From the Rheumatology Unit, Department of Medicine Solna,
Karolinska Institutet, Stockholm, Sweden

MOLECULAR AND EPIDEMIOLOGICAL INVESTIGATIONS OF LUNG INVOLVEMENT IN VERY EARLY RHEUMATOID ARTHRITIS

Guðrún Björk Reynisdóttir

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Molecular and epidemiological investigations of lung involvement in very early rheumatoid arthritis

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Guðrún Björk Reynisdóttir

Principal Supervisor:
Associate professor Anca I. Catrina
Karolinska Institutet
Department of Medicine, Solna
Rheumatology Unit

Co-supervisors:
Professor Lars Klareskog
Karolinska Institutet
Department of Medicine, Solna
Rheumatology Unit

MD, PhD Sofia Ernestam
Karolinska Institutet
Department of Medicine, Solna
Rheumatology Unit

Professor Johan Askling
Karolinska Institutet
Department of Medicine, Solna
Clinical epidemiology &
Rheumatology Unit

Opponent:
Professor Eric L Matteson
Mayo Clinic College of Medicine
Department of Medicine
Division of Rheumatology

Examination Board:
Associate professor Eeva Piitulainen
Lund University
Department of Respiratory Medicine
& Allergology

Associate professor Anna Fogdell Hahn
Karolinska Institutet
Department of Clinical Neuroscience

Associate professor Inger Gjertsson
University of Gothenburg
Department of Medicine
Department of Rheumatology
& Inflammation Research

To Diddi, Reynir, Freyja and Þórey Hanna

ABSTRACT

Rheumatoid arthritis (RA) is a systemic inflammatory joint disease with at least two distinct clinical phenotypes defined by the presence or absence of antibodies, i.e. rheumatoid factor (RF) and/or anti-citrullinated protein antibodies (ACPAs). These two phenotypes differ both with respect to risk factors and disease outcome, with seropositive disease being more likely associated with extra-articular manifestations, such as lung manifestations, and tobacco exposure. Both ACPA and RF can be detected in the blood years prior to the onset of joint inflammation suggesting that these antibodies are initially generated outside the joints. The current thesis investigates the pathogenic link between lungs and joints with a focus on the potential role of the lung as an initiating site for the RA-associated autoimmunity.

We investigated a cohort of patients with early, untreated RA, who underwent high resolution computed tomography and conducted lung function tests. A subgroup of these patients was subjected to bronchoscopy with retrieval of bronchoalveolar lavage (BAL) and bronchial biopsies. All investigations were repeated after six months of anti-rheumatic treatment.

We found more prevalent lung abnormalities, both parenchymal (54%) and airway (66%) in RA patients as compared to controls. The parenchymal abnormalities were significantly more frequent in the subgroup of ACPA-positive RA patients compared to ACPA-negative patients. The same was true after compensating for smoking. Signs of inflammation and immune activation with more lymphocytic infiltration and expression of citrullinated proteins were found in the lungs of ACPA-positive as compared to ACPA-negative patients. ACPAs were enriched in the BAL as compared to the blood compartment of ACPA-positive patients. Using mass spectrometry we were able to identify two novel citrullinated vimentin peptides that were present in a majority of bronchial (n=6) and RA synovial biopsies (n=7) tested. Immune reactivity against these targets was specifically detected in the blood of RA patients. At 6 months follow-up one third of patients with lung fibrosis (11%) at baseline had progressed and additionally 3 patients had developed new radiological changes suggestive of early interstitial lung disease. Moreover, there was an increase in airway obstruction, with a decline in forced expiratory volume in one second in all patients, more prominent in smokers.

In conclusion, lung changes in early RA are prevalent and may be progressive despite anti-rheumatic treatment. Taken together our results support the hypothesis that the lung may be an early important player in the pathogenesis of RA in a subset of patients. These findings encourage new therapeutic strategies to target the local inflammation in the lungs, with the aim to prevent the progress of autoimmunity in ACPA-positive healthy individuals.

LIST OF SCIENTIFIC PAPERS

The thesis is based on the following papers. They will be referred to in the text by their roman numerals:

I. **Reynisdottir G**, Karimi R, Joshua V, Olsen H, Hensvold AH, Harju A, Engstrom M, Grunewald J, Nyren S, Eklund A, Klareskog L, Skold CM, Catrina AI. Structural changes and antibody enrichment in the lungs are early features of anti-citrullinated protein antibody-positive rheumatoid arthritis.
Arthritis & Rheumatology (Hoboken, NJ) 2014, 66(1):31-39.

II. **Reynisdottir G**, Olsen H, Joshua V, Engstrom M, Forsslund H, Karimi R, Skold CM, Nyren S, Eklund A, Grunewald J, Catrina AI. Signs of immune activation and local inflammation are present in the bronchial tissue of patients with untreated early rheumatoid arthritis.
Annals of the Rheumatic Diseases Epub ahead of print, 2015 Nov 3.

III. Ytterberg AJ, Joshua V, **Reynisdottir G**, Tarasova NK, Rutishauser D, Ossipova E, Haj Hensvold A, Eklund A, Skold CM, Grunewald J, Malmstrom V, Jakobsson PJ, Ronnelid J, Padyukov L, Zubarev RA, Klareskog L, Catrina AI. Shared immunological targets in the lungs and joints of patients with rheumatoid arthritis: identification and validation.
Annals of the Rheumatic Diseases 2015;74(9):1772-7.

IV. Olsen H*, **Reynisdottir G***, Pawlowski J, Harju A, Karimi R, Klareskog L, Skold CM, Nyren S, Catrina AI, Eklund A, Grunewald J. Pulmonary manifestations in early rheumatoid arthritis: Features at baseline and after six months of standard treatment.
In Manuscript.
* These authors contributed equally

CONTENTS

LIST OF ABBREVIATIONS

ACPA(s)	anti-citrullinated protein antibody(-ies)
ACR	American College of Rheumatology
AID	activation-induced cytidine deiminase
BAL	bronchoalveolar lavage
BALF	bronchoalveolar lavage fluid
CCP	cyclic citrullinated peptide
CD	cluster of differentiation
CIT	citrullinated
COPD	chronic obstructive pulmonary disease
CRP	C-reactive protein
DAS	disease activity score
DLCO	diffusing capacity for carbon monoxide
DMARD	disease-modifying anti-rheumatic drugs
ELISA	enzyme-linked immunosorbent assay
ESR	erythrocyte sedimentation rate
ExRA	extra-articular rheumatoid arthritis
FACS	fluorescence-activated cell sorter
Fcγ	receptor for immunoglobulins
FEV1	forced expiratory volume in one second
FVC	forced vital capacity
HLA	human leukocyte antigen
HRCT	high resolution computed tomography
Ig	immunoglobulin
IL	interleukin
ILD	interstitial lung disease
LURA	LUng investigation in newly diagnosed RA
MTX	methotrexate
NK	natural killer
OR	odds ratio
PAD	peptidyl arginine deiminase

PFT	pulmonary function test
RA	rheumatoid arthritis
RF	rheumatoid factor
SE	shared epitope
SVC	slow vital capacity
TNFi	tumour necrosis factor inhibitor

1 INTRODUCTION

The aim of this thesis is to investigate the potential role of the lung in the pathogenesis of autoimmunity in rheumatoid arthritis and to characterize the extent of lung changes in RA at diagnosis.

1.1 RHEUMATOID ARTHRITIS

Rheumatoid arthritis (RA) is a chronic systemic inflammatory disease characterized by peripheral joint inflammation. The prevalence of RA is about 0.5-1% in developed countries and the annual incidence varies between 20-50 cases per 100,000 inhabitants depending on age and sex [1]. RA is 2-3 times more frequent in women and the usual age of onset is between 40-70 years with peak onset in the fifth decade of life [1, 2]. Patients with RA have a reduced life expectancy compared to the general population [3], mainly due to cardiovascular diseases [4, 5] and secondly due to respiratory diseases [6]. The presence of extra-articular manifestations in RA (reflecting the severity of systemic disease) has been shown to be a major contributor to the excess mortality in RA [7].

1.1.1 Clinical features and diagnosis

The clinical course of RA is variable, ranging from a mild to rapidly progressive erosive disease. The onset is usually insidious with symmetrical swelling and pain of the joints in hands and feet and morning stiffness. If left untreated irreversible joint destruction may occur with subsequent functional disability and decreased quality of life. General symptoms such as low-grade fever, malaise and fatigue can also be experienced and in some cases extra-articular symptoms may predate the joint disease [8].

The diagnosis of RA is still today based on a set of criteria due to the lack of a specific diagnostic test. The classification criteria from the American College of Rheumatology (ACR) from 1987 (Table 1), [9], originally created for research purposes, have been widely used internationally as a diagnostic tool. During 2010 updated criteria were issued by the ACR and the European League Against Rheumatism (EULAR) in order to allow for earlier diagnosis of RA (Table 2) [10]. Inclusion of all patients included in the studies presented in this thesis was performed between 2007 and 2012 and therefore the 1987 criteria were used.

Table 1. The ACR classification criteria for RA (1987)

Criterion
1. Morning stiffness
2. Arthritis of ≥3 joint areas
3. Arthritis of hand joints
4. Symmetric arthritis
5. Rheumatoid nodules
6. Rheumatoid factor
7. Radiographic changes

Patients fulfilling at least 4/7 criteria are classified as having RA. Criteria 1-4 must be present for at least 6 weeks.

Table 2. The ACR/EULAR classification criteria for RA (2010)

A. Joint involvement	**Score**
1 large joint	0
2-10 large joint	1
1-3 small joints (with or without involvement of large joints)	2
4-10 small joints (with or without involvement of large joints)	3
>10 joints (at least 1 small joint)	5
Serology (at least 1 test result is needed for classification)	**Score**
Negative RF *and* negative ACPA	0
Low-positive RF *or* low positive ACPA	2
High-positive RF *or* high-positive ACPA	3
C. Acute-phase reactants (at least 1 test result is needed for classification)	**Score**
Normal C-reactive protein (CRP) *and* normal erythrocyte sedimentation rate (ESR)	0
Abnormal CRP *or* abnormal ESR	1
D. Duration of symptoms	**Score**
<6 weeks	0
≥6 weeks	1

Target population: Patients who 1) have at least 1 joint with definite clinical synovitis (swelling) 2) with the synovitis not better explained by another disease. To be classified as rheumatoid arthritis, an overall score of ≥ 6/10 is required.

