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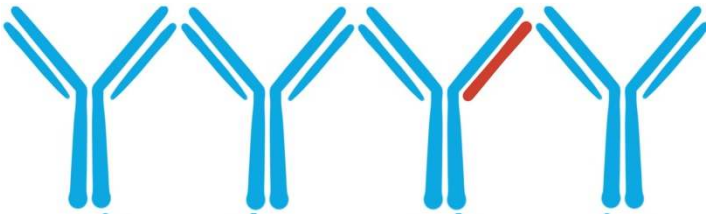
Doctorado en Biotecnología, Medicina y Ciencias Biosanitarias

MULTI-PARAMETRIC MODEL FOR THE IMPROVED DIAGNOSIS AND STRATIFICATION OF RHEUMATOID ARTHRITIS PATIENTS

Advancing precision medicine in rheumatoid arthritis



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Abstracts

English version

Introduction

Rheumatoid arthritis (RA) is one of the most prevalent autoimmune diseases. Despite several serological markers are known for this disease and two of them, rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPA), are included in the RA classification criteria, there are still many patients that are negative for these two biomarkers. For this reason, there is a need to identify novel biomarkers that can help close this serological gap in RA, as well as to improve patient stratification, prediction of prognosis and response to treatment, and disease monitoring.

Objectives

The main objective of this thesis was to develop a multi-parametric model that helps to improve RA diagnosis, patient stratification and prediction of prognosis. The specific objectives included, firstly, the assessment of the current practice, the evaluation of several immunoassays for the detection of the RA classification criteria markers, and the analysis of combinatory approaches. Secondly, among the RA novel biomarkers, the anti-protein-arginine deiminase (PAD) antibodies were selected to study their clinical and immunological relevance in RA. We then aimed to characterize the PAD enzymes as antigenic targets, to develop immunoassays for the detection of these antibodies, and to determine their clinical significance and utility in RA in several characterized cohorts.

Methods

Several methodologies were utilized to achieve the thesis objectives. These included literature screening; feasibility studies, early development and optimization of a range of immunoassays for the detection of autoantibodies in human samples; biochemical characterization of the antigens and the autoantibodies; measurement of biomarkers in different clinical cohorts using the immunoassays; data analysis; and drafting of combinatory approaches for the integration of the different data points.

Results

Combinatory approaches based on RF and ACPA showed an improved diagnostic performance over the individual markers and can help correctly classify a higher number of patients. During the study of the PAD enzymes and anti-PAD antibodies, several linear epitopes were identified on the five PAD family members, with data suggesting the presence of epitopes unique to PAD3 or to PAD4, in addition of the cross-reactive epitope. The presence of anti-PAD4 antibodies of the IgA isotype in RA was confirmed and an association with erosive disease was identified, a link also confirmed for anti-PAD3 IgG. We identified anti-PAD4 IgM and antibodies to PAD1 and 6 for the first time, and confirmed that anti-PAD4 IgG can help close the serological gap in RA and are associated with a more severe disease phenotype. We confirmed that antibodies to PAD2 are also present in the sera of RA patients, however the reported association with milder disease could not be reproduced.

Conclusions

A combinatory approach based on ACPA and RF IgM represents a promising tool to improve the diagnosis of RA. Anti-PAD3 and 4 antibodies are useful biomarkers for patient stratification. A multi-parametric model for the improvement of RA diagnosis and patient stratification based on these autoantibodies and other features has been proposed.

Spanish version

Introducción

La artritis reumatoide (AR) es una de las enfermedades autoinmunes más prevalentes en la población. A pesar de que se conocen varios marcadores serológicos para esta enfermedad y de que dos de ellos, el factor reumatoide (FR) y los anticuerpos anti-proteínas citrulinadas (ACPA), están incluidos en los criterios de clasificación de AR, existen todavía un gran número de pacientes que son negativos para estos dos autoanticuerpos. Por ello, se necesitan identificar nuevos marcadores que ayuden a cerrar esta brecha serológica, además de a mejorar la estratificación de pacientes, la predicción de pronóstico y respuesta a tratamiento y monitorización de la enfermedad.

Objetivos

El objetivo principal de esta tesis fue el desarrollo de un modelo multiparamétrico que ayude a mejorar el diagnostico de AR, la estratificación de pacientes y la predicción del pronóstico de la enfermedad. Los objetivos específicos incluyeron, en primer lugar, la valoración de la práctica actual, la evaluación de inmunoensayos para la detección de los marcadores de AR incluídos en los criterios de clasificación y el análisis de combinaciones de estos marcadores. En segundo lugar, de entre los nuevos marcadores de AR, se escogieron los anticuerpos anti-proteína-arginina deiminasa (PAD) para el estudio de su relevancia clínica e inmunológica en AR. Los objetivos fueron entonces caracterizar las enzymas PAD, desarrollar inmunoensayos para la detección de anticuerpos frente a estas proteínas y determinar su significado y utilidad clínica en AR en varias cohortes caracterizados.

Métodos

Se utilizaron varias metodologías para alcanzar los objetivos de esta tesis. Estas incluyeron el cribado de literatura científica; estudios de viabilidad, desarrollo temprano y optimización de inmunoensayos para la detección de autoanticuerpos en muestras humanas; caracterización bioquímica de los antígenos y anticuerpos; cuantificación de biomarcadores en diversas cohortes clínicas utilizando estos inmunoensayos; análisis de datos y diseño de aproximaciones basados en combinaciones de resultados.

Resultados

Combinaciones de FR y ACPA demostraron un rendimiento diagnóstico superior a los marcadores individuales y una utilidad para clasificar correctamente a un número mayor de pacientes. Durante el estudio de las enzimas PAD y anticuerpos frente a ellas, se identificaron varios epítopos lineales en los cinco miembros de esta familia de proteínas. Nuestros datos sugirieron la presencia de epítopos únicos de PAD3 o de PAD4, además del epítopo de reacción cruzada. Se confirmó la presencia de anticuerpos anti-PAD4 del isotipo IgA en AR y se identificó una asociación con enfermedad erosiva, algo que también se vio con anti-PAD3 IgG. Por primera vez, se identificaron anti-PAD4 IgM y anticuerpos frente a PAD1 y PAD2 en AR y se confirmó que anti-PAD4 IgG es un marcador útil para identificar pacientes seronegativos para FR y ACPA, y su asociación con un fenotipo de enfermedad más severa. Confirmamos que los anticuerpos frente a PAD2 están presentes en el suero de pacientes con RA, sin embargo, no pudimos reproducir la asociación de este marcador con un fenotipo más leve, como se había indicado en literatura previamente.

Conclusiones

Un modelo basado en la combinación de ACPA y FR IgM representa una herramienta prometedora para mejorar el diagnostico de AR. Los anticuerpos anti-PAD3 y 4 son biomarcadores útiles para la estratificación de pacientes. Se ha propuesto un modelo multiparamétrico basado en estos anticuerpos y otras características para la mejora del diagnóstico y la estratificación de pacientes con AR.

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In this section, the scientific data, the evidence and facts, and the objective observations move aside and allow the subjective, the intangible and the essential to get the recognition.

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Antigen design and development

Erika Darrah and her team from JHU performed the early work for the design and generation of the PAD2, PAD3 and PAD4 antigens and shared this knowledge and as well as reagents (plasmids, proteins and characterized samples) with Inova. This helped with the initiation of the PAD antigens project at Inova, initially lead by Cristina Gascon and Aaron Llanes in the case of PAD3. David Lucia later lead the PADs project from the antigen design and development side and his contributions were essential for the successful generation of these proteins in-house, their characterization, and the addition of further scientific vision in the antigen work. Lauren Aboutin, Aaron Llanes and Alejandra Espinosa generated several lots of the PAD3 antigen that were key for several studies. Alejandra Espinosa helped with the PAD4 citrullination studies.

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Abbreviations

A1AT: alpha 1 anti-trypsin
AA: amino acids
ACPA: anti-citrullinated protein antibodies
ACR: American College of Rheumatology
AI: Artificial Intelligence
ALBIA: addressable laser bead assays
AMLI: Association of Medical Laboratory Immunologists
APS: anti-phospholipid syndrome
ARD: Annals of Rheumatic Diseases
ARHP: Association of Rheumatology Health Professionals
AS: ankylosing spondylitis
AUC: area under the curve
AxS: ankylosing spondylarthritis
BD: blood donors
bDMARDs: biologic disease-modifying antirheumatic drugs
BiP: immunoglobulin binding protein
Ca++: ionized calcium.
CarP: carbamylated proteins
CCP: cyclic-citrullinated peptide
CD: celiac disease
CDAI: clinical disease activity index
CI: confidence interval
CIA: chemiluminescence immunoassay
CIAr: collagen-induced arthritis
CLEAR: Consortium for the Longitudinal Evaluation of African Americans with Early Rheumatoid Arthritis
COG6: component Of Oligomeric Golgi Complex 6
CORA: Controversies in Rheumatology & Autoimmunity
CRP: C-reactive protein
CSA: clinically suspect arthralgia
CTD: connective tissue diseases
CTLA4: cytotoxic T-lymphocyte associated protein 4
CXCL: C-X-C Motif Chemokine Ligand
Da: Dalton
DAS28: disease activity score with 28-joint counts
DELFIA: dissociation-enhanced lanthanide fluorescence immunoassay
DM: dermatomyositis
DMARDs: disease-modifying antirheumatic drugs
ELISA: enzyme linked immunosorbent assay
EORA: elderly onset RA
ESCAPE: Evaluation of Subclinical Cardiovascular Disease and Predictors of Events in Rheumatoid Arthritis
ESR: erythrocyte sedimentation rate
EULAR: European League Against Rheumatism
EURIDISS: European Research on Incapacitating Disease and Social Support

EWRR: European Workshop for Rheumatology Research
Fc: fragment crystallizable
FCGR: Fc gamma receptor
FDR: first degree relatives
FMS: fibromyalgia syndrome
FPF: false positive fraction
GFAP: glial fibrillary acidic protein
HAQ: health assessment questionnaire
HCQ: hydroxychloroquine
HI: healthy individuals
His: histidine
HLA: human leukocyte antigen
HnRNP: heterogeneous nuclear ribonucleoprotein
hr: human recombinant
HSP90: heat shock protein 90
HUPI: Hospital Universitario La Princesa index
ICA: International Congress on Autoimmunity
IFA: immunofluorescence assays
Ig: immunoglobulin
IIM: idiopathic inflammatory myopathies
IL: interleukin
IL6R: interleukin-6 receptor
ILD: interstitial lung disease
ING4: Inhibitor of growth protein 4
IP: immunoprecipitation
IRIS: Inova Research and Innovation Summit
JAK: Janus kinases
JES: joint erosion score
JHU: John Hopkins University
JNS: joint narrowing score
kDa: kilo Dalton
MAA: malondialdehyde-acetaldehyde
MBDA: multibiomarker disease activity
MBP: myelin basic protein
MDA: malondialdehyde
MFI: median fluorescence intensity
MHC: major histocompatibility complex
MLH: Marcos Lopez-Hoyos
MOA: mechanism of action
MRI: magnetic resonance imaging
MRP: myeloid-related protein
MS: multiple sclerosis
MTX: methotrexate
MTXPG: MTX polyglutamates
n.s.: non-significant
NA: not available
nbDMARDs: non-biologic disease-modifying antirheumatic drugs.
NETs: neutrophil extracellular traps

NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells
NP: not provided
NSAIDs: nonsteroidal anti-inflammatory drugs
NTR: Netherlands Trials Register
OA: osteoarthritis
OAARD: other ANA-associated autoimmune rheumatic diseases
OAID: other autoimmune diseases
OR: odds ratio
ORAR: Oslo Rheumatoid Arthritis Register
PAD: protein-arginine deiminase
PADI4: protein-arginine deiminase 4 (gene)
PAS: patient activity scale
PBS: phosphate buffered saline
PE: phycoerythrin
PM: polymyositis
PM: precision medicine
PMAT: particle-based multi-analyte technology
PMR: polymyalgia rheumatica
PPAD: P. gingivalis PAD
PsA: psoriatic arthritis
pSjS: primary sjogren's syndrome
PTM: post-translational modifications
PTPN22: Protein tyrosine phosphatase, non-receptor type 22
RA: rheumatoid arthritis
RACAT: Rheumatoid Arthritis Comparison of Active Therapies
RAD51B: RAD51 Paralog B
RADAI: rheumatoid arthritis disease activity index
RA-ILD: rheumatoid arthritis associated interstitial lung disease.
RANKL: receptor activator of NF-κB ligand
RAPID3: routine assessment of patient index data with 3 measures
RBC: red blood cell
RF: rheumatoid factor
RFU: relative fluorescent unit
RLU: relative light unit
ROC: receiver operating characteristics
RUO: research use only
SD: standard deviation
SDAI: simplified disease activity index
SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis
SE: shared epitope
SJC: swollen joint count
SjS: Sjogren´s syndrome
SLE: systemic lupus erythematosus
SNP: single nucleotide polymorphisms
SSc: systemic sclerosis
STAT4: signal transducer and activator of transcription 4
TBD: to be determined
TJC: tender joint count

TNF: tumor necrosis factor
TNFi: tumor necrosis factor inhibitor
TPF: true positive fraction
TSJ: total swollen joint:
TSS: total sharp score
TYK2: tyrosine kinase 2
UA: undifferentiated arthritis
ULN: upper limit of normal
US: ultrasound
VACSP: Veterans Affairs Cooperative Study Program
WB: western blot
XR: cross- reactive
YORA: young onset RA

Chapter 1. Introduction

1.1. The immune system and autoimmunity

The human immune system, comprised by cells, molecules and mechanisms that are responsible for the coordinated execution of the immune response, is a remarkably complex and sophisticated entity. The term "immunity" derives from the Latin word *immunitas* which refers to the protection offered to the Roman senators against any legal action during their service and to the exemption from performing public service. With time, this term would evolve to the medical sense of "protection from disease", the role of the immune system.

Immune tolerance is defined as the state of unresponsiveness of the immune system to substances or tissues that could potentially induce an immune response. Tolerance to self-antigens (autoantigens) occurs because the lymphocytes that recognize these self-antigens die, get functionally inactivated or change their specificity (the receptors get edited). This mechanism represents the homeostasis in a healthy and normal immune system, and it is important for normal physiology. Nevertheless, failure or deficits of the self-tolerance mechanisms can result in immune responses to self- cells or tissues. This is what is known as autoimmunity.

The overall estimated prevalence of autoimmune diseases is 4.5% (2.7% for males and 6.4% for females) [1] which translates to approximately 15 million affected individuals in the United States and 33 million in Europe. It is calculated that there are more than 80 autoimmune disorders [1]. They can be organ-specific or systemic and they are heterogeneous regarding prevalence, manifestations, and pathogenesis [2]. Examples of some of the most prevalent or known autoimmune diseases include systemic lupus erythematosus (SLE), multiple sclerosis, type 1 diabetes mellitus and rheumatoid arthritis (RA).

The understanding of autoimmunity and autoimmune diseases has progressed tremendously over the past decades, but there is still great misunderstanding around this type of disorders and further research is needed to better understand these conditions and their pathogenic mechanisms, and to shed more light into this fascinating field.

1.2. Rheumatoid Arthritis as an autoimmune disease

RA is one of the most prevalent and known autoimmune diseases. It is a chronic, inflammatory disease that affects mainly the joints. It is characterized by cartilage and bone damage, with resulting pain, fatigue and irreversible disability, especially if insufficiently or unsuccessfully treated, often leading to a significant reduction in health-related quality of life. A variety of extra-articular comorbidities, including cardiovascular disease or pulmonary manifestations, are also associated with this disease. The presence of autoantibodies is characteristic of this disease, with rheumatoid factor (RF) and antibodies that target citrullinated proteins, known as anti-citrullinated protein antibodies (ACPA), as the main two. However, up to 40% of RA patients do not present these biomarkers, a percentage that can be even greater in early disease. This is often defined as seronegative RA or the serological gap [3].

RA is a complex heterogeneous disease with variable clinical presentation and manifestations. The underlying pathogenic mechanisms can vary between individuals with an RA diagnosis and across the disease stages. Several genetic and environmental risk factors have been identified and described for this disease (Table 1).

RA-associated joint damage is in general irreversible; therefore, early diagnosis is a key factor for therapeutic success. The factors for poor outcomes include high disease activity, seropositivity and early joint damage. Several treatment options with different targets are currently available, however, many patients still do not respond to current therapies or achieve remission and could benefit from treat-to-target strategies and new therapies.

The conceptualization of this disease has evolved significantly in the last decades. It has moved from a destructive and disabling condition with limited therapeutic options, to a disease in which remission is an achievable goal. And although RA cannot be cured yet, control of inflammation and of other symptoms and prevention of joint destruction are feasible therapeutic objectives.

1.2.1. Clinical manifestations

The main clinical characteristics of RA are joint swelling and inflammation, tenderness, pain, stiffness and eventually, damage. Associated to these symptoms, RA patients usually suffer from fatigue, physical disability and limitations, and experience a reduction in the quality of life. Depression appears to be more common in patients with RA than in healthy individuals and in general, it is associated with poorer outcomes [4, 5]. RA symptoms can vary in severity, and flares can alternate with periods of relative remission.

Joint swelling is one of the hallmarks of this disease. It reflects synovial membrane inflammation consequent to immune activation. The cellular composition of synovitis in RA comprises a variety of innate and adaptive immune cells [6]. Smaller joints in the hands and feet tend to be affected first. As the disease progresses, symptoms often spread to other joints such as the wrists, knees, hips or shoulders, and in most cases, this happens symmetrically. As opposed to osteoarthritis, RA is characterized by a robust tissue response that promotes articular destruction [7].

Bone erosion is one of the main clinical outcomes in RA and it occurs as a result of excessive local bone resorption and inadequate bone formation. The main triggers of this process are synovitis, including the production of proinflammatory cytokines and receptor activator of nuclear factor $\varkappa$B (NF-$\varkappa$B) ligand (RANKL), as well as antibodies directed against citrullinated proteins [8]. It has been suggested that ACPA could be initiating joint damage through an interaction with citrullinated peptides that are expressed by the osteoclasts (bone-resorbing cells) and their precursors, resulting in the maturation and activation of these cells [9, 10]. Nevertheless, the underlying pathways might be different between ACPA positive and negative individuals and perhaps even for patients that present other autoantibodies.

RA does not exclusively affect the joints and extra-articular manifestations are also common. Other organs that are frequently negatively affected in the disease include the skin, the eyes, the heart and bloods vessels, and the lungs. Cardiovascular disease is common in RA and together with infection, it represents one of the main causes of mortality in these patients [11]. Particularly interesting is interstitial lung disease (ILD), an umbrella term for a large group of disorders that cause scarring

(fibrosis) of the lungs. The reported prevalence of ILD in RA is highly variable ranging from 4% to 68% depending on the methods of detection and the study population [12, 13]. And although its potential for mortality and morbidity has arguably been underappreciated for decades, it represents one of the most severe extra-articular manifestations of RA, with a patient survival estimated in 3 years [14]. Despite its severity, there is a lack of validated classification criteria and biomarkers for this condition, and there is a strong need to identify the early asymptomatic disease and define its natural history.

1.2.2. Epidemiology, etiology and pathophysiology

The prevalence of RA is known to differ between ethnicities [15, 16]. Geographical differences have been reported [17] and the prevalence of this disease is not completely understood in certain regions due to the lack of robust epidemiological studies. Despite these limitations, most studies in Europe and the United States report a prevalence ranging between 0.5% and 1.0% in the Caucasian population [1, 17, 18]. Increased morbidity and mortality are also noted in this group compared to the general population [19, 20].

The exact causes of RA are not fully understood. The initiation of the disease seems to be the result of a combination of features, including genetic susceptibility and environmental factors. RA development is determined by a genetic predisposition upon which environmental and additional genetic factors operate to ultimately trigger the disease, most likely in a combinatory and additive fashion (numerous hits).

Several genetic and environmental risk factors that increase the susceptibility to RA have been described (reviewed in [21] and summarized in Table 1). In recent years, there has been an evolution of the understanding of the genetics of RA [22]. It is estimated that the heritability in RA is between 50-60% and that this percentage is higher for ACPA and/or RF positive patients than for seronegative patients [23, 24]. The human leukocyte antigen alleles (HLA)-DRB1*04 [25], *01, and *10 [26] are the strongest genetic risk factors for the development of RA. The association between the presence of a common amino acid motif (QFRAA) encoded in these alleles, the shared-epitope (SE), and susceptibility for RA has been extensively studied [7, 26, 27]. Epigenetics, including DNA methylation and histone acetylation, are also known to play a role in RA development [28]. Moreover, the development of new technologies has enabled the detection of a number of new genes associated with RA susceptibility, which may represent potential novel therapeutic targets [22].

Gender is known to be a significant factor in the development of autoimmune disease [29]. In general, women are two- to three-fold more likely to develop RA [18, 30] and differences in disease onset and serological profiles have been described as well. The effect of hormones, specifically estrogen, could help explain these differences however, the role of hormones in the development of RA is controversial [31, 32].

Non-genetic risk factors for the development of RA comprise tobacco smoking [33], silica exposure [34], vitamin D deficiency [35], and changes in the oral microbiota, including *Porphyromonas gingivalis* [36, 37] and *Aggregatibacter actinomycetemcomitans* [38] –both very common in periodontal disease-, and in the intestinal microbiota related to the levels of *Prevotella copri* [39]. The contribution to the disease of some of these environmental factors is not fully understood, mostly attributed to the

lack of robust studies. However, the link between smoking and susceptibility to RA has been widely studied and continues to be a research area of high interest. Several mechanism that seem to be associated with the development of RA in smokers, especially in genetically predisposed individuals, have been identified, including persistent inflammation due to oxidative stress, changes in apoptosis, autoantibody production and epigenetic changes (reviewed in [40]). An effect of smoking in radiographic progression and joint damage has also been suggested [41], however, these aspects remain controversial [42] and further studies are necessary to better understand the underlying mechanisms.

Table 1 Summary of risk factors for RA. Level of scientific evidence was assigned based on history, impact and number of publications, level of validation and robustness of the results, controversy *vs.* alignment of findings, and understanding of the driving mechanism.

Risk Factors	Level of Scientific evidence	References
Genetic		
Familial risk (FDR)	High	[43-45]
HLA-DRB01, 04, 10 ('Shared Epitope')	High	[23, 26, 46, 47]
PTPN22	Medium	[22, 48-53]
TYK2	Medium	[22, 54]
STAT4	Medium	[22, 55]
NF-kB	Medium	[56, 57]
PADI4	Medium	[53, 58-63]
IL6R	Low	[64]
CTLA4	Low	[53]
COG6	Low	[65, 66]
RAD51B	Low	[67-69]
FCGR	Low	[70-72]
Polymorphisms within non-coding regions	Low	[73-75]
Epigenetics	Low	[76-78]
Non-genetic		
Female gender	High	[31, 32, 79-81]
Exposure to tobacco smoke	High	[33, 40, 82, 83]
Microbiota changes	Medium	[36, 38, 39, 84, 85]
Exposure to silica	Medium	[34, 86, 87]
Air pollution	Low	[88-90]
Body mass index/obesity	Low	[91-93]
Low vitamin D intake and levels	Low	[35, 94]
High sodium, red meat and iron consumption	Low	[95-97]
Alcohol consumption (negative association)	Low	[98-100]
Stress	Low	[101, 102]

Abbreviations: FDR: first degree relatives; HLA: human leukocyte antigen; PTPN22: protein tyrosine phosphatase, non-receptor type 22; TYK2: tyrosine kinase 2; STAT4: signal transducer and activator of transcription 4; NF-kB: nuclear factor kappa-light-chain-enhancer of activated B cells; PADI4: protein-arginine deiminase 4; IL6R: interleukin-6 receptor; CTLA4: cytotoxic T-lymphocyte associated protein 4; COG6: component of oligomeric Golgi complex 6; RAD51B: RAD51 Paralog B; FCGR: Fc gamma receptor.

The presence of ACPA, RF and other RA biomarkers can be detected years before the clinical onset of the disease [103-105], however, an additional 'hit' to the immune system is likely required to

initiate symptoms and transition into full disease. Although acute onset is possible, the pathogenesis of RA is known to begin years before the disease is clinically evident in most patients.

Based on the presence of symptoms and biomarkers, four disease stages could be defined (Figure 1). First, (1) an initial 'at-risk' phase for individuals that are seropositive and/or present familial or clinical risk factors but that do not show any signs of arthritis or other symptoms. Studies that provide additional insights and help understand the likelihood and timing of developing RA based on these risk factors will be of great utility. This initial stage would be followed by a (2) 'pre-clinical' phase (pre-RA), characterized by abnormalities in the levels of autoantibodies and other biomarkers in the absence of signs and symptoms of arthralgia or inflammatory arthritis. RF and ACPA are the major autoantibody systems investigated in the studies on this pre-clinical phase. More recently, anti-carbamylated protein (CarP) [105-107] and antibodies to specific citrullinated antigen targets [108-110] have also been examined. The study of pre-clinical RA changes in autoantibody affinity [111], avidity and isotype evolution [103, 112], as well as modifications on the immunoglobulins -such as glycosylation- and their implications are currently very active research areas. New knowledge on these topics and on novel autoantibody systems and inflammatory markers, will lead to a better understanding of RA autoimmunity and inflammation in this important phase, and might open new avenues for novel intervention strategies. After disease onset, two phases are typically differentiated based on disease duration: (3) 'early RA', usually for less than two years with the disease, and (4) 'established RA', when the disease has been present for longer periods of time. Throughout this process, different opportunities for stage- and individual-specific interventions can present.

Figure 1 RA disease stages based on the presence and duration of symptoms and biomarkers.

In this context, one important concept is the therapeutic "window of opportunity" [113], which refers to a period of time in which the disease is more manageable and the predicted outcomes are better than outside this period. This idea is supported by data that show that patients treated early show more favorable outcomes than patients treated later after symptoms onset, and that aggressive therapeutic approaches applied within this period can slow the rate of long-term structural damage [114-117]. Understanding the window of opportunity for each individual patient will allow clinicians to tailor treatment to the operating pathologic processes and represents one of the goals of precision medicine (PM) in RA.

The activation of the immune system and progression into RA is a complex process that involves interactions between components of both the adaptive and the innate immune pathways. It is believed that the loss of self-tolerance to citrullinated self-antigens is one of the key factors in the early development of RA. While in the past the focus to understand RA pathogenesis was primarily on inflammation, more recent studies suggest that autoimmunity *per se* helps modulate the disease and in particular, bone destruction. It has been suggested that autoantibodies play a key role in this process through their effect on immune cells and bone resorbing osteoclasts, facilitating bone resorption loss [8-10, 118].

Additionally, recent studies have found that mucosal and probably also systemic ACPA and RF immunoglobulin (Ig) A are commonly found in 'at-risk' individuals, sometimes in the absence of serum autoantibodies, and often in conjunction with inflammation and/or evidence of dysbiosis [119, 120]. In this context, Elliot et al. [111] recently described a persistent ACPA IgA response with continuous affinity maturation. These results suggested the presence of a persistent mucosal antigen that continually promotes the production of IgA plasmablasts, which during their affinity maturation and epitope spreading, could lead to the generation of ACPAs that bind additional citrullinated antigens. Altogether, these data support what is known as the 'mucosal origin hypothesis' [121]. This premise suggests that the disease originates in one or more sites in the mucosa and that the loss of the mucosal barrier function, together with the systemic spread of an ACPA IgG response, would represent key early events in the pre-clinical development of RA.

Finally, it was recently demonstrated for the first time that ACPA immunity can be initiated by anti-protein arginine deiminase (PAD) response. Immunization of mice with particular major histocompatibility complex (MHC) types with PAD enzymes generated a T-cell response to PAD and eventually triggered the later production of ACPA. The investigators concluded that their results suggest a hapten/carrier mechanism, in which the carrier is the PAD enzyme that citrullinates and the hapten is any protein being citrullinated and therefore bound by PAD [122]. Although replication, validation of the results in humans and further research are needed, the results of this study open new interesting ways to understand the pathogenesis of this complex disease and for novel interventional approaches.

1.2.3. Diagnosis

The diagnosis of RA is usually a complex and highly personalized process lead by the rheumatologist, in which clinical manifestations and serological results, including autoantibodies and acute-phase biomarkers, are considered. No diagnostic criteria are available due to the heterogeneity in the disease manifestations and to the potential consequences of a misdiagnosis. Instead, classification criteria for the disease were proposed for the first time in 1987 by the American College of Rheumatology (ACR) and the European League Against Rheumatism (EULAR) [123] and later revised in 2010 [124], which constitutes the current criteria. One of the weaknesses of the original criteria from 1987 is that it failed to identify patients with early disease who could benefit from early treatment. Two major changes that concern serological markers were added to the current revision: the addition of ACPA -discovered in 1998- as RA marker and the assignment of different scores based on autoantibodies levels. At least one serological result is needed for classification, but the current criteria do not contemplate the possibility of a weighted effect of the number of autoantibodies present in the score. Furthermore, RF and ACPA carry the same weight. Understanding whether this is clinically accurate would be very important. In this context, a recent letter to the editor published by Bossuyt [125] reported that the probability for RA with ACPA [cyclic citrullinated peptide (CCP) 2] increased from 3.4 to 73.6 (low versus 3× ULN value), which is significantly higher than reflected in the classification criteria (2 versus 3 points). Future refinements of the RA classification criteria may attribute a higher relative score to a high-positive ACPA compared with a low-positive ACPA and different weights to RF and ACPA.

These criteria might be used to inform the diagnostic decision making in clinical practice, but they are also intended to help stratify patients for clinical research. They were originally developed for individuals with at least one joint with definite clinically swelling that cannot be explained by any other disease. Numerical scores are assessed in four domains: joint involvement, serology, acute phase reactants and duration of symptoms (Table 2). The cut-off to classify a patient as RA is a score of 6 or higher, out of a maximum of 10 points.

Table 2 Summary of factors and associated scores considered for a RA diagnosis in the current ACR/EULAR classification criteria. Modified from Aletaha et al. *[126]*.

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

Abbreviations: RA: rheumatoid arthritis; ACR/EULAR: American College of Rheumatology/European League Against Rheumatism; RF: rheumatoid factor; ACPA: anti-citrullinated protein antibodies; ULN: upper limit of normal: CRP: C-reactive protein; ESR: erythrocyte sedimentation rate.

1.2.4. Prognosis and disease monitoring

Predicting disease prognosis is key not only for the patient but also for the clinician to make clinical and therapeutic decisions. However, unlike other diseases, one single biomarker does not reflect the severity of the disease. This, in addition to the complexity of the pathogenesis, makes it challenging to find reliable biomarkers of disease severity. To date, clinical disease activity is considered the best biomarker for this purpose.

A wide number of disease activity measures exist and are applied in clinical practice. A systematic literature review performed by the Rheumatoid Arthritis Clinical Disease Activity Measures Working Group started in 2008 resulted in the identification of a total of 63 RA disease activity measures. Six of them were selected for inclusion in the ACR-recommended RA disease activity measures published in 2012 based on accuracy, sensitivity to change, discrimination in different levels, having remission criteria and feasibility to be performed in a clinical setting [127]. The selection includes Clinical Disease Activity Index (CDAI), Disease Activity Score with 28-joint counts (DAS28), Patient Activity Scale (PAS), PAS-II, Routine Assessment of Patient Index Data with 3 measures (RAPID3), and Simplified Disease Activity Index (SDAI).

Among these, DAS28 is one of the most widely used, given the balance between its convenience for clinical practice, and its relevance and accuracy to reflect disease activity and progression. This score combines information from swollen joints, tender joints, acute phase response [measured with erythrocyte sedimentation rate (ESR) or C-reactive protein (CRP)] and patient self-report of general health. The DAS was originally developed in the early 1990s [128]. A few years later, in 1995, Prevoo et al. proposed the modified DAS28, which includes the assessment of 28 joints rather than 44 in the original DAS [129]. Despite the increasing reliance on DAS28, there is still some concern that the components of this score are subject to measurement error [130] and there is a need for higher levels of standardization and training for its use [131].

More recently, the ACR published an update of their recommendations for disease activity measures in RA [132]. An extensive systematic literature review lead to the identification of 47 RA disease activity measures this time. The screening process resulted in the selection of 11 measures that met a minimum standard for regular use and 5 measures that were preferred for regular use in most clinic settings. The measures not included in the previous recommendations include DAS (predecessor to DAS28), RAPID5 (related to RAPID3), Hospital Universitario La Princesa Index (HUPI), Multi-Biomarker Disease Activity (MBDA) score [133], Rheumatoid Arthritis Disease Activity Index (RADAI), and RADAI-5. Remarkably, for the first time, a cumulative score based on the serum concentrations of twelve serological biomarkers, the MBDA, is included in these recommendations [132, 133].

Both bone erosion and joint space narrowing are also important indicators of a bad prognosis and disability. Clinical assessment of the joints can be challenging and present with high variability. Imaging techniques such as plain film radiography, magnetic resonance imaging (MRI), X-Ray and ultrasound (US) are commonly used to evaluate this clinical feature. Numerical scores to quantify this outcome are not always calculated in clinical practice due to the difficulty, and the associated effort and cost. The total Sharp score (TSS) [134] is one of the most commonly used tool to measure disease burden. This scoring method, updated into the van der Heijde modified Sharp scoring [135], allows for separate scoring for bone erosions [joint erosion score (JES)], joint space narrowing [joint narrowing score (JNS)], and subluxation.

1.2.5. Treatment and disease management

In the current recommended therapeutic approach, captured in the ACR guidelines for the treatment of RA[136], the main therapeutic goals are remission or achievement of low disease activity, control of inflammation and symptoms, adaptation to treatment, prevention of joint and organ

damage, and improvement of physical function and overall well-being. In order to achieve these objectives, disease activity is tightly monitored and if one or more of the targets are not reached, a change of therapy is usually recommended. However, treatment assignment is commonly performed in a 'trial-error' fashion and it could significantly be improved.

In the last few years, advances in the field have allowed for a paradigm shift in the strategy towards a treat-to-target approach [137, 138]. Advancing PM in Rheumatology and in the management of autoimmune disease has become the focus of many groups and collaborative efforts. Several challenges are still present and further work is needed for its implementation in clinical practice, but with no doubt, this is the current objective and guiding principle for the management of RA.

Several treatment options with different mechanism of action (MOA) in the pathogenesis of the disease are available and new drugs are currently under development. Although there is a need for better patient stratification and more targeted strategies, the broad spectrum of therapies for the treatment and management of RA is one of the components that enables the mentioned paradigm shift towards the treat-to-target approach. There are four main categories of treatments (Figure 2):

- Nonsteroidal anti-inflammatory drugs (NSAIDs) such as ibuprofen (Advil, Motrin IB) and naproxen sodium (Aleve), that can relieve pain and reduce inflammation.
- Steroids, such as prednisone (Deltasone, Sterapred, Liquid Pred), that can help reduce inflammation and pain and slow joint damage. In most cases, they can be used temporarily to calm a symptom flare. Other steroids used in RA include hydrocortisone (Cortef, A-Hydrocort), prednisolone, dexamethasone (Dexpak Taperpak, Decadron, Hexadrol), and methylprednisolone (Depo-Medrol, Medrol, Methacort, Depopred, Predacorten).
- Non-biologic disease-modifying antirheumatic drugs (DMARDs), such as methotrexate, hydroxychloroquine (Plaquenil), leflunomide (Arava) or sulfasalazine (Azulfidine), which can slow the disease progression and prevent joint damage.
- Biologic agents, which represent the newest – first one was approved in 1998- and more complex type of DMARDs. Several agents are found in this category, and based on their target, there are five major types:
  - Tumor necrosis factor (TNF) inhibitors: certolizumab (Cimzia), etanercept (Enbrel or Benepali), Golimumab (Simponi), adalimumab (Humira), and infliximab (Remicade)
  - Interleukin (IL) blockers: IL-1 [anakinra (Kineret)], IL-6 [sarilumab (Kevzara), tocilizumab (Actemra)] and IL-17 [Secukinumab (Cosentyx)]
  - Agents that target B-cells: rituximab (Rituxan)
  - T-cell blockers: abatacept (Orencia)
  - Janus kinases (JAK) inhibitors: baricitinib (Olumiant) and tofacitinib (Xeljanz)

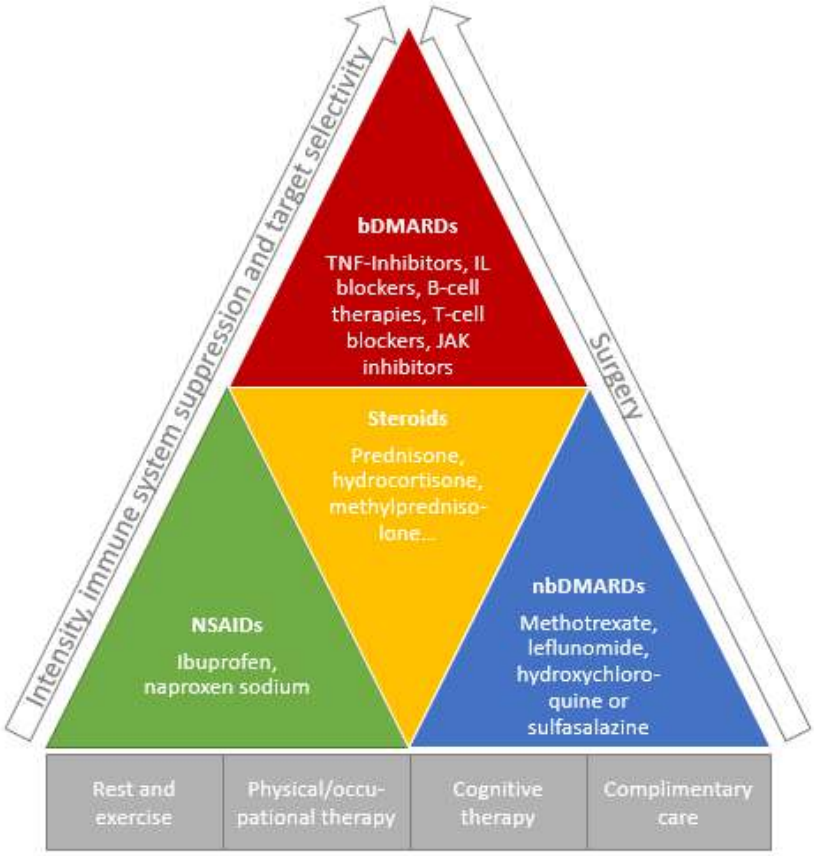

Figure 2 Overview of interventions and treatments in RA. The four main categories based on MOA and expected outcomes are shown inside the pyramid. Complimentary therapies are represented at the base.
Abbreviations: bDMARDs: biologic disease-modifying antirheumatic drugs; TNF: tumor necrosis factor; IL: interleukin; JAK: Janus kinases; MOA: mechanism of action; NSAIDs: nonsteroidal anti-inflammatory drugs; nbDMARDs: non-biologic disease-modifying antirheumatic drugs.

In general, the first line of treatment is usually methotrexate. NSAIDs are often used to help relieve the pain and inflammation. Complimentary therapies might also be recommended to the patient. Steroids can also help with this purpose and can be used temporarily to calm a flare. If these strategies are insufficient, doctors may switch to or add a different type of DMARD. When the non-biological DMARDS are not enough to easy the symptoms and inflammation, doctors may recommend a biologic agent. Combinations of therapies are a common approach in clinical practice as well as in clinical trials.

PAD inhibitors

Despite the availability of all these treatment options, many patients still do not respond or achieve remission with current therapies. Therefore, there is a need for better treat-to-target strategies and new therapies. In this respect, PAD inhibitors are emerging as a new class of drugs to treat RA. Different small molecule PAD inhibitors are currently being investigated [139, 140]. And although murine arthritis models have many limitations, the relationship between PAD4 and the incidence of induced arthritis models is currently being investigated in several animal studies.

Willis et al. first demonstrated the potential for the pan-PAD inhibitor Cl-amidine to prevent arthritis in a mouse collagen-induced arthritis (CIAr) model [141], and later on, confirmed robustly that PAD4 inhibition alone is sufficient to block murine arthritis clinical and histopathological end-points [142]. Selective PAD inhibitors are also being investigated. Mice treated with BB-Cl-amidine, a molecule that exhibits increased potency against PAD2, resulted in reduced joint inflammation and destruction to a greater degree as compared to Cl-amidine in mice with CIAr [143, 144]. Furthermore, treatment of mice with GSK199, a PAD4 selective inhibitor, significantly reduced arthritis severity in CIAr and a subset of ACPA were reduced, however, global citrulline levels and circulating anti-collagen II antibody levels were not affected [142].

Beyond small molecule PAD inhibitors, several groups are developing monoclonal antibodies specific for PAD2 or PAD4, and others are working on PAD deficient or knockout murine models. Seri et al. [145] recently showed that PAD4 deficiency resulted in reduced arthritis severity in a glucose-6-phosphate isomerase-induced arthritis model. And Suziki et al. [146] found a similar outcome in PAD4 knockout mice.

Although still in a very early phase, PAD inhibitors represent a new promising approach in the treatment and management of RA. The development of more selective PAD inhibitors will help better understand the role of the individual PAD enzymes in RA and their potential as therapeutic targets. In this context, anti-PAD antibodies may emerge as useful biomarkers to stratify patients for prediction or monitoring of response to this new therapeutic approach.

1.2.6. Health economics

RA is a disabling disease that usually encompasses a substantial economic burden on both the patient and the health care system. The burden on the patient is not only associated with increased direct medical costs but also linked to loss or reduction of employment, due to reduced work capacity and/or functional disability. It has been calculated that RA patients incur $19.3 billion in direct and indirect costs in the United States annually [147] and that this disease poses a considerable incremental burden on the United States health care, primarily driven by incremental pharmacy expenditure [148]. Although the numbers can vary significantly based on geography, the economic burden associated to this disease is broadly accepted. Remarkably, a recent study described that RA-associated total expenditures were 51% higher among ACPA positive patients compared with those without this biomarker [149], indicating that this biomarker could also be useful in patient stratification based on health economic aspects.

1.3. The PAD enzymes and citrullination in RA

Post-translational modifications (PTM), those that occur after the ribosomes translate mRNA into polypeptide chains, are known to play a central role in autoimmune diseases. They can promote the generation of neo-(auto)antigens and help trigger the autoimmune response. One of these PTM with a central role in RA is citrullination. In normal physiological conditions, it is very important in key cellular processes such as apoptosis, organization of structural proteins [150, 151] and gene regulation, especially during early embryonic development [152, 153]. Citrullination consists on the conversion of the amino acid arginine into citrulline (Figure 3), resulting in the loss of a positive charge and the gain of 1 Dalton (Da) in mass [154]. For this reason, citrullination can increase protein

hydrophobicity, and can result in a change of the conformational structure of the protein, unfolding [155], and alterations of the intra- and inter-molecular interactions.

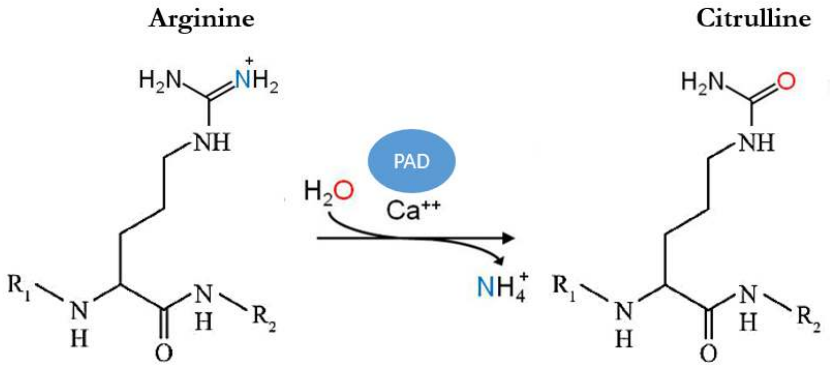

Figure 3 Citrullination of peptidyl-arginine by protein-arginine deiminase (PAD) enzyme and conversion intro peptidyl-citrulline.

This process is mediated by a member of the PAD family and it is an irreversible change. The activity of the PAD enzymes is stringently regulated in normal conditions, however, dysregulation of the citrullination pathway can occurs and it is associated with several diseases, including several types of cancer [156], neurodegenerative diseases [157-161], and autoimmunity [162]. Particularly relevant is the case of RA, characterized by hypercitrullination, accumulation of citrullinated products, and the presence of antibodies to the modified antigens present in the joints.

1.3.1. The PAD enzymes

The PAD enzymes were described for the first time in 1977 by Rogers et al. [163]. A total of five members of the PAD family have been reported in humans: PAD1, 2, 3, 4, and 6 (reviewed in [164], Table 3), with a significant protein sequence homology between them (Table 4).

Table 3 Overview of the human PAD family and their characteristics.

PAD family member (UniProt ID)	Mass (kDa)	Length (AA)	Substrate	Subcellular location	Tissue and cellular expression under normal conditions	Physiological roles	Ref.
PAD1 (Q9ULC6)	74.7	663	Keratin K1, filaggrin	Cytoplasm	Epidermis, prostate, testis, placenta, uterus, spleen and thymus	Skin differentiation, terminal differentiation of keratinocytes	[164-167]
PAD2 (Q9Y2J8)	75.6	665	Vimentin, MBP, GFAP, β and γ-actins, histone H3, fibrinogen, collagen II, alpha-enolase	Cytoplasm, may become nuclear in human mammary epithelial cells	Skeletal muscle, brain, pancreas, glial cells, macrophages, bone marrow, muscle, breast, colon, embryo, eye, kidney, epidermal, uterus, thymus	May plain a role in brain development, innate immune defense, female reproduction, gene expression	[160, 164, 168-179]
PAD3 (Q9ULW8)	74.7	664	Trichohyalin, fillaggrin	Cytoplasm	Hair follicles and keratinocytes	Skin differentiation, hair follicle formation, terminal differentiation of keratinocytes	[163, 164, 167, 180, 181]
PAD4 (Q9UM07)	74.1	663	Histones H2A, H3, H4, vimentin, p300, nucleophosmin /B23, ING4, fibrinogen, collagen II, alpha-enolase	Nucleus and cytoplasmic granules (eosinophils, neutrophils)	Eosinophils, neutrophils, granulocytes, macrophages, several cancerous tissues	Chromatin decondensation, transcription regulation, tumorigenesis, cellular differentiation, transcriptional corepressor for the estrogen receptor and p53, NETs formation	[164, 172-174, 182-190]
PAD6 (Q6TGC4)	77.7	694	Keratin	Cytoplasm	Egg, ovary, early embryo, thymus, oocyte, peripheral blood leukocytes	Embryonic development, oocyte cytoplasmic lattice formation, fertility	[164, 191-194]

Abbreviations: PAD: protein-arginine deiminase; kDa: kilo Dalton; AA: amino acids; MBP: myelin basic protein; GFAP: glial fibrillary acidic protein; ING4: Inhibitor of growth protein 4; NETs: neutrophil extracellular trap.

The members of the PAD family differ in their substrate specificities and tissue-specific expression. Studies suggest that these enzymes have the capacity to select unique protein targets and that this capability may play a role in autoantigen selection in RA [175]. To date, of the five PAD family members, three of them, PAD2, PAD3 and PAD4, have been identified as antigenic targets in RA (see section 1.4.2.).

Table 4 Protein sequence homology between the PAD2, 3, and 4 enzymes, identified as antigenic targets in RA. Data was generated by BLASTp analysis *[195]* using the FASTA sequences from UniProt (The UniProt Consortium). UniProt IDs can be found in Table 3.

Proteins compared	**PAD2 *vs.* PAD3**	**PAD2 *vs.* PAD4**	**PAD3 *vs.* PAD4**
Identities	345/667 (52%)	337/668 (50%)	374/669 (56%)
Positives	452/667 (67%)	440/668 (65%)	461/669 (68%)
Gaps	5/667 (0%)	8/668 (1%)	11/669 (1%)
E value	0.0	0.0	0.0

Abbreviations: PAD: protein-arginine deiminase.

The PAD enzymes require calcium for their catalytic activity. The Ca^{++} concentration in the extracellular fluid ranges between 0.49-0.98 mM in synovial fluid and 1.1-1.3mM in plasma [196]. On the other hand, the Ca^{++} concentration inside the cells is usually kept at approximately 1 nM [197], but it is subject to increases of 10- to 100-fold during various cellular functions. The intracellular calcium level is kept relatively low with respect to the extracellular fluid.

Daamgard et al. observed that the calcium requirement for half-maximal activities of PAD2 and PAD4 were between 0.35 and 1.85 mM, and that synovial fluid contained sufficient calcium levels for citrullination [198]. The calcium concentration typically used in *in vitro* citrullination assays for maximum PAD4 activation ranges between 5-10 mM. However, half-maximal PAD activity has been reported at calcium concentrations ranging from 40 μM [199, 200] to 3.3 mM [201]. In this context, it is important to mention that the methods for calcium quantification and for PAD activity measurement are very important factors and often, sources of discrepancies. If it was confirmed that the concentrations of calcium identified for maximum PAD4 activity *in vitro* are much higher than the extracellular calcium concentrations *in vivo*, this would suggest the existence of additional factors that modulate this process during normal physiology and in the pathogenesis of RA.

The human PAD family members

PAD1 is mainly expressed in the cytoplasm at the epidermis [165] and uterus, and keratin is the preferred substrate.

PAD2 is the most broadly expressed isotype, found in skeletal muscle, brain, spleen and secretory glands [164]. Regulated both at the transcriptional and the translational level, the known substrates for PAD2 are myelin basic protein (MBP) in the central nervous system and vimentin in skeletal muscle and macrophages [170]. Moreover, studies suggest a tissue-specific hormonal regulation of PAD2 expression [169, 202]. Although PAD2 is mainly localized in the cytoplasm, data suggest that a fraction of PAD2 can also be found in the nucleus despite lacking a nuclear translocation signal, and that this nuclear PAD2 may citrullinate histones H3 and H4 and play a role in gene regulation [179].

On the other hand, PAD3 can be found in hair follicles, it has a cytoplasmic intra-cellular localization and its natural substrate, trichohyalin, is a major structural protein of inner root sheath cells of hair follicles [155, 164].

PAD4, unlike the other members, is mainly found in the nucleus and expressed in white blood cells (granulocytes, monocytes). For this reason this protein can be detected in several tissues [164]. In addition to upregulated enzymatic activity, PAD2 and PAD4 are overexpressed by neutrophils and monocytes in the synovium of RA patients in co-expression with numerous citrullinated proteins [203]. PADI4 has been confirmed as a susceptibility gene for RA [53, 58, 59, 204] and an association between single nucleotide polymorphisms (SNPs) in this gene and RA has been reported in numerous ethnic groups [60, 205, 206]. However, these findings have not been replicated in certain populations [207, 208]. Besides, it has been reported that the PAD4 enzyme can autocitrullinate itself influencing the enzyme structure and immune response, contributing to the regulation of citrullinated proteins generation during cell activation [209]. The clinical implications of these observations need to be further explored. A relatively broad range of targets has been described for PAD4, with certain overlap with PAD2 (Table 4). The enzymes´ specificity for cellular substrates and synthetic peptides seems to be different for PAD2 and PAD4, with the latest being more restricted by the amino acid composition surrounding the acceptor arginine residue [210]. It is unclear whether one of the two proteins dominates in the generation of citrullinated self-proteins that are targeted by ACPAs. Interestingly, a recent study showed that very high-titers of ACPA preferentially bind fibrinogen citrullinated by PAD4 vs. PAD2 [211]. In a more recent publication, both enzymes seemed to be equally efficient in generating citrullinated targets for ACPAs on fibrinogen and enolase but autoantibodies to histone H3 in these patients seemed to have a preference for the antigen citrullinated by PAD4 [173].

The last member of the PAD family, PAD6 [192-194], is localized in egg cytoplasmic sheets, structures that are only found in mammalian egg cells and early embryos and that undergo reorganization in early stages of development. The knowledge on PAD6 is rather limited and in contrast to PAD1, PAD2, PAD3, and PAD4, that are catalytically active, it is unclear whether PAD6 is too [191, 212].

Porphyromonas gingivalis PAD (PPAD)

The identification of a prokaryote PAD enzyme in *P. gingivalis* [213], the most common pathogen in periodontal disease, illustrates the high conservancy of this enzyme in evolution and denotes an additional link between infection and autoimmunity. To date, this pathogen is the only prokaryote described to express a functional bacterial PAD. Several studies have demonstrated that periodontal disease is an important risk factor for RA [36, 37], and suggest that PPAD may represent a mechanistic link between periodontitis and RA [84].

Data also suggest that PPAD can perform protein citrullination at the inflamed periodontal sites, and that it can citrullinate bacterial and host proteins [214], and trigger a cascade of events that can lead to neutrophil extracellular traps (NETs) formation, generation of citrullinated neoantigens, and ACPA production [85]. These findings may suggest a potential etiological role for *P. gingivalis* in RA through the generation of citrullinated antigens that drive the autoimmune response. Recently, antibodies to PPAD were described in RA patients [215], however, the association between these antibodies, ACPA and their potential role in the development of RA needs to be further investigated.

1.3.2. Citrullination

In 1998, Schellekens and colleagues reported for the first time the presence of antibodies that targeted an –at the time- unusual amino acid, citrulline, in the sera of RA patients [216]. These antibodies are what we know now as ACPA. This finding aroused a major interest around the world to understand the clinical meaning of this new type of autoantibodies and the role of citrullination in the pathogenesis of RA. Since then, citrullination and the associated autoantibodies have been extensively studied and major advances have been made. ACPA are now recognized as one of the main biomarkers and part of the classification criteria for RA [124].

In addition to this important role of citrullinated proteins as major antigenic targets in RA, citrullination and the PAD enzymes play a role during apoptosis, autophagy and NETs formation (known as NETosis), processes well-known for their involvement in autoimmunity [217]. During infection or inflammation, PAD4 becomes activated in neutrophils resulting in the citrullination of multiple autoantigens and the ejection of chromatin from the cell, generating the so-called NETs, important in the protection against infection [218]. Citrullination of histones represents an important step in this process [219]. The release of NETs and PADs by the neutrophils is likely followed by citrullination of extracellular antigens that together with the infectious agents, the complement activation and the formation of immune complexes, could be challenging the immune system and potentially compromising the immune tolerance. Once tolerance is broken, the presence of additional 'hits' to the immune system and/or additional co-factors that increase PAD activity (such as autoantibodies[220]) could be helping to maintain efficient citrullination and contributing to trigger autoimmunity. This process could represent one of the links between infection and autoimmunity.

Moreover, smoking is highly associated with ACPA-positive RA and a link between exposure to cigarette smoke and induction of citrullination in the lungs, even prior to any inflammatory response, has been suggested [83, 221]. Smoking is known to trigger HLA-DR-restricted immune reactions to autoantigens modified by citrullination [82] and to enhance PAD2 expression and protein citrullination in the bronchial mucosal and alveolar compartment [222].

Lastly, it was recently demonstrated that PAD4 directly citrullinates NF-κB, which has a critical role in the expression of pro-inflammatory cytokines IL-1β and TNFα, providing a molecular mechanism by which citrullination propagates inflammation in RA [223]. This not only represents an interesting insight in the pathogenesis of RA, but also offers therapeutic potential.

