Antibody-Associated Inflammation In and Outside the Joint in Rheumatoid Arthritis

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Vijay Joshua

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THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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Thesis defense will take place on Thursday 7th of September 2017, 9:00 am at the Center for Molecular Medicine (CMM) Lecture hall, L8:00, Karolinska University Hospital, Solna
To my beloved parents
Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by pain, chronic inflammation and joint destruction. Epidemiological investigations in large population-based cohorts have identified both genetic (such as HLA-DRB1 Shared epitope, SE) and environmental (such as smoking) risk factors for the development of RA. One of the hallmarks of the disease is the presence of antibodies against a large array of citrullinated proteins (anti citrullinated protein antibodies, ACPA), which are present years before the onset of clinical symptoms. These findings suggest that RA-associated autoimmunity might be initiated somewhere else than the joints, possibly at the mucosal surfaces of the lungs. In the current thesis we aimed to investigate immunological events in the lungs contributing to ACPA generation and to identify novel targets for these antibodies.

Presence of ACPA associates with parenchymal lung abnormalities (as detected by high resolution computed tomography, HRCT) in early-untreated RA. Recognition of more than one citrullinated target by ACPA and specifically recognition of citrullinated fibrinogen peptides increases the odds of detecting HRCT lung abnormalities. Beside HRCT abnormalities, significantly decreased microbial richness and diversity is present in the bronchoalveolar lavage of early-untreated RA patients as compared to healthy volunteers. Interestingly, a similar microbiota dysbiosis is also detected in the presence of overt lung inflammation in the bronchoalveolar lavage of patients with lung sarcoidosis, suggesting that similar inflammatory mechanisms might be active in both lung sarcoidosis and RA. To further explore the possibility that changes in the lungs contribute to generation of autoimmunity in RA, we investigated the presence of citrullinated targets in the mucosal biopsies of early-untreated RA. We identified several novel citrullinated targets, with two citrullinated vimentin peptides detected in a majority of the biopsies. Interestingly these two peptides were also identified in the inflamed synovial tissues of RA patients showing that shared immunological targets are present in the lungs and joints in RA patients. Antibodies against these novel targets were detectable in RA patients suggesting that they could act as immunological targets during disease development. Using a similar approach, we further screened for antibody-reactivity against novel citrullinated targets identified in the synovial fluid of RA patients. Several citrullinated fibrinogen peptides specifically reacted with the antibodies in RA serum, with varying proportion of reactivity for each of these peptides. Interestingly, these antibodies differ from the classical ACPA being associated with the PTPN22 risk allele but not with the HLA-DRB1 SE. As such RA patients carrying the PTPN22 risk allele displayed higher proportion of B cells reacting with citrullinated fibrinogen loaded B cell antigen tetramers than those lacking the risk allele.
In conclusion, we provide evidence for a pathogenic link between early events in the lungs and autoimmunity in RA and identify several novel immunological targets for this autoimmunity. Our studies contribute to the understanding of the longitudinal development of RA, opening the possibility for future targeting of early pathogenic events in order to delay and/or prevent the disease.
LIST OF SCIENTIFIC PAPERS

I. Association between number and type of different ACPA fine specificities with lung abnormalities in early, untreated rheumatoid arthritis.

*Submitted.* *Contributed Equally*

II. The lung microbiota in early rheumatoid arthritis and autoimmunity.


III. Shared immunological targets in the lungs and joints of patients with rheumatoid arthritis: identification and validation.

*Annals of Rheumatic Diseases, 2015 Sep, 74(9):1772-7.*

IV. Antibody responses to de novo identified citrullinated fibrinogen peptides in rheumatoid arthritis and visualization of the corresponding B cells.

RELATED SCIENTIFIC PAPERS NOT INCLUDED IN THE THESIS

V. Mechanisms involved in triggering rheumatoid arthritis.  
Anca I. Catrina, Vijay Joshua, Lars Klareskog and Vivianne Malmström.  

VI. Identification of a novel chemokine-dependent molecular mechanism underlying rheumatoid arthritis-associated autoantibody-mediated bone loss.  
Epub 2015 Nov 26

VII. Signs of immune activation and local inflammation are present in the bronchial tissue of patients with untreated early rheumatoid arthritis.  
Epub 2015 Nov 3

VIII. Serum RANKL levels associate with anti-citrullinated protein antibodies in early untreated rheumatoid arthritis and are modulated following methotrexate.  
Arthritis Research & Therapy, 2015 Sep, 17:239

IX. Structural changes and antibody enrichment in the lungs are early features of anti-citrullinated protein antibody-positive rheumatoid arthritis.  
X. Environmental and genetic factors in the development of anticitrullinated protein antibodies (ACPAs) and ACPA-positive rheumatoid arthritis: an epidemiological investigation in twins.

XI. Monoclonal IgG antibodies generated from joint-derived B cells of RA patients have a strong bias toward citrullinated autoantigen recognition.
### LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ACPA</td>
<td>Anti-citrulline protein antibodies</td>
</tr>
<tr>
<td>ACR</td>
<td>American college of rheumatology</td>
</tr>
<tr>
<td>AKA</td>
<td>Anti-keratin antibodies</td>
</tr>
<tr>
<td>APF</td>
<td>Antiperinuclear factor</td>
</tr>
<tr>
<td>ARA</td>
<td>American rheumatology association</td>
</tr>
<tr>
<td>BAL</td>
<td>Bronchoalveolar lavage</td>
</tr>
<tr>
<td>BCR</td>
<td>B cell receptor</td>
</tr>
<tr>
<td>CarP</td>
<td>Carbamylated proteins</td>
</tr>
<tr>
<td>CCP</td>
<td>Cyclic citrullinated peptide</td>
</tr>
<tr>
<td>CII</td>
<td>Collagen type II</td>
</tr>
<tr>
<td>Cit</td>
<td>Citrullinated</td>
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<tr>
<td>CTLA4</td>
<td>Cytotoxic T lymphocyte-associated protein 4</td>
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<tr>
<td>EULAR</td>
<td>European League Against Rheumatism</td>
</tr>
<tr>
<td>Grp78</td>
<td>Citrullinated glucose-regulated protein 78</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leucocyte antigen</td>
</tr>
<tr>
<td>HRCT</td>
<td>High resolution computed tomography</td>
</tr>
<tr>
<td>iBALT</td>
<td>Inducible bronchus-associated lymphoid tissue</td>
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<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
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<tr>
<td>ILD</td>
<td>Interstitial lung disease</td>
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<tr>
<td>Lyp</td>
<td>Lymphoid phosphatase</td>
</tr>
<tr>
<td>MDA</td>
<td>Malondialdehyde</td>
</tr>
<tr>
<td>OA</td>
<td>Osteoarthritis</td>
</tr>
<tr>
<td>OTU</td>
<td>Operational taxonomic unit</td>
</tr>
<tr>
<td>PAD</td>
<td>Peptidyl arginine deiminase</td>
</tr>
<tr>
<td>PADI4</td>
<td>Peptidyl arginine deiminase 4 gene</td>
</tr>
<tr>
<td>PTPN22</td>
<td>Protein tyrosine phosphatase non-receptor type 22</td>
</tr>
<tr>
<td>RA</td>
<td>Rheumatoid arthritis</td>
</tr>
<tr>
<td>ReA</td>
<td>Reactive arthritis</td>
</tr>
<tr>
<td>RF</td>
<td>Rheumatoid factor</td>
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<tr>
<td>rRNA</td>
<td>Ribosomal ribonucleic acid</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>SCFA</td>
<td>Short chain fatty acids</td>
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<tr>
<td>SE</td>
<td>Shared epitope</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>STAT4</td>
<td>Signal transducer and activator of transcription 4</td>
</tr>
<tr>
<td>T1D</td>
<td>Type 1 diabetes</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
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<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
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<tr>
<td>TRAF1</td>
<td>TNF receptor-associated factor 1</td>
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1 INTRODUCTION

1.1 Rheumatoid Arthritis

Rheumatoid Arthritis (RA) is a chronic systemic inflammatory autoimmune disease affecting the joints which when left untreated leads to joint erosions, destruction and deformities of the joints. RA affects 0.5-1% of the world population [1]. It predominantly affects females rather than males, in the ratio of 3:1 [2]. The disease in its pathological state is characterized by inflamed synovium, accumulation of synovial fluid and destruction of the cartilage and bone.

1.2 Historical description and classification of RA

Paleontological data from skeletons obtained from sites in ancient Egypt, Greece and the Nordics have demonstrated the presence of bone lesions and joint deformities that are indicative of RA [3]. This has given rise to speculation of RA being a disease since ancient times. Evidences of RA can also be found in the art such ‘The Painter’s Family’ by Flemish painter Jacob Jordaens (1593–1678), ‘Saint Matthew’ (1609), ‘The Drunken Sleeping Satyr’ (1610), ‘Suzanna and the Elders’ (1614) and ‘Between Christ and the Virgin’ by Flemish painter Peter Paul Rubens, ‘The Sleeping Cupid’ (1608) by Italian painter Michelangelo Caravaggio and many others [4].

The first description of RA acknowledged by modern medicine was by the French surgeon Augustin Jacob Landré Beauvais in his dissertation during 1800. The term ‘Rheumatoid arthritis’ was coined by the British physician Archibald Garrod in 1890 [3]. Many efforts have been made to classify the disease and the first classification criteria were proposed by the American Rheumatology Association (ARA) in 1957 which was later revised in 1958 [5]. This was used in practice until 1987 when the revised classification criteria by the American College of Rheumatology (ACR) were formulated [6]. Since the 1987 criteria for RA are fulfilled rather late in the disease, a new criteria set was developed by ACR/European League Against Rheumatism (EULAR) in 2010 that allows the classification of RA very early in disease course [7]. The current criteria focus on the early disease and includes the presence of autoantibodies such as anti citrullinated protein antibodies (ACPA) and rheumatoid factor (RF).
1.3 Genetic risk Factors associated with RA

Research conducted decades earlier found an association between the Human Leucocyte Antigen HLA-DRB1 and RA which lead to the ‘Shared Epitope’ (SE) hypothesis for genetic susceptibility in RA [8]. The HLA-DRB1 gene encodes the beta chain of the HLA class II molecule which along with the alpha chain is involved in antigen presentation to the T cells. The susceptibility is conferred by the amino acids present in position 11, 71 and 74 that are involved in the peptide binding grove of the HLA molecule. The HLA-DRB1 allele that are considered SE include *01:01, *01:02, *04:01, *04:04, *04:05, *04:08, and *10:01 [9]. Structural analysis has shown that citrullinated peptides (which are autoantigens in RA) preferentially bind to the P4 pocket in the peptide binding grove of the HLA-DR SE compared to the native peptides [10]. Epidemiological studies have found a strong association between the HLA-DRB1 SE and development of RA [11, 12] with the SE homozygotes having a higher risk to develop RA compared to heterozygotes having odds ratios of 2.32 and 8.33 respectively [12].

