The value of rheumatoid arthritis autoantibodies in disease pathogenesis and treatment prognosis

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The Value of Rheumatoid Arthritis Autoantibodies in Disease Pathogenesis and Treatment Prognosis

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By Emma de Moel

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CHAPTER 1

Introduction

RHEUMATOID ARTHRITIS

Rheumatoid arthritis (RA) is a severe autoimmune disease of unknown etiology that symmetrically affects the small diarthrodial joints of the hands and feet. Its prevalence is estimated to affect approximately 0.5-1% of the global population, making it one of the most prevalent autoimmune diseases worldwide (1). RA typically manifests with symptoms such as joint pain, swelling, stiffness, and fatigue, profoundly impacting patients' quality of life and productivity. If left untreated, it leads to progressive joint damage and disability. Moreover, RA is associated with a range of comorbidities, including cardiovascular diseases, osteoporosis, and systemic inflammation, further exacerbating its burden on affected individuals (2). Central to the disease process is the activation of innate and adaptive immune cells, leading to the production of pro-inflammatory cytokines, chemokines, and autoantibodies. Below, the most important autoantibodies are described, with what is currently known about their role in pathogenesis and disease propagation.

AUTOANTIBODIES

RA is broadly classified into seropositive and seronegative RA based on the presence or absence of specific autoreactive antibodies in patients' serum. Seropositive RA is characterized by the presence of rheumatoid factor (RF) and/or anti-citrullinated protein antibodies (ACPAs), whereas seronegative RA lacks these conventional autoantibodies. Seropositive RA is associated with more aggressive disease phenotypes, higher disease activity, increased radiographic progression, and poorer treatment responses compared to seronegative RA (3, 4). Autoantibodies are also thought to play a crucial role in the pathogenesis of RA, and several types have been identified, each with distinct targets and clinical significance, described below.

Rheumatoid factor

RF was the first autoantibody associated with RA and is detected in approximately 60-70% of patients. RF is a hallmark autoantibody in RA, integral to major classification criteria such as the 1987 American College of Rheumatology (ACR) criteria and the 2010 ACR/European League Against Rheumatism (EULAR) criteria (5, 6). RF positivity is associated with more severe disease manifestations, including increased joint damage and extra-articular complications (7). RF targets the Fc region of IgG and comprises various isotypes, with IgM being the most prevalent (8). Although its role in RA pathogenesis is not fully elucidated, RF likely contributes to immune complex formation, particularly in the synovial fluid, where high-affinity RF may perpetuate inflammation by stimulating proinflammatory cytokine production (9, 10).

Anti-citrullinated protein antibodies

ACPAs are highly specific biomarkers for RA, are detected in approximately 70-80% of patients, and occur rarely in individuals without RA (11, 12). ACPAs target citrulline residues on proteins or peptides, a post-translational modification mediated by peptidyl arginine deiminases (PADs), converting arginine to citrulline (13). ACPAs recognize a plethora of citrullinated proteins, including fibrinogen, vimentin, α-enolase, and collagen type II, among others, and show marked isotype usage (14, 15). Their presence overlaps largely with that of RF IgM, and they are similarly incorporated with RA classification criteria mentioned above in the form of the clinical anti-CCP2 or CCP3 test, which measures ACPA directed to a diverse spectrum of citrullinated peptides (16, 17). ACPA positivity correlates with more severe disease phenotypes, increased radiographic progression, and poorer treatment responses (18, 19).

