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

FROM AUTOIMMUNITY TO INFLAMED JOINTS: STUDY OF RISK FACTORS, MEDIATORS AND CELLULAR TARGETS IN RHEUMATOID ARTHRITIS

Alexandra Cîrciumaru, MD

Karolinska Institutet

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From autoimmunity to inflamed joints: study of risk factors, mediators and cellular targets in rheumatoid arthritis

Thesis for Doctoral Degree (Ph.D.)

By

Alexandra Cîrciumaru, MD

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Principal Supervisors:
Professor Anca Catrina† and
Associate professor Bence Réthi
Karolinska Institutet
Department of Medicine, Solna
Division of Rheumatology

Co-supervisors:
Professor Lars Klareskog MD, PhD
Karolinska Institutet
Department of Medicine, Solna
Division of Rheumatology

Aase Hensvold MD, PhD
Karolinska Institutet
Department of Medicine, Solna
Division of Rheumatology

Opponent:
Professor Carl Goodyear
University of Glasgow
School of Infection and Immunity

Examination Board:
Docent Susanna Brauner, MD, PhD
Karolinska Institutet
Department of Clinical Neuroscience

Docent Jonas Wetterö, PhD
Linköping University
Department of Biomedical and Clinical Sciences
Division of Inflammation and Infection

Docent Ioannis Parodis, MD, PhD
Karolinska Institutet
Department of Medicine, Solna
Division of Rheumatology

Părinţilor mei.

To my families, old and new.

Popular science summary of the thesis

My paternal grandmother had gracious hands. I have the proof in pictures and in a pair of lace gloves that barely fit beyond the tip of my fingers. My memory of her hands in real life is very different: she had ulnar deviation, "swan neck" and "*en boutonnière*" deformities of the fingers, and chronically swollen metacarpophalangeal joints, all of which impacted the range of motion and overall mobility of her hands. For all the part of her life that I knew her she suffered from rheumatoid arthritis (RA). She tried treatment with gold salts (very effective on pain and swelling but low tolerance to the treatment led to discontinuation) and combinations of synthetic disease modifying antirheumatic drugs (sDMARD), the latter with acceptable effect on pain and swelling, better tolerance but less effective on disease progression, at least in her case. When the dawn of biologic treatment came, the long disease duration already led to other deformities of the joints that nowadays are mostly seen in rheumatology textbooks.

RA is a chronic autoimmune disease (a life-long condition where the immune systems attacks the self) mainly affecting the joints. When I set the diagnosis of RA there is always a discussion with my patients on what this really means and how it will impact their daily life. The common perspective on the subject is that RA is (still) a debilitating disease and there is always a fear of the "inevitable" outcome of being bound to the wheel chair. Luckily, with today's criteria of early detection [1] and treatment armamentarium there are good chances of achieving low disease activity (LDA) and remission (where there are no signs of disease activity). In my practice I rarely see the deformities I have seen in my grandmother.

So then why is there a need for another thesis on RA?

New therapies blocking inflammatory cytokines or disrupting immunological synapses do indeed offer potent reduction of disease activity and better overall results compared to the "old generation" of drugs. And there are patients who achieve disease remission, some even having the "luxury" of being without any type of therapy for prolonged periods of time – but not forever. On a population level, in whatever study one looks at, there is rarely more than half achieving remission and so far I haven't found a compound where LDA and/or remission reached 100%, not even in animal models, where treatments usually work better than in humans. And while articular deformities are quite extreme, the hallmark of RA in the daily practice is still joint swelling, pain and stiffness and, on the long run, irreversible radiographic changes. On top of that there are extra-articular manifestations (e.g. interstitial lung disease), secondary osteoporosis and chronic fatigue. This means that the overall costs of having RA are still high with an impact on the individual that is not negligible. Nevertheless, the development of modern therapies during the past decades also brought the advantage of a better understanding of RA and gave a glimpse into cellular and molecular mechanisms of disease. With this information at hand, it is now more than clear that the next step is to research the possibilities of prediction of who gets the disease and whether there is a way for prevention altogether.

From lab bench to bedside, the aim of this thesis is to study the role of autoimmunity in early, preclinical stages of RA in order to better characterize the prototypical individual that would progress to disease and help develop diagnostic tools and improve the standard of care.

The present work combines observations from the laboratory and the clinical setting and aims at better understanding the roles of antibodies against citrullinated proteins (ACPA) found in the majority of RA patients.

In paper I, we followed an "at-risk for RA" cohort (individuals experiencing musculoskeletal symptoms in the absence of joint swelling and who tested positive for ACPA by anti-CCP2 test at their general practitioner) and we saw that a third developed arthritis within the first year of follow-up. When looking at the baseline information from those who developed arthritis and those remaining arthritis free, we observed that tenosynovitis (inflammation of the lining sheath of the tendons), two markers from blood related to inflammation and the presence of ACPA differentiated the two groups and could predict who will progress to arthritis. Most importantly, having at least one type of the tested ACPAs stood out as the strongest factor in the proposed prediction model.

In paper II, in a collaboration with colleagues from the Netherlands and Germany, we aimed to validate a questionnaire for "self-testing" of symptoms, aimed to ease access to healthcare. Briefly, we tested whether a purely symptom-oriented questionnaire, meant to be answered by individuals with musculoskeletal complaints, can help identifying those at risk for arthritis and further help direct them to the rheumatologist. The questionnaire, although directing individuals to seek healthcare, did not perform well in predicting inflammatory arthritis outcome in the case of our at-risk individuals, suggesting the need for additional biomarkers (e.g. ACPA) in risk stratification additionally to the symptomatology.

In paper II, we were interested in understanding whether ACPA could be involved in early manifestations of RA, before the occurrence of clinical signs of joint inflammation. For this purpose we used a mouse model where we injected ACPA purified from RA patients and we observed that these antibodies induced pain-like behaviour and tenosynovitis as well as bone loss, all in the absence of joint swelling.

In paper IV, we then tested the effect of ACPA obtained from RA patients and individuals at risk for RA on a type of cell of the innate immune response, namely the neutrophil, which may be able to contribute to disease pathogenesis in relation to ACPA presence. Our results show that ACPA alone could not activate neutrophils. However, once activated, neutrophils were able to bind ACPA, suggesting that they may not play a role in initiation but may contribute in maintaining or potentially reinforcing the already activated immune processes. More interestingly, we observed that there is a broad range of binding patterns of ACPA to different targets suggesting that they may exert a variety of functions. These results together with observations that ACPA affect other cell types from the joint responsible for bone destruction may at least partly explain the heterogeneity of clinical features and response to therapy of ACPA positive RA patients.

Taken together these studies suggest there is a continuum from autoimmunity to inflamed joints, and while so far there is no definitive one single ACPA that leads to RA there is evidence of i) ACPA being important in RA development in a population at risk for the disease and ii) there is an ACPA repertoire with a broad specificity suggesting a variability of the clinical phenotypes and potentially opening the possibilities for more personalized therapy approaches. These results could be useful in the context of early intervention for disease delay and possibly prevention.

Rezumatul tezei doctorale

Bunica mea avea mâini grațioase. Am dovada în pozele de familie și într-o pereche de mănuși de dantelă pe care abia le pot trece de vârful degetelor. În amintirile mele însă, mâinile bunicii erau umflate, cu deformări ale degetelor "în gât de lebădă" și "în butonieră". De când o știam eu, bunica avea poliartrită reumatoidă. A avut pe rând tratamente cu săruri de aur și medicamente antireumatice modificatoare de boală (sDMARD), însă cu tolerabilitate scăzută și efect terapeutic relativ modest. La momentul când terapia biologică a devenit disponibilă, bunica mea avea deformări articulare ireversibile pe care în ziua de astăzi le întâlnesc preponderent în cărțile de reumatologie.

Poliartrita reumatoidă este o boală cronică a răspunsului imun care țintește structurile corpului (autoimunitate), afectând în special articulațiile. În practica mea medicală, în momentul în care pun diagnosticul de poliartrită reumatoidă, îmi rezerv timp de discuție cu pacienții deoarece am observat că cea mai mare frică legată de diagnostic nu este durerea, ci dizabilitatea fizică și "inevitabilul" scaun cu rotile. Din fericire, există în prezent criterii de clasificare timpurie a poliartritei reumatoide[1] care, împreună cu arsenalul terapeutic considerabil, ajută la obținerea unei activități reduse a bolii și chiar remisiune (unde semnele de boală lipsesc), astfel că în practică rar întâlnesc deformările pe care le avea bunica mea.

Atunci de ce este mai nevoie de încă o teză pe tema poliartritei reumatoide?

Noile terapii blochează moleculele inflamatorii și cupează sinapsele imunologice mult mai rafinat comparativ cu tratamentele din "vechea generație". Numărul pacienților care ating și mențin remisiunea clinică, uneori chiar cu perioade lungi fără a necesita terapie, a crescut considerabil în ultimii ani. Totuși nicio terapie la acest moment nu vindecă poliartrita reumatoidă. Iar deși deformarile articulare sunt rare în ziua de astăzi, semnele tipice ale poliartritei reumatoide în practica medicală rămân tumefacția și durerea articulară care în contextual unei evoluții cronice duc la modificări radiologice ireversibile. În plus există afectarea extraarticulară (spre exemplu boala pulmonară asociată poliartritei reumatoide), osteoporoza secundară și oboseala cronică. Toate acestea presupun costuri directe și indirecte deloc neglijeabile pentru individ și societate. În același timp, dezvoltarea noilor terapii a dus la progrese în înțelegerea mecanismelor intime ale bolii, astfel încât este clar că următorul pas reprezintă explorarea posibilităților de predicție a riscului de boală și, într-un viitor nu foarte îndepărtat, prevenție.

Lucrarea de față îmbină munca și observațiile din laborator cu practica în clinică și își propune să studieze rolul autoimunității în stadiile incipiente, așa-zise preclinice ale poliartritei reumatoide, cu scopul de a caracteriza prototipul individului aflat la mare risc de boală și astfel de a dezvolta strategii de screening și diagnostic cu aplicabilitate clinică. De asemenea, teza își propune studiul anticorpilor împotriva proteinelor citrulnate (ACPA), tipici în majoritatea pacienților cu poliartrită reumatoidă, și întelegerea rolului acestora în riscul de dezvoltare a bolii.