1.1.2 Autoantibodies in RA and phenotypes

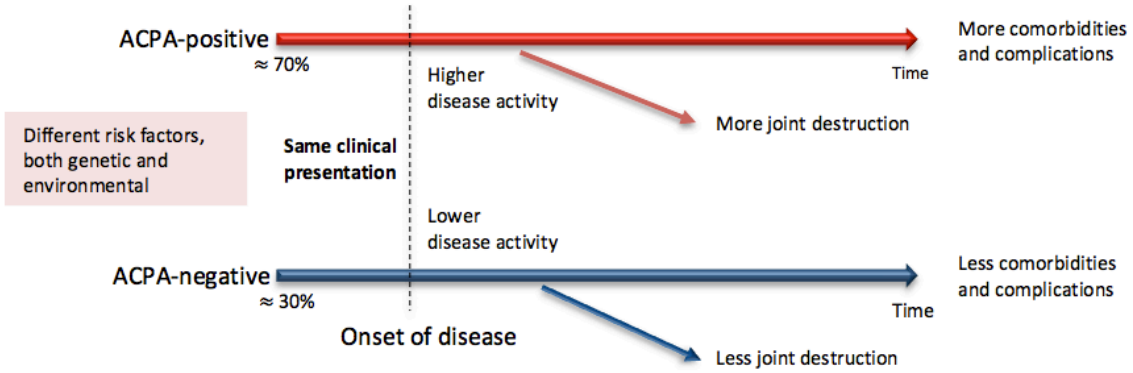
RA is an autoimmune disease traditionally divided into two subsets based on the presence or absence of autoantibodies. The classic antibody related to RA is rheumatoid factor (RF), first described in 1939 [11]. RF is antibodies against the Fc portion of IgG that are evident in about 70% of all patients with RA [12]. RF can also be detected in other autoimmune and infectious diseases as well as in healthy individuals, thereby diminishing its diagnostic specificity [13]. The later discovered and more specific biomarker in RA is anti-citrullinated

peptide antibodies (ACPAs) [14]. These antibodies can be detected in approximately 70% of RA patients, but have a much higher specificity (94-98%) than RF [15]. The ACPA pool in RA patients is heterogeneous, with antibody reactivities against multiple citrullinated proteins differing between individual patients [16]. Details about the citrullination process, the different ACPA specificities and their potential role in RA pathogenesis will be discussed further in *chapter 1.3*. The standard test to measure the presence of ACPAs in clinical practice is a commercial anti-cyclic citrullinated peptide (anti-CCP) immunoassay. The anti-CCP test covers reactivities against multiple citrullinated protein targets [17]. The second generation of anti-CCP has been demonstrated to have a high sensitivity and specificity [18] and is the test used to categorize RA patients in the studies presented in this thesis.

More recently identified are antibodies against carbamylated proteins, which are homocitrulline containing antibodies. These antibodies, not yet used in clinical practice, are present in a majority of ACPA-positive patients but also in about 15% of patients with ACPA-negative RA [19]. Interestingly, ACPAs, RF and antibodies against carbamylated proteins may be present several years before disease onset [20-23].

Following the discovery of ACPAs, given their specificity for RA as well as their presence in blood years before symptoms, research in the field of RA has focused on these antibodies. Studies have demonstrated that patients with ACPA-positive disease differ from patients with ACPA-negative disease with respect to genetic and environmental risk factors (Figure 1) [24]. In addition, presence of ACPA has been associated with a more severe disease course and more joint destruction [25-27].

Figure 1. Differences between ACPA-positive and ACPA-negative RA patients



Adapted from Klareskog et al [24].

1.1.3 Disease activity

Assessment of disease activity is crucial in the management of RA patients and needs to be done on a regular basis. Since there is no single test available the disease activity assessment includes examination of joints with respect to tenderness and swelling and measurement of inflammatory markers, such as erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP). These measurements are not without limitations, however, as many patients with RA have normal values despite existing joint inflammation [28].

The most widely used assessment tool of disease activity is the 28-joint disease activity score (DAS28), originally developed to evaluate treatment outcome in clinical trials [29]. This is a composite index that includes numbers of swollen and tender joints (28 joints), a measurement of ESR and the patient's general health assessment on a visual analogue scale (0-100). DAS28 has also been modified to include CRP instead of SR, since CRP levels may better reflect acute inflammation than levels of ESR [30]. The outcome of DAS28 is used to define disease activity as low, moderate or high [31]. The main limitation of DAS28 is that it does not include the joints in the feet and therefore may underestimate disease activity. Furthermore, ESR levels can be increased due to other medical conditions and the patient's global assessment can also be influenced by factors other than those caused by the inflammatory disease. One should thus always consider what underlies the value of DAS28.

Other assessment tools such as patient-reported questionnaires of physical function (Health Assessment Questionnaire, HAQ) and assessment of radiographic progression are also used routinely in clinical practice [32, 33].

1.1.4 Treatment

There has been a paradigm shift in the management and treatment of patients with RA during the last 20 years. A combination of factors has contributed to this, such as an increased awareness and the possibility of diagnosing patients at an earlier stage as well as the understanding of the importance to initiate anti-rheumatic treatment as soon as a diagnosis is set in order to avoid future disability. Additionally, there is increased knowledge on how to use the older medications and new biological treatments have come into use [34, 35].

Today there are two major classes of disease-modifying anti-rheumatic drugs (DMARDs); the conventional synthetic DMARDs (csDMARD) which include methotrexate (MTX), sulfasalazine and leflunomide, and the biological DMARDs (bDMARD) which include tumour necrosis factor inhibitors (TNFi) (adalimumab, certolizumab pegol, etanercept, golimumab, infliximab), a T cell costimulation inhibitor (abatacept), an anti-B cell agent (rituximab), an interleukin-6 receptor (IL-6R) blocking monoclonal antibody (tocilizumab), and an IL-1 inhibitor (anakinra).

Methotrexate is the mainstay of initial treatment but in case of intolerance or contraindications sulfasalazine or leflunomide are alternatives. Glucocorticoids have been debated for a long time due to the known detrimental effects especially related to high

doses and long-term use. In contrast, the good effect of low dose glucocorticoids on both disease activity and radiographic progression justify their use as part of the initial treatment [36]. If low disease activity is not achieved after 3 months of treatment with MTX the therapy needs to be intensified. Either addition of other csDMARDs (such as hydroxychloroquine and sulfasalazine) or bDMARD (in the first place TNFi, but also abatacept, tocilizumab or rituximab) should always be considered in the presence of poor prognostic factors, such as ACPA and/or RF positivity, high disease activity or early radiographic changes [37].

This important development in treatment enables us to set the goal high, which for the patient means achieving clinical remission or low disease activity, thereby minimizing the risk for joint destruction with functional disability and maximizing RA-related quality of life. Future hope is to be able to further individualize treatment options, for example on the basis of absence or presence of specific biomarkers.

1.1.5 Extra-articular manifestations

RA is a systemic disease with approximately 40% of patients having extra-articular manifestations (ExRA) over the course of the disease [38-40]. ExRA can affect almost any structure in the body, causing e.g. rheumatic nodules, opthalmic disease, vascular disease, cardiopulmonary manifestations, kidney disease and neurological manifestations [38, 41]. Usually these systemic manifestations are considered markers of disease severity and are associated with significant morbidity and increased mortality [7, 40, 42, 43].

Previous studies have demonstrated that age, smoking, positive serology (both RF and ACPA), presence of the genetic risk factor human leukocyte antigen (HLA)-DR shared epitope, and severe disease activity are risk factors for ExRA [8, 44-46]. The presence of RF is more strongly associated with extra-articular manifestations than anti-CCP antibodies [47]. This may be explained by the high levels of circulating immune complexes that have been detected in these patients [48] and the potential role of RF in the formation of complement activating immune complexes [47]. However, a recent study found even stronger associations with the presence of antibodies against mutated citrullinated vimentin (MCV) [49], suggesting a possible role of citrullination and specific anti-citrulline immunity in the pathogenesis of extra-articular involvement.

The optimal management of ExRA is not known and recommendations are based on case reports and expert opinions. The general approach is to treat the underlying joint disease aggressively. In cases where joint inflammation is low and/or extra-articular manifestations dominate (e.g. vasculitis) treatment usually includes glucocorticoids and immuno-suppressive agents [50].

Although the majority of ExRA manifestations occur as a complication of RA, some of these manifestations can present at or before joint inflammation. This is particularly the case with lung manifestations [8].

1.2 LUNG MANIFESTATIONS IN RA

It is common knowledge among rheumatologists and pulmonologists that lung diseases often accompany RA. The reported prevalence of lung abnormalities in RA varies between studies from 10-67%, depending on the mode of investigation (HRCT versus chest radiography), definition of lung disease as well as the RA population under study (early versus longstanding) [51-54]. Moreover, lung diseases are a significant contributor to increased mortality in RA patients, accounting for about 10% of deaths in RA and making it the second leading cause of death in this patient group [6, 47, 55-57] after cardiovascular diseases [4, 5].

The spectrum of pleuropulmonary manifestations in RA is broad, ranging from pleural disease and airways obstruction to severe interstitial lung disease (ILD) (Table 3) [58-61].

Table 3. Pleuropulmonary manifestations associated with rheumatoid arthritis

Interstitial	Airway	Pleural	Vascular	Other
RA-ILD	Bronchiectasis	Pleuritis	PH	Infections
Rheumatoid nodules	Obliterative bronchiolitis	Effusions	Vasculitis	Drug reactions
Caplan's syndrome	COPD	Empyema		Chest wall disease
	Upper airway obstruction			Lung cancer

COPD = chronic obstructive pulmonary disease, PH = pulmonary hypertension RA-ILD = rheumatoid arthritis associated interstitial lung disease.

Typically these manifestations are considered a complication of arthritis disease, but in some cases lung disease predates joint inflammation and predominates the clinical picture [56, 62]. The clinical interpretation of RA patients with lung symptoms is further complicated by the fact that many of the treatment alternatives for RA can induce pulmonary changes. In addition, patients with systemic disease are more susceptible to infections and immunosuppressive medications further increase this risk. Taken together this imposes a great challenge to attending clinicians and the importance of good history and clinical assessment cannot be underestimated [63].

The investigations most commonly used to evaluate lung involvement are chest radiography, high resolution computed tomography (HRCT) and pulmonary function test (PFT). HRCT has a clear superiority over chest radiography and PFT in detecting subtle lung changes [64]. PFTs (dynamic spirometry, lung volumes and diffusing capacity for carbon monoxide (DLCO)) are often used to follow lung function and in differentiating between obstructive and restrictive disease. The most practical parameters to have in mind are total lung capacity (TLC), forced expiratory volume in 1 second (FEV1), forced vital capacity (FVC) and the ratio between these two. A low FEV1/FVC indicates obstructive disease, whereas a normal or high ratio in the presence of low TLC is generally suggestive of restrictive disease. Diffusion capacity for carbon monoxide (DLCO) assesses gas exchange and in the context of restrictive lung disease, reduced DLCO indicates intrinsic lung disease.

1.2.1 Airway changes

The most sensitive detection method for airway changes is HRCT. Not all comparative studies have reported a correlation between HRCT and lung function testing, since the latter may not change until advanced disease [54]. Frequent features of clinically manifested airway disease are cough, dyspnea on exertion and in certain cases an increased risk for infections.

1.2.1.1 Upper airway obstruction

The main cause for this condition in RA is cricoarythenoid arthritis, which is more common in women and in longstanding disease. It can go unrecognized due to the late appearance of symptoms. Treatment aims to control inflammation and in cases of severe obstruction surgical intervention may be needed [65].

1.2.1.2 Small airway obstruction

This condition can occur as a lone manifestation or in combination with RA-ILD. Prevalence is quite high on HRCT and often without physiological abnormality. The clinical significance and natural history are unknown. Perez et al found a correlation between small airways disease and bronchiectasis and bronchial wall thickening [66].

