII[80]. Arthritis induced with homologous CXI leads to a more pronounced chronic relapsing arthritis compared to CIA[81], displaying increased titers of anti-CXI antibodies during the chronic phase. The model is also MHC-dependent, albeit in rats, the associated MHC haplotypes differs between CIA and $C^{XI}IA$ [82].

1.9.3 Collagen antibody induced arthritis (CAIA)

The antibodies induced in CIA can be isolated and used to transfer disease in a model called collagen antibody induced arthritis (CAIA)[83]. Importantly, the dominant B-cell epitopes in CII are conserved between species[67,84] and antibodies against different epitopes have been shown to be either pathogenic or protective[85], yet all tested anti-collagen antibodies induce pain[86]. The standard CAIA cocktail uses 4 mAbs specific for C1, J1, U1 and the D3 epitope and is boosted with LPS[87]. The CAIA model is B- and T-cell independent and does not involve the priming phase in contrast to other models such as CIA. Consequently, it is used to study the disease onset phase and mechanisms for antibody mediated inflammation.

1.9.4 Pristane induced arthritis (PIA)

Arthritis can also be induced in rats through the immunization of different mineral oils[88], pristane induced arthritis (PIA) is the most studied model due to high incidence (99,6%) and excellent reproducibility[89]. PIA has a quick onset starting around 10 days with a peak at around 20 days. Thereafter, the inflammation steadily decreases before the start of a chronic relapsing disease with new inflammation. Isolated $CD4^+$ T-cells can transfer the disease[90], regulated by CLRs[91]. PIA induces the production of both RF, anti-CII[92] and anti-CXI antibodies[93]. However, antibodies are not known to play a major role in the pathogenesis of PIA[94]. Interestingly, pristane induces chronic relapsing arthritis only in susceptible rat strains. In contrast, mice immunized with pristane display phenotypically different symptoms and instead develop a systemic lupus erythematosus (SLE) like disease in susceptible strains[95].

1.9.5 Other experimental arthritis models

In addition to collagens, the immunization of other cartilage associated proteins such as cartilage oligomeric matrix protein (COMP)[96] and aggrecan[97] can induce arthritis. The ubiquitous protein glucose-6-phospho-isomerase (GPI) can also induce arthritis upon immunization with an adjuvant[98]. The unlikely autoantigen was discovered in the K/BxN model as a result of transgenic T-cells specific for GPI, leading to the production of anti-GPI antibodies precipitating on the cartilage surface[99] due to an interaction with proteoglycans[100]. These antibodies induce arthritis in the K/BxN serum transfer model through similar molecular mechanisms as CAIA[101]. Another similarity between GPI and CII as autoantigens is that arthritis induction through protein immunization is MHC dependent with an association to the H-2^q haplotype[98].

Many other proteins have also been implicated as autoantigens in RA due to the cross-reactivity of ACPA including vimentin, fibrinogen, filaggrin, α-enolase and histones. There have been attempts to use these proteins to establish new arthritis models[102], yet it is debated if any of them can induce experimental arthritis in a reproducible fashion[103]. The absence of data from experimental animal models does not exclude them as potential autoantigens. None of the animal models captures the full complexity of RA, instead each of them is an attempt to explain a limited phase or pathway of the disease.

1.10 AUTOANTIBODIES IN RA

1.10.1 Rheumatoid Factors (RF)

Rheumatoid Factors (RF) are antibodies binding the Fc region of immunoglobulin G (IgG). They were identified in patients with RA more than 70 years ago[104]. The name RF was given due to the association with RA, although they are present both in other rheumatic diseases and non-rheumatic conditions, as well as in healthy individuals, especially the elderly[105]. The high prevalence of low-affinity RF during many infections points towards a key role in host defense[106] prior to the induction of the high affinity RF observed in autoimmune disorders. The reported frequency in RA is between 60-90% and has a specificity of 85%. RF was the only autoantibody to be included in the 1987 ACR RA classification criteria, and since the introduction of standardized test for ACPA it is now interpreted together with ACPA status in clinical settings as exemplified in 2010 ACR/EULAR classification criteria for RA[107]. The presence of RF is correlated with a more erosive disease and studies exploring the interaction between RF and ACPA show a significant additive effect in the double positive subset (RF+/ACPA+) of patients[108,109].

1.10.2 Anti-citrullinated proteins antibodies (ACPA)

The first observation of RA-specific antibodies was reported in 1964 and were named Antiperinuclear Factor (APK)[110]. It was followed by antikeratin antibodies (AKA)[111] which were shown to bind the protein filaggrin[112]. Monoclonal antibodies against filaggrin showed that APK and AKA were in fact the same RA-specific antibody[113]. Despite the high specificity

for RA, the inconvenient testing method required hindered their use as a serological marker for clinical use. It was not until the discovery that citrulline was the antigenic target[114] that standardized assays could be developed and ACPAs were later included in the 2010 ACR/EULAR classification criteria for RA[107]. The standard assay used for detection of ACPA is the CCP2 ELISA test which contains a set of undisclosed synthetic cyclic citrullinated peptides with a reported sensitivity of over 70% and specificity above 98%[115]. In addition, there are several reports that the standard CCP2 assay doesn't capture all ACPA, up to 20% of the CCP2 negative patients have antibodies binding other citrullinated antigens[116]. Even though ACPA have a high specificity for RA compared to RF[105], they are also present in 1-2% of the general population without RA[117]. During a 3-year follow up of non-RA ACPA positive individuals in the Swedish twin registry only 8,5% developed RA, indicating that a positive ACPA status defined by the CCP2-kit has a limited predictive value for the onset of RA in the general healthy population[118]. For this reason, ACPA are currently only used as a biomarker for individuals which have symptoms of RA or other joint-related diseases.

1.10.3 Anti-modified protein antibodies (AMPA)

In recent years, antibodies binding other posttranslational modifications (PTMs) such as homocitrulline and acetylation have also been found[119,120]. There has been a controversy if these antibodies are in fact a distinct class of autoantibodies in RA[121]. A recent report studying BCR sequences from B-cells using citrullinated or acetylated antigens revealed that most mAbs were highly cross-reactive between multiple PTMs[120]. The high cross-reactivity of antibodies between different PTMs has led to the proposed model that the term ACPA should be replaced by AMPA: anti-modified protein antibody.

1.10.4 Anti-CII antibodies

The first report of collagen as a candidate autoantigen RA was made by Steffen in 1970[122], although the initial studies in the field did not make clear distinction between different types of collagen making comparisons between studies confusing. The turning point came with the establishment of an experimental arthritis model comparing different types of collagen, identifying type II collagen (CII) as the major candidate[123]. While the link to the pathogenesis has been difficult to prove experimentally for other autoantibodies in RA, the arthritogenicity of anti-CII antibodies has been thoroughly investigated in experimental arthritis models such as CIA and CAIA[67,73,83,85,124,125]. In RA, anti-CII antibodies are associated with the acute onset and early stages of disease[126,127]. The prevalence of anti-CII antibodies in RA has yielded estimates between 3-88%[128–130], highlighting the need for a standardized testing protocol with defined reagents. Despite a clear link to the pathogenesis of RA, the detection of anti-CII antibodies has never been established as a biomarker for clinical use.