The polymorphism of the Protein tyrosine phosphatase non-receptor type 22 (PTPN22) gene is another genetic risk for RA. PTPN22 C1858T (R620W) allele was first identified as a risk factor for type 1 diabetes (T1D) [13] and subsequent studies found the polymorphism to be a risk factor for RA [14]. The PTPN22 encodes the protein lymphoid phosphatase (Lyp) which is a class 1 protein tyrosine phosphatase and is involved in the downstream T cell receptor (TCR) and B cell receptor (BCR) signaling [15]. Early studies in human samples have proposed that the PTPN22 C1858T variant is a gain of function mutation resulting in enhanced inhibition of TCR signaling [16]. However, in two knock-in mouse models expressing the analogous mutation R619W, in the murine ortholog PEST domain phosphatase (PEP), enhanced TCR signaling was observed [17]. Similarly, mice lacking the PTPN22 gene have an enhanced TCR signaling against very weak antigens and self-antigens but very few differences in TCR signaling against strong antigens [18]. Due to contradictory conclusions from these studies, the exact role of this single nucleotide polymorphism (SNP) in T cells is yet to be clarified. Studies conducted in B cells with Lyp R620W from T1D shows an altered B cell homeostasis leading to expansion of transitional and anergic B cells and decreased BCR signaling [19]. Analysis of BCR in Healthy, T1D and RA individuals with PTPN22 C1858T have found and enhancement of autoreactive B cells in the periphery [20]. A study looking into a large cohort of RA patients found a very strong gene-gene interaction between HLA-DR SE and PTPN22 to develop ACPA positive RA having an odds
ratio of 13.2 compared to 6.1 and 1.4 with individuals carrying either HLA-DR SE or PTPN22 C1858T respectively [21].

Currently there are more than 100 genetic polymorphisms associated with RA [22] including STAT4 [23], TRAF1-C5 [24], PADI4 and CTLA4 [25].

1.4 Environmental risk Factors associated with RA

Smoking is one of the best established environmental risk factors for RA, especially in ACPA positive RA [26, 27]. In a case-control study of 679 RA patients found that those that have smoked for over 20 years had a higher relative risk of 2.7 to develop ACPA positive/RF positive RA [28]. In smokers carrying one or two copies of the shared epitope risk allele, the relative risk of ACPA positive RA is 6.5 and 21.0 respectively, suggesting a complex interaction between genetic and environmental risk factors for the development of RA [11].

Another environmental risk factor for RA is exposure to silica in industries involving mining, construction, ceramics, glass and electronics. In a case-control study of 276 males with RA, those that were exposed to silica was found to have an odds ratio of 2.2 for developing RA, when adjusted for age, residential area and smoking [29]. In a subsequent study with increased sample size by the same authors found that current smokers exposed to silica were found to have a higher risk of developing RA with odds ratio of 7.36 when compared to never smokers unexposed to silica [30].

Textile dust has been implicated as one of the environmental risk factors for RA. In a case-control study comparing Malaysian women with occupational exposure to textile dust found an odds ratio of 2.8 to develop RA [31]. The odds for RA was even higher for individuals carrying any shared epitope risk allele (OR 2.5 for non-carriers vs 25.1 for carriers), which is in agreement with the gene-environment interaction for development of RA.

The exposure to air pollution assessed by outdoor levels of different size fractions of particulate matter (PM$_{10}$ and PM$_{2.5}$) and gaseous pollutants (SO$_2$ and NO$_2$) found no association for risk of RA in an American [32] or Swedish cohort [33]. However, another study where exposure to traffic pollution defined by distance to road (0–50, 50–200, ≥ 200 meters), after adjustment for age, calendar year, race, cigarette smoking, parity, lactation, menopausal status and hormone use, oral contraceptive use, body mass index, physical activity, and census-tract-level median income and house value, an elevated risk of RA was observed for women living within 50 meters from road compared to those living farther away [34].
1.5 Posttranslational Modifications in RA

Many posttranslational modifications have been identified and implicated in the pathogenesis of RA. This section gives an overview of all known modification associated with RA and their role in the disease.

1.5.1 Citrullination

Citrulline is a non-coding amino acid formed by the deimination of peptidyl arginine by the Peptidyl Arginine Deiminase (PAD) enzymes. The process converts the positively charged arginine into a neutrally charged citrulline residue creating a minor mass shift of approximately 1 Da (figure 1). The PAD enzymes catalyze this process under high calcium (Ca$^{2+}$) concentrations and human PAD cannot convert free arginine to citrulline. There are five known isoforms of this enzyme in humans namely PAD1, PAD2, PAD3, PAD4 and PAD6. PAD1 is found in the epidermis and uterus, PAD2 in skeletal muscles, brain, inflammatory cells, secretory glands, PAD3 in hair follicles and keratinocytes, PAD4 in granulocytes and PAD6 in oocytes and embryos [35]. Interestingly, the only known bacterial PAD enzyme known as PPAD is found in Porphyromonas gingivalis which is one of the pathogens causing Periodontitis [36].

Citrullination is found under normal physiological conditions in the terminal differentiation of keratinocytes which facilitates crosslinking, creating a matrix resistant to insults [37]. Upon challenge neutrophils release neutrophil extracellular traps (NETs) which contain deiminated histone H3, to entrap extracellular pathogens [38]. Analysis of different human inflammatory and non-inflammatory tissues by immunohistochemistry has shown that citrullination is an inflammation-dependent process [39]. Analysis of bronchoalveolar lavage (BAL) cells from healthy smokers and non-smokers has shown that higher expression of PAD2 and citrullination are present in smokers [40], suggesting that environmental factors such as smoking could play a role in triggering citrullination in the lungs.

Citrulline as a constituent autoantigen in RA arises from exploring the targets of antiperinuclear factor (APF) and anti-keratin antibodies (AKA), which were one of the earliest assays used to identify autoantibodies in RA [41, 42]. The targets of AKA and APF were found to be filaggrin containing citrulline residues. Presence of citrullination is not restricted to RA but also found in osteoarthritis (OA) and reactive arthritis (ReA) synovium, as shown by immunohistological analysis [43]. Analysis of bronchial biopsies have shown that citrullination is enhanced in the lungs of ACPA positive RA smokers as compared to ACPA negative RA [44]. In a study comparing synovial biopsies from healthy volunteers and RA patients before and after treatment with glucocorticoids and methotrexate found that, citrullination was
enhanced in the RA synovium and decreased after treatment with glucocorticoids but not methotrexate [45]. This shows that treatment affecting inflammation could also bring down citrullination in RA. Citrulline immunity in RA gives rise to ACPAs whose role and function will be discussed in detail later.

![Figure 1: Different post translational modifications in RA. The figure represents the structural similarities between the different post translational modifications in RA. The homo-citrulline has one more carbon atom in the backbone compared to citrulline, whereas the acetyllysine has a CH₃ compared to NH₂ in its side chain compared to homo-citrulline.](image)

1.5.2 Carbamylation

Carbamylation is the non-enzymatic post translational modification of proteins with the addition of cyanate to primary amino- or thiol groups. One of the common carbamylation reactions is the conversion of lysine to homocitrulline [46] which resembles the peptidyl-citrulline but is one CH₂ group longer (figure 1). Carbamylation decreases the activity of enzymes and hormones such as ceruloplasmin, apo-aspartate aminotransferase, matrix metalloproteinase-2, erythropoietin, human serum albumin and insulin [47]. The carbamylation equilibrium is disrupted during disease condition. Patients suffering from uremia have an increased level of carbamylation but the contribution of carbamylation to the disease condition is difficult to ascertain and is not understood. The antigenicity towards carbamylated proteins
was evaluated by measuring antibodies against carbamylated antigens in the serum of RA and it was found that 45% of the RA serum contained antibodies against carbamylated proteins (CarP) [48]. Subsequently, autoantibody-reactivity against carbamylated alpha enolase [49] and fibrinogen [50] were present in most of the ACPA positive individuals but also detected in some serum samples of the ACPA negative individuals. This reactivity was predominantly overlapping with anti citrulline reactivity, with 37% being both anti citrulline and anti carbamylation positive and 8% being only anti carbamylation positive. Studies using animal models have shown that immunization of mice with carbamylated antigens could active T cells, cause immune activation and arthritis, demonstrating its arthrogenic properties [51]. In a recent study in mice, elevated levels of carbamylated proteins and antibodies against carbamylated proteins were detected in the blood of animals exposed to cigarette smoking [52]. These results together highlight the importance of studying the immunity against carbamylation.

1.5.3 Acetylation

Recently, acetylation has been proposed as another immunogenic post translational modification in RA. Acetylation is a very common histone modification-process, carried out by the enzyme histone acetyltransferase whereby acetyl groups are added to the free amines of the lysine residues [53]. The structure of the acetylated lysine side chain closely resembles the carbamylated lysine except for the terminal amine (NH₂) group replaced by a methyl (CH₃) group (figure 1). Analysis of serum from early untreated RA patients, showed the presence of autoantibodies against acetylated vimentin. The reactivity against acetylated vimentin was confined to those that had reactivity towards citrullinated vimentin peptide, however cross reactivity assays using serum blocked by modified peptides displayed a lesser amount of cross reactivity between the different post translational modifications [54]. More studies are required to ascertain the immunogenicity of acetylation in RA.

1.5.4 Malondialdehyde Modification

Another modification that has been recently identified as immunogenic in RA is the Oxidation-associated malondialdehyde (MDA) [55]. MDA is a naturally occurring, highly reactive aldehyde, produced under oxidative stress states associated with excessive generation of reactive oxygen species (ROS). MDA modifications occur in amino acids such as lysine, arginine, and histidine and to a lesser extent asparagine and glutamine. Analysis of serum from patients demonstrated the presence of high titers of anti-MDA antibodies in RA compared to other disease controls and healthy individuals. The same study, using mass spectrometry on
synovial biopsies, MDA modifications were identified in proteins such as actin, hemoglobin, serum albumin among others. In vitro stimulation of osteoclasts (bone resorbing cells) using anti-MDA antibodies enhanced their differentiation and bone resorption capacity [55].

1.6 Autoantibodies in RA

Autoantibodies that have been identified in RA are RF, ACPA, anti-CarP antibodies, anti-MDA antibodies and anti-acetylated antibodies. Antibodies reacting against these different posttranslational modifications are known as anti-modified protein antibodies (AMPA) [56]. The most abundant of these are the RF and ACPA.

1.6.1 Rheumatoid Factor

Rheumatoid Factor (RF) are natural occurring autoantibodies against the Fc portion of IgG. RF first described in 1940 [57] and were named so due to their association with RA [58]. They are generally low affinity, poly-reactive antibodies of mainly IgM isotype, however IgA, IgG and IgE isotypes have been detected in serum of RA patients [59, 60]. IgG RF has been found in 70% of the RA patients but is not specific for the disease. The presence of RF has been reported in the healthy population, in disease conditions such as psoriatic arthritis, primary Sjögren’s syndrome, systemic lupus erythematosus, and also during bacterial and viral infections [61]. In a case-control study of blood donors, IgA RF was the earliest autoantibody to be detected before the disease onset and was a very good predictor of RA [62]. In a study analyzing the different RF isotypes and retrospective X rays in RA patients, found an association between IgA RF and periarticular bone erosion in the hands but not with IgG and IgM RF [63]. Extra-articular manifestations (vasculitis, rheumatoid nodules) were more frequent in RF positive, but not in ACPA positive, compared to negative patients [64].