1.2.1.3 Obliterative bronchiolitis (OB)

OB is a rare and severe complication of RA and should be suspected in cases of abrupt onset of severe dyspnea and cough. It is more common in women and in association with high levels of RF. It has also been described as an adverse reaction to anti-rheumatic drugs such as penicillamin and sulfasalazine [67]. The HRCT image of OB includes signs of bronchial wall thickening, centrilobular emphysema and bronchiectasis [68]. OB is usually regarded as a consequence of arthritis disease but one case report described the presence of OB in at-risk individual with high RF levels but no joint inflammation [69].

1.2.1.4 Follicular bronchiolitis

Follicular bronchiolitis consists of lymphoid hyperplasia of bronchus-associated lymphoid tissue and can be found in combination with ILD and can also accompany other rheumatic diseases [70]. High levels of RF are usually present. Interestingly, one report from Japan described one case in which follicular bronchiolitis preceded joint inflammation [71], but this condition is usually considered a complication of longstanding RA. HRCT signs are centrilobular or peribronchial micronodules, bronchial dilation and bronchial wall thickening [72].

1.2.1.5 Bronchiectasis

Bronchiectasis are apparent in about 30% of RA patients when detected by HRCT [73], and is thus one of the most frequent lung abnormalities evident in RA patients. However, symptomatic bronchiectasis is only recorded in 2.7% of RA patients [74]. Patients with RA

and co-existent clinically manifest bronchiectasis have an increased mortality rate, 7.3 times that of the general population [75]. A recent study in RA patients with concomitant symptomatic bronchiectasis found higher disease activity, disease severity and presence of anti-CCP and RF as compared to patients with RA alone [76]. Interestingly, one old study described appearances of bronchiectasis before joint inflammation [77], and this has been recently confirmed in a new study in which bronchiectasis was observed to precede RA in about half of the cases [76].

1.2.1.6 Obstructive lung disease (OLD)

A study from Nannini et al of the prevalence of OLD in RA patients demonstrated a higher risk of OLD in RA patients with associated higher mortality, as compared to non-RA subjects [78]. The diagnosis of OLD was based on FEV1/FVC < 0.7 and included the diagnosis of COPD, asthma, ILD with airway obstruction and bronchiectasis. Another study from Taiwan has also reported a significantly higher risk of developing COPD in RA patients as compared to in non-RA individuals [79].

1.2.2 Parenchymal diseases

1.2.2.1 Rheumatoid lung nodules

Studies have shown a wide prevalence of lung nodules ranging from 4% [80] to 32% [81] depending on the diagnostic mode of investigation. Generally lung nodules are more common in patients with longstanding disease, the presence of autoantibodies and concomitant subcutaneous rheumatoid nodules. They are usually asymptomatic but can rupture into the pleural cavity and cause pneumothorax, effusion and infection. The general prognosis is good in most cases with occasional spontaneous disappearance of nodules. A main concern is to differentiate rheumatoid lung nodules from lung cancer [82].

1.2.2.2 Caplan syndrome

In 1953 Caplan described the association between pneumoconiosis in coal miners and RA [83]. Since then Caplan syndrome has been described in individuals exposed to dust such as silica and asbestos [84]. The majority of patients with this association are RF-positive [84].

1.2.2.3 Pleural disease

Pleural disease is common among RA patients, autopsy studies revealing a prevalence of 38-73%, but mainly subclinical disease. This is more common in the male gender and in longstanding disease, but can precede the joint inflammation and often coexists with other RA-associated lung diseases [84].

1.2.2.4 Drug-induced lung disease

Most of the DMARDs in use today can lead to drug-induced lung diseases with impact on morbidity and mortality in RA [85]. These manifestations can present acutely or subacutely and vary in severity. The most common complications are infections (bacterial and

opportunistic) [86, 87], diffuse interstitial processes [88] and less commonly airway disease [67].

Methotrexate (MTX) is the drug most commonly implicated in drug-induced pneumonitis, progression of underlying RA-ILD or fibrosis [89, 90]. The likelihood of developing pneumonitis during MTX treatment has been estimated to be 0.3-11% and pre-existing lung disease is one of the risk factors [89, 91]. Patients usually respond quite well to drug withdrawal or treatment with glucocorticoids. However, some studies have reported about a 20% increase in mortality rate for the affected patients, especially if the toxicity occurs during the first 6 months of treatment [88, 91, 92]. Prolonged use of MTX has been related to mild reduction in lung capacity values, although this has not been found to be clinically important [63, 92]. Similar to MTX, leflunomide can also cause pneumonitis, in particular in those individuals with severe interstitial lung disease, and is not recommended to patients with previous history of lung toxicity due to Methotrexate [93, 94].

Case reports have related bDMARDs-induced pneumonitis with high mortality in RA patients [88], particularly in those with pre-existing lung disease [95]. However, the prevalence is thought to be low and some case reports have described improvement in RA-ILD following treatment with biologics [96]. Reports on the association between newer biologics, such as tocilizumab, and ILD have been conflicting [97, 98].

Overall the risk of pneumonitis due to MTX, leflunomide or TNF inhibitors has been estimated to be low (about 1%) and is particularly related to pre-existing lung disease. Controlled studies are needed regarding safety in treating RA patients with concomitant ILD [97, 98].

1.2.3 RA-associated interstitial lung disease (RA-ILD)

The most common and severe lung manifestation associated with RA is interstitial lung disease (RA-ILD). As mentioned above the reported prevalence varies and depends on the mode of investigation, the definition of lung disease as well as the RA population under study. Gabbay et al described lung abnormalities consistent with ILD in 6% using chest radiography versus 33% using HRCT in early arthritis patients receiving anti-rheumatic treatment, of which 24% had clinically evident disease (8% of all subjects) [51]. A study in patients with >10 years from diagnosis of RA yielded similar results i.e. preclinical ILD on HRCT in 33% of the patients [52]. Olson et al reported the prevalence of clinical ILD to be about 6.8% in women and 9.8% in men [99]. A study from Bongartz et al indicated a lifetime risk of clinical ILD in RA to be approximately 10% [56]. ILD is usually considered a complication of RA but in about 10-20% patients ILD predates RA [100-102].

The clinical presentation of ILD is usually insidious with cough and progressive dyspnea on exertion. Recognition may be delayed due to exercise limitations related to the joint disease. Physical examination may reveal bibasilar crackles and clubbing in advanced disease. Clinical evaluation includes laboratory testing, pulmonary function testing

(restrictive pattern and impaired gas transfer for widespread disease), HRCT and in certain cases bronchoalveolar lavage (BAL) and lung biopsy.

ILD is categorized according to the histopathological pattern that correlates quite well with the radiographic appearance on HRCT [103]. These include the following: usual interstitial pneumonia (UIP), nonspecific interstitial pneumonia (NSIP), organizing pneumonia, lymphocytic interstitial pneumonia, desquamative interstitial pneumonia and diffuse alveolar damage [60]. Usual interstitial pneumonia (UIP) is the predominant subtype in RA and has been associated with a more severe outcome [101, 104].

The diagnosis of the subtype is based on a combination of the clinical investigations mentioned above. The use of HRCT in differentiating between subtypes and determining the extent of disease is now well established [101]. The typical HRCT changes for UIP are traction bronchiectasis, honeycombing and reticular abnormalities, whereas in NSIP ground glass opacification usually dominates and honeycombing is absent [57]. A lung biopsy may be required if a definite diagnosis cannot be determined, as this can be relevant for treatment decisions. BAL is only needed in cases where other differential diagnoses are suspected, e.g. infections, eosinophilic pneumonia or malignancy [105].

Risk factors for RA-ILD have been reported to include male gender, high disease activity, high autoantibody titers (RF and/or anti-CCP) and smoking [101, 106-109].

1.2.3.1 Treatment of RA-ILD and prognosis

Due to the lack of controlled trials the treatment recommendations are generally based on treatment guidelines for idiopathic lung diseases, case series and expert opinions. When taking decisions regarding treatment several factors need to be considered, such as subtype of ILD and thereby the likelihood of response, extent of disease (> 30% of lungs on HRCT), severity and progression [110]. Currently the management of subclinical RA-ILD is not recommended, as we lack knowledge on the natural history as well as treatment response [111]. Immunosuppressive treatment can be beneficial in RA-ILD, with the exception of patients with UIP. However, some studies have demonstrated a positive effect of treatment in UIP, suggesting a better prognosis in RA-UIP as compared to in idiopathic fibrosis or a misdiagnosis of UIP instead of NSIP on HRCT [112].

Current therapy usually involves a combination of glucocorticoids and cytotoxic agents; azathioprine, mycophenolate mofetil or cyclophosphamide [113, 114]. Methotrexate can be considered after carefully considering the risk of lung toxicity [52]. Case reports on the effect of biological therapies (TNF inhibitors, rituximab, abatacept, tocilizumab) are conflicting, with some reporting beneficial effect [115] and others a worsening of lung disease [116]. They should therefore be used with caution until more evidence for their use has been published.

Given the causative role of smoking in both RA and ILD all patients should be encouraged and supported to quit smoking.

The prognosis for patients with RA-ILD is poor, with a median survival after ILD diagnosis of about 3 years [56, 99, 102]. However, the prognosis varies somewhat, depending on the underlying histopathological subtype. The poorest prognosis at five years was evident in the group with diffuse alveolar damage (20%) and UIP (37%), whereas a better prognosis was recorded for organizing pneumonia (60%) and NSIP (94%) [117]. A study of prognostic factors for progression of UIP found association with bibasilar crackles, low DLCO and extent of HRCT changes on initial presentation. Using multivariate logistic regression, a DLCO below 54% remained the only significant predictor for progressive disease [118].

During recent years there have been speculations regarding the causal link between ACPA and lung involvement. Studies have demonstrated associations between ACPA titers and the presence of RA-ILD and furthermore that lung changes can predate joint inflammation in a proportion of patients [106, 107, 109, 119, 120].

In an effort to further understand the association between lungs and joints in RA, both from a clinical and molecular viewpoint, we initiated the clinical study on LUng investigation in newly diagnosed RA (LURA).

1.3 ETIOPATHOGENESIS OF RHEUMATOID ARTHRITIS

The cause of RA is unknown but much knowledge has been gained through research during the last decades, and several etiopathogenic clues have now been identified. We do know that RA is a complex disease in which interaction between genes and environmental factors may induce immunological responses that generate autoimmunity, which subsequently results in clinical disease in certain individuals.

The main focus here is to highlight possible etiologies and immunopathogenesis in RA patients with emphasis on events prior to the appearance of clinical joint disease and leading to development of autoimmunity.

1.3.1 Etiology

1.3.1.1 Genetics

We know from twin studies that genetic factors play an important role in the pathogenesis of RA and it has been estimated that genetic susceptibility contributes about 40-60% to the risk for RA [121, 122].