Consequently, during the past few years, the PAD enzymes have started to gain more and more attention, especially as novel therapeutic targets. Despite this progress, the role of citrullination, the PAD enzymes and ACPA in the pathogenesis of the disease is still not fully understood and these topics currently represent very promising and potentially impactful research areas in RA.

1.3.3. Other post-translational modifications in RA

In addition to citrullination, other PTM and the associated antibody systems have been described in RA, including acetylation and anti-acetylated protein antibodies [224], lipid peroxidation, anti-malondialdehyde-acetaldehyde (MAA) and anti-malondialdehyde (MDA) antibodies [225], pepsin

cleavage and anti-hinge antibodies [226, 227], and probably most importantly after ACPA, carbamylation and anti-CarP antibodies [228].

Unlike citrullination, carbamylation is a non-enzymatic post-translational modification mediated by cyanate in the form of isocyanic acid, in which lysine residues get converted intro homo-citrulline, very similar in structure to citrulline, with the first one being one CH_2 group longer. In a similar way to citrullination, carbamylation also results in the loss of a positive charge, with the associated consequences in protein structure and function. It has been shown that several conditions have an involvement of carbamylated protein accumulation in their pathology, including inflammation [229], aging [230], chronic kidney disease [231], asthma [232], and cardiovascular disease [231, 233].

Antibodies that target homocitrulline containing antigens were reported for the first time in RA by Shi et al. [228] and since their discovery, many studies have been performed to understand their clinical relevance [234]. Moreover, citrullination and carbamylation at the fragment crystallizable (Fc) of IgG where RF binds were recently reported in RA [235], linking the three major autoantibody systems in this disease. The clinical relevance of anti-CarP antibodies will be discussed in section 1.4.2.

1.4. Serological markers

1.4.1. Classical markers

RF

The first and only marker included in the 1987 ACR/EULAR RA classification criteria until its update in 2010 was RF, an autoantibody that targets the Fc region of IgG. Contrary to most biomarkers in autoimmunity, the IgM isotype is the most prevalent and clinically useful marker in RA diagnosis. It is found in approximately 70-80% of patients with confirmed RA [236]. In addition, elevated levels of RF IgA and IgG have also been reported in patients with RA [237, 238]. For RF IgA a prognostic value has been proposed given its association with disease severity [239-241]. The clinical utility of RF IgG remains unclear, but some studies suggest that elevated levels of RF IgG are highly specific for RA diagnosis [238, 242]. RF is known for its low specificity due to its presence in patients with infections and other autoimmune diseases [236], as well as in a proportion of healthy individuals [243], with rates between 10-25% in elderly patients without RA [236]. It has been proposed that the detection of all three RF isotypes improves the specificity and predictive value of RF testing [244], however, this is currently not the most common approach in clinical practice and is quite variable depending on the geography.

It is now pretty well-established that RF precedes the onset of RA and can be found in the pre-clinical phase [104, 112]. The association of this marker with inflammation and disease activity has been studied for over thirty years now [245, 246] and more recently, the utility of this marker for prediction of response to treatment was suggested [247-249].

The approach in the study of the role and clinical meaning of RF in RA has evolved over time towards the analysis considering the interactions with ACPA, rather than studying the two antibodies separately. Within this context, the pro-inflammatory role of RF, in conjunction with ACPA, was recently demonstrated. The combined presence of RF IgM and ACPA mediates increased production of pro-inflammatory cytokines *in vitro* and is associated with elevated systemic inflammation and disease activity in RA [250-252].

ACPA

Since the discovery in 1998 [216], ACPA soon became the main biomarkers in RA and were included in the 2010 classification criteria [124]. Several citrullinated proteins have been identified as autoantigens targeted by ACPA, including extracellular proteins (filaggrin, collagen II, fibrinogen, and calreticulin), cytoplasmic proteins (enolase and vimentin), membrane-associated proteins (myelin basic protein), and nuclear proteins (histones) [253]. ACPA are generally detected with anti-CCP antibody assays. To date, three generations of anti-CCP assays with reported differences between them [125, 254-258] are commercially available.

The ACPA response is known to dynamically evolve before the onset of RA. Ongoing affinity maturation and epitope spreading that contribute to the propagation of the ACPA response have recently been reported in established RA [111, 259].

ACPA are known to be highly specific for RA and to have a higher diagnostic value than RF in early RA [260]. Some RA patients can present antibodies of the IgA isotype in the absence of ACPA IgG [254]. Similarly to other autoantibodies in RA, ACPA can be detected up to 10 years before the disease onset [103, 112], remarking its utility for prediction of development of RA. Interestingly, high levels of ACPA have been described in the serum [261-263] or sputum [119] of first-degree relatives (FDR) of RA patients and for this reason, an application as biomarker for screening and identification of individuals at risk of developing RA has been proposed. This is the concept behind one of the currently ongoing RA prevention trials, StopRA [264] (Table 6). However, the prevalence of RA is low in ACPA positive individuals [265], not all ACPA positive individuals will eventually develop the disease and for those that will do, the time frame at which this will occur is still unpredictable. For these reasons, additional studies that investigate these concepts will be of great interest.

Historically, the clinical utility of this valuable biomarker has gone beyond diagnosis. The presence of ACPA is associated with a more severe disease, joint damage and extra articular manifestations [266, 267], and a pathogenic role for these antibodies has been repeatedly suggested [268]. ACPA can induce TNF-α production [269-271], osteoclastogenesis and are associated with complement activation [252, 272, 273] and NETosis, inflammation [274], and oxidative stress [275]. These findings indicate that ACPA can be a useful biomarker in prognosis and patient stratification. An association between ACPA and response to treatment has also been suggested [248, 249, 276]. Nevertheless, this paradigm might be changing due to a better understanding and management of ACPA positive RA [277].

The serological gap in RA

RF and ACPA are widely used in the diagnosis and classification of RA patients and the combination of results from these markers might provide further value in the management of RA. However, despite the established clinical value of these autoantibodies, up to 40% of RA patients are seronegative for RF and ACPA. This leaves a serological gap in this disease [3], that contributes to the difficulty of the RA diagnosis and the correct classification of these patients. Moreover, patients with seronegative RA usually experience a delay in diagnosis and in treatment initiation, and are less likely to attain remission, suggesting that the window of opportunity is more frequently missed in these

individuals[278]. For these reasons, the search for novel RA markers that help to close this serological gap and improve diagnosis and patient stratification, as well as prognosis, response to treatment or monitoring of the disease, has been a very active and exciting research area [279].

1.4.2. Novel markers

Anti-PAD antibodies

Anti-PAD4 antibodies

Over the past years, it has been demonstrated that in addition to its role in citrullination, PAD4 is also an autoantigen in RA targeted by anti-PAD4 IgG [61, 280-282][1]. Several studies, in which typically enzyme-linked immunosorbent assay (ELISA) or immunoprecipitation (IP) methods were used for the detection and quantification of these autoantibodies, have examined their sensitivity and specificity for RA diagnosis with independent cohorts [61, 220, 280-282, 284-298]. A summary of these studies can be found in Table 5. Anti-PAD4 antibodies are usually found in a subgroup of RA patients with a prevalence of 20-45%[286] and are associated with the presence of ACPA [281, 288, 299]. A recent meta-analysis [300] showed a pooled sensitivity and specificity of anti-PAD4 antibodies for RA of 38.0% and 96.0% and good discrimination between RA and controls has consistently been observed. Yet, whether these antibodies have an added value to RF and ACPA for the identification of RA patients and the diagnosis of this disease remained unexplored in a systematic way until recently.

The effect of these autoantibodies on the enzyme's functionality and activity is not completely understood. Some data indicate that anti-PAD4 antibodies seem to inhibit the enzymatic activity of the protein [301]. In the same study, peptides located in both the N-terminal (211-290) and the C-terminal domains (601-650) of the protein were recognized by anti-PAD4 positive sera from RA patients [301]. Other researchers had previously investigated the epitope(s) localization of PAD4 using truncated versions of the protein and anti-PAD4 positive sera from RA patients [61]. However, neither these results nor the data from the study by Auger et al. have been reproduced. The exact location, sequence and structure of the epitopes recognized by anti-PAD4 antibodies remain unidentified.

Anti-PAD4 antibodies have been reproducibly associated with a more severe and aggressive form of RA [61, 220, 281, 286, 294]. Variable associations of these antibodies with measures of inflammations, disease activity or erosion have been repeatedly reported, indicating a clinical value of anti-PAD4 antibodies as a prognostic biomarker that could help stratify patients based on severity of the disease.

Despite this association of anti-PAD4 antibodies with disease severity and the independent link between smoking and ILD, little is known about whether there is an etiological association of cigarette smoking and the development of these antibodies. Although differences in terms of smoking profile between the anti-PAD4 positive and negative patients have been reported [220, 302], very

[1] To date, only one abstract has described the presence of anti-PAD4 IgA antibodies in serum and interestingly, in sputum, where the IgA isotype was more prevalent than the IgG.
283. Demoruelle MK, W.H., Davis RL, Marin AI, Norris JM, Holers VM, Deane KD, Darrah E. , *Anti-Peptidylarginine Deiminase-4 Antibodies Are Present in the Sputum of RA Patients and Can Activate Peptidylarginine Deiminase-4 Enzyme Activity [abstract]*. . Arthritis Rheumatol., 2017. **69** (suppl 10).

recent data have indicated that there is not a direct link between smoking and the development of anti-PAD4 antibodies [298].

Interestingly, anti-PAD4 can precede the onset of the disease and are found in a pre-clinical phase in a subset of RA patients [288], suggesting a predictive value of this biomarker. The exact timing of appearance of anti-PAD4 antibodies during the evolution of RA still requires systematic studies. In a study using a military cohort with samples collected over decades, it was demonstrated that anti-PAD4 antibodies might occur after ACPA and RF. Since immunoprecipitation -which sometimes lacks sensitivity- was used for the measurement of anti-PAD4 antibodies in this study, these findings should be verified with other methods and in other cohorts. Only then it will be possible to conclude whether anti-PAD4 antibodies arise after ACPA and RF and whether they hold value in the prediction and potential prevention of RA. The finding that anti-PAD4 antibodies might appear later is also somewhat controversial to the findings that immunization with PAD enzymes can stimulate the production of ACPA in mice [122]. However, this might be explained by the fact that animal models not always translate to human disease, especially when generated via immunization (*vs.* spontaneous models). Additional insights will also come from the sequencing of the B-cell repertoire of RA patients and the subsequent analysis of potential mutations [111, 303]. Pollman et al. [299] studied the levels of anti-PAD4 in RA patients over a period of 10 years and demonstrated that anti-PAD4 positive patients remained positive over time, and some patients that initially did not present these antibodies became positive later in the disease course.

The potential effect of treatment on the levels of anti-PAD4 is also an area of interest. Limited data have indicated that anti-TNF-α therapy does not impact the levels of anti-PAD4 antibodies over a 12-month period time. However, this could be indicative of a specific phenotype characterized by inadequate response to this treatment type [299]. Somewhat contrary to these findings, Darrah et al. recently reported that although anti-PAD4 positive patients are characterized by a worse radiographic joint damage at baseline, they respond more favourably to treatment escalation therapy, that was more effective in slowing the progression of the disease and decreasing disease activity [296]. Significant differences in study design between these two studies could explain the discrepant outcomes. Additional studies in larger cohorts treated with drugs based on different mechanisms of action are needed to further investigate a potential effect of therapy on these antibodies and their associations with treatment response.

Table 5 Summary of studies on anti-PAD4 antibodies in RA, including number and origin of patients, detection method used and diagnostic findings.

Study, year of publication	Number of RA patients	Origin of RA patients	RA stage	Number of controls	Controls composition	Detection method	Sensitivity	Specificity
Nissinen et al., 2003 [280]	57 / 51	NP	57 early (baseline and follow up 3 years later); 51 established	172	43 SLE, 19 pSjS, 20 MS, 90 HI (BD)	ELISA	Early: 88% at baseline, 70% at follow up; Established: 22%	76.2%
Takizawa et al., 2005 [284]	42	NP	Established	82	19 SLE, 23 other rheumatic diseases, 40 HI	ELISA and WB	50%	95%
Roth et al., 2006 [285]	184	Rheumatological Unit of Malmo University Hospital	Early	59	HI (BD)	ELISA	31% all patients (25% MTX, 35% non-MTX)	97%
Zhao et al., 2008 [286]	109	Department of Rheumatology and Immunology, People's Hospital, Peking University, Beijing	Established	338	67 SLE, 48 pSjS, 41 SSc, 34 OA, 23 DM/PM, 19 AS, 106 HI (DB)	ELISA	45%	94%
Harris et al., 2008 [61]	38 / 129	John Hopkins Arthritis Center / ESCAPE RA TRIAL	Established	158	32 HI, 31 myositis, 31 SSc, 32 SjS, 32 SLE	IP	42% /36%	99%
Halvorsen et al., 2008 [281]	237/177	EURIDISS RA cohort / Oslo RA Register	Established	232	84 SLE, 148 HI	ELISA	22% / 25%	91.4%
Halvorsen et al., 2009 [287]	40	NP	Established [at baseline (n=40) and follow up after one year (n=33)]	NP	NP	ELISA	42.5% at baseline, 45.5% at follow up	NP
Auger et al., 2009 [282]	116	Rheumatology Unit La Conception Hospital, Marseilla, France	Established	93	33 AS, 60 HI	ELISA	29%	98%
Kolfenbach et al., 2010 [288]	83	Military Cohort - Walter Reed Army Medical Center Rheumatology Clinic	Preclinical	83	83 HI	IP	18%	99%
Wang et al., 2011 [289]	102	NP	Established [with active disease (n=50), without active disease (n=52)]	239	84 SLE, 35 pSjS, 20 SSc, 100 HI	ELISA	32.40%	96%
Ishigami et al., 2013 [290]	32	Department of Rheumatology, Tokyo Metropolitan Geriatric hospital and Institute of Gerontology	NP	30	10 OA, 20 HI	ELISA	37.50%	100%

Study, year of publication	Number of RA patients	Origin of RA patients	RA stage	Number of controls	Controls composition	Detection method	Sensitivity	Specificity
Ferucci et al., 2013 [291]	82	Two indigenous North American populations in Canada and the United States; First Nations or Alaska Native people	Established	191	147 FDR, 44 HI	IP	29.30%	98.90%
Darrah et al., 2013 [201]	194	ESCAPE RA Cohort	Established	66	36 HI, 30 PsA	IP	37.10%	NP
Reyes-Castillo et al., 2015 [292]	170	Rheumatology service of two hospitals in Jalisco, Mexico	Early and established	103	103 HI	ELISA	24% (18% early, 29% established)	95%
Umeda et al., 2015 [293]	148	University of Tsukuba Hospital	Established	113	36 SLE, 37 SjS, 40 HI	ELISA	20%	89%
Navarro-Millan et al., 2016 [294]	192	CLEAR Registry	Early and established	NP	NP	IP	24%	NP
Martinez-Prat et al., 2018 [295]	*640*	*MTX PATH, IMPACT and CAPITAL studies*	*Early and established*	*833*	*369 SLE, 64 Sjs, 33 SSc, 29 IIM, 14 OAARD, 85 FMS, 42 OAID, 197 HI*	*PMAT*	*35% (16.% early)*	*95.4% (99.1% early)*
Darrah et al., 2018 [296]	282	VACSP, RACAT study	Established	NP	NP	IP	26%	NP
Guderud et al., 2018 [297]	745	EURIDISS cohort, ORAR and a cohort early RA patients undergoing MRI, a cohort of patients starting TNFi therapy	Early and established	70	70 HI	DELFIA	25.90%	Information not provided
Cappelli et al., 2018 [298]	274	John Hopkins Arthritis Center	Established	NP	NP	IP	25%	NP

Abbreviations: NP: not provided; ESCAPE: Evaluation of Subclinical Cardiovascular Disease and Predictors of Events in Rheumatoid Arthritis; CLEAR: Consortium for the Longitudinal Evaluation of African Americans with Early Rheumatoid Arthritis; MTX: methotrexate; VACSP: Veterans Affairs Cooperative Study Program; RACAT: Rheumatoid Arthritis Comparison of Active Therapies; EURIDISS: European Research on Incapacitating Disease and Social Support; ORAR: Oslo Rheumatoid Arthritis Register; TNFi: tumor necrosis factor inhibitor; RA: rheumatoid arthritis; SLE: systemic lupus erythematosus; pSjS: primary Sjogren's syndrome; MS: multiple sclerosis; HI: healthy individuals; BD: blood donors; SSc: systemic sclerosis; OA: osteoarthritis; DM: dermatomyositis; PM: polymyositis; AS: ankylosing spondylitis; FDR: first degree relatives; PsA: psoriatic arthritis; IIM: idiopathic inflammatory myopathies; OAARD: other ANA-associated autoimmune rheumatic diseases; FMS: fibromyalgia syndrome; OAID: other autoimmune diseases; ELISA: enzyme-linked immunosorbent assay; WB: western blot; IP: immunoprecipitation; PMAT: particle-based multi-analyte technology; DELFIA: dissociation-enhanced lanthanide fluorescence immunoassay.

Anti-PAD3 antibodies

PAD3 was also recently identified as an antigenic target in RA. This enzyme, shown to be expressed by peripheral blood neutrophils and capable of citrullinating intracellular proteins [175], is also recognized by autoantibodies in RA patients [220]. Competition experiments demonstrated that this subset of anti-PAD3 antibodies cross-react with PAD4 and a cross-reactive (XR) epitope between these two enzymes was identified [201]. Not as prevalent as anti-PAD4, the anti-PAD3/4 XR antibodies seem to be present in 10-20% of RA patients and until very recently, they had only been found in anti-PAD4 positive patients. Similar to anti-PAD4, anti-PAD3 antibodies are also associated with ACPA and the HLA-DRB1 SE [220].

Interestingly, when compared to the anti-PAD3/4 negative patients or the anti-PAD4 positive only, these subset of anti-PAD3/4XR antibodies seem to be associated with the most erosive joint disease [220, 294] and specifically correlated with JES [304]. Additionally, an association between the XR antibodies and RA-ILD has been reported, suggesting clinical utility of this biomarker for prediction of this comorbidity in RA patients [302].

Although the features of these antibodies remain to be defined, some data suggest that affinity maturation has a role in defining their function [305]. In addition, it has been shown that they increase catalytic efficiency of the PAD4 enzyme by decreasing the calcium concentration needed for its activation. By mimicking the calcium-ion binding to the enzyme, they support protein citrullination at physiologically relevant calcium concentrations. In this model, these antibodies would represent important drivers of dysregulated protein citrullination and RA pathogenesis.

Anti-PAD2 antibodies

Several factors point to PAD2 as an important driver in RA, including the association of PADI2 polymorphism with the development of the disease, the expression of this enzyme in the tissue and synovial fluid from inflamed joints and its capacity to generate citrullinated autoantigens [306, 307]. Yet, PAD2 had not been identified as a target of the immune response in RA until very recently, when Darrah et al. described for the first time the presence of antibodies targeting this protein in the sera of RA patients [308]. In contrast to anti-PAD4 or to the anti-PAD3/4 XR antibodies, anti-PAD2 antibodies seem to be characteristic of a genetically and clinically distinct subtype of RA patients with less severe baseline joint inflammation, slower joint disease progression and less lung disease. Together with the lack of association between anti-PAD2 and ACPA or anti-PAD3/4 XR antibodies that was observed in the mentioned study, these data suggest an added prognostic value of these biomarker in patient stratification.

Anti-CarP antibodies

Antibodies targeting carbamylated proteins were described in RA for the first time in 2011 and were identified in both ACPA positive and negative patients [228]. Over the past few years, several targets of these antibodies have been identified, including vimentin [309, 310], albumin [311], alpha-enolase [312], 78-kDa glucose-regulated protein [313], and alpha 1 anti-trypsin (A1AT) [314]. In addition to this variety in the target recognition, the anti-CarP antibody response seems to be

differently regulated with respect to ACPA and uses a broad spectrum of isotypes and IgG subclasses [315].

Upon their discovery, there was an initial excitement around their potential utility in early diagnosis, to help close the serological gap in RA or as a predictive biomarker, given that they could be found before the onset of the disease [105, 316, 317]. Nevertheless, their presence in other rheumatological diseases [318-322] and in other forms of arthritis [323], and the associated relatively low specificity, limit their diagnostic utility in RA. Furthermore, although some data indicate that the triple combination with RF and ACPA could help identify individuals at risk for the development of RA[316], their added value to ACPA and RF for the diagnosis of this disease still hasn't been validated [324].

In spite of this limited diagnostic value, anti-CarP antibodies have a prognostic value in RA thanks to their association with disease severity and joint damage [324-328], in particular, decreased bone mineral density [329], worse and faster radiological progression of the disease independent of ACPA [106, 330], and mortality [331].

Other novel markers

In addition to RF, ACPA, anti-PAD and anti-CarP antibodies, several novel markers and autoantigens haven been described in RA in the past few years, including mutated citrullinated vimentin, fillagrin, Ra-33 [heterogeneous nuclear ribonucleoprotein (hnRNP) A2], fibrinogen, fibronectin, alpha-enolase, type II collagen, immunoglobulin binding protein (BiP), annexin [3], 14-3-3 eta [332], C-X-C Motif Chemokine Ligand (CXCL) 13[333], the University of Hasselt peptides [334, 335] and a few more. Nevertheless, to date, none of these markers are currently being used in routine clinical practice and further work is required to elucidate and validate their clinical utility.

Special interest has arisen around circulating calprotectin, a protein reportedly found in the serum or plasma of RA patients. Known for years as a marker for inflammation in a number of rheumatic diseases [336], this heterodimeric complex formed by the two subunits S100A8/9 [also known as myeloid-related protein (MRP) 8/14] has been proposed as an important biomarker in RA. Several studies have shown significant associations of calprotectin in serum or plasma with clinical, laboratory and ultrasound assessments of RA disease activity [337-342] and prediction of remission [340, 343]. Probably more importantly, data supporting its utility as a marker for prediction of response to treatment has increased over the last few years [338, 341, 344, 345]. Although still early for this marker, the recent results and studies are promising for RA patients.

1.5. Current and emerging biomarker detection technologies

Several technologies for the detection of relevant biomarkers in autoimmunity are nowadays commercially available, including immunofluorescence assays (IFA), cell-based assays, dot-blot, ELISA, chemiluminescent immunoassay (CIA), addressable laser bead assays (ALBIA), antigen arrays on planar surfaces such as line immunoassays and dot blots, mass spectroscopy and several multi-analyte assays.

In this section, the most relevant technologies for the measurement of RA biomarkers based on utility and applicability in a clinical setting will be discussed.

1.5.1. Traditional technologies

The development of the first ELISA independently by Eva Engvall and Peter Perlman [346], as well as Bauke van Weemen and Anton Schuurs [347] in 1971, revolutionized the immunoassay field. This plate-based technique, in which an antigen or an antibody is immobilized to a solid surface, became and still is a gold standard test in many research and clinical fields, including autoimmunity.

Years after the description of the original ELISAs, the first early prototypes of a CIA were designed and its adoption rapidly grew, first within the clinical chemistry fields and later for diagnostic of infectious and autoimmune diseases. Nowadays, this technology has a notable presence in many autoimmune disease areas including celiac disease (CD), anti-phospholipid syndrome (APS), connective tissue diseases (CTD), SLE, and RA [348]. Several CIA platforms are currently commercially available, with the BIO-FLASH system (Biokit, Barcelona, Spain) as one of the most widely used in the field of autoimmunity.

The underlying principle of CIA also relies on a specific interaction between an antibody and an antigen but in this case, the detection is based on a chemical reaction that results in the emission of a photon of light that is quantified. The luminescence observed is proportional to the amount of the specific biological molecule present in the sample.

1.5.2. Emerging technologies

Multiplexed immunoassays based on flow cytometry are common tools in research settings, however, due to the work and time that these assays usually require and the associated cost, their application is very limited or almost non-existent in a clinical environment.

Multi-analyte approaches with algorithmic analyses represent one of the emerging technologies in the field of autoimmunity. Within this setting, the novel PMAT [research use only (RUO), Inova Diagnostics, San Diego, US] for the simultaneous detection of biomarkers – mostly autoantibodies- is currently under development. For this technology, the antigen is coupled to paramagnetic beads with unique signatures. The coupling procedure consists of three main steps: (1) bead activation, (2) antigen coupling and (3) bead blocking. The testing reaction is performed in an instrument based on the Aptiva® technology (Inova Diagnostics, San Diego, US, RUO). In this process, the beads are first incubated with patient samples and assay buffer. Then they are washed and incubated with a phycoerythrin (PE)-labelled anti-human IgA, IgG or IgM detector (Inova Diagnostics, San Diego, US) that will emit fluorescence. After an additional washing cycle, the particles are analyzed through digital imaging technology and the Median Fluorescence Intensity (MFI) measured on the particles is calculated. Currently a panel for the detection of several RA biomarkers, including the RF, ACPA and the anti-PAD antibodies is under development.

1.6. Current challenges in RA: unmet needs

The knowledge and understanding of RA have evolved significantly in the last decades. This has helped to improve patient care, transforming RA from a destructive and disabling disease with limited therapeutic options, to a disease in which remission is an achievable goal through early intervention, control of inflammation and prevention of joint destruction. Despite these advancements, there are still many areas where there is a need for improvement.

1.6.1. Diagnosis, patient stratification and prognosis

Successfully diagnosing RA can be very challenging due to several aspects. As previously mentioned, there is still a considerable percentage of patients that are seronegative for RF and ACPA [3]. This, together with the heterogeneity of the clinical manifestations of the disease, significantly contribute to the difficulty of the diagnosis. For these reasons, novel biomarkers that can help to close the serological gap in RA, that enable an early and accurate diagnosis, and that help to rule out other rheumatological diseases with similar clinical manifestations are strongly needed.

Furthermore, given the heterogeneity in symptoms, clinical manifestations, phenotypes and underlying pathogenic mechanism, patient stratification into more homogeneous subpopulations is also needed. Associated with the ability to foresee disease severity and activity and occurrence of comorbidities, better patient stratification will not only allow for a better prediction of prognosis, but the hope is also, that this will enable more targeted interventional approaches.

1.6.2. Response to treatment

One of the main areas where there is a strong need and opportunity for improvement in the management of RA patients is in prediction and monitoring of response to treatment. The current approach is usually based on a 'trial-error' strategy, in which the response rate is highly variable and below what patients and clinicians would desire [349]. The rather common lack of clinical response and the associated need to switch to alternative treatments, often result in patient dissatisfaction and frustration of the clinician.

In this context, the concept of 'window of opportunity' is very remarkable [113] and as previously indicated, understanding this period for each individual patient is key for therapeutic success. Numerous studies have now shown that the earlier the treatment is initiated, the better outcomes can be anticipated [114, 116, 117], and that early therapeutic intervention may significantly reduce the risk of RA onset in the first phases of the disease [350]. Furthermore, joint erosion and bone damage are inherently irreversible and progressive, therefore, early treatment becomes crucial to stop disease progression as soon as possible and to prevent further damage.

In the last few years, the concepts of companion diagnostics and complementary diagnostics have been gaining more and more attention. Used for quite some time now, the term companion diagnostics refers to tests that provide information that is essential for the safe and effective use of a corresponding drug or biological product. On the other hand, coined more recently and not as well understood yet, complementary diagnostics may inform on improving the benefit/risk ratio without

restricting drug access. In this context, novel biomarkers or newly identified clinical associations of the existing ones, might become very powerful tools for PM.

Several drugs based on different MOA and therapeutic targets are currently commercially available and in the last few years, several studies have investigated the utility of different biomarkers to predict response to each treatment type. Although some advancements have been made, further research is needed in this area. Additional biomarkers, or combinations of them, that can help stratify patients based on their disease phenotype and severity, as well as potentially, on the specific pathogenic mechanisms driving their disease will facilitate the transition from a "one-size fits all" to a "treat-to-target" approach, from the current "imprecision medicine" to PM.

1.6.3. Prediction and prevention

Because biomarkers are known to appear before the onset of the disease and certain risk factors for RA are very well understood, prediction of the development of RA and prevention through early intervention represent feasible objectives. Numerous studies are now focused in the investigation of the pre-clinical phase of RA and of several interventional approaches in this disease stage (Table 6). A better understanding of this stage in terms of evolution, biomarkers, and especially, timing with respects to disease onset, will allow for early intervention, either based on modification of certain risk factors or active therapeutic intervention, with the ultimate goal of preventing the disease.

Table 6 Summary of interventional clinical studies focused on pre-clinical RA and prevention.

Study name/ ID	Study initiator	Date of initiation/ Completion (status)	Drug	Inclusion criteria (Number of patients)	Conclusions	References
STAPRA (NTR5265) Statins to Prevent Rheumatoid Arthritis	University of Amsterdam, Netherlands	August 2017 / July 2020 (Ongoing)	Atorvastatin	RF IgM and ACPA positivity or high ACPA titer (>3x ULN), with or without current joint pain, but without current clinical synovitis (estimated enrollment n=110)	NA	NA
StopRA (NCT02603146) Strategy to prevent the onset of clinically apparent rheumatoid arthritis	University of Colorado, Denver, USA	March 2016/ March 2020 (Ongoing)	HCQ	Elevation of anti-CCP3, defined by result of anti-CCP3 ≥40 units, at Screening (estimated enrollment n=200)	NA	NA
TREAT EARLIER (NTR4853) Treat early arthralgia to reverse or limit impending exacerbation to rheumatoid arthritis	Leiden University Medical Center and Erasmus Medical Center in Rotterdam, Netherlands.	January 2015/ January 2021 (Ongoing)	Methylpredni-solone	Patients without clinically detectable arthritis but with arthralgia of small hand or feet joints of recent onset (<1 year) that according to the rheumatologist is suspect to be an early presentation of RA (CSA) (n=NA)	NA	NA
APIPPRA (ISRCTN46017566) Arthritis Prevention in the Pre-clinical Phase of Rheumatoid Arthritis with Abatacept: a randomised controlled trial	King's College London, Bristol-Myers Squibb (Abatacept's manufacturer)	April 2014/ December 2018 (Completed)	Abatacept	Arthralgia considered to be inflammatory in nature, ACPA and RF positive, or high titer ACPA	There is limited experience of the design and implementation of trials for the prevention of inflammatory joint diseases	[351]
ARIAA (NCT02778906) Abatacept Reversing Subclinical Inflammation as Measured by MRI in ACPA Positive Arthralgia	University of Erlangen-Nürnberg Medical School, Bristol-Myers Squibb (Abatacept's manufacturer)	November 2014/ December 2018 (Completed)	Abatacept	ACPA (with or without RF), joint pain present for at least 6 weeks, presence of synovitis or osteitis in MRI of the dominant hand at baseline (estimated enrollment n=98)	NA	NA

Study name/ ID	Study initiator	Date of initiation/ Completion (status)	Drug	Inclusion criteria (Number of patients)	Conclusions	References
PRAIRI (NTR1969) Prevention of clinically manifest RA by B-cell directed therapy in the earliest phase of the disease	Academic Medical Center, Amsterdam	January 2010/ December 2013 (Completed)	Rituximab	Patients with arthralgia but without evidence of clinical arthritis (n=109)	A single infusion of 100 mg of Rituximab significantly delays the development of arthritis in subjects at risks of developing RA	[352, 353]
Steroids in Very Early Arthritis (STIVEA)	The University of Manchester,	NA/2010 (Completed)	Methylpredni-solone acetate	Patients inflammatory polyarthritis with symptom duration of 4–10 weeks; tenderness and soft tissue swelling of two or more joints, at least one of which had to be a wrist, metacarpophalangeal or proximal interphalangeal joint (n=268)	Treatment of patients with very early inflammatory polyarthritis methylprednisolone acetate postpones the prescription of DMARDs and prevent one in 10 patients from progressing into RA	[354]
The Stop Arthritis Very Early (SAVE) trial	Medical University of Vienna	NA/2010 (Completed)	Methylpredni-solone	Monoarthritis, oligoarthritis (≤3 swollen joints), "polyarthritis" (≥4 swollen joints), symptoms duration of <16 weeks (n=383)	Neither remission nor development of RA is delayed by glucocorticoid treatment	[355]
ADJUST (NCT00124449) Abatacept study to Determine the effectiveness in preventing the development of rheumatoid arthritis in patients with Undifferentiated inflammatory arthritis and to evaluate Safety and Tolerability	Bristol-Myers Squibb (Abatacept's manufacturer)	July 2005/ November 2009 (Completed)	Abatacept	Anti-CCP2+ patients with UA (not fulfilling the ACR criteria for RA) and clinical synovitis of two or more joints (n=56)	Abatacept delayed progression of UA/very early RA in some patients	[356-358]
Primary prevention of rheumatoid arthritis (NTR133)	VUMC /Jan van Breemen Instituut	October 2005/April 2008 (Completed)	Dexametha-sone	Twice increased IgM-RF and/or anti-CCP with 4+ weeks interval; HLA-DR SE positive; no autoimmune disease (n=NA)	NA	NA

Study name/ ID	Study initiator	Date of initiation/ Completion (status)	Drug	Inclusion criteria (Number of patients)	Conclusions	References
PROMPT/Probaat (NTR73) The PRObable rheumatoid arthritis: Methotrexate versus Placebo Treatment	Leiden University Medical Center, Netherlands	March 2001 / June 2005 (Completed)	Mtx	Patients with UA who fulfilled the ACR 1958 criteria for probable RA; less than 2 years of complaints (n=110)	A 1-year course of MTX delayed and prevented RA development in high-risk UA patients.	[357, 358]

Abbreviations: RA; Rheumatoid Arthritis; NTR: Netherlands Trials Register; MRI: magnetic resonance imaging; ACPA: anti-citrullinated protein antibodies, NA: not available; RF: rheumatoid factor; ULN: upper limit of normal; UA: undifferentiated arthritis; CCP: cyclic-citrullinated peptide; HCQ: hydroxychloroquine; CSA: clinically suspect arthralgia; ACR: American College of Rheumatology; MTX: methotrexate.

1.7. PM in RA

Associated to these mentioned unmet needs in RA, several opportunities for the development and implementation of PM approaches have emerged in the last few years. Thanks to the evolution of the "-Omic technologies" (genomics, transcriptomics, epigenomics, proteomics, metabolomics) and the introduction and hatching of artificial intelligence in health care, PM is currently a reality in many fields.

The complete and successful implementation of PM in autoimmunity and RA will require a paradigm shift in several aspects, including the use and application of autoantibodies and other biomarkers. It could be anticipated that multi-parametric approaches that integrate ACPA and RF, as well as novel biomarkers, will be helpful in improving diagnosis, patient stratification, prediction of prognosis and response to treatment. Companion and complementary diagnostic tests, together with the currently available drugs and novel therapies, will be crucial in improving patients' lives. As new treatments become available, relevant and actionable biomarkers that facilitate their use in clinical trials and in clinical practice will become more and more important. The availability of new tests results, together with the increasing amount of clinical information being collected on the patients (meta-data), will facilitate the generation of a more and more individualized and broader clinical picture on each patient.

One of the immediate consequences of this progress is the generation of vast amounts of data points and information (Big Data), with the challenges that come with this. In this context, Artificial Intelligence (AI) plays a crucial role, thanks to its intrinsic capacity to help manage, analyze and facilitate the knowledge extraction from big amounts of parameters and data points. Because RA is a highly complex disease, AI represents a powerful tool to integrate clinical data and different test results from each patient and help the clinician or even the patient, get a broader understanding of the disease. Patient empowerment and data ownership represent two of the key aspects of PM as well, and AI as a tool for clinicians and researchers is becoming more and more important in this process.

Biomarkers and AI facilitate the slow but robust implementation of PM approaches in the field and in RA. Although many challenges are currently being faced and new ones can be anticipated, this transition from "classical" medicine to PM will also translate into a conversion from what Eric Topol coined in his recent book [359] as "shallow medicine", characterized by patient dissatisfaction and clinicians' burnout as a result of a damaged patient-doctor relationship, into "deep medicine", in which the patient care becomes the focus again and medicine gets more human.

In this chapter, the current understanding of RA has been reviewed, focusing on the clinical, pathological and serological perspectives, and in the implications and potential for PM in this disease. Within this context, this thesis aimed to investigate the clinical significance of novel RA biomarkers, specifically the anti-PAD antibodies, and to integrate this knowledge with the current understanding of this disease, in order to develop a new model that helps to improve the diagnosis, stratification and management of RA patients. In the next chapters, we will summarize this work and its implications and contribution to the advancement of PM in RA.

Chapter 2. Objectives

Despite significant advances in the past decades, there are still many unmet needs and areas of improvement in the diagnosis, patient stratification, management, treatment and prevention of RA. The overall objective of this thesis was to develop a model that contributes to improvements in these areas and that ultimately, has a positive impact in RA patients. In order to achieve this general goal, several specific objectives were defined.

The first one was to identify the main actionable areas for improvement in RA management, with special focus in diagnosis and patient stratification. For this purpose, an assessment of the current diagnostic method was performed, including the evaluation of the process in clinical routine and the biomarkers utilized in this context. Next, we interrogated whether a combinatory interval approach based on the currently available markers in clinical practice could help improve the diagnosis.

The second specific goal was to identify and select novel RA biomarkers through literature screening and in-house expertise, to study their clinical significance and to evaluate their added value to the existing ones. The anti-PAD antibodies, including anti-PAD4, anti-PAD3 and anti-PAD2, were selected for further investigation. To study these antibodies, immunoassays for the detection and quantification of these autoantibodies were designed, developed and optimized. To that end, biochemical characterization of the PAD enzymes and the autoantibodies to these proteins was performed. Once robust assays were available, they were utilized in several clinical studies with human samples to investigate the clinical significance of the anti-PAD antibodies in RA, focusing on their potential to close the serological gap, their utility in predicting joint erosion and disease severity, their associations with pulmonary manifestations and their utility for patient stratification.

Fundamentally, the RA classification criteria markers and combinations thereof, as well as novel biomarkers in this disease were studied to select the building blocks of a novel RA model.

To accomplish these objectives, the methodologies involved included:

- Literature screening
- Feasibility studies, early development and optimization of a range of immunoassays for the detection of autoantibodies in human samples
- Biochemical characterization of the antigens and the autoantibodies
- Measurement of serological markers in different clinical cohorts using the immunoassays
- Data analysis of the autoantibodies results in conjunction with the information available for the clinical samples
- Drafting of algorithmic approaches for the integration of the different data points

Chapter 3. Publications

3.1. Comparison of Serological Biomarkers in Rheumatoid Arthritis and Their Combination to Improve Diagnostic Performance

frontiers in Immunology

ORIGINAL RESEARCH
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Comparison of Serological Biomarkers in Rheumatoid Arthritis and Their Combination to Improve Diagnostic Performance

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Introduction: The diagnosis of rheumatoid arthritis (RA) is based on a combined approach that includes serological markers such as rheumatoid factor (RF) and anti-citrullinated peptide/protein antibodies (ACPA). The goal of this study was to evaluate the clinical performance of several RF and ACPA immunoassays for the diagnosis of RA, as well as the diagnostic value of a combinatory approach with these markers.

Methods: The study cohort included 1,655 patients from the Swiss Clinical Quality Management registry with sera from 968 patients with RA and 687 disease controls, including patients with axial spondyloarthritis (n = 450) and psoriatic arthritis (n = 237). ACPA were determined by anti-CCP2 IgG enzyme-linked immunosorbent assay (ELISA), QUANTA Flash® CCP3 IgG [chemiluminescent immunoassay (CIA)], and QUANTA Lite® CCP3 IgG ELISA. RF was determined by ELISA (QUANTA Lite® RF IgM, RF IgA, and RF IgG) and with two research use only CIAs (QUANTA Flash® RF IgM and RF IgA).

Results: All three ACPA assays showed good discrimination between RA patients and controls and good clinical performance. Overall, CCP3 performed better than CCP2. More pronounced differences were observed between the RF assays. We observed that CIA platforms for both RF IgM and RF IgA showed better performance than the ELISA platforms. Excellent and good total agreements were found between ELISA and CIA for CCP3 (total agreement 95.3%, kappa = 0.90), and between CCP2 and CCP3 ELISA (total agreement 86.6%, kappa = 0.73), respectively. RF IgM CIA and ELISA had a good qualitative agreement (86.5%, kappa = 0.73); RF IgA CIA and ELISA showed a moderate total agreement (78.5%, kappa = 0.53). When combinatory analyses were performed, the likelihood of RA increased with dual positivity and triple positivity and combining different markers resulted in higher odds ratio than the individual markers in all cases.

Conclusion: ACPA and RF showed good clinical performance in this large Swiss cohort of RA patients and controls. Overall, the performance of CCP3 was superior to CCP2. The combination of these biomarkers in an interval model represents a potential tool for the diagnosis of RA patients.

Keywords: rheumatoid arthritis, RA, diagnosis, anti-citrullinated peptide/protein antibodies, CCP3, CCP2, rheumatoid factor, RF

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic autoimmune disease that is characterized by pain, inflammation, and joint destruction and affects up to 1.0% of the general population (1, 2). Early diagnosis and treatment in RA is crucial as it can prevent disease progression and irreversible joint damage (3–5). RA diagnosis is based on a combined approach that consists of history acquisition, clinical examination, imaging modalities, and testing of acute-phase and serological markers such as rheumatoid factor (RF) and anti-citrullinated peptide/protein antibodies (ACPA) (2).

Rheumatoid factor IgM, the main isotype identified by RF assays, is found in approximately 70–80% of patients with confirmed RA (6–8). In addition, elevated levels of RF IgA and IgG have been reported in patients with RA (9, 10). Studies suggest that elevated levels of RF IgG are highly specific for RA diagnosis (10, 11). It has been proposed that the detection of all three RF isotypes improves the specificity and predictive value of RF testing (12).

A caveat of RF testing is its low specificity and that it can be found in patients with infections and other autoimmune diseases, as well as in a proportion of healthy individuals (13), with rates between 10 and 25% in elderly patients without RA (14). Despite this, RF was the only serological marker included in the 1987 American College of Rheumatology (ACR) classification criteria.

In 1998, the presence of autoantibodies specific to citrulline-containing antigens was reported in RA patients (15). Clinical studies confirmed that ACPA were more specific than RF for a diagnosis of RA. ACPA have a higher sensitivity than RF in early RA, an improved specificity, and good positive predictive value (16). Consequently, ACPA were included in the 2010 revised ACR/European League Against Rheumatism (EULAR) criteria for RA. These new classification criteria differentiate between low- and high-positive ACPA and RF levels, with greater weight given to higher autoantibody levels (2).

Anti-citrullinated peptide/protein antibodies are generally detected using anti-cyclic citrullinated peptide (CCP) antibody assays (17). The first generation of the CCP test used a peptide derived from the filaggrin protein as the antigen. The second and third generation CCP (CCP2 and CCP3, respectively) are no longer based on the filaggrin-derived native sequences, but on peptides specifically designed and optimized (mimotypes) to detect ACPA. These improvements enhance the immunoreactivity of the citrulline-containing epitope (18–20). Serum samples from a subset of RA patients that report negative for the anti-CCP2 assay (second generation) can react to other citrullinated proteins (21–24). This suggests that there are additional epitopes that are not present in the second generation CCP antigen sequence. The third generation CCP antigen was developed by testing a large number of RA patients and control subjects on various citrullinated peptides (25, 26).

Anti-citrullinated peptide/protein antibodies and RF are widely used aids in the diagnosis and classification of RA. The combination of results from these markers might provide further value in the management of RA. Furthermore, it was recently demonstrated that the combined presence of RF IgM and ACPA mediates increased production of pro-inflammatory cytokines *in vitro* and is associated with elevated systemic inflammation and disease activity in RA (27, 28).

Nevertheless, many patients are seronegative for ACPA and RF, and there is a need for novel serological biomarkers to help close this serological gap (29) and improvement of early diagnosis, classification of RA subtypes, and patient stratification.

Enzyme-linked immunosorbent assay (ELISA) and bead-based chemiluminescent immunoassays (CIAs) are two frequently used tests for the quantification of ACPA and RF. Several differences between these two platforms have been described (30). One of those differences includes potentially improved sensitivity due to the larger surface binding area of the bead-based assay. Although it is possible that laboratories use both technologies, ELISA and CIA, the majority of laboratories prefer to run all tests on the same platform when possible, due to increased efficiency. Therefore, we decided to focus on the combinations of assays on the same platform.

This study aimed to evaluate the clinical performance of several immunoassays (plate-based ELISA- and bead-based CIA-) for the detection of RF and ACPA as aids in diagnosis of RA, as well as the diagnostic value of an approach based on combinations of outcomes of these serological biomarkers.

MATERIALS AND METHODS

Patients and Sera

All patients included in this study originated from the national Swiss registry established in 1997, the Swiss Clinical Quality Management (SCQM), which collects data from patients with inflammatory rheumatic diseases (http://scqm.ch) (31, 32). The registry longitudinally collects clinical, safety, and radiological data from patients with RA, axial spondyloarthritis (axSpA), and psoriatic arthritis (PsA). The diagnosis is based on the opinion of board-certified rheumatologists. In 2010, a biobank situated at the Department of Genetic and Laboratory Medicine of the University Hospital in Geneva (HUG) was established. This biobank includes serum samples of patients participating in the SCQM registry. Participation with the SCQM registry is on a purely voluntary basis. All patients provided signed informed consent prior to inclusion in the SCQM registry. An additional separate signed informed consent following Institutional and State regulations was collected for the biobank prior to blood acquisition. All biological samples are stored and used anonymously in this study. The study protocol received approval of the local ethics commission of the University Hospital of Geneva (protocol 10-089) and of the SCQM Biobank Scientific Advisory Board. All serological samples available at the time of the study were included in the

Abbreviations: RA, rheumatoid arthritis; AS, ankylosing spondylitis; axSpA, axial spondyloarthritis; ASAS, Assessment of Spondyloarthritis International Society; PsA, psoriatic arthritis; CASPAR, classification criteria for psoriatic arthritis; ACPA, anti-citrullinated peptide/protein antibodies; CCP, cyclic citrullinated peptide; RF, rheumatoid factor; ELISA, enzyme-linked immunosorbent assay; CIA, chemiluminescent immunoassay; QF, QUANTA Flash®; QL, QUANTA Lite®; AUC, area under the curve; ROC, receiver-operating characteristic; FDR, false discovery rate; ACR, American College of Rheumatology; EULAR, European League Against Rheumatism; CU, chemiluminescent units; RLU, relative light units; ULN, upper limit of normal; OR, odds ratio; RUO, research use only.

analysis. The cohort comprised sera collected from 1,655 patients (968 RA and 687 controls). The controls included patients with axSpA ($n = 450$) and PsA ($n = 237$). A summary of the patients' characteristics can be found in **Table 1**. For the majority of patients in the registry, the classification criteria for the three different diseases were available. For axSpA, the Assessment of Spondyloarthritis International Society criteria were utilized (33) and for PsA the classification criteria for psoriatic arthritis were utilized (34). The majority of patients classified as negative for the correspondent disease criteria were as a result of missing data required to confirm the criteria.

Immunoassays

Anti-citrullinated peptide/protein antibodies were determined by anti-CCP2 IgG ELISA (Euro Diagnostica, Malmö, Sweden), QUANTA Flash® CCP3 IgG CIAs (Inova Diagnostics, San Diego, CA, USA), and QUANTA Lite® CCP3 IgG ELISA (Inova). RF was determined by ELISA (QUANTA Lite RF IgM, RF IgA, and RF IgG, all Inova), and by QUANTA Flash RF IgM and RF IgA, two CIAs [research use only (RUO), both Inova] designed for the BIO-FLASH® Instrument (Biokit s.a., Barcelona, Spain). The principles and protocols of the BIO-FLASH assay system have been previously described (30). The QUANTA Flash RF assays are novel CIA that use rabbit IgG as antigen, coated onto paramagnetic beads. Results obtained with CCP2 ELISA were expressed in U/mL. Results measured with CCP3 CIA IgG assay were expressed in chemiluminescent units. Results obtained with RF CIA IgM and IgA were expressed in relative light units. Results with the CCP3 IgG and RF IgM, IgA, and IgG ELISAs were expressed in Units. All tests were performed according to the manufacturers' instructions for the commercially available assays and to research protocols for the RUO assays. Cutoff values recommend in the instruction for use were applied. Preliminary cutoff values for the RF IgM and IgA RUO CIA were established using the 95% CI of the reference limit at ≥95% in a selected cohort of samples ($n = 191$) including samples from patients suffering from systemic lupus erythematosus ($n = 9$), vasculitis ($n = 5$), antiphospholipid syndrome ($n = 12$), celiac disease ($n = 8$), infectious diseases ($n = 27$) including HBV ($n = 5$), HCV ($n = 6$), HIV ($n = 9$), and syphilis ($n = 7$), apparently healthy individuals ($n = 117$), and samples with anti-nuclear antibodies ($n = 13$). A summary of the characteristics of the different assays can be found in Table S1 in Supplementary Material.

Statistical Evaluation

All statistical analyses were performed by *Analyse-it®* for Excel method evaluation software (version 4.81.1; Leeds, UK) and the Python Scipy module. Receiver-operating characteristic (ROC) analyses were carried out to analyze the discrimination between RA patients and controls. The ROC curves were plotted, with the area under the curve (AUC) as an indicator of the diagnostic value. Sensitivities, specificities, and odds ratios (OR) were calculated based on the manufacturer's cutoff for the commercially available assays and on the preliminary cutoffs for the CIA RUO assays, as well as at the 3× upper limit of normal (ULN) for each assay (**Figure 1A**). Sensitivities and OR were also calculated based on

TABLE 1 | Summary of patients' characteristics at baseline.

	Total	RA		axSpA		PsA	
	All	All	ACR-EULAR+	All	ASAS+	All	CASPAR+
	N = 1,655	*N* = 968	*N* = 780	*N* = 450	*N* = 316	*N* = 237	*N* = 185
Age (SD)	49.2 (13.7)	53.1 (13.2)	52.7 (12.8)	41.1 (12.2)	38.6 (11.1)	48.3 (12.1)	48.0 (12.2)
Gender (% male)	39.4	25.6	22.9	59.3	63.1	57.8	58.4
Disease duration (median years) (IQR)	5.65 (2.1, 13.2)	4.56 (1.6, 10.6)	4.62 (1.6, 10.9)	9.3 (4.1, 18.1)	10.7 (4.7, 19.1)	5.3 (1.8, 13.1)	5.0 (2.0, 12.1)
BMI (median) (IQR)	25.3 (22.3, 29.0)	25.2 (22.2, 29.1)	25.1 (22.1, 29.0)	24.9 (22.4, 28.1)	24.8 (22.4, 27.9)	26.7 (23.7, 29.9)	26.9 (23.9, 30.1)
Smoker (% actual or past)	58.0	57.3	58.0	57.2	60.5	63.1	64.4
Alcohol consumption (%)	72.5	70.2	69.3	–	–	82.3	82.2
Higher education (%)	57.1	57.9	58.5	57.1	58.0	54.4	54.1
CRP	9.9	10.4	11.1	9.9	10.5	7.8	7.9
Raised CRP or ESR (%)	43.5	51.0	54.9	35.1	37.3	29.1	29.7
Physician global score	3.2 (2.2)	3.3 (2.3)	3.6 (2.3)	3.1 (2.1)	3.1 (2.0)	3.2 (2.3)	3.3 (2.3)
DAS28 (SD)	–	3.8 (1.6)	4.0 (1.5)	–	–	2.8 (1.4)	2.7 (1.5)
Erosive radiographic changes (%)	–	59.6	65.0	–	–	–	–
HAQ (median, IQR)	–	0.75 (0.25, 1.375)	0.875 (0.375, 1.375)	–	–	0.5 (0.125, 1)	0.5 (0.125, 1)
RADAI	–	3.63	3.82	–	–	–	–
BASDAI	–	–	–	4.43 (2.4)	4.4 (2.4)	–	–
BASFI	–	–	–	3.1 (2.6)	3.0 (2.6)	–	–
BASMI	–	–	–	2.0 (1.8)	1.9 (1.9)	–	–
ASDAS-CRP	–	–	–	2.68 (1.2)	2.75 (1.2)	–	–

Mean values (±SD) unless otherwise indicated.

RA, rheumatoid arthritis; axSpA, axial spondyloarthritis; ASAS, Assessment of Spondyloarthritis International Society; PsA, psoriatic arthritis; CASPAR, classification criteria for psoriatic arthritis; IQR, inter quartile range; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; DAS28, disease activity score 28; HAQ, health assessment questionnaire; RADAI, rheumatoid arthritis disease activity index; BASDAI, Bath ankylosing spondylitis disease activity index; BASFI, Bath ankylosing spondylitis functional index; BASMI, Bath ankylosing spondylitis metrology index; ASDAS-CRP, ankylosing spondylitis disease activity score-C-reactive protein; ACR, American College of Rheumatology; EULAR, European League Against Rheumatism.

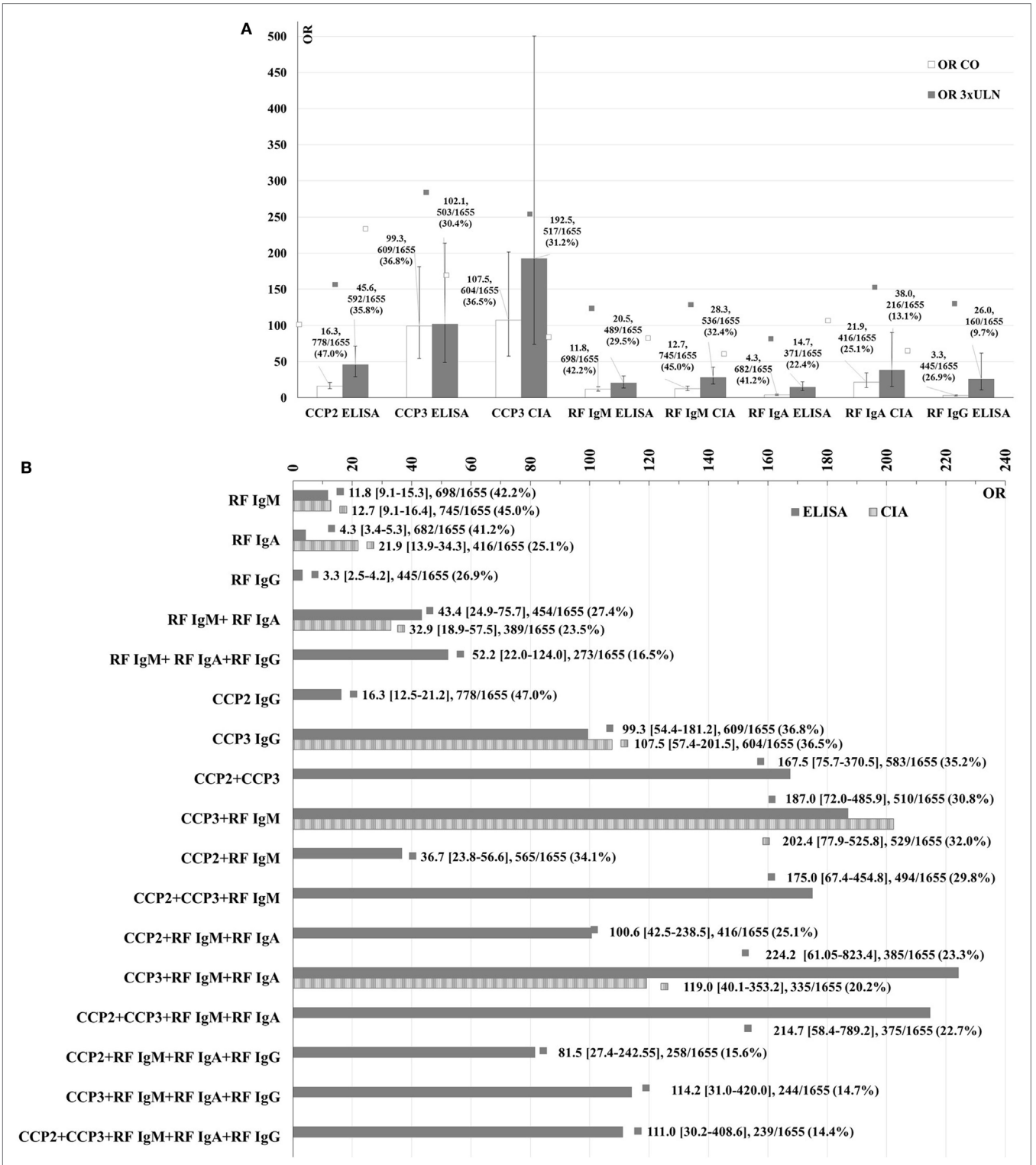

FIGURE 1 | Association of single markers with RA [OR] on the ELISA and CIA platforms at the manufacturers cutoff or preliminary cutoffs for the research use only assays versus at the 3× ULN **(A)** and association of single markers and combinations of markers with RA (OR) on ELISA (solid) and CIA (dashed) at the cutoffs **(B)**. OR values, 95% CI, number of patients positive for each condition, and % over the total number of patients included in the study are shown.

the 95% specificity cutoffs. A *t*-test and Benjamini–Hochberg correction, false discovery rate set at 5%, were run for the individual markers. Likelihood plots were generated to compare the change of OR at the assay cutoff point versus the 3× ULN for the seven assays and also, to note if any optimal cutoffs with higher OR could be observed. Method comparison between platforms for the markers available on ELISA and CIA was performed. Combinations of markers were created at the manufacturer's cut-offs and at 3× ULN (**Figure 1B**). ORs and the number of patients within each condition were calculated. Differences between likelihood ratios for the combinations of markers were calculated using BDTcomparator as described previously (35, 36). For these differences, *p*-values <0.05 were considered significant.

