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BY
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AALBORG UNIVERSITY
DENMARK

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by

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Giordano R, Saii Z, Fredsgaard M, Hulkko LSS, **Poulsen TBG**, Thomsen ME, Henneberg N, Zucolotto SM, Arendt-Nielsen L, Papenbrock J, Thomsen MH, Stensballe A. Pharmacological Insights into Halophyte Bioactive Extract Action on Anti-Inflammatory, Pain Relief and Antibiotics-Type Mechanisms. *Molecules. 2021 May; 24;26(11)*.

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Poulsen TBG, Karamehmedovic A, Aboo C, Jørgensen MM, Yu X, Fang X, Blackburn JM, Nielsen CH, Kragstrup TW, Stensballe A. Protein array-based companion diagnostics in precision medicine. *Expert Rev Mol Diagn. 2020 Dec; 20(12)*.

Birkelund S, Bennike TB, Kastaniegaard K, Lausen M, **Poulsen TBG**, Kragstrup TW, Deleuran BW, Christiansen G, Stensballe A. Proteomic analysis of synovial fluid from rheumatic arthritis and spondyloarthritis patients. *Clin Proteomics. 2020 Aug 6; 17:29*.

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CONFERENCE ACTIVITY

7th International Caparica Conference on Analytical Proteomics, 2021, *oral*.

1st International Electronic Conference on Biomedicine, 2021, virtual. *Poster*.

European League Against Rheumatism (EULAR) 2020, virtual. *Two posters*.

The Danish Proteomics Society 2019, University of Southern Denmark, Denmark. *Poster*.

The Danish Proteomics Society 2017, University of Southern Denmark, Denmark. *Poster*.

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The Danish Proteomics Society 2016, University of Southern Denmark, Denmark.

ENGLISH SUMMARY

Dysregulation of the human immune system may result in the systemic appearance of autoantibodies, adverse immune reactions, and the development of autoimmune diseases. The etiology of many autoimmune diseases remains largely unknown but multiple contributing factors that increase the risk of developing an autoimmune disease have been identified, such as genetics, obesity, infections, and smoking. Rheumatoid arthritis (RA) is one of the most prevalent autoimmune diseases worldwide. It is characterized by symmetrical inflammation of the joints, especially the small joints in the hands and feet, leading to pain, swelling, and bone erosions. A high number of RA patients produce anti-citrullinated protein antibodies (ACPAs), which are autoantibodies directed against neoepitopes in proteins that have undergone citrullination. ACPAs may be present years before clinical symptoms develop in RA patients, demonstrating their possible involvement in the early pathogenesis and their usefulness as an early biomarker for RA. Not all patients respond to or benefit from the same medical treatment even though the disease presentation may seem identical. As of now, no single serological or clinical test exists to determine if the patient will respond well to the treatment or not or if there is an increased risk of relapse. Therefore, we aimed to investigate the use of autoantibody profiling to further differentiate RA patients in order to improve prognostic and diagnostic outcomes.

The possibility of subdifferentiating RA patients based on their autoantibody fingerprint was investigated through two studies. In the first study, we investigated autoantibodies from healthy and RA patient subgroups against proteins in their native configuration using a protein microarray consisting of more than 1600 protein targets. In our search for potentially important RA autoantigens, we identified several autoantigens shown to be present in synovial fluid. In the second study, we modified the protein microarray platform used in Study I using PAD enzymes and investigated autoantibodies against citrullinated proteins. We showed that on-array protein citrullination is possible and enables the detection and quantification of ACPAs in RA patients and we identified new potential autoantigens not previously associated with RA.

In conclusion, these two exploratory studies show that we can measure and quantify the global autoantibody landscape in healthy and RA patients, both against native and modified proteins, and demonstrate differences in the autoantibody profiles of the two current subgroups of RA, ACPA-positive and ACPA-negative RA, using both native and citrullinated autoantigens. Further studies using individual RA patient samples incorporating leads from our studies combined with currently known autoantigen targets in RA are needed to shed light on individual autoantibody patterns and their links to treatment outcomes.

DANSK RESUME

Dysreguleringen af det menneskelige immunsystem kan resultere i systemisk tilstedeværelse af autoantistoffer, negative immunreaktioner og udviklingen af autoimmune sygdomme. Ætiologien for mange autoimmune sygdomme er stadig ukendt, men mange medvirkende faktorer, der øger risikoen for at udvikle en autoimmun sygdom, er blevet identificeret som f.eks. genetik, fedme, infektioner og rygning. Reumatoid arthritis (RA) er en af de mest prævalente autoimmune sygdomme i verden. RA er karakteriseret ved symmetrisk inflammation af leddene, specielt de små led i hænder og fødder, hvilket medfører smerte, hævelse og knogleerosioner. Mange RA-patienter producerer anti-citrullineret proteinantistoffer (ACPAs), som er autoantistoffer mod citrullinerede proteiner. ACPAs kan være til stede flere år før, der udvikles kliniske symptomer på RA, hvilket gør, at de muligvis er involveret i den tidlige patogenese samt er brugbare biomarkører for RA. Ikke alle patienter responderer lige godt på den behandling de får, på trods af sygdomsbilledet er ens. Der er lige nu ingen serologisk eller klinisk test, der kan vise, om en patient vil respondere godt på behandling eller ej. Derfor vil vi undersøge brugen af autoantistof-profilering for at differentiere RA-patienter yderligere for derved at forbedre prognostiske og diagnostiske resultater.

Muligheden for at subdifferentiere RA-patienter baseret på deres autoantistof-profil blev undersøgt gennem to studier. I det første studie undersøgte vi autoantistoffer fra raske og RA-patienter mod proteiner i deres native konfiguration ved brugen af protein mikroarrays, som består af mere end 1600 forskellige proteiner. Her kunne vi identificere adskillige autoantigener, som også er til stede i ledvæske. I det andet studie modificerede vi proteinerne på mikroarrayet ved brug af PAD-enzymer og derved undersøgte autoantistoffer mod citrullinerede proteiner. Dette studie viste, at vi kunne citrullinere direkte på mikroarrayet og derved muliggøre detektionen og kvantificeringen af ACPAs i RA patienter. Samtidig identificerede vi nye potentielle autoantigener, som ikke tidligere har været associeret med RA.

Disse to eksplorative studier viser, at vi kan måle autoantistof-landskabet i RA patienter både mod native og modificerede proteiner og samtidig demonstrere forskelle i patienters autoantistof-profil hos ACPA-positive og ACPA-negative RA patienter ved brug af både native og citrullinerede autoantigener. Fremtidige studier, der benytter sig af individuelle RA patientprøver, der inkorporerer resultaterne fra disse studier og kombinerer dem med nuværende kendte autoantigener i RA, er nødvendige for at belyse de individuelle autoantistofmønstre og deres sammenhæng med behandlingsudfaldet.