The strongest genetic risk factor identified to date is genes coding regions within the human leukocyte antigen (HLA) class II system (surface receptors expressed mainly on antigen-presenting cells), locus HLA-DRB1. There are several HLA-DRB1 alleles (HLA-DRB1*0401, *0404, *01) that have been associated with RA and the products of these alleles share a region of highly similar amino acid sequence at positions 70-74 of the beta1 chain of HLA-DRB1 molecule, termed the shared epitope (SE). These residues belong to the peptide-binding (antigen) site and might therefore be implicated in antigen presentation to CD4+ T cells, suggesting its potential role in the pathogenesis of RA [123].

Hill et al demonstrated that citrullinated peptides were more efficiently presented in the context of HLA class II SE, contributing to enhanced T-cell response [124]. Notably, the shared epitope only correlates with an increased risk for ACPA-positive RA, but not with ACPA-negative RA [125, 126]. This relationship appears to be more important for the progression from being ACPA-positive healthy individual to developing ACPA-positive RA rather than for becoming ACPA-positive [122]. Furthermore, a study with 20,000 individuals of European descent characterized additional amino acid sites within HLA-DRB1 at positions 11 and 13 that are related to an increased risk for RA. These positions are also within the DRB1 binding groove (though not part of shared epitope) and are involved in antigen binding [127]. Investigators have estimated that HLA-DR contributes to less than 50% of the genetic susceptibility in RA and vast numbers of other potential risk genes have been suggested [128].

The second strongest genetic risk factor in RA appears to be the protein tyrosine phosphatase, non-receptor type 22 (PTPN22). PTPN22 is expressed in both hematopoietic tissue and cells of the immune system, and is thought to be involved in T- and B-cell regulation [129]. Several others risk alleles for RA have been identified through genome-wide studies, some of which also confer susceptibility to other autoimmune diseases. One such polymorphism is for the peptidyl arginine deiminase type 4 (PADI4) gene, where association was first demonstrated in the Asian population and subsequently in an European Caucasian population [130-132].

Although important knowledge regarding genes and their potential associations to RA pathogenesis has been gained the last decades, much about RA heritability remains unclear.

1.3.1.2 Environmental factors

Airway exposures

The most established risk factor for RA is smoking [133, 134] and studies have further indicated an increased risk with higher intensity and duration of smoking [135, 136].

However, the most striking findings are from studies investigating the interaction between genetic and environmental risk factors, where smoking and the presence of two alleles of the HLA-DRB1 shared epitope leads to an over 20-fold increased risk for developing seropositive and in particular ACPA-positive RA [125, 126, 137-140].

Similar results have been reported regarding silica exposure and risk of ACPA-positive RA [141]. In addition, other airway exposures, such as asbestos, aerosolized dust and air pollution have been implicated as risk factors for RA [142-144]. Results from a study on oral nicotine-containing tobacco did not show any association with increased risk for RA, suggesting that it is probably the irritants and not the nicotine that contribute to the increased risk [145].

Infectious agents

Infections have long been suspected to be a causative factor in RA and much research has focused on attempting to define the responsible pathogenic virus or bacteria. Epstein Barr virus, cytomegalovirus, retroviruses and human parvovirus B19 have all been suspected, but the current data are conflicting [146-149]. Regarding bacterial infections, some studies have indicated an association with serological findings of *Proteus mirabilis* and *Mycoplasma* species and an increased risk for RA [150-152]. However, a recently published large population-based case-control study determined no association between prior infections in respiratory-gastrointestinal- or urogenital tracts and an increased RA risk [153].

Several studies have reported an association between RA and periodontal disease, in which the major etiologic agent is the *Porphyromonas gingivalis* bacterium. This bacterium contains the enzyme peptidyl arginine deiminase (PAD) which may lead to the generation of intrinsic citrullinated proteins or potentially induces citrullination of host proteins. Either way this could lead to the first breaking of immune tolerance, and in genetically susceptible individuals (e.g. HLA-DR SE) the evocation of immune reactivity with subsequent antibody production (ACPAs) [154-157]. Another potential mechanism may be that antibodies are initially produced against the bacterial citrullinated proteins and through molecular mimicry lead to the breaking of immune tolerance. Further support comes from a study of RA patients and their non-RA relatives, revealing an association between antibodies against *Porphyromonas gingivalis* and the presence of ACPA in genetically susceptible patients and at-risk individuals [158].

The gut microbiome contains, as the name implies, commensal, symbiotic and pathogenic microorganisms [159]. They usually live in harmony but the equilibrium can be disrupted due to external factors such as infections, diet and antibiotic use, leading to diseases. Further, the gut microbiome is believed to influence the development of both the innate and adaptive immune systems and has been implicated in the pathogenesis of autoimmune diseases [160-162]. Several studies have demonstrated differences in the gut microbiome between RA patients and controls [163, 164]. The study from Scher et al found more *Prevotella copri* in untreated new-onset RA patients compared with chronic, treated RA patients [164] and Liu et al reported a greater abundance of *Lactobacillus* in DMARD naïve, early RA patients compared to in healthy controls [163]. However, the implications of these studies are only tentative. Larger studies are needed to fully evaluate the microbiome in both gut and other mucosal surfaces in RA patients.

Other risk factors

Given the unequal gender distribution hormonal factors probably play a role in the pathogenesis of RA. Additional support is the higher peak of RA incidence postpartum and subsequent to menopause when hormonal levels fluctuate. Studies regarding the protective role of oral contraceptives against RA have been conflicting [165-167]. Recent studies have also shown an association between obesity and an increased risk of developing RA [168, 169]. Lu et al observed an association between obesity and both

seropositive and negative RA in women while Wesley et al only determined an association with seronegative RA in women [170]. Other environmental factors, such as the potential protective role of dietary factors and alcohol on the risk of RA are inconclusive and need to be studied further [167].

1.3.2 Immunopathogenesis

It is known that RA is caused by a complex interaction between genes and the environment, leading to a breaking of immune tolerance with consecutive generation of autoantibodies such as RF and/or ACPA. Interestingly, ACPAs, RF and/or antibodies against carbamylated proteins can be detected in blood many years prior to the appearance of the articular disease [20-23] suggesting that autoimmunity develops years before disease onset. This gives us unique opportunities to not only investigate the longitudinal development of RA, but also to potentially interfere with this development in order to prevent joint inflammation. Importantly, a proportion of ACPA-positive healthy individuals might never develop joint inflammation or RA [122, 171, 172] and we need to identify the differences between these groups. A first step is to gain knowledge on how and why autoimmunity occurs and secondly identify the factors that determine which individuals will progress and develop joint inflammation, while others will remain healthy.

The following sections will explain the citrullination- and carbamylation processes and the immune responses evoked against these modifications.

1.3.2.1 Post-translational modifications

Citrullination is a post-translational modification, during which the positive charged amino acid arginine is deiminated to the neutral amino acid citrulline. This process is catalyzed by enzymes called peptidyl arginine deiminases (PADs), whose activities are dependent on higher levels of calcium (Ca^{2+}) than normally occur in living cells [173]. Five isoforms of PAD have been identified, but PAD2 and PAD4 are particularly relevant to RA as these have been demonstrated in the inflamed synovium of RA patients [174, 175] and are active in myeloid cells [176, 177]. Citrullination is a normal physiological process that associates with inflammation [178] and supposedly takes place both intra- and extracellularly. Given the required high levels of Ca^{2+} for PAD activation it has been suggested that cell death in the context of infections or inflammation will allow extracellular leakage of PAD enzymes and high Ca^{2+} levels, leading to extracellular citrullination [179]. However, this is not completely true in the case of joint inflammation in which only very low levels of cell death occur despite inflammation. Possibly other redundant *in vivo* mechanisms regulating PAD activity even at lower Ca^{2+} levels exist, and these still need to be characterized. Interestingly, one recent study demonstrated that antibodies that cross-react with PAD3 and PAD4 can contribute to an enhanced citrullinating capacity of PAD4 by lowering the threshold for Ca^{2+} required for activation [180]. Citrullination of proteins induces structural and polarity changes that might eventually uncover new antigens or neoepitopes. Citrullination may thus render the protein 'foreign' and possibly antigenic, but it is only in the context of genetic susceptibility that immune reactivity against these proteins occurs [181].

Carbamylation is another post-translational modification relatively recently linked to RA pathogenesis, where lysine is converted into homocitrulline through a chemical reaction by cyanate. Cyanate is naturally present in the body. Smoking enhances carbamylation by increasing the cyanate concentration [182]. Carbamylation, as with citrullination, is predominantly believed to occur during inflammation [183]. Anti-carbamylated antibodies can be found in ACPA-positive RA and also in about 15% of ACPA-negative RA patients [19, 184].

1.3.2.2 Antibodies against post-translational modifications

Even though citrullination and carbamylation appear to be ubiquitous processes that are not unique to RA, the immune responses evoked by these modified proteins are highly specific for RA and are not evident in other inflammatory diseases [14, 19].

The ACPA response is very heterogeneous and contains a repertoire of diverse antibodies with fine specificities against different citrullinated epitopes of different proteins. This pool of antibodies differs between individual patients and they can be partly cross-reactive [14, 16, 185, 186]. Studies have demonstrated antibody reactivities against citrullinated extracellular and/or cell surface bound targets, such as collagen II, vimentin, alpha-enolase and fibrinogen, which have all been localized in inflamed joints in RA [16, 156, 187-190]. In addition, ACPAs directed against intracellular molecules such as histones have been identified [191]. Recent studies have demonstrated a breaking of tolerance to one citrullinated epitope initially, with subsequent gradual epitope spreading and increasing reactivities, such that the ACPA response becomes more diverse and the levels of antibodies higher [189, 190, 192]. This initial breaking of tolerance seems to differ between individuals, but no causative associations have yet been reported. Shortly before onset of clinical symptoms there is a rather rapid rise in diversity and titers of ACPAs and certain patterns of reactivities emerge, suggesting that certain combinations of antibodies may be more likely to induce arthritis [190]. Moreover, the ACPAs diverse immune response is fully developed at the onset of articular disease and very few individuals become ACPA-positive at later stages of the disease course [27, 181, 185]. Parallel to the rise and diversity of autoantibodies an increase in cytokine and chemokine levels occurs, probably reflecting activation of adaptive immunity and the presence of systemic pre-clinical inflammation [189, 193].

The increasing knowledge of the possible associations of different ACPA specificities to different clinical manifestations and their relation to risk factors is of particular interest. Associations with antibodies against cit-vimentin and cit-alpha enolase and smoking in the presence of certain HLA-DRB1 SE alleles and PTPN22 have been reported [155, 194]. As previously mentioned, antibodies against cit-vimentin have also been strongly associated with severe ExRA [49].

1.3.2.3 Potential role of ACPAs

Besides the role of ACPAs in diagnosis of RA they may also contribute to important pathophysiologic processes in RA:

1. ACPAs are detectable in the blood years before symptoms of joint inflammation and predict development of RA in patients with undifferentiated arthritis or arthralgia [21, 22, 195].
2. ACPAs associate with higher disease activity and joint destruction as compared to ACPA-negative patients [25, 27].
3. RA patients have a sustained production of IgM ACPA [196].
4. Animal studies have demonstrated that ACPAs, while not able to induce arthritis by themselves, worsen disease course in susceptible mice [197].