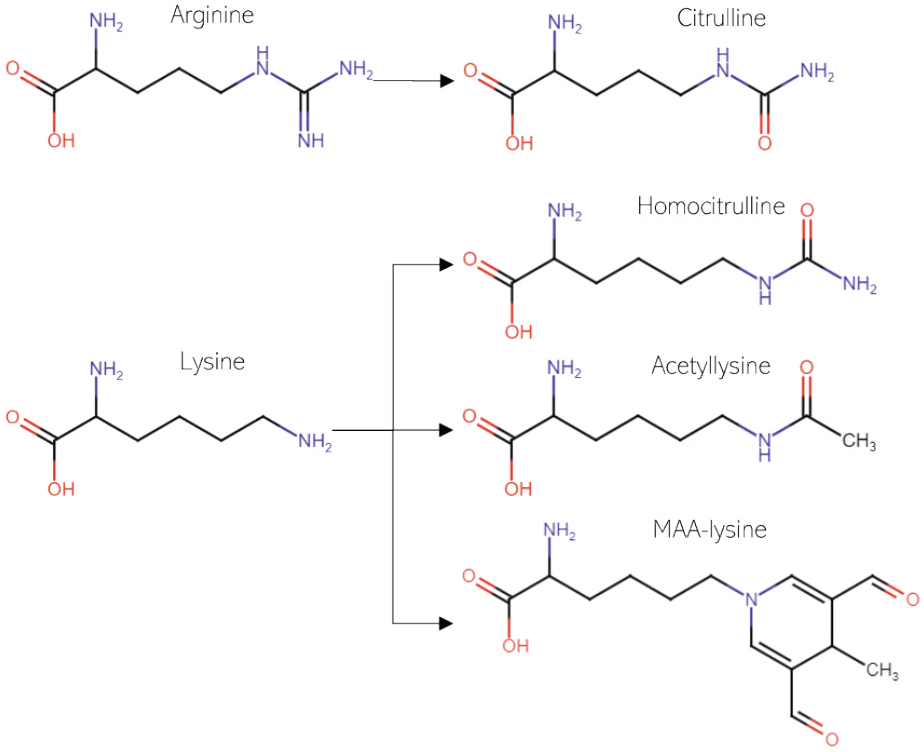
Other anti-modified protein antibodies

Besides RF and ACPAs, a growing number of anti-modified protein antibodies (AMPAs) continue to be discovered which target other post-translation modifications of peptides (Figure 1). Anti-carbamylated protein antibodies (anti-CarP) have emerged as novel autoantibodies in RA and are detected in approximately 30-40% of patients (20). Carbamylation is a chemical modification involving the conversion of lysine residues to homocitrulline in proteins, mediated by myeloperoxidase and other reactive oxygen species in diverse states of inflammation (21). Anti-CarP antibodies recognize carbamylated proteins and have been implicated in RA pathogenesis, although their precise mechanisms of action remain to be elucidated. Anti-CarP positivity has been associated with more severe joint damage and radiographic progression, independent of ACPA status, suggesting their potential as prognostic biomarkers in RA (20, 22, 23).

Anti-acetylated protein antibodies (AAPA) are antibodies targeting acetylated proteins, which are formed by the acetylation of lysine residues (24). Acetylation is a reversible post-translational modification regulated by histone acetyltransferases and histone deacetylases. AAPA positivity has been observed in RA patients, although its clinical significance and functional role in disease pathogenesis is unclear.

Anti-malondialdehyde-acetaldehyde adduct antibodies (anti-MAA) are antibodies targeting adducts formed by the reaction of malondialdehyde (MDA) and acetaldehyde with lysine in proteins(25). These adducts are generated under conditions of oxidative stress and lipid peroxidation, which are prevalent in RA synovium(26, 27). Anti-MAA antibodies have been detected in RA patients, particularly those with severe disease and extra-articular manifestations (25, 28, 29). However, anti-MAA/MDA antibodies are not RA-specific and can be found in many other conditions as well. Nonetheless, they are thought to contribute to RA pathogenesis by promoting inflammation, endothelial dysfunction, and tissue damage, although further studies are needed to determine their exact role.

Figure 1: Post-translational modifications on arginine and lysine



AUTOANTIBODY CHARACTERISTICS IN RA

A growing body of evidence points to the importance of AMPAs and especially ACPAs in the pathogenesis of RA. ACPAs can be detected in the sera of RA patients years before the onset of clinical symptoms, implicating their involvement in the preclinical stages of disease development (30, 31). ACPAs have also been identified in individuals with recent-onset clinically suspect arthralgia, with a positive predictive value exceeding 60%(32). However, at this early stage, ACPA titers are typically low, and their peptide recognition profile is limited(33, 34). As individuals progress towards disease onset, ACPA titers and peptide-recognition profiles undergo substantial expansion, indicative of an active immune response and suggesting a role for ACPAs in precipitating disease onset. The efficacy of selective B-cell depletion therapies in treating RA provides compelling evidence for the involvement of B cells and possibly autoantibodies(35), including ACPAs, in driving the chronicity of RA.

The recognition profile of ACPAs is diverse, with these antibodies targeting a variety of citrullinated antigens. Epitope spreading, characterized by an increase or shift in the antigen recognition profile, may have important pathophysiological consequences as it

does in other autoimmune disease like pemphigus (36), potentially exacerbating disease severity and progression. While specific anti-citrullinated epitope or protein reactivity predictive of disease course in RA have not been conclusively identified, the breadth of ACPA recognition profiles may offer insights into disease heterogeneity and progression.

Isotype switching further enhances the functional diversity of AMPAs in RA patients before disease onset (37-39). Interestingly, the avidity maturation of ACPAs and anti-CarP differs from conventional antibody responses, with these autoantibodies exhibiting relatively low avidity despite extensive isotype switching (40, 41). Little is as of yet known about other AMPA families in this regard. ACPA also exhibit an additional unusual feature of containing additional glycans in the variable antibody domains, notably increased in RA and predictive for disease progression (42, 43). These discrepancy suggests that the regulation of ACPA responses may differ from that of conventional antibody responses, highlighting the unique immunological characteristics of ACPAs in RA. It is expected that similar investigations will following for the characteristics in the other AMPAs.