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LIST OF MANUSCRIPTS

This PhD thesis is based on the following two peer reviewed journal articles and one expert review:

Manuscript I: **Poulsen TBG**, Damgaard D, Jørgensen MM, Senolt L, Blackburn JM, Nielsen CH, Stensballe A. Identification of novel native autoantigens in rheumatoid arthritis. *Biomedicines 2020 May 27; 8(6).*

Manuscript II: **Poulsen TBG**, Damgaard D, Jørgensen MM, Senolt L, Blackburn JM, Nielsen CH, Stensballe A. Identification of potential autoantigens in anti-CCP positive and anti-CCP negative rheumatoid arthritis using citrulline-specific protein arrays. *Sci Rep. 2021 August 27; 11(1).*

Review I: **Poulsen TBG**, Karamehmedovic A, Aboo C, Jørgensen MM, Yu X, Fang X, Blackburn JM, Nielsen CH, Kragstrup TW, Stensballe A. Protein array-based companion diagnostics in precision medicine. *Expert Rev Mol Diagn. 2020 November 26; 20(12).*

LIST OF ABBREVIATIONS

ACPA	Anti-citrullinated protein antibody
ACR	American college of rheumatology
Anti-carP	Anti-carbamylated protein
Anti-CCP	Anti-cyclic citrullinated peptides
BCR	B-cell receptor
bDMARD	Biological DMARD
CDAI	Clinical disease activity index
CDx	Companion diagnostics
csDMARDs	Conventional synthetic DMARD
DAS28	Disease activity score 28-joint
DMARD	Disease-modifying antirheumatic drugs
EULAR	European league against rheumatism
FLS	Fibroblast-like synoviocyte
JAK	Janus kinase
MLS	Macrophage-like synoviocyte
MMP	Matrix metalloproteinase
MTX	Methotrexate
PAD	Protein arginine deiminase
PTM	Post-translational modification
RA	Rheumatoid arthritis
RF	Rheumatoid factor
RTX	Rituximab
SDAI	Simplified disease activity index
SE	Shared epitope
T2T	Treat-to-target
TCR	T-cell receptor
tsDMARD	Targeted synthetic DMARD

CHAPTER 1.INTRODUCTION

The human body is protected against external pathogens by the complex biological network known as the immune system. The first layer of defense consists of different surface barriers such as the skin and mucous membranes (e.g. lung and gut) that prevent pathogens from entering the body. If the pathogen successfully breaches these barriers, it encounters the innate immune system and the adaptive immune system. The innate immune system is fast-acting but non-specific, while the adaptive immune system is slower but specific and acquires immunological memory, allowing for a faster and stronger immune response the next time it encounters the same pathogen. The immune system may mistakenly identify the body's own proteins (self-proteins) as foreign proteins (non-self-proteins), thus directing its immune response against otherwise healthy cells and tissue. This may initiate the production of antibodies against self-proteins (autoantibodies), consequently contributing to the development and detrimental effects of autoimmune diseases such as rheumatoid arthritis (RA). These autoantibodies are interesting as serological biomarkers due to their presence several years before disease presentation (1,2).

This PhD thesis is centered around an initial idea of investigating the application of complex protein arrays for global and personalized profiling of autoantibodies in selected pathologies. Initially, I researched the options for global profiling of the repertoire of autoantibodies against native autoantigens in RA patients and healthy subjects. Next, the study was extended by developing a method to introduce post-translational modifications (PTMs) on the protein arrays by creating citrullinated antigens for the detection of anti-citrullinated protein antibodies (ACPAs). Finally, a review focusing on the technology platforms available and the applications for protein microarrays, moving toward the use of companion diagnostics (CDx) in other diseases than the well-established oncology area, was conducted. The following chapter will introduce the autoimmune disease RA and the immunopathological triggers of autoimmunity, describe how treatment of RA is approached, highlight important autoantibody classes in RA, and describe the initial steps needed to investigate the potential of CDx implementation within rheumatology.

1.1 RHEUMATOID ARTHRITIS

Rheumatoid arthritis is a systemic inflammatory autoimmune disease characterized by chronic inflammation of the joints and synovial tissue, leading to swelling, pain, erosion of bone, and disability (3). Eventually, disease progression may reduce quality of life, lead to substantial medication costs and potentially lead to increased mortality (4). RA is estimated to affect 0.5-1% of the general population worldwide, with approximately 40 new cases each year (per 100,000 population) in the US and Northern European countries, and it predominantly affects women (twice as often as men) (5–8). RA primarily affects the synovial joints but extra-articular manifestations

such as vasculitis, accelerated atherosclerosis, and nodules, are also evident (9–11). Extra-articular manifestations, however, rarely accompany initial disease presentation. Early presentation of RA symptoms may include symmetric morning stiffness, swelling, and pain in the small joints of the hands and feet. Without proper treatment, RA can lead to irreversible structural joint damage, emphasizing the need for early diagnosis and treatment (12–15).

Several risk factors have been identified for RA, such as smoking, vitamin D deficiency, obesity, silica exposure, the female gender, and several genetic variations (16–20). The possible link between smoking and RA development may lie in a potential increase in the expression of the peptidylarginine deiminases (PADs) enzymes due to smoking, thus facilitating protein citrullination and the generation of neoepitopes triggering the immune system (21). Another explanation involves epigenetic modulation, such as the DNA methylation seen in smokers developing RA (22). Both mechanisms, however, seem to be somewhat reversible upon quitting smoking (22,23). The etiology of RA, however, remains unknown. It is believed that a molecular trigger breaches the self-tolerance several years prior to the patient developing an RA phenotype, resulting in e.g. the production of autoantibodies escalating to higher levels and more targets. This is usually referred to as “the first hit” (24). It is speculated that environmental pollutants such as smoking or bacterial infections may initiate this initial breach (25–28). However, additional factors are needed as some healthy people produce ACPAs (2,29). This leads to the idea of a “second hit” (or multiple hits) that drives disease from healthy (or asymptomatic) ACPA-positive individuals to the onset of arthritis or early RA (30). Here, it is suggested that the major genetic risk factor, HLA-shared epitope (HLA-SE), plays a role in transforming the ACPA response toward established disease, while another genetic variation (HLA-DRB1*13) seems to exert a protective role against RA (31–33). Furthermore, it is speculated that the second hit initiates several immunological mechanisms such as somatic hypermutation, epitope spreading, and class-switching, all contributing to evolving the ACPA immune response and driving the disease toward established RA (34–40). It should be noted that this is simply a hypothesis of how RA develops and even though the products of these mechanisms have been observed in the RA population, such as the rise in ACPA levels and the expansion of immunoglobulin isotypes, it is still not understood what exactly triggers these events and leads to RA (2,35,41,42).