To further investigate the model based on the RF IgM and CCP3 combination, scatter plots were created for the results of these two markers on each platform (**Figure 2A** for ELISA and **Figure 2C** for CIA). The plots were sectioned into nine groups using the manufacturer's cutoffs or the preliminary cutoffs for the RUO assay and the 3× ULN. The OR was calculated in each section. In addition, based on clinical significance, several combinations of sections were created for both platforms, assuming three intervals of patients: patients with a low likelihood of RA (interval I),

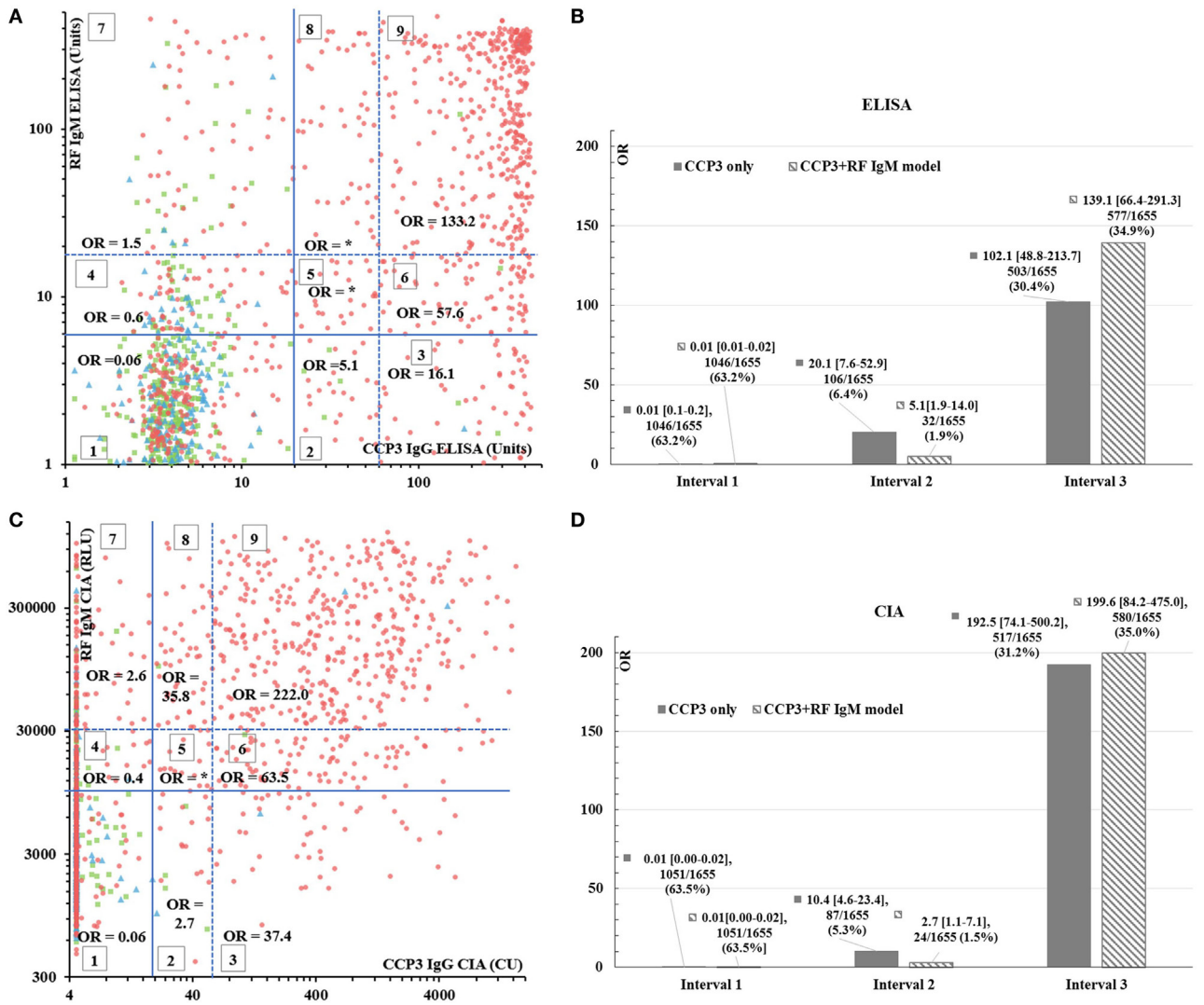

FIGURE 2 | RF IgM and CCP3 combinatory model. Panels **(A,C)** represent the scatter plots for the RF IgM and CCP3 combination on ELISA **(A)** and CIA **(C)**. The red dots represent patients with RA, the green squares are patients with axSpA and the blue triangles show patients with PsA. The lines sectioning the graphs into nine groups represent the manufacturer's cutoffs (solid line) or the preliminary cutoffs for the RUO assay and the 3× ULN (dashed). The different sections of the graph are indicated by numbers (1–9). The *x* and *y*-axes are shown in logarithmic scale. Panels **(B,D)** show the OR, 95% CI, number and percentage of patients for the individual CCP3 assays (solid) versus the combined model of CCP3 and RF IgM (dashed) in the three intervals on ELISA **(B)** and CIA. **Note: OR could not be calculated since no controls were found in those groups.* Abbreviations: CU, chemiluminescent units; RLU, relative light units; CO, cutoff; ULN, upper limit of normal; OR, odds ratio; RF, rheumatoid factor; CIA, chemiluminescent immunoassay; ELISA, enzyme-linked immunosorbent assay; RA, rheumatoid arthritis; axSpA, axial spondyloarthritis; RUO, research use only.

patients within an area of uncertainty (interval II), and patients with a high likelihood of RA (interval III). The OR and the number of patients within each combination were calculated. The selection of the best combination was made based on two factors: the OR for RA and the number of patients correctly classified. These combinations were compared with the individual performance of CCP3 below the cutoff (interval I), between the cutoff and the 3× ULN (interval II), and above the 3× ULN (interval III).

RESULTS

Comparison of the Clinical Performance

All three ACPA assays (CCP2 ELISA and CCP3 ELISA and CIA) showed good discrimination between RA patients and controls, with AUC of 0.82, 0.83, and 0.82, respectively. At the cutoff values provided by the manufacturer, the CCP2 ELISA showed a high sensitivity (71.1%) and a moderately high specificity (86.9%) with a corresponding OR of 16.3 (95% CI 12.5–21.1). The two CCP3 assays showed lower sensitivities (61.8% for ELISA and 61.4% for CIA), but significantly higher specificities (98.4 and 98.5%, respectively), resulting in much higher predictive values, with OR of 99.3 (95% CI 54.4–181.2) and 107.5 (95% CI 57.4–201.5), respectively. The clinical performance characteristics for all assays are summarized in **Table 2**.

For RF, more pronounced differences between assays were observed. The AUC derived from ROC analysis ranged from 0.61 (RF IgG ELISA) to 0.82 (RF IgM CIA). At the cutoffs provided by the manufacturer for the ELISAs and at the preliminary cutoffs established for the CIAs, the sensitivities ranged from 35.6% (RF IgG ELISA) to 67.1% (RF IgM CIA), and the specificities were between 77.9% (RF IgA ELISA) and 96.9% (RF IgA CIA). The RF IgM CIA was the most sensitive, while the RF IgA CIA was the assay with the highest specificity. The RF IgG ELISA showed the lowest discrimination value (AUC = 0.61), with the lowest sensitivity at the recommended cutoff (35.6%). Differences were also observed between the predictive values of the RF assays, with OR that ranged from 3.3 (95% CI 2.5–4.2) for the RF IgG ELISA to 21.9 (95% CI 13.9–34.3) for RF IgA CIA. Interestingly, the OR for the RF IgA CIA was more than five times higher than the OR for the ELISA of this RF isotype (OR = 4.3, 95% CI 3.4–5.3). Similar results were obtained for both ACPA and RF when analyses were performed only including the patients for which diagnostic criteria was available (data not shown).

When all assays were compared at the same specificity (95%), the sensitivities varied from 27.6% (RF IgG ELISA) to 68.1% (CCP3 IgG CIA). As results greater than 3× ULN carry more clinical weight for the diagnosis and classification of RA as seen in the 2010 ACR/EULAR classification criteria (2), the clinical performance at the 3× ULN was also analyzed. At this threshold, the sensitivities ranged from 16.0% (RF IgG ELISA) to 59.0% (CCP2 ELISA) and the OR varied from 14.7 (95% CI 9.7–22.4, RF IgA ELISA) to 192.5 (95% CI 74.1–500.2, CCP3 IgG CIA).

In the RF IgM and CCP3 IgG negative population based on ELISA ($n = 858$), the RF IgA ELISA had an 18.5% sensitivity with 76.9% specificity and an OR of 0.8 (95% CI 0.5–1.1) and the RF IgG ELISA reported a 10.6% sensitivity, an 88.0% specificity and

TABLE 2 | Summary of clinical performance characteristics of the assays used in this study.

	CCP2 IgG ELISA	CCP3 IgG ELISA	CCP3 IgG CIA	RF IgM ELISA	RF IgM CIA	RF IgA ELISA	RF IgA CIA	RF IgG ELISA
Cutoff	25 U/mL	20 U	20 CU	6 U	10,344 RLU	6 U	7,425 RLU	6 U
# Positive samples/total # samples (%)	778/1,655 (47.0)	609/1,655 (36.8)	604/1,655 (36.5)	698/1,655 (42.2)	745/1,655 (45.0)	682/1,655 (41.2)	416/1,655 (25.1)	445/1,655 (26.9)
# Positive RA samples/total # RA samples (%)	688/968 (71.1)	598/968 (61.8)	594/968 (61.4)	611/698 (63.1)	650/968 (67.1)	530/968 (54.8)	395/968 (40.8)	345/968 (35.6)
# Positive samples/total # axSpA samples (%)	59/450 (13.1)	8/450 (1.8)	6/450 (1.3)	56/450 (12.4)	60/450 (13.3)	103/450 (22.9)	14/450 (3.1)	68/450 (15.1)
# Positive PA samples/total # PsA samples (%)	31/237 (13.1)	3/237 (1.3)	4/237 (1.7)	31/237 (13.1)	35/237 (14.8)	49/237 (20.7)	7/237 (3.0)	32/237 (13.5)
Sensitivity (%)	71.1	61.8	61.4	63.1	67.1	54.8	40.8	35.6
Specificity (%)	86.9	98.4	98.5	87.3	86.2	77.9	96.9	85.4
OR (95% CI)	16.3 (12.5–21.2)	99.3 (54.4–181.2)	107.5 (57.4–201.5)	11.8 (9.1–15.3)	12.7 (9.9–16.4)	4.3 (3.4–5.3)	21.9 (13.9–34.3)	3.3 (2.5–4.2)
AUC (95% CI)	0.82 (0.80–0.84)	0.83 (0.81–0.85)	0.82 (0.81–0.84)	0.79 (0.77–0.81)	0.82 (0.80–0.84)	0.70 (0.67–0.72)	0.79 (0.77–0.81)	0.61 (0.59–0.64)
Cutoff for 95% specificity	53.0	8.3	6.6	14.6	24,767	14.2	5,252	8.3
Sensitivity at 95% specificity	61.3	68.1	66.1	50.2	55.0	39.7	48.1	27.6
OR at 95% specificity (95% CI)	29.7 (20.7–42.7)	39.9 (27.7–57.5)	36.5 (25.4–52.6)	18.8 (13.1–27.0)	22.1 (15.4–31.5)	12.6 (8.7–18.2)	17.3 (12.1–24.8)	7.2 (5.0–10.4)
Cutoff 3× ULN	75	60	60	18	31,032	18	22,275	18
Sensitivity at 3x ULN (%)	59.0	51.2	53.0	47.5	52.7	35.7	21.8	16.0
OR at 3× ULN (95% CI)	45.6 (29.1–71.5)	102.1 (48.8–213.7)	192.5 (74.1–500.2)	20.5 (13.9–30.4)	28.3 (18.8–42.6)	14.7 (9.7–22.4)	38.0 (16.0–90.4)	26.0 (10.9–62.1)
p-Value	7.31e−29	1.36e−105	2.87e−25	8.09e−58	1.23e−48	4.44e−36	2.53e−13	2.29e−15
FDR	True	True	True	True	True	True	True	True

CCP, cyclic citrullinated peptide; RF, rheumatoid factor; CIA, chemiluminescent immunoassay; RUO, research use only; CU, chemiluminescent units; RLU, relative light units; OR, odds ratio; AUC, area under the curve; ULN, upper limit of normal; FDR, false discovery rate; RA, rheumatoid arthritis; axSpA, axial spondyloarthritis; ELISA, enzyme-linked immunosorbent assay.

TABLE 3 | Agreement between the CCP2 and CCP3 ELISA assays (A), and between ELISA and CIA for CCP3 (B), RF IgM (C) and RF IgA (D).

Kappa = 0.73 (95% CI 0.70–0.76)		**CCP3 IgG ELISA**			**Percent agreement (95% confidence interval)**	
		Negative	**Positive**	**Total**		
CCP2 IgG ELISA	**Negative**	851	26	877	Neg. agreement	97.0% (95.7–98.0%)
	Positive	195	583	778	Pos. agreement	74.9% (71.8–77.9%)
	Total	1,046	609	1,655	Total agreement	86.6% (84.9–88.2%)
Kappa = 0.90 (95% CI 0.88–0.92)		**CCP3 IgG CIA**			**Percent agreement (95% confidence interval)**	
		Negative	**Positive**	**Total**		
CCP3 IgG ELISA	**Negative**	1,010	36	1,046	Neg. agreement	96.6% (95.3–97.5%)
	Positive	41	568	609	Pos. agreement	93.3% (91.0–95.0%)
	Total	1,051	604	1,655	Total agreement	95.3% (94.2–96.3%)
Kappa = 0.73 (95% CI 0.69–0.76)		**RF IgM CIA**			**Percent agreement (95% confidence interval)**	
		Negative	**Positive**	**Total**		
RF IgM ELISA	**Negative**	822	135	957	Neg. agreement	85.9% (83.5–88.0%)
	Positive	88	610	698	Pos. agreement	87.4% (84.7–89.7%)
	Total	910	745	1,655	Total agreement	86.5% (84.8–88.1%)
Kappa = 0.53 (95% CI 0.49–0.57)		**RF IgA CIA**			**Percent agreement (95% confidence interval)**	
		Negative	**Positive**	**Total**		
RF IgA ELISA	**Negative**	928	45	973	Neg. agreement	95.4% (93.9–96.5%)
	Positive	311	371	682	Pos. agreement	54.4% (50.6–58.1%)
	Total	1,239	416	1,655	Total agreement	78.5% (76.4–80.4%)

CCP, cyclic citrullinated peptide; RF, rheumatoid factor; CIA, chemiluminescent immunoassay; ELISA, enzyme-linked immunosorbent assay.

OR of 0.9 (95% 0.5–1.4). In the RF IgM and CCP3 IgG seronegative population defined by CIA ($n = 835$), the RF IgA showed a 5.2% sensitivity with a 98.6% specificity and an OR of 4.0 (95% CI 1.7–9.5).

To investigate if the combination of both ACPA tests with different citrullinated antigens (CCP2 and CCP3) provides better clinical performance, we looked at the performance of the CCP3 assay in the CCP2 negative population and vice versa. In the CCP2 negative population ($n = 877$), the CCP3 ELISA reported a sensitivity of 7.5%, a 99.2% specificity, and an OR of 9.6 (95% CI 3.7–24.9) and the CCP3 CIA had a 6.4% sensitivity with a 99.5% specificity and OR of 13.6 (95% CI 4.2–43.6). On the other hand, the CCP2 ELISA had a 30.0% sensitivity with an 87.6% specificity in the CCP3 ELISA negative population ($n = 1,046$) and a 29.9% sensitivity and 87.7% specificity in the CCP3 CIA negative population ($n = 1,051$), with OR of 3.0 (95% CI 2.2–4.2) and 3.1 (95% CI 2.2–4.2), respectively.

Likelihood plots were generated to compare the OR at the assay cutoff point versus the 3× ULN for the different assays and also, to note if any optimal cutoffs with higher OR could be observed (see Figure S1 in Supplementary Material). The association of single markers with RA on the CIA and ELISA platforms at the cutoffs versus at the 3× ULN was also analyzed (**Figure 1A**). As expected, higher OR were observed at the 3× ULN than at the cutoffs for all markers.

Agreement Between Different Methods

An agreement greater than 95% was considered excellent, between 80 and 95% was good and between 50 and 80% was moderate. Excellent qualitative agreement was found between ELISA and CIA for CCP3, with a total agreement of 95.3% (95% CI 94.2–96.3%) and a kappa of 0.90 (95% CI 0.88–0.92). When the CCP2 and CCP3 ELISAs were compared, a good total agreement (86.6%, 95% CI 84.9–88.2%), moderate positive agreement (74.9%, 95% CI 71.8–77.9%), and excellent negative agreement (97.0%, 95% CI 95.7–98.0%) were observed and a kappa of 0.73 (95% CI 0.70–0.76) was found. The RF IgM CIA and ELISA showed a good qualitative agreement, with a total agreement of 86.5% (95% CI 84.8–88.1%), a positive agreement of 87.4% (95% CI 84.7–89.7%), a negative agreement of 85.9% (95% CI 83.5–88.0%), and a kappa of 0.73 (95% CI 0.69–0.76). For the RF IgA assays, although the negative agreement was high (95.4%, 95% CI 93.9–96.5%), the positive agreement was moderate (54.4%, 95% CI 50.6–58.1%) resulting in a moderately high total agreement (78.5%, 95% CI 76.4–80.4%) (kappa = 0.53, 95% CI 0.49–0.57). A summary of these agreement analyses can be found in **Table 3**.

Combinations of Markers

When combinatory analyses were performed, the likelihood of RA increased with dual and triple positivity. Combining different markers resulted in higher OR than the individual markers in all cases (**Figure 1B**). Nevertheless, 95% CI of the OR overlapped in some cases indicating that the differences may not be clinically significant.

The predictive value of the RF test was increased by the detection of multiple isotypes. Combining ACPA and RF IgM resulted in higher OR than the individual markers. Differences were observed between the combinations that included CCP2

and/or CCP3 ELISA assays. The combination of CCP3 and RF IgM resulted in a higher OR (OR = 187.0, 95% CI 72.0–485.9) than in the combination of CCP2 with RF IgM (OR = 36.7, 95% CI 23.8–56.6). The triple combination of CCP2, RF IgM, and CCP3 IgG on ELISA resulted in a lower OR (OR = 175.0, 95% CI 67.4–454.8). Out of all the combinations analyzed, the highest OR was observed for CCP3 IgG, RF IgM, and RF IgA on ELISA (OR = 224.2, 95% CI 61.0–823.4), followed by CCP2, CCP3, RF IgM, and RF IgA on ELISA (OR = 214.7, 95% CI 58.4–789.2). The addition of RF IgG to these combinations resulted in a lower OR (see **Figure 1B**).

Receiver-operating characteristic analysis showed that the combination of CCP2 and CCP3 ELISA did not result in better discrimination between RA and controls than with individual CCP assays (AUC 0.79, 95% CI 0.78–0.81). Nevertheless, this combination had a higher specificity (99.1%) with a lower sensitivity (59.6%) and resulted in a higher OR (OR = 167.5, 95% CI 75.7–370.5).

In the model based on the RF IgM and CCP3 combination (**Figure 2A** for ELISA and **Figure 2C** for CIA), higher ORs were observed as the antibody levels of CCP3 and RF IgM increased. This is in accordance to the different weights assigned to different antibody levels in the 2010 ACR/EULAR classification criteria (2).

When the sections within the scatter plots for the RF IgM and CCP3 models were combined, for both platforms, the combination with highest OR and better classification based on number of patients in each group was identified as the following: patients with a low likelihood of having RA were defined as those that were negative for both RF IgM and for anti-CCP3 and those with positive RF IgM but who were negative for anti-CCP3 (groups 1, 4, and 7 = interval I); patients within an area of uncertainty were defined as those who were negative for RF IgM and demonstrated anti-CCP3 levels between the cutoff and the 3× ULN (group 2 = interval II); and finally patients with a very high likelihood of having RA were defined as the remaining patients (groups 3, 5, 6, 8, and 9 = interval III). These combinations were compared with the individual performance of CCP3 below the cutoff (interval I), between the cutoff and the 3× ULN (interval II), and above the 3× ULN (interval III) (**Figures 2B,D**). A higher number of patients could be correctly classified with the combinatory models than with the individual CCP3 markers.

DISCUSSION

Anti-citrullinated peptide/protein antibodies and RF are important serological markers for the diagnosis and classification of RA (2). RF was the first well-known marker in RA; however, it is known to have low specificity and to be present in various inflammatory diseases (37), which is consistent with the data in our study. When ELISA and CIA for the detection of RF IgM were compared, a high qualitative agreement was found. The RF IgM CIA showed a better discrimination between RA patients and the controls than the RF IgM ELISA, with a higher sensitivity and very similar specificity. Overall, the RF IgM CIA showed a better performance than the ELISA for the detection of these antibodies. This might be attributed to the technological advantages of the CIA as illustrated in a recent review article (30).

In addition to RF IgM, raised levels of RF IgG and IgA have been reported in patients with RA (10, 38, 39). At the preliminary cutoff, the RF IgA CIA showed a much higher specificity (the highest of all RF assays) compared with the ELISA. When the two assays were compared, a high negative agreement but a low positive agreement was found, resulting in a moderate total agreement. This could be due to the differences between the two platforms (30). While the RF IgA ELISA uses a polyclonal anti-human IgA antibody as conjugate, the CIA uses a monoclonal antibody for detection, which could explain the higher specificity of this platform compared with ELISA. It has been described that the detection of RF IgA in early disease suggests a poor prognosis and justifies more aggressive treatment (10, 38). However, most samples included in this study were derived from patients with established disease; therefore, the utility of RF IgA in early RA compared with advanced disease is outside the scope of this study. Nevertheless, our results suggest that the detection of RF IgA, when measured by CIA, can help to close the serological gap, due to some RA patients presenting RF IgA in the absence of RF IgM and ACPA.

Regarding RF IgG, the diagnostic value of this isotype in our cohort is not clear. RF IgG has a very low prevalence in RA patients, compared with the other two isotypes. In this study, the RF IgG ELISA showed a moderately high specificity similar to the RF IgM assays, with a very low sensitivity. Although this assay reported a very low sensitivity at the 3× ULN, the OR was moderately high. Since the performance of RF IgG on ELISA is limited, no RF IgG CIA was developed. However, RF IgG might have clinical utility in aspects other than the diagnosis, such as clinical association with vasculitis (11) or prediction for erosive disease and radiographic progression (40).

It has been suggested that the specificity and predictive value of the RF test is substantially increased by the detection of all three RF isotypes (12). Although we did not test for total RF, we saw that the dual and triple combinations of the RF isotypes showed higher OR than the individual markers, with the combination of the three isotypes measured by ELISA being the highest, among all the RF combinations analyzed.

Consistent with the results of several clinical studies, we observed that ACPA are more specific diagnostic markers than RF for RA, with the exception of RF IgA CIA in our study that also displayed a very high specificity (higher than CCP2). Significant differences between ACPA assays have been reported (41) and in our study, although the CCP3 CIA and ELISA showed equivalent performance and a high qualitative agreement, differences were observed between the CCP2 and the CCP3 assays, especially in the predictive value, with CCP3 outperforming CCP2.

Anti-citrullinated peptide/protein antibodies significantly improves the diagnosis of RA, especially in the RF negative population. Surprisingly, when analyzed at the 3× ULN, the CCP3 ELISA reported a very similar OR to that obtained at the manufacturers cutoff, as opposed to the CCP2 ELISA and the CCP3 CIA that showed a significant increase in OR at the 3× ULN. Out of all assays tested, the CCP3 CIA showed the highest OR at the assay cutoff as well as at the 3× ULN, confirming that the CCP3 CIA is a reliable test for the fully automated and rapid detection of ACPA, as previously described (20).

For the classification criteria, RF and ACPA carry the same weight, which is not confirmed by our findings, where great differences are observed in the OR of these two markers. In this context, a recent letter to the editor published by Bossuyt (42, 43) reported that the probability for RA using CCP2 ELISA (Euro-Diagnostica) increases from 3.4 to 73.6 (low versus 3× ULN value), which is significantly higher than reflected in the classification criteria (2 versus 3 points). This is somewhat contradictory to our findings where we saw an increase from 16.3 to 45.6 in the CCP2 OR at the cutoff and at the 3× ULN, respectively, and with regards to the CCP3 CIA where the OR increases from 107.5 to 192.5, which corresponds well to the weighting of the classification criteria. By contrast, the CCP3 ELISA did not show a significant increase. Future refinements of the RA classification criteria may attribute a higher relative weight to a high-positive ACPA compared with a low-positive ACPA. Future studies are needed to evaluate whether cutoff points for ACPA assays (especially for the 3× ULN) are aligned between different manufacturers.

Currently, the combination of ACPA with RF by turbidimetry which detects all RF isotypes is the most commonly used approach (44–46). In our study, the combination of CCP3 with RF IgM, on both platforms, showed a very high OR, higher than the OR with the individual markers at the cutoffs and equivalent to the CCP3 CIA OR at the 3× ULN. The addition of RF IgA to the CCP3 and RF IgM ELISA combination resulted in a higher OR. By contrast, the addition of RF IgG to this model did not seem to be valuable from a diagnostic perspective. Interestingly, the combination of both ACPA tests with different antigens (CCP2 and CCP3), did not give a better discrimination between RA and controls when compared with the models based on CCP3 only. However, a higher specificity was observed with the CCP2 and CCP3 ELISA combination compared with the individual CCP assays. These results are in agreement with what was recently published by Vos et al. (17), where the positivity for both CCP2 and CCP3 resulted in the most specific identification of the RA patients. Although the samples were tested for RF IgM in that study, this marker was not analyzed in combination with CCP. In addition, it has been previously reported that ACPA can be found in PsA patients with a prevalence between 5 and 13% (47–49) and that they are linked to erosive disease (49, 50). In our cohort, ACPA were detected in 13.1% of PsA patients with anti-CCP2, however, only in 1.3 and 1.7% of patients with the CCP3 IgG ELISA and CIA, respectively. This would suggest a higher specificity of the CCP3 assay compared with CCP2 in this group of patients. No data were available about erosive disease in PsA patients.

Besides the OR, it is also important to assess how many patients can be captured using a single test or a combination of tests. By combining test results, often the specificity and consequently the OR increase substantially. However, this usually in turn reduces the number of patients that can be correctly classified. Although the added value of the CCP3 and RF IgM combination versus the CCP3 individual assays is limited, a higher number of patients could be correctly classified with the combinatory model compared with the individual CCP3 assays, with fewer patients found within the areas on uncertainty (interval II) (**Figures 2B,D**). These data suggest a potential diagnostic utility of a combinatory model approach using combinations of biomarkers, especially RF IgM and anti-CCP3.

Even though ACPA have significantly contributed to improve the diagnosis of RA, there is an unquestionable need for novel biomarkers to enhance the early diagnosis of RA, especially in patients currently classified as seronegative, as well as, to define the different RA subclasses and to stratify patients according to different disease phenotypes (29). Once more diagnostically relevant biomarkers have been established, modern multianalyte techniques for the simultaneous detection of a wide spectrum of markers, may provide additional benefits in diagnosis, classification of RA subtypes, stratification of patients to start early treatment, and potentially lead to prevention. The inclusion of novel RA-associated markers in multiplex assays and prediction models may facilitate the use of profiling in diagnostic routine laboratories.

One limitation of this study was that ACPA and RF are used in the diagnosis of RA in clinical practice. This could have influenced the observed association between these antibodies and the predictive value of RA. This is an inherent problem of any study that investigates the performance of RF and ACPA in the diagnosis of RA. Furthermore, the findings of these studies would have to be validated in independent cohorts that include additional disease controls.

In the context of precision medicine, the combination of biomarkers represents a very promising tool to improve the diagnosis of RA patients (17) and to predict the possible association with disease development (51, 52) and therapeutic response (53).

CONCLUSION

Anti-citrullinated peptide/protein antibodies and RF CIA showed good clinical performance in this large cohort. RF IgA performance is very platform dependent, with the CIA demonstrating superior performance compared with the ELISA. Overall, the performance of CCP3 was superior to CCP2, when analyzed individually as well as in combination with RF IgM. CCP2 and CCP3 complement each other and have a better predictive value than the individual assays but did not show a better discrimination between RA and controls. The combination of several of these biomarkers, in particular CCP3 IgG and RF IgM, seems to be useful for the clinical diagnosis of RA patients and to help correctly classify a higher number of patients.

AUTHOR CONTRIBUTIONS

LM-P performed the statistical analyses and drafted the manuscript. MN designed the study, helped with the statistical analyses, and drafted the manuscript. LM-P and MN contributed equally to this paper. CL and PR-L helped design the study. CL collected and managed the samples. PR-L performed the CCP2 testing. CB designed the study. LC carried out the CCP3 and RF testing and compiled the results. CG designed the study. MM designed the study and reviewed the statistical analyses. All authors read, reviewed, and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fimmu.2018.01113/full#supplementary-material

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3.2. Autoantibodies to Protein-Arginine Deiminase (PAD) 4 in Rheumatoid Arthritis: Immunological and Clinical Significance, and Potential for Precision Medicine

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REVIEW

Autoantibodies to protein-arginine deiminase (PAD) 4 in rheumatoid arthritis: immunological and clinical significance, and potential for precision medicine

Anti-PAD4 antibodies in RA

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ABSTRACT

Introduction: The protein-arginine deiminase (PAD) 4 enzyme plays an important role in the pathogenesis of rheumatoid arthritis (RA) and also represents an antigenic target. Anti-PAD4 antibodies can be present in RA and are associated with specific clinical features.

Areas covered: This review aims to analyze the current knowledge and recent findings on anti-PAD4 antibodies in RA and their clinical and immunological significance.

Expert opinion: Anti-PAD4 antibodies are not currently used in clinical practice for the management of RA. Nevertheless, there is growing evidence of their relevance in RA, and of their potential utility to improve diagnosis, patient stratification, and prognosis.

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1. Introduction

Rheumatoid Arthritis (RA) is a chronic, inflammatory disease that affects 0.5–1% of the population [1]. It is a complex heterogeneous condition with variable clinical manifestations, characterized by chronic synovial inflammation, and articular cartilage and bone damage. Several risk factors have been identified [2], including genetic and environmental factors, as well as lifestyle. Nevertheless, the underlying pathogenic mechanisms are still not fully understood.

The presence of autoreactive T- and B-cells and the production of autoantibodies are key features of RA, with Rheumatoid Factor (RF) and anti-citrullinated protein antibodies (ACPA) being the main two biomarkers. Yet, up to 60% of the early RA patients do not present these antibodies, often referred to as the serological gap [3]. Although the combination of ACPA and RF can increase the diagnostic efficiency [3], seronegative patients persist. Consequently, numerous studies aimed to identify novel biomarkers to further improve the diagnosis of RA [4]. In addition, biomarkers that aid in establishing prognosis, in patient stratification, in disease activity monitoring and in predicting response to treatment are desired. In this context, several novel autoantibodies have been described in RA patients over the past few years [4], including antibodies targeting carbamylated proteins (CarP) [5–8] and the protein-arginine deiminase (PAD) enzymes [9–12]. The PAD proteins are enzymes that catalyze the conversion of peptidyl-arginine into peptidyl-citrulline, a post-translational modification (PTM) known as citrullination or deimination, which is an important part in the pathogenesis of RA. In addition to their role in citrullination, three members of the PAD family of enzymes have been recently identified as autoantigens in RA, including PAD2, PAD3, and PAD4 [11–13], and among them, antibodies to PAD4 are the most characterized. In the last few years, PAD enzymes captured growing attention due to the cumulative understanding of their role in the pathogenesis of RA and the potential to block PAD activity as a novel treatment strategy [14–16]. The objective of this review is to provide an overview of the current understanding on anti-PAD4 antibodies in RA, including their role in pathogenesis, their clinical significance and their potential for precision medicine (PM) approaches.

2. The PAD enzymes and citrullination

Citrullination is an important PTM in normal physiological conditions of key cellular processes such as apoptosis, organization of structural proteins [14,17] and gene regulation, especially during early embryonic development [18,19]. The activity of the PAD enzymes is stringently regulated in normal conditions; however, dysregulation of the citrullination pathway can occur in association with several diseases, including several types of cancer [20], neurodegenerative diseases [21], and

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Article highlights

- Five members of the PAD family have been described in humans, and three of them, PAD2, 3 and 4, have been identified as autoantigens in RA.
- Anti-PAD4 antibodies are found in 30–40% of the RA patients with specificity of >95%, and in 2-18% of the RF and ACPA seronegative individuals.
- Anti-PAD4 antibodies are associated with joint erosions and a more severe disease phenotype.
- Testing for anti-PAD4 antibodies might provide diagnostic and prognostic value.

autoimmune diseases [22]. Particularly relevant is the case of RA, characterized by hypercitrullination and accumulation of citrullinated products and antibodies to the modified antigens present in the joints. Thus, citrullination can promote generation of neo-(auto) antigens and help trigger the autoimmune response [23].

Citrullination and the PAD enzymes also play an important role during apoptosis, autophagy and the formation of neutrophil extracellular traps (NETs) [24], processes well known for their involvement in autoimmunity. During infection or inflammation, PAD4 becomes activated in neutrophils resulting in the citrullinating of multiple autoantigens [25–27] and the ejection of chromatin from the cell, generating the NETs, important tools in the protection against infection [28]. Citrullination of histones represents an important step in this process [29]. Little is known about the role of PAD2 in NETs formation; limited data seems to indicate that PAD2 is required for tumor necrosis factor alpha (TNFα)-induced citrullination and arthritis, but that it is not required for the generation of NETs [30]. The release of NETs and PADs by neutrophils is likely followed by citrullination of extracellular antigens [31] that together with the infectious agents [2], the complement activation [32] and the formation of immune complexes [33] could challenge the immune system and potentially compromise the immune tolerance. Once tolerance is broken, the presence of an additional 'hit' to the immune system and/or additional co-factors that increase PAD activity (such as autoantibodies [11]) could be helping maintain efficient citrullination and contributing to autoimmunity. Moreover, it was recently demonstrated that PAD4 directly citrullinates nuclear factor κB (NF-κB) which has a critical role in the expression of pro-inflammatory cytokines IL-1β and TNFα [34]. Therefore, citrullination can help propagate inflammation in RA.

Citrullination is an irreversible process resulting in conformational structure changes of the modified protein, that can lead to unfolding [17], and alterations of the intra- and intermolecular interactions. The PAD enzymes require calcium for their catalytic activity; however, the calcium concentration identified for maximum PAD4 activity *in-vitro* is higher than found in the synovial fluid [27,35,36], suggesting the existence of additional factors that modulate this process during normal physiology and in the pathogenesis of RA.

The PAD enzymes were first described in 1977 by Rogers and colleagues [37]. Today, a total of five isotypes are known (reviewed in [27], summarized in Table 2) of which PAD2, PAD3, and PAD4 have been identified as antigenic targets in RA. As expected by the similar cellular function, there is a significant protein sequence homology between these three enzymes (Table 1). The members of the PAD family differ in their substrate specificities and tissue-specific expression and studies suggest that the PAD enzymes have the capacity to select unique protein targets and that this capability may play a role in autoantigen selection in RA [26].

Table 1. Protein sequence homology between the protein-arginine deiminase (PAD) 2, 3, and 4 enzymes. Data were generated by BLASTp analysis [38] using the FASTA sequences from UniProt (The UniProt Consortium). The UniProt IDs of each isoform can be found in Table 2.

Proteins compared	PAD2 *vs.* PAD3	PAD2 *vs.* PAD4	PAD3 *vs.* PAD4
Identities	345/667 (52%)	337/668 (50%)	374/669 (56%)
Positives	452/667 (67%)	440/668 (65%)	461/669 (68%)
Gaps	5/667 (0%)	8/668 (1%)	11/669 (1%)
E value	0.0	0.0	0.0

PAD, protein-arginine deiminase.

PAD2 is the most broadly expressed isoform. Many proteins have been identified as substrates of PAD2, with the main being myelin basic protein (MBP) in the central nervous system, and vimentin in skeletal muscle and macrophages [26,27,35,36]. Studies suggest a tissue-specific hormonal regulation of PAD2 expression [64,65]. Although PAD2 is mainly localized in the cytoplasm, data suggest that a fraction of PAD2 can also be found in the nucleus despite lacking a nuclear translocation signal, and that this nuclear PAD2 may citrullinate histones H3 and H4 and play a role in gene regulation [66].

On the other hand, PAD3 is expressed in hair follicles and has a cytoplasmic intra-cellular localization [27]. Its natural substrate, trichohyalin, is a major structural protein of inner root sheath cells of hair follicles [67].

PAD4 is mainly found in the nucleus and expressed in white blood cells (granulocytes, monocytes) and can be detected in several tissues [27]. In addition to upregulated enzymatic activity, PAD2 and PAD4 are overexpressed by neutrophils and monocytes in the synovium of RA patients in co-expression with numerous citrullinated proteins [68]. PADI4 has been confirmed as a susceptibility gene for RA [69–71] and an association between single nucleotide polymorphisms (SNPs) and RA has been reported in numerous ethnic groups [72–74], although these findings have not been replicated in certain populations [75,76]. Besides, it has been reported that PAD4 can autocitrullinate itself influencing the enzyme structure and immune response and that this process contributes to the regulation of citrullinated proteins generation during cell activation [77]. Nevertheless, the clinical implications of these observations need to be further explored.

A relatively broad range of targets has been described for PAD4, with certain overlap with PAD2 [78–83]. The enzymes´ specificity for cellular substrates and synthetic peptides seems to be different for PAD2 and PAD4, with the latest being more restricted by the amino acid composition surrounding the acceptor arginine residue [36]. It is unclear whether one of the isoforms dominates in the generation of citrullinated self-proteins that are targeted by ACPAs. A recent study showed that very high-titers ACPA preferentially bind fibrinogen citrullinated by PAD4 vs. PAD2 [84]. However, in a more recent

Table 2. Overview of human protein-arginine deiminases (PAD) isoforms and their characteristics.

Isoform (UniProt ID)	Mass (kDa)	Length (aa)	Substrate	Subcellular location	Tissue and cellular expression under normal conditions	Physiological roles	Ref.
PAD1 (Q9ULC6)	74.7	663	Keratin K1, filaggrin	Cytoplasm	Epidermis, prostate, testis, placenta, uterus, spleen and thymus	Skin differentiation, terminal differentiation of keratinocytes	[27,39–41]
PAD2 (Q9Y2J8)	75.6	665	Vimentin, MBP, GFAP, β and γ-actins, histone H3, fibrinogen, collagen II, alpha-enolase	Cytoplasm, may become nuclear in human mammary epithelial cells	Skeletal muscle, brain, pancreas, glial cells, macrophages, bone marrow, muscle, breast, colon, embryo, eye, kidney, epidermal, uterus, thymus	May plain a role in brain development, innate immune defense, female reproduction, gene expression	[26,27,35,42–48,64,66,81,83]
PAD3 (Q9ULW8)	74.7	664	Trichohyalin, fillaggrin	Cytoplasm	Hair follicles and keratinocytes	Skin differentiation, hair follicle formation, terminal differentiation of keratinocytes	[27,37,41,49,50]
PAD4 (Q9UM07)	74.1	663	Histones H2A, H3, H4, vimentin, p300, nucleophosmin/B23, ING4, fibrinogen, collagen II, alpha-enolase	Nucleus and cytoplasmic granules (eosinophils, neutrophils)	Eosinophils, neutrophils, granulocytes, macrophages; several cancerous tissues	Chromatin decondensation, transcription regulation, tumorigenesis, cellular differentiation, transcriptional corepressor for the estrogen receptor and p53, NETs formation	[27,45,51–59,81,83]
PAD6 (Q6TGC4)	77.7	694	Keratin	Cytoplasm	Egg, ovary, early embryo, thymus, oocyte, peripheral blood leukocytes	Embryonic development, oocyte cytoplasmic lattice formation, fertility	[27,60–63]

PAD, protein-arginine deiminase; kDa, kilo Dalton; aa, amino acids; MBP, myelin basic protein; GFAP, glial fibrillary acidic protein; NETs, Neutrophil Extracellular Traps.

publication, both enzymes seemed to be equally efficient in generating citrullinated targets for ACPAs on fibrinogen and enolase, but autoantibodies to histone H3 in these patients seemed to have a preference for the antigen that had been citrullinated by PAD4 [81].

3. Anti-citrullinated protein autoimmunity in the pathogenesis of RA

3.1. Animal models

Experimental models of inflammatory arthritis have proven essential to understanding the underlying mechanisms and developing therapeutics for the treatment of RA. Findings in multiple mouse models of RA support a key role for PADs and citrullinated antigens in the pathogenesis of RA.

Animal models that recapitulate certain clinical and mechanistic characteristics of RA have been developed over the years. Collagen induced arthritis (CIA) is the classical animal model of RA, first established in rats [85] and later adapted to mice [86]. CIA develops following immunization with type II collagen that was derived from either bovine or chicken. In addition to recapitulating certain histologic features of RA, including inflammation, pannus formation, and cartilage and bone damage, pathogenic antibodies including ACPA have been described to develop in mice with CIA [87–90]. These findings support the possibility that ACPA and anti-citrullinated protein autoimmunity could contribute to the pathogenesis of this model.

Two common citrullinated autoantigens targeted in RA include citrullinated fibrinogen and citrullinated histone 2B (H2B). A significant proportion of ACPA+ patients express antibodies targeting these two antigens [89,91–93]. Immunization of mice with citrullinated fibrinogen or citrullinated H2B both resulted in inflammatory arthritis [89,92,94]. Further, fibrin-induced arthritis (FIA) in mice was associated with the development of RF as well as antibodies against other citrullinated proteins.

There are also mouse models in which antibody transfer results in inflammatory arthritis. Examples of such antibody transfer models of RA include anti-collagen antibody-induced arthritis (CAIA), the K/BxN serum transfer model in which anti-glucose-6-phosphate isomerase (GPI) antibodies induce inflammatory arthritis, and other models in which transfer of serum antibodies from arthritic mice results in arthritis in the recipients. These antibody transfer models enable characterization of the role of antibodies against particular targets in the induction or exacerbation of inflammatory arthritis. For example, in the first study to demonstrate that an antibody against a citrullinated target could contribute to inflammatory arthritis in mice, a recombinant antibody specific for citrullinated fibrinogen exacerbated suboptimal CAIA [87]. Subsequently, it was shown that transfer of polyclonal antibodies derived from mice immunized with either citrullinated fibrinogen or citrullinated H2B induced arthritis in naïve recipients [89,92]. Together, these studies demonstrate that citrullinated fibrinogen and citrullinated H2B can be pathogenic autoantigens that mediate autoimmune arthritis in mice.

3.2. PAD inhibition

Genetic and pharmacologic inhibition of PADs in mouse models of RA has been used to further define the roles and mechanisms of PAD2 and PAD4, citrullination and NETosis in inflammatory arthritis. Mice deficient for PAD4 have been studied for development of both CIA [95] and K/BxN serum transfer arthritis [96], as well as in other models of arthritis

[97]. Global PAD as well as PAD4-selective inhibitors have also been tested in mouse models of RA, and the results provide further insights into the mechanisms by which PADs contribute to inflammatory arthritis.

PAD4-deficient DBA1J mice exhibited modestly reduced arthritis severity following immunization with type II collagen (CII) to induce CIA [95]. Although an initial study reported that genetic PAD4 deficiency did not protect mice against K/BxN serum transfer arthritis despite reducing levels of citrullination in arthritic joints [96], and it is possible that PAD4 does not play a role in the effector phase of the anti-GPI passive transfer model. In contrast, a subsequent study demonstrated that genetic PAD4-deficiency protected mice against GPI-immunization-mediated inflammatory arthritis [97]. In addition to inhibiting clinical arthritis following GPI-immunization, PAD4-deficiency also was associated with reduced serum IL-6 and reduced synovial myeloid and Th17 cells [97]. Together, these data suggest that PAD4 plays an important pathogenic role in immunization-induced autoimmune arthritis.

In a series of studies using TNF-transgenic mice that spontaneously develop inflammatory arthritis, PAD2-deficient mice exhibited reduced inflammatory arthritis [30]. The authors further demonstrated that TNFα-induced arthritis resulted in increased citrullination in inflamed ankle joints, and that genetic deficiency in PAD2 reduced this citrullination while the levels of citrullination in inflamed ankle joints were not significantly altered by PAD4 deficiency. The authors also demonstrated that PAD2-deficient neutrophils appeared to undergo normal NETosis, while PAD4 was critical for neutrophil NETosis [30].

The importance of PADs in RA mouse models has been corroborated by studies utilizing small molecule PAD inhibitors. Cl-amidine, a pan-PAD inhibitor, reduced the severity of inflammatory arthritis in the CIA model [98]. Further, the CII-immunized Cl-amidine-treated mice exhibited reduced synovial and serum citrullination, and decreased anti-CII antibody titers in these mice [98]. While Cl-amidine also significantly reduced arthritis severity following K/BxN serum transfer, it had no significant impact on ACPA titers [99].

While Cl-amidine globally inhibits PADs (including both PAD2 and PAD4), BB-Cl-amidine exhibits increased potency against PAD2 [100,101]. Treatment of mice with BB-Cl-amidine-reduced joint inflammation and destruction to a greater degree as compared to Cl-amidine in mice with CIA [101]. While a shift from pro-inflammatory Th1 and Th17-type responses to pro-resolution Th2-type responses were associated with BB-Cl-amidine treatment, minimal change in antibodies to collagen or citrullinated peptides was observed [101].

More recently, treatment of mice with GSK199, a PAD4-selective inhibitor significantly reduced arthritis severity in CIA. In this study, a subset of ACPA was reduced by GSK199 treatment, but global citrulline levels and circulating anti-CII antibody levels were not affected [16].

In addition to development of small molecule PAD inhibitors, several groups are developing monoclonal antibodies specific for PAD2 or PAD4. Although such reagents will inhibit specific targeting of PAD2 or PAD4, due to their relatively large size as monoclonal antibodies they will not fully penetrate joint tissues and/or cells and thereby likely be most effective at examining inhibition of extracellular PAD2 and/or 4 released by neutrophils, macrophages and/or other cells in animal models of RA.

4. Anti-PAD antibodies in RA

4.1. Anti-PAD4 antibodies

Anti-PAD4 antibodies were first described in patients with RA by Nissinen and colleagues in 2003 [13] (Figure 1). Since this initial description, numerous studies over the past years have

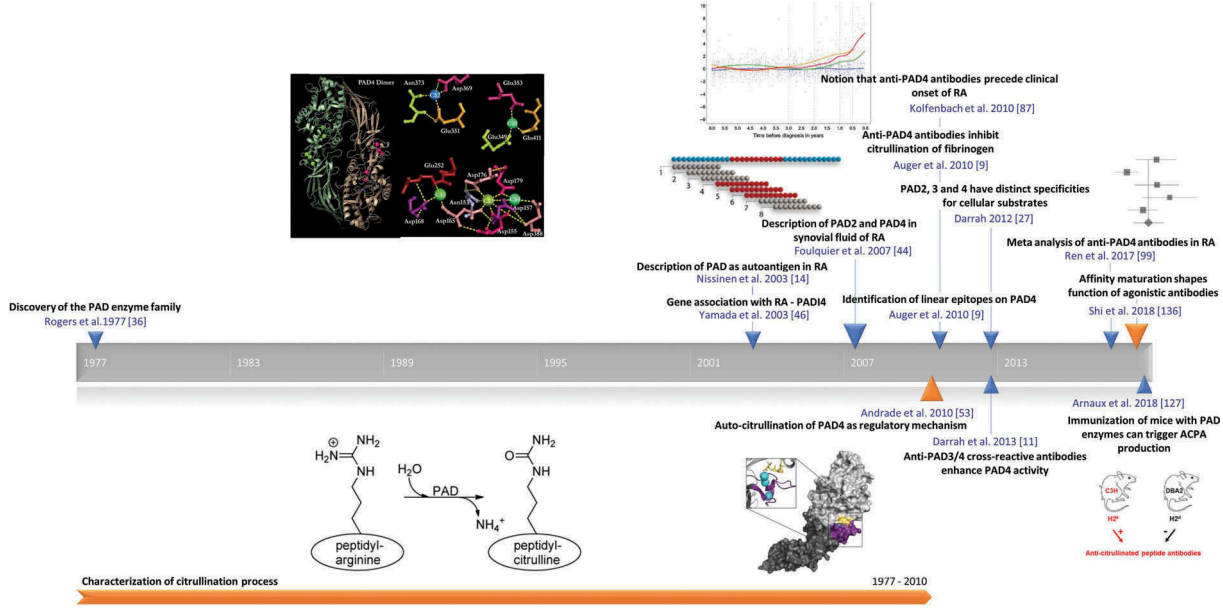


Figure 1. History of anti-protein arginine deiminase (PAD4) antibodies in rheumatoid arthritis (RA). The history of anti-PAD4 antibodies started with the first description of the PAD enzyme family in 1997 [37]. Later on in 2003, a study by Nissinen et al. reported PAD as a target of autoantibodies in RA patients [13]. Several additional studies led to the conclusion that anti-PAD4 antibodies might present value in the diagnosis and management of RA patients.

Table 3. Summary of studies on anti-PAD4 antibodies, including number and origin of patients, detection method used and diagnostic findings.

Study, year of publication	Number of RA patients	RA patients origin	RA stage	Number of controls	Controls composition	Detection method	Sensitivity	Specificity
Nissinen et al., 2003 [13]	57/51	NP	57 early (baseline and follow up 3 years later); 51 established	172	43 SLE, 19 pSjS, 20 MS, 90 HI (BD)	ELISA	Early: 88% at baseline, 70% at follow up; Established: 22%	76.2%
Takizawa et al., 2005 [103]	42	NP	Established	82	19 SLE, 23 other rheumatic diseases, 40 HI	ELISA and WB	50%	95%
Roth et al., 2006 [104]	184	Rheumatological Unit of Malmo University Hospital	Early	59	HI (BD)	ELISA	31% all patients (25% MTX, 35% non-MTX)	97%
Zhao et al., 2008 [105]	109	Department of Rheumatology and Immunology, People's Hospital, Peking University, Beijing	Established	338	67 SLE, 48 pSjS, 41 SSc, 34 OA, 23 DM/PM, 19 AS, 106 HI (DB)	ELISA	45%	94%
Harris et al., 2008 [106]	38/129	John Hopkins Arthritis Center/ESCAPE RA TRIAL	Established	158	32 HI, 31 myositis, 31 SSc, 32 SjS, 32 SLE	IP	42%/36%	99%
Halvorsen et al., 2008 [102]	237/177	EURIDISS RA cohort/Oslo RA Register	Established	232	84 SLE, 148 HI	ELISA	22%/25%	91.4%
Halvorsen et al., 2009 [107]	40	NP	Established [at baseline (n = 40) and follow up after one year (n = 33)]	NP	NP	ELISA	42.5% at baseline, 45.5% at follow up	NP
Auger et al., 2009 [108]	116	Rheumatology Unit La Conception Hospital, Marseilla, France	Established	93	33 AS, 60 HI	ELISA	29%	98%
Kolfenbach et al., 2010 [109]	83	Military Cohort – Walter Reed Army Medical Center Rheumatology Clinic	Preclinical	83	83 HI	IP	18%	99%
Wang et al., 2011 [110]	102	NP	Established [with active disease (n = 50), without active disease (n = 52)]	239	84 SLE, 35 pSjS, 20 SSc, 100 HI	ELISA	32.4%	96%
Ishigami et al., 2013 [111]	32	Department of Rheumatology, Tokyo Metropolitan Geriatric hospital and Institute of Gerontology	NP	30	10 OA, 20 HI	ELISA	37.5%	100%
Ferucci et al., 2013 [112]	82	Two indigenous North American populations in Canada and the United States; First Nations or Alaska Native people	Established	191	147 FDR, 44 HI	IP	29.3%	98.90%
Darrah et al., 2013 [11]	194	ESCAPE RA Cohort	Established	66	36 HI, 30 PsA	IP	37.1%	NP
Reyes-Castillo et al., 2015 [113]	170	Rheumatology service of two hospitals in Jalisco, Mexico	Early and established	103	103 HI	ELISA	24% (18% early, 29% established)	95%
Umeda et al., 2015 [114]	148	University of Tsukuba Hospital	Established	113	36 SLE, 37 SjS, 40 HI	ELISA	20%	89%
Navarro-Millan et al., 2016 [115]	192	CLEAR Registry	Early and established	NP	NP	IP	24%	NP
Martinez-Prat et al., 2018 [116]	640	MTX PATH, IMPACT and CAPITAL studies	Early and established	833	369 SLE, 64 Sjs, 33 SSc, 29 IIM, 14 OAARD, 85 FMS, 42 OAID, 197 HI	PMAT	35% (16% Early)	95.4% (99.1% early)

(*Continued*)

Table 3. (Continued).