1.11 THE PATHOGENICITY OF AUTOANTIBODIES

Epidemiological studies show that seropositive RA patients, in particular the double positive RF+/ACPA+ subset, have a more severe disease outcome[108,109]. One proposed mechanistic link for the additive effect is that RF forms immune complexes with ACPA which stimulate the release of pro-inflammatory cytokines from macrophages to stimulate osteoclasts[109]. There are reports indicating that ACPA in healthy individuals correlate with bone loss before clinical onset, but the specificity of ACPAs is not known[131]. Since the citrullinated cyclic peptides used in commercial kits are undisclosed and may not be expressed in vivo, one approach to find the mechanistic link has been to study fine specificities of ACPA against citrullinated peptides from different putative autoantigens. The proteins that have been studied most extensively include fibrinogen, vimentin, α-enolase, filaggrin and CII, although with mixed results[14,132–135]. Several animal models have reported arthritogenicity of ACPA, although low antibody titers and lack of appropriate non-citrullinated control in most studies make the causative link inconclusive[136–138]. The production of ACPA is however not the feature of any of the established animal models for arthritis, and B-cells recognizing citrulline are negatively selected in the mouse[139]. There are also reports of purified polyclonal and monoclonal human ACPA with arthritogenic properties[140–142].

Finally, the ACPA isolated from mice have been shown to induce (ACC1) or enhance (ACC4) arthritis[143,144]. A recent publication of the first crystal structure of a human ACPA revealed how it was able to cross-react between different citrullinated peptides[145]. In contrast, the crystal structures of the two mouse ACPA revealed how they could either cross-react to native CII (ACC1) or be specific to one citrullinated epitope on CII (ACC4)[143,144]. The different cross-reactivity of ACPA in relation to antibodies known to induce arthritis in animal models[96,99,124,143], led to proposing a model of three different classes of ACPA where their functional roles are determined by their specificity (Figure 4). In this proposed model the antibodies which cross-react with joint proteins are arthritogenic and have an important role in the onset of arthritis, while the ACPAs that are widely cross-reactive to citrullinated peptides may have a role in the pathogenesis after the inflammation in the joint has been established. However, they may also be non-functional or even regulatory[146].

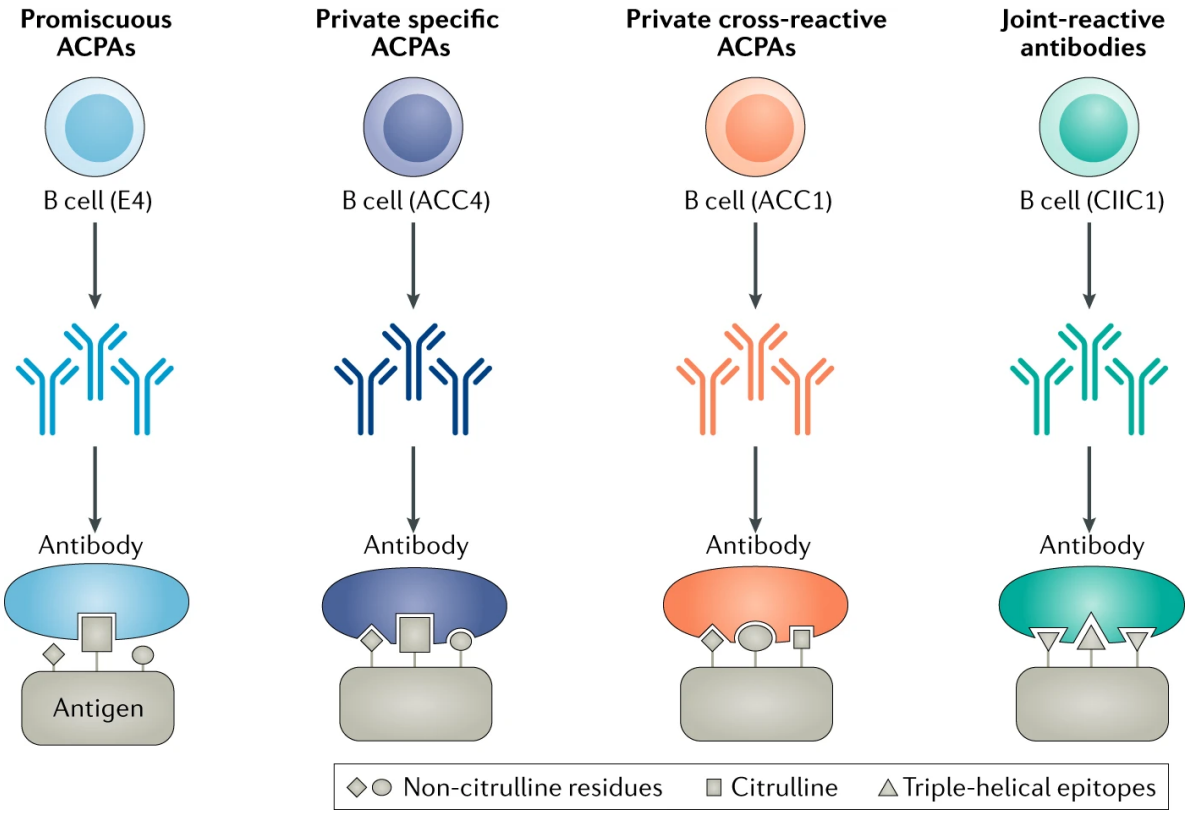

Figure 4. Examples of different types of ACPAs and joint-reactive autoantibodies in RA. Illustration of the binding of antigen by different types of autoantibodies in rheumatoid arthritis (RA), each exemplified by a crystallized antibody–peptide complex. *Figure from Ge et. al. Nat Rev Rheumatol* ***15,*** *503–508 (2019). Reprinted with permission from Nature.*

1.12 PATHOGENESIS OF RA

To summarize the etiology of RA, a model for disease development in RA divides it into three separate stages; priming, disease onset and chronic joint inflammation[147] (Figure 5).

At stage one, autoimmunity emerges from exposure to environmental risk factors such as cigarette smoke and pathogens in genetically pre-disposed individuals. While epidemiological studies show a strong epistatic effect between genetic and environmental risk factors in the development of RA, a study of 12,590 twins suggests that induction of ACPA in the general population correlates more strongly with environmental exposures rather than genetic risk factors[117]. The exact location is not known, although it is postulated to occur in the mucosal membranes such as periodontium, gut, or the lung tissue[148]. The initial stage is characterized by the production of RF and ACPA which can be detected in the serum several years before the diagnosis in a subset of patients[14,149]. The antibody profile of these autoantibodies during the pre-symptomatic phase shows low titers and autoantibodies shown to induce arthritis are not present.

In the second stage around the onset of RA, the autoantibody profile changes drastically with a rise in titer, affinity, epitope spreading and with antibodies targeting joint proteins[14]. The joint protein that has been most extensively studied is CII, which is the major protein in the joint cartilage and antibodies against CII in RA are associated with an early inflammatory and

destructive phenotype[127]. Why only a minority of individuals that are ACPA positive develop arthritis is not known; the current dogma around this switch to joint reactivity argues for a genetical predisposition, most notably HLA-DRB1 alleles and other T-cell activating genes such as PTPN22.