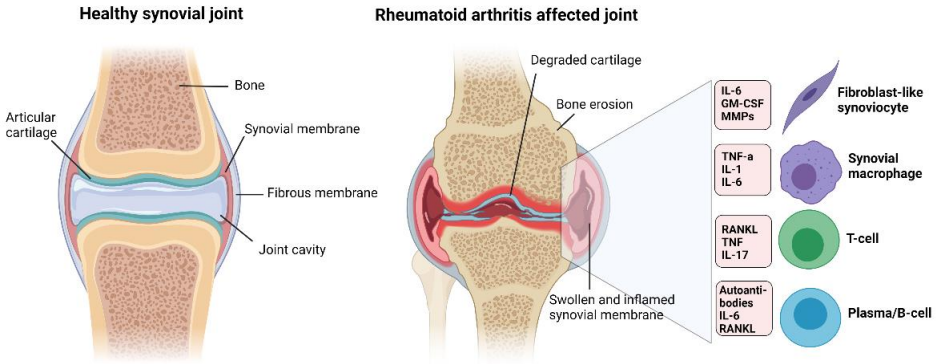

Figure 1. Healthy and rheumatoid arthritis-affected joint. Schematic representation of a joint showing a healthy state and an RA-affected state, including degraded cartilage, bone erosion, and inflamed synovial membrane. The figure also shows examples of cell types involved in RA and their contribution to disease e.g. expression of cytokines and autoantibodies (43–52). Created with Biorender.com.

The healthy synovial joint consists of the ends of two bones (termed epiphysis in long bones) covered by articular cartilage supported by the joint capsule (Figure 1). The joint capsule consists of two layers: the outer fibrous membrane and below it the inner synovial membrane (synovium). The fibrous layer connects the articulating bones and supports the synovium, which is responsible for secretion of synovial fluid to the joint cavity. In preclinical RA, there is no clear sign of infiltration or inflammation of the joints; however, autoantibodies, i.e. ACPAs or rheumatoid factor (RF), are produced at detectable levels. The presence of ACPAs has been shown to precede clinical symptoms of RA by up to a decade and coupled with the detection of pro-inflammatory cytokines before clinical onset of RA it points toward an immune activation happening during the pre-clinical phase of RA (2,53). Today, ACPAs are used as both a diagnostic marker for seropositive RA and as a prognostic marker for disease severity (54–56). Transitioning from preclinical RA to early RA, mononuclear cells infiltrate the joint. marking the start of the development of articular inflammation. Additionally, an expansion of the autoantibody repertoire unfolds, leading to higher levels of already present autoantibodies such as ACPAs and RF but also several new targets such as type 2 collagen, proteoglycans, and nuclear antigens (48,57). In established RA, there is an activation of the synoviocytes lining the inner surface of the joint. Macrophage-like and fibroblast-like synoviocytes (MLS and FLS) produce pro-inflammatory cytokines (IL-1, IL-6, TNF-α) but especially the production of matrix metalloproteinases (MMPs) by FLS plays a dominant role in RA as the MMPs contribute to cartilage degradation (58). High infiltration of immune cells (e.g. CD4+ memory T-cells, B-cells, and plasma cells) to the synovial lining is also seen in this stage of RA (59,60). The constant activation of T- and B-cells acting on self-antigens, recruitment of immune cells to the joint, and activation of the

inflammatory response resulting in expression of destructive cytokines and MMPs are all contributors to the destruction of the joint seen in RA.

RA patients can be clinically classified into two groups according to the serological presence or absence of ACPAs. These autoantibodies can be detected in patients several years before the clinical onset of symptoms and are highly specific for RA (37,61). The anti-cyclic citrullinated peptides (anti-CCP) test is used to identify autoantibodies against citrullinated peptides and classify patients as having anti-CCP positive RA or anti-CCP negative RA. These two patient groups can be considered as two different disease entities with differences in, among other things, risk factors, disease severity, and prognosis (62,63). Testing positive for anti-CCP usually indicates a more severe form of RA disease and it is suggested to treat it more aggressively compared to anti-CCP negative diagnosed RA (64–66). However, RA cannot be diagnosed based on the anti-CCP test alone: a combination of several other clinical features and tests is needed. The American College of Rheumatology (ACR) and the European League Against Rheumatism (EULAR) created a set of classification criteria based on a scoring system to evaluate the possibility of a patient suffering from RA (56). These classification criteria consider the number of swollen or tender joints, serology status (both ACPA and RF), acute-phase reactants (CRP and erythrocyte sedimentation rate), and the duration of symptoms.

1.1.1 PROTEIN CITRULLINATION AS A TRIGGER IN RHEUMATOID ARTHRITIS

The non-standard amino acid citrulline is a product of a post-translational modification known as citrullination or deimination created by converting the amino acid arginine into citrulline. Citrulline was first isolated from watermelon in 1914 (without naming the amino acid) but it was not until 1930 that its chemical formula and structure were determined, in addition to it being named citrulline, based on the Latin name for watermelon, *Citrullus vulgaris* (67–69). In the following decades, citrulline was demonstrated to be enzymatically generated by the enzyme family known as PADs by side-chain conversion of peptidylarginine to peptidylcitrulline in a calcium-dependent process (70–72). The process converts the primary ketimine group to a ketone group. This results in a charge net loss (from positively charged arginine to neutrally charged citrulline), altering protein conformation, function, and interactions (73,74).

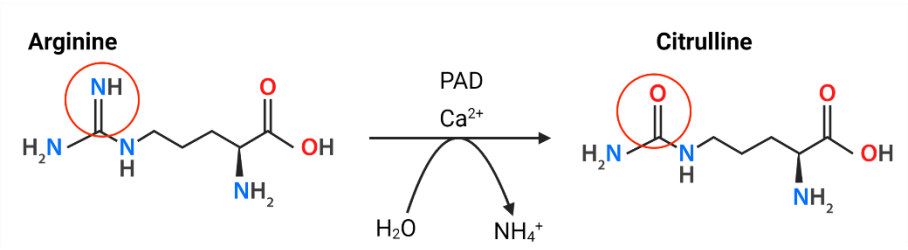

Figure 2. Conversion of arginine to citrulline. Arginine is converted to citrulline in the reaction known as citrullination. Peptidylarginine deiminases are responsible for this by replacing the ketimine group with a ketone group, highlighted by the red circle. The process is calcium dependent, uses a water molecule, and yields ammonia. Created with Biorender.com.