Study, year of publication	Number of RA patients	RA patients origin	RA stage	Number of controls	Controls composition	Detection method	Sensitivity	Specificity
Darrah et al., 2018 [118]	282	VACSP, RACAT study	Established	NP	NP	IP	26%	NP
Guderud et al., 2018 [119]	745	EURIDISS cohort, ORAR and a cohort early RA patients undergoing MRI, a cohort of patients starting TNFi therapy	Early and established	70	70 HI	DELFIA	25.9%	Information not provided
Cappelli et al., 2018 [117]	274	John Hopkins Arthritis Center	Established	NP	NP	IP	25%	NP

NP, Not Provided; ESCAPE, Evaluation of Subclinical Cardiovascular Disease and Predictors of Events in Rheumatoid Arthritis; CLEAR, Consortium for the Longitudinal Evaluation of African Americans with Early Rheumatoid Arthritis; MTX, Methotrexate; VACSP, Veterans Affairs Cooperative Study Program; RACAT, Rheumatoid Arthritis Comparison of Active Therapies; EURIDISS, European Research on Incapacitating Disease and Social Support; ORAR, Oslo Rheumatoid Arthritis Register; TNFi, Tumor Necrosis Factor inhibitor; RA, Rheumatoid Arthritis; SLE, Systemic Lupus Erythematosus; pSjS, primary Sjogren's Syndrome; MS, Multiple Sclerosis; HI, Healthy Individuals; BD, blood donors; SSc, Systemic Sclerosis; OA, Osteoarthritis; DM, Dermatomyositis; PM, Polymyositis; AS, Ankylosing Spondylitis; FDR, First Degree Relatives; PsA, Psoriatic Arthritis; IIM, Idiopathic Inflammatory Myopathies; OAARD, Other ANA-Associated Autoimmune Rheumatic Diseases; FMS, Fibromyalgia Syndrome; OAID, Other Autoimmune Diseases; ELISA, Enzyme-linked immunosorbent assay; WB, Western Blot; IP, Immunoprecipitation; PMAT, Particle-Based Multi-Analyte Technology; DELFIA, Dissociation-Enhanced Lanthanide Fluorescence Immunoassay.

confirmed the presence those antibodies in RA patients (Table 3) [9–11,102–119]. Anti-PAD4 antibodies are usually found in a subgroup of RA patients with a prevalence of 20-45% and are associated with the presence of ACPA [11,102,117,120]. Meta-analysis [121] showed a pooled sensitivity of 38.0% with 96.0% specificity of anti-PAD4 antibodies in RA, and good discrimination between RA patients and controls has consistently been observed. Several studies have looked into the presence of anti-PAD4 antibodies in independent cohorts, summarized in Table 3. Interestingly, anti-PAD4 antibodies have been found in ACPA negative patients, suggesting a utility to help close the serological gap in RA [116].

The effect of the autoantibodies on the enzyme functionality and activity is not completely understood (Figure 2). Some data indicate that anti-PAD4 antibodies seem to inhibit the enzymatic activity of the protein [9]. In the same study, peptides located in both the N-terminal domain (211–290) and the C-terminal domain (601–650) of protein were recognized by anti-PAD4 positive sera from RA patients (Figure 3). Other researchers had previously looked into the epitope(s) location in PAD4 using truncated versions of the protein and anti-PAD4 positive sera from RA patients and localized several epitopes on the protein [106]. However, the exact location, sequence, and structure of the epitopes recognized by anti-PAD4 antibodies still need to be fully understood.

Interestingly, anti-PAD4 can precede the onset of disease and are found in the pre-clinical phase in a subset of RA patients [109]. The exact timing of appearance of anti-PAD4 antibodies during the evolution of RA still requires systematic studies. In a study using a military cohort with samples collected over decades, it was demonstrated that anti-PAD4 antibodies might occur after ACPA and RF. Since immunoprecipitation was used for the measurement of anti-PAD4 antibodies which sometimes lacks sensitivity, those findings should be verified with newer methods such as ELISA or the recently developed PMAT system [116]. Only then it will be possible to conclude whether anti-PAD4 antibodies arise after ACPA and RF and whether they hold value in the prediction and potential prevention of RA [122]. The finding that anti-PAD4 antibodies might appear later is also somewhat controversial to the findings that immunization with PAD enzymes can stimulate the production of ACPA in mice [123]. However, this might be explained by the fact that animal models not always translate to human disease, especially when generated via immunization (vs. spontaneous models). Additional insights will also come from the sequencing of the B-cell repertoire of RA patients and the subsequent analysis of potential mutations [124,125]. Pollman et al. [120] studied the levels of anti-PAD4 in RA patients over a period of 10 years and demonstrated that anti-PAD4 positive patients remained positive over time, and some patients that initially did not present these antibodies became positive later in the disease course.

The potential effect of treatment on the levels of anti-PAD4 is also an area of interest. Limited data have indicated that anti-TNF-alpha therapy does not impact the levels of anti-PAD4 antibodies over a 12-month period time and the researchers suggested that this could be indicative of a specific phenotype characterized by inadequate response

to this treatment type [120]. Somewhat contrary to these findings, Darrah et al. recently reported that anti-PAD4 positive patients are characterized by a worse radiographic joint damage at baseline, but a more favorable response to treatment escalation therapy, which was more effective in slowing the progression of the disease and decreasing disease activity [118]. Significant differences in study design between these two studies could explain the discrepant outcomes. Additional studies in larger cohorts treated with drugs with different mechanism of action are needed to further investigate a potential effect of therapy on these antibodies and their associations with treatment response.

The discovery and characterization of a PAD enzyme expressed by the primary periodontal pathogen *Porphyromonas gingivalis*, known as the as PPAD, together with the fact that periodontitis is a known RA risk factor RA, formed the basis for the hypothesis that PPAD may represent a mechanistic link between periodontitis and RA [126]. Several studies have suggested that PPAD can perform protein citrullination at the inflamed periodontal sites and trigger a cascade of events that can lead to NET formation, generation of citrullinated neoantigens, and ACPA production [127]. The link between these events and the clinical onset of RA remains an area of interest. Recently, antibodies to PPAD were described in RA patients [128]; however, the association between these antibodies, ACPA and their potential role in the development of RA needs to be further investigated.

4.2. Antibodies to other PAD enzymes

Besides PAD4, PAD2 and PAD3 have also been described as targets of autoantibodies in patients with RA. Compared to anti-PAD4 antibodies, the body of literature on the autoantibodies to other PAD enzymes is scarce. In addition, although no studies have described antibodies targeting PAD1 or PAD6, it is likely that those proteins serve as autoantigens in RA. In the section below, the current knowledge on anti-PAD2 and anti-PAD3 antibodies is summarized.

4.2.1. Anti-PAD2 antibodies

Several factors point to PAD2 as an important driver in RA, including the association of PADI2 polymorphism with the development of the disease, the expression of this enzyme in the tissue and synovial fluid from inflamed joints and for its capacity to generate citrullinated autoantigens [81,129–131]. Yet, PAD2 had not been identified as a target of the immune response in RA until very recently, when Darrah et al. described for the first time antibodies targeting this protein in the sera of RA patients [12]. In this pilot study, in contrast to anti-PAD4 or the anti-PAD3/4XR antibodies, anti-PAD2 antibodies seemed to be characteristic of a genetically and clinically distinct subtype of RA patients with less severe baseline joint inflammation, slower joint disease progression, and less lung disease. Together with the lack of association between anti-PAD2 and ACPA and the low overlap with anti-PAD3/4XR antibodies observed, these data suggest that these antibodies define a different subpopulation of RA patients, potentially with milder progress and better outcome. Further studies are needed to verify the interesting but preliminary findings.

4.2.2. Anti-PAD3 antibodies

In 2013 PAD3 was also identified as an antigenic target in RA [11]. Anti-PAD3 antibodies are present in 10-20% of the RA patients. Similar to anti-PAD4, anti-PAD3 antibodies are also associated with ACPA and the HLA-DRB1 SE [11]. Competition experiments demonstrated that this subset of anti-PAD3 antibodies cross-react with PAD4, leading to the identification of a cross-reactive epitope in these two enzymes. Although the characteristics of these antibodies remained to be further understood, data suggests that affinity maturation has a role in defining the function of these antibodies [132]. It was demonstrated that these anti-PAD3/4 cross-reactive (anti-PAD3/4XR) antibodies mimic the calcium-ion binding to the enzyme and increase the catalytic efficiency of PAD4 by decreasing the calcium concentration needed for its activation (Figure 2). Thus, they support protein citrullination at physiologically relevant calcium concentrations and therefore, represent important drivers of dysregulated protein citrullination and RA pathogenesis.

5. Clinical associations of anti-PAD4 antibodies

The high number of 'seronegative' RA patients, with negative RF and/or ACPA, have driven the demand for novel diagnostic biomarkers. In the last few years, anti-PAD4 antibodies have been identified in between 2% and 17.7% of seronegative RA patients in numerous cohorts [105,116,133].

However, the utility of biomarkers goes beyond diagnosis, and several clinical studies have recently focused on the clinical associations and the prognostic value of anti-PAD4 antibodies (Table 4). Nevertheless, the majority of these studies were cross-sectional in design, limiting their power to determine a temporal association between the clinical feature and the presence of anti-PAD4 antibodies.

5.1. Erosions, radiographic progression, and clinical activity

Out of the 13 identified studies, 10 described the presence of erosive disease [11,102,105–107,109,115,117,118,120,134] and/or longitudinal radiographic progression [102,107,115,118,120] in patients with anti-PAD4 antibodies. Additionally, seven studies found a higher modified Sharp score and overall more erosive disease at the time of recruitment in patients with anti-PAD4 antibodies when compared to PAD4 negative patients [102,105,106,117,118,120,134]. In the three studies focused on the cross-reactive anti-PAD3/4XR antibodies, the presence of the cross-reactive antibodies was strongly associated with baseline erosive disease and more severe radiographic progression [11,115,117].

Two studies evaluated response to treatment (DMARDs, biologics, and glucocorticoids) in terms of radiographic progression of anti-PAD4 and anti-PAD3 positive versus negative patients [11,118]. In 2013 Darrah and colleagues suggested that patients with both anti-PAD4 and anti-PAD3 antibodies represent a subset individuals with more erosive disease and severe radiographic damage despite therapy [11]. A few years later, the very same group conducted a prospective study and demonstrated that anti-PAD4 antibodies may represent

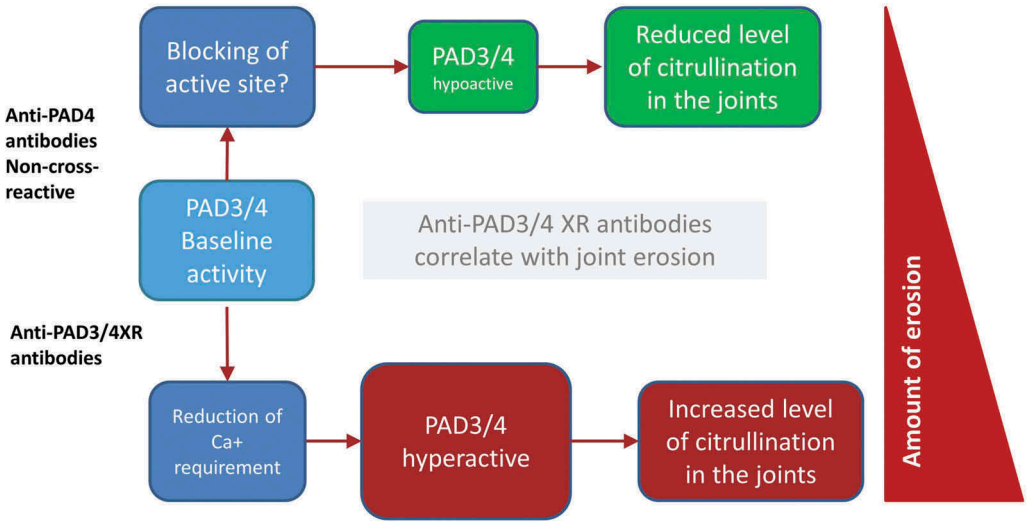
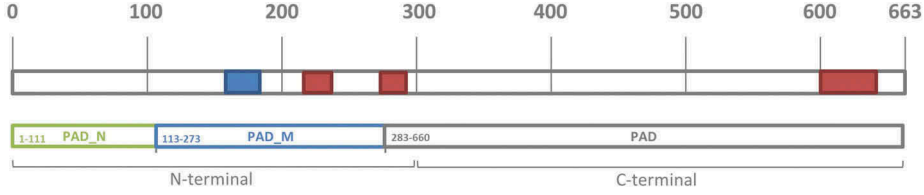


Figure 2. Illustrated model of the impact of anti-protein-arginine deiminase (PAD)4 antibodies on PAD enzyme activity, citrullination and rheumatoid arthritis (RA) pathogenesis. Depending on the epitope specificity, anti-PAD4 antibodies can either inhibit or stimulate the activity of PAD enzymes thereby reducing or enhancing the burden on joint erosions in RA. Anti-PAD3 anti-PAD4 cross-reactive antibodies (anti-PAD3/4XR) have been shown to reduce the Calcium requirement for PAD which is a requirement for the enzymes activity in the low Calcium concentrations present in the synovium fluid of RA patients.

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        10         20         30         40         50
MAQGTLIRVT PEQPTHAVCV LGTLTQLDIC SSAPEDCTSF SINASPGVVV
        60         70         80         90        100
DIAHGPPAKK KSTGSSTWPL DPGVEVTLTM KVASGSTGDQ KVQISYYGPK
       110        120        130        140        150
TPPVKALLYL TGVEISLCAD ITRTGKVKPT RAVKDQRTWT WGPCGQGAIL
       160        170        180        190        200
LVNCDRDNLE SSAMDCEDDE VLDSEDLQDM SLMTLSTKTP KDFFTNHTLV
       210        220        230        240        250
LHVARSEMDK VRVFQATRGK LSSKCSVVLG PKWPSHYLMV PGGKHNMDFY
       260        270        280        290        300
VEALAFPDTD FPGLITLTIS LLDTSNLELP EAVVFQDSVV FRVAPWIMTP
       310        320        330        340        350
NTQPPQEVYA CSIFENEDFL KSVTTLAMKA KCKLTICPEE ENMDDQWMQD
       360        370        380        390        400
EMEIGYIQAP HKTLPVVFDS PRNRGLKEFP IKRVMGPDFG YVTRGPQTGG
       410        420        430        440        450
ISGLDSFGNL EVSPPVTVRG KEYPLGRILF GDSCYPSNDS RQMHQALQDF
       460        470        480        490        500
LSAQQVQAPV KLYSDWLSVG HVDEFLSFVP APDRKGFRLL LASPRSCYKL
       510        520        530        540        550
FQEQQNEGHG EALLFEGIKK KKQQKIKNIL SNKTLREHNS FVERCIDWNR
       560        570        580        590        600
ELLKRELGLA ESDIIDIPQL FKLKEFSKAE AFFPNMVNML VLGKHLGIPK
       610        620        630        640        650
PFGPVINGRC CLEEKVCSLL EPLGLQCTFI NDFFTYHIRH GEVHCGTNVR
       660
RKPFSFKWWN MVP
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Auger et al. 2010 [9],
Seaman et al. 2016 [142]

Darrah et al. 2013 [11]

Figure 3. Protein-arginine deiminase (PAD) 4 primary structure and sequence. The domains and epitopes described in literature are marked in the structure.

a biomarker for better response to therapy despite more aggressive erosive disease at baseline [118].

Clinical activity in RA was generally assessed by erythrocyte sedimentation rate (ESR), C-reactive protein (CRP) and/or disease activity score (DAS) 28 [102,105,113,115,118,119,134]. Overall, only Zhao and colleagues found higher levels of ESR and DAS-28 in patients with anti-PAD4 antibodies, whereas the other studies found no differences between the two groups, except for higher CRP in patients positive for both anti-PAD4 and anti-CCP antibodies [102]. Surprisingly, Cappelli et al. found lower CDAI score in patients with anti-PAD3/4XR antibodies [117].

Table 4. Summary of studies on anti-PAD4 antibodies and clinical associations.

Study, year of publication	Erosive disease	Radiographic progression	CRP	ESR	DAS28	Rheumatoid nodules	Physical disability	Lung involvement	Response to treatment
Anti-PAD4									
Harris et al., 2008 [106]	More	NA	NA	NA	NA	NA	NA	NA	NA
Halvorsen et al. 2008 [102]	More	Yes	Higher, but related to ACPA presence	NA	NA	NA	More severe	NA	NA
Zhao et al., 2008 [105]	More	NA	No difference	Higher	Higher	More	NA	No difference	NA
Halvorsen et al., 2009 [107]	More	More	No difference	No difference	Higher	NA	NA	NA	NA
Kolfenbach et al., 2010 [109]	No difference	NA	NA	NA	NA	NA	NA	NA	NA
Pollmann et al., 2012 [120]	More	Yes	NA	NA	NA	NA	NA	NA	NA
Darrah et al., 2013 [11]	More, but only for anti-PAD3/4XR	Yes, but only for anti-PAD3/4XR	NA	NA	NA	NA	NA	NA	Worse
Giles et al., 2014 [134]*	More	NA	NA	NA	No difference	NA	No difference	More, but only of anti-PAD3/4XR; no difference for anti-PAD4	NA
Reyes-Castillo et al., 2015 [113]	NA	NA	No difference	No difference	No difference	NA	NA	NA	NA
Navarro-Millàn et al., 2016 [115]*	More, but only for anti-PAD3/4XR	Yes, but only for anti-PAD3/4XR	No difference	NA	NA	NA	NA	NA	NA
Cappelli et al., 2018 [117]	More, but only for anti-PAD3/4XR	NA	NA	NA	NA	NA	NA	NA	NA
Darrah et al., 2018 [118]	More at baseline	Better response to therapy	NA	No difference	No difference	NA	NA	NA	Better
Guderud et al., 2018 [119]	NA	NA	No difference	No difference	No difference	NA	No difference	NA	NA
Anti-PAD3									
Seaman et al., 2016 [138]	More, but only for high serum levels	No difference	NA	NA	NA	NA	NA	NA	NA
Anti-PAD2									
Darrah et al., 2018 [12]	No difference, but few swollen joints	No difference	No difference	NA	No difference	No difference	No difference	Less, but without statistical significance	No difference

The items 'more', 'higher' and 'no difference' are referred to the comparison with anti-PAD negative patients. When multivariate analysis is present, only this item is taken into consideration, while crude analysis was excluded.
CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; DAS28, disease activity score 28; NA, not analyzed; ACPA, anti-citrullinated protein antibodies.

The presence of rheumatoid nodules may be associated with a more aggressive disease [135]. Zhao et al. evaluated this extra-articular manifestation of RA in their cohort, finding a positive association of rheumatoid nodules with anti-PAD4 antibody positivity [105].

More than 25% of the RA patients are at risk of a certain grade of physical impairment and identification of high-risk patients needing early and aggression therapy is needed [136]. Three studies assessed the correlation between anti-PAD4 levels and the presence of physical disability, with conflicting results [102,119,134]. However, longitudinal studies that look into this aspect are still lacking.

It is important to mention that certain differences between the studies could represent a limitation in these comparisons. These include the use of different methods for the measurement of anti-PAD antibodies, different scales for measurement of erosions and radiographic progression and even different scores for the evaluation of joint erosion. Joint space narrowing score and modified Sharp score were used in the majority, whereas some used ad hoc erosion scores.

5.2. Lung involvement

Lung involvement is common in RA and might affect the airways, pleura, parenchyma, and vasculature. Interstitial lung disease (ILD) in patients with RA is the second most common cause of death, after cardiovascular disease [137]. To date, two studies have investigated the relationship between anti-PAD4 antibodies and lung involvement, and one focused on ILD. Zhao and colleagues found no differences among patients positive or negative for anti-PAD4 for 'pulmonary fibrosis'. A cross-sectional study by Giles and colleagues found that anti-PAD3/4XR antibodies were associated with ILD; however, no correlation with abnormalities of pulmonary function tests or the reported respiratory symptoms was observed [134]. No correlation was found for patients with anti-PAD4 monospecific antibodies. It is important to note, however, that anti-PAD3/4XR positive individuals presented a longer standing disease, limiting any ability to establish temporality in this association.

Little is known about whether there is an etiological association of cigarette smoking and the development of these antibodies. Although differences in terms of smoking profile between the anti-PAD4 positive and negative patients have been reported [11,134], very recent data have indicated that there is not a direct link between smoking and the development of anti-PAD4 antibodies [117]. The data around this topic are quite controversial and systematic studies are needed to better understand the link between smoking and the development of anti-PAD antibodies.

5.3. Clinical associations of antibodies to other PADs

5.3.1. Anti-PAD3

Only one cross-sectional study investigated the presence of anti-PAD3 alone in RA patients, showing that only higher levels of these antibodies correlated to the presence of erosion [138]. Nevertheless, disease duration at the time of sampling was not assessed in this study and very limited clinical information was available on these patients.

5.3.2. Anti-PAD2

Very recently, Darrah and colleagues reported that higher levels of anti-PAD2 antibodies are associated with fewer swollen joints, but no difference was found regarding the presence of erosions. Only five patients positive for anti-PAD2 antibodies presented ILD at CT-scan and their ILD-score assessed by an expert radiologist resulted significantly lower than the control group [12].

6. Anti-PAD4 antibodies and precision medicine

The knowledge and understanding of RA have evolved significantly during the last decades which help to improve patient care transforming RA from a destructive and disabling disease with limited therapeutic options, to a disease for which remission is an achievable goal through early intervention, control of inflammation and prevention of joint destruction [139,140]. Opportunities for PM approaches in RA are emerging but its complete implementation will require a paradigm shift in several aspects, including the use and application of autoantibodies and other biomarkers.

In this context, anti-PAD4 antibodies represent very interesting serological tools. Although more studies are needed, similarly to RF IgM and ACPA, they seem to precede onset of RA, so they could be useful in disease prediction and earlier diagnosis [109]. Furthermore, anti-PAD4 antibodies can be found in ACPA negative patients and can help to close the serological gap [116,133]. Consequently, it could be anticipated that multi-parametric approaches that integrate ACPA and RF, and that this novel biomarker will be helpful in improving diagnosis. In addition, anti-PAD4 antibodies can help stratify patients based on risk for erosion and ILD potentially guiding therapeutic decisions [11,118,134]. However, whether they can help predict or monitor response to treatment still needs to be investigated.

Several treatment options targeting different pathways are currently available; however, many patients still do not respond or achieve remission with current therapies [139]. Therefore, there is a need for new treat-to-target strategy and new therapies. In this respect, PAD inhibitors are emerging as a new class of drugs to treat RA [15,16,98,141,142], and anti-PAD antibodies could represent useful biomarkers to stratify patients for prediction of response to this new therapeutic approach.

Hence, anti-PAD4 antibodies can be helpful in addressing several needs in RA and represent a useful biomarker to facilitate the implementation of PM approaches in RA.

7. Conclusion

In conclusion, there is a growing body of evidence that anti-PAD4 antibodies represent a promising biomarker in the diagnosis and stratification of RA. In particular, the presence in ACPA/RF negative individuals holds promise to help to close the serological gap. In addition, the validated association with joint erosions indicates that anti-PAD4 antibodies might have utility beyond the diagnosis and allow for stratification of patients according to the disease severity. Less is known about autoantibodies to the other PAD enzymes. While some studies have looked at antibodies targeting PAD2 and PAD3,

no study has reported antibodies to PAD1 or PAD6 until today. Further research is needed to better understand the role of anti-PAD antibodies in RA and their clinical significance.

8. Expert opinion

Although known for almost 20 years now, anti-PAD4 antibodies are not used in clinical practice for the diagnosis and management of RA patients. This is mostly related to the lack of standardized and validated immunoassays for the detection of anti-PAD antibodies. However, based on the growing body of evidence on the relevance of anti-PAD antibodies in RA and their potential utility in the diagnosis of RA, it is likely that commercial assays will become available. From the recent data, the main benefit of anti-PAD4 antibodies lies in the detection of early RA patients that are negative for the classification criteria markers ACPA and RF as well as in the identification of patients with more aggressive disease the manifest in joint erosion and damage. These findings were recently summarized in meta-analysis performed by different groups and using different approaches [121,143]. It was concluded that anti-PAD4 antibodies can be found in about 35% of the RA patients accompanied with a specificity of more than 95%. In the patients negative for ACPA and RF, the sensitivity decreases, but still provides high OR for the differentiation between RA and controls. In light of the trend toward precision medicine, it is important to mention that testing for anti-PAD4 antibodies also has the potential to define a subset of RA patients that benefit from treatment escalation. Based on the different associations of antibodies to the other PAD isoforms (e.g. PAD2 and PAD3), multi-analyte testing for anti-PAD antibodies might provide a useful tool for patient stratification and PM. According to current knowledge, patients with anti-PAD4 and especially anti-PAD3/4XR might benefit from more aggressive treatment, whereas patients positive for anti-PAD2 antibodies might present a more benign form which would require less attention. The recent study by Darrah et al. [118] indicated that treatment escalation through additional DMARDs including TNFα inhibitor provided benefits to anti-PAD4 positive patients. Whether this also applies for other DMARDs available on the market today or under development remains unclear. Of special interest could be the combination of anti-PAD4 testing with potential anti-PAD inhibitors as a companion or complementary diagnostic approach. However, further studies are required and assays need to be developed and taken through regulatory scrutiny (including but not limited to CE mark and FDA clearance).

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Declaration of interest

L Martinez-Prat and M Mahler are employees of Inova Diagnostics. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

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3.3. Antibodies Targeting Protein-Arginine Deiminase 4 (PAD4) Demonstrate Diagnostic Value in Rheumatoid Arthritis

Antibodies targeting protein-arginine deiminase 4 (PAD4) demonstrate diagnostic value in rheumatoid arthritis

Although anti-citrullinated protein antibodies (ACPA) and rheumatoid factor (RF) are widely used as part of the diagnosis of patients with rheumatoid arthritis (RA), they leave a serological gap.[1] Recently, several novel autoantibodies have been described in RA including antibodies targeting protein-arginine deiminases (PAD).[2 3] Similar to ACPA and RF, anti-PAD4 antibodies also precede the clinical onset of RA.[4] A subset of autoantibodies cross-reactive to PAD3 and PAD4 decrease the enzyme's calcium requirement into the physiologic range, thereby increasing the catalytic efficiency of PAD4.[5] Here we aim to evaluate the diagnostic relevance of anti-PAD3/4 antibodies in a large cohort (n=1473) of patients with RA and relevant controls (details in figure 1, table 1).

The median age (min/max) of the patients with RA was 60 (18/90) years and 510 (79.7%) were female. For 528 patients with RA and 582 controls, data on disease duration were available. Median (min/max) disease duration was 8.0 (0.0/68.0) years. Anti-PAD3 and anti-PAD4 IgG were measured using a particle-based multianalyte technology (research use only, Inova Diagnostics, USA). Cut-off values for anti-PAD3 and anti-PAD4 antibodies were established using the 95th percentile of healthy individuals and set to 200 and 1000 units, respectively. ACPA IgG and RF IgM were measured by EliA (Phadia, Germany).

Anti-PAD3 and anti-PAD4 antibodies were observed in 14% (88/640) and 35% (223/640) of patients with RA , respectively, as well as in 0%–21% and 0%–9% of controls (see online supplementary table S1) and found in ACPA+ and ACPA− patients, even at high titres (figure 1). The prevalence of the double positivity in patients with RA was 12% (77/640). Similar results were found in the ACPA/RF double-negative patients (data not shown). Consequently, the OR for RA in the ACPA− population was 5.9 for anti-PAD4 and 2.3 for anti-PAD3 antibodies. In

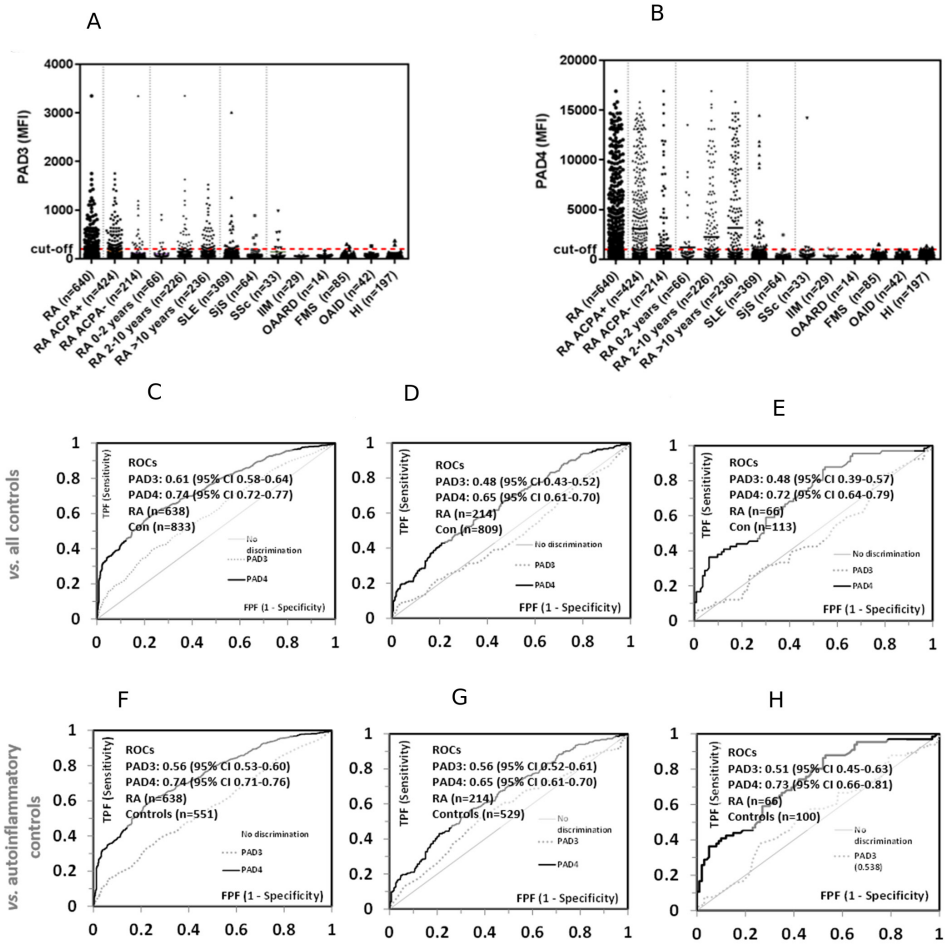

Figure 1 Distribution and diagnostic performance of anti-PAD3 and anti-PAD4 antibodies in patients with RA and controls. The prevalence and titres of both anti-PAD3 (A) and anti-PAD4 (B) antibodies were significantly higher in RA versus controls. Both anti-PAD3 and anti-PAD4 antibodies were found in ACPA+ and in ACPA− patients as well as in early RA. Cut-off values are indicated by the dotted red line (200 MFI for PAD3 and 1000 MFI for PAD4). Receiver operating characteristic (ROC) analyses of anti-PAD3 and anti-PAD4 antibodies are shown in (C)–(H). (C) and (F) show the ROC for the total study population, (D) and (G) for the ACPA negative group, and in (E) and (H) when considering disease duration and focusing on patients with early RA (0–2 years of disease duration), including all controls or only autoinflammatory conditions, respectively. ACPA, anti-citrullinated peptide/protein antibodies; FMS, fibromyalgia syndrome; HI, healthy individual; IIM, idiopathic inflammatory myopathy; MFI, median fluorescent intensity; OAARD, other ANA-associated autoimmune rheumatic disease; OAID, other autoimmune disease; PAD, protein-arginine deiminase; RA, rheumatoid arthritis; SjS, Sjogren's syndrome; SLE, systemic lupus erythematosus; SSc, systemic sclerosis.

Table 1 Diagnostic performance of anti-PAD3 and anti-PAD4 antibodies compared with the classification criteria markers anti-CCP2 antibodies and RF

	Anti-CCP2	RF IgM	Anti-PAD3	Anti-PAD4
Entire cohort	(n=1471)	(n=1469)	(n=1473)	(n=1473)
Sensitivity, % (95% CI)	66.5 (62.7 to 70.0)	67.3 (63.6 to 70.8)	13.8 (11.3 to 16.6)	35.0 (31.4 to 38.8)
Specificity, % (95% CI)	97.1 (95.7 to 98.1)	87.7 (85.3 to 89.8)	95.7 (94.1 to 96.9)	95.4 (93.8 to 96.7)
LR+, % (95% CI)	23.1 (15.6 to 34.3)	5.5 (4.5 to 6.6)	3.2 (2.2 to 4.6)	7.7 (5.5 to 10.7)
LR–, % (95% CI)	0.3 (0.3 to 0.4)	0.4 (03 to 0.4)	0.9 (0.9 to 0.9)	0.7 (0.6 to 0.7)
OR (95% CI)	66.8 (43.2 to 103.2)	14.7 (11.3 to 19.1)	3.5 (2.4 to 5.3)	11.3 (7.8 to 16.2)
Youden's index	0.6	0.6	0.1	0.3
Cut-off for 95% specificity	5.8	12.1	175	980
Sensitivity at 95% specificity, % (95% CI)	69.4 (65.7 to 72.8)	60.5 (56.6 to 64.2)	15.8 (13.2 to 18.8)	35.3 (31.7 to 39.1)
OR at 95% specificity (95% CI)	42.7 (30.4 to 59.8)	29.5 (20.8 to 42.0)	3.5 (2.4 to 5.1)	10.3 (7.3 to 14.6)
CCP2 negative population	(n=1473)	(n=1020)	(n=1023)	(n=1023)
Sensitivity, % (95% CI)	NA	22.9 (17.8 to 29.0)	8.9 (5.8 to 13.4)	19.2 (14.4 to 25.0)
Specificity, % (95% CI)	NA	88.5 (86.1 to 90.5)	95.9 (94.3 to 97.1)	96.2 (94.6 to 97.3)
LR+, % (95% CI)	NA	2.0 (1.4 to 2.7)	2.2 (1.3 to 3.7)	5.0 (3.2 to 7.7)
LR–, % (95% CI)	NA	0.9 (0.8 to 0.9)	0.9 (0.9 to 1.0)	0.8 (0.8 to 0.9)
OR (95% CI)	NA	2.3 (1.6 to 3.3)	2.3 (1.3 to 4.1)	5.9 (3.6 to 9.7)
Youden's index	NA	0.1	0.05	0.2
Cut-off for 95% specificity	NA	11.1	173	940
Sensitivity at 95% specificity, % (95% CI)	NA	19.2 (14.4 to 25.0)	9.3 (6.1 to 14.0)	19.6 (14.9 to 25.5)
OR at 95% specificity (95% CI)	NA	4.7 (2.9 to 7.5)	2.0 (1.1 to 3.5)	4.7 (3.0 to 7.4)
Early RA population (0–2 years)	(n=178)	(n=179)	(n=179)	(n=179)
Sensitivity, % (95% CI)	60.0 (47.9 to 71.0)	56.1 (44.1 to 67.4)	7.6 (3.3 to 16.5)	16.7 (9.6 to 27.4)
Specificity, % (95% CI)	94.7 (88.9 to 97.5)	83.2 (75.2 to 89.0)	93.8 (87.8 to 97.0)	99.1 (95.2 to 99.8)
LR+, % (95% CI)	11.3 (5.3 to 25.0)	3.3 (2.1 to 5.3)	1.2 (0.4 to 3.5)	18.8 (3.2 to 112.2)
LR–, % (95% CI)	0.4 (0.3 to 0.6)	0.5 (0.4 to 0.7)	1.0 (0.9 to 1.1)	0.8 (0.7 to 0.9)
OR (95% CI)	26.8 (10.4 to 68.4)	6.3 (3.2 to 12.6)	1.2 (0.4 to 3.9)	22.4 (3.6 to 138.2)
Youden's index	0.5	0.4	0.01	0.2
Cut-off for 95% specificity	15.1	10.1	226	620
Sensitivity at 95% specificity, % (95% CI)	53.0 (41.2 to 64.6)	54.5 (42.6 to 66.0)	7.6 (3.3 to 16.5)	28.8 (19.3 to 40.6)
OR at 95% specificity, (95% CI)	24.4 (9.0 to 65.6)	25.9 (9.6 to 69.7)	1.8 (0.5 to 6.0)	8.7 (3.2 to 24.0)

CCP, cyclic-citrullinate peptide; LR, likelihood ratio; NA, not applicable; PAD, protein-arginine deiminase; RA, rheumatoid arthritis; RF, rheumatoid factor.

the entire cohort, receiver operating characteristic curve analysis showed significant discrimination between RA and controls for both markers (figure 1C). In the ACPA− subgroup (figure 1D), anti-PAD4, but not anti-PAD3 showed significant discrimination. However, in the clinically relevant area (90%–100% specificity), significant discrimination was observed (for details see figure 1). Combining ACPA and anti-PAD4 antibodies increased the sensitivity by 6.2%–72.7% (95% CI 69.1% to 76.0%) with a decrease in specificity by 3.7%93.4% (95% CI 91.5% to 94.9%). Double positivity yielded 28.4% (95% CI 25.1% to 32.1%) sensitivity and 99.2% (95% CI 98.3% to 99.6%) specificity.

When analysing antibody expression in relation to disease duration (0–2, >2–10 and >10 years), in early disease (0–2 years), the prevalence of anti-PAD3 antibodies was comparable in RA and controls. In patients with more established disease, anti-PAD3 antibodies were significantly more prevalent in RA versus controls. In contrast, anti-PAD4 antibodies were significantly more prevalent in patients with early RA versus controls. For both antibodies, the prevalence increased with disease duration in patients with RA.

Recently, it was demonstrated that immunisation with PAD enzymes can trigger ACPA production in mice[6] which might change the understanding of RA pathogenesis and increase the importance of anti-PAD antibody testing. Although several studies indicated diagnostic value of anti-PAD (especially anti-PAD4) antibodies, these antibodies are not currently used in clinical practice due to lack of commercial assays for their detection. To date, this is the largest study on the diagnostic relevance of anti-PAD3 and anti-PAD4 antibodies in RA versus a broad range of controls. Both anti-PAD3 and anti-PAD4 antibodies showed discrimination between RA and controls, with anti-PAD4 antibodies being superior. In the ACPA− group, anti-PAD4 antibodies showed a significant discrimination. This was even more pronounced in early RA (disease duration 0–2 years). Our data provide strong evidence that anti-PAD4 antibodies represent a useful biomarker in the diagnosis of RA.

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the study, performed the statistical analysis and helped write the manuscript. All authors read, reviewed and approved the final manuscript.

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Competing interests MM, LMP and DL are employed at Inova Diagnostics selling autoimmune diagnostic assays. TD and CI are employed at Exagen Diagnostics offering clinical testing.

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3.4. Clinical Utility of Anti-peptidyl Arginine Deiminase Type 4 Antibodies

Clinical Utility of Antipeptidyl Arginine Deiminase Type 4 Antibodies

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Clinical Utility of Antipeptidyl Arginine Deiminase Type 4 Antibodies

To the Editor:

Rheumatoid factor (RF) and anticitrullinated protein antibodies (ACPA) are important biomarkers in the diagnosis of rheumatoid arthritis (RA) but leave a gap of > 50% seronegative in early RA[1]. In addition, there is marked clinical heterogeneity in the seropositive group, precluding the use of RF and ACPA alone as prognostic biomarkers. These characteristics drive the demand for novel diagnostic and prognostic markers in RA[1]. In this context, we read the recent paper by Guderud, *et al* on the clinical utility of antipeptidyl arginine deiminase type 4 (anti-PAD4) antibodies with great interest[2]. The authors studied anti-PAD4 antibodies in 745 patients with RA using a dissociation-enhanced lanthanide fluorescence immunoassay (DELFIA) and found 26% to be positive. In addition, the study also investigated the genotype of *PADI4* using TaqMan assays in 945 patients and 1118 controls. Based on the results, the authors concluded that anti-PAD4 antibodies are not useful clinical biomarkers in RA. Unfortunately, there are some significant questions and concerns regarding this conclusion.

Importantly, the authors did not specify what they meant by "no current clinical utility in RA," and their conclusion was based on a limited clinical dataset composed of disease activity measures (modified Health Assessment Questionnaire and 28-joint count Disease Activity Score), swollen and tender joint counts, and systemic markers of acute inflammation (C-reactive protein and erythrocyte sedimentation rate). *Clinical utility* is a broad term that may apply to many aspects of the journey of a patient with RA, including disease prediction and prevention[3], diagnosis, stratification, prognosis, and monitoring. Therefore, care needs to be taken in declaring that a putative biomarker has no clinical utility without addressing this range of potential applications.

This is especially true in the case of anti-PAD4 antibodies, which have been reproducibly shown to be associated with radiographic joint damage in 4 independent cohorts of patients with RA (Table 1)[4,5]. Importantly, these and other cohorts have revealed variable associations of anti-PAD4 antibodies with acute measures of inflammation and disease activity scores, such as those measured by Guderud, *et al*[2]. Thus, the findings of this current manuscript are consistent with the published literature but failed to determine the association of anti-PAD4 antibodies with joint damage, the singular clinical aspect of RA that has been reproducibly associated with this putative biomarker. These factors suggest that although anti-PAD4 antibodies may be of questionable utility in measuring acute inflammation in RA (a feature readily measured by existing laboratory tests and physician examination), they may have clinical value as a prognostic biomarker related to the accumulation of radiographic joint damage[5,6,7,8].

Regarding the potential utility of anti-PAD4 antibodies in the diagnosis of RA, Guderud, *et al* report that 6% of the total RA patient population is anti-PAD4+/ACPA–. This equates to 15.8% of the ACPA– subgroup and is consistent with the 2–17.7% PAD4+/ACPA– patients described in other RA cohorts. Further, 2 recent review articles analyzed the prevalence and performance characteristics of anti-PAD4 antibodies[9,10]. Both were consistent in their conclusion that anti-PAD4 antibodies showed good discrimination between RA and controls, and the more recent article presents a metaanalysis[10] with a pooled sensitivity and specificity of anti-PAD4 antibodies for RA of 38.0% (95% CI 30.0–46.0%) and 96.0% (95% CI 93.0–98.0%), respectively. This suggests that anti-PAD4 antibodies may also be clinically useful for RA diagnosis even within traditionally seronegative individuals.

This recent report also raises several questions regarding the method used to detect anti-PAD4 antibodies and the need for consistency in the field. First, the DELFIA method used by Guderud, *et al* is not a validated technology for the detection of autoantibodies, has not been compared directly to ELISA or immunoprecipitation for the detection of anti-PAD4 antibodies, and does not play a significant role in diagnostic settings. In addition, it is unclear which controls have been included to assess the performance of the test, and no information is provided regarding batch effects, standardization, or calibration. This is particularly important because the authors combined data from 2 different cohorts, in which antibody testing was performed at drastically different timepoints, and came to markedly different conclusions. Of the total subjects, 366 of 745 patients were from a study by Halvorsen, *et al*[5] from 2008 that was among the first to determine that anti-PAD4 antibodies are associated with markers of disease severity, including radiographic joint damage, in patients with RA (Table 1).

The conclusion that anti-PAD4 antibodies are not clinically useful is not supported by the data presented by Guderud, *et al*[2] and contrasts with a body of published literature. We acknowledge that anti-PAD4 antibodies might not have diagnostic utility when compared to ACPA, but they hold clinical promise in assessing disease prognosis as it relates to the accumulation of radiographic joint damage and in disease diagnosis of RA, especially in ACPA-negative individuals.

Table 1. Summary of studies reporting the prognostic value of anti-PAD4 antibodies in RA.

Study	Cohort	Anti-PAD4 Assay	Association with Anti-PAD4 Antibodies								
			Anti-CCP	Duration	DAS28	HAQ	SJC	TJC	CRP	ESR	Joint Damage
Guderud 2018	EURIDISS	DELFIA	NR	NR	NR	NR	NR	NR	NR	NR	NR
	ORAR	DELFIA	+	+	±	–	±	–	±	–	NR
Navarro-Millan 2016	CLEAR	IP	+	+	NR	NR	NR	NR	–	NR	+
Reyes-Castillo 2015	JMR	ELISA	+	+	–	–	NR	NR	–	–	NR
Darrah 2013	ESCAPE	IP	+	+	–	–	NR	NR	–	NR	+
Kolfenbach 2010	Pre-RA	IP	+	NR	NR	NR	NR	NR	NR	NR	NR
Harris 2008	ESCAPE	IP	+	+	NR	NR	NR	NR	NR	NR	+
Halvorsen 2008	EURIDISS	ELISA	+	+	NR	NR	NR	NR	+	–	NR
	ORAR	ELISA	+	+		+	+	–	+	–	+
Zhao 2008	PUPH	ELISA	+	NR	+	NR	NR	NR	–	+	+

+: yes; –: no; ±: associated in univariate but not multivariate models. RA: rheumatoid arthritis; anti-CCP: anticyclic citrullinated peptide antibodies; DAS28: 28-joint count Disease Activity Score; HAQ: Health Assessment Questionnaire; SJC: swollen joint count; TJC: tender joint count; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; DELFIA: dissociation-enhanced lanthanide fluorescence immunoassay; EURIDISS: European Research on Incapacitating Disease and Social Support; ORAR: Oslo RA Register; CLEAR: Consortium for the Longitudinal Evaluation of African Americans with Early Rheumatoid Arthritis; JMR: Jalisco, Mexico Rheumatology; ESCAPE: Evaluation of Subclinical Cardiovascular Disease and Predictors of Events in Rheumatoid Arthritis; PUPH: Peking University People's Hospital; NR: not reported; anti-PAD4: antipeptidyl arginine deiminase type 4; IP: immunoprecipitation.

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3.5. The Utilization of Autoantibodies in Approaches to Precision Health

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The Utilization of Autoantibodies in Approaches to Precision Health

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Precision health (PH) applied to autoimmune disease will need paradigm shifts in the use and application of autoantibodies and other biomarkers. For example, autoantibodies combined with other multi-analyte "omic" profiles will form the basis of disease prediction allowing for earlier intervention linked to disease prevention strategies, as well as earlier, effective and personalized interventions for established disease. As medical intervention moves to disease prediction and a model of "intent to PREVENT," diagnostics will include an early symptom/risk-based, as opposed to a disease-based approach. Newer diagnostic platforms that utilize emerging megatrends such as deep learning and artificial intelligence and close the gaps in autoantibody diagnostics will benefit from paradigm shifts thereby facilitating the PH agenda.

Keywords: precision medicine, precision health, biomarkers, autoantibodies, autoimmunity

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INTRODUCTION: TENETS OF MEDICINE AS A FRAMEWORK FOR PRECISION HEALTH (PH)

The three actionable tenets of clinical medicine are: (1) disease prediction and prevention; (2) early and accurate diagnosis; (3) effective and timely treatment (1, 2). The term Precision Health (PH) was deliberately chosen here to focus on the first tenet and emphasize disease prevention and healthy living or "wellness," which refers to maintaining longer and healthier lives, a medical imperative that is shaping global research and health policy. This has been articulated as an integrated, systems approach referred to as "P4 medicine": Predictive, Preventive, Personalized, and Participatory (3). The paradigm shift to PH is fostered by the emergence of multi-analyte diagnostic technologies, individual, real-time health information, deep learning (DL) and artificial intelligence (AI) approaches to "big data" (4–7). DL and AI are a part of our daily lives; their utility has enabled optimized travel, efficient exercise, social networking, finance management, and is beginning to permeate into healthcare. By definition, AI is comprised of supervised learning algorithms varying in complexity, which are able to profile patterns in data sets that are not immediately obvious using uni-variate approaches. The rapid growth and accessibility of AI-related technologies allows abstraction of information that may not have been immediately obvious, enabling profiling, personalizing, and delivering improved health care to each patient. Taken together, it is anticipated that these approaches to PH will translate into decreased healthcare expenditures (HCE) where the "VALUE PROPOSITION" is expressed as markedly improved clinical OUTCOMES as the numerator and COSTS as the denominator (8). Because of this "value proposition," HCE in PH become an investment rather than a cost (9).

A successful PH paradigm is dependent on disease prediction and prevention through timely intervention (10, 11). A crucial challenge is how to shift the clinical focus to timely intervention in individuals who will progress from pre-clinical to undifferentiated autoimmune disease and then to clinically active systemic autoimmune diseases (SAIDs), the latter with attendant high morbidity and/or mortality. Keys to this approach will not only focus on healthy living and earlier, more accurate diagnosis, but curbing HCE due to decreased hospital admissions and readmissions, evidence-based decisions on expensive therapeutics, and decreased physician visits because the individual will have a more clearly defined participatory clinical care pathway (12). We touch on evidence-based and informed approaches to interventions for pre-clinical autoimmune disease that have emerged and are evolving.

TABLE 1 | Components of Precision Health: a P8 approach.

1	PATIENT (individual) is the focus
2	PROACTIVE approach
3	PREDICTION of disease
	SSSMAARTT biomarkers*
	Risk factors
4	PREVENTION of disease
5	PERSONAL WELLNESS is emphasized
6	PROSPECTIVE
	Real time history, physical exam
	Real time biomarkers
7	PERSONALIZED database
8	PARTICIPATORY surveillance

SSSMAARTT = (Sensitive, Specific, Stable, Measurable, Actionable, Added value, Realistic, Timely, and Titratable.

COMPELLING REASONS FOR PRECISION HEALTH

There are many compelling reasons why healthcare professionals attendant to SAIDs should embrace PH. First, it is well established that advanced disease and accompanying morbidity is often present at the time of diagnosis of SAIDs [e.g., systemic lupus erythematosus (SLE), systemic sclerosis (SSc), rheumatoid arthritis (RA), Sjögren's syndrome (SjS)]. For example, approximately 30% of SLE have kidney disease at inception or at the time they are first seen by a specialist (13) and 13% of SSc will die prematurely within 3 years of diagnosis (14). In RA, several studies have shown that all features of chronic synovial inflammation can be found within weeks to months after the first clinical evidence of arthritis, providing evidence that asymptomatic synovial inflammation may precede the development of clinical signs and symptoms in RA (15, 16). A key factor responsible for advanced morbidity and early mortality at the time of diagnosis is the time that has elapsed from the onset of symptoms to diagnosis and initiation of therapy (10, 17–19). Depending on the SAID, the interval from first symptom to diagnosis ranges from 2 to 20 years and may even be longer if the onset is in the elderly (20, 21).

Second, increased HCE on SAIDs are highly correlated with advanced disease. For example, the annual direct per patient costs for SLE was as high as $71,334.00 (2015 US dollars) in those with lupus nephritis (reviewed in (13)). A review spanning 2000–2009 found the direct costs were related to inpatient (16–50%) and outpatient (24–56%) services, followed by medications (19–30%). This is in stark contrast to HCE on a SLE patient without kidney disease where the HCE ∼$5,000 USD/year (22) [reviewed in (1, 13)].

Third, clinicians will be able to make more informed therapeutic choices based on pathogenesis-based approaches that inform clinical trials (23) and individual therapies (24). The clinical misadventures and attendant HCE in "one size fits all" or "trial and error" approaches are replaced by evidence or big data based methodologies focused on the "right patient," "right drug," "right dose," at the "right time" (2). Lastly, SAIDs are typically chronic conditions comprised of heterogeneous disease phenotypes. Understanding the underlying molecular mechanisms and the drivers of specific SAIDs will open new avenues for targeted treatment and improved outcomes.

KEY COMPONENTS OF PH

To effectively implement PH, attention should be given to eight key components of the "healthcare system," which we refer to as the "P8" (**Table 1**). Our proposed P8 health paradigm is based in part on the P4 medicine approach proposed by Hood and colleagues (25). In effective PH, the approach is PROACTIVE, the individual PATIENT is the focus and they must be active participants (PARTICIPATORY) in promotion of wellness in addition to monitoring and surveillance of the condition (26). A meaningful impact on PERSONAL WELLNESS and HCE will require the identification of risk factors, clinical parameters, and biomarkers that are PREDICTIVE of and then used as an approach to case finding followed by timely interventions and PREVENTION of disease. The identification of relevant and clinically meaningful biomarkers should meet criteria included in the SSSMAARTT biomarkers acronym (Sensitive, Specific, Stable, Measurable, Actionable, Added value, Realistic, Timely, and Titratable) (27).

The role of healthcare providers in SAID should be PROSPECTIVE in nature by incorporating real time history, physical exam(s), biomarker results, and, where needed and available, molecular imaging (28, 29), wearable devices (30), and other investigational tools. Taken together, comprehensive

Abbreviations: aCL, anti-cardiolipin; ACPA, anti-citrullinated peptide antibodies; AI, artificial intelligence; ANA, anti-nuclear antibody; DL, deep learning; dsDNA, double-stranded DNA; HCE, healthcare expenditures; IIF, indirect immunofluorescence; MSPA, multi-analyte solid phase assays; PH, precision health; RA, rheumatoid arthritis; SAID, systemic autoimmune disease; SjS, Sjögren's syndrome; SLE, systemic lupus erythematosus; Sm, smith; SSc, systemic sclerosis; UCTD, undifferentiated connective tissue disease.

TABLE 2 | Overview of prevention trials for rheumatoid arthritis.

Trial name	Drug	Initiator/Comment
StopRA (Strategy to prevent the onset of clinically apparent rheumatoid arthritis)	Hydroxychloroquine	University of Colorado, Denver, USA
PRAIRI (Prevention of RA by Rituximab)	Rituximab	Academic Medical Center, Amsterdam
ARIAA (Arthritis prevention in the preclinical phase of rheumatoid arthritis with Abatacept)	Abatacept	Sponsored by abatacept's manufacturer, Bristol-Myers Squibb
APIPPRA (Abatacept reversing subclinical inflammation as measured by MRI in ACPA positive Arthralgia)	Abatacept	Sponsored by abatacept's manufacturer, Bristol-Myers Squibb
TREAT EARLIER (Treat early arthralgia to reverse or limit impending exacerbation to rheumatoid arthritis)	Methylprednisolone	Leiden University Medical Center and Erasmus Medical Center in Rotterdam, both in the Netherlands.
STAPRA (Statins to Prevent Rheumatoid Arthritis)	Atorvastatin	University of Amsterdam

individualized information is captured in PERSONALIZED electronic medical records (31, 32). The P8 approach inevitably leads to the generation of "big data." This introduces unique computational and statistical challenges that require a new paradigm (4, 5, 7) and innovative thinking such as the use of DL and AI to reclassify disease and stratify patients based on "molecular taxonomy" or the "immunobiome" (33, 34).

Despite the promise and imperative of PH, it will likely take at least a decade before healthcare providers fully embrace and implement these concepts. One of the reasons could be the perception that the PH agenda clashes with the "less is more" movement (35, 36). However, we believe that the reticence, for the most part, is due to lack of training in PH and of clearly defined clinical care pathways focusing on prevention through timely interventions. Indeed, actionable strategies for SAID prevention have already been proposed for SLE (13), SSc (37), and RA (38–41) (**Table 2**). Even then, many healthcare providers are more comfortable focusing on "curing" advanced disease rather than preventing it. Hence, effective P8 PH requires extraordinary efforts to educate healthcare providers and administrators (42).

One of the challenges that needs to be addressed is having a proper screening or triage system for individuals at risk of developing SAIDs (43, 44). In addition, based on the risk level, a sequential and timely approach for intervention is desirable to maximize effectiveness while maintaining safety. This approach to primary prevention includes the removal or modification of lifestyle risk factors (e.g., smoking, weight loss, dietary supplement intake) or the initiation of pharmacological interventions such as timely treatment in high risk cases (e.g., with hydroxychloroquine) if prevention trials prove successful (34, 35) (secondary intervention).