Pathogenic effect of ACPAs in joints of RA patients

Higher titers of ACPAs in synovial fluids than in serum indicate that ACPAs are generated within the joint during established disease [198] and the ultimate proof came from the demonstration that B cells from inflamed joints of RA patients produce ACPAs. Furthermore, these authors determined that these antibodies had undergone multiple mutations and thus probably received help from T cells [186]. Less is known about the T cell reactivities, although some studies have reported reactivities against collagen type II [199], cit-fibrinogen [200] and cit-vimentin [201].

ACPA bound to its cit-protein (antigen) can form immune complexes, and immune complexes have been shown in animal models to be central players in mediating synovial inflammation [202]. These immune complexes can activate macrophages and other immune cells through toll-like receptors and Fcγ receptors, thereby mediating effects such as TNF alpha production and activation of the complement system (Figure 2) [203-205].

Antibodies against cit-vimentin promote release of neutrophil-derived extracellular traps (NET:s), which are thereby able to activate macrophages [206]. Additionally, ACPAs can bind cit-proteins present in NET:s and consequently activate fibroblasts [205, 207].

Interestingly, Harre et al recently reported that antibodies to cit-vimentin are able to contribute to activation of osteoclasts [208], which may account, at least partly, for the joint erosions and could possibly contribute to the initiation of joint inflammation. We have recently described in our laboratory that both polyclonal affinity purified ACPAs as well as certain monoclonal ACPAs are able to directly interact with targets on the osteocyte surface to induce an intracellular activation resulting in secretion of IL-8 that further increases osteocyte activation in an autocrine manner (Krishnamurthy et al, Annals of the Rheumatic Diseases, in press).

Altogether this leads not only to increased production of immune mediators and inflammation but may also add to the generation of more cit-proteins and increased immune responses, so-called epitope spreading. Following increased production of immune

complexes (ACPAs bound to cit-protein), RF may subsequently be produced given their probable role in complement activation [192, 209, 210].

ACPAs are generated within the joint during established disease but probably not during the pre-clinical period as studies have not found any signs of inflammation or immune activation in the joint prior to joint inflammation [211]. Since ACPAs are present in the blood before onset of disease, then where do ACPAs originate from and how may the immune tolerance to citrullinated proteins be broken?

1.3.2.4 Potential origin of ACPAs

Most of the environmental risk factors mentioned above originate at mucosal surfaces. Furthermore, one study reported that the proportion of IgA ACPA was higher than IgG in individuals at risk for RA [212]. Other studies have demonstrated the presence of IgA ACPA early in the disease course and in addition found associations with smoking [213, 214]. Altogether, this suggests that the production of ACPA might originate from mucosal surfaces. As smoking is the best established risk factor for ACPA-positive RA, and bearing in mind that RA patients often have lung changes, the lung could be a potential initiating site in a subset of patients.

Lung immunity

The lungs are in immediate contact with the outside environment through the upper and lower airways and are thereby exposed to various stimuli, irritants and infections. They are equipped with different defense mechanisms. The epithelium lining the bronchial tree contains ciliated epithelial cells and mucus-producing goblet cells. On the luminal surface there are immunoglobulins, complement proteins, cells of the innate (e.g. macrophages, dendritic cells and neutrophils) and adaptive immune systems (T- and B-cells) [215]. Alveolar macrophages are the most abundant cells in airways of healthy lungs and are essential for its non-specific defense. Besides the macrophages role in phagocytosis, they are able to recruit other cells of the immune system, secrete pro-inflammatory mediators and act as antigen presenting cells [216]. In the lung ectopic lymphatic tissue called bronchus-associated lymphoid tissue can form in response to inflammation or infections. This allows for local production of antibodies in the lung and class switching, thereby efficiently eliminating the pathogen [217, 218]. Thus the lungs are exposed to various stimuli and have an efficient immune system and the potential to generate antibodies locally. The lung also has a microbiome in which subtle changes may contribute to initiation of autoimmunity.

Possible mechanisms underlying initiation of autoimmunity and generation of antibodies

The innate immune system recognizes agents via pattern recognition receptors. Their role is to eliminate potential pathogens, recruit immune cells and activate the adaptive immune system [219]. Below follows a description of potential steps in activation of innate and adaptive immunity, ultimately leading to the generation of antibodies against citrullinated proteins. Smoking will be used as an example of a risk factor.

Step 1. Repeated stimuli from irritants such as smoking (or infections) can lead to activation of the innate immune system. This can occur by several mechanisms, for example smoke activation of dendritic cells, macrophages and other cells of the innate system via pattern recognition molecules [220]. Smoking can also induce alveolar macrophage apoptosis [221]. In addition, smoke enhances bacterial colonization of the lungs and alters the balanced microbiome, which can activate the innate system and in case of infections, adaptive immunity [222].

Step 2. Increased activation of the innate system and increased cell death may exceed the phagocytic capacity of phagocytes. PAD enzymes and citrullinated proteins can leak out into the extracellular space, leading to increased extracellular citrullination and rendering them visible to the immune system.

Step 3. The antigen presenting cells (dendritic cell, macrophages, B-cells) (now activated by smoke) present the citrullinated antigen bound to HLA class II molecule to CD4+ T cells. Usually does this antigen presentation not lead to immune reactivity. However, individuals that possess the susceptible genes, such as shared epitope, may bind those citrullinated antigens more efficiently, contributing to enhanced T cell activation [124]. T cells present these antigens to B cells, which in the presence of additional co-stimulatory factors may differentiate into plasma cells and generate antibodies (ACPA).

Step 4. After the first breaking of tolerance with antibody generation a following cascade of events may occur: ACPA bound to citrullinated protein can form immune complexes, which can further activate innate immune cells through toll-like receptors as well as the complement system through Fcγ receptors [205]. This (in combination with the original stimuli) contributes to increased activation of immune cells, more inflammation, increased citrullination and formation of more immune complexes. A vicious cycle occurs and if the immune system cannot control this, perhaps due to some defect in regulatory T-cells and in the presence of continuous stimulation, this leads to epitope spreading and diversification of the ACPA response.

The lungs are thus immunologically active organ and exposed to various stimuli. The knowledge that lung manifestations can appear before joint inflammation [56, 62, 76] and an association has been found between the presence of ACPA and interstitial lung disease even in the absence of joint inflammation further strengthen this relationship [119].

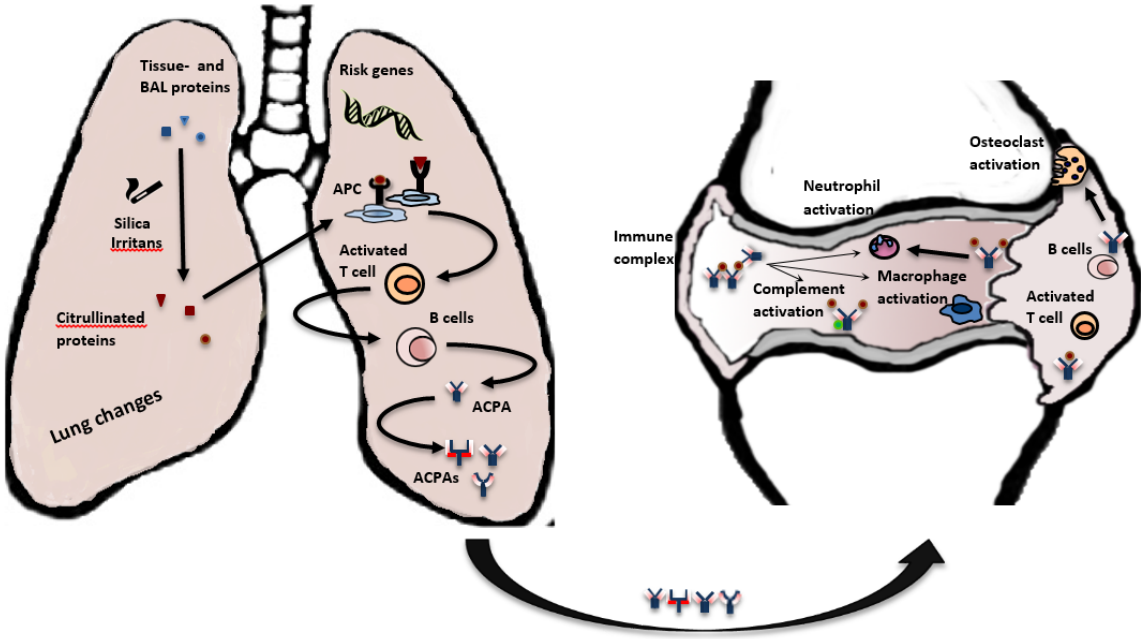
Moreover, Rangel-Moreno et al found ectopic lymphoid tissue in the lungs of patients with longstanding RA and concomitant lung disease and B cells which were able to bind citrullinated proteins, indicating a local antibody production in the lungs [223].

Many other questions about the involvement and the role of the lungs in early RA are still not addressed and in the current studies I have explored part of these questions. My focus has been on the extent of lung involvement, and whether there are any signs of immune activation in the lungs of early RA patients. The results will be presented and discussed in the next chapters.

Given that autoimmunity is generated outside the joint we miss a potential link between presence of ACPA in serum and inflammation in joints, that is the transition from a healthy ACPA-positive individual to a patient with ACPA-positive joint inflammation. Speculations have been about a minor trauma or infection that could lead to inflammation in the joint and thereby induce citrullination of proteins [178, 181]. Usually this would go by unrecognized, but perhaps as the ACPAs are already available these citrullinated proteins could attract antibodies into the joint, and with the right combination these may induce generation of immune complexes, inflammation, recruitment of cells of the immune system, angiogenesis, which ultimately leads to joint inflammation and onset of clinical disease.

For this to occur the same targets must be present in the joints and in the potential initiating organ (this issue has been addressed in the current thesis, Paper III).

Figure 2. A schematic presentation of the lung-joint hypothesis and potential effector mechanisms of ACPAs in joint.



Adapted from Catrina et al [224]. ACPAs = anti-citrullinated protein antibodies, APC = antigen presenting cells, BAL = bronchoalveolar lavage.

Smoking, silica and other possible irritants can lead to activation of the innate immune system and increased expression of citrullinated proteins. In genetically susceptible individuals these cit-proteins when presented by APC lead to activation of T-cells, which may thereby induce maturation of B-cells and subsequently production of antibodies. Initially only one specificity of ACPA is produced but with subsequent epitope spreading the diversity and titers of ACPA increase.

ACPA may exert its pathogenic effect by forming immune complexes that can activate macrophages, neutrophils (leading to NET:osis), and the complement system through toll-like and Fcγ receptors. ACPA can further activate osteoclasts. B-cells are found in joints that produce ACPAs.

2 AIMS

The overall aim of this thesis was to characterize the temporal relationship between lung manifestations and joint disease onset and to investigate whether autoimmunity in RA may be generated in the lungs.

The specific aims were:

i) to characterize lung involvement in early RA patients and to compare it to healthy individuals.

ii) to characterize expression of citrullinated proteins as well as the degree of inflammation and immune activation in BAL and bronchial tissue of patients with early RA.

iii) to study the link between the lungs and joints in RA patients by assessing shared immunological targets in these organs.

iv) to characterize the clinical and immunological lung response to anti-rheumatic treatment in RA patients.