The interest in PAD enzymes in RA research began back in 1998 when citrullinated peptides were recognized by antibodies from RA patient sera (75). Today, these autoantibodies are known as ACPAs and serve as a hallmark in RA diagnosis. There exist five different PAD enzymes in humans (PAD1-4, -6), widely distributed throughout the body (76). Especially PAD2 and PAD4 are deemed to be interesting in RA due to their presence in macrophages and neutrophils in the RA-affected synovium (77,78). Today, it is still not fully understood how each PAD enzyme impacts RA and what differences they each contribute with; however, it seems both enzymes possess distinct citrullination specificities with some degree of overlap (79–81). Both human PAD2 and PAD4 enzymes have also been identified as autoantigens in RA patients. Anti-PAD4 antibodies were identified in 2005 by Takizawa and colleagues and were later shown to be present in up to 45% of RA patients (82). Thirteen years later in 2018 antibodies against human PAD2 were reported (83). Anti-PAD4 antibodies seem to correlate with ACPA positivity but have also been detected in some ACPA-negative RA patients (84–86). Furthermore, anti-PAD4 antibodies are associated with higher baseline joint damage in RA patients in several studies (87–90). However, it is not clear if the presence of these antibodies is linked to the progression of radiographic joint damage over time (87). Studies of antibodies against human PAD2 are scarce but one study showed an association between anti-PAD2 antibodies and less severe joint and lung disease in RA patients (83).

1.1.2 TREATMENT APPROACHES IN RHEUMATOID ARTHRITIS

The approach used to treat RA patients has changed dramatically in the last few decades and many therapeutic options are currently available. The improvement in treatment options has made it possible to aim for clinical remission in most patients if they are diagnosed and treated early (91). The improvement in RA outcomes may be credited to both the introduction of the highly effective biologic agents introduced in the late 90s and the implementation of a treat-to-target (T2T) approach in RA care in 2010 (92,93). The principles of the T2T concept in RA set remission or low disease score as the end goal in treatment and include several steps for reaching this (93). After the decision on what the target of the treatment should be (e.g. remission), it is important to choose a way to achieve this. Examples of disease activity measures to use include the 28-joint disease activity score (DAS28), clinical disease activity index (CDAI), or the simplified disease activity index (SDAI). Next, it must be decided when and how often the disease needs to be accessed to identify any improvement or lack thereof in disease activity. This could be anywhere from a few weeks to several months depending on the severity of the disease. If the desired target has not been met

after the appropriate time, it is necessary to adjust treatment. Several options are available to the clinician when changing the treatment plan, including increasing the dosage of the drug, changing to another drug, or the addition of a second drug in combination with current treatment. Although the T2T approach was not formally implemented within the field of rheumatology until 2010, the first trial to investigate the main idea behind T2T was published around 10 years earlier in 1999 (93,94). A few years later in 2004 another trial compared intensive management of RA patients (T2T approach) with routine care and demonstrated that the T2T approach resulted in the best outcome (e.g. related to disease activity and progression) for the patients at no additional cost (95). A list of widely used drugs for treatment of RA can be found in Table 1.

As mentioned above, the treatment options available for RA patients are numerous. There exist several different classes of drugs with different modes of action. Disease-modifying antirheumatic drugs (DMARDs) are a class of drugs used for treating RA. Conventional synthetic DMARDs (csDMARDs) and targeted synthetic DMARDs (tsDMARDs) are two categories of chemically synthesized drugs while biological DMARDs (bDMARDs) are produced by genetic engineering in living organisms such as bacteria and yeast. Guidelines developed and recommended by EULAR describe the drug selection decisions at the start of clinical diagnosis of RA and which type of drug to use if improvements are not achieved after 3-6 months (66). Here, DMARDs should be the first drug prescribed and they suggest the use of the csDMARD methotrexate (MTX) unless any contraindications are present, in which case they suggest the use of different csDMARDs: leflunomide or sulfasalazine. If there is an improvement 3 months after DMARD start and the target is achieved after 6 months, the treatment should continue until sustained remission is achieved. In the sustained remission stage (a minimum of 6 months remission according to the index-based remission or Boolean remission) dose reduction is suggested (96). Failure to achieve improvements or the treatment target for the patient results in changing from the first csDMARD used, e.g. MTX, to either a new csDMARD or a combination therapy of two csDMARDs. If any poor prognostic factors (high RF/ACPA, high disease activity, early joint damage, or failure of two or more csDMARDs) are present EULAR recommend adding a bDMARD or a tsDMARD (e.g. a Janus kinase (JAK) inhibitor). An evaluation of improvement in disease state and achieving the treatment goal is again carried out after 3 and 6 months, respectively. Finally, if the patient does not seem to benefit from the treatment it is advised to change the bDMARD or tsDMARD until the patient benefits from the treatment.

Table 1. Examples of drugs used in RA treatment and their target. Most of these drugs are highlighted due to being recommended as first choices in their respective categories by the Danish Council for the Use of Expensive Hospital Medicines

(RADS) or EULAR recommendations. The year of approval is per the European Medicines Agency.

Drug	Target/mechanism	Year approved for RA treatment in EU
csDMARDs		
Methotrexate	Inhibits purine metabolism	1980s or earlier
Sulfasalazine	IL-1 and TNF-alpha suppressor	1980s or earlier
Leflunomide	Inhibits pyrimidine synthesis	1999
tsDMARDs		
Baricitinib	Inhibits JAK1 and JAK2	2017
Filgotinib	JAK inhibitor	2020
Tofacitinib	JAK inhibitor	2017
bDMARDs		
Abatacept	Inhibits T-cell costimulatory signal	2007
Adalimumab	TNF inhibitor	2003
Etanercept	Decoy TNF receptor	2000
Rituximab	Targets CD20 on B-cells triggering cell death	2006
Tocilizumab	IL-6 receptor blocking	2009

1.1.3 DRUG RESPONSES IN RHEUMATOID ARTHRITIS

Despite the broad selection of drugs targeting different pathways in RA, no clear pattern seems to exist that shows which patient will benefit from which drug prior to administering the drug. Being able to predict treatment outcomes in patients will make it possible to treat patients both early on and effectively, which are both critical goals in managing RA and reaching remission without patients suffering from irreversible damage of the joints. The misuse of currently available drugs not only affects the wellbeing of the patient by prolonging the ineffective treatment period, thereby increasing the risk of irreversible damage; it also burdens the healthcare system economically due to the high cost of biologics (97,98). The following paragraphs will give short descriptions of how selected drugs with different mechanisms of action in RA treatment work and how well patients respond to them.