În primul articol al tezei am urmărit o populație la risc pentru poliartrită reumatoidă: indivizi având un test anti-CCP[1] pozitiv și simptome musculoscheletale (durere și redoare articulară) dar fără tumefacție articulară (artrită). O treime din acești indivizi au dezvoltat boala în aproximativ un an de la înrolarea în studiu. Analiza datelor clinice și paraclinice (date ecografice și de laborator) la intrarea în studiu a arătat

[1] ACPA reprezintă o familie de anticorpi, țintind diverse proteine citrulinate. În practica medicală, prezența și nivelul ACPA se detecteaza printr-un test de sânge care se numește anti-CCP.

că factorii predictivi pentru dezvoltarea artritei sunt: prezența ACPA în sânge, inflamația membranei sinoviale a tendonului (tenosinovita) vizualizată ecografic și două molecule implicate în inflamație. Dintre aceștia, prezența a cel puțin unui tip de ACPA din cele nouă variante testate a reprezntat cel mai puternic factor de predicție a bolii.

Al doilea articol este o colaborare cu două centre din Olanda respectiv Germania unde am testat validitatea unui chestionar de a identifica și tria rapid indivizii cu simptome musculoscheletale și astfel de a facilita accesul acestora la specialist. Chestionarul este dedicat pacienților, este interactiv și poate fi accesat online iar, pe baza răspunsurilor, este menit să orienteze respondentul către medicul de familie sau direct către specialist. Pentru a testa validitatea acestuia am folosit date clinice și anamnestice ale participanților din centrul nostru cât și din celelalte centre. Simularea a constat în a răspunde chestionarului bazat pe simptomele și anamneza participanților la intrarea în studiul prezentat în primul articol. În cazul cohortei din centrul nostru, chestionarul nu a putut determina corect cine va dezvolta artrită și implicit cine ar trebui să consulte direct specialistul. Aceste rezultate indică faptul că simptomatologia în stadiul incpient al bolii este relativ nespecifică și de aceea este necesar și screeningul de biomarkeri din sânge (spre exemplu ACPA) pentru a determina riscul de dezvoltare a bolii.

În continuare, în al treilea articol, am investigat în ce măsură pot ACPA contribui la simptomatologie în stadiile preclinice de boală. În acest scop, ACPA purificați de la pacienți cu poliartrită reumatoidă au fost injectați în șoareci. Animalele au dezvoltat comportament asemănător durerii, tenosinovită și au prezentat semne de scădere a masei osoase la nivel articular, toate în absența tumefacției articulare. Aceste rezultate sugerează că ACPA pot fi implicați în stadiile precoce ale bolii, înaintea apariției artritei.

În al patrulea articol, am testat efectele ACPA de la pacienți și indivizi la risc pentru poliartrtă reumatoidă asupra unui tip de celulă implicată în răspunsul imun, mai exact neutrofilul. Din rezultatele obținute reiese că ACPA nu pot activa neutrofilele. Totuși, odată activate, neutrofilele pot exterioriza componente intracelulare care pot lega ACPA, sugerând că acest tip de celulă nu inițiază ci mai degrabă contribuie la perpetuarea proceselor immune. Mai mult, ACPA au prezentat afinități variable față de țintele celulare atât în cazul neutrofilelor cât și într-un alt tip de celulă, osteoclastul, care este implicat în remodelarea osoasă. Aceste rezultate sugerează o heterogenitate a efectelor ACPA și pot explica observațiile din practica medicală unde pacienții cu poliartrită prezintă simptome clinice și răspunsuri terapeutice variabile.

În concluzie, rezultatele acestei lucrări doctorale sugerează că există un continuum între fazele inițiale ale activării immune și apariția tumefacției articulare, și deși nu am putut identifica un singur tip de ACPA care să inducă poliartrită reumatoidă, există dovezi că i) ACPA are rol predictiv pentru dezvoltarea bolii într-o populație considerată la risc și ii) există un spectru de ACPA care exercită efecte diverse ce poate explica simptomatologia și aspectele clinice. O bună parte a rezultatelor lucrării de față prezintă potențial de aplicabilitate imediată: implementarea unui program de screening a indivizilor la risc pentru poliartrită reumatoidă care să includă ecografie musculoscheletală, crearea unui panel de testare a afinităților ACPA (similar cu testele care se folosesc în clinică pentru alte afectări autoimmune, ex: sclerodermia), ca o completare la testul anti-CCP. De asemenea, rezultatele acestei teze sugerează posibilitatea modulării răspunsului imun în stadiile preclinice și implicit un anumit potențial de prevenție a poliartritei reumatoide. De aceea, în perspectivă, susținem colaborarea între centrele de cercetare și sugerăm harmonizarea criteriilor de includere în studii a indivizilor la risc pentru boală cu scopul de a implementa intervenții precoce.

Abstract

Rheumatoid arthritis (RA) is a chronic autoimmune disease affecting mainly the joints and leading to disability. Circulating antibodies against citrullinated proteins (ACPA) are present in the majority of patients and are considered important to disease pathogenesis, however the mechanisms triggered by these antibodies remain unclear. Although significant progress has been made in refining disease modifying therapies during the last decades, to date there is still no curative treatment. In this context identification of individuals likely to develop the disease from those being at risk can help rethink the therapeutic strategy towards prevention and personalized medicine.

The main objective here was to study the role of ACPA in disease pathogenesis in the very early stages of RA in order to a) set up a predictive model for arthritis development and b) have a better understanding of ACPA effects on cellular targets, especially of the immune system.

This work followed a translational approach: the observations from the clinics were assessed in experimental models, in a laboratory setting.

In **Paper I** we followed-up a cohort of individuals at risk for developing RA (Karolinska RiskRA cohort) i.e. individuals positive for CCP2-antibody test and musculoskeletal symptoms (joint stiffness and pain) in the absence of clinical or ultrasound signs of arthritis or any other rheumatic diagnosis. Baseline data was collected, including clinical and ultrasound assessments of the joints and blood samples were analyzed for 9 ACPA reactivities, a panel of 92 inflammation-related markers and HLA shared epitope. 101/267 individuals progressed to arthritis within a median of 14 months of follow-up. The multivariate analysis showed that the presence of at least one ACPA reactivity, tenosynovitis detected by ultrasound, high levels of IL-6 and low levels of IL-15Rα had independent and significant predictive value in a model for arthritis progression.

Next, in **Paper II** we tested whether a symptom-based e-questionnaire called "*Rheumatic?*" could be used as a tool for prediction of arthritis development. This was an international collaboration with two other centers, in Leiden, The Netherlands and in Erlangen, Germany. The tool was primarily aimed to be used by patients and, based on their answers, to suggest visit to the general practitioner or directly to the rheumatologist. In the case of the Karolinska RiskRA cohort the tool had limited usability regarding discrimination for an inflammatory outcome, suggesting that symptomatology alone is not sufficient for prediction. This confirms our previous observations from our cohort where clinical parameters were not significant for risk prediction in univariate analysis.

In **Paper III** we studied the effects of ACPA on pain-like symptoms, tenosynovitis and bone loss in the preclinical stages of RA. Briefly, ACPA obtained from RA patients were injected in healthy mice. MRI of the joints at 28 days post injection showed signs of tenosynovitis, which was further confirmed by histological analysis. Moreover ACPA-treated mice showed pain-like behavior and had signs of bone resorption in Xray microscopy.

In **Paper IV** we aimed to further characterize the citrullinated targets of ACPA in neutrophils, cells potentially important in disease pathogenesis. For this purpose we tested ACPA obtained from RA patients and an at-risk individual. Some but not all ACPA could bind to citrullinated targets released by activated neutrophils, suggesting that neutrophils could be important sources for citrullinated antigens. However, ACPA could not induce neutrophil activation nor bind to intact cells, suggesting a limited role in perpetuating ACPA response via boosting autoantigen release from neutrophils. When testing

polyclonal ACPA preparations from individual RA patients we observed a highly variable binding capacity to neutrophil-derived antigens and similarly, a variable effect on another target cell for ACPA, specifically the osteoclast. These results suggest high patient-to-patient variability in the ACPA effects in line with the clinical observations on the heterogeneity in RA clinical phenotypes.

In conclusion, on the timeline from systemic autoimmunity to clinical diagnosis of RA, we propose a particularly high at-risk for disease phase, characterized by the presence of ACPA, subclinical tenosynovitis and inflammation-related factors. Digital tools such as *Rheumatic?* e-questionnaire show promising use and should be further developed to help arthritis prediction. ACPA could directly contribute to symptomatology of the risk phase, where a heterogeneity in targeting different cell types may contribute to the various clinical presentations.

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List of scientific papers

I. Cîrciumaru A, Kisten Y, Hansson M, Mathsson-Alm L, Joshua V, Wähämaa H, Haarhaus ML, Lindqvist J, Padyukov L, Catrina SB, Fei G, Vivar N, Rezaei H, Af Klint E, Antovic A, Réthi B, Catrina AI, Hensvold A. Identification of early risk factors for anti-citrullinated-protein-antibody positive rheumatoid arthritis-a prospective cohort study. Rheumatology (Oxford). 2024 Mar 8:keae146. Epub ahead of print. PMID: 38457608. https://doi.org/10.1093/rheumatology/keae146

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List of scientific papers not included in the thesis

I. Chatzidionysiou, K., Cîrciumaru, A., Rethi, B., Joshua, V., Engstrom, M., Hensvold, A., Af Klint, E., & Catrina, A. (2021). Tocilizumab decreases T cells but not macrophages in the synovium of patients with rheumatoid arthritis while it increases the levels of serum interleukin-6 and RANKL. *RMD open*, *7*(2), e001662.

II. Sun, M., Rethi, B., Krishnamurthy, A., Joshua, V., Cîrciumaru, A., Hensvold, A. H., Ossipova, E., Grönwall, C., Liu, Y., Engstrom, M., Catrina, S. B., Steen, J., Malmstrom, V., Klareskog, L., Svensson, C., Ospelt, C., Wähämaa, H., & Catrina, A. I. (2019). Anticitrullinated protein antibodies facilitate migration of synovial tissue-derived fibroblasts. *Annals of the rheumatic diseases*, *78*(12), 1621–1631.