The third and final stage is chronic inflammation of the joints. This phase leads to tissue destruction and remodeling; the patients that have reached this stage represent the vast majority seen by clinicians. This is also the focus for the current therapies. The most efficient treatments at this stage target cytokines produced by macrophages such as TNF-α and interleukin-6 (IL-6), although we currently lack biomarkers for predicting which patients will respond to the treatment. For seropositive patients, therapies targeting T and B-cells have also shown to be efficient, indicating that adaptive immunity also plays a role in chronic phase for this subset of patients[150]. While anti-B-cell therapy works in a subset of RA patients, the functional role of autoantibodies in the chronic phase is still not clarified. Once the disease is established, new cartilage proteins/epitopes normally hidden to the immune system may be exposed. One candidate autoantigen to illustrate this established phase is type XI collagen which is postulated to be located inside the collagen fibers, and to which antibodies are seen at a late stage of disease in experimental arthritis[151]. The exposure of neoantigens and cross-reactivity between these and other joint proteins[152] could further perpetuate the disease and have an impact on long term remission.

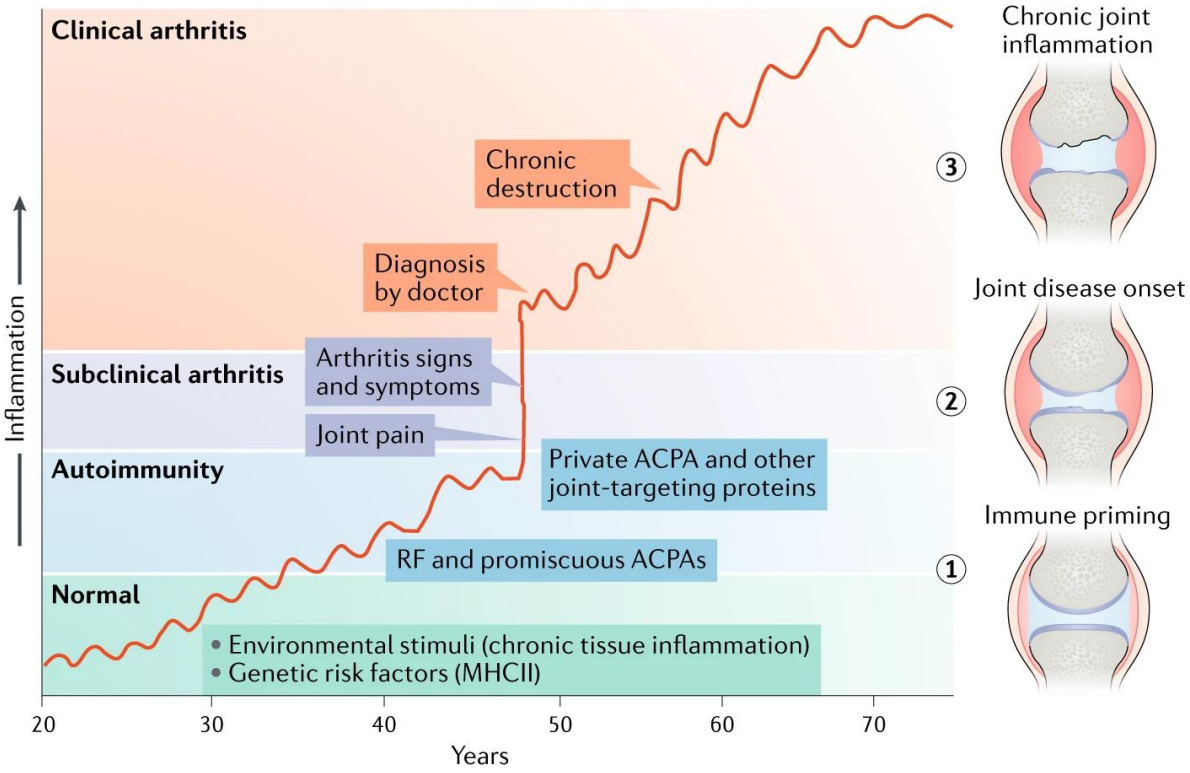

Figure 5. The three stages of the development of RA. *Figure from Ge et. al. Nat Rev Rheumatol* ***15,*** *503–508 (2019). Reprinted with permission from Nature.*

2 AIMS

The original goal of this PhD project was to investigate environmental risk factors of RA in animal models of arthritis, with a focus on the origin and function of ACPA. Out of the 5 projects included in the original research plan, two of them are published in this thesis (**Study I** and **Study IV**). The remaining original projects were not included in the thesis due to limitations in reagents, genetically susceptible mice strains and access to facilities. Instead, the PhD project took a new turn and led to establishing a platform for studying joint-reactive autoantibodies and using it to analyze animal models of arthritis, monoclonal antibodies and human patient cohorts with inflammatory joint diseases (**Study II-V**).

Study I examined the age-old theory of a link between periodontitis and arthritis. Our investigation focused on the hypothesis by Rosenstein *et. al.* that the immune response to the oral pathogen *P.gingivalis* may be involved in the etiology of RA[46]. While there are several mechanisms by which a *P.gingivalis* induced periodontitis can influence arthritis development, the cornerstone in this theory is that *P*.PAD enzyme could induce autoimmunity in RA through molecular mimicry of citrullinated antigens.

In **Study II,** our goal was to address a major bottleneck for characterizing CII as an autoantigen in RA: a library of high-quality triple helical peptides. Once established, the library would be instrumental for investigating the role of anti-CII as biomarkers and pathogenic factors in arthritis.

For **Study III** the aim was to examine CXI as an autoantigen in arthritis by immunization and isolating anti-CXI clones to characterize their function, specificity and arthritogenicity.

An ACPA-clone of mouse origin that binds both cyclic citrullinated peptides and non-citrullinated CII-peptides has been an enigma since its first report[143]. In **Study IV** the quest was to solve this puzzle and determine the structural basis for the cross-reactivity and determine its function.

Finally, in **Study V**, we searched to further explore the cross-reactivity of ACPA with a series of human clones to elucidate their role in the pathogenesis of RA.

3 METHODOLOGICAL CONSIDERATIONS

This section provides a summary of selected key methods used in the projects included in this thesis. More detailed methodology can be found in the original manuscripts of **Study I-V.**

3.1 PATIENT MATERIAL

3.1.1 TIRA-2

In Study **II**, **IV and V,** we analyzed the antibody reactivities in serum samples from the prospective cohort second Swedish Early Intervention in RA Trial (TIRA-2)[31]. Early RA patients with symptom duration of at least 6 weeks and up to 12 months were recruited from 6 rheumatology units in mid- or southeast Sweden between 2006-2009. Most patients (84%) fulfilled the 1987 criteria from ACR[19]. Two smaller groups were recruited, either having morning stiffness with a duration of >60 minutes, symmetric arthritis, and small-joint engagement of the hands or feet (5% of patients), or ≥1 joint with palpable synovitis and anti-CCP positive (11% of patients). At the time of first serum sampling, all patients were naïve for Disease-modifying antirheumatic drugs.

3.1.2 BARFOT

In **Study II**, we analyzed antibody reactivities in serum samples from the Better Anti-Rheumatic PharmacOTherapy (BARFOT) cohort[153]. This is an observational prospective multicenter study of patients with early RA that were recruited between 1992-2006. To be included in the study, participants had to fulfill the revised 1987 criteria from ACR[19] and have a disease duration of up to 12 months.