1.1.3.1 Methotrexate

MTX is categorized as a csDMARD and acts via multiple mechanisms that all contribute to the total therapeutic efficacy seen in treatment of RA. Inhibition of aminoimidazole-4-carboxamide ribonucleotide transformylase (ATIC) leads to increased levels of adenosine, which suppresses inflammation (99). Inhibition of the enzyme dihydrofolate reductase leads to increased reactive oxygen species (ROS) levels, ultimately resulting in increased sensitivity to apoptosis, as seen in e.g. T cells (100,101). Furthermore, MTX also inhibits TNF-stimulated nuclear factor-κB (NF-κB) transcriptional activity, further contributing to MTXs anti-inflammatory effects (102). A recent study including more than 1000 RA patients from the UK demonstrated that more than 40% were non-responders to MTX (103). This is further supported by another study which showed that more than 50% of participants were non-responders in an RA cohort of more than 100 patients (104).

1.1.3.2 Baricitinib

Baricitinib is a new tsDMARD approved for use in RA treatment in 2017. It is a JAK inhibitor acting on JAK1 and JAK2 essential for cytokine signaling via the STAT pathway, resulting in inhibition of several proinflammatory cytokines such as IL-6, IL-12, and IFN-γ (105,106). Multiple studies including several hundred RA patients investigating baricitinib's effect demonstrate that approximately 60-70% of patients achieved at least ACR20 improvement after 12 weeks of treatment (107–109). These studies were conducted on RA patients who had an inadequate treatment response to either csDMARDs or bDMARDs.

1.1.3.3 Abatacept

Abatacept is a modified antibody against CD80 and CD86 on antigen-presenting cells that blocks the co-stimulatory signal to immune cells. This prevents e.g. T-cells from being fully activated, thus stopping the subsequent proinflammatory cascade. The ACTION study published in 2014 studied among other things the effectiveness of abatacept in RA patients from both Europe and Canada (110). Including

approximately 1000 patients in their study, the researchers demonstrated that just under 70% of patients administered abatacept demonstrated a good or moderate response according to EULAR response criteria after 6 months. A more recent study including 2700 patients demonstrated that 60% achieved good or moderate EULAR response after 12 months of abatacept treatment, while a third study demonstrated that approximately half of the included patients achieved remission (according to CDAI) after 6 months (111,112).

1.1.3.4 Rituximab

Rituximab (RTX) is another antibody drug targeting a surface protein on immune cells, namely CD20 expressed on B-cells. The function of CD20 remains unclear but the binding of rituximab to CD20 results in depletion of B-cells (113,114). The REFLEX study published in 2006 investigated response to RTX in approximately 500 RA patients who had shown an inadequate response to at least one TNF inhibitor (115). The study demonstrated that 35% of the RA patients receiving RTX did not obtain a moderate or good response according to EULAR response criteria (116). This is further supported by other studies showing approximately 40% non-responders to RTX in patient cohorts of 20 and 500 (117,118).

1.2 ANTIBODIES AND THEIR PRESENCE IN DISEASE

One of the first mentions of what later became known as antibodies dates back to the late 19th century. In 1890 Behring and Kitasato showed that the transfer of serum from diphtheria-immunized animals could cure other animals suffering from the infection (119). Behring's work on serum therapy later won him the Nobel Prize in Physiology or Medicine in 1901. In 1891, the year after Behring and Kitasato's study was published, another German scientist, Paul Ehrlich, published a paper about immunity and for the first time used the term antibody (Antikörper in German) (120). Today, we have expanded the knowledge about antibodies and how they function considerably. We know that antibodies consist of two identical heavy chains and two identical light chains. There exist five classes of antibodies in humans, denoted by their heavy chain type (alpha, gamma, delta, epsilon, and mu): IgA, IgD, IgE, IgG, and IgM. These antibody classes each serve different purposes, which are summarized in Table 2. The initial step in antibody production is the binding of a foreign substance, an antigen, to a naïve B-cell via its surface B-cell receptor (BCR), resulting in internalization of the bound antigen. The antigen is then presented to a CD4+ T-cell via MHC-II on the B-cell. This results in the production and secretion of lymphokines (e.g. IL-2 and GM-CSF) by the T-cell, which activates the B-cell (121,122). The activated B-cell proliferates to numerous clonal daughter cells that, followed by additional stimulation by T-cell cytokines, differentiate into B-memory cells and plasma cells (123). B-memory cells will quickly recognize the same antigen and act accordingly if exposed to it again, while the plasma cells secrete antibodies. Initially,

the naïve B-cell expresses membrane-bound IgM and IgD (124,125). Upon activation (by presentation of an antigen), the B-cell can undergo genetic rearrangement of the immunoglobulin heavy chain locus, changing the class of antibody produced to IgG, IgA or IgE (126). Afterward, it may differentiate into a plasmablast (a stage between the B-cell and a mature plasma cell) or lastly into an antibody-secreting plasma cell (127). The change in immunoglobulin production is also known as class switching (128). Since the discovery of RF, an autoantibody against the Fc portion of IgG was found to be predominantly IgM mediated and ACPAs were found to be predominantly of the IgG isotype: these two antibody isotypes have been the main focus in autoantibody research in RA and both are examined in routine clinical tests (129–131).

Table 2. Immunoglobulin isotypes found in humans and their function, levels, and structure.

Antibody class	Function	Percentage of total antibody serum levels	Structure	References
IgA	Involved in immune functions of mucous membranes.	15%	Dimer and monomer	(132–134)
IgD	Mostly found as membrane bound on B-cells.	0.2%	Monomer	(135,136)
IgE	Mainly serves as a defense against parasite infections and associated with hypersensitivity in e.g. allergic asthma.	<0.01%	Monomer	(133,136,137)
IgG	Binds many different pathogens. Predominantly involved in the	75%	Monomer	(132,133,136)

	secondary immune response.			
IgM	First antibody produced in response to antigen. Binds complement C1 and activates the classical complement pathway. Found as membrane bound on B-cells.	10%	Monomer and pentamer	(132,133,136)