AUTOANTIBODIES AND OTHER BIOMARKERS AS PREDICTORS OF DISEASE AND EFFECTIVE THERAPY

In current practice, autoantibodies are most often used to confirm the diagnosis and classify SAIDs but are also becoming increasingly important biomarkers that predict complications and/or comorbidities. Take for instance the 2012 Systemic Lupus International Collaborating Clinics classification criteria where patients must fulfill at least one clinical as well as immunologic criteria, which include antinuclear antibodies (ANA), anti-double stranded DNA (dsDNA), anti-Smith (Sm), and anti-phospholipid antibodies (45). In addition to a classification criterion, anti-dsDNA is associated with SLE nephritis (46) and can be used to monitor disease activity (47, 48). Being able to identify a patient's risk for disease complications or organ involvement based on their autoantibody profile allows clinicians to be more vigilant about screening and monitoring patients who have established disease. This has implications for the management of patients with SAIDs including SSc and myositis, where the presence of certain autoantibodies should prompt a more aggressive approach to investigation (e.g., cardiopulmonary testing, malignancy screening) and therapy (33, 49, 50).

An understanding of which autoantibodies are predictive of disease is derived from studies of patients with undifferentiated connective tissue disease (UCTD) (51). These patients generally have a milder form of a SAID that may eventually evolve into a defined SAID. In a retrospective study of 148 patients with UCTD and anti-SSA/Ro60 antibodies, ∼25% developed a well-defined SAID within a short time (4.5 years) (52). Most of these patients developed SjS (50%) or SLE (30.5%). In the same study, the presence of anti-dsDNA along with anti-SSA/Ro60 antibodies was predictive of SLE development. Other small cohort studies have also shown that UCTD patients who eventually developed SLE had one of anti-dsDNA or anti-SSA/Ro60 antibodies at baseline and at diagnosis of SLE, they had developed the other antibody (53). In a more recent retrospective review of 98 UCTD patients, 14% developed into a defined SAID (54) in which the presence of an ANA titer ≥1/640 (OR 7.00 [1.99–24.66], $p = 0.002$) and anti-centromere positivity (OR 3.77 [1.03–13.79], $p = 0.045$) at baseline as well as other clinical features were associated with the development of definite SAID.

A subtype of UCTD patients with some manifestations of SLE who did not meet full classification criteria were referred to as incomplete SLE (ILE). Between 10 and 60% of patients with ILE progressed to complete SLE, usually within 5 years of disease onset (55–57). In a seminal study of 130 former military SLE patients whose sera were available from the USA Department of Defense Serum Repository, Arbuckle et al. (58) detected at least one SLE-related autoantibody before the diagnosis of complete SLE and first clinical manifestations of ∼90% of subjects. ANA, anti-SSA/Ro60 and anti-SSB/La antibodies were the earliest autoantibodies to appear, while others such as anti-Sm and anti-U1RNP antibodies appeared only months prior to diagnosis. The mean interval from earliest autoantibodies detected to diagnosis of complete SLE ranged from 3.7 years

for anti-SSA/Ro60 to 0.9 years for anti-U1RNP. Other studies have shown that anti-cardiolipin (aCL) antibodies are significant predictors of complete SLE development (56, 59). McCain et al. demonstrated that 24/130 (18.5%) SLE patients were positive for IgG and/or IgM aCL prior to SLE diagnosis (59). The antibodies appeared as early as 7.6 years prior to SLE diagnosis with a mean onset occurring 3.0 years before SLE diagnosis. The presence of aCL also seemed to predict a more severe clinical outcome including more frequent renal disease, central nervous system disease, thrombocytopenia, and clotting events.

Patients with features of SSc but not meeting classification criteria have been referred to as very early systemic sclerosis (60–62). Once again, the idea of an earlier diagnosis of this disabling disease is to allow earlier interventions designed to block or slow progression to severe morbidity. The current diagnostic and classification criteria limit the ability to detect early disease because they typically depend on features that are the sequelae of the disease. In one study of 60 early SSc patients, the presence of autoantibodies (anti-Scl-70, centromere, and/or anti-RNA polymerase) was the most important predictor of faster progression to SSc, particularly in those with preclinical internal organ (heart or lung) involvement at baseline (63).

Using RA as another example, <50% of anti-citrullinated peptide antibodies (ACPA) positive individuals will develop RA within 3 years of follow-up (64). The risk of developing RA therefore depends on many factors that can be divided into three categories: the modifiable (e.g., behavioral: smoking, dietary, environmental), fluctuating or progressive (e.g. autoantibodies and other biomarkers), and the constant (e.g. germline genetics) (65–69). The pre-clinical phase of RA and other SAIDs may be mitigated by modification of the risk profile (70). It has been established that the presence of three biomarkers (ACPA, rheumatoid factor, and anti-carbamylated protein antibodies) are highly discriminatory in correctly identifying RA in a cohort of early arthritis (71–73). When comparing the presence of one, two or three autoantibodies to the patients with zero autoantibodies, the odds ratio (OR) of having the diagnosis RA significantly increased from 3.8 (95 % CI 2.9–5.0) for the patients with one autoantibody, to 20.9 (95 % CI 12.7–34.3) for the group with two and finally to 112.2 (95 % CI 52.4–240.5) for the individuals with three autoantibodies (74). It should be emphasized that additional studies need to determine the predictive value of autoantibody profiles in prospective, longitudinal cohorts of individuals with/without early clinical features of RA.

A rapidly emerging area of evidence indicates that the glycosylation of antibodies can also provide added value in prediction of RA. It has been known for more than two decades that glycosylation of antibodies is associated with autoimmunity (75, 76). Several studies have demonstrated that the Fc portion of IgG shows different glycosylation patterns in patients with autoimmune diseases when compared to healthy individuals [reviewed in (76)]. In healthy individuals, the glycosylation site is typically fully glycosylated IgG. In addition, there is growing evidence that the differences in glycosylation also reflect variations in disease activity. Lastly, the ratio between G0/G1 glycosylation type also changes during the conversion from pre-clinical to clinical phase. In contrast to the Fc de-glycosylation, the Fab regions of antibodies exhibit increased levels of glycosylation in RA compared to healthy individuals which alters properties of the antibody in a variety of ways. Importantly, the glycosylation occurs as part of the somatic hypermutation of the complementary-determining regions and might have impact on the antigen binding characteristics. In a recent study, it was shown that the affinity of ACPA can either increase or decrease based on the glycosylation of their variable domains (77, 78). In addition, the stability of the antibody can increase after Fab glycosylation which also can lead to the formation of immune complexes. Therefore, the glycome status of an individual might provide valuable insights in the prediction of SAIDs (79, 80). Furthermore, this might even pave the way for new treatment approaches through glycoengineering of antibodies (81, 82).

TABLE 3 | Current and emerging autoantibody detection technologies.

- Indirect immunofluorescence (IIF)
- Cell based assays
- Enzyme-linked immunoassays (ELISA)
- Multi-analyte solid phase assays (MSPA)
  - Antigen arrays on planar surfaces: line immunoassays, dot blots
  - Addressable laser bead assays (ALBIA)
  - Particle-based multi-analyte technologies (PMAT)
  - Chemiluminescence (CIA)
  - Multiplexed point of care diagnostics: *"Lab on a chip"*
  - Multi-analyte arrays with algorithmic analysis (MAAAA)
- Mass spectroscopy
- Electrochemiluminescence arrays
- Nanotechnology—nanobarcodes

In addition to autoantibodies, other biomarkers included in omics technologies (e.g., genomics, ribonomics, proteomics, glycomics, metabolomics, etc.) are also becoming key components of PH (83, 84). An important contribution of these multi-analyte technologies is to help understand the pathogenic processes throughout the entire course of the disease. This understanding will be key to guide patient stratification for clinical trials and evidence-based interventions as opposed to the "trial and error" approaches that are prevalent today (23). In this context, advances in molecular medicine are increasingly based on the use of biomarkers as drug development tools (85). Companion diagnostics are expected to identify individuals with a higher chance to benefit from a particular treatment and in this way, biomarkers can increase the success rate of drug development programs and accelerate the availability of new therapeutics (86, 87).

FILLING KNOWLEDGE AND TECHNOLOGICAL GAPS

To make progress in the use of autoantibodies in the PH agenda, knowledge and technological gaps must be addressed. First, the parameters and mechanisms that chart or trigger the evolution

of very early or undifferentiated SAIDs to full clinical disease must be clarified (88). Examples have been discussed above but longitudinal studies of larger early SAID and UCTD cohorts, or even apparently healthy cohorts using newer multi-analyte technologies are required to more accurately classify and predict disease.

In addition to clinical gaps, there are technological gaps that also need to be addressed. With respect to autoantibody testing, universally accepted, standardized, and cost-effective follow-up testing algorithms need to be developed. Although the ANA indirect immunofluorescence (IIF) test is regarded as the screening test of choice for some SAIDs, recent evidence suggests that follow-up testing algorithms might be inverted wherein multi-analyte solid phase assays (MSPA) (**Table 3**) that detect antibodies to disease-specific autoantibody targets is a more cost-effective approach (89–91). Samples that have negative multi-analyte array test results might then be tested by ANA IIF to determine if antibodies to targets not included in the MSPA are detected. Although older thinking indicated that only a single autoantibody was required to confirm a diagnosis, this can lead to a diagnostic dilemma because many sera from SAID patients typically have multiple autoantibodies. Furthermore, each antibody could have added value not only for accurate diagnosis but also for disease stratification and on evidence-based approaches to therapy and prognosis. The sensitivity of many autoantibody assays today, including MSPAs, is limited to well-characterized targets although it is known that the B-cell repertoire in a given SAID is often much more complex. Hence, a significant proportion of SAIDs are potentially incorrectly labeled "sero-negative" (92). It is anticipated that the sensitivity, specificity and predictive value of autoantibody testing can be increased by including AI approaches to MSPA, a paradigm shift referred to as multi-analyte arrays with algorithmic analyses (MAAAA) (93).

In summary, PH applied to SAID is anticipated to create paradigm shifts in the use and application of autoantibodies. Autoantibodies combined with other multi-analyte "omic" profiles will form the basis of disease prediction, and subsequently, earlier intervention linked to disease prevention strategies. As medical intervention moves to disease prediction and intent-to-prevent, diagnostics will include an early symptom/risk-based approach, as opposed to a disease-based approach. Newer platforms and technologies adaptable to the paradigm shifts that help close the gaps in autoantibody profiling will facilitate the PH agenda. In this setting, DL and AI will be key to interpretation of "big data" from multi-analyte autoantibody arrays and other biomarker testing. The current status and future of PH in RA and SLE has recently been discussed at the Precision Medicine in Autoimmunity (PMA) meeting (http://www.precisionmedicineautoimmunity.org/) which will likely provide the basis for new collaborative work towards a more precise approach to medicine in autoimmune conditions.

AUTHOR CONTRIBUTIONS

MF: Conceived topic, wrote outline, and parts of manuscript and primary edits. LM-P, MM, and MC: Literature review, wrote parts of and edited the manuscript.

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Conflict of Interest Statement: MF is a consultant to Inova Diagnostics, (San Diego, CA, USA) and Werfen International (Barcelona, Spain). MM and LM-P are employees of Inova Diagnostics, a company that manufactures and sells autoantibody assays.

The remaining author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Chapter 4. Summary of results and discussion

4.1. Combinatory models with the RA classification criteria biomarkers

As previously introduced, the diagnosis of RA is usually based on a combinatory approach that integrates clinical symptoms assessed by the rheumatologist in a clinical exam, and serological results, including markers of inflammation (CRP and ESR) and autoantibodies (RF and ACPA), as outlined in the current ACR/EULAR classification criteria [124].

The heterogeneity and the serological gap in RA combined make the clinical diagnosis very challenging. RF is known to have a relatively low specificity as it can be present in other rheumatological diseases, several of which have clinical manifestations that could be mistaken for characteristic of RA. Although these biomarkers can be present before the disease onset allowing for early diagnosis [103, 104, 112], symptoms in early phases of the disease can be unclear, and flares and phases of relative remission can also contribute to the difficulty of early diagnosis.

The common practices for testing of these antibodies can vary between geographies, hospitals, clinics and laboratories. One or both RF and ACPA, and more than one isotype, can be tested and reported to the clinician. This scenario can lead to the generation of multiple results for each patient. Moreover, it has been demonstrated that taking into account antibodies levels can improve diagnosis [125] and the current classification criteria assigns different weights based on biomarkers titers [124]. The physician will usually combine these results from laboratory testing with the clinical features following the classification criteria scoring system. However, since those criteria have been developed for classification and not for diagnosis, the clinician might deviate and use personal experience to establish the diagnosis. Here, combinatory scores that integrate the results from different laboratory tests based on the currently used RA biomarkers in a systematic and data-driven manner would be helpful to improve diagnosis.

Additionally, several technologies for the detection of RA biomarkers are nowadays commercially available. In general and depending on the geography, total RF is usually tested on clinical chemical analyzers based on latex fixation assays [360]. More recently, the testing for the individual RF isotypes has started to be adopted by clinical laboratories. Regarding ACPA, three generations of the anti-CCP test are currently available and differences in performance have been reported [254, 256-258, 361].

For all these reasons, in the manuscript entitled **"Comparison of serological biomarkers in rheumatoid arthritis and their combination to improve diagnostic performance"** published in the June 2018 issue of 'Frontiers in Immunology' [362] (chapter 3.1), we aimed to evaluate and compare the clinical performance of several RF and ACPA immunoassays on two different platforms, of two anti-CCP tests (second and third generation), as well as to investigate the diagnostic value of combinatory strategies based on these biomarkers.

In this study, a total of five different RF assays were compared (RF IgM, IgA and IgG ELISA and RF IgM and IgA CIA) and pronounced differences were observed. The AUC derived from ROC analyses to differentiate RA from controls ranged from 0.61 (RF IgG ELISA) to 0.82 (RF IgM CIA). The sensitivities ranged from 35.6% (RF IgG ELISA) to 67.1% (RF IgM CIA), and the specificities were between 77.9% (RF IgA ELISA) and 96.9% (RF IgA CIA). The RF IgM CIA was the most sensitive, while the RF IgA CIA was the assay with the highest specificity. Differences were also observed in the predictive values, with OR ranging from 3.3 (95% CI 2.5-4.2) for the RF IgG ELISA to 21.9 (95% CI 13.9-34.3) for RF IgA CIA.

When the ELISA and CIA platforms were compared for the detection of RF IgM, a high qualitative agreement was observed, with a better discrimination, higher sensitivity and very similar specificity for the CIA. This was likely attributed to characteristics of this technology (see review article by Mahler and Bentow et al. [348]).

With respect to RF IgA, CIA vs. ELISA showed higher specificity and high negative agreement, but low positive agreement. Interestingly, the OR of RF IgA CIA was more than five times higher compared to the corresponding ELISA (OR=4.3, 95% CI 3.4-5.3). This finding was also likely attributed to the differences between the two platforms, mainly the detection method, given that the ELISA uses a polyclonal anti-human IgA antibody as conjugate, while the CIA uses a monoclonal antibody for detection. The utility of RF IgA in early RA diagnosis and in assessing poor prognosis [238, 239, 245] could not be evaluated in this study due to the nature of the cohort, that comprised patients with established RA and for whom disease severity information was not available.

In the RF IgM and CCP3 IgG seronegative population defined by ELISA (n=858), the RF IgA ELISA had an 18.5% sensitivity with 76.9% specificity and an OR of 0.8 (95% CI 0.5-1.1). In the same seronegative population defined by CIA (n=835), the RF IgA CIA showed a 5.2% sensitivity with a 98.6% specificity and an OR of 4.0 (95% CI 1.7-9.5). Based on these data we concluded that RF IgA measured by CIA could help to partially close the serological gap in clinical practice.

The RF IgG test was available only on ELISA at the time of testing the cohort. This marker, with a very low prevalence in RA patients compared to the other two isotypes, showed the lowest discriminatory value out of all the RF tests (AUC=0.61), with a low sensitivity and a moderate specificity (35.6% and 85.4%, respectively). In the RF IgM and CCP3 IgG negative population based on ELISA (n=858), the RF IgG ELISA reported 10.6% sensitivity and 88.0% specificity resulting in OR of 0.9 (95% 0.5-1.4) and therefore no added diagnostic value was observed. Whether this isotype can have a clinical utility in aspects other than diagnosis as suggested in some studies [363, 364], was outside the scope of this study and remains to be confirmed.

In agreement with several studies [365-367], ACPA were confirmed to be more specific than RF for the diagnosis of RA. All three ACPA assays (CCP2 ELISA and CCP3 ELISA and CIA) showed very good discrimination between RA patients and controls. In our study, CCP3 CIA and ELISA showed equivalent performance and a high qualitative agreement. However, differences were observed between the second and third generation CCP tests on ELISA, with CCP3 outperforming CCP2. The two CCP3 assays showed lower sensitivities (61.8% for ELISA and 61.4% for CIA), but

significantly higher specificities (98.4% and 98.5%, respectively) *vs.* CCP2 (71.1% sensitivity, 86.9% specificity). This also resulted in much higher predictive values, with OR of 99.3 (95% CI 54.4-181.2) for CCP3 ELISA, 107.5 (95% CI 57.4-201.5) for CCP3 CIA, and 16.3 (95% CI 12.5-21.2) for CCP2 ELISA. These results illustrate the reported differences between these two generations of ACPA assay and the controversy around this topic [254, 256-258, 361]. Out of the three CCP assays evaluated, CIA showed the highest OR at the assay cut-off as well as at the 3 x ULN, confirming that the CCP3 CIA is a reliable test for the detection of ACPA.

While in the RA classification criteria the same weight is assigned to RF and ACPA, our results showed significant differences between the OR for the diagnosis of RA of these two biomarkers, suggesting that a higher emphasis should be assigned to ACPA. When we investigated the different weights assigned to these markers based on their levels, we observed an increase from 16.3 to 45.6 in the CCP2 OR at the cut-off vs. 3 x ULN respectively, and from 107.5 to 192.5 for CCP3 CIA. These results confirm the importance of taking into account antibody levels in RA diagnosis [125], as captured in the classification criteria. In contrast, the OR for CCP3 ELISA was not significantly different at 3 x ULN. Future studies might be needed to evaluate whether cut-off points for ACPA assays (especially for the 3 x ULN) are aligned between different manufacturers [368].

In the combinatory analyses, the likelihood of RA increased with dual and triple positivity, with higher OR for the combinations of markers than with the individual markers in all cases. However, the 95% CI of the OR overlapped in some cases indicating a need to further investigate whether these differences are statistically significant.

For RF combinations, the OR of the RF test was increased by the detection of multiple isotypes. The dual and triple combinations of RF IgM, IgA and IgG showed a higher OR than the individual markers. The combination of the three RF isotypes on ELISA yielded the highest OR (52.2, 95% CI 22.0-124.0) confirming that the specificity and predictive value of the RF test is increased by the detection of all three isotypes.

Analyses of the combination of ACPA and RF demonstrated higher OR for the combinatory models compared to the individual markers at the cut-off values on both platforms, and equivalent to the CCP3 CIA at the 3 x ULN. In this model, higher ORs were again observed with increasing CCP3 and RF IgM levels, consistent with the different weights assigned based on antibody levels in the 2010 ACR/EULAR classification criteria [124]. Of all the combinations analyzed, the highest OR was observed for CCP3 IgG, RF IgM and RF IgA on ELISA (OR=224.2, 95% CI 61.0-823.4). The addition of RF IgA to the CCP3 and RF IgM ELISA combination resulted in a higher OR, nevertheless, the addition of RF IgG to this model resulted in a lower OR and did not seem to add value from a diagnostic perspective. Differences were observed between the combinations that included the CCP2 and/or CCP3 ELISAs.

Besides the diagnostic value for the disease (captured in the OR) it is also important to assess how many patients can be correctly classified based on a single test or combinations. Therefore, we compared the OR and number of patients of a combinatory model based on RF IgM and CCP3 to the CCP3 individual assays. Three intervals of patients were defined: patients with a low likelihood of

having RA, patients within an area of uncertainty, and patients with a very high likelihood of RA. In this model, a higher number of patients were correctly classified with the combinatory model compared to the individual CCP3 assays, with fewer patients found within the areas of uncertainty, suggesting an improvement in the diagnosis of RA with this approach.

In the context of precision medicine, the combination of biomarkers represents a very promising tool to improve the diagnosis of RA patients. This research can help understand what the contribution of the RA 'classical' biomarkers in this approach should be and how to model it. Along these lines, the Inova Advanced Technologies team initiated the feasibility work to design algorithmic models with these biomarkers, using the data set and the insights from this study.

Despite this progress, there is still an unquestionable need for novel biomarkers to enhance the early diagnosis of RA, especially in patients currently classified as seronegative, as well as, to stratify patients according to different disease phenotypes. In the next sections, we will describe the work that has been done in this direction.

4.2. Novel biomarkers in RA – The anti-PAD antibodies

Extensive literature screening and preliminary internal data resulted in the selection of anti-PAD antibodies as novel biomarkers in RA for further evaluation. At the initiation of this thesis, several publications on anti-PAD4 IgG, but only two manuscripts on anti-PAD3 IgG were available, and PAD2 had not been identified as an autoantigenic target in RA [308]. A longstanding collaboration on the anti-PAD3/4 XR antibodies with Erika Darrah's group from Johns Hopkins University (JHU), pioneers in the anti-PAD antibody work, stimulated the research interest in anti-PAD2 antibodies. In addition, all the literature available at the moment was focused on the IgG isotype of these antibodies and only one abstract presented at the 2017 ACR/ARHP annual meeting had described the existence of the anti-PAD4 IgA in human serum and sputum [283]. No reports of anti-PAD antibodies of other isotypes or autoantibodies to PAD1 or PAD6 exist to this day.

To study these biomarkers, biochemical characterization of the PAD enzymes as autoantigens and of anti-PAD antibodies was carried out, in parallel to feasibility, optimization and early development of immunoassays for anti-PAD antibody detection. The novel assays were then used to measure these autoantibodies in numerous clinical cohorts, as summarized in Table 11.

4.2.1. The PAD enzymes as autoantigens

The PAD antigens represented key elements in the immunoassays for the detection of anti-PAD antibodies. Consequently, considerable effort was invested in carefully selecting and characterizing these proteins as autoantigens in RA.

All five human PAD proteins were assessed and evaluated as part of this thesis with different degrees of detail, with special emphasis on PAD4 and PAD3, followed by PAD2. A summary of the proteins utilized in in our studies can be found in Table 7.

Table 7 Summary of the different PAD proteins evaluated. Details on the protein expression system, tags and origin are shown. The studies in which the immunoassays built with the different proteins were used are also indicated.

Protein	Exp. system	Tags	Origin	Studies (see Table 11)
PAD1 (full length)	*E. coli*	N-terminal His tag	Commercial vendor	Epitope Mapping, RA samples screening
PAD2 (full length)	V1: Insect cells (cell line not disclosed)	V1: N-terminal His-tag	V1: Commercial vendor	Epitope Mapping, JHU PAD2, MLH, Florence RA-ILD, CHU Bichat
	V2: *E. coli*	V2: N-terminal His (cleaved) and T7 tags	V2: JHU	Epitope Mapping, JHU PAD2, MLH, Florence RA-ILD, CHU Bichat
	V3: *E. coli*	V3: N-terminal His and T7tags	V3: JHU	Epitope Mapping, JHU PAD2, MLH, Florence RA-ILD, CHU Bichat
PAD3 (full length)	*E. coli*	N-terminal His tag	Inova Diagnostics (JHU plasmids)	Swiss RA
	Insect cells (*Sf9*)	N-terminal His tag	Inova Diagnostics (JHU plasmids)	Epitope Mapping, Swiss RA, Exagen, MLH, RA-ILD Florence, Rome Seronegative RA, CHU Bichat, Benucci, CIA *vs.* PMAT comparison
PAD4 (full length)	*E. coli*	N-terminal His tag	Commercial vendor	Epitope Mapping, Exagen, MLH, RA-ILD Florence, Rome Seronegative RA, CHU Bichat, Benucci, JHU PAD2
PAD4 (full length)	*E. coli*	C-terminal His tag	Inova Diagnostics	Citrullination studies
PAD6 (full length)	Insect cells (cell line not disclosed)	N-terminal His tag	Commercial vendor	Epitope Mapping, RA samples screening

Abbreviations: CIA: chemiluminescence immunoassay; His: histidine; JHU: John Hopkins University; MLH: Marcos Lopez-Hoyos, PAD: protein-arginine deiminase; PMAT: particle-based multianalyte technology; RA: rheumatoid arthritis RA-ILD: rheumatoid arthritis associated interstitial lung disease.

Commercially available PAD4 was used for all studies, except for the citrullination experiments, that were performed with the protein generated in-house. With regards to PAD3, generated in-house, significant efforts were invested in the optimization and troubleshooting of the expression and purification protocols for this antigen. Two expression systems, bacteria (*E. coli*) and insect cells (*Sf9*) were evaluated in parallel for the expression of the full-length N-terminal his-tagged human recombinant PAD3. Differences in reactivity between these two antigen versions were observed with the anti-PAD3 immunoassay in several internal studies. These discrepancies might be explained by purity differences of the two antigenic constructs as a result of the respective purification protocol. Additional underlying reasons might include different folding, epitope exposure and/or differences in the PTM of the antigen (e.g. glycosylation or citrullination -see section on citrullination below-) associated to these two expression systems, that could play a role in the immunogenicity of the antigen.

Technical challenges in the expression and purification of the PAD3 antigen were encountered. In consequence, high variability between different lots of antigen was repeatedly observed, making the extraction of conclusions very challenging. After extensive studies, antigen production and assay optimization efforts, the PAD3 protein expressed in insect cells was selected based on the following criteria:

- Better specificity measured by CIA on the Swiss RA cohort (sensitivity and specificity were 10.2% and 98.7% for insect cells antigen *vs.* 18.5% and 89.7% for the *E. coli* protein), as well as a stronger association with worse radiographic damage at baseline (appendix 1)
- Replication in the Benucci cohort (Table 11) (n=43, spearman's *rho*=0.37, *p*=0.0157) of the previously published correlation with JES described by Seaman et al. (n=39, spearman's *rho*=0.36, *p*=0.0226) [304].
- Optimization of the insect cells PAD3 protein expression and purification protocols that resulted in successful production of four consistent lots of PAD3 protein (purity and performance, data not shown)

With regards to PAD2, a total of three versions of this protein were evaluated (Table 7) and differences in reactivity when used in the anti-PAD2 assay were also observed (commented later in this section). Commercially available human recombinant PAD1 and PAD6 were used for the screening and preliminary studies on antibodies to these two members of the PAD family.

For assay optimization purposes and to better understand the PAD enzymes as antigens and autoantibody targets, basic molecular and biological characterization on these proteins was performed, including linear epitope mapping and study of the citrullination status of the enzymes.

Epitope mapping

To date, the knowledge on the epitopes of the PAD antigens is very limited. Based on the initial work performed by Harris at al. [18], Auger at al. identified four linear epitopes on PAD4 [301] located both in the N-terminal (aa 211–230, 271-290) and the C-terminal domains (aa 601–620, 621-640) of the enzyme. The antibody binding was associated with different effects on the PAD4 function, mainly resulting in inhibition, but also, in activation and no effect.

A few years later, the observation of anti-PAD3 antibodies being present only in anti-PAD4 positive patients and the inhibition studies performed by Darrah at al. [220, 302] lead to the identification of a PAD3/4 XR epitope. The investigators proposed a conformational structure and localized the XR epitope in the calcium binding region at the interface between the N- and the C-terminal domains of the PAD4 protein.

When the peptides described by Auger et al. were evaluated at Inova Diagnostics (prior to this thesis), although IgG binding to some of the peptides was detected, no significant correlation between the peptides reactivity and the signal obtained with the full length PAD3 protein was observed.

Despite the limitations of this study (potential technical limitations associated to the use of ELISA methods and the fact that the PAD4 full length protein was not included), these results may indicate that the four peptides might not be part of the XR epitope or/and that the target epitope of the PAD3/4 XR antibodies is indeed conformational. While Auger et al. observed that most antibodies to the four PAD4 linear epitopes inhibited the enzyme's activity [301], Darrah et al. showed that the anti-PAD3/4 XR antibodies enhanced it [201]. These data could indicate that the four peptides could be part of linear epitopes unique to PAD4.

Contrary to the studies by Darrah et al. [220, 302] where anti-PAD3 antibodies were only found in anti-PAD4 positive patients, we have identified for the first time anti-PAD3 antibodies in anti-PAD4 negative patients (anti-PAD3 monospecific) (see section 4.2.3. "Additional insights from the Exagen study"). These novel observation lead to the hypothesis that additional epitopes in the PAD3 enzyme could be being recognized by these monospecific anti-PAD3 antibodies.

On the other hand, Darrah et al. recently reported a minimum overlap between anti-PAD2 and anti-PAD3/4 XR antibodies (1.6%, 3/184 RA patients) using IP [308]. In our hands, a more pronounced overlap between these two autoantibodies was observed (appendix 2). In our feasibility studies including samples (n=40) from JHU (Table 11) characterized for anti-PAD2 reactivity, we observed strong quantitative and qualitative differences in immunoreactivity between the three versions of the PAD2 antigen (Figure 4). Qualitative analysis was based on preliminary cut-offs to match the PAD2 status of these samples defined by JHU. As previously indicated, the three versions of this protein differed in the expression system utilized for their generation and in the presence or absence of a his-tag (Table 7). Anti-PAD2 were found to have a prevalence of 45.0% (18/40) when using PAD2 v2 as the antigen in the immunoassay, and 30.0% (12/40) with v3. The twelve patients that were positive with PAD2 v3, were also positive with PAD2 v2, but only three of them seemed to be positive with v1. This last version showed a very distinct pattern and seemed to be the least sensitive of all three (15.0%, 6/40). Of the six patients that were identified with PAD2 v1, four were positive with PAD2 v2 and three with v3.

Spearman correlation analysis revealed non-significant low association between anti-PAD2 v1 and v2 (Figure 4A) and a moderate significant association between anti-PAD2 v1 and v3 (Figure 4B). Higher agreement was observed between the other two versions (v2 and v3, both generated at JHU) (Figure 4C), which only differ in the presence or absence of a his-tag. The cleavage of the tag after purification could explain the discrepancies, as a result of conformational changes of the protein and potential differences in epitope(s) exposure.

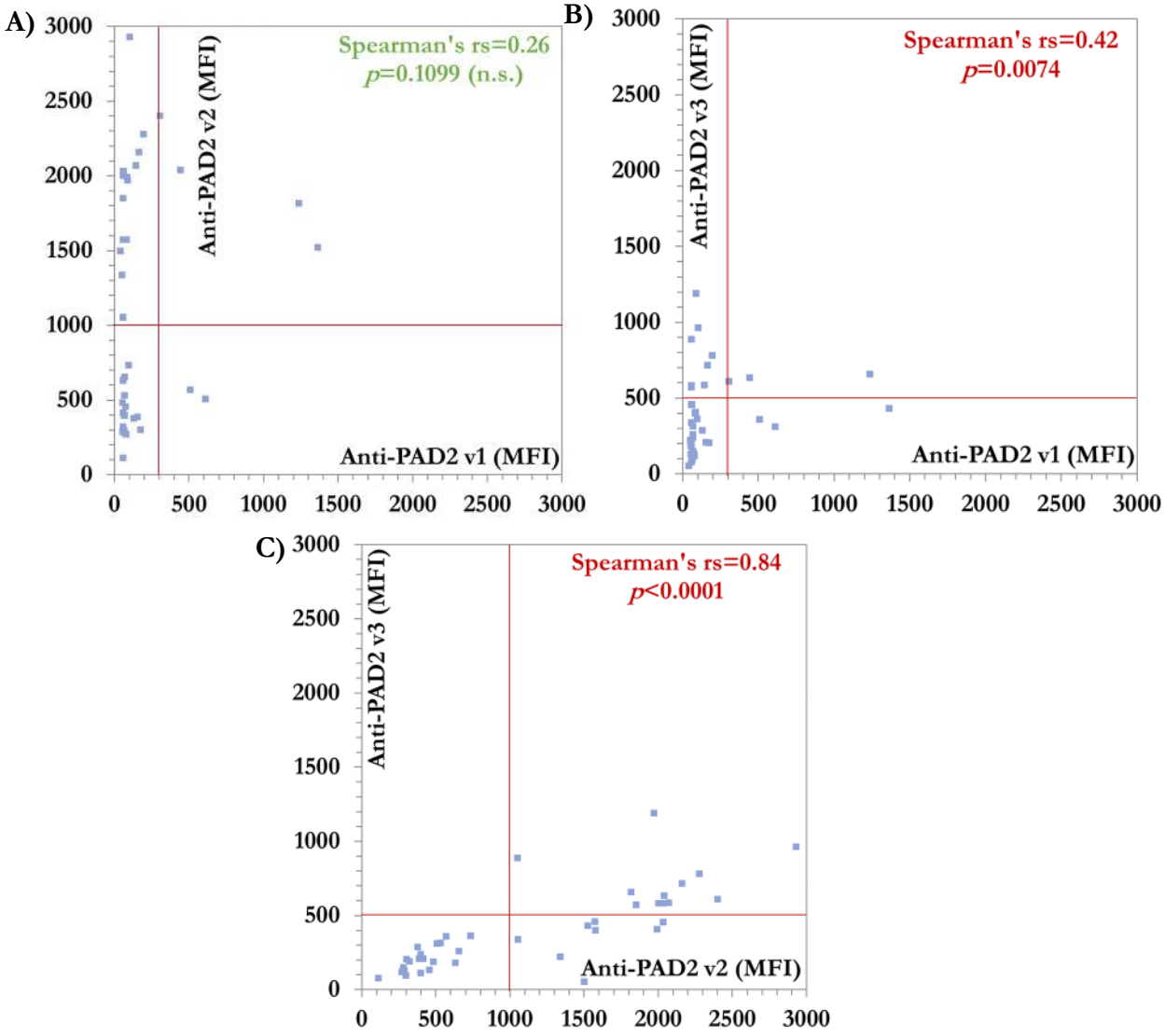

Figure 4 Spearman correlation analysis between the three versions of the PAD2 antigen assayed with the forty samples from JHU with characterized anti-PAD2 status. Spearman's rs and *p*-value are shown in each graph. Red lines indicate the preliminary cut-offs.
Abbreviations: MFI: median fluorescence intensity; PAD: protein arginine deiminase.

Although cohort bias or size and technical limitations could be contributing factors, these findings might indicate that several PAD2 epitopes could be recognized by the autoantibodies in the sera of RA patients. Different technologies for the generation of the antigens and the detection of these antibodies could result in different conformations and/or epitope exposure and explain the differences observed. It could also be hypothesized that there might be a monospecific anti-PAD2 positive population, probably the one reported by Darrah at al., associated with the clinical features described by these research group (less severe disease, less radiographic damage and lower risk of ILD) [308] and distinct from the one characterized by the presence of anti-PAD3 and anti-PAD4 antibodies.

Inhibition experiments

Following the protocol described by Darrah et al. [220], inhibition experiments were performed with human sera of different anti-PAD3 and 4 profiles based on PMAT reactivity (Figure 5). In short, the samples were incubated with either human recombinant PAD3 or PAD4, or a control buffer [phosphate buffered saline (PBS)]. The anti-PAD3 and PAD4 IgG reactivity was then measured on the samples under the different conditions. The results are summarized in Figure 5 below. A decrease in the signal was observed for anti-PAD3 and anti-PAD4 double positive patients after incubation with either enzyme (samples 1-3). A stronger effect on anti-PAD4 signal was in general observed with the incubation with this enzyme *vs.* with PAD3 (samples 1-3). This effect could be due to higher levels of anti-PAD4 antibodies in the samples potentially resulting in a saturation of the bead with antibodies and/or lack of linearity. The presence of an anti-PAD4 monospecific subpopulation of antibodies in these samples would also help explain these results. Interestingly, the reactivity of anti-PAD3 monospecific (sample 4) or anti-PAD4 monospecific (sample 6) antibodies was only inhibited by the respective PAD family member. These results point towards the co-existence of the PAD3/4 XR epitope, with additional unique epitopes in the PAD3 and PAD4 enzymes.

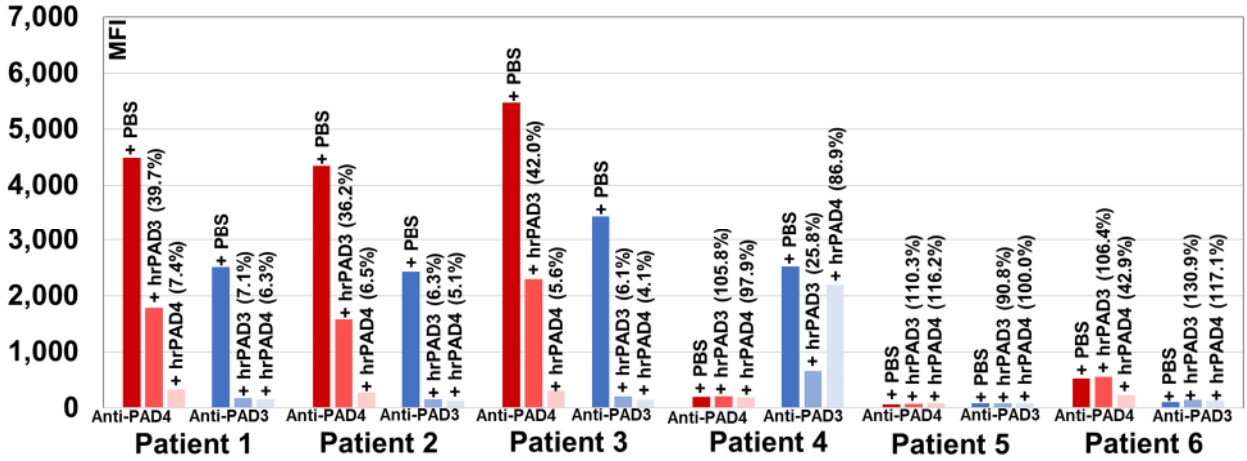

Figure 5 Anti-PAD3 and anti-PAD4 IgG inhibition experiments. Signal is expressed in MFI. Red and blue bars represent anti-PAD4 and anti-PAD3 IgG reactivity, respectively. Inhibition reagent used in each condition (PBS -control-, hrPAD3 or hrPAD4) is indicated at the top of the bar. Percentage recovery of the MFI for each condition over the corresponding control (PBS) is shown in brackets.
Abbreviations: PBS: phosphate buffered saline; hr: human recombinant; MFI: median fluorescence intensity; PAD: protein-arginine deiminase.

The PEPperPrint Epitope Mapping study

To shed more light on the epitope distribution and location on the PAD proteins, we performed a linear epitope mapping study on PAD1, PAD2, PAD3, PAD4 and PAD6. This study was carried out in collaboration with PEPperPrint (Heidelberg, Germany), with the PEPperCHIP® technology. To create the microarrays, the sequences of the PAD enzymes were linked and elongated with neutral GSGSGSG linkers at the C- and N-termini to avoid truncated peptides. The linked and elongated antigen sequences were translated into 15 amino acid peptides with a peptide-peptide overlap of 13 amino acids. The resulting peptide microarrays contained 1,689 different peptides printed in duplicate (3,378 peptide spots), and were framed by additional HA (YPYDVPDYAG, 60 spots) and polio (KEVPALTAVETGAT, 60 spots) control peptides.

A total of four pools of samples with different anti-PAD profiles based on the results with a preliminary PMAT anti-PAD IgG panel were selected, including three pools with RA samples and 1 control pool (other rheumatological diseases). The microarrays were incubated with the sample pools and then stained with the secondary antibody [goat anti-human IgG (Fc) DyLight680]. Finally, quantification of spot intensities and peptide annotation were performed. Based on averaged median foreground intensities, intensity maps were generated.

The peptides in each enzyme with the highest reactivity were identified and are illustrated in Figure 6. A selection of them was then ordered from two commercial peptide synthesis vendors for further evaluation. Feasibility studies were performed with some of the selected linear peptides on ELISA and PMAT, however, further optimization of the assays is required and will be performed in the near future. Screening of immunoreactivity to these linear peptides, in parallel with the full-length PAD proteins, will be performed in a multiplex format on the PMAT.

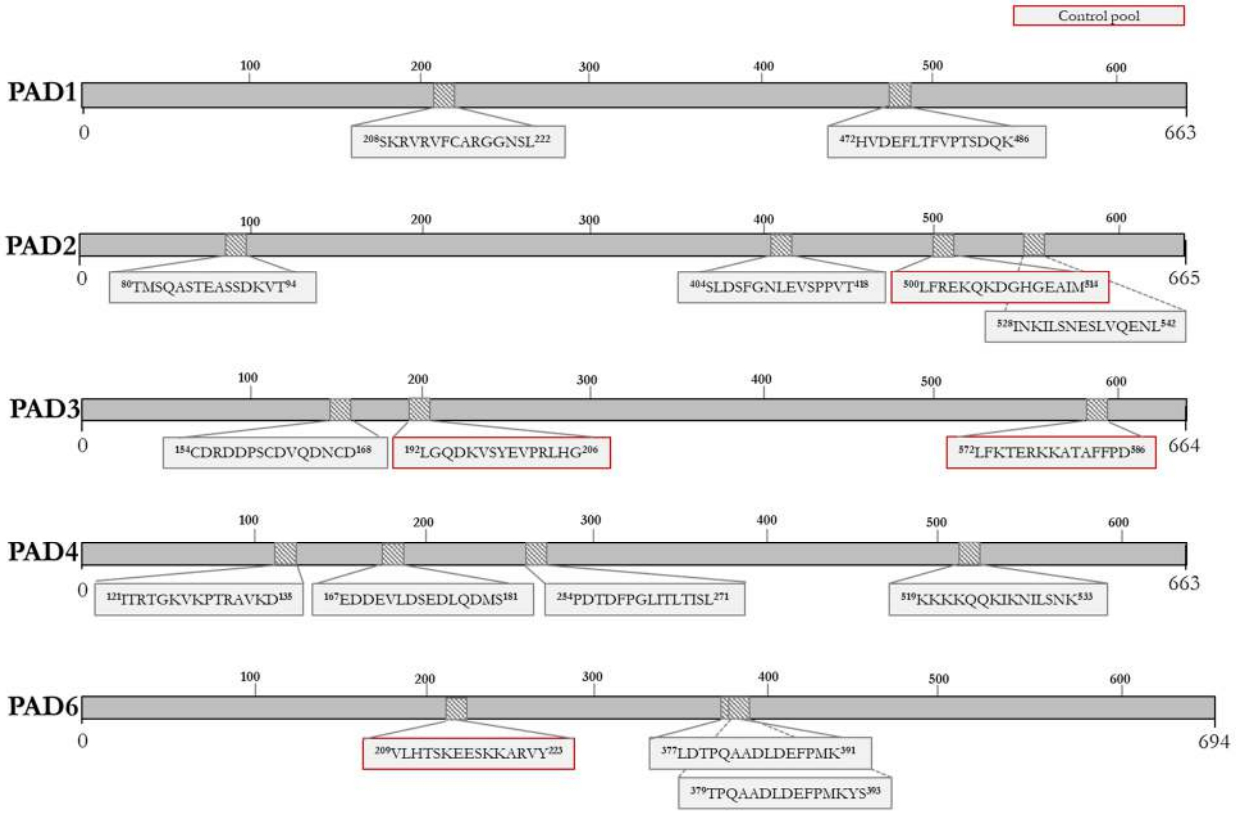

Figure 6 Selection of peptides with the highest intensity in each PAD enzyme as a result of the PEPper Print linear epitope mapping study. Candidate linear epitopes and location are indicated in the boxes. Boxes with red outline indicate peptides with high intensity identified with the control pool.
Abbreviations: PAD: protein-arginine deiminase.

Since some data on the epitope distribution of PAD4 was available, we generated a summary of the linear epitopes described in literature as well as the candidates identified in the PEPper Print study (Figure 7).

Figure 7 PAD4 linear epitopes described in literature [220, 282, 304] and candidates newly identified in our study. Primary structure (boxes) and amino acid sequence of the PAD4 protein is illustrated. Proposed linear epitopes are marked and color-coded based on the study. The protein domains are represented underneath the primary structure.
Abbreviations: PAD: protein-arginine deiminase.

Citrullination status of the PAD enzymes

The notion that the PAD4 enzyme could autocitrullinate *in-vitro* [209] triggered the investigation of the citrullination status of the PAD enzymes and an associated impact on their immunoreactivity. In the study by Andrade et al., autocitrullination of PAD4 resulted in an inactivation of the enzyme through change in the structure of the protein, which could represent a potential control mechanism of enzyme activity. Additionally, citrullination had a significant impact on the antigenicity by annulling its recognition by multiple rabbit antibodies and interestingly, increasing recognition of human anti-PAD4 antibodies from RA patients. Nonetheless, only four human anti-PAD4 positive

patients were used in this study. Furthermore, strong limitations in the detection of citrullination methods used (anti–modified citrulline immunoblotting) have recently been reported [369]. While certain commercially available antibodies might be able to successfully distinguish between carbamylation (homocitrulline) and citrullination (citrulline), a considerable degree of unspeficificity and cross-reactivity with certain anti-homocitrulline antibodies has been reported [369]. More refined methods, such as mass-spectrometry, are still essential to accurately differentiate between these two important PTM in RA in proteins from complex biological samples.

On the other hand, a study recently reported a direct relationship between the degree of citrullination of the heat shock protein 90 (HSP90) and the level of *ex-vivo* recognition by antibodies to this protein associated to ILD in RA patients[370]. These data supported the idea that changes induced by citrullination in the protein structure generated cryptic epitopes capable of bypassing B-cell tolerance in the appropriate genetic context.

Uncontrolled autocitrullination of the PAD enzymes could be occurring during their expression and purification given that the co-factor calcium is present at various steps during these processes. We then hypothesized that this potential modification and the associated shifts in the conformational structure of the PAD enzymes as reported for PAD4 by Andrade at al. [209], could result in alterations to the epitope(s) exposure and consequently, differences in their immune recognition. Different degrees of citrullination could explain variability in the reactivity of the different batches of enzymes, especially relevant for PAD3, given the technical challenges faced.

To study the citrullination status of the PAD enzymes used for the immunoassays, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western Blot (WB) analyses were performed. A mouse monoclonal anti-poly histidine alkaline phosphatase (Sigma, Cat.#A5588), and two unlabeled rabbit polyclonal anti-citrulline antibodies [Abcam (Cat.#100932) and Origene Technologies (Cat.# AP54847SU-N)] were used as primary antibodies in these studies. Being aware of the previously described limitations in the detection of citrullination by WB [369], the goal of this study was to gain preliminary insights about (1) whether the PAD proteins being used in our immunoassays could be citrullinated, whether there could be (2) differences between the citrullination patterns on the different PAD enzymes, (3) between different vendors or (4) batches of protein, or (5) based on the expression system.

The appearance of bands with the anti-citrulline antibodies at the expected molecular weights for all PAD proteins tested seemed to indicate that the PAD2, PAD3 and PAD4 utilized for our immunoassays were citrullinated. Different band profiles were observed between the PAD family members and between the same protein generated in different expression systems, suggesting differences in the citrullination profiles (data not shown). Nevertheless, as relatively expected, the results obtained with this method and these antibodies were inconclusive (experiment controls not performing as expected, lack of reproducibility). We confirmed that WB is not the ideal method to study citrullination.

In parallel, during the optimization of the protocol for the generation of a PAD4 protein in-house by the Inova Biotechnology team, low binding of the protein -high elution in the flow through-

was observed with anion exchange chromatography (separation by net negative surface charges) (data not shown). This technique was being tested as a protein polishing step. Because citrullination results in the loss of positive charges in the protein (arginine is positively charged at a neutral pH, whereas citrulline has no net charge), we hypothesized the existence of differently citrullinated species of PAD4 during the generation of the protein. Anion exchange chromatography could then be separating the hypothesized differently citrullinated PAD4 species, based on differences in charge. The less citrullinated species would be less negatively charged, resulting in weaker binding to the positively charged chromatography resin and their appearance in the flow through or earlier eluted fractions. The more citrullinated species would be more negatively charged and bind in a stronger manner to the resin, and therefore, these species would elute in later fractions of the chromatographic profile.

These observations could then indicate that several species of PAD4 might be present as a result of the protein in-house generation protocol, due to autocitrullination of the enzyme, enabled by the presence of the co-factor calcium during the process. Understanding whether there are differences in reactivity in the detection of anti-PAD4 antibodies using these different species in the immunoassays would be of great interest. In this context, it would also be important to evaluate the weight and relevance of the peptide backbones *vs.* the citrullinated motifs in the PAD enzymes in their recognition by the autoantibodies that target them. This point could help understand better the anti-PAD antibodies, in comparison to the ACPA response, in which there is a specific citrulline recognition that is backbone-mediated [371, 372], allowing for the promiscuous recognition that is characteristic of this response.

Studies that investigate in more detail these findings with methods such, as mass spectrometry, *in vitro* citrullination assays and additional functional experiments with very well characterized PAD proteins in terms of PTM, will be useful for a better understanding of anti-PAD immune response.

4.2.2. Characterizing the anti-PAD antibodies

In order to better characterize the antibodies that target the PAD enzymes in RA, several studies were performed, including experiments designed to investigate the isotype usage and IgG subclasses distribution, and the existence of autoantibodies to the other PAD family members never described as RA antigenic targets until now.

Isotype usage and IgG subclasses distribution

Different isotypes (IgM, IgG, or IgA) [373] and IgG subclasses (IgG1, IgG2, IgG3, or IgG4) [374] differ in their capacity to recruit immune effector mechanisms. The isotype usage of the RF [242, 252], ACPA [365, 375, 376], as well as anti-CarP [315] response have been studied, showing a broad usage of isotypes in RA patients. To better understand the anti-PAD4 immune response in RA, the isotype and IgG subclass usage were evaluated. For this purpose, modified immunoassays for the detection of anti-PAD4 IgA and IgM, and anti-PAD4 IgG1, IgG2, IgG3 and IgG4 were created. The principle of these modified assays was identical to the standard assays for the measurement of anti-PAD4 IgG previously described. In short, the PMAT particles coupled with PAD4 protein were used with different detectors compatible with the PMAT system: anti-human IgA or IgM (Inova

Diagnostics, San Diego, USA) and anti-human IgG1, 2, 3 and 4 (anti-IgG1, 2 and 3 from MyBiosource, Cat.# MBS674030, MBS674040 MBS674086, respectively; anti- IgG4 from Abcam, Cat.# ab99825).

The described immunoassays for the detection of the three isotypes were used for the screening and detection of these anti-PAD4 biomarkers in 110 human sera from the Inova Biobank, including samples from individuals with RA (n=63) -with joint erosion information available (absence or presence) for 62 of them-, and controls (n=47), comprising healthy individuals (n=15), infectious disease (n=12), SLE (n=9), SSc (n=10), and SjS (n=1).

In addition to IgG, anti-PAD4 IgA and IgM were also identified in the serum of RA patients. These results confirm the presence of anti-PAD4 IgA [283] in the serum of RA patients and expand the finding to the IgM isotype, that to date, has not been reported in literature. ROC analysis showed better overall discrimination of the IgA and IgM isotypes than IgG between RA and controls, however, at the relevant diagnostic area (>90% specificity), IgG outperformed the other two markers (Figure 8A). With regards to erosive disease, the best discrimination was observed with anti-PAD4 IgA, closely followed by IgG, and finally IgM (Figure 8B).

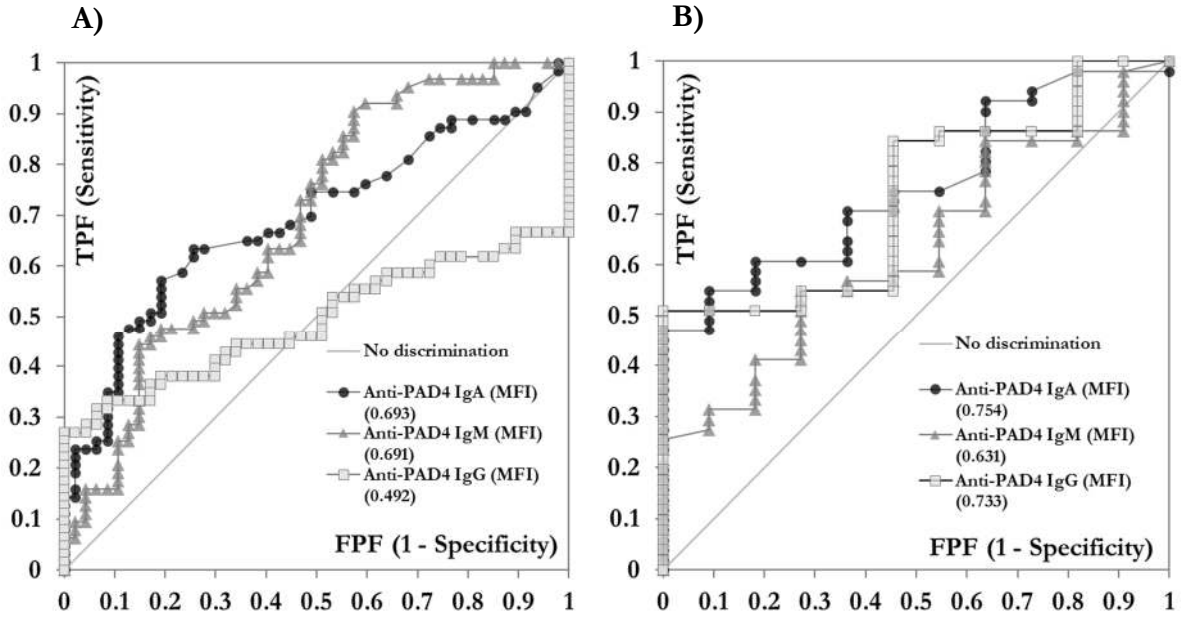

Figure 8 ROC analysis of the discrimination between RA and controls (A) and RA erosive disease (B) of anti-PAD4 IgG, IgA and IgM. The AUC is shown in brackets for each biomarker.
Abbreviations: AUC: area under the curve; FPF: false positive fraction; MFI. Median fluorescence intensity; PAD: protein-arginine deiminase; RA: rheumatoid arthritis; ROC: receiver operating characteristics; TPF: true positive fraction.

With a fixed specificity at the 95%, preliminary cut-off values were identified at 1247, 171, 536 MFI for anti-PAD4 IgG, IgA, and IgM, respectively, reporting sensitivities of 23.8%, 15.9% and 28.6%. Based on these cut-offs, patients with the presence of one, two or the three isotypes were observed. The prevalence of each isotype and the overlap between the three are shown in the Venn Diagram illustrated in Figure 9.