RISK FACTORS FOR RA AND AUTOANTIBODY FORMATION

Understanding the temporal evolution of autoantibodies described above and its potential role in RA development requires consideration of both genetic and environmental risk factors. Among the various genetic risk factors implicated in RA, human leukocyte antigen (HLA) is the most prominent. Specifically, a common amino acid sequence at position 70-74 of the HLA-DRB1 molecule, referred to as the “shared epitope” (HLA-SE), has been strongly linked to the development of ACPA-positive but not ACPA-negative RA (44). Notably, HLA-SE also does not predispose to the rare state of ACPA-positivity in the absence of RA, but rather associates only with ACPA-positive RA (45). Additionally HLA-SE is specifically associated with the presence of variable-domain glycosylated ACPA during the pre-disease phase (46), which may have various implications (beyond the scope of this introduction) in the process of autoreactive ACPA B-cell survival. Conversely, certain alleles of HLA-DRB1, such as HLA-DRB1*13, have been identified as protective against seropositive RA development (47), suggesting a complex interplay between HLA alleles and disease susceptibility.

Environmental risk factors, such as tobacco smoking and inhalant exposures, also demonstrate stronger associations with ACPA-positive RA(48-50). Of particular interest is the gene-environment interaction between HLA-SE alleles and smoking (51), suggesting a potential role for smoking-induced citrullination in RA pathogenesis. Other evidence implicates mucosal sites like the periodontium and the microbiome of the intestinal tract(52, 53), as well as female sex hormones(54) as potential factors in autoimmune initiation in RA.

TREATMENT CHALLENGES

RA is not a new disease. RA has been demonstrated in paleopathological studies of human remains dating back to 4000BCE, and findings suggestive of RA appear in 17th century Dutch art (55, 56). Despite its long history, the disease processes underlying RA remained virtually untreatable until as recently as 50 years ago, and patients could expect only limited symptomatic relief from the debilitating pain, loss of function, and systemic complications that the disease causes. Since then, huge advances have been made in effective therapies that not only address symptoms, but also halt the persistent, uncontrolled inflammation and associated disease progression, termed disease modifying anti-rheumatic drugs (DMARDs) (57). Novel biologic and small-molecule agents have further augmented the rheumatologists therapeutic arsenal, together with a treat-to-target approach, and disease remission is achievable in an increasing numbers of patients (58).

However, all of these treatments have side-effects that are often not insignificant. A growing need exists to allow patients to attempt to taper their DMARDs but remain symptom free, termed drug free remission (DFR)(59). However, only 10-20% of patients are able to achieve and retain this lofty treatment goal (60), and disease flare following unsuccessful DMARD tapering may exacerbate disease burden, though thankfully, patients who experience a disease flare are virtually always able to recapture remission (61). Identifying factors predicting disease flare post-DMARD tapering could optimize clinical decision-making.

Sustained DFR is also interesting from a pathophysiological standpoint, since it most closely approximates the holy grail of RA treatment outcomes: a cure. If DFR is sustained, perhaps it identifies a form of a true state of disease remission, without the need for treatment, in which the underlying autoimmune processes that initiated and propagated disease, have been durably dampened and immune tolerance has been re-established. Identifying factors which are prognostically favorable for this outcome, could then shed light on which mechanisms in RA are important in perpetuating disease, and improve treatment by targeting those mechanisms (62).

AMPA AND TREATMENT OUTCOMES

Given their presence and evolution over time in pre-diseases states, it is understandable that there is much interest in the prognostic value of autoantibody characteristics in RA once RA has manifested. Autoantibody seropositivity is associated with poorer treatment outcomes and radiographic damage (18, 19, 63). However, given the diversity of the RA autoantibody profile, with its varied autoantigen recognition and extensive isotype switching, the breadth of this profile may also be important. Furthermore, serum autoantibody levels may fluctuate. In other autoimmune diseases

like systemic lupus erythematosus, these fluctuations correlate with disease activity, and are monitored over time to inform clinical decision-making (64). If such fluctuations in serum autoantibody concentrations also occur in RA, they may hold promise as accessible biomarkers for disease course prediction.

Unfortunately, conflicting studies regarding the relationship between autoantibody fluctuations and disease activity, often not accounting for immunosuppressive treatment intensity, complicate interpretation. Most studies have shown that RF (IgM, IgA, and IgG) levels decrease after treatment initiation with different DMARD classes, while anti-CCP2 (IgG) levels decrease only marginally, rebound after decreasing, or do not decrease at all, but did not account for treatment effects (65-71). Virtually nothing was known, at the start of the studies described in this thesis, about the prognostic value of changes in AMPA characteristics (isotype usage, epitope recognition, and seroconversion to negative, to name a few) over time, and newer autoantibodies like anti-CarP, Similar reflections apply to AAPAs, and anti-MAA and also these also warranted consideration.