T- and B-cells that mistakenly identify self-proteins as foreign are known as autoreactive cells and are constantly produced in the human body. Luckily, there exist processes early in cell development to clear these cells to avoid them causing any harm, namely central and peripheral tolerance. Central tolerance takes place in the thymus and bone marrow for T- and B-cells, respectively, while peripheral tolerance takes place in the immune periphery when the cells exit the primary lymphoid organs (138). In the thymus, T-cells are presented with self-peptides by thymic epithelial cells (139). If the T-cell receptor (TCR) successfully recognizes and binds to the MHC-presented self-peptide, the T-cell will receive a survival signal, begin differentiation into a CD4+ or CD8+ T cell and leave the thymus (positive selection) (140). If the TCR and MHC do not bind, the T-cell will die by neglect, while if the TCR-MHC binding is too strong the cell can undergo anergy, receptor editing to delete autoreactive receptors and develop new non-autoreactive receptors, clonal diversion (development into regulatory T-cells) or clonal deletion (apoptosis) (140). This is known as negative selection. Similar mechanisms exist for B-cell development in the bone marrow i.e. positive selection resulting in B-cell maturation and migration to secondary lymphoid organs, receptor editing, or apoptosis (141). However, self-reactive B- and T-cells may escape the protective mechanisms of central tolerance in the bone marrow and thymus. Therefore, additional selection occurs in the periphery, also known as peripheral tolerance. Here, clonal deletion, development into or suppression by regulatory T-cells, or induction of anergy due to the absence of costimulatory signals keeps the population of escaped self-reactive lymphocytes in check (142,143). Failure of these two branches of immunological tolerance leads to the escape of autoreactive B- and T-cells that may populate the body with autoantibodies, laying the foundation for autoimmune diseases.

1.2.1 RELEVANCE OF AUTOANTIBODY FAMILIES IN RHEUMATOID ARTHRITIS

Several autoantibody classes or families are present in the circulation of RA patients (144). ACPAs and anti-carbamylated protein (anti-carP) antibodies target citrullinated and carbamylated epitopes, respectively, and their presence in RA patients has been known for many years. More recently, anti-acetylated protein antibodies targeting acetylated epitopes have also been found in RA patients (145). However, antibodies against unmodified epitopes have also been identified, including but not limited to vimentin, keratin, aggrecan, and RA33 (146–148). The following paragraphs will give a short introduction to the most well-known autoantibody classes in RA.

1.2.1.1 Antibodies against unmodified epitopes

Native or unmodified epitopes have not been a major focus in RA research since the important discovery of ACPAs. While it may seem logical to credit any reactivity toward native epitopes to cross-reactivity from their citrullinated counterpart this may not necessarily be the case. IgG antibodies against native peptides in seropositive RA patients were found against peptides not containing arginine or lysine; thus, it seems unlikely that the reactivity identified may be credited to cross-reactivity against citrulline (ACPA) or homocitrulline (anti-carP) (149). It has also been proposed that native epitopes are mostly targeted in early RA with low radiographic erosion, while citrullinated epitopes are identified later in the disease course (148). A subset of patients presenting with intermediate severity of RA showed reactivity toward both native and citrullinated epitopes. Thus, this may represent a transitioning from early/mild RA associated with antibodies against the native epitope to a more advanced RA disease state associated with antibodies against the citrullinated epitope. However, this was only demonstrated using a single autoantigen, namely RA33. Nevertheless, it is still likely that the citrullinated antigen is responsible for breaking self-tolerance, thus leading to antibody reactivity against native sites due to i.e. epitope spreading (150). For now, it is still unknown what exactly the role of native autoantigens is in RA, and if the break of immune tolerance can be contributed to citrullination only; this knowledge gap alone warrants continuing research into autoantibodies against unmodified epitopes.

1.2.1.2 Antibodies against modified epitopes

ACPAs are probably the most researched family of autoantibodies in RA. The significant level of interest in citrullination and ACPAs in RA began when Schellekens et al. (1998) demonstrated that citrulline was the antigenic constituent that was recognized by autoantibodies in RA patients (75). Several decades prior to this, autoantibody reactivity from RA sera was shown to bind to granules in buccal mucosa cells (151). The antigenic target was later identified as citrullinated filaggrin (152–155). The presence of ACPAs several years prior to clinical manifestation of arthritis alone makes them highly interesting; however, as already mentioned, a second trigger seems to be needed since not everyone with ACPAs develops RA.

Furthermore, the pathogenic role of ACPAs has been studied extensively and it has been shown that ACPAs were able to activate macrophages *in vitro* and point the macrophage toward the M1 proinflammatory phenotype (156–158). Several other properties of ACPAs related to pathogenic effects seen in RA have also been identified, such as macrophage and osteoclast activation, modulation of synovial fibroblasts, and exacerbation of arthritis in combination with other triggers such as lipopolysaccharide (159–164).

Similar to ACPAs, anti-carP antibodies seem to be present several years before patients develop any clinical signs of RA (165). Anti-carP antibodies have been shown to be present in both ACPA-positive and ACPA-negative RA patients and may be associated with a more severe disease course (166,167). Anti-carP antibodies targets carbamylated epitopes which is created by the conversion of lysine to homocitrulline by cyanate (167,168). Smoking has been shown to induce carbamylation, most likely by indirectly increasing the amount of cyanate by increasing thiocyanate, which can be oxidized to cyanate by myeloperoxidase in neutrophils, which are highly present in synovial fluid (169–172). Recently, it was shown that autoantibodies in RA patients recognize acetylated vimentin (145). Not much is known about the role of acetylation in RA yet; however, an intriguing proposal has been voiced concerning the ability of bacteria to acetylate host proteins (24,173,174). This provides a link between bacteria and the breach of immune tolerance against modified self-proteins in RA, which is something that is still unclear.

1.2.2 FROM AUTOANTIBODY DISCOVERY TO COMPANION DIAGNOSTICS

It is well established by now that levels of certain autoantibody classes (e.g. ACPAs) rise several years prior to clinical manifestation of RA (1,2). Utilizing this knowledge, we have been able to detect RA disease in patients earlier than was previously possible and with a higher degree of confidence. The idea of identifying patterns in this early stage of autoantibody reactivity and be able to correlate it to treatment outcome or the like is intriguing. Succeeding in this regard will increase the likelihood to introduce CDx assays to the field of rheumatology. Initially, to pursue this idea, establishing a platform and method to screen many autoantibodies simultaneously is critical. Currently, numerous technologies able to detect the presence of autoantibodies exist, and recently, we described the introduction of CDx assays within protein array-based platforms that can do so (175). For now, protein microarrays may not be suited for point-of-care devices, as described in Review I; however, they seem ideal for the initial discovery phase. Applying the potential identified biomarker findings to a simpler and cheaper device may ensure easier implementation in clinical settings.