TABLE OF CONTENTS

List of abbreviations

ACPA	anti-citrullinated protein antibodies
APC	antigen presenting cell
ATP	adenosine triphosphate
AUC-ROC	area under the reciever operating curve
BMI	body mass index
CCL2	C-C motif chemokine ligand 2
CCL17	C-X-C motif chemokine ligand 17
CCL22	C-X-C motif chemokine ligand 22
CCP	cyclic-citrulllinated peptide
CI	confidence interval
CSA	clinically suspicious arthralgia
CT	computer tomography
CXCL1/2	C-X-C motif chemokine ligands 1/2
CXCL8	C-X-C motif chemokine ligand 8
DMARD	disease modifying anti-rheumatic drugs
EULAR	European league against rheumatism
FDR	first degree relative
GM-CSF	granulocyte-macrophage colony-stimulating factor
HLA	human leukocyte antigen
HR	hazard ratio
IA	inflammatory arthritis
Ig	immunoglobulin
IL-10	interleukin 10
IL-15Rα	interleukin 15 receptor alpha
IL-17	interleukin 17
IL-23	interleukin 23
IL-6	interleukin 6
im	intramuscular (related to drug administration)
iv	intravenous (related to drug administration)
LPS	lipopolysaccharides
M-CSF	macrophage colony-stimulating factor
MRI	magnetic resonance imaging

MSK	musculoskeletal
NET	neutrophil extracellular traps
PAD	peptidyl arginine deiminase
qw	every week (related to drug administration)
RA	rheumatoid arthritis
RAMRIS	rheumatoid arthritis magnetic resonance imaging score
RF	rheumatoid factor
sc	subcutaneous (related to drug adminsitration)
SC-ACPA	secretory ACPA
SE	shared epitope
Th17	T helper 17 cells
TNFα	tumor necrosis factor alpha

1 Introduction

An overview on rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic autoimmune disease with high socioeconomic impact due to the potential for joint destruction and extra articular manifestations which contribute to increased comorbidity. There is a broad arsenal of therapeutic options in the established disease phase, with a proportion of patients achieving remission. However, the majority of the patients will need lifelong therapy, some also struggling to maintain a low disease activity.

The pathogenesis of RA is complex. Genes and environmental factors may give a predisposition to the disease, but the events until arthritis diagnosis are not fully characterized. The current understanding is that in predisposed individuals a breach in immune tolerance at mucosal sites may lead to occurrence of local autoimmunity which, under certain circumstances, may become systemic with the formation of circulating autoantibodies. Next, individuals with systemic autoimmunity experiencing musculoskeletal symptoms such as joint pain are considered to be at an increased risk for disease and some will develop arthritis (Figure 1).

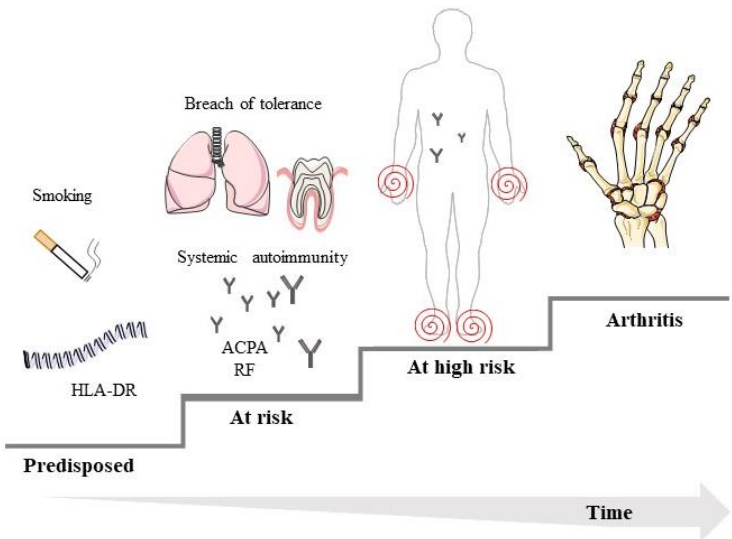

Figure 1. "The pathogenesis ladder of RA" - a possible model for RA pathogenesis comprising intermediary steps of risk and allowing, at least theoretically, the possibility of moving up and down the risk phases through early interventions; ACPA - anti-citrullinated protein antibodies; RF – rheumatoid factor; *This figure has been partly generated using Servier Medical Art, provided by Servier, licensed under a Creative Commons Attribution 3.0 unported license*

This approach suggests two important aspects:

a) not all individuals progress towards the disease and there is a need for further understanding the role of immune processes and autoantibodies in the early phases of RA. Moreover, this implies the potential for better stratification of the "at risk for disease" phases.
b) there may be a "window of opportunity" where life-style changes and/or timely therapeutic interventions could delay or even prevent the disease.

With this in mind, the scope of this thesis is to explore the role of autoimmunity in early, preclinical stages of RA in order to better understand disease progression and explore possibilities for improved risk stratification. In doing so we aim to contribute with knowledge for early therapeutic interventions.

The following literature review is aimed to guide the reader on current understanding of the field and present the knowledge gaps and research questions this thesis addresses.

2 Literature review

RA is a chronic inflammatory disease phenotypically divided in two clinical entities according to the presence of antibodies against citrullinated proteins (ACPA) and/or rheumatoid factor (RF). The prevalence ranges between 0.1-2.7 % worldwide with variations according to geographical locations [2-4]. In Sweden the cumulative prevalence is estimated at 0.77% with an annual incidence of 41 per 100 000 individuals [5, 6]. There is a gender disparity (male:female 1:2) that tends to decrease with older age [6].

The immune response to citrullinated proteins is highly specific for RA and ACPA have been considered to play a role in disease pathogenesis[7, 8]. However the exact mechanisms by which ACPA could lead to disease are still not well understood.

2.1. Citrullination in health and disease

Citrullination is a posttranslational protein modification in which an arginine residue is converted to citrulline. This reaction is catalysed by members of the peptidyl arginine deiminase (PAD) family. Five PAD isoforms (PAD1, PAD2, PAD 3, PAD4 and PAD6) have been described, which are expressed in cells with a broad tissue distribution (e.g. immune, epithelial, embryonic cells). All PADs have cytoplasmic localization, with the exception of PAD4 which is found in the nucleus and is able to citrullinate histones. The consequence of citrullination is the change in the protein charge leading to changes in intra- and intermolecular interactions and folding.

Citrullination is physiologically important in processes such as keratinisation, brain plasticity, and embryonic development [9]. Since PADs are expressed in immune cells, citrullination may also be involved in immunity and inflammation. One example is by regulating immune cell differentiation (e.g. osteoclastogenesis from progenitors of the myeloid line such as monocytes or dendritic cells) [10-13]. Another example is in activated neutrophils where citrullination of histones by PAD4 is needed for the formation of neutrophil extracellular traps (NETs), in response to certain stimuli, in a process named NETosis, which is important in trapping and immobilizing pathogens [14].

PAD activity requires certain calcium levels. *In vitro* citrullination occurs at high calcium concentrations which *in vivo* may occur in the event of cellular membrane disruption (i.e. cell lysis) where the influx of the extracellular calcium could activate PADs. However, since PADs are also involved in processes such as gene regulation or cytoskeletal citrullination (as part of keratinization), the high calcium concentration needed for PAD activity might disturb other pathways. This implies that there must be alternative activation ways at lower calcium concentrations, or even potentially calcium independent [9, 15]. The latter is an interesting possibility since bacterial PADs are able to citrullinate substrates in the absence of calcium.

Excessive citrullination of physiological substrates or citrullination of targets usually not subjected to this posttranslational modification have been described in several autoimmune diseases, and citrullinated targets were observed in inflamed synovia of rheumatic arthropathies such as RA and spondylarthritis, as well as osteoarthritis [16-19]. However, relative to other rheumatic diseases, in RA there is a high disease-specific immune response to citrullinated substrates with autoantibody formation (i.e. ACPA), clinically relevant for diagnostic purposes [1]. Therefore, in the context of RA,

citrullination may be responsible for neo-antigen formation that could a) trigger adaptive immune responses via activation of autoreactive T and B cells, thus leading to antibody formation (i.e. ACPA) in genetically predisposed individuals and b) form immune complexes with the ACPA, activate Fc receptor positive cells of the innate immune system and promote pro-inflammatory cytokine production [20].

2.2. Genetic and environmental factors contribute to progression towards arthritis

The strongest genetic association has been described with the HLA allele group and in particular with HLA-DR-SE (shared epitope) alleles [21]. Presence of shared epitope genetic variants confers a higher risk towards development of the ACPA positive RA [22-24]. Non-HLA alleles associated with increased risk for RA include variants of *PTPN22*, *PADI4*, *CTLA4*, *IL6R, STAT4*. These and others previously described genetic associations are involved in native immune response, leukocyte activation and migration but also other metabolic and signalling pathways suggesting that RA pathogenesis is not restricted to the immune system [24-26].

Genetic contribution in ACPA positive RA development varies between 40-60% as described in previous twin studies [27-29] suggesting that exposure to certain environmental factors are partly responsible for disease progression.

Smoking status together with the presence of shared epitope alleles have been suggested to increase the risk for disease progression [30, 31]. In a case-control study smoking was found to be associated with ACPA positive RA but not with ACPA negative disease variant [32]. ACPAs have been identified in bronchoalveolar fluid from early seropositive RA patients and anti-CPP positive individuals without arthritis [33, 34]. Moreover, PAD2 and PAD4 were significantly more expressed at bronchial mucosa and alveolar compartment in smokers suggesting a possible association between citrullination and smoking [35, 36] .

Since the incidence of RA seems to be higher in urban compared to rural areas [4] ambient noxious particles may contribute to disease pathogenesis. Interestingly, prospective studies on pollution related to living in industrialised/urban areas did not find a significant difference for the risk of disease progression [37, 38]. Nevertheless, air pollutants such as silica or textile dust have been linked to higher risk for developing ACPA positive RA [39, 40] and again, when adjusted for smoking status, results showed higher odds ratio for smokers. This could suggest an additive effect of noxious factors leading to persistent local mucosal injury to which citrullination, in the context of local inflammation, may be a defensive response, at least in the initial stages.

Moderate alcohol intake has been negatively associated with arthritis development [41, 42]. This effect seems to be dose-dependent according to a recent study and remains significant even after adjusting for gender, educational status, body mass index, physical activity, cardiovascular disease and smoking status. Interestingly, the association between smoking and RA incidence seems to be lower if concomitant alcohol consumption suggesting a possible interaction in the case of double exposure [41, 43]. At a cellular level, ethanol and its metabolite were reported to alter the functionality of follicular T helper cells, by decreasing cytokine production and impairing accumulation and organization of immune cells in germinal centres [44].