3.1.3 EIRA-1

In **Study III** and **V,** we analyzed antibody reactivities in serum samples of RA patients and controls from the Epidemiological Investigation of RA (EIRA-1) cohort[32]. This population-based case-control study started in 1996 and diagnosed RA patients using the revised 1987-criteria from ACR[154]. Patients between 18-70 years old were recruited within 12 months from the first symptoms of arthritis from rheumatology clinics in southern and central Sweden. Controls for the study were recruited at random from the national population registry, matched for gender, age and residential area. The patients included in EIRA-1 partially overlaps with the previously described BARFOT cohort.

3.1.4 Population controls

For both TIRA-2 and BARFOT, no case controls were recruited as part of the original study design. Instead, controls recruited for other patient cohorts were used.

3.1.4.1 WINGA

In **Study IV** and **V**, controls recruited to be used in the Western Region Initiative to Gather Information on Atherosclerosis cohort (WINGA) were used. In addition, controls diagnosed

with a rheumatic disease in the patient registry were excluded. A limitation with these controls were that they were recruited to match atherosclerosis patients which have a different gender and age distribution compared to RA.

3.1.4.2 Malmö Diet and Cancer Study

In **Study II**, age and gender matched controls for BARFOT and TIRA2 were obtained from population controls used in the Malmö Diet and Cancer Study. Individuals diagnosed with a rheumatic disease were excluded.

3.2 EXPERIMENTAL ANIMALS

In **Study I** and **III**, rats from the inbred DA strain were included. For **Study II**, **III** and **IV** mice strains susceptible to arthritis carrying either the $H2^q$ or $H2^r$ MHC-haplotype were used. All animals were bred and kept under specific pathogen free conditions (FELASA II) at the animal facility of Medical Inflammation Research. Water and pellets were provided *ad libitum* and they were housed in individually ventilated cages.

3.3 LIGATURE-INDUCED EXPERIMENTAL PERIODONTITIS

For the induction of periodontitis in **Study I**, silk ligatures were tied around the second upper molar on both sides under anesthesia[155]. The periodontitis was evaluated every 10 days by a trained dentist, and after each examination swabs of the oral pathogens *P.gingivalis* and *F.nucleatum* were applied to facilitate the development of periodontitis.

3.4 EXPERIMENTAL ARTHRITIS

3.4.1 Pristane Induced Arthritis (PIA)

The PIA model used in **Study I** and **III** was induced by a single intradermal injection of 100 µl pristane (2,6,10,14-tetramethylpentadecane) at the dorsal side of the tail base. The disease progression was measured by weighing and clinical scoring on a 60-point scale[156].

3.4.2 Collagen Induced Arthritis (CIA)

In **Study II** and **III** the CIA model was used. Arthritis was induced by an intradermal injection of emulsion made from rat CII and complete Freund's adjuvant (CFA). At day 35, a booster injection with CII and incomplete Freund's adjuvant was injected intradermally. The disease progression was measured by clinical scoring on a 60-point scale. In addition, for **Study III** a modified protocol substituting CII with CXI was also used.

3.4.3 Collagen Antibody Induced Arthritis (CAIA)

To evaluate the arthritogenicity of monoclonal antibodies, the CAIA model was used in **Study III** and **IV**. The monoclonal antibodies were injected intravenously, either alone or as a cocktail together with other monoclonal anti-CII antibodies. The mice were boosted with an intraperitoneal injection of LPS on day 5. Clinical scoring was done on a 60-point scale.

3.4.4 Bead-based multiplex assay

In **Study II-V**, we analyzed antibody responses using the Luminex platform. For **Study II**, a platform established at Medical Inflammation research with a Bio-plex 200 was used. For **Study IV** and **V**, a platform established at the Plasma Profiling division at Sci-Life was used. For **Study III**, both plaforms were used. Magnetic beads with unique dyes were first coated with NeutrAvidin, thereafter coupled with biotinylated peptides. The process was optimized testing different concentrations of NeutrAvidin and biotinylates peptides. Next, human or rodent samples were diluted and pre-incubated in a blocking buffer for 1 h at RT on a shaker. Different serum concentrations and blocking buffers were evaluated during optimization. The most important modification from the original protocol was the addition of NeutrAvidin to reduce unspecific binding. Thereafter, the beads with different peptides were mixed with the serum samples and incubated for 75 minutes. beads were washed on a plate washer and then resuspended in a solution containing the secondary anti-human, anti-rat or anti-mouse IgG Fcγ-PE. After 40 min of incubation, the beads were washed and the fluorescence intensity was measured. The median fluorescence intensity (MFI) was used to quantify the interactions of the antibody with the given peptides. For the comparison of responses to peptides in human serum samples in **Study II**, **III** and **V**, the ratio value (fold change), calculated by dividing the MFI value for the peptide of interest by the MFI value of either the median value of cyclic controls peptides or the THP control peptide (described in **Study II**), was used. In **Study IV** the THP control had not yet been synthesized.

3.5 STATISTICAL METHODS

To compare numerical data between two independent groups where normal distribution could be assumed, analysis was made with the student's t test (two-tailed) (**Study I**). In contrast, the non-parametric method Mann-Whitney U test (also called Wilcoxon rank-sum test) was used for independent group comparisons of numerical data where normal distribution could not be assumed (**Study I-V**). To compare categorial data of two independent groups, Fischer's Exact test was applied (**Study II-IV**). Pearson correlation test was used for calculating correlations for independent numerical variables (**Study III**). Statistical analyses were performed with IBM SPSS Statistics 21.0 (**Study I**), GraphPad Prism v8.0.1 Software (**Study II-III**) or R software using several packages (**Study IV-V**). P-values <0.05 were considered statistically significant.

3.6 ETHICAL CONSIDERATIONS

All studies in the thesis have been approved by local ethical committees.

That being said, scientists must reflect on all aspects of their research, including ethical. For this PhD project, the animal experiments are obviously the focal point.

The first question is why? RA is a severe autoimmune disease affecting up to 1% of the population, and there is no cure. Looking back at history, the use of animal research has been instrumental in development and testing of several biologically based therapeutics targeting

pro-inflammatory cytokines (IL-1[76], IL-6[77], anti-TNF-α[78]). The introduction of these has revolutionized the care and treatment of RA patients.

The downside of performing animal experiments is of course the suffering caused. Comparing the suffering caused by RA to the suffering caused in arthritis experiments is no easy task, although an attempt to quantify would probably be in favor for performing experiments. Unfortunately, for complex disease such as RA, it's not possible to replace animal experiments with cells, "organs-on-a-chip" or *in silico* studies.

Although if rodents have the similar capacity to suffer and feel joy, why don't they have unalienable rights just like us? Judging by how the emancipation of our own species has evolved over time and only recently come to include all members, future generations will probably make different judgements than we make today.

4 PRESENT INVESTIGATIONS

4.1 STUDY I: EFFECTS BY PERIODONTITIS ON PRISTANE-INDUCED ARTHRITIS IN RATS

In **Study I** we explored the role the oral pathogen *P.gingivalis* in the pathogenesis of RA. The proposed link between the two[46] was the result of two independent observations reported within a year of each other; the identification of ACPA as a biomarker in RA[114] and the *P.*PAD enzyme in *P.gingivalis*[44]. Since the first report of cross-reactivity between citrullinated human alpha-enolase (CEP-1) and bacterial enolase[157] in RA, the scientific community has yielded numerous (Figure 6) epidemiological[158,159] and experimental[160,161] evidence in support of the link[47].