3 PATIENTS AND METHODS

3.1 SUBJECTS

The studies presented in this thesis were approved by the local ethics committee in Stockholm. Written informed consent was obtained from all patients and controls.

LURA cohort: All the studies presented in this thesis are based on data from the **LU**ng investigation in newly diagnosed **RA** patients (**LURA**) cohort.

These patients were recruited at the early arthritis clinic at Karolinska University Hospital in Solna and Huddinge between 2007 and 2012. Individuals with new onset of joint pain were referred from primary care centers in the Stockholm area to the arthritis clinic at Karolinska. Those that fulfilled the ACR criteria from 1987 and had a symptom duration of less than a year were invited to participate in the study. Exclusion criteria were previous treatment with disease-modifying anti-rheumatic treatment and/or oral glucocorticoids, pregnancy or plans for pregnancy, severe debilitating disease, alcohol and/or drug abuse. All included patients were investigated according to the clinical routine at the arthritis clinic at Karolinska. Thorough clinical examination was conducted, joint assessment, DAS28, conventional blood samples and X-ray of the lungs, hands and feet taken. Participating in the study also included the following investigations: high resolution computed tomography (HRCT), dynamic spirometry, body plethysmography and measurement of diffusing capacity for carbon monoxide (DLCO), research blood samples and answering questionnaires about respiratory symptoms and previous history of pulmonary diseases. We included a total of 143 patients, of which 105 patients underwent HRCT (not included in the protocol from the beginning). Patients were followed prospectively and the same investigations were repeated after six months of treatment. Information on demographic characteristics from the EIRA study (a large population-based epidemiological investigation in newly diagnosed RA patients) is included in Table 4 to show the external validity of the study cohort [225].

Bronchoscopy cohort: The 105 patients that had a HRCT were asked to undergo a bronchoscopy. Twenty-four patients agreed to this at inclusion whereby bronchoalveolar lavage (BAL) and bronchial biopsies were taken. Twenty-one patients agreed to repeat the investigation after 6 months.

COSMIC (Chronic Obstructive pulmonary disease and Smoking OMIC Perspective) cohort: A control group of healthy individuals from the same geographical area and similar median age and sex distribution as LURA cohort. These individuals were recruited by advertisement at the Lung clinic at Karolinska University Hospital Solna as a control group to the COPD study at the pulmonary clinic. Seventy-nine included individuals underwent HRCT, spirometry and bronchoscopy with BAL. As a control group to LURA in Paper I we used 43 individuals from the COSMIC cohort that had a similar age and sex distribution as well as smoking history. BAL from all 79 individuals in COSMIC were used as a control to the LURA bronchoscopy cohort in Paper II. See patient characteristics for all cohorts in Table 4.

Table 4. Characteristics of the subjects included in Papers I-IV and EIRA cohort

	EIRA	LURA	COSMIC	Broncho-scopy	BAL Controls	Biopsies Controls
Numbers included	1430	105	43	24	79	15
Female, %	70	67	72	46	51	40
Median age (range)	54 (44-61)	56 (22-84)	55 (44-65)	59 (23-76)	56 (44-65)	35 (21-56)
Never-smokers, %	30	26	33	21	49	60
Current-smoker, %	27	29	67	42	51	40
ACPA+, %	63	67	2	75	nd	nd
RF+, %	66	68	nd	75	nd	nd
Median DAS28	5.3	5.4	na	4.9	na	na

na, not applicable; nd, not done

3.2 METHODS

Investigations were performed at baseline before any anti-rheumatic treatment was started (Paper I-III) and after 6 months of treatment (Paper IV).

High resolution computed tomography (HRCT): HRCT was performed using a Siemens Sensation CT instrument at full inspiration and contiguous 2mm images were reconstructed. Images were independently reviewed for abnormalities by two blinded investigators in accordance with the International Classification of HRCT for Occupational and Environmental Respiratory Diseases criteria [226]. Pathological changes were categorized as i) parenchymal abnormalities; nodules larger than 3 mm, opacities, ground glass opacities, fibrosis and/or emphysema, ii) airway abnormalities; bronchiectasis, air trapping and/or bronchial wall thickening.

Lung function tests: patients performed a dynamic spirometry and body plethysmography. Diffusing capacity for carbon monoxide (DLCO) was measured using the single-breath method and corrected for hemoglobin.

Bronchoscopy was performed to obtain bronchial biopsies and bronchoalveolar lavage fluid (BALF). Bronchial biopsies were taken from segmental and subsegmental septa of the left lung. For the BALF 50 ml x 5 of sterile, phosphate buffered saline solution was instilled and

immediately aspirated. The handling of BAL fluid was completed as previously described in detail [227].

Enzyme-Linked ImmunoSorbent Assay (ELISA) (Papers I and III)

In Paper I we measured the levels of IgG ACPAs in both serum and BAL using a commercial anti-CCP-2 kit (Immunoscan RA Mark 2; Euro-Diagnostica) according to the manufacturer's instructions. We used the same kit with some modifications to the protocol to measure the level of IgA ACPAs in serum and BAL as previously described [213].

In Paper III we established an in-house ELISA to detect antibodies against newly identified citrullinated vimentin. The cut-off for positivity was determined using a population of 152 healthy individuals and was set at 98% specificity in arbitrary units per milliliter (AU/ml).

Immunohistochemistry (Papers I and II)

Bronchial biopsies were stained with biotinylated anti-CCP antibodies generated in-house by affinity purification from a pool of synovial fluid of ACPA-positive patients. Briefly, fluids were first loaded onto a G column to isolate IgG and then purified on a CCP affinity column to obtain CCP eluates (IgG anti-CCP) and flow-through fraction (IgG other than anti-CCP). Both fractions were biotinylated and then used for detection of citrullination, the flow-through being used as an irrelevant control. To further characterize the bronchial tissues we performed immunohistochemistry for various cell type markers (T cells, B cells, plasma cells, dendritic cells), immune activation markers (HLA-DQ, HLA-DR, activation-induced cytidine deiminase (AID)) and PAD2. See Supplementary Table 1 in Paper II for details on the antibodies used.

We used formaldehyde- or acetone-fixed cryostat sections of bronchial biopsies. We started by blocking with hydrogen peroxide and then incubated with the primary antibody according to the protocol for the antigen of interest. After washing we blocked with 1% serum from the same species as the secondary antibody is produced in order to reduce background signal. Thereafter the peroxidase-labeled secondary antibody was added. After subsequent washes the slides were incubated with avidin-biotin-horseradish peroxidase (Vectastain, ABC-HP) kit. Colour reaction was developed by either diaminobenzidine (DAB) substrate kits or AEC peroxidase (ImmPACT vector SK-4205). Counterstaining with hematoxylin revealed the nuclei of the cells and slides were mounted with glycerol. In those cases that we wanted to detect intracellular antigens we used saponin as a permeabilizing detergent. Results were analysed by a double-blind, semi-quantitative evaluation mainly using a four-point scale: 0-no staining, 1-low amount of staining, 2-intermediate amount of staining and 3-high amount of staining.

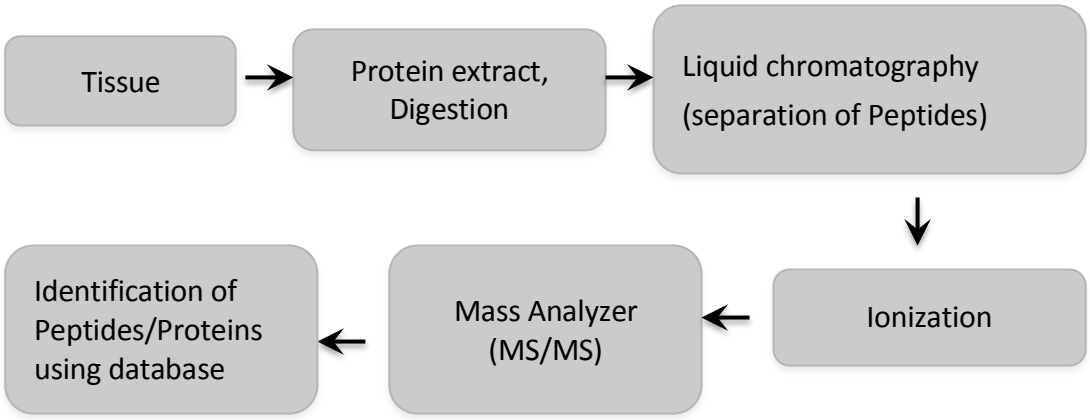
Flow cytometry (Papers II and IV)

The expression of cell surface molecules was analysed using a fluorescence-activated cell sorter (FACS) CANTO II. BAL fluids were strained through a Dacron net (Millipore, Cork, Ireland) and centrifuged. Cell pellets were then stained for CD4, CD8, CD25, CD27, CD28,

CD69, CD103, CXCR3, HLA-DR (antibodies from BD Biosciences, San Jose, CA, USA) and an intracellular staining kit for FOXP3 (eBioscience) as previously described [228].

Mass spectrometry-based proteomics (Paper III)

The combination of liquid chromatography (LC) and mass spectrometry (MS) is a technique that allows us to identify and characterize peptides and posttranslational modifications with high specificity. We utilized this technique in Paper III to detect citrullinated peptides in bronchial and synovial tissue from RA patients. A typical experiment comprised the following steps [229]:



At first the tissues were homogenized by shaking with ceramic beads, after which the homogenate was sonicated and centrifuged (protein extraction). After reduction and alkylation the proteins were digested by a sequencing grade endoproteinase Lys-C (fragmentation). LC-MS/MS were performed on an Easy-nLC system (Thermo-Scientific) on line coupled directly to the mass spectrometer. The results from the mass spectrometer were searched against a concatenated version of the human IPI database V.3.86 using the Mascot search engine (Matrix Science).

Statistical analyses

All the statistical analyses were performed using Statistica version 10 (Paper I) and Graph Pad Prism 5 or 6 (GraphPad Software Inc., San Diego, CA, USA)(Papers I-IV). In the case of descriptive statistics, data were presented as mean with standard deviation for normally distributed variables and median with interquartile range (IQR) for nonparametric variables. For comparison between groups with continuous variables the Mann-Whitney U test (nonparametric) or unpaired t-test (normal distribution) were used. Wilcoxon signed rank test and paired t-test were used where appropriate for paired observations. Correlations were analysed using the Spearman's rank correlation test. When assessing categorical variables we performed Chi-square test when 80% of the expected frequencies were at least 5, otherwise Fisher's exact test was used.

In Paper I, which is a case-control study, we used logistic regression to assess the influence of potential predictive variables on the outcome (HRCT changes). The association is presented as an odds ratio (OR) with 95% confidence interval (CI). We started by performing univariate logistic regression analysis to assess the causal role of various factors on the presence of HRCT abnormalities. Subsequently we conducted a multivariate analysis to examine which variable remained significant.

Comparisons between multiple groups were performed using analysis of variance, with post-hoc analysis using Tukey, Dunnett and/or Sidak test.

In all studies a p value <0.05 was considered to be significant.