Other modifiable risk factors such as low social status, obesity or sedentary lifestyle have been associated with risk for RA [45]. By contrast oestrogen exposure (multiparity, prolonged breastfeeding) and higher physical activity are considered protective, as reviewed here [46].

Microbial taxa has been implicated in the development of RA. Associations between periodontitis, oral or intestinal dysbiosis (with some of the most cited bacteria being *Porphyromonas gingivalis, Aggregatibacter acntinomycetemcomitans* and *Prevotellaceae spp.*) and seropositive RA have been reported in tandem with shared epitope in several populations with different ancestry [47] suggesting a gene-environment interaction in disease progression. Recent reports on recurrent transient translocation of citrullinated oral bacteria in the blood of RA patients and their association with disease flares support the mucosal origin hypothesis for RA pathogenesis [48]. Oral dysbiosis and higher prevalence of periodontitis were observed also on ACPA positive individuals compared to healthy controls [49, 50]. Restoring local microbiota and periodontitis treatments may improve RA outcomes [51] thus potentially even interventions on the oral health could be beneficial in risk stages as well.

Although genetic predisposition together with environment related factors seem to be necessary for further progression to seropositive RA, many of the individuals having the above-mentioned risk will never develop arthritis. This suggests that there may be additional factor(s) and possibly the next step up the "pathogenesis ladder" may be the breach of immune tolerance (Figure 1).

2.3. Loss of immune tolerance and development of systemic autoimmunity

Circulating ACPA can be present years before clinical signs of the disease [52]. Citrullination has been described to occur elsewhere than in the joints as suggested by the observation that ACPA are present in individuals who do not develop arthritis (see Table 2. summarizing the available studies on disease progression in ACPA positive individuals without clinical arthritis). Citrullination at mucosal sites has been proposed based on epidemiological data and the identification of citrullinated targets in both lungs and joints [30, 36]. Indeed, structural lung changes on high resolution computer tomography, local protein citrullination and higher levels of ACPA in bronchoalveolar fluid were observed and were more prevalent in early diagnosed untreated ACPA positive RA patients compared to ACPA negative patients and healthy controls [33]. Local injury (by noxious airborne agents) could disrupt the epithelial barrier, expose subepithelial structures and trigger local inflammation. In a study by Li et al, smoking induced oxidative stress in lung macrophages with consequent citrullination of vimentin [53]. Furthermore, smoking has been reported to reduce ciliary motility and decrease mucous secretion which may contribute to bronchial dysbiosis [54]. In this context, newly generated citrullinated proteins as well as formation of damage associated molecular patterns (consequent to irritation from particulate pollution i.e. cadmium and carbon black from cigarette smoke) and generation of pathogen associated molecular patterns (occurring due to local dysbiosis) could be potential sources of activation of innate immunity locally. More concretely, the process may start at the cellular level with dendritic cells, local macrophages and other antigen presenting cells (APCs) generating and processing the citrullinated antigens, and presenting them to the CD4+ T cells. At the same time, neutrophil activation may result in NETosis, a process in which intracellular material is externalized with the main purpose of immobilizing pathogens (a more detailed explanation of the role for NETs is provided in the following section on cellular targets for ACPA). NET products may be the source for citrullinated compounds that could bind to local citrulline reactive B cells and induce antibody production. Alternatively, these NET

derived compounds may be internalized by local APCs and presented to reactive T cells engaging the immune response.

Similar events could disrupt other mucosal sites. Altered oral and gut microbial milieu influenced the severity of RA symptoms in animal models [55] and reports on disease modifying anti-rheumatic drug (DMARD) therapy regulating intestinal flora (reviewed here [56]) show circumstantial evidence for the contribution of microbiome to autoimmunity at digestive tract mucosa. At the gingival level, local citrullination and neo-antigen formation could occur through bacterial enzyme activity. *Porphyromonas gingivalis*, a bacterium linked to periodontitis, expresses PAD enzymes (PPAD) that enhance local citrullination in a calcium-independent way, contrary to the PAD activity of human cells. PPADs are also able to citrullinate C-terminal arginine residues generated by gingipain, an arginine specific protease secreted by *Porphyromonas gingivalis* [47] leading to local occurrence of autoimmunity and production of IgA antibodies against *Porphyromonas gingivalis* arginine-specific gingipain B (RgpB). A study comparing RA patients with and without periodontitis and healthy controls showed higher saliva levels of IgA anti-RgpB in RA patients regardless of periodontal disease status compared to healthy controls, suggesting a predisposition for developing autoimmunity in RA [57].

At the intestine level, inflammation could lead the tight junctions between epithelial cells to disassemble creating a barrier breach and facilitating the influx of pathogens and bacterial products (e.g. ATP, LPS, and fatty acids) into the blood stream. As a response to increased ATP, dendritic cells prime T cells to become Th17 and secrete IL-17, while macrophages become activated in response to LPS and fatty acids and secrete pro-inflammatory cytokines and chemokines. In addition, direct activation of neutrophils by microbes leads to increased degranulation and NET generation with externalisation of citrullinated histones [47, 56].

Supporting the theory of local immunological responses, secretory ACPA (SC-ACPA) which are mainly of the IgM class have been described at mucosal sites (lung and oral cavity) and seem to associate with serum SC-ACPA levels in early disease as well as in populations at risk for RA [57-60]. In early RA, serum SC-ACPA have been shown to correlate with the levels found in the bronchoalveolar lavage fluid and to associate with cigarette smoking and lung abnormalities on high resolution computer tomography [59]. Serum levels SC-ACPA were found to be prognostic for arthritis development in individuals at risk for RA [60] and also associate with disease activity in established RA [61], supporting the link between a mucosal immunological response and RA development.

To summarise, external factors could contribute to insults at mucosal level that generate and maintain an inflammatory environment. In predisposed individuals such processes may be able to challenge the immune response to an extent that creates the premises for development of persistent systemic autoimmunity.

2.4. Cellular targets for ACPA

ACPA potentially exert their roles by directly binding to their cellular targets or by forming immune complexes which are able to bind to Fc receptors on the cells' surface. The clinical sign of RA is joint swelling due to synovial tissue inflammation with immune cell infiltration and pannus formation. ACPA may activate macrophages in an immune-complex mediated way and induce production of pro-inflammatory cytokines, thereby maintaining the inflammatory environment. Alternatively, ACPA may

promote further cell differentiation at the articular level such as osteoclastogenesis from monocyte-macrophage precursors, thus contributing to bone loss [10-12, 62].

The RA synovium has a complex cellular composition of stromal and immune cell repertoire, where cytokine production likely model the local microenvironment and contribute to joint destruction [63, 64]. Innate immunity seems to play a bigger role in RA pathogenesis than previously thought, and in particular neutrophils may be interesting in the context of ACPA generation and/or ACPA effector properties as well as mediators of the overall immune response and interaction with stromal structures. As part of the innate immune response, neutrophils have a quick reaction to external pathogens in the form of phagocytosis, granule release, reactive oxygen species generation and NETosis, a process in which the intracellular material is extruded in the extracellular environment in web-like structures. The main role for NETosis is pathogen entrapment. However, dysregulation of NET clearance and/or exaggerated NET formation may lead to increased NET material in the systemic circulation which may be exposed to other immune cells [65, 66]. Consequently, such NETs, containing citrullinated and/or acetylated autoantigens, could be recognized by anti-modified protein antibodies further perpetuating the immune response by either directly triggering autoreactive B cells or forming immune complexes with these autoantibodies. Previous reports on identification of citrullinated proteins together with PAD enzymes and myeloperoxidase in inflamed synovial tissue suggest that neutrophils contribute to local immune activation and pannus formation [67, 68]. NET contents could also be internalized by resident fibroblast-like synoviocytes and further presented on the major histocompatibility complex as neo-epitopes. Concomitantly, neutrophil elastase, which is released together with the NETotic material, can directly degrade the extracellular matrix and generate cartilage fragments which might be an additional substrate for PADs, undergo citrullination and be further recognized by autoreactive lymphocytes [69, 70]. It is unclear however whether ACPA can directly activate neutrophils either alone or via immune-complexes [70, 71].

ACPA positive individuals may experience arthralgia in the absence of clinical inflammation. This leads to the question whether ACPA are able to elicit pain. Mice injected with CCP-eluates but not CCP-free IgG (flow through) from ACPA positive RA patients presented pain-like symptoms as defined by reduced tactile threshold [72]. Furthermore, in this study on a murine model of arthritis, ACPAs bound to CD68+ macrophage-like cells and induced expression of CXCL1/2 (the equivalent of the human IL-8). Intra-articular injection of CXCL1/2 reproduced pain-like symptoms while blocking its receptor increased the tactile threshold [72]. Alternatively, ACPAs could bind to fibroblasts and osteoclasts and promote the release of pro-nociceptors such as soluble phospholipase A which activates acid-sensing ion channels in sensory nerve endings at the joint level [73]. Taken together, these observations suggest an indirect effect on pain from ACPA. Studies on murine arthritis models show that antibodies against cartilage can form immune complexes and bind to the Fcγ receptor of nociceptors inducing pain before the typical signs of joint inflammation [74]. Similar studies using purified ACPA from RA patients are currently lacking.

Pain may be related to other structural changes and may be due to immune infiltration at extra-articular sites in the absence of clinical signs of arthritis. One such site could be the tendon sheath since it is structurally close to the synovial capsule. Moreover, tendons are subjected to mechanical strains which can trigger inflammation. Indeed, tenosynovial inflammation with pannus formation was histologically described in murine arthritis models (human tumor necrosis transgenic mice) before signs of articular damage occurred [75]. In a small prospective cohort on ACPA positive individuals with arthralgia,

tenosynovitis was detected on magnetic resonance imaging and it correlated with arthritis development suggesting that extra-articular involvement could precede structural changes at the joint level [76].