1.6.2 Anti-citrulline protein antibodies

ACPAs are antibodies that recognize antigens that contains the non-coding amino acid citrulline. The first ELISA to detect ACPA was developed using peptides derived from filaggrin, which has a sensitivity of 76% and specificity of 96% [42] and by using a cyclic variant of the peptide (CCP) gives a moderate sensitivity of 68% and an increased specificity of 98% [65]. Currently a second generation of this cyclic citrullinated peptides (CCP2), which has a better diagnostic and prognostic ability than CCP is being used commercially in detection of ACPAs [66]. ACPAs recognize citrullinated epitopes present on several proteins namely
filaggrin, fibrinogen [67], vimentin [68], alpha-enolase [69], collagen type II [70], Histone H4 [71], tenascin-C [72], apolipoprotein E, myeloid nuclear differentiation antigen and β-actin [73]. ACPA in diagnostics is typically measured as IgG, however APCAs can be present as IgM, IgA, IgG1, IgG2, IgG3 and IgG4 isotypes [74]. Analysis of serum from patients with undifferentiated arthritis towards RA, revealed that although the number of isotypes did not change rapidly at follow up, the presence of more isotypes correlated with high levels of ACPAs and were significant predictors of radiographic progression [75].

Retrospective studies analyzing serum from blood donors have shown the presence of ACPAs as early as 10 years prior to the onset of the clinical symptoms of RA [62, 76]. The presence of ACPAs is associated with severe disease course and radiological progression [77]. In a study of 242 patients with RA, it was observed that the prevalence of joint erosions, assessed using high-resolution peripheral quantitative computer tomography (HR-pQCT) was more in the ACPA and RF positive group as compared to only ACPA or RF positive and RF negatives, suggesting an additive effect of both ACPA and RF in bone erosion in RA [78]. The presence of ACPA against antigenic targets present in different proteins is termed as the ACPA fine specificity repertoire. In studies analyzing blood samples from individuals predating the disease, an increase in ACPA fine specificity was seen very close to onset of clinical RA [79, 80]. This indicates a process of antibody maturation and epitope spreading leading to the disease onset.

1.7 Effector Functions of autoantibodies in RA

Over the years, studies have been carried out to understand the effector functions of the autoantibodies in RA (figure 2). Earlier studies, using monoclonal antibodies against citrullinated fibrinogen injected into mice did not show development of arthritis, however when injected along with an ‘arthrogen’ (like antibodies against CII) significantly increased the severity of arthritis compared to mice injected with arthrogen alone or in combination with a control antibody [81]. This study demonstrates the ability of ACPA to enhance the disease and propagate inflammation. There are growing number of in vitro studies investigating the ACPA effects on different cell types. ACPA-containing immune complexes act on macrophages via the Fc gamma Receptor (FcγR) IIa to enhance the production of proinflammatory cytokines such as TNF [82]. Similar study using ACPA immune complexes containing citrullinated fibrinogen were able to stimulate macrophages via both the Toll-like receptor 4 (TLR-4)/myeloid differentiation protein (MyD88) and the FcγR pathway to produce TNF [83]. Analysis of ACPA from RA serum showed that it can activate the complement system via the
classical and alternative pathway but not the lectin binding pathway [84]. Studies using U937 cells (monocyte cell line) revealed that ACPA bound to surface-expressed citrullinated glucose-regulated protein 78 (Grp78) enhanced the production of TNF [85]. This mechanism was mediated through the ERK1/2 and JNK signaling pathways to enhance IKK-α phosphorylation, which leads to the activation of NF-κB and the production of TNF [86]. These studies highlight the role of ACPA in promoting inflammation in- and outside the joint in RA.

Studies comparing polyreactive (low affinity) and monoreactive (high affinity) IgM RF derived from RA patients have shown that the monoreactive IgM RF has a higher complement activating capacity [87]. In a study using IgM RF derived from a patient with type II cryoglobulinemia, on monocyte derived macrophages found that, IgM RF by itself did not promote TNF secretion but together with ACPA-citrullinated fibrinogen immune complex (ACPA-IC) was able to enhance proinflammatory cytokine production by macrophages [88]. Similar results were observed using IgM and IgA RF derived from RA patients, where IgM and IgA RF in combination with ACPA-IC had different dose-dependent effects on macrophages [89].

Recent studies have focused on understanding the bone destruction-capacity of ACPA. Affinity purified antibodies against mutated citrullinated vimentin (MCV) were used to stimulate in vitro generated osteoclasts and found that anti-MCV antibodies enhanced both osteoclastogenesis and bone destruction. The authors showed that this was mediated by the antibodies binding to citrullinated vimentin on the surface of osteoclasts and downstream induction of TNF [90]. Similarly, ACPAs isolated from patients by affinity chromatography against CCP2, were used to study the effects on osteoclasts. The purified polyclonal ACPAs enhanced the osteoclast maturation and bone-destruction capacity [91]. Osteoclasts stimulated with monoclonal antibodies generated from single B cell cloning showed that only specific ACPAs could induce osteoclastogenesis. This mechanism of enhanced osteoclast activity was found to be dependent on the PAD enzymes and IL-8 in an autocrine manner. Furthermore, intravenous injection of ACPAs in mice decreased trabecular bone mineral density compared to controls, which was reversed by subcutaneous administration of reparixin (IL-8 antagonist) [91]. Mice injected with ACPAs also exhibited long-lasting pronounced pain-like behavior in the absence of inflammation compared to control animals injected with non-ACPA IgGs. This pain like behavior was dependent on osteoclast-derived release of CXCL1/2 (Mouse IL-8 analogue) which activates the sensory neurons through the receptor CXCR2 [92]. This could explain the arthralgia that commonly precedes the onset of synovitis in individuals who develop ACPA positive RA [93, 94].
Figure 2: Functions of autoantibodies in RA. The figure summarizes the functional role of ACPA, RF and Immune complexes on different immune cells in the joints namely Osteoclasts, Macrophages, Neutrophils, and the role of other inflammatory mediators in the joints (adapted from [148]).

1.8 Role of Lungs in RA

The lungs are one of the primary organs that have been implicated as both the cause and consequence of RA. The lung involvement amongst extra-articular manifestations of RA is well documented. Studies have shown that lung disease such as pulmonary fibrosis is the second leading cause of mortality in RA [95]. There are a spectrum of lung manifestations in RA, that are classified as Airway disease (Cricoarytenoid arthritis, Emphysematous type, Bronchiolitis/obliterative bronchiolitis, Bronchiectasis), Parenchymal disease (Non-specific interstitial pneumonia, Usual interstitial pneumonia, Acute interstitial pneumonia/diffuse alveolar damage, Organizing pneumonia, Nodules including rheumatoid nodules and Caplan’s syndrome), Vascular disease (Rheumatoid vasculitis, Pulmonary hypertension, Thromboembolic disease), Pleural disease (Pleuritis/pleural thickening, Pleuropericardial
effusion, Bronchopleural fistula, Pneumothorax) and others (Medication/drug toxicity, Infection, Malignancy including pulmonary lymphoma) [96].

A population-based study comparing RA patients and controls found that the life time risk of developing interstitial lung disease (ILD) was 7.7% for RA patients compared to 0.9% for non-RA controls and the risk of death for RA patients with ILD was 3 times higher than in RA patients without ILD [97]. In a cohort study analyzing the lungs using high resolution computed tomography (HRCT) found bronchiectasis in 35%, bronchial wall thickening in 50%, ground-glass opacification in 18%, and reticular changes in 12% of the early RA patients [98]. It was also found that concentration of ACPA correlated with lung function parameter such as D_LCO (impaired gas transfer) and bronchial wall thickening observed by HRCT. In a study comparing HRCT lung changes between early and longstanding RA patients, the most frequent HRCT abnormality was the bronchial dilation (which is a hallmark of bronchiectasis) found in 33.8 % and 49.2 % of early and long-standing RA patients respectively. There was a significant difference in bronchial wall thickening (6.2 % in early RA vs 18 % in longstanding RA) and parenchymal micronodules (7.7 % vs 21.3 %). However no significant difference was observed in radiological lung abnormalities corresponding to interstitial pulmonary disease suggesting that the changes could be independent of disease duration [99]. In a study designed to identify the risk factors for ILD and airway disease (AD) in RA, found high titers of RF and anti-CCP, age (≥65), HLA DRB1*1502, sex and smoking as significant risk factors for ILD whereas high titers of RF and anti-CCP were a risk factor for developing AD [100]. There was a negative association between HLA DRB1*1502 allele and AD. Another finding of prominent interest is the analysis of ACPA fine specificity and its association with ILD, found that the RA patients with ILD had significant higher number of ACPA fine specificities compared to non-ILD [101]. Other autoantibodies such as antibodies against PAD3/4 which enhance citrullination have also been implicated in RA-associated ILD. Interestingly, RA patients having anti-PAD3/4 antibodies have a higher frequency of ILD (68%) compared to non-ILD RA (29%) [102]. These studies highlight the importance of lung as a secondary organ affected by the disease in RA and this could involve an interplay between environmental factors, genetics and autoantibodies.

Aside from the macroscopic changes seen by HRCT, lungs are a site of inflammation and microscopic changes in RA. Analysis of bronchoalveolar lavage (BAL) cells and bronchial biopsies have shown the presence of citrullinated proteins [40, 44]. Analysis of bronchial biopsies from the lungs of early RA patients have shown a presence of increased lymphocyte infiltrates, inducible bronchus-associated lymphoid tissue (iBALT) structures, increase in
activation markers like CD3, HLA-DR and HLA-DQ compared to healthy controls [103]. ACPAs have been detected in the BAL and sputum of early RA and were found to be enriched in the lungs compared to circulation [44, 104]. Analysis of lungs from RA patients with lung disease indicates the presence of iBALT structures and presence of B cells capable of producing ACPAs [105] suggesting that lungs could be an organ for maturation and production of pathological autoantibodies in RA.

1.9 Microbiome in RA

Microbiome refers to the collection of microorganisms that are present in an environmental niche. In the human body, it has been proposed that there are approximately 10 times more bacteria than the number of human cells and 100 times more bacterial genomic content than the human genome [106]. However, recent revised estimates suggest that approximately equal number of bacteria and human cells to be preset in the body [107].

1.9.1 Methods to study microbiome

Historically, the study of microbiome has been performed using labor-intensive culture based techniques, where only relatively small proportion of the bacteria could be studied. With the advent of sequence based technologies, it has been show that 80% of the bacteria present in our body have never been cultured before, because the laboratory conditions and nutrients do not favor the growth of all the organisms (especially anaerobes) [108]. The sequencing can be performed by two different methods. The first method is to use 16S rRNA sequencing that provides information on the identity of the bacterial communities present in an environmental niche and enables us to understand the complexity of the bacterial communities. This is based upon the sequencing of the 16S ribosomal RNA (16S rRNA) of the bacteria which is highly conserved and serves as the signature of a bacterial species. The 16S rRNA contains hypervariable regions that provide specific signatures, which enable the identification of bacteria present in a sample. The second approach is the whole genome shotgun sequencing, which allows us to study the various pathways the bacterial communities might be involved in and what they perform in the environment [109]. Using these approaches several sites in the human body including the airways, oral cavity, skin, gastrointestinal tract (gut) and urogenital tract have been studied [110].
The gut microbiome is the most commonly studied and its role in health and disease has been understood using human samples and animal models. The homeostasis (and protection) between the gut microbiome and immune system is maintained by a physical barrier provided by the tight epithelial cells and mucosal layer, biochemical products such as enzymes and antimicrobial proteins and immunological factors such as IgA and epithelia-associated immune cells [111]. The gut microbiota provides the host with a wide range of nutrients that the host produce in low amounts or is incapable of producing. The microbial flora produces short chain fatty acids (SCFAs) that are absorbed and used by the cells in various cellular processes. Lactic acid bacteria can support the production of vitamin B12 and *Bifidobacterium* facilitate the folate production [106].