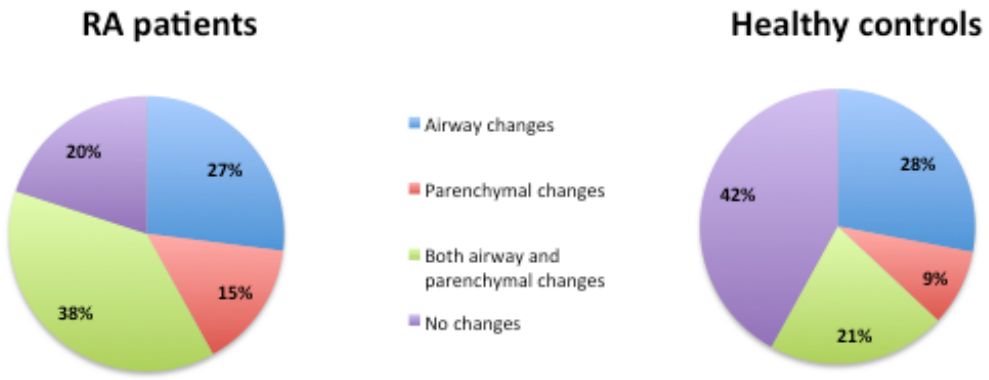
4 RESULTS AND DISCUSSION

When we started the LURA study there was limited knowledge available regarding lung involvement in early RA patients. As mentioned in the *Introduction*, lung changes often accompany RA, due to the disease itself, infections or reactions following anti-rheumatic treatment. Previous studies in relatively early RA patients included patients that were already receiving anti-rheumatic treatment, thereby complicating the interpretation [51, 53, 54, 230]. We initiated the **LU**ng investigation in newly diagnosed **RA** (**LURA**) to shed light onto the temporal relationship between lung involvement and onset of articular disease before any treatment is started, and to investigate whether lung involvement differs between subsets of RA. In addition we included a healthy control group to account for the various changes detected by HRCT, which may not be of clinical relevance. Secondly, we wanted to investigate our hypothesis of a potential role of the lungs in the generation of autoimmunity/ACPA and thereby their role in the pathogenesis of RA.

4.1 PARENCHYMAL AND AIRWAY ABNORMALITIES ARE DETECTABLE EARLY IN PATIENTS WITH UNTREATED RA (PAPER I)

We determined a higher frequency of both parenchymal and airway changes in our cohort of newly diagnosed RA patients compared to the control group, 54% of RA patients having parenchymal changes, while only 30% of controls did. Fibrosis (11%) and ground glass opacities (7%) were only recorded in RA patients. Airway changes were apparent in 66% of RA group versus 42% of the controls (Figure 3). When stratified for ACPA there was a significant difference between the presence of parenchymal changes in ACPA-positive patients (63%) as compared to ACPA-negative patients (37%) and controls (30%). These differences remained after compensation for smoking. No difference was evident regarding ACPA positivity and negativity in terms of airway changes. However, the frequency of bronchiectasis was slightly higher in ACPA-positive patients compared to ACPA-negative patients although not statistically significant.

Figure 3. Pie charts representing the frequency of lung changes in RA patients and healthy controls



A higher proportion of RA patients (though not significant) had airflow limitation (FEV1/FVC<70%) compared to controls (36% versus 21%), and reduced DLCO (defined as <80% of predicted), 52% as compared to 45% in the control group. No difference was found between ACPA-positive and -negative patients and no correlation was determined between the findings of HRCT and lung function.

Using univariate analysis an association was found between parenchymal changes and age ≥ 65 years as well as the presence of ACPA. However, using multivariate logistic regression ACPA remained the only significant predictor of parenchymal lung abnormalities.

The main drawback of this study was the somewhat inadequate matching of the control group (Table 4 in section 2.1) regarding smoking due to inclusion criteria in the COSMIC study. Thus more current smokers were included in the control group and they also had more pack years (median 27) as compared to only 11 median pack years in the RA cohort. Despite this, the control group did not have a worse ratio of FEV1/FVC, but they did report more frequent cough and dyspnea than patients in our cohort during the last 12 months prior to inclusion. As previously stated, no correlation was found between pulmonary symptoms, HRCT changes or lung function tests but overall the difference in history of smoking may affect our results and underestimate the differences of lung manifestations between the groups.

Our results are in accordance with other previously mentioned studies of early RA patients, which all described prevalent lung changes in these patients [51, 53, 54, 230]. The studies are not quite consistent with respect to types of lung abnormalities, associations with ACPA and duration of RA. Mori et al determined an association between interstitial abnormalities and early RA, whereas airway abnormalities were more prominent in longstanding disease. The study from Wilsher et al of 60 patients with symptom duration < 1 year and receiving DMARDs recorded bronchiectasis in 35% of patients and ground glass changes in 18%. Furthermore, Wilsher et al only reported an association of ACPA with bronchiolar disease but not with interstitial disease. This concords with the study from Demoruelle et al, who reported a higher prevalence of lung abnormalities, particularly airway inflammation, in APCA-positive healthy individuals at-risk for RA as compared to ACPA-negative at-risk individuals [231]. Other studies both in patients with established RA and in patients with lung disease and no joint inflammation have found strong correlations between titers and numbers of ACPA specificities and lung disease, both interstitial and bronchiolar involvement [107, 119, 120].

It is difficult to compare these studies as most of them are performed with patients of longer duration of RA receiving anti-rheumatic treatment. One possibility is that airway disease is more associated with smoking status and parenchymal disease with ACPA status, but this remains to be further investigated in even larger studies with careful consideration of the smoking status. Further support of a specific association between ACPA and parenchymal disease is that it has been shown that RA patients with associated ILD have more ACPA specificities and higher ACPA titers [120]. In accordance with this, our preliminary results suggest that the more fine specificities exist, the more parenchymal

changes are detectable on HRCT (Table 5) (Joshua et al, poster ACR 2015). This is not the case, however, for airway changes.

Table 5. Number of ACPA specificities associates with parenchymal changes

ACPA specificities (n)	Patients (n)	p-value	OR (95% CI)
0	24	Ref	Ref
1-3	30	0.03	4.09 (1.16 - 14.38)
4-6	22	0.02	5.52 (1.40 – 21.76)
> 6	27	0.01	5.75 (1.53 – 21.63)

Similarly, anti-citrullinated fibrinogen and anti-citrullinated vimentin reactivities appear to be connected to parenchymal changes but not to airway changes (Table 6) (Joshua et al, poster ACR 2015).

Table 6. Specific ACPA fine specificity associates with parenchymal lung changes

	Parenchymal changes		Airway changes	
ACPA specificity	OR	p	OR (95% CI)	p
Citrullinated fibrinogen	**5.0 (1.8-14.0)**	**0.002**	1.1 (0.4-2.8)	0.89
Citrullinated vimentin	**2.9 (1.2-7.4)**	**0.03**	1.5 (0.6-3.7)	0.34
Citrullinated enolase	1.1 (0.5-2.5)	0.78	2.0 (0.8-5.1)	0.15

The lung function test results did not correlate with neither HRCT involvement nor symptoms, which probably reflects the extent of pulmonary disease and is in accordance with other studies that have shown at most modest correlation between airway disease and lung function [54]. In addition, another study reported no value of lung function tests in predicting development of RA [232].

Taken together, this part of the study demonstrates a high frequency of airway and parenchymal abnormalities in RA patients already at diagnosis, and of particular interest is the association between the presence of ACPA and parenchymal changes. The fact that no correlation was found between the HRCT changes, lung function and symptoms supports the notion that these abnormalities are subclinical. However, this also emphasizes the importance of learning more about the natural development of these lung abnormalities as well as responses to treatment. Additionally, these findings underscore the need for awareness of potential early lung involvement and for rheumatologists to have a low threshold to actively look for potential lung disease (preferably by conducting HRCT). This knowledge also highlights the importance of implementing smoking cessation programs for patients with RA.

4.2 SIGNS OF INFLAMMATION AND IMMUNE ACTIVATION ARE PRESENT IN THE LUNGS OF EARLY UNTREATED RA (PAPERS I AND II)

Previous studies of lung histology and immunology in RA have focused on patients with longstanding RA and concomitant lung disease. These studies have reported more lymphocyte infiltrates in the lungs of these patients as compared to lung biopsies from patients with solely lung disease but not RA [223, 233, 234]. In addition, signs of inducible bronchus-associated lymphoid tissue (iBALT) with germinal centers and B cells that bound to citrullinated proteins have been demonstrated, indicating the possibility that antibodies against citrullinated proteins may be produced in the lungs [223].

As discussed in the *etiopathogenesis chapter*, multiple factors point to the lung as an initiating site of autoimmunity in RA. Smoking and other airway exposure can induce citrullination and activate the innate immune system [220, 221, 235], changes in microbiome may contribute to autoimmunity [160, 161], and an IgA ACPA response is apparent early during the development of ACPA immunity [212-214]. Furthermore, as we demonstrated in Paper I, the strong association between ACPA and early lung manifestations may suggest the lung as an early player in the development of RA.

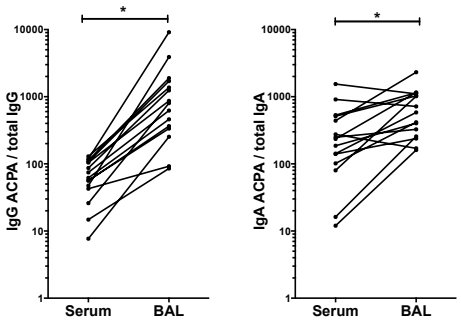
We found more lymphocytic infiltration in the lungs of ACPA-positive RA patients (9/18) as compared to ACPA-negative patients (1/6), $p>0.05$ and controls (2/15), $p<0.05$. In 2/9 ACPA-positive patients the lymphocytic infiltration resembled germinal-like structures and these were positive for B cells, activation-induced cytidine deaminase (AID) and biotinylated citrullinated enolase. B cells and plasma cells were only found in the lungs of ACPA-positive patients and T cells were somewhat more common in ACPA-positive versus ACPA-negative patients. The expression of immune activation markers such as HLA-DR and HLA-DQ was significantly higher in ACPA-positive patients compared to in ACPA-negative.

In BAL we found higher relative numbers of lymphocytes and neutrophils and lower numbers of macrophages in ACPA-positive patients compared to in ACPA-negative patients. The same was true when we compared smoking ACPA-positive patients to smoking controls. More activated T cells were identified in BAL of ACPA-positive patients compared to controls.

We detected increased expression of PAD2 in BAL of ACPA-positive patients and higher expression of citrullinated proteins in the lungs of ACPA-positive patients compared to in ACPA-negative patients, being especially high in ACPA-positive smokers. This is partially in concordance with previous results from our group, whereby higher expression of PAD2 and citrullinated proteins was detected in the lungs of smokers compared to never smokers [235]. However, here we also recorded an association between citrullination and presence of ACPA in the non-smokers, suggesting that other factors than smoking (such as local injuries due to irritants and/or inflammation) might be responsible for this finding.

We identified higher relative levels of ACPAs in BALF as compared with paired serum in our patient cohort with early, untreated RA (Figure 4).