Although ACPA are hypothesized to be major drivers of RA pathology recent studies suggests a more complex picture where certain ACPA may have anti-arthritogenic effects. Engineered antibodies that bind to citrulline at position 3 in histone 2A and histone 4 were shown to inhibit NET formation and increase the uptake of NET products by macrophages in murine models of arthritis, sepsis, peritonitis, lung fibrosis and colitis [77]. In murine models of collagen induced arthritis, monoclonal and polyclonal ACPAs from RA patients seemed to reduce joint inflammation and in some cases even blocked arthritis development altogether [78, 79]. In a study by Gomez et al., ACPA effect was preventive rather than therapeutic and seemed to be dependent on the phase of arthritis [80]. Although seemingly paradoxical, these recent results underline the high variability of ACPA functions and the complexity in disease pathogenesis.

It is important to be aware of the limitations of the experimental models: the caveats of translating *in vitro* data to *in vivo*, as well as the species limitations. Nevertheless, taken together these observations point to the possibility that different ACPA may have different effects depending on their reactivities and which cells they bind to. This may also contribute to the heterogeneity of the clinical presentation and variations of response to therapy [81]. Further studies are needed to confirm these observations and better characterise the ACPA repertoire responsible for different cellular effects.

2.5. Soluble mediators in RA

Cytokine- and chemokine-mediated pathways are pivotal to RA development, contributing to initiation and perpetuation of inflammation, and modulating immune-stromal tissue interactions with consequences in the disease development [82-84]. Therapies blocking IL-6 and TNFα fundamentally revolutionized RA treatment, while blockade against IL-23 or IL-17 showed less effect in RA but are extremely useful in other autoimmune diseases such as psoriasis or atopic dermatitis. TNFα inhibition was reported to lead to reduced CXCL8 and CCL2 expression [85], indirectly suggesting a benefit of chemokine modulation in RA. Cytokines and chemokines usually share receptors creating a redundancy in the system with possible compensatory mechanisms, as suggested by studies where CCL2 and CXCL8 blockade did not demonstrate therapeutic benefits [86]. These observations may point to a different taxonomy of autoimmune diseases based on the soluble mediators, where for example RA could be considered a TNFα and IL-6 –axis disease. To add to the complexity of the system, soluble mediators may have different roles at distinct stages of disease evolution [87] and therefore more important than just blocking is choosing the optimal timing which may be relevant for prevention.

2.6. Rethinking the “pathogenesis ladder” and moving towards prevention

RA is a complex systemic disease with insidious debut and a variable prodromal symptomatology both in duration and characteristics, making early diagnosis challenging. Importantly, the pathologic process predates the clinical arthritis so by the time of diagnosis the immunological cascade and the leukocyte-stromal interactions may already have induced structural damage. Clinical tools for early disease classification exist [1], however it is evident that risk stratification for disease development is needed.

ACPA positive individuals experiencing musculoskeletal symptoms are at high risk for progression to arthritis and their symptom burden oftentimes matches that of already diagnosed patients [88].

Previous attempts at delaying the disease onset showed, in their majority, promising results (summarized in Table 1). Although complete prevention was not achieved, variable times of disease delay were observed as well as milder disease outcomes after diagnosis. Longer prospective studies are needed, however these results open the possibility to "step-down the pathogenesis ladder" (Figure 1) and lower disease risk in predisposed individuals.

Tabel 1. A summary of studies on therapeutic interventions in the risk phase of RA.

Year	Study name	N	Treatment	Serological status	Results on disease outcome	Ref.
2010	-	83	Dexametasone im x 2	37% ACPA (+) 27% RF(+) 36% ACPA and RF (+)	No delay/prevention.	[89]
2019	PRAIRI	81	Rituximab 1000mg iv x1	All ACPA (+) and RF (+) as part of inclusion criteria	Delay of arhtritis by 12 months in patients receiving treatment vs placebo group.	[90]
2021	STAPRA	62	Atorvastatin 40 mg/day	100%ACPA (+) 64% RF (+)	No protective effect of statins was observed.	[91]*
2021	ARIAA	98	Abatacept 125 mg sc qw 6 months	100% ACPA (+)	Arthritis prevention at 12 months (8.2% vs 34.7%, treatment vs placebo) Improvement in subclinical synovitis on MRI according to RAMRIS score (61%vs 31%, treatment vs placebo)	[92]
2022	TREAT-ERALIER	236	Methyprednisolon 120mg im x1 followed by Mehtotrexate 25 mg po qw 12 months	23% ACPA (+) 29% RF (+) 33% ACPA or RF (+)	No delay/prevention, but lower disease burden in the group treated with Methotrexate.	[93]
2022	StopRA	144	Hydroxychloroquine 200-400mg/day 12 months	100%ACPA (+) No data on RF status.	No delay/prevention	[94]**

2024	APIPPRA	213	Abatacept 125 mg sc qw 12 months	13% ACPA(+) 80%RF (+) 86% ACPA and RF (+)	Delay of arthritis by 12 months in patients receiving treatment vs placebo group. Effect sustained by 24 months.	[95, 96]

Abbreviations: N, number of study individuals, ACPA, anti-citrullinated protein antibodies, positive (+), negative (-); RF, rheumatoid factor; im, intramuscular; iv, intravenous; sc, subcutaneous; po, orally administered; qw, every week; MRI, magnetic resonance imaging; RAMRIS, rheumatoid arthritis magnetic resonance Imaging score; Ref, reference. *Stopped due to inclusion problems. **Stopped due to futility.

Perhaps the greatest challenge in conducting such studies is knowing which individuals would benefit from prevention programs. So far prospective studies in ACPA positive cohorts have shown the contribution of several risk factors in further progression (as summarized in Table 2) though with inconsistent results possibly due to different definitions of inclusion criteria.

Therefore, there is a need to develop ways to identify these individuals as early as possible, provide better risk stratifications for predicting RA development and implement clinical guidelines for treatment of their actual pain and musculoskeletal symptoms as well as for reducing their risk to develop arthritis.

Tabel 2. Prevalence of arthritis progression in other at-risk cohorts

Location	**N**	**Inclusion criteria**	**Arthritis progression,** **n (%)**	**Significant risk factors for arthritis progression**	**Ref.**
Amsterdam, Netherlands	374	ACPA(+) and/ or IgM-RF(+) at two timepoints and arthralgia	131 (35%)	High anti-CCP titers RF and ACPA positivity Moderate alcohol consumption Presence of HLA-SE Symptom duration <1 year Morning stiffness>60 min VAS pain >50	[42, 97, 98];
Rotterdam, Netherlands	174	CSA and/or FDR with RA	31 (18%) IA ACPA (+): 7 (23%)	RF and ACPA positivity Morning stiffness>60 min	[99]
Groningen, Netherlands (Lifelines)	308	ACPA(+) general population out of which 75 were defined as CSA	10 (5.6% from general population and 13.3% from CSA individuals)	High CCP titers RF and ACPA positivity	[100]

Leiden, Netherlands	241	CSA in small joints, duration <1 year	45 (19%) RA ACPA (+): 20 (44%)	High CRP levels RF and ACPA positivity Morning stiffness>60 min	[101-103]
Guadalajara, Mexico	819	First and second degree relative of RA patients	17 (2.1%) RA ACPA(+): 14 (82%)	Serologic group (anti- CCP+, RF+ vs others) Anti-CCP positive vs negative RF positive vs negative Relative status (offspring vs other)	[104] ‡
Leeds, UK	455	ACPA(+) and incident MSK symptom	148 (33%)	High anti-CCP titers RF and ACPA positivity Presence of HLA-SE History of smoking	[105-108]
Leeds, UK	5791	Incident MSK symptoms, primary health care	121 (2%) ACPA(+): 68 (56%)	High anti-CCP titers Pain in hands Pain in feet	[109]
Linköping, Sweden (TiRx)	82	ACPA(+) and MSK pain	39 (48%)	High CRP levels RF and ACPA positivity	[60, 110]
Manitoba, Canada	374	FDR RA	18(5%) ACPA(+): 10 (55%)	High anti-CCP titers RF and ACPA positivity	[111]
Colorado, USA (TIP-RA)	86	ACPA (+) subjects without IA	18 (20.9%)	BMI Alcohol intake, dose-dependant	[112]
Colorado, USA (SERA)	131	FDR of RA probands positive for either ACPA or RF and with at least one follow-up visit	20 (15%) ACPA(+): 14 (70%)	High anti-CCP titers	[113]

Abbreviations: N, number of study individuals; n (%), case prevalence; RA, rheumatoid arthritis; ACPA, anti-citrullinated protein antibodies, positive (+), negative (-); CCP, cyclic citrullinated protein; CSA clinically suspect arthralgia; FDR, first degree relative; IA, inflammatory arthritis; MSK, Musculoskeletal; BMI, body mass index; Ref, reference. *In Leiden, Netherlands the study endpoint was defined as rheumatoid arthritis not arthritis as in the other cohorts. ‡ In this paper the authors look at the positive predictive value of antibody status showing comparable risk for arthritis in ACPA+/RF+ and ACPA+/RF- positive FDR RA

3 Research aims

The present work aimed to study the role of autoimmunity in early stages (preclinical features) of RA in order to better understand disease progression and further develop diagnostic tools and improve the standard of care.

More specific aims were:

- In **study 1** we aimed to study factors involved in arthritis progression in a population at risk i.e. ACPA positive individuals having musculoskeletal complaints, without arthritis - Paper I.
- In **study 2** we aimed to validate a digital tool for risk prediction in a clinical setting - Paper II.
- In **study 3** we aimed to characterize the citrullinated targets of ACPA in relation to disease progression by using ACPA from RA patients and individuals identified in study 1, and study their effects on specific cell types (i.e. osteoclasts and neutrophils) - Papers III and IV.

4 Materials and methods

An important aspect of this work was to be able to translate observations from the clinical setting to *in vitro* and *in vivo* models. Information on materials and methods used for the projects is available in more detail in the respective papers. However, some ethical considerations need to be addressed.

Regarding working with individuals at risk for developing RA and healthy volunteers: participants were provided with oral and written information prior to recruitment and documentation of participation and written consent were recorded according to Swedish laws and Karolinska Institutet's rules and instructions (in line with the Helsinki declaration). Participants have the right to withdraw from the study at any time without providing explanation on their decision and without any consequences to their potential future care. We collect biological samples from RA patients, at-risk individuals and healthy controls. These samples include human cells and blood samples for further use in our experimental models. We purify ACPAs from blood, bronchoalveolar lavage (done for research purposes from newly diagnosed RA patients and anti-CCP positive at-risk individuals), synovial fluid (collected upon therapeutic extraction), and bone marrow (obtained at therapeutic arthroplastic procedures). Detailed information on procedural steps and associated risks was given in verbal and written format prior to each intervention and participants had the possibility of reflection and to ask questions before giving their consent. Samples were handled according to the Swedish legislation on biobanking.