The gut is mostly dominated by bacteria from two phyla namely Firmicutes and Bacteroidetes [108]. Though the gut is continuous organ system, studies using mice have identified that the microbiome composition varies along the gastrointestinal tract with the gastric, duodenal, and large intestine having higher phylogenetic diversity than the jejunum and ileum [112]. Also, the communities found in the small intestine and stomach were different from those seen in the large intestine and fecal samples suggesting the existence of location-based microbial ecosystem in the gut. Factors affecting the gut microbiome include diet, smoking, antibiotic-use, drugs, probiotics, prebiotics and enteric infections [113]. Age and sex are also factors that determine the microbiome composition. The change in bacterial community causing an imbalance is termed as dysbiosis and this has been studied in Obesity [114], Ankylosing Spondylitis [115], Inflammatory bowel disease [116], Type 1 diabetes [117] and Type 2 diabetes [118].

Gut dysbiosis in RA has been previously studied from fecal samples using culture-based techniques demonstrating that the presence of *Clostridium perfringens* was higher in RA compared to healthy controls [119]. Another study using computerized gas-liquid chromatography (GLC) to investigate bacterial cellular fatty acids (CFAs) in the fecal samples found differences between RA and healthy controls suggesting dysbiosis [120]. Furthermore, a study comparing the fecal microbiome from healthy individuals, psoriatic arthritis (PsA), new onset RA and RA patients on treatment, found that the relative abundance of *Prevotella* increased and *Bacteroides* decreased in new onset RA as compared to healthy, PsA and treated RA [121]. The authors were able to identify *Prevotella copri* as a strain that was significantly abundant in new onset RA. Comparison between treated RA and early RA showed that, treatment was effective in bringing the balance between *Prevotella* and *Bacteroides* similar to
that seen in healthy individuals which the authors speculate could be due to treatment-dependent decrease of inflammation. Another large study using fecal samples from a large cohort of RA and healthy individuals obtained similar results showing dysbiosis in the gut of RA compared to healthy, with enrichment of gram positive bacteria in the RA gut. Bacteria such as *Haemophilus* were over-represented in the healthy individuals whereas *Lactobacillus salivarius* was enriched in RA. In this study, the authors were able to compare the fecal samples from the same patient before and after treatment and found that treatment was able to restore the microbial balance [122].

Some of our understanding on the role of microbiome in RA comes from studies using animal models. In an adjuvant-induced (*Mycobacterium bovis* - BCG) arthritis model germ free rats developed more severe arthritis compared to specific pathogen fee rats which was enhanced by colonization with *Bifidobacterium, Propionibacterium acnes, Lactobacillus casei, L. fermentum, L. murini, and L. acidophilus* [123]. However, in a K/BxN mice model, germ free conditions attenuated the arthritis, and was enhanced by the colonization with segmented filamentous bacteria through a Th17 response [124]. Similarly, in an IL-1 receptor antagonist knockout (IL1rn<sup>−/−</sup>) mice model, germ free conditions did not favor the development of arthritis while colonization with *Lactobacillus bifidus* lead to the development of arthritis by a Th17 and TLR4 dependent mechanism [125]. These studies suggest that altering the microbial flora may trigger or exacerbate the diseases in RA.

### 1.9.3 The Oral Microbiome

The oral cavity is the entry portal of the gastrointestinal tract and is colonized by different microbial species. Although the oral microbiome is studied as a whole, many different habitats exist in the oral cavity and bacterial diversity different among these sites. The most prevalent phyla in the oral cavity are Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, Fusobacteria, and abundant species being *Streptococcus* [126]. Bacteria present in the oral cavity such as *Porphyromonas gingivalis*, a periodontopathic bacterium found in the gums is implicated in RA. This is because periodontitis is associated with RA and *P. gingivalis* is the only known bacterial to have its own PAD enzyme that can generate citrullinated proteins [127]. In a recent study using gingival cervical fluid (GCF) from individuals with and without periodontitis found that enrichment of *Aggregatibacter actinomycetemcomitans* in periodontitis patients compared to controls [128]. *A. actinomycetemcomitans* induces hypercitrullination via Leukotoxin A which forms pores creating an influx of extracellular calcium favoring the activity of PAD enzymes. Analysis of saliva and dental plaques from RA
patients and healthy individuals showed that bacteria such as *Lactobacillus salivarius*, *Atopobium* spp. and *Cryptobacterium curtum* were over-represented in the RA group. Interestingly *P. gingivalis* was over-represented in the healthy individuals in both the saliva and dental plaques, in this study [122].

1.9.4 The Lung Microbiome

Similar to the gut mucosa the airways are also colonized by bacteria, but unlike the gut the airways have a low bacterial burden. The upper respiratory tract has a higher bacterial burden of about 100 to 10,000 times more bacteria than the lower respiratory tract [129], which makes it challenging to study lung microbiome due to high noise and low signal. Differences in microbiome can be observed in different regions of the upper airways with studies displaying that phyla present in the nasopharynx (nasal cavity) were Firmicutes (73%), Proteobacteria (12.6%), Bacteroidetes (7%), and Actinobacteria (5.6%); in the oropharynx (oral cavity) the principal phyla were Bacteroidetes (36.4%), Firmicutes (27.7%), Proteobacteria (12.6%), and Fusobacteria (12.3%) [130]. In a study comparing microbiome of nasal cavity, oral cavity, lungs and stomach (gastric juice) of healthy individuals found that the oral microbiota but not nasal microbiota, overlapped with that of the lungs though with a very low relative abundance [131]. One of the ways of delivery of microbiota to the lower airways could be due to microaspirations which is common in both health and disease states [132].

In a study comparing the BAL of healthy individuals versus COPD patients, it has been demonstrated that the diseased lungs have lower diversity and abundance of bacteria [133]. The study also highlights some of the core pulmonary bacterial microbiome present in lower airways such as *Pseudomonas*, *Streptococcus*, *Prevotella*, *Fusobacterium*, *Haemophilus*, *Veillonella*, and *Porphyromonas*. In an investigation comparing oral wash and BAL from healthy smokers and non-smokers showed that though the overall bacterial community in the lung resembles that of the oral cavity, there were distinct bacterial species that were overrepresented in the lung [134]. Though there was no significant decrease in diversity of bacterial communities, a decrease in relative abundance of Bacteroidetes and Proteobacteria in the BAL of smokers compared to non-smokers was observed. Differences in lung microbiome can be observed during disease conditions such as Chronic Obstructive Pulmonary Disease (COPD) [135] and Asthma [136] with some taxa correlating with the disease parameters. Not much is understood about the microbiome perturbation caused by smoking and inflammation in the lungs of RA and studies looking into this aspect with feasible designs are important in understand how they play a role in the etiology in RA.
1.10 Model for etiology and pathogenesis of RA

The disease manifestation in RA is different in ACPA positive compared to ACPA negative individuals, with the known environmental (such as smoking) and genetic factors (HLA-DR SE) associated to the ACPA positive and not ACPA negative disease [11]. There is a complex interplay between genetic and environmental factors that leads to immunity, which breaches a ‘tolerance threshold’ leading to the onset of the disease. Here I have summarized a model for the etiology and pathogenesis of ACPA positive RA based on existing knowledge.

Epidemiological investigation in a large population-based twin cohort suggests that the environmental factors (such as smoking and silica) play an important role in the development of ACPAs whereas the genetic factors (HLA-DR SE) might play a larger role in development of ACPA positive RA, called step wise development of RA model [137]. The presence of ACPA (detected by CCP2) [62, 76], anti-CCP (IgA, IgG, IgM) [138], RF (IgA, IgG and IgM) [139] in circulation long before the onset of the disease might suggest that immunity in RA arises in organs other than the joints. Possible organs could be the mucosal surfaces such as the gut, gums and/or lungs. Epidemiological data suggests a strong association between smoking and the development of ACPA and ACPA positive RA, makes the lungs one of the strong candidates for the site for initiation of autoimmunity in RA.

The lung faces a lot of environmental insults and offers wide range of physical, innate and adaptive immune protection. The epithelial cell surface of the lungs are directly exposed to environmental and external pollutants such as dust, silica and cigarette smoke which affects the physical barrier provided by the epithelial layer by increasing its permeability [140]. Investigation of the bronchial mucosal biopsies and bronchoalveolar lavage (BAL) cells from healthy smokers and non-smokers, showed that smoking increases citrullination and expression of PAD2 enzymes [40]. Cigarette smoke affects the alveolar macrophages to become more apoptotic [141] and activates them to produce pro-inflammatory mediators [142]. In vitro stimulation of bronchial epithelial cells with cigarette smoke extract increased the production of chemoattractant IL-8 that attracts neutrophils. This correlated with in vivo data from human BAL samples where an elevation in IL-8 among smokers, correlated with the number of neutrophils present in the BAL [143]. Neutrophils produce NETs which are a source of different citrullinated autoantigens in RA [144]. A recent study using transgenic mice carrying RA-susceptible *0401- genes exposed to cigarette smoke, displayed high levels of PAD expression and antibodies against native and citrullinated vimentin compared to RA resistant
These studies show that environmental stimuli in the lungs lead to the generation of modified autoantigens (figure 3).

Figure 3: Early immune events in the lungs of RA. Environmental triggers (such as smoke, silica, dust and agents such as microorganisms) can lead to an increase in PAD enzymes and citrullination. The generation of the autoantigens leads to activation of antigen presenting cells (APCs with subsequent activation of T cells (T) and B cells (B) and local production of ACPAs (adapted from [148]).

Then generation of autoantigens could lead to local production of autoantibodies in the lungs. Autoantibodies (ACPA and RF) were found in the sputum of individuals at risk of developing RA [104]. Studies in BAL from early untreated RA have shown the presence of ACPA (IgA and IgG) in the lungs [44]. The ACPA that are generated in individuals that are at risk to develop RA might then undergo affinity maturation. Analysis of blood donor samples predating the disease onset found expansion of ACPA fine specificities close to onset of RA and the epitope expansion was closely correlated with the appearance of preclinical inflammation [101]. Another feature before the onset of disease is the arthralgia that is observed in many ACPA positive subjects [146]. Studies in mice have shown that ACPA injected intravenously cause pain-like behavior in mice without arthritis [92].
tomography (CT) to study the joints of these mice it was observed that ACPAs significantly reduced the trabecular bone mineral density and bone volume fraction compared to control antibodies [91]. This observation falls in line with studies on ACPA positive and ACPA negative healthy individuals undergoing micro-CT, finding a significant decrease in bone mineral density in ACPA positive healthy individuals [147]. This suggest that bone loss in RA starts prior to the onset of the clinical disease symptoms (longitudinal events summarized in figure 4).