Understanding the clinical implications of changes in autoantibody levels is crucial. If autoantibody levels predict future disease activity, measuring pre-treatment values or monitoring changes over time could guide treatment decisions. Additionally, investigating the association between autoantibody changes, immunosuppression, and disease activity could provide insights into the role of B cell autoimmune response in RA persistence.

CALPROTECTIN: A NEW PROGNOSTIC BIOMARKER

Not only AMPAs have been investigated as possible biomarkers for predicting RA course: calprotectin, a heterodimeric complex of S100 calcium-binding proteins (MRP-8 and MRP-14), shows promise as a biomarker in RA (72, 73). Although conceptually similar to conventional inflammatory indices like erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP), it may serve as a more sensitive marker of the disease activity in rheumatic illnesses because it directly reflects inflammation in synovium and significantly increases during active inflammation (74, 75). Multiple studies recently reviewed suggest its potential in various inflammatory disorders, including RA, ankylosing spondylitis, psoriatic arthritis, and systemic lupus erythematosus (76). Interestingly, calprotectin has also been implicated in predicting disease relapses in juvenile idiopathic arthritis (77, 78). Theoretically, its presence in synovial tissue and its ability to enter systemic circulation may make it a more specific and reliable marker for residual joint inflammation than hepatocyte-dependent acute-phase reactants like CRP (79). Perhaps by measuring calprotectin in patients in stable remission under DMARDs, we may patients at risk of future disease flare after DMARD tapering and facilitate risk stratification to predict whether DMARD tapering will be successful.

CANCER IMMUNOTHERAPY & AUTOIMMUNITY

While the field of rheumatology and RA boomed with new autoantibody discoveries, breakthroughs were also being made in a completely different field: cancer immunotherapy, in the form of immune checkpoint inhibitors (ICIs). Broadly speaking, ICIs work by blocking the body's natural mechanisms for T-cell inhibition, be it via cytotoxic T-lymphocyte–associated protein 4 (CTLA-4), programmed cell death protein 1 (PD-1), or its ligand PD-L1, to promote an antitumor immune response. Ipilimumab, an anti-CTLA-4 monoclonal antibody, was the first of its kind in advanced metastatic melanoma (80), and it and its brothers have provided significant survival benefits over traditional chemotherapies for a wide range of advanced malignancies (81).

What is intriguing about this anti-cancer therapy is that by inducing immune antitumor effects, ICIs also induce unintended autoimmune side-effects in as many as 80% of patients, termed immune-related adverse events (irAEs)(82, 83). These irAEs can occur in virtually any organ system, can be severe enough to warrant ICI cessation or even cause long-term damage, and are difficult to predict(84). Because of this phenomenon, irAEs draw comparisons to many traditional autoimmune diseases, such as vitiligo, ulcerative colitis, Hashimoto thyroiditis, and even rheumatoid arthritis. In irAEs following ICI treatment, we have, in effect, a human model for induction of autoimmunity, and its potential overlap in the field of rheumatic autoimmune studies is promising indeed. At the start of the studies described in this thesis, the nature of the autoimmune response induced following ICI in general, and the autoantibody response more specifically, was still ill defined and therefore important to investigate both from a clinical- and basic/translational scientific view.

OUTLINE OF THIS THESIS

Main aims

- To characterize the relationship of genetic and environmental risk factors with the AMPA profile in different populations of RA
- To describe the longitudinal changes in characteristics of the AMPA profile, and investigate its prognostic value regarding early treatment outcomes and drug-free remission
- To assess calprotectin as a marker of residual inflammation in patients in disease remission and as clinical tool in tapering DMARDs
- To investigate autoantibody formation and its relation to irAEs in ICIs

As described above, RA autoantibodies play diverse roles in the pathogenesis of RA, reflecting the complex interplay between genetic predisposition, environmental factors, and dysregulated immune responses. Understanding the intricate mechanisms

underlying RA pathogenesis, heterogeneity, and autoantibody profiles is crucial for advancing precision medicine approaches, improving patient outcomes, and developing targeted therapies tailored to individual disease subtypes and phenotypes.

In **Chapter 2,** we examined AMPAs in four different seropositive RA populations from across the world. These populations were vastly different in terms of genetic background, smoking habits, and environmental exposures. By studying the differences in the AMPA profile of these patient – or, perhaps more interestingly, their commonalities – and the relationship of AMPA to known RA risk factors, we were able to investigate whether divergent or common pathways are at play in the development of seropositive RA.