Our research involves handling and storage of personal sensitive data. This is obtained in the context of the Swedish national registry for rheumatic diseases (SRQ) with given consent from the participants. Data is encrypted and coded and the code for identification is kept very strictly according to Karolinska Institutet regulations on data storage and data management.

Some aspects of the doctoral projects involve working with animals with the aim to evaluate the effects of ACPA in an animal model and further understanding the pathogenic process in humans. Animal work was planned and experiments were designed in accordance with Swedish regulations on animal research and following the 3Rs (replace, reduce, refine). We are constantly striving to minimize discomfort by ensuring optimal housing conditions. The experimental animal work was planned at all times to be as efficient as possible while using the lowest number of animals needed and by maximizing the amount of information per experiment.

5 Results and discussion

5.1. Prediction of arthritis progression in a population at risk

In **Paper I** we aimed to identify factors involved in arthritis progression in a population at risk for the disease. For this purpose we used data from the Karolinska RiskRA cohort, which was started in 2014 and at the moment of writing of this thesis comprised 435 individuals (ongoing enrollment). Briefly, inclusion criteria in the RiskRA cohort are referral to the rheumatology clinic due to musculoskeletal complaints such as arthralgia and/or joint stiffness, and ACPA positivity (positive anti-CCP2 test), in the absence of clinical or ultrasound detected arthritis. Exclusion criteria are prior diagnosis of rheumatic disease, arthritis at the time of recruitment and unwillingness to participate in the study.

Baseline data from the first 267 individuals was included in the analysis. The mean age of study participants was 48 years old (SD 14) and the majority were female (79%). One hundred and one (38%) individuals developed arthritis within a median of 14 months of follow-up (IQR: 6-27), in line with previous prospective cohort studies (Table 1). The main clinical differences at baseline between progressors and those remaining arthritis free were the prevalence of RF positivity (51/101 in individuals progressing to arthritis compared to 37/166 who did not, $p< 0.0001$) and HLA-SE which was present in 73/95 individuals who developed arthritis compared to 92/159 individuals who did not ($p = 0.003$). Univariate Cox regression analysis showed significant differences in a large number of investigated variables (n = 32) between those developing arthritis and those remaining arthritis free. Next, collinearity was investigated and candidate variables having a correlation coefficient <0.3 [114] (n = 10) were further used for the multivariate analysis (Table 3).

Table 3. Variables used for multivariate analysis

Number of HLA-SE (0-1-2 allele)
Anti-CCP2 levels
Presence of any ACPA reactivity
RF status and anti-CCP2 levels, 3 categories*
Ultrasound detected tenosynovitis
Interleukin-6 levels
Interleukin-17C levels
Delta and Notch-like epidermal grown factor-related receptor levels
Interleukin-15 receptor-alpha levels
C-X-C motif chemokine 6 levels

** RF positive versus RF negative & High anti-CCP2 versus RF negative & Low anti-CCP2.*

Multivariate analysis of the 10 candidate variables (Table 3) using Cox regression with backward and forward selection identified the presence of ≥1 ACPA reactivity, ultrasound detected arthritis and levels of IL-6 and IL-15Rα as significant independent predictors for arthritis development.

Predicting disease onset is crucial to early initiation of therapy. Similar cohorts exist (see Tabel 1) but there is still a lack of consensus regarding criteria for risk stratification. In our study we included individuals who 1) actively seek health care due to musculoskeletal symptomatology, 2) have a positive anti-CCP2 test, and 3) lack clinical and ultrasound evidence of arthritis. We were thus able to have data collection at an early stage of the risk phase thanks to stringent ultrasound exclusion criteria (we excluded those presenting at baseline with synovial hypertrophy on gray scale ≥2 and/or colour Doppler positive signal) while at the same time ensures homogeneity of the data (all participants are positive on an anti-CCP2 test and all patients have musculoskeletal symptomatology).

There are several major findings in this study. First, the importance of having at least one ACPA reactivity was a prognostic factor in arthritis progression (96% in progressors versus 62% in those remaining arthritis free), as compared to the conventional clinically used anti-CCP2 test with its cut-off for high positivity. Second, we found significant differences in inflammation-related proteins between healthy individuals and the RiskRA cohort as well as within the at-risk individuals, especially regarding the levels of IL-6 and IL-15 Rα. Third, we show that ultrasound-detectable tenosynovitis is strongly associated with arthritis development making it clinically useful when triaging ACPA positive individuals for further follow-up. Based on these results, we propose a high-risk for RA phase characterized by the presence of at least one ACPA reactivity, high IL-6 and low IL-15 Rα levels, and ultrasound detectable tenosynovitis.

The presence of ACPA is observed years before disease onset but their role in pathogenesis is not yet clear. Previous studies on pre-RA cohorts (data collected retrospectively in regards to the RA diagnosis) support the possibility of epitope spreading, increasing antibody levels and reactivity closer to diagnosis [115-117]. We could show that the tested ACPA reactivities were more prevalent and had higher levels in those developing arthritis. Similar to previous reports by Stadt et al. [42], we were able to demonstrate that ACPA is a risk factor for arthritis development. The predictive value of any of the ACPA reactivities tested was higher compared to having an anti-CCP2 positive test even at high levels (>3x upper limit of normal) (HR 2.1., 95%CI 1.2-3.54, p 0.006) suggesting that at least mechanistically there is a spectrum of ACPA with relevance to disease progression. This may also have implications from a therapeutic approach and is in accord with a very recent study showing that ACPA profiles rather than the clinically used test (CCP3 in the case of the mentioned study) was able to discriminate treatment response outcomes in a RA cohort [118]. Compared to other studies [97, 101, 110, 113], in our hands the presence of RF, even in high concentrations, did not contribute together with ACPA in an overall better prediction of risk.

Other antibodies against posttranslational modifications such as carbamylation have been previously described in the context of both severity of established RA [119-121] as well as risk prediction for disease onset [100, 101, 110, 113]. Although our study lacks this type of data, based on the literature the available information on the risk contribution of these antibodies seems to be low as compared to ACPA.

An important aspect of our results is the systematic approach of on the inflammatory-related proteins. Significant differences between those progressing to arthritis and those remaining arthritis free were seen in the levels of IL-6 and IL-15Rα. Similar to other studies in pre-RA cohorts, IL-6 levels were

elevated in Risk-RA individuals compared to healthy controls, suggesting an incipient stage of inflammation and immune activation [122-124]. Our results show that decreased levels of IL-15Rα predict arthritis progression. IL-15 is a pro-inflammatory cytokine involved in survival, proliferation and differentiation of adaptive and innate immune cells [125] and is known to be upregulated in autoimmune diseases such as rheumatoid arthritis, psoriasis or Crohn's disease [126]. It elicits its roles mainly through trans-presentation, a process where IL-15 binds to its receptor IL-15Rα on a cell and then the complex is transferred to the neighboring cells, further activating IL-15 signaling components on the receiving cells [126]. The low levels of IL-15Rα in the individuals progressing to arthritis compared to those remaining arthritis free could suggest the consumption of the free form of the receptor and support the notion of immune activation early in the risk phase. Moreover, our findings on IL-6 and IL-15Rα could be also interpreted in the context of finding a "window of opportunity" for immune modulation in the at risk phase.

Among the ultrasound findings, tenosynovitis was significantly associated with progression to arthritis (HR 4.2, 95%CI 2.4-7.3, $p < 0.001$). Put differently, in those having tenosynovitis at baseline, 16 out 19 individuals progressed towards arthritis as compared to 79 out of 234 without tenosynovitis who progressed towards arthritis (84% as compared to 34%, respectively). As to the remaining three without arthritis diagnosis, it is worth mentioning that the absence of diagnosis refers to the time-frame of the study data cut-off. Tenosynovitis was common in hands (flexor digitorum) and wrist compartments, sites of mechanical strain. The presence of tenosynovitis suggests an incipient stage of inflammation taking place outside the joint. This finding is in accord with previous imaging studies on both ultrasound and MRI (the latter less operator dependent) where tenosynovitis and subclinical synovitis closely preceded arthritis diagnosis. [102, 127] In comparison to previous studies and due to stringent inclusion criteria of our study, our findings on tenosynovitis come in the lack of synovial hypertrophy. ACPA may play a causative role in this scenario, as suggested by results from our group where, in a murine model, monoclonal ACPA induced tenosynovitis detected by MRI in the absence of clinical signs of arthritis[128].

In this research, we examined the baseline factors which may further contribute independently to arthritis progression. This may create an oversimplified picture of the complex biological mechanisms behind disease initiation and the results need to be interpreted in the appropriate context. The inflammation-related proteins IL-6 and IL-15Rα do not have yet a place in the clinical praxis as diagnostic or screening tests. However, their presence in the risk phase and more importantly the differences in levels in the progressors compared to those remaining arthritis free suggest an early inflammatory status and immune activation, and should be considered for future studies on disease mechanisms and timely intervention. Conversely, both ACPA specificity and ultrasound detected tenosynovitis have the potential to be measured in the clinical setting. Screening for ACPA reactivities as a complement to the existing CCP-test could improve risk stratification and may be easily available similarly to the investigations used as diagnostic tools for systemic diseases (e.g. ANA/ENA, myositis panels). Moreover, ultrasound assessment of tenosynovitis can effectively stratify the risk for arthritis development in an at-risk individual, with a positive predictive value (PPV) of 89% (95%CI 74-100) and a negative predictive value (NPV) 64% (95%CI 57-70]).

Although beyond the scope of our study, longitudinal data of antibodies and inflammatory biomarkers as well as antibodies against other post-translational modifications or other structures (e.g. anti-PAD) could add to the characterization of the risk phase and should be used for future investigations.

Limitations to our study are the lack of analyses of bone erosions and cell phenotyping. Nevertheless, these can be mitigated by further studies of the cohort.