The breach of tolerance first leading to systemic autoimmunity and later to disease in RA might require an additional hit [148]. The exact nature of what causes the trigger to the disease is yet to be deciphered. Another aspect that requires further understanding is the propagation of immunity arising at the mucosal sites and transitioning to the joints. We aimed to address that by searching for shared immunological targets in the lungs and joints, which will be discussed in detail in this thesis.

**Figure 4: Model for longitudinal development of RA.** The figure summarized a model for the development of ACPA positive RA.

Other models for the etiology and pathogenesis of the disease might exist that could be independent of the HLA-DR SE, PTPN22, smoking or lungs. But the fascinating aspect of the disease is that, no matter when the immunity starts, it finally ends up targeting the joints which might suggest that there might be a common factor that could link all this different initiating events in RA.
2 AIMS

The general aim is to characterize the anti citrulline immune responses (both antibodies and antibody-producing cells) and its impact on inflammation, within and outside the joint of RA.

Specific aims are:

- To investigate ACPA-response in relation to lung abonormalities in early-untreated RA (paper I).
- To investigate the lung microbiome in in early-untreated RA (paper II).
- To investigate the immunolgical link between the lungs and joints in RA (Paper III).
- To identify new articular and extra-articular ACPA targets (paper III and IV).
- To characterize immunity against new discovered targets (paper III and IV).
3 MATERIALS AND METHODS

This section summarizes the biological materials obtained from various cohorts and the methods used for the different analysis. More detailed description can be found in each paper.

3.1 Subjects

Different biological material was obtained from patients and controls. The ethical review board of the Karolinska University Hospital approved all the studies included in this thesis and all patients and controls gave informed consent.

3.1.1 LURA Cohort (Paper I, II, III)

The lung investigation in newly diagnosed RA (LURA) cohort is a unique cohort of newly diagnosed RA patients (n=134) according to the ACR 1987 classification criteria [6] with patient reported symptom of less than 1 year, naïve to treatment with oral glucocorticoids and disease modifying anti-rheumatic drugs (DMARDs). Pregnancy, alcohol and/or drug abuse were exclusion criteria. Serum was obtained during the time of visit to the clinic and clinical parameters such as DAS28 were measured. HRCT and spirometry was performed on 106 (consecutive) patients. Some of the patients (n=20) underwent bronchoscopy during which bronchial biopsy and bronchoalveolar lavage (BAL) was obtained. The patient demographics of the different sample groups is summarized in table 1.

Table 1: LURA Cohort. Demographics of patients from whom serum, bronchoalveolar lavage and bronchial biopsies were obtained.

<table>
<thead>
<tr>
<th></th>
<th>Serum</th>
<th>HRCT</th>
<th>Bronchoscopy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Serum</td>
<td>BAL</td>
</tr>
<tr>
<td>Number</td>
<td>134</td>
<td>106</td>
<td>20</td>
</tr>
<tr>
<td>Age (years), Median</td>
<td>57 (20-84)</td>
<td>61 (23-85)</td>
<td>59 (28-76)</td>
</tr>
<tr>
<td>Range</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female, n (%)</td>
<td>91 (67.9 %)</td>
<td>73 (68.9 %)</td>
<td>8 (40 %)</td>
</tr>
<tr>
<td>ACPA positive, n (%)</td>
<td>88 (65.7 %)</td>
<td>71 (67.0 %)</td>
<td>16 (80.0 %)</td>
</tr>
<tr>
<td>Smoking</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-smokers, n (%)</td>
<td>38 (28.4 %)</td>
<td>29 (27.4 %)</td>
<td>4 (20.0 %)</td>
</tr>
<tr>
<td>Ex-smokers, n (%)</td>
<td>58 (43.3 %)</td>
<td>46 (43.4 %)</td>
<td>7 (35.0 %)</td>
</tr>
<tr>
<td>Current smokers, n (%)</td>
<td>38 (28.4 %)</td>
<td>31 (29.2 %)</td>
<td>9 (45.0 %)</td>
</tr>
</tbody>
</table>
3.1.2 RA Cohort (Paper II, IV)

Serum was obtained from an early RA cohort called EIRA which has been described previously [11] and was used in the analysis for paper III. Serum was obtained from a cohort of established RA patients undergoing joint arthrocentesis and was used for analysis in paper IV. Synovial biopsies were obtained from patients with long standing RA undergoing orthopedic surgery. The patient demographics of the different sample groups is summarized in table 2.

Table 2: RA Cohort. Demographics of patients from whom serum and synovial biopsies were obtained. NA denotes data not available.

<table>
<thead>
<tr>
<th></th>
<th>EIRA Serum</th>
<th>Established RA Serum</th>
<th>Synovial Biopsy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>259</td>
<td>347</td>
<td>7</td>
</tr>
<tr>
<td>Age (years), Median (Range)</td>
<td>56 (20-70)</td>
<td>58 (21-94)</td>
<td>40 (30-63)</td>
</tr>
<tr>
<td>Female, n (%)</td>
<td>187 (72.2 %)</td>
<td>277 (79.8 %)</td>
<td>5 (71.4 %)</td>
</tr>
<tr>
<td>ACPA positive, n (%)</td>
<td>135 (52.1 %)</td>
<td>250 (72.0 %)</td>
<td>5 (71.4 %)</td>
</tr>
</tbody>
</table>

3.1.3 Disease Controls (Paper II, III, IV)

Serum was obtained from patients with Psoriatic arthritis (PsA) or Ankylosing spondylitis (AS) to be used as disease controls in paper III and IV.

Table 3: Disease Controls. Demographics of the serum and BAL samples obtained from patients with PsA, AS and Sarcoidosis to be used as disease controls. NA denotes data not available.

<table>
<thead>
<tr>
<th></th>
<th>PsA / AS Serum</th>
<th>Sarcoidosis BAL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>236</td>
<td>10</td>
</tr>
<tr>
<td>Age (years), Median (Range)</td>
<td>47 (18-85)</td>
<td>40 (30-63)</td>
</tr>
<tr>
<td>Female, n (%)</td>
<td>115 (48.7 %)</td>
<td>3 (30.0 %)</td>
</tr>
<tr>
<td>ACPA positive, n (%)</td>
<td>10 (4.2 %)</td>
<td>0 (0 %)</td>
</tr>
</tbody>
</table>

Smoking

| Non-smokers, n (%) | NA | 4 (40 %) |
| Ex-smokers, n (%)  | NA | 4 (40 %) |
| Current smokers, n (%) | NA | 2 (20 %) |
BAL and serum was obtained from patients with sarcoidosis to be used as disease controls in our study of the lung microbiome. Detailed characteristics of the disease status have been described in paper II. The patient demographics of the different sample groups is summarized in table 3.

3.1.4 Healthy Controls (Paper I, II, III, IV)

Serum and BAL was obtained from healthy volunteers. For paper II, the study of the lung microbiome, BAL was obtained from healthy individuals with normal x-ray and no clinically relevant lung disease. The demographics of the different sample groups is summarized in table 4.

*Table 4: Healthy Controls. Demographics of the serum and BAL samples obtained from healthy individuals. NA denotes data not available.*

<table>
<thead>
<tr>
<th></th>
<th>Serum</th>
<th>BAL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>152</td>
<td>28</td>
</tr>
<tr>
<td>Age (years), Median (Range)</td>
<td>57 (23-71)</td>
<td>28 (19-50)</td>
</tr>
<tr>
<td>Female, n (%)</td>
<td>107 (70.4 %)</td>
<td>14 (50 %)</td>
</tr>
<tr>
<td>ACPA positive, n (%)</td>
<td>5 (3.3 %)</td>
<td>1 (3.6 %)</td>
</tr>
<tr>
<td>Smoking</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-smokers, n (%)</td>
<td>NA</td>
<td>14 (50 %)</td>
</tr>
<tr>
<td>Ex-smokers, n (%)</td>
<td>NA</td>
<td>0</td>
</tr>
<tr>
<td>Current smokers, n (%)</td>
<td>NA</td>
<td>14 (50 %)</td>
</tr>
</tbody>
</table>

3.2 High Resolution Computed Tomography and Spirometry (Paper I)

HRCT was performed on RA patients (n=106) of the LURA cohort. The HRCT images were blindly assessed by an experienced radiologist and a pulmonologist. The HRCT abnormalities were categorized as the following:

- Airway abnormalities - Bronchiectasis, air trapping and air wall thickening
- Parenchymal abnormalities - Nodules larger than 3mm, ground glass opacities, opacities, fibrosis and emphysema

Spirometry was performed to assess the working capacity of the lungs. The forced expiratory volume in 1 second (FEV₁), vital capacity (VC), forced vital capacity (FVC) and diffusing capacity for carbon monoxide (DLco) were measured and used for analysis. Detailed description of the method can be found in paper I.
3.3 HLA-DR and PTPN22 Genotyping (Paper I, III, IV)

The RA patients were genotyped for the HLA-DRB1 allele as described previously [149] and HLA-DRB1*0101, *0102, *0401, *0404, *0405 or *1001 alleles were classified as HLA-shared epitope (HLA-SE) alleles [9]. In total 322 of the 347 established RA patients (in paper IV) were genotyped to identify the PTPN22 R620W risk allele carriers as described previously [150].

3.4 Microbiome Analysis (Paper II)

Microbiome analysis was performed on the BAL to identify the dysbiosis of the lungs. This was performed using the 16S rRNA V4 gene sequencing which acts as a unique signature to identify different taxa present in any given sample.

3.4.1 DNA Extraction and Sequencing

Bacterial DNA was extracted from the BAL samples using the Qiagen DNA extraction kit. High throughput sequencing of the 16s rRNA V4 region was performed using the MiSeq Illumina sequencer.

3.4.2 Microbiome sequence Analysis

The 16s rRNA sequences obtained were analyzed using the Quantitative Insights into Microbial Ecology (QIIME) pipeline for analysis of community sequence data. The reads were de-multiplexed and quality filtered with default parameters using prinseq. Sequences were then clustered into operational taxonomic units (OTUs) using a 97% similarity threshold with USEARCH and the Greengenes 16S reference dataset and taxonomy [151]. To avoid bias due to the different number of sequences per sample, 3687 sequences (minimum number of sequences found in a given sample) were used per sample in order to define OTUs and taxonomic composition and ade4 package in R was used to construct Principal Coordinate Analysis (PCoA) based on unweighted UniFrac distances.

3.5 Mass Spectrometry (Paper III)

Mass spectrometry was used to identify citrullinated peptides in the synovial and bronchial biopsies. Briefly, the samples were homogenized by shaking with ceramic beads followed by sonication. After reduction and alkylation, the proteins were digested with Lys-C.
The samples were then passed through Liquid chromatography (LC) and MS/MS analysis was performed. The peptides were identified using the Mascot search engine using the human IPI database. All citrullinated peptides identified had a Mascot score of at least 20. Detailed description of the methodology can be found in paper III.