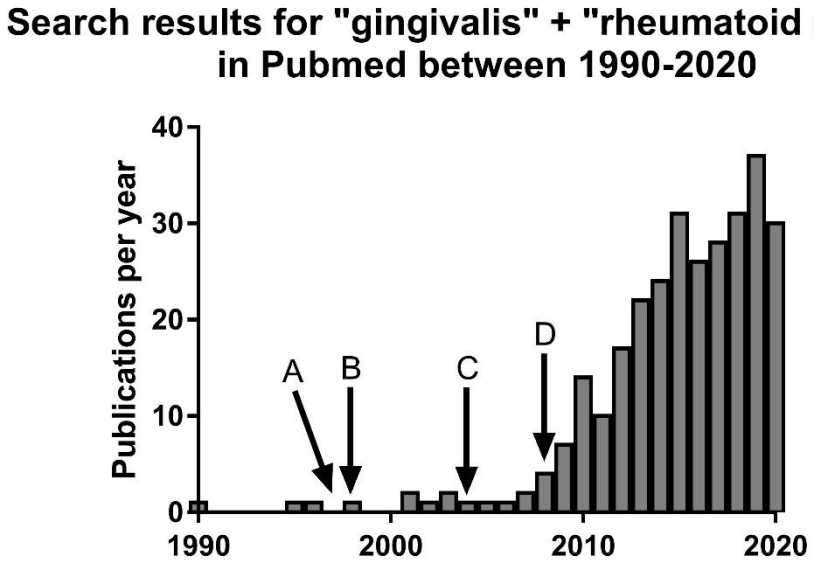

Figure 6 Number publications per year with the keywords "gingivalis" and "rheumatoid arthritis" in Pubmed between 1990-2020. A: Identification of ACPA as a biomarker in RA. **B:** Identification of PAD enzyme in P.gingivalis**. C:** Proposed link between P.gingivalis and RA. **D:** Report of antibody cross-reactivity between citrullinated alpha-enolase and bacterial enolase in serum of RA patients.

The experimental periodontitis model with ligatures and pathogens showed early clinical signs in most rats already 3 weeks into the experiment; at week 6, severe periodontitis was established in all rats. The periodontitis measured by a tooth mobility score, reached a plateau after 8 weeks. The alveolar bone level was measured through micro-CT, confirming a significant bone loss in the periodontitis group after 15 weeks. In contrast, the group with only arthritis had no significant alveolar bone loss or tooth mobility score compared to healthy controls. Arthritis observed in rats after pristane immunization at week 8 was comparable across all groups. No significant difference was observed in the acute phase protein α-1-AGP or the 20-plex cytokine test between the experimental groups.

Antibodies against the RgpB were found in 8 of 9 rats with periodontitis. In contrast, no response to RgpB was found in rats without periodontitis (Figure 7a). To characterize a citrulline specific antibody response, the citrullinated peptides CPP3 and CEP-1 with their corresponding controls RPP3 and REP-1 were examined revealing a significant increase in the

concentration of anti-CPP3 antibodies in the periodontitis group (Figure 7b). No difference was observed between the groups in the antibody titers against RPP3, CEP-1 or REP-1.

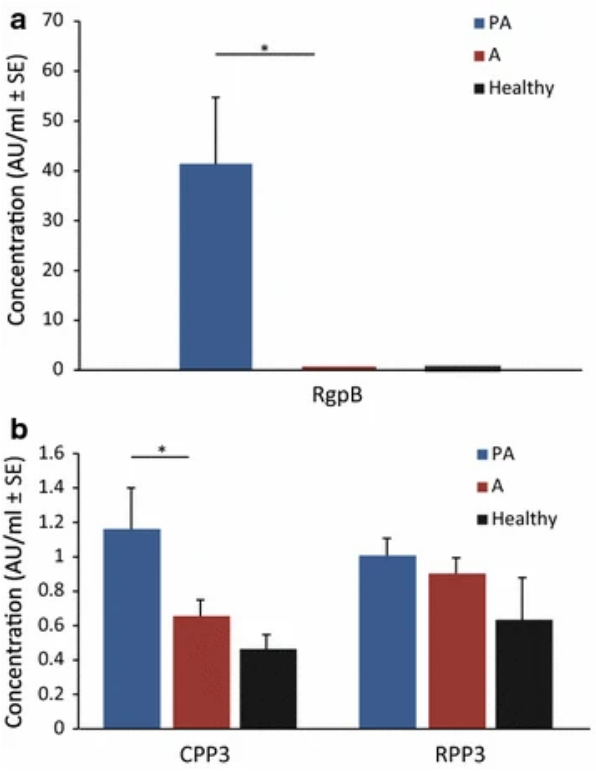

Figure 7 Effect of pre-existing periodontitis on the antibody response to RgpB and PPAD. Plasma RgpB, CPP3 and RPP3 levels. Mean plasma concentrations (AU/ml ± SE) of antibodies against **(a)** RgpB, **(b)** CPP3 and RPP3 in arthritis rats with and without periodontitis and healthy control animals at endpoint of the experimental period of 15 weeks. *P<0.05 was considered statistically significant. *PA* rats with experimental periodontitis and PIA (n=9), *A* rats with PIA without periodontitis (n=12), *Healthy* control rats without induced arthritis or periodontitis (n=4), *RgpB* arginine gingipain B, *CPP3* citrullinated peptide derived from PPAD, *RPP3* arginine-containing (uncitrullinated) PPAD peptide

Our investigation combined two established animal models for periodontitis and arthritis: ligature-induced periodontitis and PIA. To promote the progression of periodontitis, swabs of *P.gingivalis* and *F.nucleatum* were applied after each oral examination. The combination was chosen based on earlier reports demonstrating that *F.nucleatum* can promote the colonization of anaerobic bacteria such a P.gingivalis[162] and display an epistatic effect in the induction of bone loss in experimental periodontitis[163]. Chronic periodontitis evaluated by a tooth mobility score was established before the induction of arthritis at week 8. The inability to reproduce previous findings, that *P.gingivalis* promotes the development of arthritis in experimental models[160,161], may be explained by the experimental models chosen. For instance, CIA is the most frequently used animal model of arthritis where anti-CII antibodies are an important effector mechanism[164]. In contrast, the PIA model is a T-cell driven model. While PIA does induce both RF[165], anti-CII[92] and anti-CXI[93] antibodies, their role in the pathogenesis has not been clarified. Another limitation of using PIA in DA rats is the very high severity of joint inflammation which could mask any potential additive effect of periodontitis. The choice of experimental periodontitis model could also be a contributing factor explaining conflicting results. However, our study spanning over 15 weeks allowed evaluation of both early and chronic stages in both periodontal and arthritic disease. At the time of publication, this was the longest animal study exploring the relationship between periodontitis and arthritis.

While no animal study can ever capture the full complexity of a human disease, we might need to ask ourselves if all animal experiments are equally informative? For instance, one may question the scientific relevance of using a method described as *"Chronic inflammatory lesions were induced by the implantation of polyurethane sponges impregnated with heat-killed Porphyromonas gingivalis into the backs of DA rats."*[160] to explore a link between periodontitis and RA in humans. The correlation found in this particular study rather implies any chronic inflammation in the body may exacerbate disease severity in experimental arthritis models.