Figure 4. Enrichment of ACPAs in BAL fluids of ACPA-positive early RA patients



This enrichment suggests a local production of antibodies in the lungs of these patients and in support of this is a study from Willis et al that detected the presence of ACPAs and RF in the sputum of at-risk RA patients, some of whom did not yet have these antibodies in the serum [236]. In addition, an association between presence of antibodies to citrullinated-heat shock protein 90 (hsp90) in both BAL fluid and serum, and RA-ILD has been reported. These antibodies against hsp90 were not as frequent in RA alone and were not detected at all in patients with idiopathic pulmonary fibrosis [237, 238]. We know that ACPAs are probably not present in an uninflamed joint (no signs of immune activation or inflammation in pre-clinical studies) although they are present in serum before the clinical onset of disease [211]. Thus the production of ACPAs is initiated outside of the joint and in certain groups of patients this could be the lung. Independently of the origin and source of ACPAs early on, the production of ACPAs eventually takes place in the joints of patients. Finally, these indications of ACPA production in the lungs need to be further proven by isolation of ACPA-producing B cells from BAL. We are currently attempting to do this using our previous strategy of isolating single B cells and cloning of their antibodies [186].

Taken together, our findings further support the hypothesis that the lung might be an initiating organ of autoimmunity in a subset of individuals, which may ultimately lead to clinical RA. Hopefully we are getting closer to an initiation of interventional studies to try to suppress the possible progression of inflammation, citrullination and anti-citrulline immunity in the lungs.

4.3 SHARED CITRULLINATED TARGETS ARE PRESENT IN THE LUNGS AND JOINTS OF RA PATIENTS (PAPER III)

The aim of Paper III was to look for a shared antigen between lung and joint tissue that could further strengthen our hypothesis of a disease initiated in the lung and subsequently leading to inflammation in the joints.

We detected 10 citrullinated peptides from 7 proteins by MS/MS. Eight citrullinated peptides were found in synovial biopsies and 7 in bronchial biopsies with 5 peptides being shared between the two tissue samples sites. Two out of these five identified citrullinated peptides were present in the majority of both synovial and bronchial biopsies: cit-vim 446-466 was found in all bronchial biopsies and 6/7 synovial samples, cit-vim 440-445 was

identified in 5/6 bronchial samples and all synovial tissue samples. The 10 citrullinated peptides identified by MS/MS are presented in Table 7.

Table 7. Quantification of citrullinated peptides in lung and joint tissue.

Protein	Actin	Annexin A2	Cysteine-rich protein 1	Fibrinogen-α			Hemoglobin subunit α	Histone H3.1t	Vimentin	
Aminoacid	62-68	47-65	10-22	449-463	559-575	582-599	129-142	25-37	440-455	446-466
Syn 1	0	0	N.D.	0.012	N.D.	0	N.D.	N.D.	0.019	0
Syn 2	0	0	0	0.007	0.050	0	0	0	0.003	0.002
Syn 3	0	0	0	0	0.130	0	0	0	0.002	0.004
Syn 4	0	0	0	0	0.102	0	0	0	0.011	0.012
Syn 5	0.003	0.072	0	0	0	0	0	0	0.014	0.006
Syn 6	0	0.064	0	0	0	0	0	0	0.001	0.002
Syn 7	0.003	0	0	0.048	0.387	0.220	0	0.363	0.024	0.054
Lung 1 NS	0	0.085	0	N.D.	N.D.	0	N.D.	N.D.	0.026	0.039
Lung 2 NS	0	0.099	0	N.D.	0	0	N.D.	1.360	0.006	0.012
Lung 3 NS	0.003	0.074	0.113	N.D.	N.D.	N.D.	0	N.D.	0.180	0.568
Lung 4 S	0	0.145	0	N.D.	0	0	N.D.	3.654	0.030	0.025
Lung 5 S	0.089	0.130	0	N.D.	N.D.	N.D.	0.163	N.D.	0.281	1.260
Lung 6 S	0	0.132	N.D.	N.D.	N.D.	N.D.	0.048	N.D.	0	2.052

ND-neither citrullinated nor unmodified were detected, 0-only unmodified peptide detected, NS-nonsmoker, S-smoker

Detecting a citrullination in a tissue is a major challenge as this post-translational modification causes a mass shift of 0.9840 Da, which is the same mass shift caused by deamidation, which occurs quite commonly both *in vivo* and *in vitro*. Furthermore, the results from our study as well as others have determined that citrullination is of low abundance in tissues, which poses even bigger challenges as we know that the quality of MS/MS is correlated to the abundance of the peptide of interest [239].

We could not detect any of the 10 citrullinated peptides identified in RA patients in the bronchial biopsies from non-RA non-smokers, while a weak signal for cit-vim 446-466 was observed in non-RA smoker bronchial biopsies. We detected a higher ratio of citrullinated/unmodified cit-vim 446-466, although this was not significant, in the bronchial biopsies from RA smokers compared to non-smokers, which agrees with our and others previous results showing a higher expression of citrullination in the lungs of smokers compared to non-smokers [235, 240].

The detection of cit-vimentin as the only citrullinated peptide present in non-RA smokers is an interesting finding. It does not rule out the presence of lower amounts of other citrullinated peptides, but one can speculate that smoking first induces citrullination of vimentin and subsequently, with continuous stimulation and activation of the innate immune system, other peptides may become citrullinated. This can add to the inflammatory burden by formation of immune complexes, more tissue destruction and production of cytokines, which may lead to an immune response in genetically susceptible individuals.

But do these newly identified citrullinated peptides provoke an immune response? As mentioned earlier, citrullination is a non-specific physiological process and without an

immune response against the citrullinated peptides it probably remains undetectable. To investigate the relevance of these shared cit-vim peptides we set up an ELISA against both native and citrullinated forms of the peptide. The cut-off was set at the 98 percentile of reactivity using a healthy population of 152 individuals. In our LURA cohort 14.9% of the patients showed immune reactivity against the citrullinated peptide, 23.4% of the anti-CCP positive group whereas only 2.6% of the anti-CCP negative group. Regarding association with the shared epitope, 85% of the patients that showed reactivity to this peptide had the shared epitope compared to 71% of patients that did not show reactivity. This is in agreement with other studies which have reported an association between the presence of the shared epitope, smoking and ACPA specificities [189, 194].

The main drawback of this study was the lack of paired lung and synovial tissue samples from the same patients and limited numbers of samples. This is obviously due to the challenge of recruiting patients to interventional studies. Nevertheless, these are unique bronchial tissue samples from patients with very early RA, naïve to anti-rheumatic treatment (neither DMARD nor glucocorticoids). Regarding the potential bias by using synovial tissue from patients with longstanding and treated RA, previous studies of patterns of citrullination in synovium have shown similar results in new onset and longstanding patients [175, 177, 178, 241].

The main finding here is that we identified citrullinated vimentin that was shared between the majority of lung and joint tissue samples, and immune responses against this antigen in RA patients. This indicates a potential link between the origin of autoimmunity in the lung, which eventually induces pathology in another target organ of effect, the joints. These findings of different citrullinated peptides in the lungs and joints, further emphasizes the need to identify and compare the presence of citrullinated proteins in different organs and to look for associations to possible etiological factors.

4.4 HRCT ABNORMALITIES DETECTED AT DISEASE ONSET ARE STILL PRESENT AFTER 6 MONTHS OF TREATMENT AND OCCASIONALLY PROGRESS (PAPERS I AND IV)

The 6-month follow-up study gave us the opportunity to gain knowledge of the longitudinal history of subclinical lung abnormalities in RA, both before and after initiation of treatment. A longer follow-up would of course be even more informative and we are currently planning for the 10-year follow-up.

The majority of patients in LURA started treatment with methotrexate (MTX) (92%) in combination with glucocorticoids (80%) in accordance with clinical guidelines [37]. Of the 105 patients that underwent HRCT at baseline, 93 patients (89%) repeated it at six months. Lung function tests were conducted in 108 patients at follow-up (out of 135 at baseline). Twenty-one of the 24 patients at baseline agreed to undergo another bronchoscopy at 6 months.

At baseline 12 patients had evident fibrotic changes on HRCT and at 6 months every third patient (4/12) had evidence of radiological progression. An additional three patients had

developed fibrosis at follow-up. This is in accordance with other studies that have reported progressive RA-ILD in 34-57% of patients within 2 years of follow-up [52, 118]. Patients that had progressive fibrosis (n=4) were treated with the target MTX dose (20mg/week) and presented with ground glass changes at baseline. Despite the fact that all patients with progressive fibrosis received high doses of MTX, no conclusions can be made for a potential role of MTX. In this respect it is important to mention that a large majority of the patients were treated with this high dose although they never developed signs of fibrosis at follow-up. Despite a previous study implied that MTX might be a potential risk factor for lung fibrosis, this was not statistically significant [52]. Notably, a causal association between MTX and chronic pulmonary fibrosis was not apparent in a large study from Dawson et al, although the mean MTX dose in that study was lower (10.7 mg/week) than recommended doses for RA [92]. Previous studies have shown an association between RA-ILD and high titers of ACPA [101, 108, 109]. Two out of four progressive cases of fibrosis presented herein had high levels of ACPA and all patients were treated with high MTX doses. These numbers are still too low to enable drawing any causality conclusion. No other differences in changes on HRCT between baseline and follow-up were observed.

We found a large decline in FEV1 in all patients at six months follow-up. No association with the presence or absence of ACPA was observed. Most prominent was the reduction in smokers (mean decline 150 ml), but even in the group of non-smokers a significant loss of function occurred (30 ml). Normally, a decline in FEV1 occurs with aging, with an annual decline in smokers of about 50-60 ml, while in non-smokers this is around 20 ml [242]. This large decline in FEV1, particularly in smokers, may imply an ongoing airway inflammation and is a concern as many patients may eventually develop COPD, which will further add to the morbidity and increased mortality observed in these patients [78, 243].

Analysis of the immunological phenotype in BAL fluids revealed a predominance of the lymphocytes and neutrophils at baseline that was not significantly changed following therapy, consistent with a picture of immune activation and inflammation in the lungs that might continue despite successful anti-rheumatic treatment with conventional DMARD (here MTX) and oral glucocorticoids.

5 CONCLUSIONS OF THE THESIS

- Lung involvement is prevalent in early, untreated rheumatoid arthritis patients with both airway and parenchymal abnormalities being more frequent compared to matched healthy controls.
- Lung involvement in early RA may already be progressive at 6 months follow-up, despite successful anti-rheumatic treatment.
- The presence of ACPA strongly associates with subclinical parenchymal lung abnormalities independently of smoking.
- Signs of inflammation and immune activation with concomitant increased citrullination and ACPA enrichment are present in both lung compartments of ACPA-positive early untreated RA patients.
- Shared novel citrullinated-vimentin targets are present in a majority of the lung and synovial tissue samples investigated.
- Specific immunity against the novel citrullinated-vimentin peptides is specifically detected in the blood of RA patients.

- Taken together our results raise awareness for the potential lung involvement in early RA and give further support for the role of lungs as an initiating organ of autoimmunity in RA.

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