3.6 Detection and Measurement of Autoantibodies

Enzyme-Linked Immunosorbent Assay (ELISA) based methods allow for quantitative and qualitative measurement of autoantibodies in the serum.

The ACPAs were detected and measured using commercial CCP2 ELISA kit (Eurodiagnostica, Malmö, Sweden) following the protocol provided by the manufacturer. The cut-off for positivity was 25 Au/ml as recommended.

Fluoroenzyme immunoassay EliA (Phadia, Uppsala, Sweden) was used to measure IgA and IgA anti-CCP2 antibodies and IgM and IgA RF. The assay was performed according to the instructions provided by the manufacturer and positivity for the assay was determined by the cut-off provided by the manufacturer.

3.6.1 Multiplex peptide chip-based assay (Paper I, II)

Detection of ACPA fine specificities against different citrullinated peptide antigens were performed using a custom peptide array method based on the Immuno Solid-phase Allergen Chip (ISAC) (Phadia, Uppsala, Sweden) platform that is used to screen for immunity against allergens.

Peptides both in the citrullinated and native form are printed in spots onto a glass slide (table 5). Serum samples, diluted 1:40 in dilution buffer (Phadia, Uppsala, Sweden), were incubated on the slides and bound autoantibodies were detected using a Cy3 conjugated goat anti-human IgG (Jackson ImmunoResearch Laboratories, Newmarket, UK). The fluorescence-intensity was measured and converted to arbitrary units (Au/ml) based on an internal calibrator present in each chip. The cut-off values were determined as the 98th percentile of the healthy control reactivity.
Table 5: Peptides in ISAC peptide array. The sequence and citrulline positions of the different peptides used in the ISAC peptide array. * denotes Cyclic Peptides.

<table>
<thead>
<tr>
<th>Name of Peptide</th>
<th>Protein</th>
<th>Amino acids</th>
<th>Amino acid sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>CEP-1</td>
<td>α-Enolase</td>
<td>5-21</td>
<td>CKIHA(cit)EIFDS(cit)GNPTVEC*</td>
</tr>
<tr>
<td>CCP-1</td>
<td>Filaggrin</td>
<td>307-324</td>
<td>SHQEST(cit)GRSRGRSRGGS*</td>
</tr>
<tr>
<td>Vim 2-17</td>
<td>Vimentin</td>
<td>2-17</td>
<td>ST(cit)SVSSSSY(cit)(cit)MFGG</td>
</tr>
<tr>
<td>Vim 60-75</td>
<td>Vimentin</td>
<td>60-75</td>
<td>VYAT(cit)SSAV(cit)L(cit)SSVP</td>
</tr>
<tr>
<td>Fiba 36-50</td>
<td>Fibrinogen α-chain</td>
<td>36-50</td>
<td>GP(cit)VVE(cit)HQSACKDS</td>
</tr>
<tr>
<td>Fiba 563-583</td>
<td>Fibrinogen α-chain</td>
<td>563-583</td>
<td>HHPGIAEFP(cit)KSSSYSKQF</td>
</tr>
<tr>
<td>Fiba 580-600</td>
<td>Fibrinogen α-chain</td>
<td>580-600</td>
<td>SKQFTSSTSYN(cit)GDSTFESKS</td>
</tr>
<tr>
<td>Fiba 621-635</td>
<td>Fibrinogen α-chain</td>
<td>621-635</td>
<td>(cit)GHAKS(cit)PV(cit)GIHTS</td>
</tr>
<tr>
<td>Fibβ 36-52</td>
<td>Fibrinogen β-chain</td>
<td>36-52</td>
<td>NEEFFSA(cit)GHRPLDKK</td>
</tr>
<tr>
<td>Fibβ 60-74</td>
<td>Fibrinogen β-chain</td>
<td>60-74</td>
<td>(cit)PAPPISSGGY(cit)A(cit)</td>
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</tbody>
</table>

3.6.2 In-house Enzyme-Linked Immunosorbent Assay (Paper III, IV)

Antibodies to various other citrullinated antigens were measured using in-house ELISA. The different antigens used is summarized in Table 6.

Table 6: Peptides used for the in-house ELISA. Fib are peptides from protein fibrinogen α-chain and Vim is peptide from vimentin protein. O represents biotin, Z represents 6-aminohexanoic acid.

<table>
<thead>
<tr>
<th>Name of Peptide</th>
<th>Amino Acid</th>
<th>Sequence of the Peptide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fib α 35</td>
<td>29-41</td>
<td>AEGGGV(Cit)GPRVVE-ZO</td>
</tr>
<tr>
<td>Fib α 216,218</td>
<td>201-225</td>
<td>KDLLPS(Cit)D(Cit)QHLPLIK-ZO</td>
</tr>
<tr>
<td>Fib α 263,271</td>
<td>256-278</td>
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<tr>
<td>Fib α 425,426</td>
<td>419-432</td>
<td>NVSPGT(Cit)(Cit)EYHTEK-ZO</td>
</tr>
<tr>
<td>Vim 435-455</td>
<td>435-455</td>
<td>O-DTHSK(Cit)TLLIKTVET(Cit)DGQVI</td>
</tr>
</tbody>
</table>

The biotinylated peptides both in the citrullinated and native form, were incubated in a streptavidin coated high binding capacity plates (Thermo Scientific, Gothenburg, Sweden). Serum was diluted 1:100 for detection of citrulline reactive autoantibodies. The bound antibodies were detected with horseradish peroxidase-conjugated goat anti-human IgG F (ab)_2 (Jackson ImmunoResearch, Newmarket, UK). Detection was performed using the chromogenic substrate, 3, 3', 5, 5'-tetramethyl-benzidine (Sigma-Aldrich, Stockholm, Sweden),
and absorbance was measured at 450 nm with reference at 650 nm. A standard was included in each plate to convert absorbance into arbitrary units (AUs). The cut-off values were determined as the 98th percentile of the healthy control reactivity.

3.7 Tetramer Production and Flow cytometry (Paper IV)

Antigen-specific B cells were detected using peptide tetramers. It consists of an R-Phycoerythrin (PE) labelled streptavidin (SA) core connected to four identical biotinylated peptides. The tetramers were assembled and used for B cell detection as described previously [152].

![Figure 5: B cell antigen tetramers.](image)

**Figure 5: B cell antigen tetramers.** Representation of the B cell tetramer, where SA is the streptavidin core, PE is the R-Phycoerythrin fluorochrome, AF647 is the Alexa Fluor 647 fluorochrome and cit and arg are the biotinylated citrulline and arginine peptides. The native, arg fibrinogen tetramer servers as the decoy.

The biotinylated citrullinated fibrinogen (cit-fib) peptides used in ELISA were incubated with SA-PE (Prozyme, Hayward, CA, USA) at a molar ratio of 10:1 and the tetramer fraction was purified using a 100-kD molecular weight cut-off Amicon Ultra filter (Millipore, Stockholm, Sweden). After measuring the concentration of PE by Nanodrop (Thermo Fischer, Gothenburg, Sweden), the molarity of the tetramer was calculated with the supplier-determined ratio of SA to PE. The decoy tetramer was prepared using the same protocol as described above, by incubating the biotinylated native, non-citrullinated fibrinogen (fib) peptides with SA-PE pre-conjugated to Alexa Flour 647 (Molecular Probes Invitrogen). The four α-chain derived Fib peptides were assembled as tetramers separately and then pooled at the time of sample staining. The decoy tetramers were assembled and used in the same manner with the corresponding native fib peptides.

To detect the tetramer positive B cells, PBMCs were isolated from blood of RA patients by Ficoll-paque (GE Healthcare, Uppsala, Sweden), and stained with the decoy and cit-fib
tetramers in buffer containing Fc blocking solution (FcR Blocker Miltenyi Biotec, Lund, Sweden), followed by incubation with anti-PE magnetic beads (Miltenyi Biotec) then passed over a magnetized LS column (Miltenyi Biotec) to enrich for tetramer-binding cells. Both the bound and flow-through fractions were stained with APC-H7 labelled anti-CD3, APC-H7 labelled anti-CD14, APC-H7 labelled anti-CD16, BV421 labelled anti-CD19, V500-C labelled anti-CD20, PE-Cy7 labelled anti-CD27 and FITC labelled anti-IgD (all antibodies from BD Biosciences, Stockholm, Sweden). The samples were analyzed by flow cytometry using a 4 laser (405nm, 488nm, 561nm and 640nm) LSR Fortessa (BD Biosciences, Stockholm, Sweden) and analyzed with FlowJo software (Tree Star). Fluorescent AccuCheck counting beads (Invitrogen, CA, USA) were used to calculate total numbers of live lymphocytes in the column-bound and flow-through suspensions. The gating strategy for tetramer staining was based on a FSC/SSC lymphocyte gate and removal of doublets followed by elimination of CD3, CD14 and CD16 positive cells before assessment of the B cell subset.

### 3.8 Statistical Analysis

All statistical analyses were performed using GraphPad Prism (version 6.0) and SPSS software. Comparison between two groups of continuous variables was performed using t-test (parametric) and Mann Whitney U test (non-parametric) and between multiple groups by analysis of variance. Chi-square test (or Fischer’s exact) was performed to analyze the association between categorical variables (example: antibody reactivity vs genetic risk factors). Binomial logistic regression analysis was performed to assess the relationship between categorical variables and a set of predicating variables (example: HRCT lung abnormalities vs age, sex, presence of ACAP fine specificities). The p values less than 0.05 were considered significant.
4 RESULTS AND DISCUSSION

4.1 Signs of Immune activation and inflammation are present in the lungs of early untreated RA

As discussed earlier, the association between smoking and ACPA, the presence of ACPA in circulation long before the onset of RA and the expansion of ACPA repertoire occurring close to the onset of joint inflammation [80] suggest that immunity in RA might be triggered in the mucosal surfaces such as the lungs. Investigation of the lungs can be performed by imagistic methods (HRCT), microscopic analysis of lung/bronchial biopsies and biochemical methods of BAL or induced sputum using ELISAs and flow cytometry. Here we employed some of these methods to study the lungs in early untreated RA patients.

Figure 6: Signs of inflammation and ACPAs in the lungs of early untreated RA. The left panel shows the representative images of the bronchial biopsy histology from ACPA negative and positive RA patients. The first row shows the presence of infiltrates using a hematoxylin and eosin staining. The second row is the CD3 staining for T cells, the third row for HLA-DR and the last row is the identification of citrullination using ACPA isolated from serum of RA patients (affinity purification using CCP2 column). The panel on the right shows the CCP2 IgG and IgA measurement normalized to total IgG and IgA in paired serum and BAL samples from early RA (adapted from [44, 103]).
Previous understanding of the RA lung pathology/histology originates from studying biopsies from patients with long standing RA [105] and ACPA positive individuals with lung disease [153]. Both these studies have showed the presence of ectopic lymphoid structures in some of the ACPA positive individuals. Using bronchial biopsies from early untreated RA patients we found that 50% (9/18) of ACPA positive RA has presence of lymphocyte infiltrates compared to 17% (1/6) in ACPA negative and 13% (2/15) in healthy individuals. CD3⁺ T cells were more frequent in the ACPA positive RA and CD19⁺ B cells were present (4/18) only in the ACPA positive bronchial biopsies. Investigation of immune activation markers like HLA-DQ and HLA-DR was upregulated in the lungs of ACPA positive compared to ACPA negative RA. These results are indicative of subtle inflammatory events in the lungs of early RA and especially in the lungs of ACPA positive RA patients (figure 6).