The analysis of ACPA production revealed a CPP3 specific response in the group with periodontitis while no difference was observed in the response towards CEP-1 or their corresponding control peptides. This is the first report of antibodies against a citrullinated *P*.PAD peptide in an experimental animal model. While we could not detect any other ACPA reactivity, an immune response towards a citrullinated foreign antigen would be the first step in a proposed mechanism of molecular mimicry between *P*.PAD and an unknown self-antigen. A limitation in the experimental setup is the duration of the priming phase of disease which in RA is thought to span over years rather than months. A longer study period may eventually have yielded epitope spreading toward other citrullinated antigens. Numerous animal studies have in contrast to our results reported more general ACPA-reactivity based to the CCP2 kit[166]. However, the CCP2-kit does not provide a negative control and is subsequently of limited value to determine if the response is citrulline specific. Response to CPP3 has also been found in elevated levels in RA, both in pre-symptomatic samples and in established-RA[167]. However, these findings were not reproduced in a smaller cohort[168]. There is also a report that *P*.PAD cannot auto-citrullinate[169], implying that citrullinated *P*.PAD and any peptides derived from it (CPP3) are not unique antigens providing the mechanistic link between *P.gingivalis* and RA. However, this study is disputed[170] and quest to find the proof of a mechanistic link continues.

In summary, periodontitis induced by *P.gingivalis* did not affect the development or disease severity of pristane-induced arthritis in DA rats. However, in rats with periodontitis we were able to detect a citrulline specific response towards a *P*.PAD derived peptide.

4.2 STUDY II: SYNTHESIS OF AN ARRAY OF TRIPLE-HELICAL PEPTIDES FROM TYPE II COLLAGEN FOR MULTIPLEX ANALYSIS OF AUTOANTIBODIES IN RHEUMATOID ARTHRITIS

In **Study II** we set out to establish a library of triple helical peptides of the major B-cell epitopes in CII to use as biomarkers for screening anti-CII antibodies. Collagen has been a proposed autoantigen in RA for over 50 years[122]. Since then, the pathogenic properties of anti-CII antibodies have been extensively studied in experimental animal models[171] and their potential role in the onset of RA has been confirmed in several studies[126,127]. However, the use of anti-CII antibodies as a biomarker has never been established for clinical use. In contrast, RF and ACPA are included in the 2010 ACR/EULAR classification criteria for RA[107]. The rapid introduction of ACPA as a classification criterion was possibly due to the commercial availability of standardized assay[13]. At the same time, there is no standardized diagnostic assay for anti-CII antibodies, illustrated by the reported prevalence in RA varying between 3-88%[128–

[130]. In addition, different subsets of anti-CII antibodies may have different function and diagnostic value depending on their epitope specificity. For instance, while mAbs directed against the C1, U1, J1 and D3 epitopes induce arthritis[87], the F4 epitope has been reported to be protective[172] and is not associated with RA[173]. The binding of anti-CII antibodies is conformation dependent, requiring a triple helical structure[174]. To dissect the different subsets of anti-CII antibodies, triple helical peptides with different inserts are therefore needed which are very laborious to synthesize compared to linear peptides[175]. Earlier, we reported a recombinant library of CII peptides expressed in *E.coli* using the foldon trimerization domain to create a triple helical structure[67]. The foldon-library was used to map new CII-epitopes in CIA. Unfortunately, the foldon domain originates from a bacterial phage[176] to which humans have antibodies against. This highlights the need for synthetic peptides with defined epitopes to explore anti-CII antibodies as potential biomarkers in RA.

A library of covalently branched THPs covering the 6 major B-cell epitopes in CII was synthesized. In addition to the native CII-sequence, citrullinated variants were made replacing the amino acid arginine with citrulline. The structure and purity of the THPs were confirmed by HPLC, Circular Dichroism (CD) spectroscopy, melting curve analysis and mass spectrometry. The mass spectrometry analysis showed that these are the largest THPs known to have been successfully characterized to date, showing molecular weights consistent with the theoretical weight both for the full THP and fragments after trypsin cleavage. The melting curve analysis demonstrated considerable variability in the melting temperature varying between 33.3-56.6 °C for the major epitopes. However, the differences could be explained by the individual peptide sequences, the F4 peptide in particular predicted to be more flexible[177]. The structural basis for forming a stable triple helix was dissected through introducing two different modifications to the C1 THP revealing that the simultaneous substitution of hydroxyproline (O) to proline (P) and the reduction of 5 to 2 GPO repeats abrogated the triple helical structure. Each modification independently did not have an effect on the ability of conformational anti-CII mAbs or serum from CII immunized mice to bind the C1 epitope.

Next, the biotinylated THPs were coupled to Neutravidin coated magnetic beads and made into a multiplex array; comprised of all synthesized THPs and their cyclic controls. Subsequently, the triple helical structure of the peptides was confirmed through the binding of conformational dependent mAbs specific for C1, U1, F4 and D3 epitope. In addition, strong binding to all peptides was observed with serum from mice immunized with CII 21 days earlier but not from non-immunized mice. Our results from the mAb and CIA screenings determined a strong binding to all the major epitopes both in mAbs and mice sera, showing no cross-reactivity between THP and cyclic peptides confirming their triple helical structure.

Finally, the THPs were evaluated in early RA patients cohorts TIRA-2 and BARFOT (n=2075) demonstrating a significantly higher antibody reactivity to C1, U1 and E10 epitope in RA patients compared to population controls (n=935) (Figure 8).

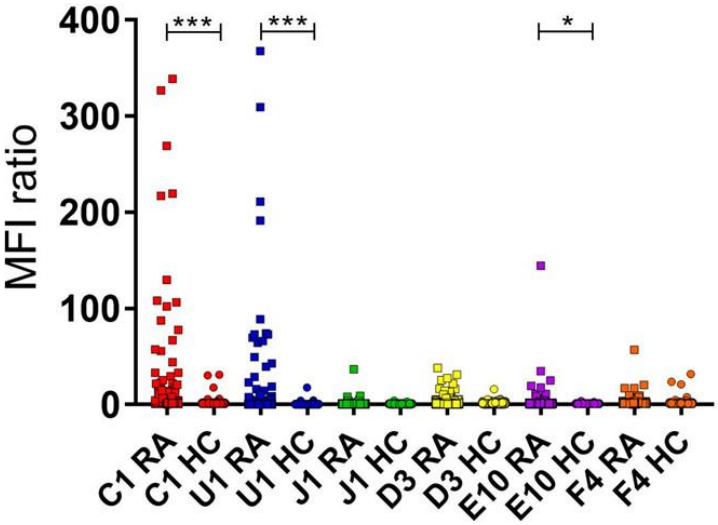

Figure 8 Antibody responses in sera from early-RA patients (*n*= 2075) and healthy controls (*n*= 935) to THPs containing the six native major CII epitopes. The MFI ratio has been calculated by dividing the MFI raw value with the median response to THP-control peptide (T_GPO_11). Cut-off for positivity for each peptide was set at a median of healthy controls +3 standard deviations. [MFI = mean fluorescence intensity.]