The studies described in **chapters 3 through 5** concern the theragnostic value of various elements of the AMPA profile. Because seropositivity is such an evident poor prognostic factor for various treatment outcomes, we measured various aspects of AMPAs in seropositive RA in an attempt to understand which part of the AMPA profile most strongly confers this poor prognosis. For this, we used data and serum from patients from the Induction therapy with Methotrexate and Prednisone in Rheumatoid Or Very Early arthritic Disease (IMPROVED) study, a multicentre, randomized controlled trial that enrolled 610 patients with untreated RA or undifferentiated arthritis (85). We measured fourteen different RA autoantibodies, comprising eight isotypes and six fine specificities within four AMPA families, and attempted to dissect whether the presence of one, many, or a specific combination of many AMPAs was related to prognosis. Specifically, we focused on early response to DMARD therapy and long-term ability to achieve drug-free remission.

The investigations in **Chapter 4 and 5** characterize the changes of autoantibodies over time, and the possible prognostic value thereof. In these chapters, the concept of immunological remission, described above, is central. At the moment, DFR is the closest proxy for disease cure available in RA, but true curation has not yet been achieved. Autoantibodies, intimately linked with disease initiation and pathogenesis, may change over time, but details are lacking. It is possible that changes in these AMPAs may set apart a group of patients in whom the underlying immunopathology has been favourably modulated, that is, patients in true immunological remission (86), and that this state of remission is favorable for achieving long term DFR. Such a relationship could yield a meaningful prognostic marker for drug tapering decisions, and it could elucidate pathways that lead to long-term resolution of the pathophysiology underlying RA.

wIn **Chapter 4**, this relationship was examined in the form of seroconversion from positive to negative for the fourteen autoantibodies mentioned above, while in **Chapter 5**, emphasis was put in level changes over time, and a direct relationship with immunosuppression was also examined.

In a similar theme, **Chapter 6** focused on the value of calprotectin as a marker of residual inflammation in RA patients in remission to identify patients with favorable chances for DFR. This study made use of the previously mentioned IMPROVED study and of similarly designed RETRO study (acronym for the Reduction of Therapy in Patients with Rheumatoid Arthritis in Ongoing Remission)(87). We evaluated whether calprotectin alone, or its addition to other clinical predictors associated with disease flare within 1 year of study-randomized tapering decisions.

At the time of the publication of **chapter 7**, little was known about autoantibody production in irAEs, and only case studies post-ICI treatment had been published. We set about characterizing the presence of a wide spectrum of organ-specific autoantibodies before and after ICI treatment, and analyzed whether the development of autoantibodies was related to treatment outcomes or toxicity. Understanding how irAEs develop has two-fold significance: first, organ-specific autoantibodies have the potential to serve as biomarkers for the development of irAEs, and second, investigating their development in patients with irAEs may provide insights into the role of autoantibodies in the onset of new autoimmune conditions, such as rheumatoid arthritis.

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CHAPTER 2

Geo-epidemiology of autoantibodies in rheumatoid arthritis: comparison between four ethnically diverse populations

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ABSTRACT

Background

Rheumatoid arthritis (RA) occurs across the globe in different ethnic populations. Most RA patients harbor anti-modified protein antibodies (AMPA); however, it is unclear whether differences exist in autoantibody responses at different geographic locations and between different ethnic groups, which could provide new clues regarding factors underlying autoantibody development. We therefore investigated AMPA prevalence and association with HLA DRB1 alleles and smoking in four ethnically diverse populations on four different continents.

Methods

Anti-carbamylated (anti-CarP), anti-malondialdehyde acetaldehyde (anti-MAA), and anti-acetylated protein antibodies (anti-AcVim) IgG were determined in anti-citrullinated protein antibody-positive Dutch (NL, $n = 103$), Japanese (JP, $n = 174$), First Nations Peoples in Canada (FN, $n = 100$), and black South African (SA, $n = 67$) RA patients. Ethnicity-matched local healthy controls were used to calculate cut-offs. Risk factors associated with AMPA seropositivity in each cohort were identified using logistic regression.

Results

Median AMPA levels were higher in First Nations Peoples in Canada and especially South African patients, as reflected by percentage seropositivity: NL, JP, FN, and SA: anti-CarP: 47%, 43%, 58%, and 76% ($p < 0.001$); anti-MAA: 29%, 22%, 29%, and 53% ($p < 0.001$); and anti-AcVim: 20%, 17%, 38%, and 28% ($p < 0.001$). Total IgG levels also differed markedly, and when autoantibody levels were normalized to total IgG, differences between cohorts became less pronounced. Although there were some associations with AMPA and HLA risk alleles and smoking, none was consistent across all four cohorts.