5.2. The contribution of digital diagnostic tools for efficient triage

Easy access to rheumatologist is essential when discussing possibilities for early intervention. Digital diagnostic tools promise increased efficiency of identification and referral to specialist. In this context, in **Paper II** we looked at *Rheumatic?*, a web-based tool developed by a multidisciplinary team including rheumatology specialists and patients. This digital tool aims to identify individuals at risk for developing rheumatic diseases and differentiate rheumatic conditions from other causes of musculoskeletal symptoms. It comprises a set of questions with different scores according to the relevancy in the clinical assessment for identification of six rheumatic diseases (rheumatoid arthritis, ankylosing spondylitis, systemic lupus erythematosus, myositis, systemic sclerosis, Sjögren's syndrome). The questionnaire is interactive and results are set to advice a visit to a general practitioner (so called threshold-1) or a rheumatologist (threshold-2).

This was a validation study where we tested the ability of *Rheumatic?* to a) predict arthritis development in a population at risk (dataset A- Karolinska Institutet) and b) discriminate between rheumatic diseases from non-rheumatic conditions in populations with early arthritis (dataset B– Erlangen University Medical Care and dataset C -Leiden University Medical Care). All three datasets included information on symptoms from 50, 51 and 72 participants respectively.

Results showed that individuals progressing towards arthritis or progressing to a rheumatic disease had higher *Rheumatic?* scores at recruitment compared to reference group. There was a significant difference between the median scores in those developing arthritis or progressing towards a rheumatic disease and those who did not in all three datasets (in dataset A: 245 vs 163 $p < 0.0001$; dataset B: 191 vs 107, $p < 0.0001$ and dataset C: 262 vs 212 $p < 0.0001$). The discriminatory capacity for progression calculated as AUC-ROC was good for datasets A and B (75.3% [95%CI 61.8-88.8] and 79% [95%CI 66.2-91.8] respectively) and lower in dataset C (53.6% [95%CI 39.2-67.9]).

Sensitivity of distinguishing those that should be recommended to visit a general practitioner (threshold-1) was in dataset A 0.67, B 0.61 and C 0.67. Specificity for the same recommendation was 0.72 and 0.87 in datasets A and B respectively and lower in dataset C (0.23). Sensitivity for the recommendation to refer to a rheumatologist (threshold-2) was low (5%, 7% and 14% for datasets A, B and C respectively) and specificity for the same threshold was high (100%, 96%, 91% for datasets A, B, and C, respectively). It is important to mention that in datasets B and C osteoarthritis was the final diagnosis in 45% and 23 % cases respectively, while gout was diagnosed in 2% and 7% of the cases respectively. This may explain the low sensitivity for threshold-2 since the clinical presentation of such diagnoses can mimic other immune-mediated conditions.

In dataset A the final outcome was the development of immune-mediated arthritis which occurred in 21/50 individuals. Here the low sensitivity for threshold-2 may be explained by the early presentation lacking the typical clinical characteristics of an immune-mediated disease. Moreover, other parameters such as ACPA reactivities or inflammation related markers may be needed for better stratification.

The use of digital tools for diagnostic purposes and ease of access to healthcare is not new. Previous attempts of computer-based diagnostic tools produced diagnosis likelihoods based on data input from patient symptoms and signs. These tools were intended primarily for internal medicine diagnoses and

although could be used in medical education proved to be limiting in the clinical praxis [129]. More than two decades later, a systematic review assessed the performance of 25 expert systems (computer programs designed to support medical decision) with different designs and developed for use in the rheumatology field. The results showed a wide range in the proportion of correctly diagnosed cases (43-99.9%) as well as variability in sensitivity and specificity (62-100% and 88-99% respectively) [130]. There are several explanations for this variability in results: a) the similar presentation in immune-mediated diseases especially in the early stages; b) differences in design of the expert systems and in close connection to this is the c) lack of validation.

EULAR guidelines for standardization of digital diagnostic decision support systems suggest the inclusion of clinical experts as well as patient representatives[131]. *Rheumatic?* was developed in a collaboration between designers, engineers, clinical experts, patients and at-risk individuals. It is a part of a larger project called Joint Pain Assessment Scoring Tool (JPAST), a program supported by European Union Institute of Innovation and Technology, aiming at early detection of autoimmune inflammatory rheumatic diseases by compiling clinical, genetic and biomarker data [132]. *Rheumatic?* was specifically developed as a digital interface for patient symptoms and includes single- and multiple-choice buttons, image area and pain questions, with individualized follow-up questions depending on the previous answers.

The aim of the present study was to test the utility and validate *Rheumatic?* in predicting immune-mediated outcomes covering three stages of disease development: a population having musculoskeletal symptoms in the absence of clinical arthritis (dataset A) and in individuals with early unclassified joint swelling without a clear diagnosis (dataset B) or without the suspicion for an inflammatory autoimmune condition i.e. osteoarthritis or gout (dataset C).

An advantage of *Rheuamtic?* is that it's multilingual (Swedish, English, Dutch, German). Moreover it has been developed by incorporating data from patients and input from rheumatologists from multiple countries, meaning that not only the language barrier has been surpassed but also the cultural aspects of describing symptoms have been to a large extent mitigated. This facilitated the use in an international context and validation in multiple centers.

In the case of the Risk-RA cohort, despite several limitations to the study (low number of individuals per dataset which impacts statistical power, questions answered retrospectively leading to potential bias and some individuals classified as non-progressors who might have developed an immune-mediated arthritis later on) these results show that there is certain potential of using digital tools for identification of individuals at risk. Further validation in larger prospective cohorts and potentially adding other biomarkers (serological, inflammation-related and/or clinical) could further improve *Rheumatic?* ability for risk stratification and disease prediction.

5.3. Identification of citrullinated targets for ACPA

5.3.1. The effects of ACPA in preclinical phases of RA.

Individuals at risk for RA often experience arthralgia with burden comparable to that of patients [88]. In **Paper III** we aimed to test whether ACPA can contribute to symptoms before disease onset. A visual abstract of the paper is shown in Figure 2.

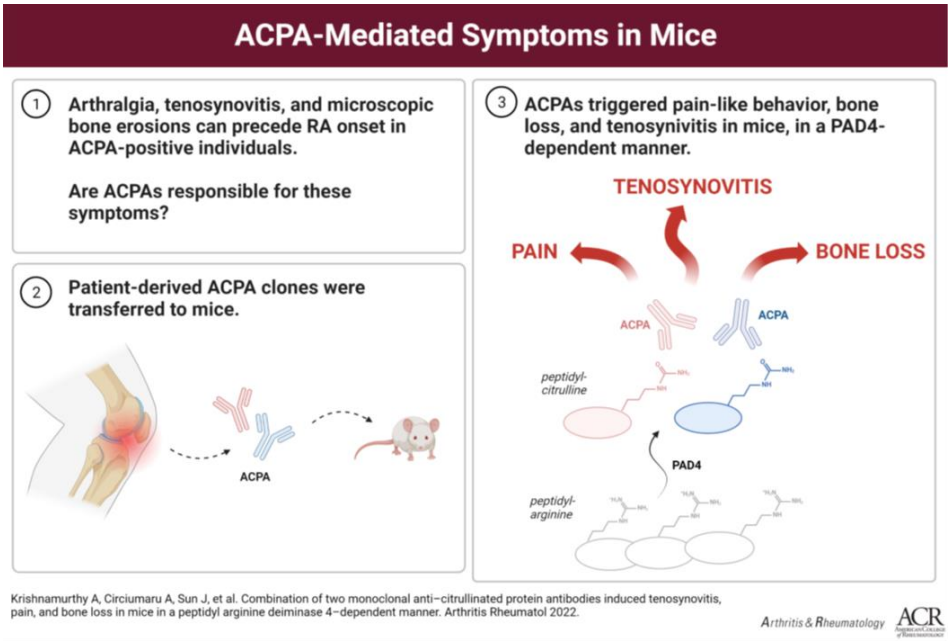

Figure 2. A murine model for the ACPA positive risk phase preceding RA.

Monoclonal ACPA from RA patients (clones C03 and B09, characterized in detail here [133]) were injected in wild-type and PAD4$^{-/-}$ mice. Pain and macroscopic inflammation were assessed over a period of four weeks, after which MRI, microCT and histology studies of the ankle joint were performed in order to assess tenosynovitis and bone erosion. Tenosynovitis was scored based on structural alterations of the tenosynovia at the anterior region of the ankle joint of the mouse hindlimb. We developed an MRI scoring system for post-gadolinium (contrast enhancement) images based on thickness and brightness of tendon sheath regions in the Z-plane: 0, 1, and 2 represented <0.5, 0.5-1 and >1mm of signal enhancement, respectively. Similarly, histological studies were based on the number of tendon sheath layers, where 0 was used for a tendon sheath composed of predominantly one layer of cells, 1 was used for two layers and 2 for three or more layers.

Systemically injected ACPA lowered the tactile threshold in the absence of macroscopic signs of inflammation and induced tenosynovitis as seen in MRI and further confirmed by histology studies, whereas control antibodies and saline did not. In addition, ACPA induced trabecular bone loss as observed by micro-CT. These results were significantly reduced in PAD4$^{-/-}$ animals suggesting that ACPA effects are dependent on citrullination.

The main finding of this study is that ACPA originating from RA patients and transferred to healthy mice induces tenosynovial inflammation in the absence of clinical arthritis, suggesting a role in the preclinical stages of disease. Previous studies showed a higher prevalence of microscopic bone loss and tenosynovitis on CT and MRI scans respectively in ACPA positive individuals, close to arthritis diagnosis and tenosynovitis seemed to be an intermediary step in the disease progression [76, 134].

Furthermore, similar to previous studies using *in vitro* and *in vivo* models [10-12, 72, 73, 135], our findings support the role of ACPA in inducing bone erosion and pain behavior. More importantly, the effect seems to be dependent on citrullination and specifically on the activity of PAD4 enzyme. B09

and C03 recognize other posttranslational modifications (homocitrullinated and acetylated antigens) [133, 136], however the observation that pain-like behavior, bone loss and tenosynovitis were reduced in PAD4$^{-/-}$ animals strongly supports the citrullination-dependent mechanism. A limitation of the study is that we did not test the impact of other PAD enzymes, notably PAD2 which, together with PAD4 but not other PAD isoforms, has been previously reported to be upregulated in the periarticular bone marrow of an arthritis murine model upon challenge with antibodies against citrullinated vimentin [137].