The results are consistent with our previous report that C1 and U1 are immunodominant B-cell epitopes both in primates, rodents and humans with arthritis[67]. Results from CIA, CAIA and cartilage explants have consistently demonstrated that antibodies targeting the C1 and U1 epitopes are highly pathogenic[171]. For E10, this was the first report comparing the prevalence in RA and controls. We also confirmed that anti-CII antibodies specific for the F4 epitope are not associated with RA[173,178], potentially explained by a regulatory function[179].

A cornerstone in the evaluation of biomarkers is consistent quality of the reagents to confirm results in independent cohorts. Reproducibility of the peptide synthesis was tested through the re-synthesis of three peptides; both batches of peptides showed consistent results in capturing anti-CII antibodies in serum.

A limitation of this study is that it doesn't analyze the response to specific epitopes with the clinical data for the patients. For instance, comparing the overlap to RF and ACPA may identify biomarkers for the "sero-negative" subset of patients. Finally, correlations to the disease development or treatment response may also yield new clinical biomarkers in RA.

In summary, a library of THP covering the major B-cell epitopes in CII was synthesized and evaluated using anti-CII mAbs, serum from CIA and RA patients. Results confirmed their triple helical structure and revealed a more frequent response to the C1, U1 and E10 epitope in RA patients compared to population controls.

4.3 STUDY III: A SHARED EPITOPE OF COLLAGEN TYPE XI AND TYPE II IS RECOGNIZED BY PATHOGENIC ANTIBODIES IN MICE AND HUMANS WITH ARTHRITIS

CXI is a minor cartilage component which forms heterotrimers from three different chains, one of which is shared with CII. Rats immunized with homologous CXI in adjuvant develop a chronic relapsing arthritis phenotypically distinct from CIA[81]. As expected, increased titer of anti-CXI antibodies is observed in the chronic phase of the disease. Interestingly, antibodies against CXI have also been detected in RA patients[93]. However, the specificity and function of anti-CXI antibodies is not well understood.

We first assessed the arthritogenicity of immunization with CII or CXI in mice with the MHC H-2^q haplotype. Immunization with CII leads to a severe arthritis with antibody response to CXI, particularly during the chronic stage. In contrast, CXI immunization induced only mild arthritis, with antibodies binding to CII detected primarily during the chronic stage. A panel of 19 monoclonal antibodies specific for CXI were generated and assessed for cross-reactivity. This revealed that only the L10D9 clone binds both CXI and CII with similar affinity. Next, we demonstrated by screening with our CII-peptide library a strong binding of L10D9 to D3, a shared epitope between CXI and CII. Of 3 CXI clones evaluated in the CAIA model, only the CXI/CII cross-reacting clone L10D9 induced arthritis. In addition, L10D9 binds strongly to cartilage both *in vivo* and *in vitro* while the other 18 CXI clones displayed weak or no binding. Finally, the antibody response to the D3 epitope was tested in mice (CIA), rats (CIA, PIA) and the early RA cohort EIRA-1. Results showed significantly increased prevalence of anti-D3 antibodies in both RA patients and arthritic rodents compared to controls. The arthritis score in CII immunized mice was also shown to be positively correlated with the antibody response to the D3 epitope.

Based solely on their structural similarity and co-localization in joints, it is surprising that immunization with CII and CXI or adoptive transfer of antibodies targeting them lead to such different phenotypes. The most plausible explanation is that CXI is hidden inside the collagen fibrils[180], which is consistent with the very weak or non-existent binding to cartilage for 18 out of 19 tested CXI clones. As implicated in the chronic relapsing disease observed in rats, CXI cartilage may be exposed in the joints during chronic inflammation. If indeed CXI is hidden from the immune system, auto-reactive B and T-cells may not be efficiently negatively selected, further perpetuating the disease as new antigens are exposed during chronic stages of disease. Therefore, the major limitation in determining the arthritogenicity of the anti-CXI clones was that they were not tested in a chronic relapsing arthritis model. The unique characteristic of the L10D9 clone to induce arthritis is clearly linked to its ability to bind CII exposed on the cartilage surface. In addition, the results from this study confirm an earlier report that monoclonal antibodies directed against D3 epitope are pathogenic[87]. The finding that anti-D3 antibodies are present both in experimental arthritis models and RA argues for a potential role in the RA pathogenesis.

In summary, this study has identified a shared epitope between CII and CXI, demonstrating the cross-reactivity between CII and CXI antibodies. The antibodies against the D3 epitope were recognized both in arthritic rodents and in RA patients.

4.4 STUDY IV: ANTI-CITRULLINATED PROTEIN ANTIBODIES CAUSE ARTHRITIS BY CROSS-REACTIVITY TO JOINT CARTILAGE

The immune response against citrulline starts several years before clinical onset of RA[14]. The production of ACPA is believed to originate at a mucosal tissue, eventually targeting the joints through a process of molecular mimicry and epitope spreading [148]. There are several candidate autoantigens proposed to be involved during onset of the disease[178,181], yet the structural basis of the cross-reactivity and the function of ACPA is not known.

To investigate the role of ACPA in the pathogenesis of RA, we examined the autoantibodies targeting the C1 epitope in CII. The C1 epitope contains two different arginine residues (R360, R365), the first shown to be citrullinated *in vitro* by PAD2[178]. The screening of the TIRA-2 early RA cohort against a panel of triple helical and cyclic C1 peptides showed a significant response to all C1 peptides compared to controls, particularly against citrullinated variants of C1. The correlation between cyclic and THP citrullinated C1 response revealed a large overlap between them, with almost no patients exclusively binding to the triple helical citrullinated C1. Correlation between cyclic citrullinated C1 on the first position (Cit360) and CEP-1 showed 43% of the patients to be double positive, although 22% of the patients were exclusively responding to only one of them.

The major limitation in characterizing polyclonal antibodies in serum is that the cross-reactivity of individual clones cannot be determined. As a result, the correlation between two peptide responses in serum may be caused by two distinct subsets of antibodies. To examine the cross-reactivity of ACPA binding citrullinated C1 we used the ACC1 monoclonal antibody reported earlier[143].

To determine the specificity of ACC1, we screened it against a library of CII-peptides, cyclic citrullinated peptide derived from filaggrin (CCP1) and the CCP2-kit. The screening revealed cross-reactivity to both native and citrullinated peptides of CII in addition to CCP1 and CCP2. The structural basis of the cross-reactivity was determined through crystalizing ACC1 with several different peptides, revealing a conserved peptide motif (RG-TG) present in all peptides. The motif is also found in other collagens. Examining this potential cross-reactivity revealed ACC1 binds CXI, identifying another shared epitope between CII and CXI as discussed in **Study III**. Finally, the arthritogenicity of ACC1 was determined by CAIA, showing it is one of the few reported clones which can induce arthritis when injected alone.

In contrast to ACC1, most anti-CII antibodies are conformation dependent requiring a triple helical structure as shown in **Study II** and **III**. The ACPA binding THP in RA patients most likely target one of the α-chains in the THP which could explain the correlation between responses to triple helical and cyclic citC1. This is also the structural basis for how ACC1 can bind both triple helical and cyclic peptides as shown in the crystal structure complexes. While there are predictions for how flexible triple helical structures are based on their sequence[177], how common "flexed out" α-chains are in the cartilage is not known. However, the strong binding of ACC1 to cartilage both *in vitro* and *in vivo* indicates it is a common feature, suggesting perhaps CII alternates between different conformational states. Measurements by CD-spectroscopy and melt-curve analysis in **Study II** also show that substituting arginine for citrulline destabilizes the peptides. As a result, citrullination *in vivo* will destabilize the triple helical structure exposing it to be targeted by ACPA.