Previous studies by our group have shown the presence of increased citrullination in the BAL cells of smokers compared to non-smokers [40]. To identify citrullination in the lung of early RA, we used ACPAs isolated from serum of RA patients by affinity purification using CCP2 columns [154]. Using these antibodies, we found increased citrullination in the lungs of ACPA positive (median histological score 1, range 0-2) compared to ACPA negative (median histological score 0, range 0-1) RA patients. To further explore if the anti-citrulline immunity existed in the lungs, we analyzed the BAL of RA patients for the presence of ACPA. After normalizing for the total IgG and IgA content, we found an enrichment of ACPA (IgG and IgA) in the BAL compared to paired serum samples from patients. These results were similar to the observation of ACPA and RF in the sputum of individuals at risk of RA and early RA [104]. This higher ratio of ACPA in the mucosal compartment supports the notion of local production of antibodies in the lungs of RA patients.

4.2 HRCT Parenchymal lung abnormalities are associated with increased in ACPA fine specificities in early untreated RA

The presence of macroscopic abnormalities assessed by HRCT abnormalities are common among early RA patients compared to controls [44] and expansion of ACPA fine specificities is linked to RA-associated ILD [101]. We assessed the role of ACPA fine specificities and its influence on the HRCT lung abnormalities in a cohort of early untreated RA patients.

Among the 106 RA patients included in the study, 58 (54.7%) had parenchymal abnormalities, 68 (64.2%) had airway abnormalities and a considerable proportion of patients,
41 (38.7%) had both parenchymal and airway abnormalities. Individuals with parenchymal and airway abnormalities were significantly older. Those having parenchymal abnormalities were mostly smokers (% of smokers among those without vs with parenchymal abnormalities, 62.5% vs 81.0%) and had significantly higher pack years (Median 3.0 vs 17.3) of smoking. There was a significant association between the presence of ACPA (anti-CCP2) and HRCT parenchymal abnormalities, whereas no such association was observed for airway abnormalities. Those having airway or parenchymal abnormalities had significantly decreased FEV$_1$ whereas the FVC was significantly decreased in those having airway abnormalities and FEV1/FVC was significantly decreased in those having parenchymal abnormalities (Table 2, Paper I).

We performed a univariate logistic regression and found that: age greater than 65 years [OR (95%CI): 2.4(1.1-5.6) p value: 0.04], ever smoking [2.6(1.1-6.2), 0.04], pack years greater than 23 [6.3 (1.9-21.4), 0.003] and presence of anti CCP2 antibodies [2.9 (1.3-6.8), 0.01] were all significant associated with parenchymal abnormalities (Supplementary table 2, Paper I). After adjusting for age, sex and smoking, the HRCT parenchymal abnormalities were found to be associated with RF (both IgM and IgA) and CCP2 IgG, antibodies against any one cit vimentin peptide, especially cit vimentin 60-75, or antibodies against any one cit fibrinogen peptide, especially cit fibrinogen alpha 563-583 and cit fibrinogen beta 60-74. After stratifying based on smokers, RF IgA, CCP2 IgG, cit vimentin reactivity, cit fibrinogen reactivity still remained as good predictors of parenchymal abnormalities (Table 3, Paper I). The presence of high number of ACPA fine specificities (greater than 5) increased the risk for parenchymal abnormalities, especially among ever smokers, 7 times in case of more than 5 ACPA fine specificities and 4 times in case of less than 5 ACPA fine specificities. No such significant associations were observed for airway abnormalities (Figure 7).

Lung changes using CT has been previously demonstrated in early RA [98, 99], early untreated RA [44], long standing RA [155] [99] and in ACPA positive subjects [156]. In this study, our data demonstrates an association of some ACPA fine specificities with parenchymal but not airway abnormalities in early untreated RA. Association between ACPA fine specificity and ILD has been demonstrated previously in a cohort of RA patients, under medication. The strength of the current studies lies in the fact that the patients in our cohort have a disease duration less than one year and have not been under DMARDs which might influence the lungs [157]. The association between parenchymal abnormalities might suggest a more intrinsic immune mechanism developing in the lungs during the early disease onset. The understanding on what the ACPA fine specificity mean in causing these changes needs careful exploration.
and interpretation. The unique RA cohort provides new insights of lungs and airway involvement in RA with more research needed to further explore the link between the influence of antibody-mediated lung-immunity and RA.

**Figure 7: Association between number of ACAP fine specificities and HRCT lung abnormalities.** The forest plot represents the odds ratio (95% CI), between the number of ACPA fine specificities (0-reference, 1-5 and >5) and presence of Parenchymal or Airway abnormalities. The significant OR (p Value < 0.05) are represented by red circles (●).

### 4.3 Distal airway dysbiosis is present in the early untreated RA

The dysbiosis (microbial imbalance) at mucosal sites leading to the trigger and exacerbation of RA has been previously hypothesized and reviewed [109]. Prior knowledge of the role of microbiome in RA is based on animal [123, 124] and human studies [121, 122, 158] involving the gut microbiome. Studies have revealed that the gut microbiome in RA patients is perturbed compared to healthy controls and can be normalized after treatment [121, 122]. In study II we planned to explore whether there is a difference in the lung microbiome in RA compared to healthy or another disease condition. We used BAL fluid obtained by research bronchoscopy from individuals with RA, Sarcoidosis and healthy, to look into the lung microbiome.

The 16srrRNA V4 gene sequences was used to identify the different taxa present in the samples. We first analyzed the alpha diversity of the sample groups, which measures the richness (number) and evenness (distribution) of the taxa. Compared to the healthy controls,
the BAL microbiome alpha diversity was significantly reduced in the RA and sarcoidosis (figure 8). The Healthy BALs had significantly higher number of OTUs as compared to RA and sarcoidosis. Whereas no difference in alpha diversity was observed between RA and sarcoidosis as calculated by the number of OTUs, Simpson’s diversity index and Faith’s phylodiversity Index displayed in figure 8.

![Figure 8: Alpha diversity of lung microbiome.](image)

The figure represents the alpha diversity of the BAL microbiome calculated using (a) the number of OTUs (b) Simpson’s diversity index and (c) Faith’s phylodiversity index.

We then analyzed the beta diversity, which measures the between-sample taxonomic diversity. Using PCoA to cluster the samples and ANOSIM test, the beta-diversity plot (figure 9) demonstrated significant difference between healthy compared to RA and sarcoidosis (p value = 0.002 and 0.021) whereas no significant difference was observed between the RA- and sarcoidosis patients (p value = 0.265). The lower abundance of microbiome in RA compared to healthy is in line with previous study investigating the gut microbiome in RA [121]. Interestingly the microbiome abundance in RA was similar to that of sarcoidosis, which is an inflammatory disease of the lungs. These findings support the hypothesis that changes in the lung microbiome might contribute to the disease pathogenesis in the early stages of RA.

In order to understand which bacterial taxa were different among healthy and the disease groups, we analyzed the relative abundance of the most abundant taxa. Families Actinomycetaceae, Spirochaetaceae, genus Burkholderia, Treponema were enriched in the healthy compared to RA. Very few taxonomic differences were observed between RA and sarcoidosis. For example, the relative abundance of the OTU aligned to the genus Pseudonocardia was higher in RA compared to healthy and sarcoidosis (figure 10). By further stratification of RA patients based on the presence of joint erosions, we found a higher relative
abundance (p value = 0.0093) of this taxa in patients having an erosive joint disease. *Pseudonocardia* is known to be an antifungal commensal microorganism [159] but its presence in relation with the fungal profile of the BAL was not explored in this study.

![Figure 9: Beta diversity of lung microbiome. The PCA plot represents the beta diversity between the healthy (Blue), RA (Red) and Sarcoidosis (Green). The RA are more clustered together with the sarcoidosis compared with the healthy individuals.](image)

We further investigated the association between the alterations in microbial composition in early RA with phenotypic characteristics at the time of sampling. We found a positive correlation between DAS28 and the genus *Megasphaera* and *Renibacterium* and OTUs belonging to *Pseudonocardia*, *Streptococcus*, and *Xanthomonadaceae* but was negatively correlated with genus unclassified *Oxalobacteraceae*. Serum and BAL anti-CCP2 positively correlated to genus unclassified *Comamonadaceae*. Presence of eosinophils in the BAL positively correlated with some OTUs belonging to the genus Pseudomonas. The exact nature of contributing of these taxa to the decrease or increase of various disease or immune related parameters requires additional studies with larger study cohorts.

Through this study we were able to show that the lung microbiome in early untreated RA is different compared to healthy individuals but similar to lung inflammatory conditions such as sarcoidosis. We have previously demonstrated the presence of subtle lung inflammation and presence of antibodies in the lungs of RA [44, 160], if the dysbiosis is a consequence or an enhancer of these immune events in the lungs of RA requires further investigation. To our knowledge, this is the only study performed to investigate the distal airway microbiome in RA. One of the drawbacks of this study could be the small sample size. Inclusion of paired samples
from the gut might also add further insight into the relationship between the lung and gut microbiome in early RA.

**Figure 10: Pseudonocardia OUT (630556) in the lungs of RA.** The bar graph (left) represents the difference in relative abundance of Pseudonocardia OUT (630556) in healthy compared to RA and Sarcoidosis and (right) the RA patients stratified based on the presence of joint erosions.

### 4.4 Shared immunological targets are present in the lungs and joints of RA

The role of mucosal sites, especially the lungs as an initiation site for RA has been described [161] but very little is known how the immunity can be transferred from the lungs to the joints, which is the central site of disease manifestation in RA. One possibility is the presence of shared immunological targets in the lungs and joints, whereby the immunoreactions initiated in the lungs could be propagated to the joint. In study III, we set out to identify shared immunological targets in the lungs and joints of RA.

We analyzed 7 synovial biopsies and 6 bronchial biopsies from RA patients using mass spectrometry to identify citrullinated proteins. We identified 5322 unique peptides and 1200 proteins in the synovial and bronchial biopsies. With a MS/MS spectral identification score of at least 20, we were able to identify 10 citrullinated peptides corresponding to 7 proteins (table 7). Of these, two citrullinated vimentin peptides were present in most of the samples: cit-Vim 440-455 in all 7 synovial and 5 out of 6 bronchial biopsies, cit vim 446-466 in 6 out of 7 synovial and in all 6 bronchial biopsies. A higher ratio of citrullinated/unmodified peptides was present in smokers compared to non-smokers.

Using mass spectrometry, we were able to identify shared citrullinated peptides in the lungs and joints of RA patients. Identifying citrullination is challenging due to the very small mass shift of ~1 Da and to discriminate it from the frequently occurring deamidation with the same mass shift. Proteins such as Actin, Fibrinogen and Vimentin have been previously
suggested as immune targets in RA. However, in our samples only vimentin peptides were identified both in the lungs and joints. Due to the invasive sampling procedure, this study lacks paired synovial and bronchial samples from the same patient and control samples from healthy donors.