Conclusions

AMPA against various post-translational modifications could consistently be detected on different continents across ethnically diverse RA populations. Differences in AMPA levels corresponded to differences in total serum IgG levels. This suggests that, despite differences in risk factors, a common pathway may be involved in AMPA development across geographic locations and ethnicities.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic autoimmune disease of unknown etiology that mainly affects the joints and is associated with circulating autoantibodies. Several RA-associated autoantibodies recognize protein epitopes that have been post-translationally modified, and are therefore known as anti-modified protein antibodies or AMPAs. The best-known post-translational modification (PTM) in RA is the enzymatic conversion of arginine to citrulline, which is recognized by anti-citrullinated protein antibodies or ACPA. More recently, other autoantibody systems have also gained attention, including anti-carbamylated protein antibodies (anti-CarP) recognizing homocitrulline-containing antigens, anti-acetylated peptide antibodies (AAPA) recognizing acetylated lysine, and anti-malondialdehyde-acetaldehyde antibodies (anti-MAA) recognizing proteins that are modified by adducts formed under oxidative stress (1-3).

The discovery of AMPA, and in particular ACPA, has had a great impact on current pathophysiological hypotheses regarding RA, due to ACPA's striking association with classical RA risk factors. The most important genetic risk factor for RA, the HLA shared epitope (SE) alleles (or the amino acids in distinct HLA DBR1 positions of these alleles) is now known to be primarily associated with the ACPA-positive subset of disease (4). This has given rise to hypotheses of different pathophysiological mechanisms underlying ACPA-positive versus ACPA-negative RA. Although a similar predilection for ACPA-positive RA has also been described with regard to smoking, more recent reports support the view that smoking is more strongly associated with rheumatoid factor and concurrent presence of multiple antibodies rather than ACPA (5-8).

Despite these insights, the exact etiological pathways leading to autoantibody formation are still unclear. It is conceivable that environmental factors may increase the risk for the development of autoantibodies in a geographic or population-specific manner, as is the case in other autoimmune diseases. For example, research in a specific population of Amerindians in Brazil has led to remarkable insights in fogo selvagem, a blistering autoimmune dermatological disease with resemblance to pemphigus. In this population, a high percentage of healthy individuals have low levels of anti-desmoglein 1 antibodies which has been found to be strongly associated with infestation with black flies. Only in individuals carrying HLA susceptibility alleles does epitope spreading occur, leading to pathogenic antibodies directed against particular subdomains of desmoglein 1 (9). Because black flies occur only endemically and only induce disease in genetically susceptible locals, this disease is an example of an exceptional interplay between population-specific environmental and genetic risk factors. In this way, a rare environmental factor may be intimately involved in disease initiation and can possibly clarify underlying pathophysiological mechanisms of disease.

This example illustrates that investigating autoantibody responses in diverse locations and ethnic groups can lead to the elucidation of novel factors playing a role in the development of the autoantibody response and of the resulting disease. RA occurs in many ethnically diverse populations around the world, with different genetic frameworks and environmental exposures. Therefore, we set out to characterize the AMPA response in four ethnically diverse RA populations originating from four different continents, since differences in autoantibody presence could point to novel risk factors and shed more light on the development of AMPA in RA.

METHODS

Study population

The study population consisted of four cohorts of patients fulfilling the 1987 revised ACR criteria for RA (10). It was not possible to obtain information on the fulfillment of the 2010 RA criteria for all cohorts. All patients supplied informed consent, and study protocols were approved by the relevant local ethics committee.

The Dutch RA patients (NL) were part of the Early Arthritis Cohort (EAC), a prospective cohort initiated in 1993 at the Leiden University Medical Center (LUMC) (11, 12). Patients were of white European ancestry. RA was diagnosed at 1-year follow-up and sera used in this study were collected at this time point. Healthy controls were recruited from the Leiden area.

The Canadian patients and controls came from an Indigenous North American population in Manitoba: First Nations Peoples in Canada (FN), who have an unusually high prevalence of RA (13, 14). All patients and controls were of self-reported Cree and Ojibway descent. Serum was collected either at the baseline visit or at a subsequent visit.

In Japan (JP), RA patients and healthy controls of Japanese descent were recruited in a cross-sectional manner from 5 hospitals in the Kyoto University area (15).

The South African population (SA) consisted of black RA patients with less than 2 years of disease duration recruited from two tertiary hospitals in South Africa participating in the Gauteng Rheumatoid Evaluation Assessment Trial (GREAT) (16). South African controls were healthy black laboratory and clinical personnel at the University of Witwatersrand.