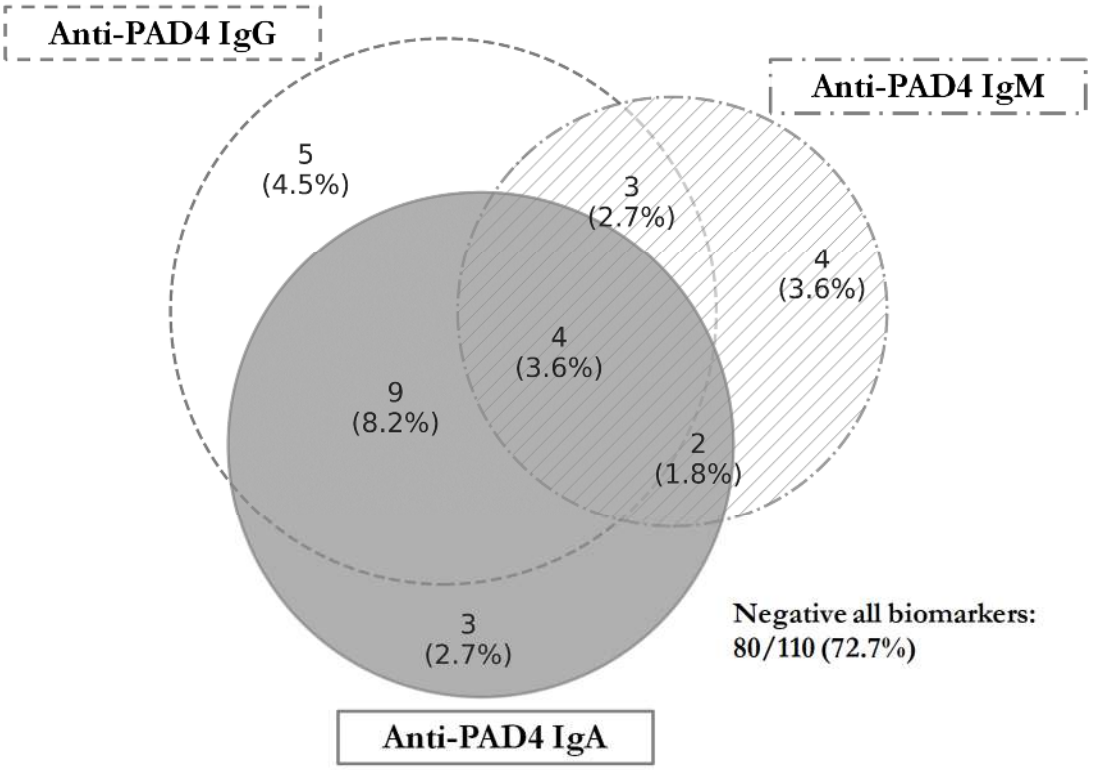

Figure 9 Three-way Venn Diagram representing the prevalence and overlap of the anti-PAD4 IgG, IgA and IgM isotypes based on the preliminary cut-offs established in this study. The Venn Diagram was generated with a software library for plotting area-proportional three-way Venn diagrams in Python obtained from the library definition (https://pypi.org/project/matplotlib-venn/).
Abbreviations: PAD: protein-arginine deiminase.

Higher levels of anti-PAD4 IgA and IgM were observed in RA *vs.* controls (p=0.0006 for both). Surprisingly, higher levels of anti-PAD4 IgG were found in controls *vs.* RA in this study, although this finding was not significant (p=0.8823). This unexpected result seemed to be driven by several controls with positive results for this isotype, that were all around the cut-off point. Higher titers of the three isotypes were observed in RA patients with erosive disease *vs.* those with absence of joint erosion (p=0.0162, p=0.0086, and p=0.1756 -n.s.- for IgG, IgA and IgM, respectively).

Anti-PAD4 IgG and IgA were measured in a larger cohort, in which the associations between the IgG and IgA isotypes and erosive disease and biological treatment use were investigated in more detail (see section 4.2.3. "Anti-PAD antibodies and erosive disease").

With regards to the IgG subclasses distribution, it has been shown that ACPA are heterogeneous in terms of citrullinated target, isotype and IgG subclass, and that the ACPA response is initially restricted and it expands with time and disease progression [375, 376]. Studies suggest a continuous activation of the RA-specific ACPA response during the course of seropositive disease [377]. On the other hand, the broad distribution of anti-CarP isotypes and IgG subclasses also indicate an ongoing immune response and the participation of several effector mechanisms, however, data here point towards different regulatory mechanisms in the anti-CarP and ACPA responses [315].

In our study, a total of nine sera from anti-PAD4 IgG positive RA patients were tested for total IgG and the four subclasses. High reactivity was observed for at least one or more of the subclasses (Figure 10), indicating that similarly to ACPA and anti-CarP [315], a broad spectrum of IgG subclass usage can be found for anti-PAD4 antibodies. Despite the technical limitation of the lack of proper IgG subclasses controls in our study, as previously reported in literature [289], IgG1 and IgG3 seem to be the predominant subclasses in the anti-PAD4 immune response. Functionally, IgG1 and IgG3 are known to be inducers of Fc-mediated effector mechanisms, such as antibody-dependent cellular and complement dependent cytotoxicity, and antibody-dependent cellular phagocytosis [378]. Our observations could then be indicating that in the anti-PAD4 response, the complement system, natural killer cells and macrophages might play an important role. This heterogeneity in the regulatory and effector mechanisms associated to the ACPA, anti-CarP and anti-PAD responses may be relevant for the immunopathogenesis of RA.

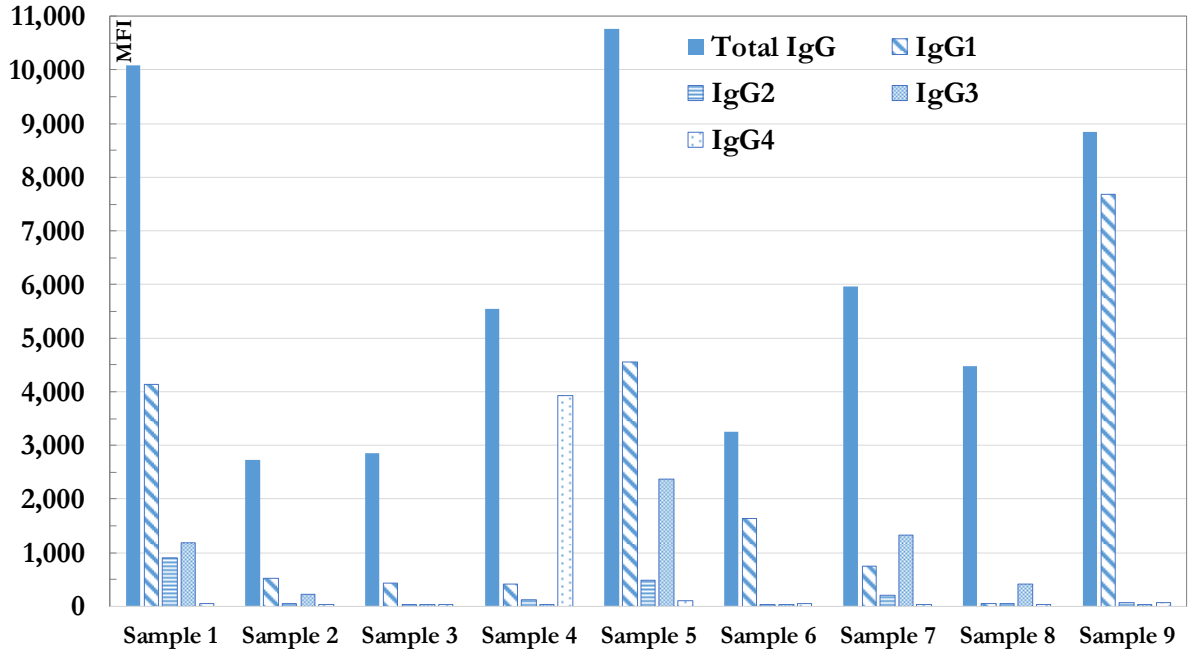

Figure 10 Anti-PAD4 IgG subclasses distribution study. Results are expressed in MFI. Total IgG and IgG1, IgG2, IgG3 and IgG4 were quantified in a total of nine RA patients. IgG1 and IgG3 seem to be predominant. Reactivity to each of the subclasses was observed in at least one patient.
Abbreviations: MFI: median fluorescence intensity; PAD: protein-arginine deiminase; RA: rheumatoid arthritis.

In summary, the anti-PAD4 response uses at least three isotypes in RA (IgG, IgA and IgM), and a broad spectrum of IgG subclasses, with an apparent predominance of IgG1 and IgG3. This is the first time that antibodies to PAD4 of the IgM isotype are reported in RA.

Optimization of these assays, testing of larger numbers of samples and additional studies in this space will be very interesting to understand the connection with immune events and clinical features in patients with different disease status or distinct anti-PAD profiles. Further research of the isotype usage and the implications on the pathogenesis of the disease, the evolution of isotype usage and IgG subclass distribution of the anti-PAD response with disease progression, as well as the clinical implications of these findings will be of great interest.

Antibodies to PAD1 and PAD6

To screen for the presence of antibodies to PAD1 and PAD6, a set of samples (n=79) from the Inova Biobank, including RA (n=36) and controls (n=43) were tested with the PMAT anti-PAD IgG panel, built for the detection of IgG to the five human PAD family members (PAD1, PAD2, PAD3, PAD4 and PAD6). The controls comprised apparently HI (n=21), SSc (n=10), SjS (n=2) and SLE (n=10).

Interestingly, good discrimination between RA and controls, as shown by the AUCs, was observed for all five antibodies (Figure 11). At the most relevant area of the curve (specificity >90%), anti-PAD4 IgG, followed by anti-PAD3 IgG, were the biomarkers with the best diagnostic performance.

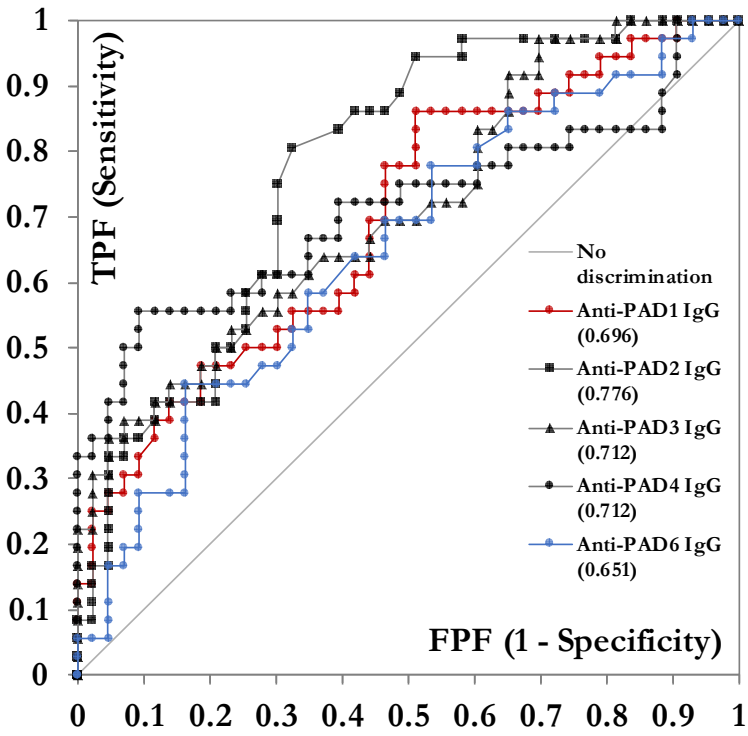

Figure 11 ROC analysis of the discrimination between RA and controls of IgG antibodies to PAD1, PAD2, PAD3, PAD4 and PAD6. The AUC is shown in brackets for each biomarker.
Abbreviations: AUC: area under the curve; FPF: false positive fraction; PAD: protein-arginine deiminase; RA: rheumatoid arthritis; ROC: receiver operating characteristics, TPF: true positive fraction.

Significantly higher levels of all five antibodies were observed in RA *vs.* controls in our cohort (Figure 12). Although cut-offs were not available at this point, based on raw signal, we identified patients with the apparent presence of multiple anti-PAD antibodies (Figure 13), as well as monospecific individuals for anti-PAD1, 3 and 4 (not shown). Spearman correlation analysis showed the highest association between anti-PAD1 and 4 (Spearman's rs=0.88, p<0.0001) and the lowest between anti-PAD4 and 6 (Spearman's rs=0.40, p=0.0003).

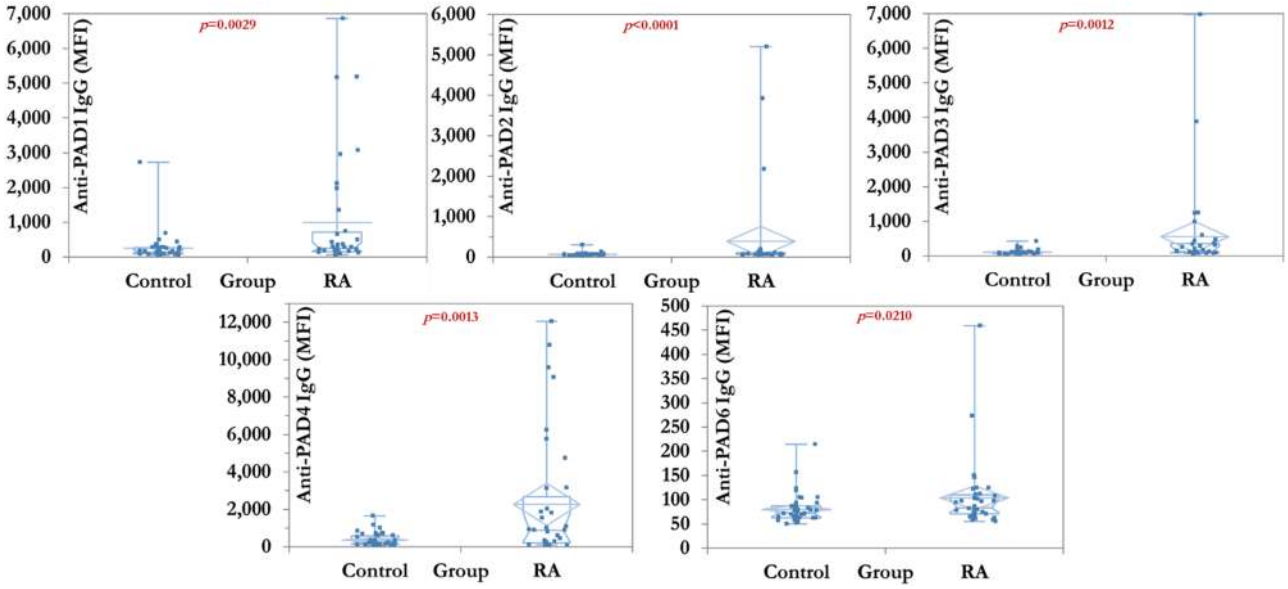

Figure 12 Descriptive plots with the comparison of the levels of the five anti-PAD antibodies in RA *vs.* controls. Results are expressed in MFI. *P*-values are shown each graph.
Abbreviations: MFI: median fluorescence intensity; PAD: protein-arginine deiminase; RA: rheumatoid arthritis.

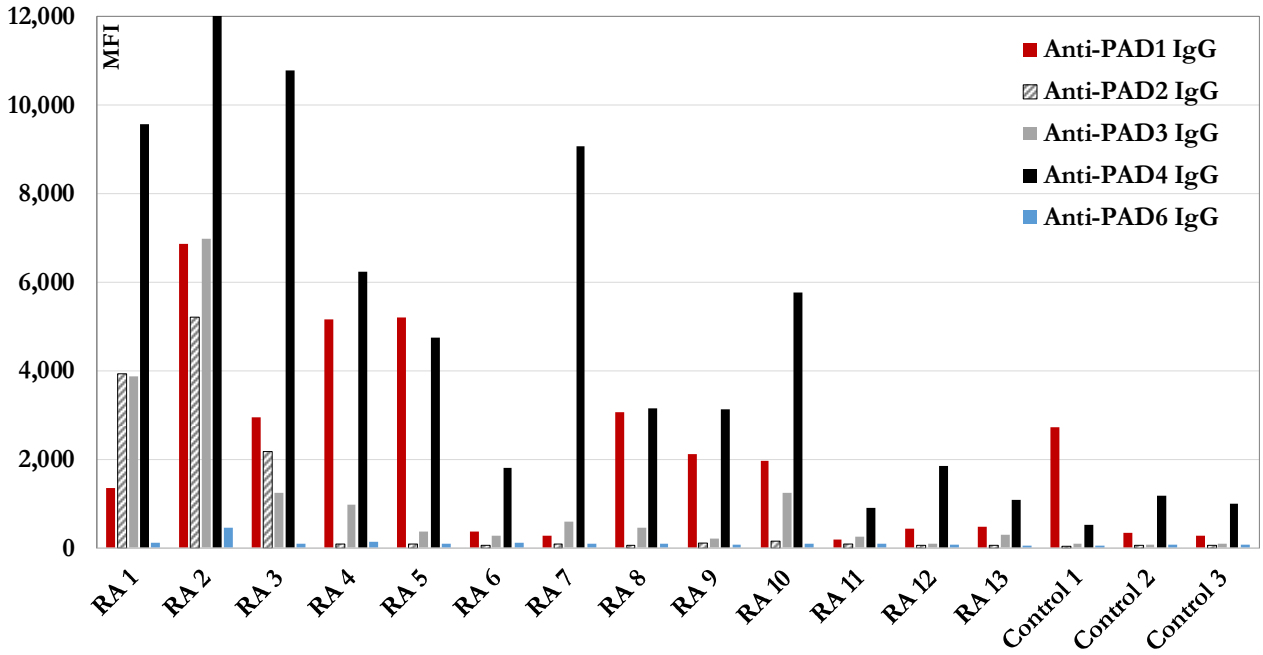

Figure 13 Bar chart illustrating the measured reactivity of IgG to PAD1, 2, 3, 4 and 6 in patients with the apparent presences of multiple anti-PAD antibodies. Signal is expressed in MFI.
Abbreviations: MFI: median fluorescence intensity, PAD: protein-arginine deiminase, RA: rheumatoid arthritis.

This is the first time that antibodies that target PAD1 and PAD6 are reported. Evaluating and characterizing these biomarkers in RA and potentially other diseases will be of great interest. It will also be important to analyze the overlap and potential new cross-reactivities between these antibodies. These novel data identifying PAD1 and PAD6 as novel autoantigens in RA and describing the isotype

distribution of the anti-PAD4 response were recently submitted as an abstract to the 2020 EULAR Congress (appendix 3).

4.2.2. Feasibility, optimization and early development of anti-PAD immunoassays

Using the PAD enzymes as antigens, PMAT immunoassays for the detection of the anti-PAD antibodies were designed and optimized. Prior to the beginning of this thesis, other colleagues at Inova had designed an assay for the quantification of anti-PAD3 IgG for the BIO-FLASH system which was used for the study by Seaman at al. [304] in which an association with joint erosion score (JES) was found. In addition, very preliminary work had been conducted to design the PMAT immunoassay for the detection of anti-PAD3 and 4 antibodies in a panel approach. However, the assays had been minimally optimized at this stage, and the anti-PAD3 assay was affected by major stability challenges.

Optimization experiments on this PAD 2-plex panel with special focus on PAD3 (due to the stability challenges) were performed. These included coupling procedure optimization during the three main steps of the protocol -activation, coupling and blocking-, as well as testing conditions optimization. Within the first one, the conditions that were studied and modified were: concentrations of activation reagents, activation incubation time, coupling buffers screening, antigen concentration on the beads, temperature controlled coupling incubation (room temperature *vs.* 4°C and 37°C), blocking buffer screening and post coupling treatment (desiccation, lyophilization, freezing). To optimize the testing conditions, an extensive beads storage buffer screening was carried out, and the detector antibody concentration and system fluid were also evaluated.

The mentioned studies were performed over the course of this thesis and resulted in several changes in the formulations of the assays that lead to an improved stability and robustness of the assay. Additionally, to ensure the reproducibility of the previously published, a direct comparison of the anti-PAD3 CIA and PMAT assay with the antigen expressed in insect cells was performed. A total of 198 samples from the Swiss RA cohort (Table 11) were tested on both assays, including RA patients (n=157) and non-RA controls (n=41), comprised of patients with AxS (n=21) and PsA (n=20). A very high and significant correlation was observed (Figure 14). PMAT was then selected as the preferred platform for further studies, given the advantage of multiplexing that this technology presents, allowing for the simultaneous detection of other autoantibodies in the same reaction.

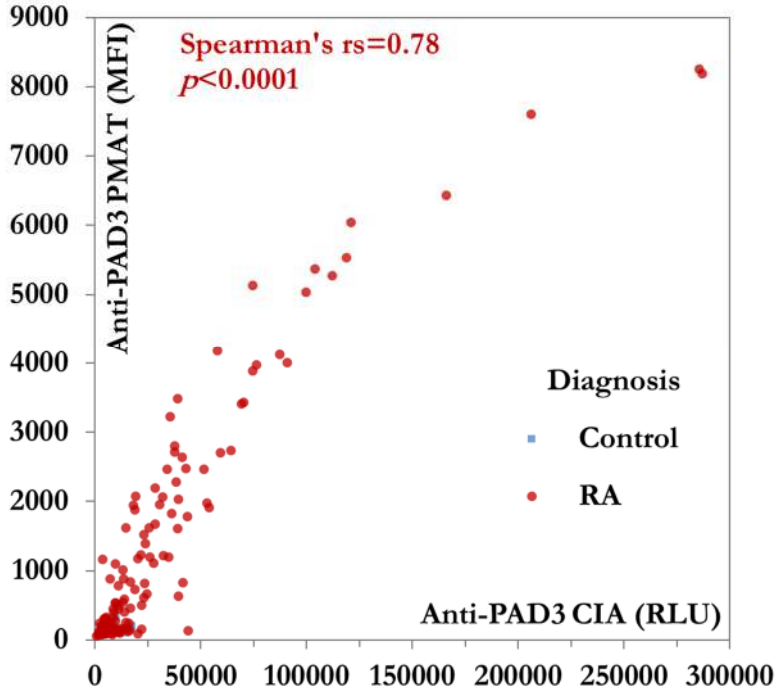

Figure 14 Spearman correlation analysis of the anti-PAD3 PMAT *vs.* CIA. Spearman's *rs* and *p*-value are shown in red. Samples are labeled based on their diagnosis (RA and controls).
Abbreviations: CIA: chemiluminescence immunoassay; MFI: median fluorescence intensity; PAD: protein-arginine deiminase; PMAT: particle-based multi-analyte technology, RA: rheumatoid arthritis; RLU: relative light unit.

Early feasibility work was also performed with the immunoassays for the detection of anti-PAD2, as well as anti-PAD1 and 6.

The Inova PMAT Assay Development team is currently continuing the development of the anti-PAD panel to eventually make them commercially available as part of the PMAT RA panel. This panel will represent an important tool in research settings as well as in clinical routine.

4.2.3. Understanding the clinical significance of anti-PAD antibodies in RA

The feasibility and optimization efforts allowed the utilization of these immunoassays in the detection of anti-PAD antibodies in several clinical studies (Table 11), with especial emphasis on anti-PAD3 and anti-PAD4 IgG. The results of those studies, summarized in this section, provide novel insights into the clinical relevance of these autoantibodies. The current knowledge and recent findings on anti-PAD4 antibodies in RA and their clinical and immunological significance were summarized in a recent review entitled **"Autoantibodies to protein-arginine deiminase (PAD) 4 in rheumatoid arthritis: immunological and clinical significance, and potential for precision medicine"**, published in the October 2019 issue of 'Expert Review of Clinical Immunology' (chapter 3.2.).

<u>Anti-PAD4 antibodies help to close the serological gap in RA</u>

Exagen Study

To study whether the anti-PAD3 and 4 IgG could help close the serological gap in RA and to evaluate their diagnostic relevance, a large cohort (n=1473) of 640 RA and 833 controls (636 with other rheumatological diseases and 197 healthy individuals) were tested for the presence of these autoantibodies using the PMAT assays. ACPA IgG and RF IgM were also measured in these patients by EliA (Phadia, Germany).

Importantly, this study lead to the identification of the preliminary cut-off values for anti-PAD3 and PAD4 antibodies (95th percentile of healthy individuals) which were later utilized in other studies. The cut-off values were set at 200 and 1000 MFI for anti-PAD3 and anti-PAD4, respectively.

In our cohort, 13.75% (88/640) and 34.8% (223/640) of RA patients, as well as 0-21% and 0-9% of controls were positive for anti-PAD3 and anti-PAD4 antibodies, respectively. The prevalence of the presence of both antibodies in RA was found to be at 12.0% (77/640) and resulted in a 28.4% (95% CI 25.1-32.1%) sensitivity with 99.2% (95% CI 98.3-99.6%) specificity. ROC curve analysis showed significant discrimination between RA and controls for both markers. Our data was consistent with previous studies such as the recent review article by Reyes-Castillo et al. [379], and the meta-analysis performed by Ren et al. [300], that indicated a pooled sensitivity and specificity of anti-PAD4 of 38.0% (95% CI 30.0-46.0%) and 96.0% (95% CI 93.0-98.0%), or the Giles at al. [302] and Darrah at al. [220] studies, in which the prevalence of anti-PAD3 antibodies was found to be 11% and 18%, respectively.

Interestingly, elevated titers were found in both ACPA positive and ACPA negative patients and the OR for RA in the ACPA negative population were 5.9 for anti-PAD4 and 2.3 for anti-PAD3. In this subgroup, only anti-PAD4 showed significant discrimination. Combining ACPA and anti-PAD4 antibodies increased the sensitivity by 6.2% to 72.7% (95% CI 69.1-76.0%) with a decrease in specificity by 3.7% to 93.4% (95% CI 91.5-94.9%). All together, these data suggested diagnostic utility especially for anti-PAD4 IgG, associated to its contribution to close the serological gap in RA.

Because it has been reported that anti-PAD4 antibodies, similarly to ACPA and RF, precede the clinical onset of RA [288], the association with disease duration was also investigated in those patients for whom this information was available (528 RA patients and 582 controls). The patients were subcategorized into 0-2, 2–10 or >10 years of disease duration. For both antibodies, the prevalence increased with disease duration. However, while in early disease (0-2 years), the prevalence of anti-PAD3 antibodies was comparable in RA and controls, in individuals with more established disease, anti-PAD3 antibodies were significantly more prevalent in RA vs. controls. On the other hand, anti-PAD4 were significantly more prevalent in early RA patients vs. controls.

Up to the present time, several studied had suggested a diagnostic value for anti-PAD antibodies, in particular anti-PAD4, however, no commercial assays are currently available, and these biomarkers are not currently used in clinical practice. Our study, the largest on the diagnostic relevance of these antibodies in RA, provided strong evidence that especially anti-PAD4 antibodies represent a useful biomarker to improve the diagnosis of this disease.

The results of this study were summarized in the **manuscript entitled "Antibodies Targeting Protein-Arginine Deiminase 4 (PAD4) Demonstrate Diagnostic Value in Rheumatoid Arthritis",** published in the March 2019 issue of 'Annals of Rheumatic Diseases' (chapter 3.3.). Combinatory approaches with ACPA and RF were evaluated and presented by our collaborators in this study (Dervieux et al.) as oral presentations at the 2018 and 2019 ACR Annual Meetings (appendices 4 and 5, respectively).

Rome seronegative RA study

The findings described in the previous subsection were validated in a second independent cohort that included a total of 133 subjects, with 41 controls and 92 early RA patients that were triple seronegative for IgG-ACPA. Anti-PAD4 IgG, measured by PMAT, were observed in 15.2% (14/92) of seronegative ERA patients and in 2.4% (1/41) of the controls. ROC analysis revealed a moderate discrimination of these antibodies in this population, with an AUC of 0.60 (95% CI 0.50-0.71). At the study cut-off, anti-PAD4 reported a sensitivity of 15.2%, with a 97.6% specificity, and an OR of 7.2 (95% CI 1.1-44.1). Therefore, anti-PAD4 IgG demonstrated diagnostic value in this cohort of early RA triple-seronegative patients and controls, validating that this biomarker represents a useful tool to help close the seronegative gap in RA. These results were presented as a poster at the 2019 Dresden Symposium on Autoantibodies (appendix 6).

Additional insights from the Exagen study

In a second phase of the Exagen study (Table 11), new patient information was made available to Inova. Additional questions were interrogated in this new data set and the outcomes of these analyses are summarized in this section. Here, we focused on the utility of anti-PAD3 and anti-PAD4 for RA patient stratification, investigated the specific characteristics associated to the different anti-PAD profiles, analyzed the evolution of these antibodies over time with disease progression and investigated a potential impact of treatment on the anti-PAD3 and 4 levels.

To this end, the new data were evaluated using version 5.01 of Analyse-it (Analyse-it Software, Ltd, Leeds, UK). Fisher exact test, student's T-test and Wilcoxon Mann-Whitney analysis were used to analyze differences between groups and titers and to test for significance. Spearman correlation analysis between the different markers was performed. *P*-values < 0.05 were considered significant.

Anti-PAD3 monospecific antibodies

The prevalence of anti-PAD3 and 4 IgG in this cohort (Table 8, Figure 15) was in line to previous studies [201, 300, 304]. Interestingly, anti-PAD3 antibodies were observed frequently in anti-PAD4 positive patients (anti-PAD3/4 XR), but also in the absence of anti-PAD4 (anti-PAD3 monospecific), at high levels. To date, anti-PAD3 antibodies had only been reported in anti-PAD4 positive patients, known as the anti-PAD3/4 XR antibodies [16]. These anti-PAD3 monospecific antibodies were observed in both RA and, even at a higher prevalence, in controls, indicating a low specificity for isolated anti-PAD3 antibodies. Several factors could help explain the identification of this new subset of anti-PAD3 monospecific patients. A possible explanation is the much larger sample size of our study compared to previous ones. In addition, as indicated in section 4.2.1. "Epitope Mapping", it is likely that epitope(s) distinct from the PAD3/4 XR epitope are recognized by anti-

PAD3 antibodies. The use of a different technology in our study could have favored the exposure of these other epitope(s), allowing for the identification of these antibodies.

Table 8 Summary of prevalence of the anti-PAD IgG profiles in the different study subpopulations. Differences in prevalence between RA and controls on one hand, and between ACPA positive and ACPA negative RA patients were tested for significance**.**
Note: ACPA results not available for two RA patients (both double negative for the anti-PAD markers).

Biomarker	All (n=1473)	RA (n=640)	ACPA+ RA (n=424)	ACPA- RA (n=214)	Controls (n=833)
Total anti-PAD4	17.7% (261/1473)	35.0% * (224/640)	42.9% (182/424)	19.2% (41/214)	4.6% (38/833)
Total anti-PAD3	13.8% (124/1473)	13.8% * (88/640)	16.3% * (69/424)	8.9% (19/214)	4.3% (36/833)
Anti-PAD3/4 XR	5.8% (85/1473)	12.0% * (77/640)	14.4% * (61/424)	7.5% (16/214)	1.0% (8/833)
Monospecific anti-PAD4	12.0% (176/1473)	22.8% * (147/640)	28.7% * (122/424)	11.7% (25/214)	3.6% (30/833)
Monospecific anti-PAD3	2.6% (39/1473)	1.7% (n.s.) (11/640)	1.9% (n.s.) (8/424)	1.4% (3/214)	3.4% (28/833)

**p<0.05, n.s.: non-significant*

Abbreviations: ACPA: anti-citrullinated protein antibodies; anti-PAD4: anti-protein arginine deiminase 4 antibodies; anti-PAD3: anti-protein-arginine deiminase 3 antibodies; RA: rheumatoid arthritis.

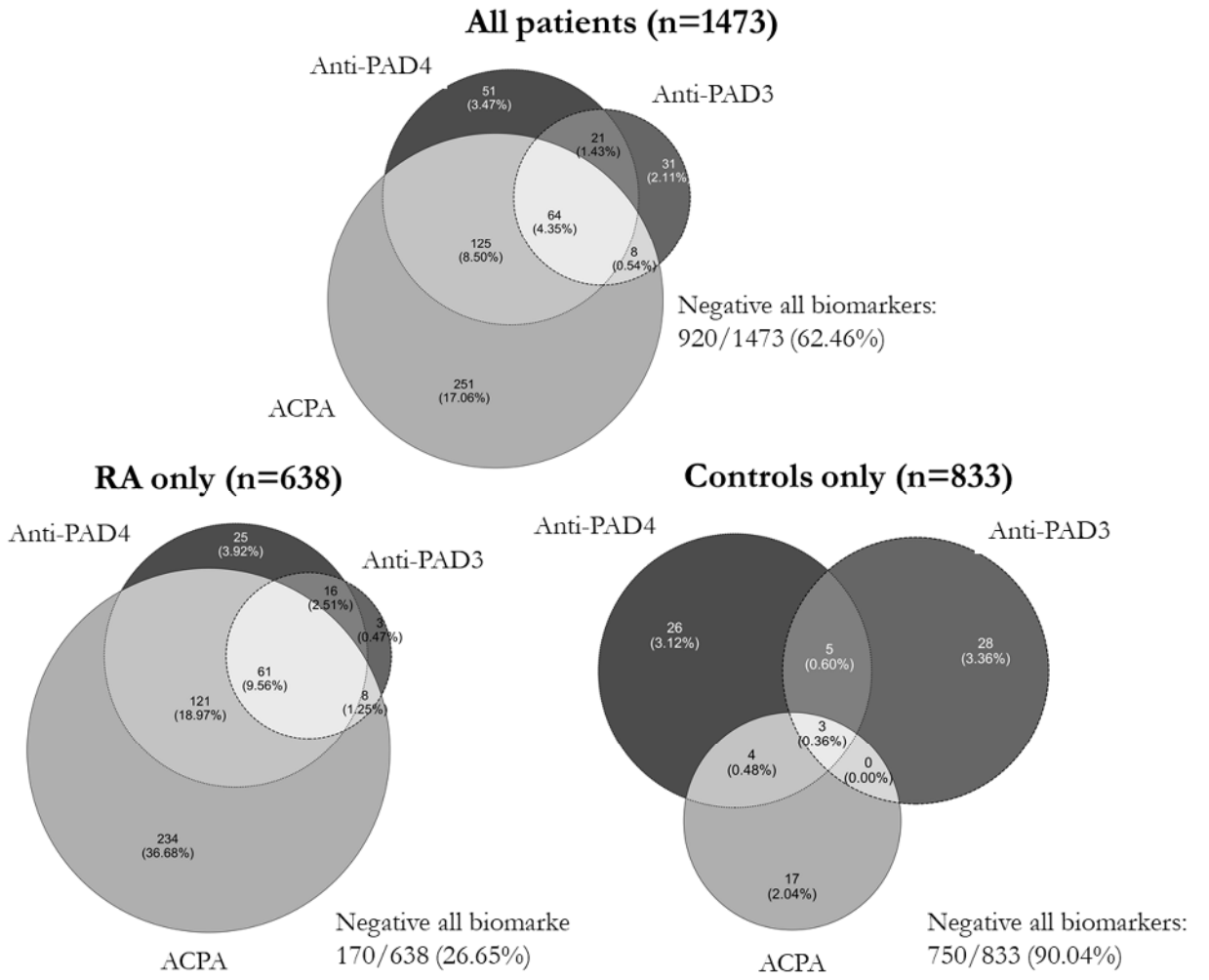

Figure 15 Three way Venn Diagrams representing the overlap between ACPA, anti-PAD3 and anti-PAD4 in the total population (a), in RA patients (b) and in controls (c). Number of patients and percentage over the population represented are shown. Venn diagrams were generated using a software library for plotting area-proportional three-way Venn diagrams in Python obtained from the library definition (https://pypi.org/project/matplotlib-venn/).
Abbreviations: ACPA: anti-citrullinated protein antibodies; PAD: protein-arginine deiminase; RA: rheumatoid arthritis.

Anti-PAD3 and anti-PAD4 antibodies as biomarkers for patient stratification in RA

The RA patients' characteristics were analysed based on distinct anti-PAD IgG profiles (anti-PAD3 and 4 double positive, anti-PAD3 monospecific, anti-PAD4 monospecific or anti-PAD3 and 4 double negative) and significant differences were observed for several features (Table 9). Patients that were double positive for anti-PAD3 and anti-PAD4, were characterized by a higher prevalence of ACPA and RF IgM, and a higher disease duration than the anti-PAD double negative patients. When RA patients with anti-PAD3 monospecific antibodies were compared with anti-PAD4 monospecific individuals, a significant difference in disease duration was observed, remarkably lower for anti-PAD3. In the total anti-PAD3 monospecific population, only 28.2% individuals had RA and 20.5% were ACPA positive. In contrast, 83.1% and 71.2% of the anti-PAD4 monospecific patients were diagnosed with RA and ACPA positive, respectively.

Table 9 Characteristics of RA patients by anti-PAD3 and anti-PAD4 status and comparisons between anti-PAD profiles. Significant differences are highlighted in bold.
Note: RF IgM result was missing for 1 RA patient (anti-PAD3 and 4 negative) and ACPA results were missing for 2 RA patients (both anti-PAD3 and 4 negative).

	All RA	Anti-PAD4 Monospecific (P4M)	Anti-PAD3/4 (PXR)	Anti-PAD3 Monospecific (P3M)	Anti-PAD3 and 4 Negative (PN)	*P4M vs PN*	*PXR vs PN*	*P3M vs PN*
n	640	147	77	11	405	*552*	*482*	*416*
Age, mean, SD (years)	59.9±13.2	62.6±13.1	61.4±12.9	59.7±9.8	58.6±13.2	***0.0017***	*0.0932*	*0.7785*
Female gender, n (%)	510 (79.7%)	120 (81.6%)	62 (80.5%)	9 (81.8%)	319 (78.7%)	*0.5508*	*0.8786*	*1.0000*
Weight, mean, SD (Kg)	79.0 ±21.0	79.6±21.6	75.4±21.5	80.0±21.7	79.4±20.7	*0.9341*	*0.1611*	*0.9307*
Caucasian, n (%)	564 (88.1%)	130 (88.4%)	67 (87.0%)	10 (90.9%)	357 (88.1%)	*1.000*	*0.8483*	*1.000*
RA duration, mean, SD (years)	11.7 ±10.9	13.2±10.5	15.5±10.8	5.8±6.4	10.7±10.9	***0.0342***	***0.0011***	*0.1418*
Treatment								
MTX Mono, n (%)	239/486 (49.2%)	52/111 (46.8%)	24/61 (39.3 %)	5/11 (45.5 %)	158/303 (52.1%)	*0.3752*	*0.0916*	*0.7635*
MTX + TNFi, n (%)	247/486 (50.8%)	59/111 (53.2%)	37/61 (60.7%)	6/11 (54.5%)	145/303 (47.9%)			
RBC MTXPG, mean, SD (nmol/L)	38.6±23.7	43.3±25.9	38.2±23.4	44.6±24.7	36.5±22.7	***0.0124***	*0.6132*	*0.2935*
RF seropositivity, n (%)	430/639 (67.3%)	123/147 (83.7%)	69/77 (89.6%)	8/11 (72.7%)	230/404 (56.9%)	***<0.0001***	***<0.0001***	*0.3663*
ACPA seropositivity, n (%)	424/638 (66.5%)	122/147 (83.0%)	61/77 (79.2%)	8/11 (72.7%)	233/403 (57.8%)	***<0.0001***	***0.0003***	*0.3723*

Abbreviations: ACPA: anti-citrullinated protein antibodies; MTX: methotrexate; PAD: protein-arginine deiminase; RA: rheumatoid arthritis; RBC MTXPG: red blood cell methotrexate polyglutamates; RF: Rheumatoid Factor; SD: standard deviation; TNFi: Tumor Necrosis Factor alpha inhibitor.

Anti-PAD3 monospecific patients seemed to less likely have RA or carry ACPA positivity and were characterized by a shorter RA duration. Whether this is due to an association with more aggressive phenotype that leads to a faster diagnosis remains to be investigated and the clinical significance of these antibodies in RA or in other rheumatological disease needs to be further elucidated.

Double positivity for anti-PAD3 and anti-PAD4 (the anti-PAD3/4 XR antibodies) was very specific for RA. This feature, in addition to the presence of these antibodies in 7.5% (16/214) of the RA ACPA negative patients suggests a diagnostic value of this combination. When RA patients with the XR antibodies were compared to anti-PAD negative patients, associations with a higher disease duration and a higher prevalence of ACPA and RF IgM were observed. This is in line with the correlation observed between the levels of these markers and the repeatedly reported association between anti-PAD4 and ACPA [281, 288, 299].

The overlap between anti-PAD4 and ACPA, RF IgM and anti-PAD3 was examined in deeper detail (Figure 16). When analysed in all samples, anti-PAD3 antibodies were present in 32.6% (85/261) of anti-PAD4 positive and in 3.2% (39/1212) of anti-PAD4 negative patients. When comparing the distribution in RA *vs.* controls, significant differences were seen for the double positivity (12.0% *vs.* 1.0%, $p<0.0001$), as well as for the anti-PAD4 monospecific profile (22.8%, 3.6%, $p<0.0001$). The differences for anti-PAD3 monospecific individuals (1.7% *vs.* 3.4%) were not significant ($p=0.0704$). In the RA population, a similar pattern was observed with significant differences between ACPA positive and ACPA negative patients for the double positivity (14.4% *vs.* 7.5%, $p=0.0140$) and anti-PAD4 monospecific (28.7% *vs.* 11.7%, $p<0.0001$), but not significant for anti-PAD3 in the absence of anti-PAD4 (1.9% *vs.* 1.4%, $p=0.7587$).

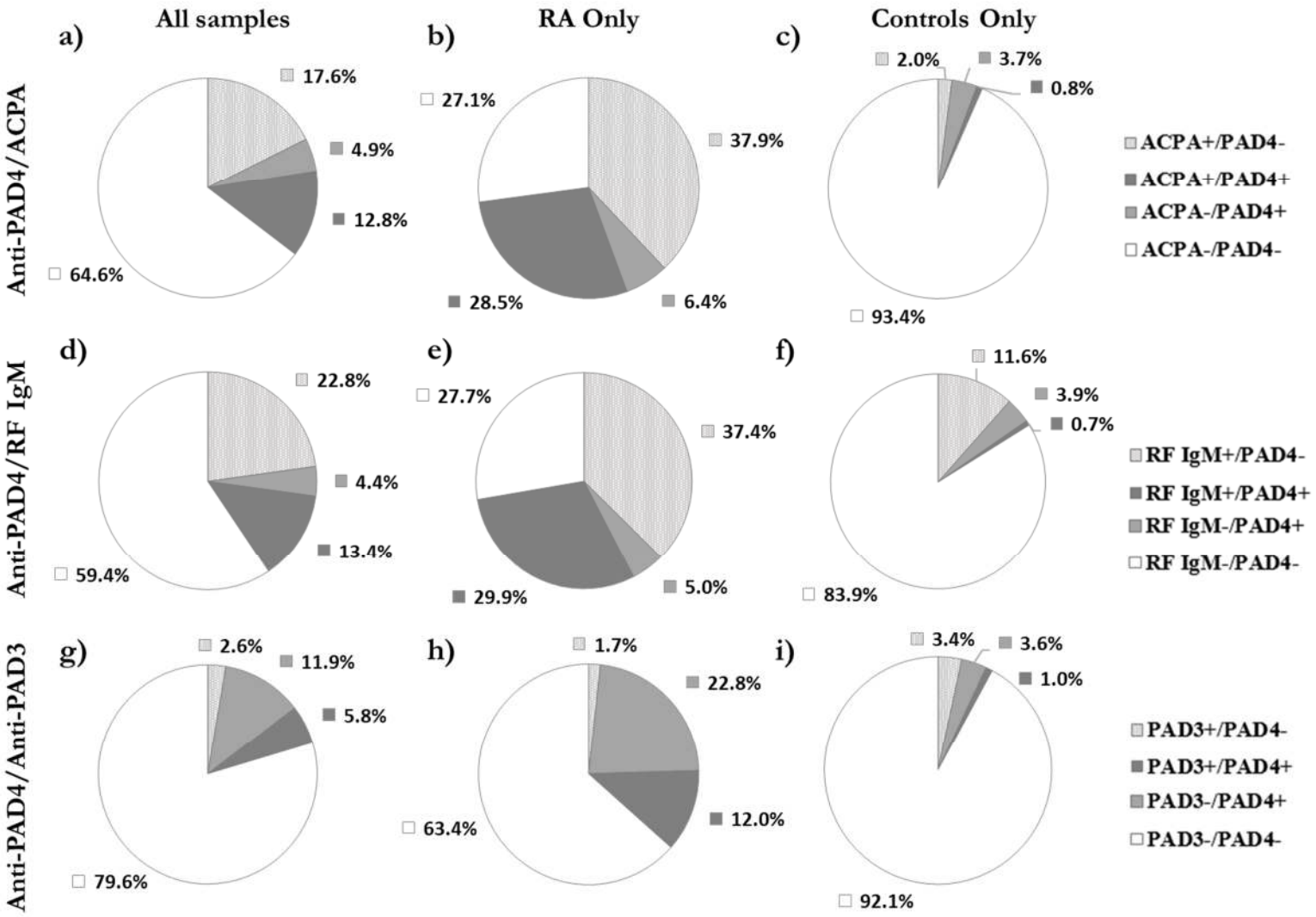

Figure 16 Biomarker profiles in all samples – a), d), g)-, in RA patients –b), e), h)- and in controls –c), f), i). The overlap between ACPA IgG and anti-PAD4 IgG is shown in a) through c), between RF IgM and anti-PAD4 IgG in d) through f), and between anti-PAD3 IgG and anti-PAD4 IgG is represented in g) through i).
Abbreviations: ACPA: anti-citrullinated protein antibodies; PAD: protein-arginine deiminase; RA: rheumatoid arthritis; RF: rheumatoid factor.

Antibody levels and treatment

When compared to the anti-PAD3 and 4 negative patients, anti-PAD4 monospecific antibodies were associated with an older age, a higher disease duration, a higher ACPA and RF IgM prevalence, and interestingly, higher levels of red blood cell (RBC) MTX polyglutamates (MTXPG). The relationship between anti-PAD4 and RBC MTXPG levels is of special interest because associations between MTXPG levels, MTX effects [380, 381], and better clinical response to this drug [382] has been shown. To analyse this in more detail, the levels of RBC MTXPG were compared between different subpopulations of patients (Table 10). A strong association between anti-PAD4 antibodies and higher titers of RBC MTXPG was consistently observed. Significantly higher levels of RBC MTXPG levels were observed in anti-PAD4 positive *vs.* negative patients (p=0.0431), in anti-PAD4 monospecific *vs.* anti-PAD3 and 4 double negative patients (p=0.0124) and *vs.* the non-anti-PAD4 monospecific individuals (p=0.0169). This effect was stronger in the ACPA negative population and in the patients under MTX monotherapy.

Although the same trend was observed in ACPA positive individuals and patients under MTX+TNFi combination therapy, interestingly, the differences in these subpopulations were not significant. Possible explanations for this finding include a potential impact of the TNFi agents on anti-PAD levels and/or the fact that a proportion of the patients under this treatment scheme were non-responders to MTX monotherapy, which was part of the inclusion criteria for one of the studies [383].

Measurement of RBC MTXPG levels is costly and labor intensive [384]. Therefore, if validated, these results would point towards anti-PAD4 as a potential useful tool for monitoring response to MTX, dose adjustment, and for patient stratification based on prediction of response to this widely used treatment agent. Anti-PAD4 could then be used as an aid to understand whether MTX treatment is optimal before making the decision to move towards more aggressive and expensive approaches.

On the other hand, differences in the treatment scheme based on the anti-PAD profiles were also identified. In the case of patients with both anti-PAD3 and anti-PAD4 antibodies, while 39.3% (24/61) were being treated with MTX monotherapy, 60.7% (37/61) were under MTX+TNFi combination therapy. When the autoantibodies levels were compared based on these treatment regimens, significantly higher titres of ACPA, anti-PAD4 and anti-PAD3 antibodies were observed in the group treated with the combination therapy of MTX + TNFi (p<0.0001, p=0.0119, p=0.0146, respectively) compared to the subgroup under MTX monotherapy. These results could indicate an association between positivity for these markers and the use of a more aggressive therapeutic approach, which in general is associated with a more aggressive disease phenotype. Interestingly, the opposite effect was observed with RF IgM, with significantly higher levels in the group under MTX monotherapy (p<0.0001).

In this context, the recent study by Darrah et al. is of special interest. Patients who failed MTX monotherapy and that were escalated into triple DMARD (MTX, sulfasalazine and hydroxychloroquine) or MTX-Etanercept combination therapy, were screened for the presence of anti-PAD4 antibodies. Interestingly, anti-PAD4 positive individuals responded more effectively to treatment escalation, with stronger reduction in disease activity and progression of the joint damage.

Unfortunately, this question could not be investigated in our study, given that information regarding treatment response was not available. Further studies analyzing the potential impact of biological treatment on the anti-PAD antibodies levels, and the utility of these biomarkers in monitoring of response to MTX and in prediction of response to therapy general, will be of great interest.

Table 10 Comparison of RBC MTXPG levels in different subpopulations of patients. Population size mean of each group and *p*-values are shown. *P*-values <0.05 are highlighted in bold.

Groups compared		RA Population	# samples	Mean Group1	Mean Group2	*p*-value
Group1	Group2					
PAD4+	PAD4-	All	434	41.5	36.7	**0.0431**
PAD4+	PAD4-	ACPA+	287	40.8	37.4	0.2340
PAD4+	PAD4-	ACPA-	145	44.5	35.9	0.0656
PAD4+	PAD4-	Patients under MTX	187	45.7	39.1	0.0796
PAD4+	PAD4-	Patients under MTX + TNFi	247	38.5	34.9	0.2271
PAD4+	PAD4-	ACPA- Patients under MTX Mono	69	40.9	34.0	0.2438
PAD4+	PAD4-	ACPA+ Patients under MTX Mono	116	47.3	43.3	0.4053
PAD4+	PAD4-	ACPA- Patients under MTX + TNFi	76	50.4	37.4	0.0886
PAD4+	PAD4-	ACPA+ Patients under MTX + TNFi	171	37.0	33.0	0.2485
ACPA+	ACPA-	All	432	39.0	37.6	0.5675
ACPA+	ACPA-	PAD4 Mono	107	41.9	51.3	0.1793
ACPA+	ACPA-	PAD3 and 4 +	58	38.7	36.1	0.7195
ACPA+	ACPA-	PAD3 Mono	9	49.2	28.5	0.3279
ACPA+	ACPA-	PAD3/4 Double Negative	258	36.8	36.1	0.7892
ACPA+	ACPA-	Patients under Mtx monotherapy	185	45.1	35.8	**0.0133**
ACPA+	ACPA-	Patients under Mtx+TNFi	204	33.8	39.4	0.0967
RF IgM+	RF IgM-	All	433	39.2	37.3	0.4505
PAD3 Mono	PAD3 and 4 -	All	269	44.6	36.5	0.2935
PAD3 Mono	Rest	All	434	44.6	38.4	0.4419
PAD3 and 4 +	PAD3 and 4 -	All	318	38.1	36.5	0.6132
PAD3 and 4 +	Rest	All	434	38.1	38.6	0.8904
PAD4 Mono	PAD3 and 4 -	All	367	43.3	36.5	**0.0124**
PAD4 Mono	Rest	All	434	43.3	37.0	**0.0169**
PAD4 Mono	PAD3 and 4 -	ACPA+	235	41.9	36.8	0.1217
PAD4 Mono	Rest	ACPA+	287	41.9	37.7	0.1790
PAD4 Mono	PAD3 and 4 -	ACPA-	130	51.3	36.1	**0.0122**
PAD4 Mono	Rest	ACPA-	145	51.3	35.9	**0.0091**
PAD4 Mono	PAD3 and 4 -	Patients under MTX mono	163	49.9	38.5	**0.0074**
PAD4 Mono	Rest	Patients under MTX mono	187	49.9	38.7	**0.0062**
PAD4 Mono	PAD3 and 4 -	ACPA- Patients under MTX Mono	60	48.8	34.0	0.0646
PAD4 Mono	Rest	ACPA- Patients under MTX Mono	69	48.8	33.9	**0.0493**

Groups compared		RA Population	# samples	Mean Group1	Mean Group2	*p*-value
Group1	Group2					
PAD4 Mono	PAD3 and 4 -	ACPA+ Patients under MTX Mono	101	50.10	42.34	0.1449
PAD4 Mono	Rest	ACPA+ Patients under MTX Mono	116	50.10	42.51	0.1375
PAD4 Mono	PAD3 and 4 -	Patients under MTX + TNFi	204	38.0	34.9	0.3761
PAD4 Mono	Rest	Patients under MTX + TNFi	247	38.0	35.8	0.5197
MTX Mono	MTX + TNFi	PAD4 Mono	107	49.9	38.0	**0.0174**
MTX Mono	MTX + TNFi	PAD3 Mono	9	62.8	35.5	0.1226
MTX Mono	MTX +TNFi	PAD3 and 4 +	58	36.0	39.3	0.6108
MTX Mono	MTX + TNFi	PAD3 and 4 -	260	38.5	34.9	0.1964
MTX Mono	MTX + TNFi	ACPA+	287	45.1	34.9	**0.0005**
MTX Mono	MTX + TNFi	ACPA-	145	35.8	39.3	0.3501
MTX Mono	MTX + TNFi	ACPA+ PAD4 Mono	91	50.1	35.7	**0.0093**
MTX Mono	MTX + TNFi	ACPA- PAD4 Mono	16	48.8	54.6	0.6189

Abbreviations: ACPA: anti-citrullinated protein antibodies; MTX: methotrexate; PAD: protein-arginine deiminase; RA: rheumatoid arthritis; RBC MTXPG: red blood cell methotrexate polyglutamates, TNFi: Tumor Necrosis Factor alpha inhibitor.

Evolution with disease progression

The levels of anti-PAD3 and anti-PAD4 IgG were analysed in 50 longitudinally followed RA patients. For these individuals, relative fluorescence units (RFU) were calculated dividing the MFI results at each time point of each assay by the respective cut-off. Percent recovery of each time point over the median for each individual was calculated and percent recoveries greater than 30% were considered noteworthy. At baseline, three of these patients were positive for both anti-PAD3 and anti-PAD4, three presented anti-PAD4 only, and two additional individuals showed anti-PAD3 monospecific antibodies. Of the anti-PAD negative individuals at baseline, four were positive for anti-PAD4 in at least one of the other time points measured. Three patients converted from positive to negative for anti-PAD3, but, in all cases, when positive, the results were in the low positive range. In the case of anti-PAD4, a total of seven patients converted from positive to negative or vice versa, with changes in both directions observed within the same individual in two cases. When the percent recovery of each time point over the median for each patient was analysed, a total of 8 and 28 patients reported changes greater than 30% in the anti-PAD3 and anti-PAD4 reactivity, respectively. Thus, fluctuations were observed for both antibodies, with a higher frequency and greater changes for anti-PAD4. Patients with the greatest percent changes for the different time points over the median for each individual and/or with changes in anti-PAD status are illustrated in Figure 17.

In addition to the previously mentioned limiting factor of the unknown effect of treatment on the anti-PAD3 and anti-PAD4 levels, another important question around these biomarkers has remained unaddressed: the timing of appearance of these antibodies and their evolution with disease progression. This represents a limitation of our and most other studies, as no data was available on treatment naïve patients and therefore, baseline levels are unknown. In accordance with the study by Pollman et al. [299] where the levels of anti-PAD4 were studied in RA patients under TNFi therapy over a period of 10 years, in our study, anti-PAD4 positive patients remained positive and some

patients negative for these antibodies became positive later during the disease course. However, in the mentioned study, only results at baseline, where average disease duration for these patients was already 2.5 years [Standard Deviation (SD)±1.2], and after 10 years were reported. Although it was known that the majority of the patients in that cohort were under treatment [281, 385], the exact nature of treatment was not disclosed. The observation the anti-PAD4 antibodies remained stable or went up in these patients might indicate that the treatments used on those patients did not affect levels of anti-PAD4 antibodies.