CHAPTER 2. OBJECTIVES

The reason why patients suffering from the same disease respond differently to the same type of medication is unknown. Autoantibodies are present many years prior to any clinical sign of RA, thus their potential use in diagnostics but also in predicting treatment outcome prior to administering any drug is intriguing. Therefore, this PhD thesis aimed to investigate the autoantibody repertoire in RA patients using high-density protein microarrays. This was done by first investigating native autoantigens in both RA patients and healthy donors in Study I. Next, we wanted to investigate the presence of ACPAs in RA patients, which we did in Study II. The potential of using complex protein microarrays in early biomarker discovery phases and transitioning to a simpler setup compliant with a clinical setting was examined in Review I.

Study I: In this study, we investigated the presence of autoantibodies from anti-CCP positive and anti-CCP negative RA plasma pools and healthy donors against native (non-modified) proteins.

Study II: In this study, we introduced the post-translational modification, citrullination, to a microarray platform and investigated the presence of autoantibodies from anti-CCP positive and anti-CCP negative RA plasma pools against citrullinated proteins.

Review I: Here, we presented different technical platforms available for CDx-focused protein array platforms and discussed current predictive biomarkers within a range of different disease areas, including RA. We also touched on the implementation of such an assay in a clinical setting or as a point-of-care test.

CHAPTER 3.RESULTS

3.1 STUDY I

Identification of novel native autoantigens in rheumatoid arthritis

Thomas B. G. Poulsen[1,2], Dres Damgaard[3], Malene Møller Jørgensen[4a], Ladislav Senolt[5], Jonathan M. Blackburn[6,7], Claus H. Nielsen[3], Allan Stensballe[1]

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Main findings

In response to the growing evidence of autoantibodies' importance within RA, we investigated the repertoire of autoantibodies in RA patient plasma against non-modified epitopes. We identified 102 proteins bound by autoantibodies of the IgG isotype. 86 of these were targeted by autoantibodies from seropositive RA patients, while 76 were targeted by autoantibodies from seronegative RA patients. Cross-referencing the new targets with synovial fluid proteome datasets, we found 24 of the 102 proteins had previously been identified in synovial fluid.

3.2 STUDY II

Identification of potential autoantigens in anti-CCP-positive and anti-CCP-negative rheumatoid arthritis using citrulline-specific protein arrays

Thomas B. G. Poulsen[1,2], Dres Damgaard[3], Malene Møller Jørgensen[4,5], Ladislav Senolt[6], Jonathan M. Blackburn[7], Claus H. Nielsen[3,8], Allan Stensballe[1,8]

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Main findings

In this study, we present 844 citrullinated proteins recognized by ACPAs. Furthermore, we identified high-intensity binding of autoantibodies from seropositive RA plasma to 87 and 99 proteins citrullinated by PAD2 and PAD4, respectively, compared to the corresponding non-modified proteins. The corresponding numbers for seronegative RA plasma were 29 and 26 proteins. Four proteins showed higher binding to PAD2-citrullinated proteins compared to PAD4, while autoantibodies against one protein preferred citrullination by PAD4. We demonstrate that PAD2 and PAD4 are equally efficient in generating citrullinated epitopes capable of ACPA binding. Lastly, we demonstrate a method for introducing citrullination on-slide to formerly native proteins.

3.3 REVIEW I

Protein array-based companion diagnostics in precision medicine

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Main findings

In this review, we investigated the role of protein microarrays in CDx assays. For this purpose, we examined studies investigating new potential predictive biomarkers within different diseases, including RA, and discussed the technology platforms available within protein microarrays. CDx-focused research is increasing in several disease areas and is not limited to oncology. High density and complex protein microarrays seem more suited for initial biomarker discovery phases than for implementation in the clinic as a finalized CDx assay.

CHAPTER 4. DISCUSSION

The initial aim of this PhD thesis was to investigate the potential of a protein microarray platform to expand the repertoire of disease-associated autoantibodies in autoimmune diseases focusing on RA. Furthermore, using this high throughput analytical platform to elucidate potential patterns in autoantibody reactivity for future multiplex biomarkers was also something we wanted to look into. This discussion will first focus on the technical aspects of the data analysis and results before touching upon the PAD enzymes and their differences in RA.

With the increasing improvements in sensitivity and throughput of technological platforms available for serological screening of autoantibodies, it has become even more important to discuss appropriate statistical approaches for presenting valid data. We need to process and filter the abundance of data we generate for optimal selection of prognostic disease biomarkers. More precisely, how we define specific reactivity against potential antigens is important, as is whether a high reactivity is required before we consider the specific interaction to be of interest in comparison to e.g. low binding.

In Study I and Study II we established a processing pipeline incorporating several different statistical cut-offs to ensure high-quality data (176,177). Besides using different quality controls such as the coefficient of variation to ensure a high degree of reproducibility between replicates, we decided to incorporate a Z-score cut-off to efficiently filter our protein array data. This filtration removed the autoantibody-antigen bindings with the lowest reactivity. It could be speculated that the low reactivities measured may be due to cross-reactivity of ACPAs in the RA plasma pool used in studies I and II or due to the pooling of multiple individual biofluid samples (178,179). Furthermore, one could speculate that potential autoantigens may demonstrate a strong binding and high concentration, and thus exhibit a high reactivity toward their target. It has been postulated that low-affinity natural IgM antibodies exert and maintain immune homeostasis by clearing e.g. cell debris, thereby preventing potential immune activation toward self-proteins, while high-affinity IgG antibodies serve a more pathological role (179–183). Thus, at least in the search for pathological autoantibodies, it may seem reasonable to disregard low reactivity interactions. When investigating a potential pattern in autoantibody reactivity that correlates with e.g. treatment outcome or a specific disease course, high reactivity (and potentially pathological) bindings may not be the only relevant interactions to investigate. Low reactivity autoantibody targets or the absence of activity toward a specific target may reflect an important marker in disease just as well as high reactivity (184). It should be noted, however, that even healthy individuals show some sort of baseline reactivity toward self-antigens (185–187). This has been shown numerous times and current literature suggests that at least for IgG antibodies the level of reactivity is consistent over time (186). This knowledge should be taken into

consideration when interpreting future results and perhaps also when deciding how to filter the generated data.