Previous studies characterizing the two tested ACPA clones show that C03 induces osteoclast differentiation, B09 targets synovial fibroblasts and induces pain, and both clones bind to activated neutrophils [72, 73, 133, 136, 138, 139]. Interestingly, observations coming later compared to this study suggest that ACPA may have a blocking effect on collagen-antibody-induced arthritis in mice [140, 141], further adding to the complexity of the picture. Nevertheless, the exact molecular mechanism by which the two tested clones induce pain, tenosynovitis and bone loss was beyond the scope of this study and more studies are needed for a better understanding of the pathogenic pathways.

Taken together these results show that monoclonal antibodies from RA patients can mediate pain-like behavior, tenosynovitis and bone loss in the absence of clinically apparent arthritis in murine models. These observations could be extrapolated to polyclonal ACPAs as suggested by the symptomatology seen in individuals at risk for developing arthritis. It is important to point out here that compared to Paper I where there was an outcome of arthritis, in this study the focus was on ACPA effect in the risk phase. These two studies contribute with knowledge on the prodromal stages of RA.

5.3.2. The role of neutrophils in ACPA mediated changes

Neutrophils isolated from blood and synovial fluid of RA patients may be more prone to undergo extracellular trap formation (NETs) as compared to healthy individuals [71]. NET products may contain citrullinated antigens that could promote ACPA production by autoreactive B cells. Alternatively, an impaired clearance of NET-derived products could expose citrullinated targets that are further recognized by ACPA.

In **Paper IV** we aimed to understand whether neutrophils are sources for citrullinated antigens and if so, under which conditions. We were also interested in the role of PAD2 and PAD4 in the generation of antigens to ACPA.

To this aim we isolated human neutrophils from the peripheral blood of healthy volunteers and murine neutrophils from the bone marrow of PAD4$^{-/-}$, PAD2$^{-/-}$ and wild-type mice. Cells were activated using calcium ionophore (A23187), phorbol 12-myristate 13-acetate (PMA), nigericin, zymosan or IL-8. The choice of activating factors comes from previous reports suggesting that neutrophil activation and composition of NET products varies according to stimuli [142, 143]. Additionally, we chose to test whether ACPA can stimulate neutrophil activation based on claims from previous reports on their potential to induce NETs [71]. We studied the binding of different monoclonal (3 derived from synovial tissue, 4 from blood, 4 from bronchoalveolar lavage fluid and 2 from bone marrow) or polyclonal ACPA preparations from individual RA patients to the activated neutrophils using flow cytometry and confocal microscopy. We analyzed NETosis using IncuCyte. PAD4 enzyme activity was inhibited using the compound BMS-5.

Our results show that ACPA could bind to neutrophil-derived antigens produced via neutrophil activation, but no binding was observed to intact cells. The tested stimuli influenced the level of neutrophil activation and the morphology of the extruded DNA-protein complexes, which in turn impacted on the extent of ACPA binding. Calcium ionophore, PMA and nigericin were strong activators, however in flow-cytometry studies there was a limited recovery of cellular-NET structures in PMA and nigericin activated cells but not in the calcium ionophore treated samples, likely due to morphological characteristics associated with the respective treatments. For this reason further experiments were done using the calcium ionophore.

Starting from the observation that ACPA bound to nuclear antigens, which in physiological conditions are not available extracellularly, we were interested whether intact cells may be targeted. The process of NETosis initiated by ACPA as previously suggested [71] would mean that there may be citrullinated epitopes on the neutrophil surface. We therefore tested a broad range of monoclonal ACPA in calcium ionophore treated cells with or without fixation and permeabilization. The majority of binding was observed in samples that were both fixed and permeabilized suggesting that the citrullinated targets are not easily available to ACPA. As previously mentioned, the tested ACPA clones originate from different compartments (blood, synovia, bone marrow, bronchoalveolar fluid), however their origin did not impact the staining pattern.

The production of citrullinated autoantigens was largely PAD4-dependent in most of the tested clones with the exception of C04 and G09 (phylogenetically related clones [144]) whereas the lack of PAD2 had little effect. Results were consistent both in murine models of PAD knock-out animals as well as *in vitro* blocking of the PAD4 enzymes. Interestingly the two clones display a stronger reactivity towards acetylated and carbamylated targets and the relatively lower dependency on PAD4 activity may reflect a more citrulline-independent binding.

In terms of ACPA reactivity, we observed different binding affinities of the monoclonals and in some cases no binding at all. Interestingly, we observed that some single patient-derived polyclonal ACPA preparations bound to NET compounds while others did not, suggesting a heterogeneity among individuals that could be explained by the different specificity patterns of their ACPA repertoires. Moreover, in a different cell system - the monocyte-derived osteoclast, the same polyclonal ACPA preparations displayed variable effects on osteoclast generation as well. We therefore face several scenarios: ACPA target either neutrophils or osteoclast, or both or none, adding to the already complicated picture of possible ACPA effector functions in the disease continuum.

Taken together these results suggest that neutrophils may be important players in the pathogenesis of RA by producing citrullinated autoantigens through a PAD4 dependent mechanism. Moreover, the variations in binding affinity suggests that the role of ACPA and neutrophils in pathogenicity may be different from patient to patient.

6 Preliminary results

CCL22 effects on bone homeostasis

In an ongoing project we are focusing on the effects of CCL22 in bone homeostasis and whether there may be potential implications in RA. CCL22 is a pro-inflammatory cytokine with a chemotactic effect on several types of immune cells. It shares a receptor with that of CCL17 which has been shown to mediate the effects on pain and inflammation of GM-CSF in arthritis models [145]. Other components of the GM-CSF axis have been studied in the context of inflammation and autoimmunity in RA [145-147].

The aim of this study was to analyze the expression and potential roles of CCL17 and CCL22 in the RA. For this purpose, we compared cytokine levels in sera of individuals at risk for RA, patients with early disease and healthy controls. Additionally, we measured the cytokine levels in synovial fluid of RA patients and compared with other arthropathies.

We observed elevated CCL22 levels in sera from individuals at risk for RA and from RA patients as compared to healthy controls. By contrast, no significant difference was observed in CCL17 levels. In RA patients, CCL22 levels associated with smoking status and bronchial wall thickening detected by high resolution computer tomography. CCL22 and M-CSF levels were higher in synovial fluid of seropositive RA patients as compared to other seronegative arthropathies. Furthermore, CCL22 levels correlated with GM-CSF and M-CSF concentrations in synovial fluid and both these cytokines induce CCL22 production in macrophage or osteoclast cultures. Blocking CCL22 in osteoclast progenitor cultures lead to decreased osteoclastogenesis suggesting a potential contribution of this chemokine in bone resorbtion.

Further studies are needed to understand the role of CCL22 in RA and whether it may be a candidate for therapeutic intervention.

7 Conclusions and points of perspective

The results from this work add to the knowledge on the role of ACPA in the very early stages of RA.

In **Paper I** we explore predicting factors for arthritis progression in ACPA positive individuals with musculoskeletal complaints in the lack of arthritis. We show that the presence of at least one ACPA reactivity, certain inflammation related proteins levels, and ultrasound tenosynovitis are independently predictive factors for arthritis in a population at risk. ACPA reactivities showed superior informative value compared to anti-CCP2 testing and these findings could be further implemented in creating a screening panel for clinical use, similar to those already existing for myositis or scleroderma. Ultrasound screening of ACPA positive individuals with musculoskeletal complains can be easily implemented in the clinical praxis enabling support for follow-up decisions. The findings on IL-6 and IL-15Rα levels suggest that there is ongoing inflammation and immune modulation in the preclinical stages of RA.

In **Paper II** we explore the utility of digital diagnosis support tools in arthritis prediction. The e-questionnaire *Rheumatic?* did not perform well in the "at-risk" stage compared to the other cohorts reflecting the particular differences in the symptomatology of the risk phase compared to the established disease. Moreover, these results suggest that symptom screening in the preclinical stage of the disease needs to be complemented with other biomarkers such as those described in **Paper I**.

In **Paper III** we showed that patient derived ACPA injected in healthy animals lead to development of tenosynovitis, pain-like behavior and bone loss in the absence of arthritis. Although the studies have different aims, the findings from this paper complement the observations from our Risk-RA cohort regarding pain and tenosynovitis in ACPA individuals in the absence of arthritis. More importantly, these results show that ACPA elicit effects in very early, preclinical stages of disease.

In **Paper IV** some but not all tested ACPAs could bind to citrullinated targets from activated neutrophils, suggesting that neutrophils may be sources for citrullinated antigens and an interesting cellular target for further study. ACPA preparations from individual RA patients had a highly variable binding capacity both to neutrophil-derived antigens and to osteoclasts, suggesting high patient-to-patient variety of the ACPA effects. These results are in line with the clinical observations on the heterogeneity in RA phenotypes.

These results from this thesis have to a good extent direct applicability for improving the standard of care for individuals at risk for RA by setting-up a program which should comprise i) a standardized practice of ultrasound screening for arthritis in rheumatology out-patient clinics and ii) a clinically usable screening panel for ACPA specificities, similar to the diagnostic tools used for systemic sclerosis or idiopathic inflammatory myopathies. Such program could be used for risk stratification and help in follow-up decisions.

Further studies on the early stages of disease are needed in order to validate prediction models and test early intervention options. As presented in this work, there is an acute need for standardization of the definition for being at risk for RA. To this end it is essential to establish national and international collaborations and harmonize definitions and inclusion criteria for studying at-risk for RA individuals.

Future translational studies should focus on dissecting the molecular mechanisms of ACPA effects on various cell types as well as the the role of cytokines such as (but not restricted to) IL-6 and IL-

15Rα in the early stages of RA. Further work on CCL22 should focus on the identification of possible sources and targets in relation to arthritis progression.

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I consider myself privileged for having had the opportunity of doing my PhD abroad and at Karolinska Institutet. This is not necessarily for the knowledge I acquired or for the prestige of the institution itself but rather for how this journey has transformed me as an individual.

My Swedish experience was supposed to be a "short" scientific exchange program in Anca Catrina's lab. Instead I got "infected" by the enthusiasm in Anca's team and the prospect of working in translational research definitely "sealed the deal". I am therefore thankful for the trust my late main supervisor **Anca Catrina** has put in me when she offered me a PhD position in her lab. I have always considered that clinical work and research go hand in hand and I found Anca's dedication to be so refreshing and inspirational at a time when I was so close to "choose just one". I am grateful that I was her student, even if for a short time.

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