**Table 7: Quantification of citrullination in the lungs and joints of RA.** The values represent the corrected citrullinated/unmodified ratios in the lung and joint biopsies. ND denotes that neither citrullinated nor unmodified peptide was identified. NS and S implying non-smokers and smokers respectively. 0 is when only the native peptide was identified.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Actin</th>
<th>Amazing A2</th>
<th>Cysteine-rich protein 1</th>
<th>Fibrinogen α</th>
<th>Hemoglobin</th>
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<td></td>
<td>25-37</td>
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<td></td>
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<td>446-466</td>
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</table>

We then aimed to investigate if a systemic immune response could be detected against the cit vimentin peptides that were present both in the lungs and the joints. In order to study that, we set up an in-house ELISA to analyze autoantibodies in the serum of RA. The two vimentin peptides that were identified by mass spectrometry were citrullinated at amino acid position 440 and 450. One peptide (vim 435-455) containing both the citrullinated amino acids were used to detect the citrulline-reactive antibodies in serum.

Using a cohort of healthy volunteers, the cut-off for positivity for the ELISA was set at 98th percentile of the reactivity. We analyzed serum from two RA cohorts and found 14.9% positive in the LURA cohort and 14.3% positive in the EIRA cohort. The disease controls displayed higher anti-citrulline positivity (3.4%) than the healthy controls (figure 11). Most of cit Vim 435-455 reactive sera was also anti-CCP2 positive (91.1%). Among the RA group,
85% of those displaying reactivity to cit vim 435-455 were HLA-DR SE positive. However, no association was found between smoking and the presence of antibodies against cit vim 435-455. We also performed cross-reactivity ELISA in serum showing reactivity towards cit vim 435-455 and other well studied vimentin epitopes namely cit vim 60-75. Although cross reactivity among ACPAs is not uncommon [162], we found that serum blocked with cit vim 435-455 peptide did not interfere with reactivity against cit vim 60-75 suggesting no cross reactivity in patient serum that were tested.

Figure 11: Antibodies against cit vim 435-455. Serum samples form Healthy controls, RA cohorts LURA and EIRA and Disease controls were analyzed for reactivity against cit vim 435-455. The dotted line indicates the cut-off for the ELISA. The shaded portion of the pie charts represent the proportion of positive individuals in each group with the number in the middle indicating the sample size.

Presence of citrullination in the lungs is not restricted to RA but can also be present in some healthy as well as COPD patients [40, 163]. One of the vimentin peptides containing citrulline at position 450 was also identified in lungs of COPD patients [163] and two of the healthy biopsies (data in paper III), while antibodies against these peptides were present only in RA (ELISA data) suggesting that other factors that might be present in RA, are required for immune response against citrullinated antigens. Our data demonstrate that the citrullinated peptides present both in the lungs and joints are indeed immunogenic eliciting a B cell response.
and might possible be one of the ways by which antibodies potentially generated at the lungs find their targets in the joints causing inflammation.

4.5 Systemic response to de novo identified citrullinated fibrinogen is associated with PTPN22 risk allele.

Using an unbiased mass spectrometry approach, elevated levels of citrullinated fibrinogen peptides were identified in the synovial fluid of RA patients compared to disease controls [164]. In this study (IV), we aimed to analyze the immune reactivity against these de novo identified citrullinated fibrinogen alpha peptides.

We intended to study if general B cell response against cit fibrinogen peptides existed, by analyzing the presence of antibodies. Using an in-house ELISA, we analyzed serum of RA patients and compared against healthy and disease controls (DC) that included patients with Psoriatic (PsA) and Spondylarthritis (SpA).

![Figure 12: Reactivity against different fibrinogen peptides in different cohorts. The bars chart represents the percent of positive reactivity in healthy (blue), RA (red) and disease controls (green).](image)

Using the 98th percent reactivity against healthy controls to set the cut off for the ELISA, we found 20.2% reactivity towards the cit-fib α 35, 12.5% towards cit-fib α 216,218, 21.0% towards cit-fib α 263,271 and 17.0% towards cit-fib α 425,426. The citrulline reactivity in the disease cohort was less than 1% for all the four peptides (figure 12). This confirms the presence of circulating antibodies against the citrullinated peptides, identified in the synovial fluid, and their occurrence being specific for RA.
Most of RA sera presented non-overlapping reactivity, with 106 samples (30.5%) displaying positivity to only one of the cit fibrinogen peptides, 48 (13.8%) to any of the two peptides, 12 (3.5%) to any of the three peptides and only two (0.6%) showing positivity to all 4 cit peptides. The multiple reactivities could mean that either multiple autoantibody response could exist against different cit antigens or some degree of cross reactivity could occur the autoantibody response. More detailed studies and specific assays would be required to address this.

Next, we analyzed the association between the reactivity against the different cit peptides and the two most studied genetic risk factors for RA, namely HLA-DR SE and PTPN22 C1858T. Interestingly, no association was found between the HLA-DR SE (table 8) while an association and significant odds ratios was observed between PTPN22 C1858T and reactivity towards cit-fib α 35 and cit-fib α 263,271. This data suggests that the reactivity against some of the cit antigens might not entirely be dependent on the classical HLA-DR SE but might involve other autoimmune anomalies arising due to PTPN22. PTPN22 promotes the survival of autoreactive B cells in RA and type-1 diabetes by evading the tolerance checkpoints [20]. Additional factors as BAFF could also play a role in B cell activation and survival, but was not analyzed in this study.

**Table 8: Association between HLA-DR SE and PTPN22 C1858T and the different cit fibrinogen peptide reactivity.** Columns one to three are results (chi-square test) for association and columns four and five are odds ratio for the presence of cit-fib antibodies in individuals with the different RA risk alleles.

<table>
<thead>
<tr>
<th></th>
<th>HLA-DR SE (326)</th>
<th></th>
<th>PTPN22 C1858T (322)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No</td>
<td>Yes</td>
<td>p Value</td>
</tr>
<tr>
<td>Cit-Fib α 35</td>
<td></td>
<td></td>
<td>0.21</td>
</tr>
<tr>
<td>Cit-Fib α 216,218</td>
<td>11 (15.1)</td>
<td>55 (21.74)</td>
<td>0.30</td>
</tr>
<tr>
<td>Cit-Fib α 263,271</td>
<td>13 (17.8)</td>
<td>57 (22.53)</td>
<td>0.39</td>
</tr>
<tr>
<td>Cit-Fib α 425,426</td>
<td>10 (13.7)</td>
<td>46 (18.18)</td>
<td>0.37</td>
</tr>
</tbody>
</table>
Based on the data suggesting the role of PTPN22 C1858T allele carriers may have an inefficient peripheral tolerance, we hypothesized that the risk allele carriers in our cohort may have an expanded population of cit fibrinogen reactive B cells. We constructed cit fibrinogen antigen tetramers to identify such autoreactive B cells in risk allele carrier (CT) and non-allele carrier (CC) RA patients. We first analyzed frozen PBMCs from 5 RA patients (three CC and two CT) demonstrating a trend of increased frequency of cit fibrinogen tetramer positive B cells among the risk allele carriers. Further analysis of PBMCs from 10 RA patients (five CC and five CT) displayed the same trend.

**Figure 13. Cit fibrinogen reactive B cells identified using antigen tetramers.** The upper panel represents the gating strategy to identify the tetramer positive B cells from PBMCs. The lower panel is the quantification of the tetramer positive B cells in a pilot and validation RA cohort.

Using tetramer technology, we were able to identify the cit fibrinogen reactive B cells that were present in higher frequency among the PTPN22 risk allele carriers (figure 13). These results are in line with previous observation demonstrating increased autoreactive B cells among the PTPN22 risk allele carriers. Our data did not reach statistical significance which might depend on to the low number of patients included in the analysis. Sorting the tetramer positive B cells and sequencing and analyzing the immunoglobulin genes for germline or somatic mutations would have shed light on the T cell-dependent or independent origin of B cell activation/reactivity.
5 CONCLUSION AND FUTURE PERSPECTIVES

Through my thesis I aimed to fit in small pieces into the large puzzle, which is the understanding of the etiology and pathogenesis of RA. From being the secondary manifestation of RA, lungs have come into the spotlight and could instead be the origin of the disease, at least in some RA patients.

In paper I, we investigated the relationship between the HRCT lung abnormalities and circulating ACPA fine specificities, where we found an association between some of the ACPAs and parenchymal abnormalities in early RA. More interestingly, the higher the number of ACPA fine specificities the higher were the odds of having parenchymal abnormalities. These findings suggest a potential role for the lungs in generation of these antibodies. In order to prove this, we are currently working to clone single B cells from the BAL fluid of ACPA positive RA patients as well as ACPA positive healthy individuals at risk for developing RA but not yet having joint inflammation. Another interesting aspect is the possibility that ACPA themselves are pathogenic and contribute to the observed lung abnormalities and this still remain to be elucidated in the future. In order to better investigate a causal role for the lung in RA we need to shift our studies towards earlier time points to include healthy individuals at risk for developing RA.

In paper II, we examined the microbiome differences in early untreated RA and compared it with healthy individuals and sarcoidosis patients. From the study, we could conclude that dysbiosis exists in the lungs of early RA and is similar to that found in lung inflammatory condition such as Sarcoidosis. Like the previous study (paper I), this fails to address the causal relationship between the lung microbiome and the disease. This study would also benefit from studying individuals at risk of developing RA which is practically challenging due to the uncomfortable bronchoscopy procedure. Though some questions can be answered through studying induced sputum, these samples have a different bacterial relative abundance compared to that present in the lower airways. Another potential future development of the microbiome studies in RA could be use of probiotics in the treatment of the disease by modulating inflammation. Recent study in mice have demonstrated that Prevotella histicola, a commensal bacteria present in the human gut, had probiotic effect in attenuating arthritis in mice [165]. Additional studies are required to understand the role of the myriad of bacteria present in the lungs and gut and to find novel effective ways to interfere with it in order to attenuate inflammation.
In paper III, we were able to identify shared citrullinated targets in the lungs and joints of RA patients and found immune reactivity against them. The results from this study could postulate that the immunity arising in the lungs are propagated via the circulation to the joints where they elicit their pathogenic roles. To understand the exact mechanisms that lead to the inflammatory joint manifestations, more studies are required to understand the triggering events and local changes in the lungs (and/or other mucosal surfaces) and how and when these changes ultimately lead to joint inflammation.

In paper IV, we demonstrated that immune reactivity towards four de novo identified citrullinated fibrinogen peptides are present and specific for RA. We also demonstrated that the reactivity against some of these citrullinated fibrinogen peptides are associated to the PTPN22 risk allele and not the HLA-DR. In addition, individuals with the PTPN22 risk allele tend to have higher frequency of citrullinated fibrinogen reactive B cells. We further demonstrated the use of B cell tetramer in identifying citrulline reactive B cells. Previous studies were able to isolated single B cells and clone citrulline reactive antibodies [166] and use them in vitro systems to study their effects [91]. Using B cell antigen tetramers would allow us to isolate such citrulline reactive B cells and to generate more specific antibodies and to understand their functional implications in RA.
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