In summary, we demonstrate the molecular mechanism for cartilage binding and arthritis induction by an ACPA that binds a wide range of cyclic citrullinated peptides (CCP1, CCP2, citC1).

4.5 STUDY V: STRUCTURAL BASIS OF CROSS-REACTIVITY OF ANTI-CITRULLINATED PROTEIN ANTIBODIES

The current method for determining the presence of ACPA to diagnose RA patients uses a set of undisclosed synthetic peptides, where response above or below a certain cut-off determines if they are ACPA positive or negative[13]. However, the quest to identify citrullinated autoantigens involved in the pathogenesis has revealed that ACPA are a very heterogenous class of antibodies, ranging from a promiscuous binding pattern recognizing peptides of diverse origin[182], to a single citrullinated epitope[143]. This cross-reactivity between citrullinated epitopes of different proteins is a pillar in the current dogma that molecular mimicry and epitope spreading starting in the mucosal tissue leads to an autoimmune disease targeting the joints. At the same time, the basis for this cross-reactivity has not been understood.

In **Study V**, we set out to characterize the structural basis of cross-reactivity in ACPA. First, clones isolated from RA patients were screened on a library of cyclic citrullinated peptides and corresponding controls. The E4 and F3 clones showed a partially overlapping binding profile, with E4 being more diverse. In contrast, the D10 and B2 clones did not show binding to any peptides included in our screen. Next, the E4 clone was crystalized together with three different citrullinated peptides; two citrullinated CII peptides and CEP-1 derived from α-enolase. The crystal structure revealed a "pocket" for the non-polar citrulline residue. In contrast, the polar amino acid arginine is a is not modelled to fit in this pocket due to its positive charge. The crystal structure also showed other interactions predicting which adjacent amino acids were preferred, explaining the specific peptide binding pattern observed for E4. On the other hand, the crystal structures of D10 and B2 without peptide displayed a very different structure, explaining why they do not bind any citrullinated peptides in our assay.

The "pocket" for a citrulline residue in E4 also provides a model for how an ACPA may cross-react to the structurally similar homocitrulline, which is the target for anti-carbamylated protein antibodies (anti-CarP), shown to cross-react with citrullinated peptides[183]. Accordingly, our report supports the growing body of evidence that many ACPA in RA are probably best described as AMPA: anti-modified protein antibody.

Finally, the prevalence of antibodies against the three crystallized peptides with E4 were evaluated in the TIRA-2 and EIRA-1 early RA cohorts. The individual peptides were recognized in between 40-68% of RA patients, with around 1/3 positive for all three peptides.

In summary, we demonstrate for the first time the structural basis for how a human ACPA can cross-react between citrullinated peptides derived from different proteins.

5 CONCLUSIONS

In **Study I**, periodontitis induced *P.gingivalis* did not affect the development or disease severity of PIA in rats. On the other hand, the group with established periodontitis showed increased levels of antibodies against a citrullinated *P*.PAD peptide. This finding supports the first step in the hypothesized link between *P.gingivalis* and RA; a citrulline specific immune response against a bacterial protein.

A library of THP covering the major B-cell epitopes in CII was successfully synthesized in **Study II**. The synthesized peptides confirmed earlier reports of C1 and U1 as the two immunodominant CII-epitopes for antibodies in RA.

In **Study III**, the L10D9 clone binding CXI was shown to induce arthritis by cross-reacting to the D3 epitope shared with CII. Antibody reactivity against the D3 epitope was observed both in serum of arthritic rodents and RA patients.

In **Study IV**, an ACPA was demonstrated to induce arthritis by binding a conserved structural motif on native CII shared with both CCP1 and CCP2.

In **Study V,** a human ACPA was crystallized with citrullinated peptides from different proteins demonstrating that the cross-reactivity was due to a specific recognition of citrulline in a "pocket" where the non-polar citrulline residue fits perfectly into.

6 FUTURE PERSPECTIVES

Hundreds of articles investigating RA are published annually, yet the growing body of evidence indicates that we are just beginning to understand the complexity of RA and other autoimmune diseases. The number of genetic risk loci associated with RA are now over 100[23] and it would be naïve to think we have identified all of them. From the perspective of environmental risk factors, our work support a potential role of P.gingivalis in RA pathogenesis, although it is unclear if animal models will ever be able to confirm the theory. This idea of a microbial origin of autoimmunity isn't new. In fact, this elegant theory has captured the imagination of countless scientists over the years. The scientific curiosity ignited by this concept has resulted in studies such as the X-ray examination of ancient mummies in search for the earliest signs of RA[58].

A recent review of the association of RA and the two oral pathogens *P.gingivalis* and *Aa*. proposed to induce citrullination cited 233 papers with majority of them less than a decade old[47]. Sorting through the wealth of data that this single hypothesis has generated since 2004 is staggering, let alone trying to integrate it with all the other theories about the etiology of RA[184]. How is it possible to reconcile all the implicated genes, environmental risk factors and epistatic interactions between them in one model the human mind can grasp? Not to mention all the proposed autoantigens being targeted both in their native form and with PTMs such as citrulline, homocitrulline and acetyl[119,120]. One explanation may be that we are not dealing with a single disease with a distinct etiology: it is rather a collection of diseases with common clinical endpoint[185].

The solution may in that case be in redefining the very concept of RA. Just as the classification criterion in 1957, 1987 and 2010 led to an updated view of what RA was, the insights gained during the last decade may soon warrant a new update. One striking observation is that the ACPA+ subset of patients appears to be separate disease with a different etiology compared to ACPA- RA. Although even within the ACPA+ subset there is large degree of phenotypic variation, in particular during the early stages. The heterogeneity observed indicates more subtypes of RA may exist, possibly linked to different initiating causes and genetic risk factors. The identification of additional subtypes is also likely to provide more efficient treatment strategies for separate subtypes. This is by no means a unique observation, much of the research today is directed towards explaining the phenotypic variation by correlating them to a wide range of biomarkers, genes and environmental risk factors.

Finding new distinct subtypes is by no means an easy task. Based on observations in this thesis an attractive target may be to define the subsets of antibodies associated with the onset of disease. To date, only antibodies binding cartilage has been conclusively shown to induce arthritis. For instance, antibodies associated with the onset of disease may be used as a biomarker to define a subtype of patients for new therapeutic strategies aimed at depleting[186], neutralizing[187] or modulating[188] arthritogenic antibodies which could reduce symptoms such as antibody mediated pain[86]. While the role B-cells during the onset of disease is not clarified, the subset of patients with joint-reactive antibodies may also be of benefit of B-cell depletion

therapy[189] currently mainly used during a later stage of disease after other treatments have failed. If the success of anti-B-cell therapy is related to depletion joint-reactive B-cells it may explain why clinical effect is not seen in all patients.

Finally, in addition to addressing symptoms such as of acute pain during the onset of disease, early therapeutic interventions also have the potential to prevent RA from becoming established. However, the window of opportunity for such interventions is likely to be narrow, and specific biomarkers to identify the patients and the appropriate treatment strategies in time will be of the essence.

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