Clinical data

All included patients were ACPA-positive, determined by clinical second- and third-generation anti-CCP enzyme-linked immunosorbent assays (ELISAs) (11, 12, 14-16).

For the Dutch cohort, smoking habits were recorded at the time of sera collection (or at baseline if 1-year data was not available) by a trained research nurse or physician. For

the Canadian cohort, patients filled in an extended smoking history questionnaire at inclusion. For the South African and Japanese cohorts, smoking history (ever or never) was recorded at inclusion.

HLA four-digit genotyping data was available for most patients of each cohort. The SE alleles were defined as described previously (4). Patients were considered SE or HLA-DRB1*03-positive (which may be related to anti-CarP positivity) if they were homo- or heterozygous for these alleles.

Detection of serum autoantibodies by ELISA

By ELISA, total IgG and four AMPAs were tested: anti-CarP fetal calf serum (FCS) IgG, anti-malondialdehyde acetaldehyde (MAA) FCS IgG, anti-acetylated-lysine vimentin IgG (anti-AcVim of the AAPA family), and anti-CCP2 IgG.

Total IgG levels were determined using Bethyl Laboratories reagents and protocol (Bethyl Labs E80-104). In-house ELISAs were conducted essentially as previously described for anti-CarP FCS IgG (1) and anti-CCP2 IgG (17). Anti-AcVim IgG was tested using an OrgenTec kit (Orgentec Diagnostika GmbH, Germany) (3). Anti-MAA FCS IgG used the same in-house protocol as anti-CarP IgG except coating with MAA-FCS and sham-FCS protein, serum dilutions at 1:1000, and detection by rabbit anti-human IgG-HRP antibody. MAA-FCS and sham-FCS were prepared as follows: a solution of 0.5 M tetramethoxypropane and 0.3% hydrochloric acid was incubated at 37 °C for 12 min with agitation. A new solution of 20% of the above, 4% of acetaldehyde, and 1–5 mg/ml FCS was prepared. pH was brought to 4.8, and this solution and sham-FCS were incubated at 37 °C for 2 h. Finally, both samples were dialyzed for 32 h against phosphate-buffered saline, refreshing multiple times, yielding MAA-FCS and sham-FCS at around 4 mg/mL.

For all AMPA ELISAs, absorbance values were converted to arbitrary units per milliliter (aU/mL) using a titration curve of pooled, serially diluted Dutch patient sera positive for that AMPA. We established cohort-specific cut-offs using each cohort's respective healthy controls' mean aU/mL plus two standard deviations, resulting in four distinct cut-offs (**Supplementary Figure 1**). Additionally, for a patient sample to be considered positive, the specific OD on the modified peptide had to be more than 0.1 optical density (OD) above the signal on the non-modified peptide.

Statistical analysis

Chi-squared tests and Kruskal–Wallis tests were used to examine differences between cohorts in the prevalence and levels of AMPAs, as well as cohort characteristics. Logistic regression was used to identify whether smoking was associated with AMPA seropositivity in each cohort, corrected for gender. Logistic regression was also used to examine the association of HLA DRB1 alleles with AMPA seropositivity. Analyses were performed with Stata 14.1: Special Edition (StataCorp LP, TX, USA).

RESULTS

Cohort characteristics

The cohort characteristics of the four geographically and ethnically diverse ACPA-positive RA populations are summarized in **Table 1.**

Cohorts differed significantly for all baseline characteristics except RF status. Notably, patients in the FN and SA cohorts were younger and the NL and SA cohorts had shorter disease durations, while the JP and SA cohorts had few smokers.