In a previous study on the same cohort, Halvorsen et al. [287] concluded that anti-TNF therapy did not impact the levels of these antibodies over a 12-month period time. The investigators suggested that this behavior could be indicative of a specific phenotype characterized by inadequate response to this treatment type for suppression of disease activity and prevention of radiographic progression. However, in that study, samples from only two time points with a 12-month interval were measured.

Our longitudinal data on a subset of RA patients under MTX monotherapy with average intervals of 1 month revealed strong fluctuations of anti-PAD4 antibodies over time. Nevertheless, whether these fluctuations correlated with changes in RBC MTXPG levels or are associated with clinical changes or response to treatment could not be analyzed because of unavailability of this information. In addition, although anti-PAD4 antibodies, similarly to ACPA and RF [103, 104, 112], have been described in the pre-clinical phase of RA [299], additional studies are needed to better understand the exact timing of appearance of anti-PAD4 antibodies. The recent study by Arnoux et al. [122] showing the generation of anti-PAD4 antibodies, earlier than ACPA, after immunization of mice with PAD, increases the interest around this topic.

In conclusion, the data analysis from this second phase of the Exagen study provided evidence that, beyond their diagnostic utility, antibodies to PAD4 and PAD3 (cross-reactive and monospecific) represent useful biomarkers for patient stratification in RA. Further studies are required to better understand the clinical phenotypes associated to the different anti-PAD antibodies profiles, the potential utility of anti-PAD4 for prediction of response and monitoring of MTX therapy, as well as to investigate the effect of treatment on these biomarkers, the timing of their appearance and their evolution with disease progression.

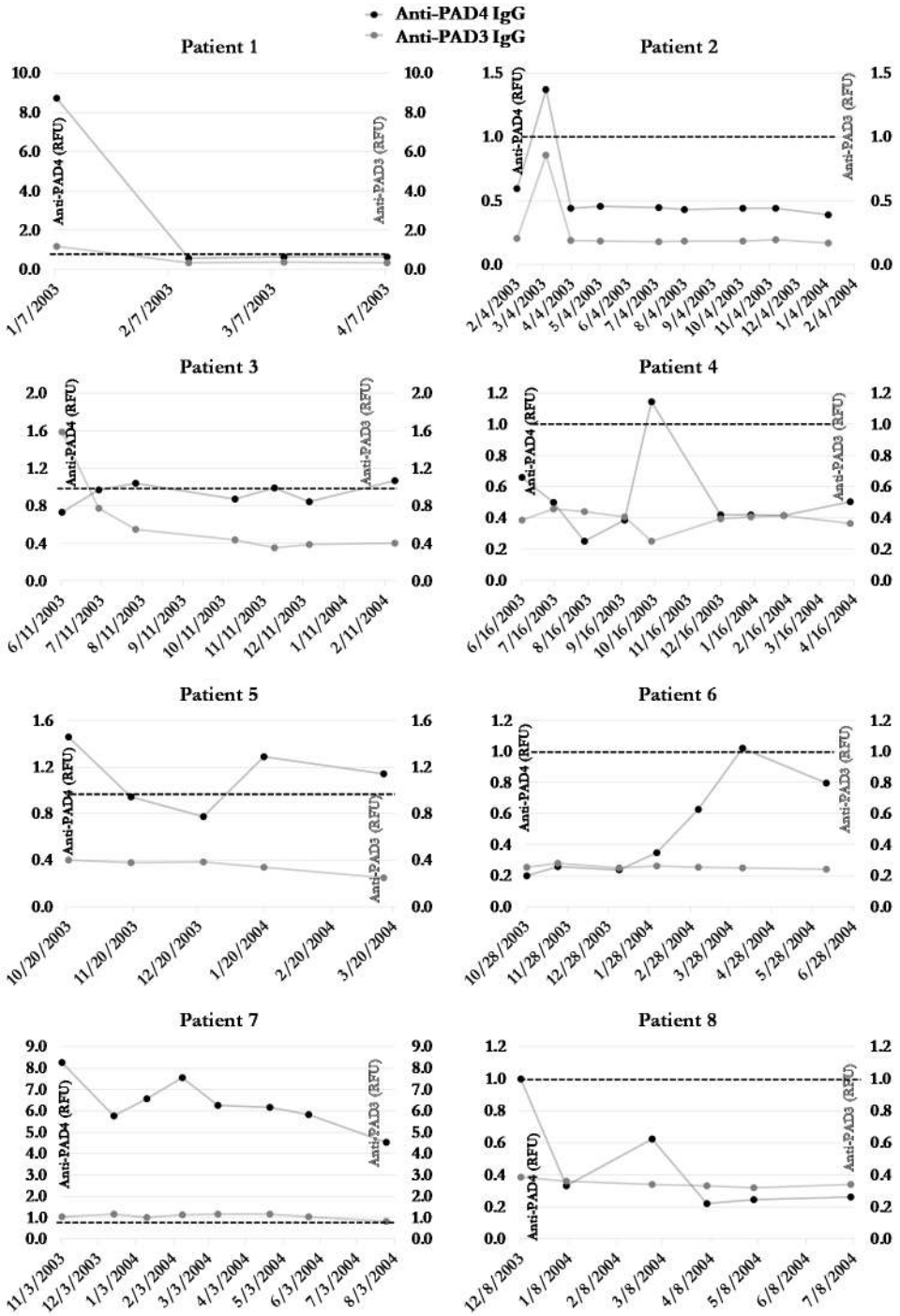

Figure 17 Fluctuation of anti-PAD3 and anti-PAD4 IgG levels over time in eight RA patients under MTX monotherapy. For these individuals, RFU were calculated dividing the MFI results at each time point of each assay by the respective cut-off. Percent recovery of each time point over the median for each individual was calculated and percent recoveries greater than 30% were considered noteworthy. Patients represented in this figure were selected based on qualitative changes in

anti-PAD status or greatest % changes for the different time points over the median for each patient. Dashed line indicates the assays cut-off.
Abbreviations: MFI: median fluorescence intensity; MTX: methotrexate; PAD: protein-arginine deiminase; RA: rheumatoid arthritis, RFU: relative fluorescent unit.

Anti-PAD antibodies and erosive disease

The Swiss RA Study

During previous studies carried out at the Inova Research and Development laboratories, anti-PAD3 IgG had been measured with the CIA assay using both the *E. coli* and insect cells expressed versions of the antigen in the sera of a total of 851 RA patients (Swiss RA study, Table 11). Information on the change in radiographic progression was available for the RA patients included in this study. In these individuals, radiographic damage was assessed with a validated scoring method, the Ratingen (Rau) score. In addition to IgG antibodies to PAD3, RF IgM, RF IgA, anti-CarP and anti-CCP3 (ELISA and CIA) were tested on these samples as well. The outcomes of this study were preliminary reported at the 2017 Dresden Symposium on Autoantibodies as an oral presentation at the Inova sponsored session (abstract not shown), and later as an abstract submitted to the 2019 EULAR Congress and published online (appendix 1).

In this cohort, anti-PAD3 positive patients showed significantly higher mean baseline Ratingen scores compared with anti-PAD3 negative individuals (14.9 *vs.* 8.8 respectively), both in the univariable and multivariable analyses (p=0.0002 and p=0.0200, respectively). In the ACPA negative subgroup (n=308), baseline Ratingen scores were significantly higher in anti-PAD3 positive patients (p=0.0100). These results indicated that the presence of anti-PAD3 antibodies was associated with significantly higher radiographic damage at baseline, in both the overall population and in the ACPA negative subgroup. Interestingly, the two subpopulations based on anti-PAD3 profile showed a similar progression overtime, as opposed to anti-CarP, which presence has been shown to be predictive for a faster radiographic progression [228]. In this scenario, anti-PAD3 IgG would then represent a useful biomarker to more accurately assess joint damage at baseline, and help justify early aggressive treatment, whereas anti-CarP would be useful as a predictive marker of radiographic damage.

Along these lines, the recent study by Darrah et al. showed that antibodies to PAD4 were also associated with worse radiographic joint damage at baseline [296], which might indicate delayed diagnosis and/or a faster progression or more active disease. Remarkably, the anti-PAD4 positive patients responded more effectively to treatment escalation, with stronger reduction in disease activity and progression of the joint damage. This finding would add more weight towards the argument of the use of more aggressive therapeutic approaches earlier in the disease in anti-PAD3 or 4 positive patients. Unfortunately, anti-PAD4 antibodies were not measured in the Swiss RA cohort and therefore, this marker could not be analyzed in this context.

The presence of multiple autoantibodies in the Swiss RA patients was generally associated with higher baseline Rau scores, particularly the combination of RF IgM, RF IgA, CCP3 ELISA, and anti-PAD3, with a baseline Rau score of 16.1 (p<0.0001 compared to those with no autoantibodies). The presence of at least three of the following antibodies, RF IgM, CCP3, anti-CarP IgG or anti-PAD3 IgG, was associated with significantly greater radiographic progression over 10 years than if these autoantibodies were absent (p=0.0300). Combinations of autoantibodies (including anti-CarP and anti-

PAD3) predicted both higher baseline radiographic damage and greater radiographic progression over time, therefore, proving to be informative to understand the prognosis of the disease.

These data have been summarized in a paper entitled "Predictive value of anti-CarP and anti-PAD3 antibodies alone or in combination with RF and ACPA on the severity of rheumatoid arthritis", that is currently under submission (Lammachia C. et al. 2020).

Other studies

Several additional studies were performed with the anti-PAD PMAT assays with the objective to investigate the association between antibodies to the PAD enzymes and severe erosive disease previously reported in literature [61, 201, 281, 287, 294].

One of the key clinical cohorts for this purpose was the one comprised of the RA samples from Dr. Marcos López-Hoyos (MLH study, Table 11). These RA patients had been categorized based on the presence or absence of erosive disease by expert rheumatologists. In these individuals, anti-PAD4 IgG and IgA, but not ACPA or anti-PAD3 IgG, were associated with erosive disease and biological treatment use in RA (appendices 7 and 8), therefore, confirming an association with severe RA. Somewhat surprisingly, the association of anti-PAD3 antibodies with erosive disease could not be reproduced in these samples. Several factors might explain this discrepancy, including sample selection bias or that a number of patients within this group were on biological treatment, and whether therapy could have had an effect on these antibodies remains unknown. Moreover, in the studies that report this association between anti-PAD3 IgG and joint damage, different technologies were employed to measure this biomarker, including CIA in the Swiss RA cohort or in the publication by Seaman et al. [304], IP in the studies by Darrah et al. [201], while the PMAT assay was used in this cohort. Despite the good correlation between the CIA and PMAT platforms, batch differences or other technical variables associated to the different technologies could have had an impact. It could also be hypothesized that with PMAT, the PAD3 antigen could be exposing and potentially favoring different epitopes compared to the other two technologies.

Fifty-four of the RA MLH samples with erosive disease and biological treatment status were then tested in an extended study that included a total of 259 patients, comprising sera from RA patients (n=104) and controls (n=155), from the Inova Biobank. All samples were tested for anti-PAD4 IgG and IgA on PMAT. The cut-off used for anti-PAD4 IgG was the one previously established in the Exagen study [295] and the anti-PAD4 IgA cut-off was established at the 95th percentile of the controls. ACPA IgG was also measured in this subset of RA patients CCP3 CIA. Although anti-PAD4 IgG and IgA levels were correlated (Spearman's rs=0.60, p<0.0001), and a significant portion of the patients expressed both isotypes, individual patients that were positive for only one of the two were identified. Anti-PAD4 IgG and IgA levels were significantly higher in RA patients *vs.* controls (p=0.0004 and p<0.0001, respectively). ROC analysis showed significant discrimination for both IgG and IgA. Anti-PAD4 reported a sensitivity and specificity of 25.0%/94.2% for IgG and 21.2%/94.8% for IgA. Interestingly, significantly higher levels of anti-PAD4 IgG and IgA, but not ACPA were found in the RA patients with erosive disease *vs.* individuals without erosions (p=0.0166, p=0.0176, and p=0.7883, respectively), and in patients under biological treatment *vs.* those that were not on biologics (p=0.0002, p=0.0009, and p=0.7752, respectively).

It would be important to mention that this cohort, however, presented several limitations. Although the patients were categorized based on the presence or absence of joint damage, and the collaborator confirmed that erosive disease had been assessed with different scores, these scores were not available for analysis. Although standardized procedures for assessment of erosive disease are available, the methods and scores to assess joint damage can be highly variable between clinicians and/or hospitals and the choice of scoring method usually depends on the time and staff available, and the required degree of accuracy and sensitivity to change [386]. Furthermore, variability in scoring radiographic abnormalities in RA patients has also been reported [387]. Additionally, the patients included in this study presented diverse disease durations, and baseline or longitudinal samples were not available.

In any event, in this study, we confirmed an association of anti-PAD4 IgG and IgA with erosive RA and biological treatment use, and the consequent link with a more severe disease phenotype. Therefore, in addition to its diagnostic value, these biomarkers represent useful prognostic tools, with potential added value to ACPA in the understanding of the disease course, that could even provide meaningful information when ACPA fails to do so. Historically, RF and ACPA seropositive RA has been known as a more aggressive disease phenotype [11, 12]. However, this might be changing due to a better understanding and management of ACPA positive RA [13]. Consequently, novel markers that help to differentiate disease phenotypes, such as anti-PAD4 IgG and IgA, are important to further improve management of RA. These findings will be summarized in a manuscript that is under preparation.

The association between anti-PAD4 IgG and erosive disease was also confirmed in the RA-ILD cohort from Florence (appendix 9). Lastly, a patent on the association with erosive disease with the title "Compositions and methods for diagnosing and assessing rheumatoid arthritis" was filed in February 2019 (US Patent Application No.: 62/806,607).

Effect of treatment on anti-PAD antibodies

Although the effect of treatment on anti-PAD antibodies could not be studied in a systematic way due to unavailability of appropriate patient samples (drawn before and after treatment initiation and ideally several follow-up time points), some of our studies provided insights into the understanding of a potential impact of therapy on the levels of this biomarkers. Some of these results have already been commented in section 4.2.3. "Additional insights from the Exagen study".

Additionally, in the Benucci study (Table 11) that included patients under several treatment types, a significantly lower prevalence of RF IgG (p=0.0441), anti-CarP (p=0.0009), and anti-PAD4 IgG (p=0.0034) was found for patients under Rituximab treatment, in comparison with the other treatment groups. Remarkably, no patients with this therapy were found to be anti-PAD4 positive. Rituximab targets and depletes B-cells, the antibody producing cells, providing a plausible explanation for the lower levels of autoantibodies. However, it remains unclear if Rituximab treatment might differentially impact the production and secretion of certain autoantibodies (i.e. anti-PAD4 *vs.* RF IgM). Sample selection bias in this cohort that could have somehow enriched the population for Rituximab responders could explain this observation. The results of this study were presented as a poster at the 2018 International Conference in Autoimmunity in Lisbon (appendix 10).

Overall, the substantial differences between cohorts limit greatly the extraction of firm conclusions on this topic. Treatment might influence the levels of antibodies to the PAD enzymes and it might represent an important con-founding factor that should not be overlooked in studies on these biomarkers. This impact might be differential based on the MOA of the different therapies. Whether changes in anti-PAD levels might reflect a particular response profile needs to be systematically investigated. Within this topic, the progress in the development of PAD inhibitors represents a very exciting area of research and it will be very interesting to evaluate the anti-PAD antibodies in association with this novel therapeutic approach

Clinical utility of anti-PAD4 antibodies

In the context of the prognostic value of anti-PAD antibodies in RA, Guderud et al. [297] recently concluded that there was no association between anti-PAD4 antibodies and clinical characteristics in RA and that therefore, these antibodies are not useful clinical biomarkers in RA. In this study, anti-PAD4 were measured in 745 RA patients using a dissociation-enhanced lanthanide fluorescence immunoassay (DELFIA) and 26% were found to be positive. This prevalence, although on the lower end, is consistent with the results stated in the meta-analysis by Ren et al. [300] and our results [295]. Unfortunately, several significant questions and limitations were identified in the study by Guderud et al. and are commented in a letter to the editor entitled **"Clinical Utility of Anti-Protein-Arginine Deiminase (PAD) 4 Antibodies"**, published in the issue of January 2019 of 'The Journal of Rheumatology' (chapter 3.4.).

Although "clinical utility" is a broad term that can refer to many different aspects, including disease prediction and prevention, diagnosis, stratification, prognosis and monitoring, the authors did not specify what they meant by "no current clinical utility in RA". Their conclusion was based on a limited clinical data set comprised of disease activity measures (HAQ and DAS28), swollen and tender joint counts (SJC and TJC), and systemic markers of acute inflammation (CRP and ESR). In the case of anti-PAD4 antibodies, an association with radiographic joint damage has been reproducibly shown in four independent cohorts [388]. In these cohorts, associations of anti-PAD4 antibodies with measures of inflammation and disease activity scores, such as those measured by Guderud et al., were observed. Although the findings of this manuscript were consistent with the published literature regarding the association with disease activity, they failed to investigate the association with joint damage, which is a validated link of these antibodies.

Additionally, Guderud et al. reported the identification of anti-PAD4 antibodies in the absence of ACPA in a 6% of the RA patients, representing 15.8% of the ACPA negative subgroup, in agreement with 2-17.7% PAD4+/ACPA- reported in other RA cohorts, reviewed in [389]. This suggests that, in addition to their value as a prognostic biomarker related to radiographic joint damage, anti-PAD4 antibodies may also be clinically useful for RA diagnosis even within traditionally seronegative individuals.

Several questions around the methods were also raised. The DELFIA method used by Guderud et al. is not a validated technology for the detection of autoantibodies, has not been compared directly to ELISA or immunoprecipitation for the detection of anti-PAD4 antibodies, and does not play a significant role in diagnostic laboratory settings. Furthermore, it is unclear how the

performance of the test was assessed and no information on standardization, calibration or potential batch effects was provided. This point is especially relevant given that the results from two different cohorts tested at different time points were combined. Interestingly, 366/745 patients were from a study by Halvorsen et al. from 2008 [281], which was one of first to determine that anti-PAD4 antibodies are associated with radiographic joint damage.

For all these reasons, we believe that the conclusion that anti-PAD4 antibodies are not clinically useful is not supported by data presented by Guderud et al. and contrasts with a body of published literature [61, 220, 281, 286, 287, 294, 296, 300]. Our data supports the fact that anti-PAD4 antibodies hold clinical promise in the assessment of disease severity in terms of joint damage and represent a useful biomarker for patient stratification and might help to close the serological gap in RA.

All these arguments and the data found in the scientific literature were reviewed in our recently published manuscript entitled **"Autoantibodies to Protein-Arginine Deiminase (PAD) 4 in Rheumatoid Arthritis: Immunological and Clinical Significance, and Potential for Precision Medicine"** [389], part of the October 2019 issue of 'Expert Review of Clinical Immunology' (chapter 3.2.). This review aimed to summarize and analyze the current knowledge, recent findings and growing evidence on anti-PAD4 antibodies in RA and their clinical and immunological significance, and their utility to improve diagnosis, patient stratification, and prognosis.

Anti-PAD2 antibodies are present in the serum of RA patients

Very recently, antibodies of the IgG isotype targeting the PAD2 were described in RA patients for the first time [308]. In that study, a negative association with disease severity and risk of ILD was observed, suggesting that anti-PAD2 might identify a distinct subpopulation of RA patients with milder disease. For these reasons, it has been proposed that the detection of autoantibodies to this member of the human PAD family, together with PAD3 and PAD4, could be very useful for patient stratification.

We generated and evaluated a PMAT assay for the detection of anti-PAD2 antibodies and confirmed its correlation with the method used in the published manuscript [308]. Following the same structure and coupling procedure as for anti-PAD3 and 4 antibodies, an assay for the detection of anti-PAD2 IgG based on the PMAT was designed. Sera from a total of 38 RA patients and 2 apparently HI (JHU samples, Table 11) were tested with this novel immunoassay (PAD2 v2, JHU non-his tagged), in addition to anti-PAD4. The anti-PAD2 IgG status of these samples had previously been characterized by our collaborators at the JHU by ELISA, and the samples had been selected to include 20 positives and 20 negatives. The results of this study were presented as a poster at the 2019 International Congress on Controversies in Rheumatology and Autoimmunity (CORA) (appendix 2).

A high correlation (Spearman's *rs*=0.81) and excellent qualitative agreement (*kappa*=1.00, 95% CI 1.00-1.00) was observed between ELISA and PMAT for the detection of anti-PAD2 antibodies. An overlap of 25.0% (10/40) between anti-PAD2 and anti-PAD4 antibodies was observed (*kappa*=0.20, 95% CI -0.10-0.50). Out of the 40 patients, 10.0 (25%) were anti-PAD2 positive and anti-PAD4 negative. Additionally, 15.0% (6/40) of the samples were anti-PAD4 positive and anti-

PAD2 negative. A total of 35.0% (14/40) of patients were double negative. The correlation between anti-PAD2 and anti-PAD4 was moderately low (Spearman's *rs*=0.37). A total of 50.0% (10/20) anti-PAD2 positive patients were positive for anti-PAD4 antibodies.

In agreement with the study by Darrah et al., we confirmed the presence of anti-PAD2 IgG in RA patients by a second independent method. We demonstrated that anti-PAD2 and anti-PAD4 antibodies can be simultaneously detected in an anti-PAD IgG panel. Nonetheless, a higher overlap between antibodies to PAD2 and 4 was observed in this cohort, in comparison to the data from JHU. Moreover, as it will be outlined in the next subsection, we failed to reproduce the association with a lower risk of ILD in two independent cohorts (Florence RA-ILD and CHU Bichat, Table 11). This, together with the strong differences observed between different PAD2 antigen versions commented in section 4.2.1. "Epitope Mapping", point towards a need to better characterize the PAD2 antigen. This would include the identification and characterization of the epitope(s) on the enzyme, as well as assay optimization to ensure the correct exposure. Additionally, understanding whether there could be several subpopulations of anti-PAD2 antibodies (monospecific vs. cross-reactive with other PAD enzymes) with potentially distinct clinical associations remains an area of interest.

Anti-PAD antibodies and lung involvement

With the objective to study the relationship between anti-PAD3 and anti-PAD4 antibodies and lung involvement in RA, in particular ILD, two independent cohorts that included RA patients with pulmonary manifestations were measured for the presence of anti-PAD antibodies.

The University of Florence RA-ILD Cohort

The first cohort comprised 71 RA patients from the University of Florence, 11 of which (11/71, 15.5%) had ILD (Table 11). IgG antibodies to PAD2, 3 and 4 were quantified in the sera of these individuals. Comparisons between the patients with and without ILD were performed and, contrary to previous publications [302], anti-PAD4 antibodies were found to be significantly lower in patients with ILD (p=0.0430). Although no significant differences were observed between these two subgroups of patients for anti-PAD3 levels (p=0.4177), there was also a trend towards higher levels in the non-ILD subpopulation. Anti-PAD2 IgG were also measured in these patients and when the levels were compared between subgroups (ILD vs non-ILD), no significant associations or trends were identified, with any of the three versions of the PAD2 antigen (data not shown). Therefore, the association between anti-PAD3 and 4 antibodies and ILD, and the negative association of this pulmonary manifestation with anti-PAD2 IgG, could not be reproduced in this cohort. One of the limitations of this study is that the patients were under treatment when the samples were collected.

Associations between anti-PAD4 levels and erosive disease (p=0.0430) and morning stiffness (p=0.0310), and a correlation between anti-PAD3 and anti-PAD4 and DAS28-ESR at the time of sampling (r=0.34, p=0.0040, and r=0.34, p=0.0040, respectively) were observed. Similarly to previous -although controversial- findings [298], we did not observe associations between anti-PAD3 and 4 antibodies and smoking. Anti-PAD3 antibodies were significantly higher in non-smokers (p=0.0042) and anti-PAD4 showed a trend towards higher titers in non-smokers but it was not significant (p=0.2202).

In conclusion, in this cohort, IgG to PAD4 were correlated with joint erosions and disease activity, whereas a negative association with ILD was found. Smoking history was not associated with the presence and levels of anti-PAD IgG. Further studies that consider relevant potential confounders -like therapy- and larger RA ILD cohorts are needed. The outcomes of this study were presented as a poster at the 2019 EULAR congress (appendix 9). Next steps with this cohort will include additional testing with the IgA isotype.

The CHU Bichat Cohort

The second cohort analyzed to study the reported associations between anti-PAD antibodies and pulmonary manifestations in RA comprised sera from individuals with idiopathic pulmonary fibrosis (IPF) (n=75), RA with the presence of ILD (n=75) and RA without ILD (n=75). These samples were measured for the presence of anti-PAD2 (the three versions), anti-PAD3 and anti-PAD4 IgG. In short, although ANOVA analyses showed trends towards differences in the levels of these antibodies between the three subpopulations, no significant differences were observed in pairwise comparisons for any of the biomarkers. Only the diagnosis was available for these patients, therefore, the limited clinical information available difficulted the interpretation of these results.

In summary, the previously reported association between the anti-PAD3/4 IgG XR antibodies and a higher risk of ILD [302] and the link between anti-PAD2 IgG and a lower risk of ILD could not be reproduced in our hands in two independent cohorts. Further evaluation of these and previous findings around this topic and additional studies are required.

Antibodies in Pre-RA

In 2015, a collaboration with Dr. Axel Finckh and his team was established with the objective to study the RA biomarkers profiles, their timing of appearance and evolution with disease progression, and to identify markers that can help predict the disease and define the transition from the "at-risk" healthy stage, to pre-RA and to full blown disease. The hope is that this, together with more targeted treatment approaches, will allow for prediction of the disease, earlier diagnosis and initiation of therapeutic strategies and will eventually enable the prevention of RA.

In this study, sera from FDR of RA patients ("at-risk" healthy stage) are being collected, including longitudinal timepoints for ACPA positive individuals (Table 11). The samples are periodically sent to Inova for screening of anti-CCP3 IgG and later testing of additional markers. An initial screening of several biomarkers, including the anti-PAD2, 3 and 4 IgG antibodies was performed on a selection of samples. The collection of samples is currently ongoing and further testing will continue to be performed.

4.3. Summary of studies, cohorts and associated clinical findings

Table 11 Summary of studies, clinical cohorts and collaborations, cohort composition and clinical information available, biomarkers measured on these samples, main findings, and associated scientific publications.

Study name	Collaborators/Origin	Cohort composition	Clinical info. available	Biomarkers measured	Main findings	Scientific publications
Swiss RA	Dr. Cem Gabay, Dr. Michael Nissen, Dr. Celine Lamacchia. University Hospitals of Geneva (Geneva, Switzerland)	968 established RA and 687 disease controls (450 AxS and 237 PsA)	Diagnosis	Anti-CCP3 ELISA and CIA, RF IgM and IgA ELISA and CIA, RF IgG ELISA, anti-PAD3 IgG CIA, circulating calprotectin ELISA	An interval and combinatory model based on RF IgM and ACPA can improve RA diagnosis. Anti-PAD3 IgG are associated with a higher radiographic damage at baseline.	Martinez Prat L. et al. (2018) [362] – Manuscript, Front Immunol. Nissen M. et al. (2019) - Abstract published online, 2019 EULAR congress Lamacchia C. et al (2020) – In submission
Exagen	Dr. Thierry Dervieux, Claudia Ibarra Exagen Diagnostics (Vista, California, US)	640 RA (early and established), 636 other inflammatory diseases and 147 healthy individuals.	Diagnosis, ethnicity, age, gender treatment, disease duration	Anti-CCP2 ELISA, RF IgM and IgA ELISA, anti-PAD3 and anti-PAD4 IgG PMAT	Anti-PAD4 IgG can help close the serological gap in RA, and anti-PAD3 and 4 IgG can improve patient stratification. Anti-PAD3 monospecific antibodies are present in RA and other rheumatological diseases. Anti-PAD3 and 4 fluctuate overtime in patients under MTX monotherapy. Anti-PAD4 monospecific antibodies might be a marker of response to MTX. Combinatory approaches including ACPA, RF and	Martinez-Prat L. et al. (2019) [295] – Manuscript, ARD Dervieux T. et al. (2018) – Oral presentation at the 2018 ACR Annual Meeting Dervieux T. et al. (2019) – Oral presentation at the 2018 ACR Annual Meeting

Study name	Collaborators/Origin	Cohort composition	Clinical info. available	Biomarkers measured	Main findings	Scientific publications
					anti-PAD4 are useful to improve RA diagnosis.	
Rome Seronegative RA	Dr. Barbara Tolusso, Dr. Elisa Gremese Fondazione Policlinico Universitario "A. Gemelli"-IRCCS, (Rome, Italy)	92 early RA patients triple seronegative for ACPA and RF IgM and IgA (41 internal disease controls were added)	Diagnosis, gender, smoking, DAS28, joint erosion, Sharp score, symptom duration	Anti-CCP2 IgG ELISA, RF IgM and IgA ELISA, anti-PAD4 IgG PMAT	Anti-PAD4 IgG can help close the serological gap in RA	Martinez-Prat L. et al. (2019) – Poster at the 14th Dresden Symposium on Autoantibodies
Marcos López-Hoyos (MLH)	Dr. Marcos López-Hoyos Marqués de Valdecilla University Hospital – IDIVAL (Santander, Spain)	53 EORA, 69 YORA, 135 PMR, 21 apparently HI >60 years old	Diagnosis, gender, age, erosive disease, treatment use, disease duration	Anti-CCP3 ELISA and CIA, anti-CarP ELISA, anti-PAD3 IgG CIA, anti-PAD2, 3 and 4 IgG PMAT (and IgA for some of the patients)	Anti-PAD4 IgG are associated with erosive and the use of biological treatment. Anti-PAD4 IgA are present in the serum of RA patients and are also associated with erosive disease and biological treatment use.	Martinez-Prat L. et al. (2019) – Poster at the 2019 EULAR Congress Martinez-Prat L. et al. (2019) – Poster at the 2019 ACR Annual Meeting Martinez Prat L. et al. (2020) – Abstract submitted to the 2020 EULAR Congress Manuscript in preparation
Benucci RA Under Treatment	Dr. Maurixio Benucci Dr. Mariangela Manfredi Dr. Maria Infantino New Hospital "San Giovanni di Dio" (Florence, Italy)	231 RA patients under diverse biological treatments	Diagnosis, treatment, duration of treatment at sample collection and treatment initiation, disease duration, age, gender, ESR, CRP,	CCP3 CIA, RF IgM, IgA, and IgG ELISA, anti-CarP ELISA, anti-PAD3 and 4 PMAT, circulating calprotectin ELISA	Differences in the prevalence of the markers between subpopulations under different treatments. Potential effect of different therapies on autoantibodies levels.	Martinez-Prat L. et al. (2018) – Poster at the 11th ICA

Study name	Collaborators/Origin	Cohort composition	Clinical info. available	Biomarkers measured	Main findings	Scientific publications
			DAS28, TSJ, JES, JNS, smoking, fast vs slow progressors			
Florence RA-ILD	Dr. Boaz Palterer University of Florence (Florence, Italy)	71 RA (11 of them with ILD)	Diagnosis, age, gender, smoking, disease duration, treatment, erosive disease, pulmonary manif., ther	Anti-CCP3 CIA, RF IgM, IgA, IgG ELISA, anti-PAD2, 3 and 4 IgG PMAT	Anti-PAD 4 antibodies were correlated with joint erosions And disease activity, but a negative association with ILD was found. Smoking history was not associated with the presence and levels of anti-PAD antibodies	Palterer B. et al. (2019) – Poster at the 2019 EULAR Congress
CHU Bichat	Dr. Sylvie Chollet-Martin Dr. Philippe D Dr. Bruno Hopitaux Universitaire Nor de Val de Paris (Paris, France)	75 IPF, 75 RA-ILD, 75 RA-non ILD	Diagnosis	Anti-CarP ELISA, anti-PAD2, 3 and 4 IgG and IgA	No significant associations between the ant-PAD antibodies tested and ILD observed	N/A
PAD2 JHU	Dr. Erika Darrah Johns Hopkins University (JHU) (Baltimore, MD, US)	20 anti-PAD2 positives, 20 anti-PAD2 negatives defined by IP with JHU PAD2 antigen (38 RA, 2 HI)	Diagnosis	Anti-PAD2 ELISA historical data from JHU, anti-PAD2 and 4 IgG.	Anti-PAD2 antibodies are present in RA and can be detected with the PMAT, with good correlation between with IP. Strong differences between the PAD2 antigens versions. Higher overlap with anti-PAD4 than previously reported.	Martinez-Prat L. et al. (2019)- Poster at the 5th CORA Congress
Swiss Pre-RA	Dr. Axel Finckh Dr. Celine Lamacchia	RA relatives (recruitment	Blinded	CCP3 ELISA and CIA, other biomarkers	Recruitment and testing ongoing.	Recruitment and testing ongoing,

Study name	Collaborators/Origin	Cohort composition	Clinical info. available	Biomarkers measured	Main findings	Scientific publications
	University Hospitals of Geneva (Geneva, Switzerland)	in progress) (n=TBD)		available for a subgroup of patients. Pending more testing.		

Abbreviations: ACPA: anti-citrullinated protein antibodies; ACR: American College of Rheumatology; AMLI: Association of Medical Laboratory Immunologists; ARD: Annals of Rheumatic Diseases; AxS: ankylosing spondylarthritis; CarP: carbamylated proteins; CCP: cyclic-citrullinated peptide; CIA: chemiluminescence immunoassay; CORA: Controversies in Rheumatology & Autoimmunity; CRP: C-reactive protein; DAS28: disease activity score 28; ELISA: enzyme-linked immunosorbent assay; EORA: elderly onset RA; ESR: erythrocyte sedimentation rate; EULAR: European League Against Rheumatism; EWRR: European Workshop for Rheumatology Research; Front. Immunol.: Frontiers in Immunology; HI: healthy individuals; ICA: International Congress on Autoimmunity; ILD: interstitial lung disease; IRIS: Inova Research and Innovation Summit; JES: joint erosion score; JHU: Johns Hopkins University; JNS: joint narrowing score; PAD: protein-arginine deiminase; PMAT: particle-based multi-analyte technology; PMR: polymyalgia rheumatica; PsA: psoriatic arthritis; RA: rheumatoid arthritis; RF: rheumatoid factor; TBD: to be determined; TSJ: total swollen joint: YORA: young onset RA.

4.4. Novel RA model - Autoantibodies for Precision Medicine in RA

Taking into consideration the current practices, the most recent published literature and the new knowledge generated during this research, here we propose a novel model for improved diagnosis, patient stratification and management, that could help facilitate the implementation of PM strategies. This model is based on several important features that are described below, including a central role of the ACR/EULAR classification criteria scoring system with some proposed modifications, and the consideration of RA risk factors with high level of scientific evidence.

4.4.1. Precision diagnosis

Modified multi-parametric scoring system

The 2010 ACR/EULAR classification criteria were developed by a task force with the goal of designing new guidelines to identify individuals at high risk of chronicity or erosive damage, that served as the basis for initiation of therapy and that would also allow for capturing patients later in the disease course. In three phases, this working group first identified the relevant clinical and laboratory variables that would be part of the scoring system. Then, a panel of expert rheumatologists agreed on the relative weights of the different categories and factors in the probability of developing RA. In the final phase, the group refined the scoring system and determined the optimal cut-off to define RA. The classification criteria were then extensively validated. However, they still present several limitations, such as low sensitivity in seronegative patients or low specificity [390-392]. Furthermore, there has been some concern regarding the choice of reference standard, the inclusion of both tender and swollen joints in the joint evaluation, the selection of the right target population in which the criteria should be used, and the cut-off for positive RA classification [390].

Based on the results obtained with the combinations of RF and ACPA in the Swiss RA study (chapter 3.1.) and the newly described utility of anti-PAD4 IgG to help close the serological gap in RA (chapter 3.3. and section 4.2.3. "Anti-PAD4 antibodies help to close the serological gap in RA"), a modification of this scoring system is proposed (Table 12). The four domains (joint involvement, duration of symptoms, acute phase reactants and serology) would remain intact, and changes in the serology section are proposed. In order to preserve the relative weight of the biomarkers in the total scoring system, the maximum score that could potentially be obtained from this section would be kept at 3 points. With the same purpose, the cut-off point for classification of a patient as having definite RA would remain at a score of ≥6/10.

While in the current model only one result (ACPA *or* RF) is needed, in the new model, both ACPA *and* RF IgM would be required for classification, while anti-PAD4 IgG would be recommended. This suggestion is a result of the proven added value of considering the first two biomarkers, as well as the fact that measuring and reporting both autoantibodies would be feasible and is a current practice in many laboratories. Additionally, although the current classification criteria mention RF IgM, it does not specify the RF isotype that should be considered in the scoring system, probably associated to the fact that until now, measuring of total RF was still a relatively common practice. The scientific literature comparing total RF with the different isotypes is extremely limited [393], nevertheless, the majority of studies in the past 10 years have tested RF isotypes separately and

have repeatedly shown the diagnostic value of this approach, and specifically, of RF IgM [394-397]. The field is evolving towards separate detection of the different isotypes and therefore, the proposed model would specify the use of the RF IgM isotype.

Table 12 Proposed modification of the 2010 ACR/EULAR classification criteria scoring system. The four main domains would be maintained. The proposed changes would affect the serology section that would include a total of ten possible scenarios, based on the results from at least two biomarkers.

A. Joint involvement	**Score**
1 large joint	0
2−10 large joints	1
1−3 small joints (with or without involvement of large joints)	2
4−10 small joints (with or without involvement of large joints)	3
>10 joints (at least 1 small joint)	5

B. Serology

Negative: <Cut-off; low positive: between cut-off and 3×ULN; high positive. >3×ULN; positive (>cut-off)

Current (at least 1 test result is needed for classification)

	Score
Negative RF and negative ACPA	0
Low-positive RF or low-positive ACPA	2
High-positive RF or high-positive ACPA	3

Proposed modification (ACPA AND RF IgM needed for classification; anti-PAD4 IgG recommended)

ACPA IgG	**RF IgM**	***Anti-PAD4 IgG***	**Score**
Negative	Negative	*Negative*	0
Negative	Negative	*Positive*	0.5
Negative	Low positive	-	0.5
Negative	High positive	-	1.0
Low Positive	Negative	-	1.0
Low positive	Low positive	-	1.5
Low positive	High positive	-	2.0
High positive	Negative	-	1.5
High positive	Low positive	-	2.5
High positive	High positive	-	3.0

Abbreviations: ACPA: anti-citrullinated protein antibodies; ACR: American College of Rheumatology; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; EULAR: European League Against Rheumatism; PAD: protein-arginine deiminase; RF: rheumatoid factor; ULN: upper limit of normal.

C. Acute phase reactants (at least 1 test result is needed for classification)

	Score
Normal CRP **and** normal ESR	0
Abnormal CRP **or** abnormal ESR	1

D. Duration of symptoms

	Score
<6 weeks	0
≥6 weeks	1

Based on the ACPA+RF IgM combinatory approaches generated in the Swiss RA study utilizing the cut-offs and the 3 x ULN for each biomarker, a total of nine scenarios would now be contemplated (Figure 18). The new scores assigned to each of them would be assigned based on several considerations:

- The scores in the 2010 classification criteria that would correspond to each scenario
- The OR obtained at the different intervals for the combinations of these two biomarkers on ELISA and CIA in our Swiss RA study (chapter 3.1)
- A higher emphasis of ACPA *vs.* RF IgM
- Increasing weights as the antibody levels rise
- Maximum and minimum scores of 3 and 0 points, respectively
- For simplicity, deltas of 0.5 between the scores are assigned to each quadrant

With these considerations in mind, the maximum of 3 points would be assigned to patients that are double positive for both biomarkers, while double negative individuals would be stratified based on the presence of anti-PAD4 IgG. Triple negative patients would score 0 points. However, given the OR associated to the presence of IgG to the PAD4 in ACPA+RF IgM seronegative individuals in our studies and the high specificity of this marker, a score of 0.5 is proposed for ACPA and RF negative but anti-PAD4 IgG positive patients.

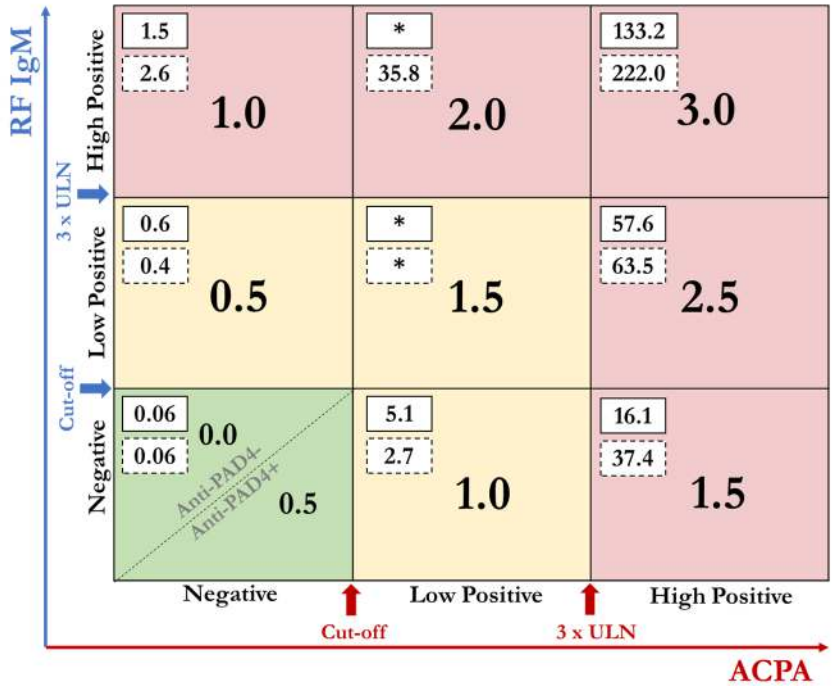

Figure 18 Modified scoring system for the serology domain of the classification criteria for RA. A total of nine scenarios are contemplated based on levels of ACPA (X axis) and RF IgM (Y axis), stratified by the cut-off and the 3 x ULN (indicated with arrows). The OR results for each quadrant obtained by ELISA or CIA in the Swiss RA study (see chapter 3.1.) are shown at the top left corner of each cell. Color of the cell indicates the score based on the 2010 ACR/EULAR classification criteria (green=0, yellow=2, red=3). The numbers in black font in the middle of the cell correspond to the proposed score for each scenario.

Abbreviations: ACPA: anti-citrullinated protein antibodies; ACR: American College of Rheumatology; CIA: chemiluminescence immunoassay; ELISA: enzyme linked immunosorbent assay; EULAR: European League Against Rheumatism; OR: odds ratio; RA: rheumatoid arthritis; RF: rheumatoid factor; ULN: upper limit of normal.

Validation of this new proposed modified scoring system would be required by analyzing its diagnostic performance in several independent cohorts, in comparison with the current criteria. This proposal could represent the first step towards an improvement in the diagnostic performance of the current classification criteria in RF and ACPA seronegative individuals, and potentially, its specificity. Furthermore, it enables better patient stratification based on these biomarkers, which might be helpful for the design and implementation of other precision medicine strategies.

Risk factors

In the new model, the consideration of RA risk factors with high level of scientific evidence (chapter 1, Table 1) is recommended. The proposed parameters to include would be gender, genetic background based on the presence of the HLA-DRB1 "shared epitope" alleles, having FDR with RA and smoking. As of now, the information relative to these parameters, when available, would be considered by the clinician during the initial assessment of the newly presented patients, following his/her own judgment. Whether these should be included as a new section in the classification criteria scoring system, and the relative weight of this domain and of each factor is outside the scope of this thesis. Strategies for prediction of the disease based on these and other risk factors are being developed [21, 398-401] and it will be a matter of time that they are somehow captured in the classification criteria.

4.4.2. Patient stratification; moving towards treat-to target

Once the diagnosis of RA was established by the clinician, the individualized patient biomarker profile could then be used to categorize the individual into a specific subgroup. The management of the different subgroups would then be tailored to their predicted disease phenotypes and expected progression. In this framework, and based on current practices, available literature and the data summarized in this thesis, three subgroups would be defined, with specific actions recommended for each of them:

- ACPA, RF IgM, anti-CarP and anti-PAD3 and 4 seronegative individuals → Standard follow-up with periodic monitoring of potential changes in their serology
- ACPA, RF and/or anti-CarP seropositive individuals → More often follow-up (variable timeframes), given that ACPA and RF are associated with a more severe disease and that anti-CarP predicts a faster radiographic progression
- Individuals that present the anti-PAD3/4 XR IgG or anti-PAD4 IgG or IgA → Early aggressive treatment,, given the association of these biomarkers with erosive and severe disease, especially at baseline (appendices 1, 7, 8, 9), as well as the link of anti-PAD4 with a better response to treatment escalation [201, 296].

This improved stratification of such heterogenous disease such as RA, will enable the implementation of treat-to-target strategies, that will hopefully lead to improved response ratios. We are starting to witnessing how the inclusion of biomarkers in clinical trials is helping reach endpoints and allowing us to learn how to treat RA better [352]. In the past few years, biomarkers, and specifically autoantibodies, have been gaining increasing attention as key elements for precision medicine, as we reviewed in the publication entitled **"The Utilization of Autoantibodies in Approaches to**

Precision Health", published in the 'Frontiers in Immunology' issue of November of 2018 (chapter 3.5.) [402]. The hope is that this situation will continue to evolve in a relatively short period of time, and that these strategies will be implemented and adopted in clinical practice and will have a direct positive impact in patients' lives.

4.4.3. Novel multi-parametric model for the improved diagnosis and stratification of RA patients

The proposed novel multi-parametric model for the improved diagnosis and stratification of RA patients is shown in Figure 19. In this model, the clinician accompanies and guides the patient throughout his or her journey. The modified ACR/EULAR classification criteria scoring system and the risk factors described in section 4.1.1. represent key elements in the establishment of the diagnosis. Once this had been done, the autoantibodies profile of each individual can then be used to assign the patient to a specific subgroup for which specific recommendations in terms of follow-up frequency and treatment are proposed. The hope is that this biomarker profiling will allow for more personalized therapeutic strategies as well as for the implementation of treat-to-target approaches.

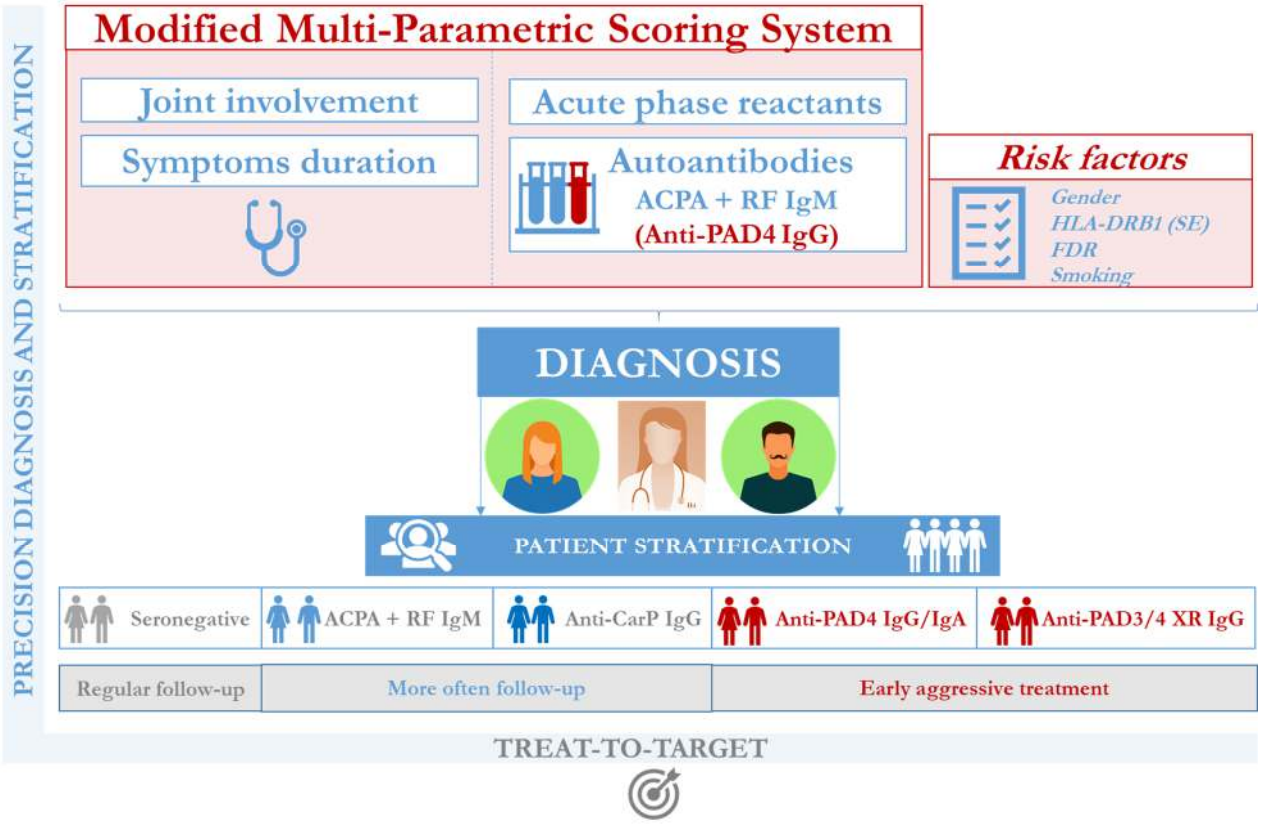

Figure 19 Novel proposed RA model in which precision diagnostics and patient stratification represent the main two features, for improvement of the diagnosis and management of RA, as well as the development of treat-to-target strategies. The main changes are the proposed modifications to the 2010 ACR/EULAR multi-parametric scoring system and the recommendation to consider risk factors with high level of scientific evidence in the clinician's assessment of new patients. **Abbreviations:** ACPA: anti-citrullinated protein antibodies; CarP: carbamylated proteins; FDR: first-degree relatives; HLA: human leukocyte antigen; PAD: protein-arginine deiminase; RF: rheumatoid factor; SE: shared epitope.

Chapter 5. Conclusions and future perspectives

In this section, the key messages and main conclusions from this thesis are summarized.

First, the evaluation and comparison of the clinical performance of several RF and ACPA immunoassays in the Swiss RA study showed an overall better diagnostic performance of CIA *vs.* the ELISA assays, as well as of CCP3 over CCP2. We also confirmed that the specificity and predictive value of the RF test is increased by the detection of all three isotypes and validated the different weights of RF IgM and ACPA based on biomarker levels that are contemplated in the classification criteria. Our results showed significant differences between the OR for the diagnosis of RA of these two biomarkers, suggesting that a higher emphasis should be assigned to ACPA. We also demonstrated that a combinatory approach based on ACPA and RF IgM represents a promising tool to improve the diagnosis of RA and can help correctly classify a higher number of patients.

Beyond the RA classification criteria biomarkers, the study of the PAD enzymes as antigenic targets lead to the identification of several linear epitopes in all five proteins and to the hypothesis that there might be epitopes unique to PAD4 or to PAD3, in addition to the previously reported PAD3/4 XR epitope [201]. We have also observed that the anti-PAD4 response uses at least three isotypes (IgG, IgA and IgM) and a broad spectrum of IgG subclasses in RA. Moreover, PAD1 and PAD6 have been discovered as autoantigenic targets in RA, which represents a completely novel finding.

The feasibility, optimization and early development of the immunoassays for anti-PAD antibodies detection in a panel format enabled the testing of several clinical cohorts for the measurement of these biomarkers. Different clinical information and parameters were available for some of this cohorts, which allowed for the interrogation of various questions, with the goal of better understanding the clinical significance of these antibodies.

We validated in two independent cohorts that anti-PAD4 IgG can help close the serological gap in RA and found an association of anti-PAD4 IgG and IgA with erosive disease and biological treatment use, corroborating a link of these antibodies with a more severe disease phenotype. The association of anti-PAD3 with higher baseline radiographic damage was confirmed in a large cohort of RA patients, and anti-PAD3 monospecific antibodies have been identified and reported for the first time. Additionally, we observed that anti-PAD3 and 4 levels can strongly fluctuate with disease progression and might be impacted by treatment, and that monospecific antibodies to PAD4 might be helpful to monitor response to Mtx monotherapy. The association of the cross-reactive antibodies with ILD described in literature [302] could not be reproduced in our hands in two independent cohorts.

Altogether, our data validate the clinical utility of anti-PAD4 IgG to improve diagnosis and indicate anti-PAD3 IgG and anti-PAD4 IgG and IgA are useful biomarkers for RA patient stratification, especially in the assessment of disease severity. Understanding the anti-PAD3/4 profile of each individual patient might provide meaningful information with utility in clinical practice.

As of anti-PAD2, we have proved that these antibodies can be detected by PMAT and that this technology is comparable to IP, and have confirmed they are present in the sera of RA patients in several independent cohorts. Nevertheless, the previously reported clinical associations of anti-

PAD2 (milder disease, lower risk of ILD) [308] and the minimum overlap between anti-PAD2 and anti-PAD3 and 4, could not be reproduced in our hands. These outcomes highlight the importance of further characterizing and understanding this protein as antigenic target in RA.

Based on our findings, a new model for the improvement of RA diagnosis and patient stratification and management has been proposed. In this model, the risk factors with the highest levels of scientific evidence and a modified scoring system based on the 2010 ACR/EULAR classification criteria have a central role. The main changes proposed include: (1) the requirement of testing for both RF and ACPA, and the recommendation for anti-PAD4 IgG testing; (2) the specification of the RF isotype to be tested (IgM); (3) a higher emphasis of ACPA over RF IgM; and (4) the contemplation of nine different scenarios (over the three current ones) based on antibodies levels and a combinatory approach. The model also includes the definition of patient subgroups based on expected disease phenotype according to the mentioned biomarkers, with specific disease management recommendations for each of them.

Although this research might have answered some questions and contributed to a better understanding of the mentioned RA biomarkers, their clinical utility, and the anti-PAD response, it has also led to additional questions and has opened new research avenues.

Regarding the classification criteria biomarkers, understanding whether the delta in the score based on RF and ACPA antibodies levels is enough remains to be further investigated. We proved that a simple algorithmic approach can be helpful in RA diagnosis, but the development and validation of more sophisticated models, including the utilization of artificial intelligence and additional biomarkers, will more likely represent a great advantage in this area.

Regarding the PAD enzymes as autoantigenic targets, future work might include deeper characterization of the PAD2 antigen and the impact of its biochemical features on its immune recognition, the investigation of the PADs candidate linear epitopes and their potential correlation with specific clinical characteristics, and the assessment of the citrullination status of the PAD antigens. This last point might be important to better understand the anti-PAD immune response, especially the potential relevance of the peptide backbones *vs.* the citrullinated motifs in the PAD enzymes in their recognition by the autoantibodies that target them.

The full development of the PMAT anti-PAD panel, including several isotypes, will allow for additional investigation of the clinical relevance of these biomarkers and of the anti-PAD response in RA. Further studying anti-PAD1 and 6 and the isotype and subclasses usage of all anti-PAD antibodies might provide additional meaningful information on the anti-PAD response in RA. Understanding whether these novel biomarkers provide an added value over the other RA markers will be important.