We performed on-slide citrullination of a protein microarray consisting of unmodified proteins immobilized on the Immunome slides in Study II. Using the protein array complexity of 1631 proteins resulted in the binding of ACPAs to 844 modified proteins on the slide. However, successful citrullination was not verified but solely assumed due to a change in IgG reactivity compared to unmodified proteins. Since it was not investigated whether citrullination was successful on a protein level and epitope level, it could be speculated that the observed change in reactivity could be the result of something else e.g. the denaturation process. However, the unmodified arrays used underwent the same procedure as the modified arrays except for the addition of the PAD enzymes (176,177). Thus, the change in reactivity observed must be the result of the enzymes responsible for citrullination, PAD2 or PAD4. One way to verify successful citrullination could be to use an antibody against the amino acid citrulline. This would also validate if every protein on the array is capable of undergoing citrullination or not. Another aspect to consider is that not every target we identified is necessarily an autoantigen involved in RA *in vivo*. Just because the proposed protein acts as an autoantigen *ex vivo* following citrullination, does not mean that this is the case *in vivo*. For these proteins to be citrullinated they need to be in physical contact with the human PAD enzymes, which themselves then again require a strict environment to facilitate citrullination, such as high calcium concentration, which is not found under normal physiological intracellular conditions (188). Increased calcium availability can be achieved by several events such as cell death or membrane disintegration in general, e.g. the bacterial pore-forming leukotoxin, leukotoxin A, the pore-forming protein perforin found in NK- and T-cells, or the membrane attack complex which is part of the complement system (189–193). Additionally, a subset of autoantibodies against PAD4 was found to lower the requirement of calcium for PAD4 activation, thus, enhancing its citrullination activity and creating a loop in which autoantibody binding results in the generation of potentially new autoantigens (194,195). Assuming these conditions are met *in vivo*, one could speculate that for the autoantigens to be of interest for the pathology of RA they must be present at the site of disease. Therefore, in Study I, we cross-referenced the identified potential autoantigens with publicly available synovial fluid proteome datasets and identified 24 out of 102 targets of IgG autoantibodies to be present in the RA joint.

Shortly after it was shown that antibodies from RA patients' sera recognized citrullinated peptides, the interest in PAD enzymes in RA research increased. PAD2 and PAD4 were shown to be expressed in the RA synovium; as a result, focus in RA research has been on these two PAD isoforms (77,78). Several proteins have been shown to be targets of citrullination in RA, such as vimentin, alpha-enolase, and fibrinogen (196). However, the individual roles of PAD2 and PAD4 in RA and in generating citrullinated epitopes that ACPAs can bind to are still not well understood

and conflicting results exist on these areas. One study found PAD2 citrullinated more arginine sites in fibrinogen compared to PAD4, while another study found that a similar number of citrullinated sites were created by the two enzymes (197,198). A third study demonstrates that PAD2 and PAD4 are equally efficient in generating citrullinated epitopes that ACPAs can bind to in both fibrinogen and alpha-enolase, while histone H3 autoantibody binding was higher after PAD4 citrullination (150). Interestingly, it was found that the antibody titer plays a role in the preferential binding of antibodies to citrullinated epitopes (199). Here, they found high dilution of RA plasma- (1:250 and 1:1000) bound fibrinogen citrullinated by PAD4 to a higher degree compared to PAD2 citrullinated fibrinogen, while they found no difference at lower titers. These observations are somewhat in accordance with our results presented in Study II where PAD2 and PAD4 generated a comparable number of IgG ACPA-binding sites using 1:200 plasma dilution (177). Another study investigated PAD substrates from different cell lines and synthetic peptides (81). The researchers found PAD4 to be more restrictive in its substrate selection compared to PAD2, while both enzymes seemed to prefer glycine and tyrosine 1 and 3 amino acids from the citrullinated arginine, respectively. Unique for PAD4, they showed several preferred amino acids in position -4 to +4 from the citrullinated arginine. The influence of amino acid compositions on PAD efficiency is difficult to elucidate in our studies since we did not have strict control of the degree to which the proteins were citrullinated or to which epitopes were modified.

Recently, a new interesting take on the development of the ACPA response in RA involving PAD enzymes was published (200,201). The researchers speculate that when citrullination occurs and the responsible PAD enzyme (PAD2 or PAD4) binds to its substrate, e.g. fibrinogen, B-cells targeting the PAD enzyme might internalize both PAD and the bound substrate. They propose that the PAD enzyme acts as the carrier while the bound substrate behaves as a hapten. Thus, the reactivity toward citrullinated proteins is a result of the internalization of a complex consisting of PAD4 and the bound citrullinated protein. They first showed the development of autoantibodies against citrullinated fibrinogen in healthy mice after immunization with PAD (200). Later, they showed antibody and T-cell response to PAD4 in ACPA-positive RA patients and proposed that this is further evidence of their hypothesis (201). This is quite interesting; however, much of it is still highly speculative. The results demonstrated do not contradict the hypothesis; however, the results do not definitive prove the existence of the proposed model either.

CHAPTER 5. CONCLUSION

The research on which this PhD dissertation is based sought to investigate the presence of different autoantibody reactivities in RA and explore potential patterns in expression that could benefit patients. Furthermore, we wanted to shed light on the role of the two RA-relevant citrullinating enzymes, PAD2 and PAD4, in the generation of ACPA-binding epitopes.

In Study I, we demonstrated IgG autoantibody reactivity against unmodified proteins from pooled RA plasma. Furthermore, we provided a list of autoantigens that could be pathologically relevant due to their presence in the joint. As expected, we also demonstrated an overall low reactivity from healthy donor plasma against unmodified proteins. In Study II, we showed that it is possible to introduce citrullination on the protein array platform and identified more than 800 ACPA-binding proteins. Furthermore, we demonstrated that PAD2 and PAD4 are equally efficient in generating epitopes to which ACPAs can bind. Lastly, we narrowed the identified targets down to 100 potential autoantigens in RA based on their high reactivity. In Review I, we highlighted technical platforms available for high-density protein biomarker identification and how we can apply these findings to suitable CDx assays that will benefit patients. In summary, we expanded the known repertoire of both unmodified and citrullinated targets of autoantibodies from RA patients and demonstrated that PAD enzymes are not restrictive in creating autoantibody-binding epitopes.

5.1 PERSPECTIVES

The studies presented in this dissertation provide grounds for additional research and tackling the following points will be a natural next step when building on these results. Performing the experiments using individualized samples instead of pooled samples will elucidate if it is possible to correlate specific autoantibody reactivity patterns with e.g. treatment response or the development of side effects. This could potentially subdifferentiate the heterogenous but grouped RA patients further. It should be noted that treatment may influence the autoantibody reactivity patterns, which should be taken into consideration when including patients in a future study. When following up on future leads, the focus should be on designing smaller arrays with only relevant targets spotted to minimize cost. If it succeeds to identify a correlation between a specific autoantibody reactivity pattern and the treatment effect, the foundation for a future CDx device will be laid and the platform used should be carefully considered in light of whether it can be easily implemented in the clinic.

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