Some other areas of interest might include the timing of appearance of the anti-PAD antibodies and isotypes in the RA immune response, with special relevance in pre-clinical disease and in RA prediction, the impact of treatment on antibody levels and their potential to predict response to therapy. Especially exciting will be to evaluate these biomarkers in the context of novel treatment approaches, such as the PAD inhibitors.

With regards to the proposed model for RA diagnosis and management of patients, testing and validation in several independent cohorts, as well as a direct comparison of its diagnostic performance with the current approach, will be essential.

Precision Medicine is already a reality in many fields, mainly in oncology. Now it's the turn for RA, the autoimmune diseases and rheumatology. Many groups have already taken the first steps and are currently actively working in this direction. The hope is that this research has contributed to the advancement of precision medicine in RA, that it has opened new research avenues towards this goal, and that it enables the implementation of new strategies that will translate into a better care of RA patients.

Appendices

Appendix 1: Abstract (online publication), Biomarkers for Radiographic Damage, Annual European Congress of Rheumatology (EULAR) (Madrid, 2019)

THE PREDICTIVE VALUE OF RHEUMATOID FACTOR, ANTI-CITRULLINATED PROTEIN ANTIBODIES, ANTI-CARBAMYLATED PROTEIN ANTIBODIES AND ANTI-PEPTIDYL ARGININE DEIMINASE TYPE-3 ANTIBODIES, ALONE OR IN COMBINATION, ON RADIOGRAPHIC DAMAGE IN RHEUMATOID ARTHRITIS

(Accepted for online publication)

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Background
Autoantibodies such as anti-citrullinated protein antibodies (ACPA), anti-carbamylated protein antibodies (CarP) and anti-peptidyl arginine deiminase 4 (PAD4) antibodies have been associated with disease severity and radiographic progression in rheumatoid arthritis (RA). However, very little is known about the anti-PAD 3 (PAD3) antibodies and of the added value of combining multiple autoantibodies to predict radiographic damage.

Objectives
To investigate the capability of rheumatoid factor (RF), ACPA, anti-CarP and anti-PAD3 antibodies to predict radiographic damage in RA, both individually and in combination.

Methods
We performed a nested cohort study within the « Swiss Clinical Quality Management » (SCQM) RA registry. Biobank samples were tested for RF [QUANTA Lite (QL), IgM and IgA], ACPA IgG [QL CCP3 IgG and QUANTA Flash (QF) CCP3IgG], anti-CarP IgG ([using carbamylated fetal calf serum as antigen by prototype ELISA, (research use only (RUO)))] and anti-PAD3 IgG antibodies [QF PAD3 insect, RUO] (all methods Inova Diagnostics). Outcome: radiographic damage assessed with a validated scoring method, the Ratingen (Rau) score. We examined the association of each autoantibody both separately and combined, with radiographic damage at baseline and over time with linear mixed-effects models. Multivariable analyses were corrected for age, sex, smoking status, disease duration, disease activity (DAS28), number of prior biologics and calendar year of biosampling.

Results
A total of 851 RA patients were included with a median of 4 Ratingen scores per patient. Autoantibodies were positive in the following proportion of patients: RF IgM 66.3%, RF IgA 56.9%, QL anti-CCP3 63.8%, QF anti-CCP3 63.3%, anti-PAD3 10.7% and anti-CarP 22.4%. Significantly

higher baseline Ratingen scores were associated with the presence of RF (IgM and IgA) and anti-CCP3 (QL and QF) and greater progression over time with RF IgM and QL CCP3 IgG (p=0.01 and p=0.04 respectively). Patients positive for anti-PAD3 demonstrated higher mean baseline Ratingen scores compared with anti-PAD3 negative patients (14.90 and vs. 8.75 8 respectively) which was significant in both univariable (Figure) and multivariable analyses (p=0.0002 and p=0.02 respectively). In the QL CCP3 negative subgroup (n= 308), baseline Ratingen scores were significantly higher in anti-PAD3 positive patients (p=0.01).There were no significant differences with regards to anti-CarP, either in the whole population or in the seronegative cohorts. The presence of multiple autoantibodies was generally associated with higher baseline Ratingen scores, particularly the combination of RF IgM, RF IgA, QL CCP3, and anti-PAD3, with a baseline Ratingen score of 16.12 (p<0.00001 compared to those with no autoantibodies). The presence of at least 3 of the following autoantibodies: RF IgM, QL CCP3, anti-CarP and anti-PAD3, was associated with significantly greater radiographic progression over 10 years (Figure) than if these autoantibodies were absent (p=0.03).

Conclusions

The presence of anti-PAD3 antibodies was associated with significantly higher scores of radiographic damage at baseline, in both the overall population and in the subgroup of ACPA-negative patients. Combinations of autoantibodies (including anti-CarP and anti-PAD3) predicted both higher baseline radiographic damage and greater radiographic progression over time.

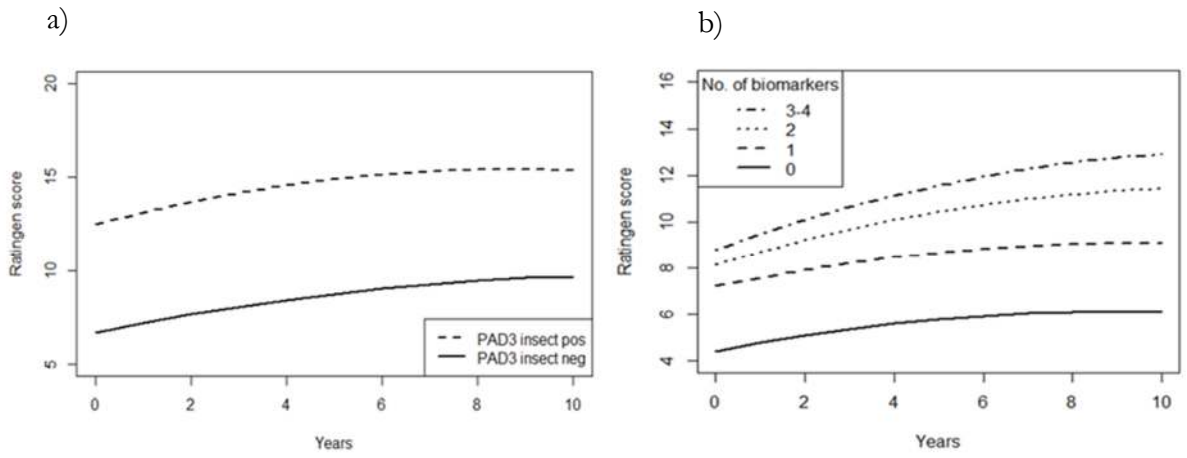

Figure 1 Radiographic damage at baseline and progression over time based on a) the presence of anti-PAD3 antibodies and b) the number of autoantibodies present (RF IgM, QL CCP3 IgG, anti-CarP and anti-PAD3). Number of patients per biomarker subgroup – 0: 177, 1: 161, 2: 335 and 3-4: 178.

Appendix 2: Poster, Anti-PAD2 antibodies, 5th International Congress on Controversies in Rheumatology & Autoimmunity (CORA) (Florence, 2019)

AUTOANTIBODIES TO PROTEIN-ARGININE DEIMINASE 2 AND 4 ARE PRESENT IN THE SERA OF RHEUMATOID ARTHRITIS PATIENTS AND CAN BE SIMULTANEOUSLY DETECTED WITH A NOVEL PARTICLE-BASED MULTI-ANALYTE TECHNOLOGY

Laura Martinez-Prat[1], Erika Darrah[2], Roger Albesa[1], Chelsea Bentow[1], Felipe Andrade[2], Michael Mahler[1]

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5th International CORA 2019 Autoimmunity Congress
March 14th-16th 2019, Florence, Italy

KEY MESSAGES

- Anti-PAD2 and anti-PAD4 antibodies can be simultaneously detected using a PMAT
- Good correlation between ELISA and the PMAT for the detection of anti-PAD2 antibodies was observed

INTRODUCTION

Novel biomarkers have been described in rheumatoid arthritis (RA) patients, including autoantibodies to the protein-arginine deiminase (PAD) enzymes. Anti-PAD4 antibodies were recently reported to help close the serological gap[1] and are associated with anti-citrullinated protein antibodies (ACPA) and more severe disease[2,3]. In contrast, anti-PAD2 antibodies do not associate with the shared-epitope (SE) or ACPA and may characterize a different subpopulation of RA patients with less risk of interstitial lung disease (ILD) and radiographic progression[4]. We aimed to evaluate a novel particle-based multi-analyte technology (PMAT) for the simultaneous detection of anti-PAD2 and anti-PAD4 antibodies.

METHODS

Sera from a total of 38 RA patients and 2 apparently healthy controls were included in this study of which 20 were positive and 20 negative for anti-PAD2 antibodies by ELISA (data from Johns Hopkins University). All samples were tested for the presence of anti-PAD2 and anti-PAD4 IgG using the PMAT. Results are expressed in median fluorescence intensity (MFI). Prevalence was calculated based on preliminary cut-offs values. Spearman correlation analysis was performed and $p<0.05$ was considered significant.

RESULTS

A high correlation [Spearman's *rho* = 0.81 (95%CI 0.66-0.90)] (Figure 1a) was observed between ELISA and PMAT for the detection of anti-PAD2 antibodies. All 20 anti-PAD2 positive patients defined by ELISA tested positive on PMAT resulting in a qualitative agreement of 100% between the two platforms (*kappa* = 1.00, 95%CI 1.00-1.00) (Figure 1).

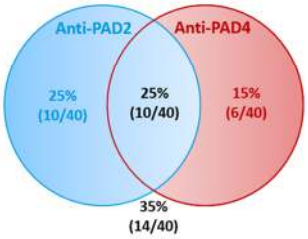

Figure 1 Venn Diagram representing prevalence and overlap of anti-PAD2 and anti-PAD4 antibodies quantified by particle-based multi-analyte technology (PMAT).

An overlap of 25% (10/40) between anti-PAD2 and anti-PAD4 antibodies was observed (*kappa*= 0.20, 95%CI -0.10-0.50). Out of the 40 patients, 10 (25%) were anti-PAD2 positive but anti-PAD4 negative. Additionally, 6/40 (15%) samples were anti-PAD4 positive and anti-PAD2 negative. A total of 14/40 (55%) of patients were double negative. The correlation between anti-PAD2 and anti-PAD4 was moderately low (Spearman's *rho* = 0.37) (Figure 2b). Lastly 10/20 (50%) anti-PAD2 positive patients were positive for anti-PAD4 antibodies.

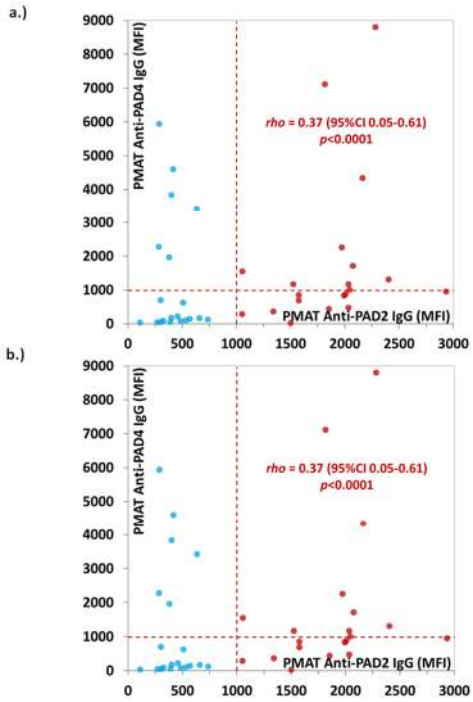

Figure 2 Spearman's quantitative correlation between (a) anti-PAD2 particle-based multi-analyte technology (PMAT) *vs.* ELISA and (b) PMAT anti-PAD4 *vs.* PMAT anti-PAD2 antibodies labeled based on anti-PAD2 ELISA qualitative status.

CONCLUSION

Anti-PAD2 and anti-PAD4 antibodies can be simultaneously detected using the novel PMAT. A good correlation was found between ELISA and PMAT for anti-PAD2 antibody detection. Further studies are needed to evaluate the clinical significance of these markers.

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Appendix 3: Abstract, Deciphering the anti-PAD response, Annual European Congress of Rheumatology (EULAR) (Frankfurt, 2020)

DECIPHERING THE ANTI-PROTEIN-ARGININE DEIMINASE (PAD) REPONSE IDENTIFIES PAD1 AND PAD6 AS NOVEL AUTOANTIGENS IN RHEUMATOID ARTHRITIS

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Background
Protein-arginine deiminase (PAD) 4 enzymes play a central role in the pathogenesis of rheumatoid arthritis (RA) and represents an antigenic target. Among the five known family members (PAD1, PAD2, PAD3, PAD4 and PAD6), only PAD2, PAD3 and PAD4 have been described to have autoantigenic properties. Furthermore, very little is known on the isotype usage of these autoantibodies. Understanding the molecular basis of the anti-PAD antibody response has the potential to open novel approaches for precision medicine in RA.

Objectives
The objectives of this study were to screen for the presence of antibodies to the five PAD family members and to evaluate the isotype usage of the anti-PAD4 response in RA.

Methods
First, we developed a panel for the detection of anti-PAD IgG based on a particle-based multi-analyte technology (PMAT), that utilized paramagnetic particles coupled with the different human recombinant PAD proteins (PAD1, PAD2, PAD3, PAD4 and PAD6) and anti-human IgG conjugate. This panel was used to test sera from RA patients (n=33) and non-RA controls (n=36). The controls were comprised of apparently healthy individuals (n=10), and patients with infectious diseases (n=10), systemic lupus erythematosus (n=7), systemic sclerosis (n=9) and Sjogren's syndrome (n=1). Next, the PAD4-coupled beads were tested with anti-human IgM, IgA and IgG conjugates on an extended cohort of RA patients (n=62) and the same non-RA controls.

Results
All five anti-PAD IgG (Figure 1) demonstrated the ability to discriminate between RA patients and controls. At greater than 90% specificity, anti-PAD4 IgG, followed by anti-PAD3 IgG, showed the best diagnostic performance. Significantly higher levels of the five antibodies were observed in RA *vs.* controls (p-values of 0.0041, <0.0001, 0.0014, 0.0039, and 0.0140 for anti-PAD1, 2, 3, 4 and 6, respectively). Significant correlation was observed between all the antibodies, with the highest between anti-PAD1 and anti-PAD4 (Spearman´s *rho*=0.87, p<0.0001) and the lowest between anti-PAD4 and anti-PAD2 (Spearman's *rho*=0.38, p=0.0015) and anti-PAD4 and anti-PAD6 (Spearman's *rho*=0.38, p=0.0011). While principal component analysis (PCA) (Figure 2) showed an association between all anti-PAD antibodies, there was further discrimination that displayed closer association between anti-

PAD1, 3 and 4 on one hand, and between anti-PAD2 and 6. For the extended testing of anti-PAD4 with IgG, IgA and IgM, all three isotypes were identified in the sera of RA patients. Higher levels of the three isotypes were observed in RA patients with erosive disease when compared with the patients without erosion, but this association was only significant for anti-PAD4 IgA (p=0.0086).

Conclusion

Our study is the first to describe PAD1 and PAD6 as novel antigenic targets in RA and to demonstrate that the anti-PAD4 B-cell immune response uses all three isotypes (IgG, IgA and IgM). The strong and significant association between anti-PAD4 IgA and joint erosion is of particular clinical relevance.

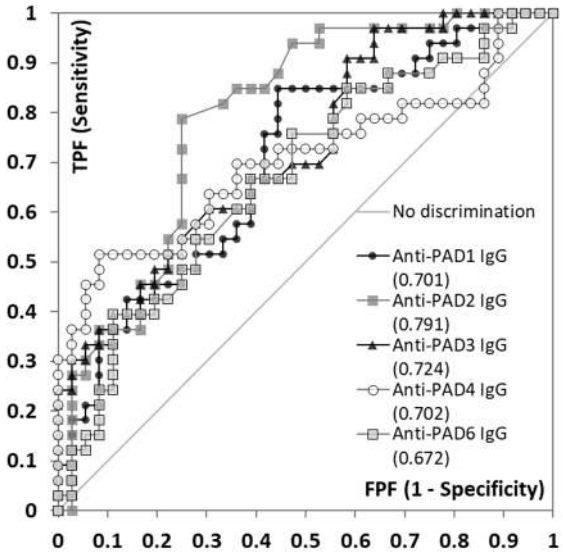

Figure 1 Receiver operating characteristics (ROC) analysis of the discrimination between rheumatoid arthritis (RA) and controls of IgG to protein-arginine deiminase (PAD) 1, PAD2, PAD3, PAD4 and PAD6. The area under the curve (AUC) values are shown in brackets for each biomarker.

Abbreviations: TPF: true positive fraction; FPF: false positive fraction

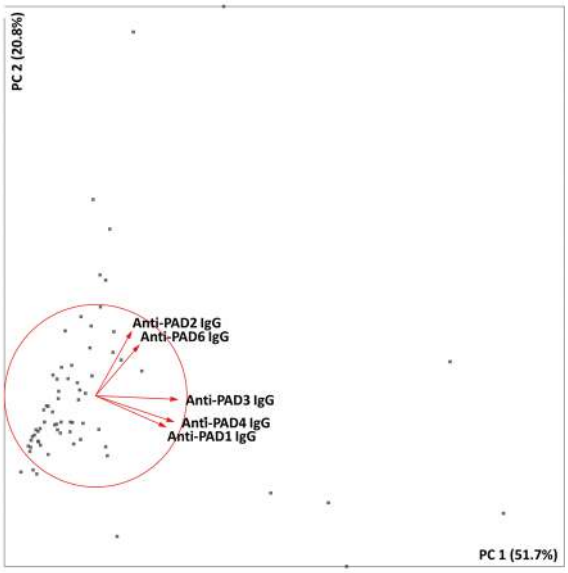

Figure 2 Two dimensional principal component analysis (PCA) plot of the anti-PAD levels in RA patients (n=33) and controls (n=36). Anti-PAD1, 3 and 4 have the main contribution to PC1, which explains 51.7% of the variance, and anti-PAD2 and 6 to PC2, that represents 20.8% of it.

Abbreviations: PC: principal component

Appendix 4: Oral presentation (abstract), Exagen Combinations Biomarkers, ACR/ARHP Annual Meeting (Chicago, US, 2018)

AN ASSAY PANEL COMBINING ANTI-PROTEIN ARGININE DEIMINASE 4 WITH RHEUMATOID FACTOR ISOTYPES DISTINGUISHES ANTI-CITRULLINATED PEPTIDE ANTIBODY NEGATIVE RHEUMATOID ARTHRITIS

Thierry Dervieux[1], Laura Martinez Prat[2], John Conklin[1], Claudia Ibarra[1], Michael Mahler[2], Michael Weinblatt[3], and Joel Kremer[4]

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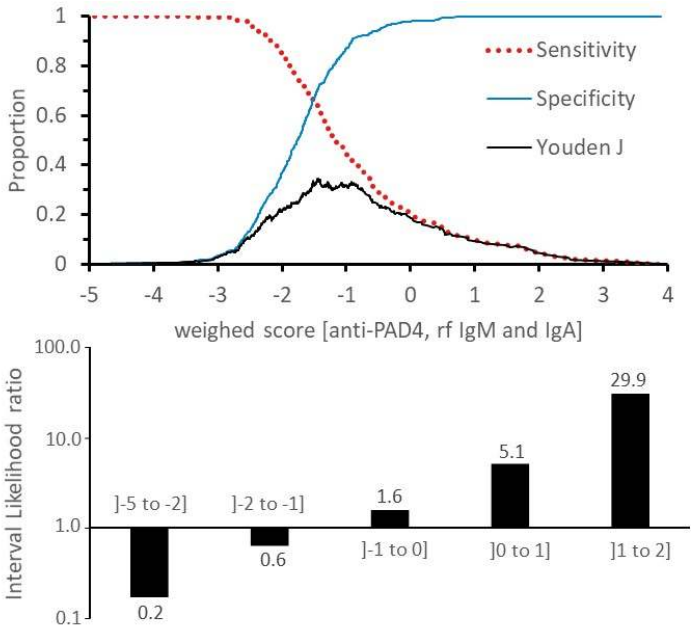
Background/Purpose: Anti-citrullinated peptide antibodies (ACPAs) are highly specific for rheumatoid arthritis (RA) but lack sensitivity. We evaluated autoantibodies to protein-arginine deiminase 4 (anti-PAD4) in distinguishing ACPA negative RA from other ACPA negative rheumatic diseases, either alone or in combination with rheumatoid factor (RF) isotypes.

Methods: ACPA negative subjects (n=1011, 205 RA and 806 non-RA [193 normal healthy individuals, NHV]) were selected from a cohort of 622 RA (34% ACPA negative, all fulfilling the 1987 or 2010 ACR criteria), and 830 non-RA (97% ACPA negative) consented subjects. ACPA status and IgM/IgA RF titers were measured using fluoroenzyme immunoassays (Thermofisher, Upsala Sweden). Anti-PAD4 titers were measured using a novel particle-based multi-analyte technology (research use only, Inova Diagnostics, San Diego, CA). ACPA negative subjects were grouped into a training set of 554 subjects (157 RA, and 397 non-RA [172 NHV]), and a subsequent independent validation set of 457 consecutive subjects (48 RA and 409 non-RA [21 NHV]). From the training set, a weighted scoring system cumulating log normalized anti-PAD4/RF isotypes titer estimates from multivariate logistic regression was calculated to distinguish ACPA negative RA from ACPA negative non-RA, and was applied to subsequent validation set. Diagnostic performances were estimated using area under the receiver operating curve (AUC), sensitivity and specificity.

Results: Overall, anti-PAD4 (>1000 Units) was 19% sensitive and 95% specific in distinguishing ACPA negative RA from ACPA negative non-RA subjects (AUC=0.65±0.02). AUCs for IgM and IgA RF isotypes were 0.67±0.02 and 0.51±0.02, respectively. From training set, multivariate logistic regression estimates for anti-PAD4 (0.36±0.11), and IgM/IgA RF isotypes (0.66±0.11 and -0.65±0.14, respectively) antibody systems were significantly and independently associated with RA ($p<0.001$; intercept=-2.59±0.66), and weighted cumulative score was 37% sensitive and 90% specific (cut-off>-0.4) (AUC=0.72±0.02, Figure). In subsequent validation set, the training set based weighted cumulative score distinguished ACPA negative RA and ACPA negative non-RA groups with 35% sensitivity and 95% specificity (AUC=0.68±0.02). Specificities in distinguishing ACPA negative RA from other ACPA negative rheumatic diseases was 92% for systemic lupus erythematosus (327/354), 97% for Sjogren's syndrome (59/61), 95% for fibromyalgia (80/84), and 91% for NHV. Positivity rate of the weighted cumulative score was 30% among RA diagnosed for less than 2 years and rose to 50% among RA diagnosed for more than 10 years.

Conclusion: These preliminary data establish the feasibility of combining anti-PAD4 with RF isotypes to distinguish ACPA negative RA from other ACPA negative rheumatic diseases.



Appendix 5: Oral presentation (abstract), Exagen Combinations Biomarkers, ACR/ARHP Annual Meeting (Atlanta, GA, 2019)

ANTIBODY SYSTEMS TARGETING CITRULLINATED, CARBAMYLATED, AND PEPTIDYL ARGININE DEAMINASE AUTOANTIGENS DISTINGUISH RHEUMATOID ARTHRITIS IN COMBINATION WITH RHEUMATOID FACTORS

Thierry Dervieux[1], Joel Kremer[2], John Conklin[1], Kelley Brady[1], Roberta Alexander[1], Tyler O'Malley[3], Jing Shi[1], Claudia Ibarra[1], Michael Mahler[4], Michael Weinblatt[5] and Arthur Weinstein[1]

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[4]Inova Diagnostics, San Diego, CA,
[5]Brigham and Women's Hospital, Boston, MA

Background/Purpose
Novel antibody systems including anti-carbamylated protein antibody (anti-CarP IgG) and anti-peptidyl arginine deiminase antibody (anti-PAD4 IgG) are emerging as independent diagnostic and prognostic biomarkers for rheumatoid arthritis (RA) and may add value to rheumatoid factor (IgM) and anti-citrullinated peptide antibody (ACPA IgG), the hallmark antibodies in RA. We evaluated the diagnostic performance of these markers in combination.

Methods
The cohort consisted of 638 consented subjects with RA (fulfilling the 1987 or 2010 ACR classification criteria, mean age: 59.8±0.5 years [SEM], 80% females) and a control group of 775 subjects (mean age: 44.7±0.5 years, 85% females, including systemic lupus erythematosus [n=369], primary Sjogren's syndrome [n=64], primary fibromyalgia [n=85], other connective tissue diseases [n=63], and a group of normal healthy donors [n=194]). Autoantibodies titers from serum were measured using fluoroenzyme immunoassays (IgM RF and anti-CCP [IgG]; Phadia, Upsala Sweden), ELISA (anti-CarP [IgG], research use only [RUO], Inova Diagnostics, San Diego) and bead-based Aptiva technology (anti-PAD4 [IgG], RUO, Inova Diagnostics) in a clinical laboratory accredited by the College of American Pathologists. For each positive antibody (above each cut-off) a score of 1 was assigned and the cumulative presence of the 4 antibodies was determined [range 0-4]. The ability of the biomarkers to distinguish RA from controls was calculated using sensitivity, specificity and interval likelihood ratio (LR). Predictive value (PPV) was estimated at 10% pre-test probability. Statistics consisted of Mann-Whitney and Chi-square test.

Results
In this cohort anti-CarP (>20 Units) yielded 33.5% sensitivity and 77.9% specificity. Anti-PAD4 (>1000 Units) yielded 35.0% sensitivity and 95.0% specificity. RF IgM (>5 Units/ml) and anti-CCP (>10 Units/ml) were 67.4% and 66.5% sensitive, respectively (87.5% and 97.0% specific, respectively). RA presented 5-fold higher 4-antibody system scores (2.02±0.05) than controls (0.42±0.02) ($p< 0.01$). Scores greater than 2 yielded 42% sensitivity and 98.8% specificity. A total of 82 subjects presented with full-house 4 antibodies (score = 4) and 81 of them had RA (99.9% specific). Interval LR and PV for each of the 4-antibody score are presented in the Table. There was no difference in the 4- antibody score between RA who fulfilled the 1987 ACR or 2010 ACR criteria (1.99±0.07 vs 2.08±0.09; $p=0.40$). In the subset of subjects newly diagnosed (less than one year), average 4-antibody system score for

RA (n=33) was 1.72±0.22 (36.3% with score greater than 2) and 0.58±0.12 for other diseases (0% with score greater than 2, 100% specific) ($p < 0.01$).

Conclusion

This cumulative combination of antibody systems targeting citrullinated, carbamylated, PAD4 and RF is highly specific for RA. It may be useful in diagnosing and classifying RA even in symptomatic patients who present early in the course of disease.

Table I: Combination of RF IgM, anti-CCP -IgG-, anti-CarP -IgG-, and anti-PAD4 -IgG

Score	RA (%, N)	CTL (%, N)	Likelihood Ratio [CI 95%]	Pre-test PPV	Post-test PPV	Change
0	20.5% (131/638)	65.8% (510/775)	0.31 [0.27 to 0.37]	10%	3.4%	-6.6%
1	11.6% (74/638)	27.2% (211/775)	0.43 [0.33 to 0.54]	10%	4.5%	-5.5%
2	25.5% (163/638)	5.8% (45/775)	4.40 [3.22 to 6.02]	10%	32.8%	22.8%
3	29.6% (189/638)	1.0% (8/775)	28.70 [14.26 to 57.77]	10%	76.1%	66.1%
4	12.7% (81/638)	0.1% (1/775)	98.39 [13.73 to 705.04]	10%	91.6%	81.6%

Appendix 6: Poster, Anti-PAD4 Rome Seronegative RA, 14th Dresden Symposium on Autoantibodies (Dresden, 2019)

ANTI-PAD4 ANTIBODIES CAN HELP CLOSE THE SERONEGATIVE GAP IN RHEUMATOID ARTHRITIS

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14th Dresden Symposium on Autoantibodies
September 10-13th, 2019, Dresden, Germany

KEY MESSAGES

- Anti-PAD4 antibodies can help to close the seronegative gap in rheumatoid arthritis (RA).
- Anti-PAD4 antibodies represent a useful biomarker for RA diagnosis.

INTRODUCTION

Novel biomarkers have been described in rheumatoid arthritis (RA) patients, including autoantibodies to the protein-arginine deiminase (PAD) enzymes. Anti-PAD4 antibodies are associated with anti-citrullinated protein antibodies (ACPA), disease severity, worse baseline radiographic joint damage, and better response to treatment escalation [1]. Very recently, it was shown that anti-PAD4 can help close the seronegative gap in RA [2]. The goal of this study was to validate these findings and to evaluate the diagnostic performance of anti-PAD4 antibodies in a cohort of seronegative early RA (ERA) patients and controls.

METHODS

Sera from a total of 133 subjects [92 Early-RA (ERA) patients and 41 controls] were included in this study (Table 1). ERA patients fulfilled the 2010 American College of Rheumatology (ACR)/European League Against Rheumatism (EULAR) criteria for RA [3]. At baseline, the ACR/EULAR core data set variables were recorded, and hand and foot radiographs were examined according to modified Total Sharp Score (mTSS). ERA patients were triple seronegative for IgG-ACPA (Axis Shield Diagnostics, Dundee, UK), IgM and IgA rheumatoid factor (RF) (Orgentec Diagnostika GmbH, Mainz, Germany), defined by ELISA. The samples were tested for anti-PAD4 IgG using a novel particle-based multi-analyte technology (PMAT) system (research use only, Inova Diagnostics, USA). The cut-off was defined in a previous study as 1171 median fluorescence intensity (MFI).

Table 1 Overview of disease groups and number of subjects included in the study.

Disease group	Number of patients	% of total
Early rheumatoid arthritis (ERA)	92	69.2%
Systemic lupus erythematosus (SLE)	10	7.5%
Sjogren's syndrome (SjS)	2	1.5%
Infectious disease	9	6.8%
Systemic sclerosis (SSc)	10	7.5%
Apparently healthy individuals (HI)	10	7.5%
	Total = 133	

RESULTS

Anti-PAD4 antibodies were observed in 15.2% (14/92) of seronegative ERA patients and in 2.4% (1/41) of the controls. Receiver operating characteristic (ROC) analysis revealed a moderate discrimination of anti-PAD4 [Area Under the Curve (AUC) = 0.60, 95%CI 0.50-0.71] (Figure 1).

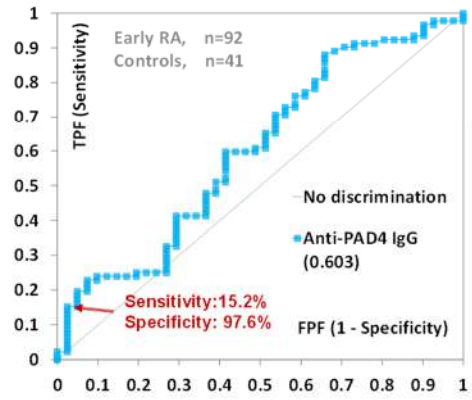

Figure 1 Receiver operating characteristic (ROC) curve analysis of anti-PAD4 IgG. The area under the curve (AUC), and the number of rheumatoid arthritis (RA) patients and controls included in the analysis are shown in the graph. The arrow indicates sensitivity and specificity at the preliminary cut-off point.

At the preliminary cut-off, anti-PAD4 reported a sensitivity of 15.2%, with a 97.6% specificity, and an odds ratio (OR) of 7.2 (95% CI 1.1-44.1). Likelihood plot for anti-PAD4 IgG in the assessment of ERA is shown in figure 2.

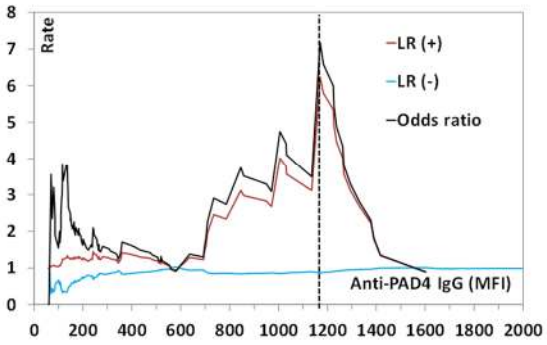

Figure 2 Likelihood plot for anti-PAD4 IgG in the assessment of triple-seronegative early rheumatoid arthritis (RA). Likelihood ratios are shown as a function of antibody titer. The black line represents the Odds Ratio (OR), the red line represents the positive likelihood ratio and the blue line represents the negative likelihood ratio. The black vertical dashed line represents the preliminary cut-off.

CONCLUSION

Anti-PAD4 IgG demonstrated diagnostic value in this cohort of ERA triple-seronegative patients and controls. These results confirm that anti-PAD4 IgG represent a useful biomarker to help close the seronegative gap in RA.

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Gemelli
Fondazione Policlinico Universitario Agostino Gemelli IRCCS
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Inova Diagnostics
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Appendix 7: Poster, MLH Erosive RA, Annual European Congress of Rheumatology (EULAR) (Madrid, 2019)

ASSOCIATION OF ANTI-PAD4 ANTIBODIES WITH EROSION AND BIOLOGICAL TREATMENT USE IN RHEUMATOID ARTHRITIS

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European Congress of Rheumatology (EULAR)
June 12th-15th 2019, Madrid, Spain

KEY MESSAGES

- Anti-PAD3, anti-PAD4, and ACPA are associated with rheumatoid arthritis onset at an early age
- Anti-PAD4 antibodies are associated with erosive disease and biological treatment use

INTRODUCTION

Novel biomarkers have been described in rheumatoid arthritis (RA) patients, including autoantibodies to carbamylated proteins (anti-CarP) and to protein-arginine deiminase (PAD) enzymes. Anti-PAD4 antibodies are associated with anti-citrullinated protein antibodies (ACPA) and worse baseline radiographic joint damage[1,2]. A subset of anti-PAD4 antibodies that cross-react with PAD3 and are associated with most erosive disease, ACPA, and progress despite treatment have also been described[3]. The goal of this study was to evaluate several novel RA markers in a cohort of RA and controls and their association with erosive disease and biological treatment use in RA.

METHODS

Sera from a total of 116 RA patients [63 young onset RA (YORA) and 53 elderly onset RA (EORA)] and 155 controls [134 individuals with polymyalgia rheumatica (PMR) and 21 healthy individuals (HI) older than 60 years old] were included in this study. Information on erosion status and biological treatment was available for 56 of the RA patients. The samples were tested for anti-PAD3 and anti-PAD4 IgG using the novel particle-based multi-analyte technology (PMAT, research use only, RUO), as well as for ACPA [CCP3 IgG ELISA and chemiluminescent immunoassay (CIA)] and anti-CarP IgG (ELISA, RUO).

RESULTS

The prevalence of each marker in the different groups is summarized in Table 1. Significantly higher levels of all markers were observed in RA vs. controls ($p<0.0001$ for anti-PAD3, ACPA (ELISA and CIA), and $p=0.0054$ for anti-CarP and $p=0.0071$ for anti-PAD4). Significantly higher levels of anti-PAD3, anti-PAD4, and ACPA (both ELISA and CIA) but not anti-CarP were observed in YORA vs. EORA ($p<0.0001$ for anti-PAD3 and ACPA ELISA and CIA, and $p=0.0016$ for anti-PAD4).

Table 1 Prevalence of the assays in the different groups and in the entire cohort (percentage and number of individuals).

Group/ Assay	PMR	HI	YORA	EORA	RA (YORA+EORA)	Total
% (N/Total)	49% (134/271)	8% (21/271)	23% (63/271)	20% (53/271)	43% (116/271)	100% (271/271)
CCP3 ELISA	7% (9/134)	0% (0/21)	92% (58/63)	23% (12/53)	60% (70/116)	29% (79/271)
CCP3 CIA	7% (9/134)	0% (0/21)	95% (60/63)	25% (13/53)	63% (73/116)	30% (82/271)
CarP ELISA	41% (55/134)	14% (3/21)	49% (31/63)	62% (33/53)	55% (64/116)	45% (122/271)
PAD3 PMAT	1% (1/134)	5% (1/21)	16% (10/63)	0% (0/63)	9% (10/116)	4% (12/271)
PAD4 PMAT	10% (13/134)	14% (3/21)	38% (24/63)	13% (7/63)	27% (31/116)	17% (47/271)
PAD3+/4+ PMAT	1% (1/134)	0% (0/21)	16% (10/63)	0% (0/63)	9% (10/116)	4% (11/271)

In the RA patients with erosion and treatment information available, anti-PAD4, but not ACPA, anti-CarP or anti-PAD3 levels, were significantly higher in patients on biologic treatment, in comparison to patients that were not on biologics ($p=0.0017$) (Figure 1a). Anti-PAD4 positive patients, were 10.1 [95% CI 2.5-52.0, $p=0.0002$] times more likely to be on biologic treatment compared to the negative group. Similarly, anti-PAD4 antibodies, but neither ACPA nor anti-CarP or anti-PAD3, were also significantly higher in patients with joint erosions ($p=0.0354$) (Figure 1b). All patients that were positive for anti-PAD4 antibodies (n=21) had erosive disease. Anti-PAD4 positive patients, were 20.2 times more likely to have erosive disease [95% CI 1.1-363.2, $p=0.0041$].

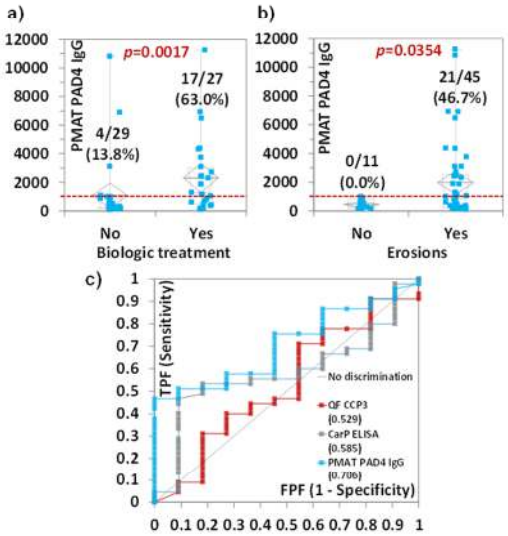

Figure 1 Association of anti-PAD4 antibodies with joint erosion status (a) and to biological treatment use (b), and ROC analysis showing the discrimination value for erosive disease of ACPA, anti-CarP, and anti-PAD4 for erosive disease (c). Dashed line indicates the preliminary cut-off of the assay. Number of positives and % within each subgroup based on preliminary cut-offs are shown.

CONCLUSION

Anti-PAD3, anti-PAD4, and ACPA are associated with disease onset at an early age. Anti-PAD4 are associated with erosive disease and biological treatment use in RA and represent a useful marker for patient stratification.

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Appendix 8: Poster, Anti-PAD4 IgA MLH Erosive RA, ACR/ARHP Annual Meeting (Atlanta, GA, 2019)

ANTI-PROTEIN-ARGININE DEIMINASE (PAD) 4 IGA ARE PRESENT IN THE SERA OF RHEUMATOID ARTHRITIS PATIENTS AND ARE ASSOCIATED WITH JOINT EROSION AND BIOLOGICAL TREATMENT USE

Inova Diagnostics
A Werfen Company

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Valdecilla IDIVAL

ACR/ARHP Annual Meeting, November 8-13th, 2019, Atlanta, GA, USA

KEY MESSAGE

- Anti-PAD4 IgA are specific for RA and are associated with erosive disease and biological treatment.
- Anti-PAD4 IgG and IgA represent useful biomarkers for prognosis and patient stratification in RA.

INTRODUCTION

Novel biomarkers have been described in rheumatoid arthritis (RA) patients, including autoantibodies to the protein-arginine deiminase (PAD) enzymes. Anti-PAD4 IgG are associated with anti-citrullinated protein antibodies (ACPA), worse baseline radiographic joint damage[1], and a better response to treatment escalation[2] suggesting a potential role in RA pathogenesis. The objective of this study was to evaluate the presence of anti-PAD4 IgG and IgA in the sera of RA patients and to investigate their association with joint erosion and biological treatment use.

METHODS

Sera from 104 RA and 155 controls were tested for anti-PAD4 IgG and IgA using the particle-based multi-analyte technology (PMAT). Information on erosive disease and biological treatment use was available for 54 of the RA patients, that were also tested by anti-cyclic citrullinated peptide (CCP) 3 chemiluminescence immunoassay (CIA). All tests were from Inova Diagnostics, CA, US. An association between the autoantibodies and these clinical features was investigated.

RESULTS

Anti-PAD4 IgG and IgA were observed in 25.0% (26/104) and 21.2% (22/104) RA patients, as well as in 5.8% (9/155) and 5.3% (8/155) of controls (Figures 1a and 1b). The prevalence of the double positivity in RA patients was 13.5% (14/104). Receiver operating characteristics (ROC) analysis showed significant discrimination between RA and controls for both isotypes (Figure 2). At the preliminary cut-offs, anti-PAD4 reported a sensitivity and specificity of 25.0%/94.2% for IgG and 21.2%/94.8% for IgA.

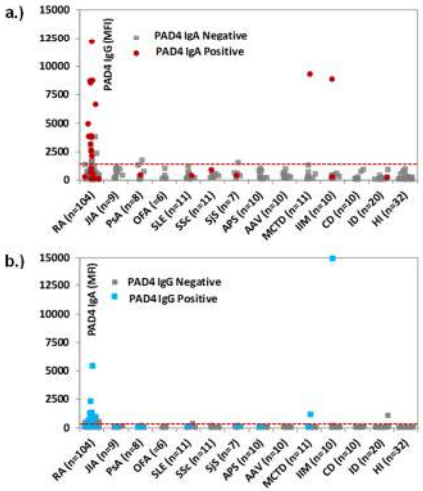

Figure 1 Levels of anti-PAD4 IgG (a) and IgA (b) in different disease groups. The positivity for the other anti-PAD4 isotype is also represented in each graph. Red dashed lines represent the preliminary cut-offs.

AAV: antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis; APS: antiphospholipid syndrome; CD: celiac disease; HI: healthy individuals; IIM: idiopathic inflammatory myopathies; ID: infections diseases; JIA: juvenile idiopathic arthritis; MCTD: mixed connective tissue disease; MFI: Median Fluorescent Intensity; OFA: other forms of arthritis; PAD: protein-arginine deiminase; PsA: psoriatic arthritis; RA: rheumatoid arthritis; SjS: Sjogren's syndrome; SLE: systemic lupus erythematosus; SSc: systemic sclerosis;

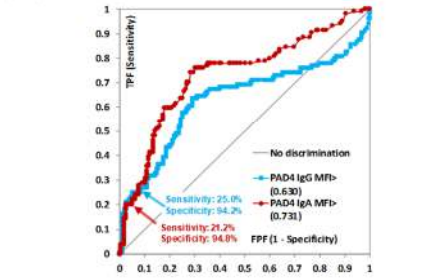

Figure 2 Receiver Operating Curve (ROC) analysis of anti-protein arginine deiminase (PAD) 4 IgG and IgA for the discrimination of rheumatoid arthritis (RA) (n=104) vs. controls (n=155). Area under the curve (AUC) is shown.

Anti-PAD4 IgG and IgA levels were significantly higher in RA patients *vs.* controls (p=0.0004 and p<0.0001, respectively). Interestingly, higher levels of anti-PAD4 IgG and IgA, but not ACPA were found in the RA patients with erosive disease *vs.* individuals without erosions (p=0.0166, p=0.0176, and p=0.7883, respectively), and in patients under biological treatment *vs.* those that were not on biologics (p=0.0002, p=0.0009, and p=0.7752, respectively) (Figure 3a and b).

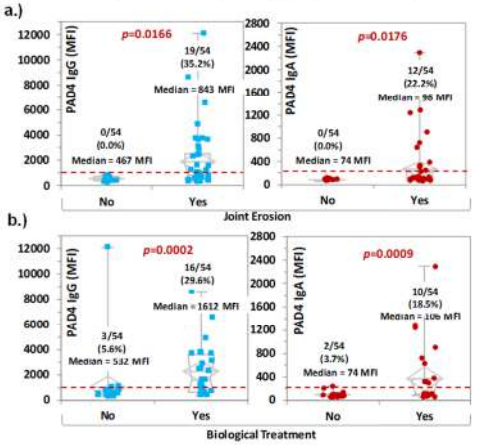

Figure 3 Pairwise comparison (Wilcoxon Mann-Whitney analysis) of anti-protein arginine deiminase (PAD) 4 IgG and IgA in RA patients based on erosive status (a) and on the biological treatment use (b). Median of each subgroup and p-values are shown in the figures.

CONCLUSIONS

Our study confirms the association of anti-PAD4 IgG with erosive RA and is the first to report anti-PAD4 IgA as a highly specific marker for RA. A strong association between anti-PAD4 IgG and IgA and erosive disease and biological treatment was observed, suggesting a potential value of these markers for prediction of prognosis and RA patient stratification.

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Appendix 9: Poster, Florence RA ILD, Annual European Congress of Rheumatology (EULAR) (Madrid, 2019)

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ANTI-PEPTIDYL-ARGININE DEIMINASE 3 AND 4 AUTOANTIBODIES IN A COHORT OF RHEUMATOID ARTHRITIS WITH INTERSTITIAL LUNG DISEASE

Boaz Palterer [1], Gianfranco Vitiello [1], Bernardo D'onofrio [1], Emanuele Vivarelli [1], Daniele Cammelli [1], Maria Grazia Giudizi [1], Laura Martinez-Prat [2], Silvia Casas [2], Chelsea Bentow [2], Michael Mahler [2], Paola Parronchi [1]

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BACKGROUND:
Interstitial lung disease (ILD) affects up to 30% of patients with rheumatoid arthritis (RA). Peptidyl-arginine deiminases (PAD) are key enzymes in RA pathogenesis as they are involved in the citrullination of proteins, targets of anti-citrullinated protein antibodies (ACPA). Although RA-ILD significantly contributes to disease burden including mortality, diagnostic and prognostic biomarkers are still lacking.

OBJECTIVES:
To measure anti-PAD3 and anti-PAD4 antibodies in a cohort of RA and compare their prevalence in patients with and without ILD. To assess the associations of anti-PAD3, anti-PAD4 and ACPA with disease activity, joint erosions, lung involvement and smoking history.

METHODS:
A total of 71 patients fulfilling the 2010 ACR/EULAR RA Classification Criteria were recruited; the mean age was 63.3±12.4 and 87% of them were females, 11 (15.5%) of them had been diagnosed with ILD. Demographic, clinical as well as radiological data were retrospectively collected. ILD was defined as usual interstitial pneumonia (UIP), non-specific interstitial pneumonia (NSIP) or indeterminate patterns on chest high-resolution computed tomography, according to ATS/ERS guidelines. Particle-based Multi-Analyte Technology (PMAT) (Inova Diagnostics, USA, research use only) was used to measure **anti-PAD3** and **anti-PAD4** autoantibodies. **ACPA IgG** were measured by chemiluminescence (QUANTA Flash CCP3, Inova Diagnostics, USA).

RESULTS:
Anti-PAD4 levels were correlated with **erosive disease** (p=0.043) and **morning stiffness** (p=0.031). Anti-PAD3 and anti-PAD4 levels were associated to **DAS28-ESR** at the time of sampling (anti-PAD3, r=0.34, p=0.004; anti-PAD4, r=0.34, p=0.004). Anti-PAD4 antibodies were significantly lower in patients with **ILD** (p=0.043). There was no association between anti-PAD4 and **smoking**, while anti-PAD3 antibodies were higher in non-smokers (p=0.004). A strong correlation was found between anti-PAD4 and anti-PAD3 levels (r=0.73, p<0.0001).

CONCLUSION:
In our cohort, anti-PAD4 antibodies were correlated with joint erosions and RA disease activity, whereas a negative association with ILD was found. Smoking history was not associated with the presence and levels of anti-PAD antibodies. Our data validate the usefulness of anti-PAD4 antibodies as a biomarker for erosive disease. Further studies that take into account relevant confounders like therapy and larger RA-ILD cohorts are needed.

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Anti-PAD4 antibodies in RA are associated with **erosive disease** and negatively associated to **Interstitial Lung Disease.**

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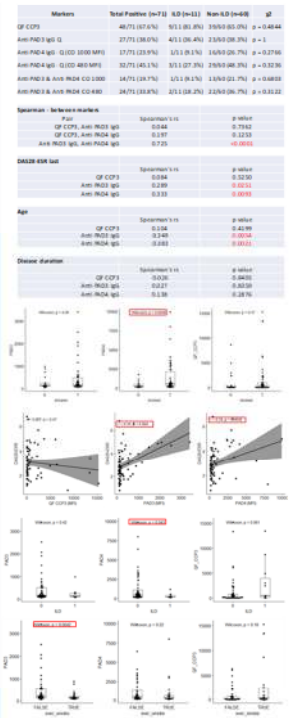

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Appendix 10: Poster, RA Under Treatment, 11th International Congress on Autoimmunity (Lisbon, 2018)

PREVALENCE OF CLASSICAL AND NOVEL MARKERS IN A COHORT OF RHEUMATOID ARTHRITIS PATIENTS UNDER TREATMENT

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11th International Congress on Autoimmunity
May 16-20th 2018, Lisbon, Portugal

KEY MESSAGES

- Differences in antibody prevalence were observed between patient subpopulations under different treatments.
- The most pronounced difference was observed for Rituximab.

INTRODUCTION

Antibodies to carbamylated proteins (anti-CarP), to peptidyl-arginine deaminases (PADs) and serum calprotectin have been reported in rheumatoid arthritis (RA) patients. We analyzed the prevalence of these novel markers together with rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPA) in RA patients under various treatments.

METHODS

The cohort consisted of 223 RA patients under various treatments (Table 1). RFs, anti-CarP antibodies [using fetal calf serum as antigen, research use only (RUO)] and serum calprotectin (RUO) were measured by ELISA. Anti-CCP antibodies were measured by QUANTA Flash CCP3 chemiluminescence immunoassay (CIA). Anti-PAD3 and PAD4 antibodies were measured using a novel particle based multi-analyte technology (PMAT) system (RUO). All methods are from Inova Diagnostics, San Diego, CA, USA. Preliminary cut-offs were used for the RUO assays. *P*-values lower than 0.05 were considered significant.

RESULTS

A strikingly high prevalence of anti-CarP was found in the total population (160/223, 71.7%) and in the RF IgM and CCP3 negative population (29/48, 60.4%). A very high prevalence of elevated calprotectin levels was also observed (180/223, 80.7%). While the prevalence of anti-PAD3 was similar to previous studies, anti-PAD4 reported a lower prevalence (Table 1).

Table 1 Prevalence of the classical and novel markers in the cohort of rheumatoid arthritis patients under treatment, grouped by treatment.

Treatment Group	N (%)	RF IgM (%)	RF IgA (%)	RF IgG (%)	Anti-CCP3 (%)	Anti-CarP (%)	Anti-PAD3 (%)	Anti-PAD4 (%)	sCP (%)
Abatacept	30/223 (13.5)	19/30 (63.3)	15/30 (50.0)	11/30 (36.7)	20/30 (66.7)	20/30 (66.7)	6/30 (20.0)	7/30 (23.3)	26/30 (86.7)
Methotrexate	49/223 (22.0)	38/49 (77.6)	29/49 (59.2)	20/49 (40.8)	39/49 (79.6)	35/49 (71.4)	15/49 (30.6)	10/49 (20.4)	44/49 (89.8)
TNF inhibitors*	80/223 (35.9)	55/80 (68.8)	38/80 (47.5)	33/80 (41.3)	51/80 (63.8)	67/80 (83.8)	17/80 (21.3)	15/80 (18.8)	61/80 (76.3)
Rituximab	31/223 (13.9)	17/31 (54.8)	17/31 (54.8)	6/31 (19.4)	20/31 (64.5)	14/31 (45.2)	4/31 (12.9)	0/31 (0.0)	24/31 (77.4)
Tocilizumab	33/223 (14.8)	18/33 (54.5)	13/33 (39.4)	10/33 (30.3)	16/33 (48.5)	24/33 (72.7)	5/33 (15.2)	6/33 (18.2)	25/33 (75.8)
Total	223/223 (100.0)	147/223 (65.9)	112/223 (50.2)	80/223 (35.9)	146/223 (65.5)	160/223 (71.7)	47/223 (21.1)	38/223 (17.0)	180/223 (80.7)

Note: patients on TNF inhibitors included 4 patients on Golimumab, 16 on Adalimumab, 27 on Certolizumab, 30 on Etanercept and 3 on Infliximab.

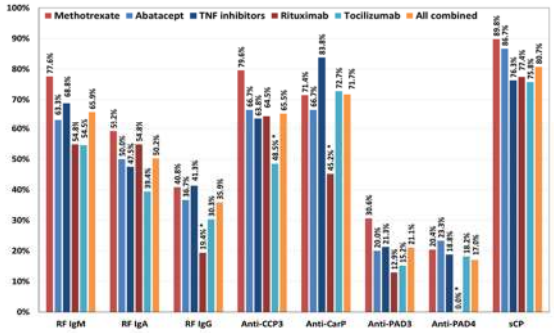

Figure 1 Prevalence of the classical and novel markers in the cohort of rheumatoid arthritis patients under treatment, grouped by treatment. Markers with a significantly lower prevalence are marked with an asterisk.

Differences in the prevalence of the markers were observed in the different treatment groups (Figure 1). A significantly lower prevalence of RF IgG (p=0.0441), anti-CarP (p=0.0009), and anti-PAD4 (p=0.0034) antibodies was found for patients under Rituximab treatment, and of anti-CCP3 antibodies (p=0.0304) for Tocilizumab treated patients. Additionally, significant quantitative correlations by Spearman's analysis were found between anti-CarP and anti-PAD3, between anti-CarP and anti-PAD4, and between anti-PAD3 and anti-PAD4 (Table 2).

Table 2 Spearman's correlation analyses between markers. Spearman's rho and *p*-values are shown. The highest Spearman's rho for each marker is highlighted in bold.

	RF IgM	RF IgA	RF IgG	Anti-CCP3	Anti-CarP	Anti-PAD3	Anti-PAD4	sCP
RF IgM	RF IgM	**0.760** <0.0001	0.735 <0.0001	0.525 <0.0001	0.229 0.0006	0.203 0.0024	0.232 0.0005	0.110 0.1016
RF IgA		RF IgA	**0.684** <0.0001	0.533 <0.0001	0.310 <0.0001	0.297 <0.0001	0.325 <0.0001	0.110 0.1022
RF IgG			RF IgG	**0.497** <0.0001	0.236 0.0004	0.224 0.0008	0.237 0.0004	0.079 0.2426
Anti-CCP3				Anti-CCP3	0.219 0.0011	0.199 0.0029	**0.227** 0.0007	-0.006 0.9269
Anti-CarP					Anti-CarP	**0.485** <0.0001	0.458 <0.0001	0.068 0.3167
Anti-PAD3						Anti-PAD3	**0.779** <0.0001	-0.005 0.9398
Anti-PAD4							Anti-PAD4	-0.013 0.8419

CONCLUSION

The novel biomarkers reported high prevalence in our RA patients under treatment. Differences in the prevalence of the markers were observed between subpopulations under different treatments. Whether these treatments might have an effect on the autoantibodies levels remains a question since no baseline information was available. Longitudinal studies are mandatory to study the potential use of those markers for patient stratification and precision medicine.

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