Table 1: Cohort characteristics

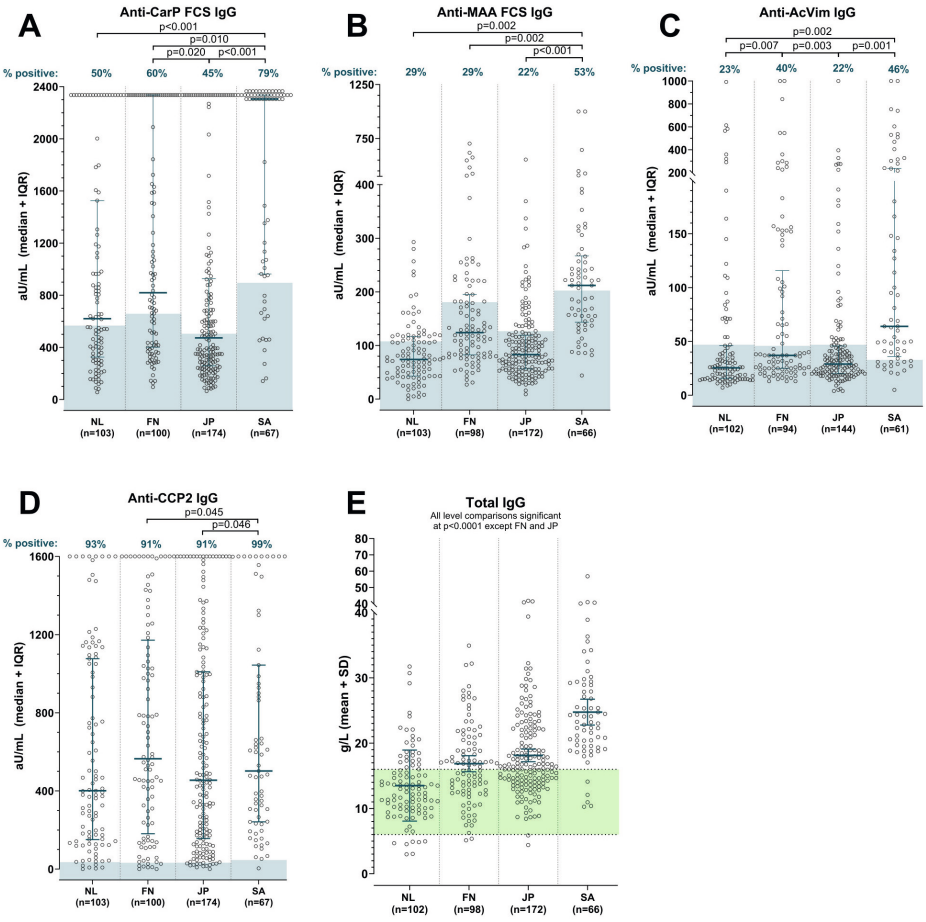
	Netherlands *N* = 103	First Nations (Canada) *N* = 100	Japan *N* = 174	South-Africa *N* = 67	*p*-value
Sex, female	66%	79%	82%	89%[1]	
Age, median years (IQR)	58 (49–65)	48 (35–55)	60 (48–67)	49 (41–56)[7]	
Disease duration, median years (IQR)	1.3 (1.1–1.8)[1]	7.7 (2.5–17.0)	7.5 (4.8–14.8)[1]	0 (0.0–0.0)[1]	
RF positive	90%	93%[1]	95%	100%	
Ever smokers	60%[5]	84%	28%[4]	12%	
HLA SE present	85%	94%[37]	78%	71%[1]	
HLA-DRB1*03 present	14%	5%[37]	0%	32%[1]	
Anti-CarP positive	50%	60%	45%	79%	
Anti-MAA positive	29%	29%[2]	22%[2]	53%[1]	
Anti-AcVim positive	23%[1]	40%[6]	22%[30]	46%[6]	
Treatment at time of sample draw	*N* data = 72	*N* data = 72	*N* data = 174	*N* data = 67	
DMARD-naïve	0 (0%)	6 (8%)	0 (0%)	58 (86%)	
Not DMARD-naïve	72 (100%)	66 (92%)	174 (100%)	9 (14%)	

Superscripted numbers indicate the number of missing data per characteristic per cohort. Significance is based on Pearson's chi-square test, t-test, or Kruskal–Wallis test, as appropriate. Disease duration refers to the time in years between the date of 1st presentation and the date of the sample draw. For South Africa, only 9 patients had samples drawn at 6 months and the rest at baseline. Symptom duration refers to the time in years between the date of symptoms and the date of the sample draw. For DMARD use, categories overlap for patients using multiple agents. Other DMARDs include azathioprine, sulfasalazine, hydroxychloroquine, leflunomide, cyclosporine, bucillamine, gold, and minocycline. p-values printed in bold were statistically significant.

Autoantibody levels and prevalence in ethnically diverse RA populations

Figure 1 displays the levels of the AMPAs in all four cohorts.

Figure 1: *AMPA levels in geographically and ethnically diverse RA populations. Levels in arbitrary units (aU/mL) for four AMPAs and grams per liter (g/L) for total IgG in the serum of four geographically and ethnically diverse RA populations. Patients clustered at the maximum were above the highest standard of the ELISA. Blue shading indicates the patients falling below the cohort-specific positivity cut-off (see Supplementary Figure 1 for data regarding cut-off determination in healthy controls and reactivity to the control peptide); green shading is the normal range for total IgG (established in European cohorts). Lines indicate the median and interquartile range. p-values correspond to chi-square tests on the proportion of patients considered positive, indicated in percentages above graphs.*



Remarkable differences were seen between the cohorts with levels generally being higher in FN and SA RA patients. This pattern of higher reactivity against the PTM antigens could also be seen to a lesser extent in the healthy controls of these two cohorts, although in each population, responses of healthy controls were clearly lower than those of RA patients (**Supplementary Figure 1**). Reactivity against the non-modified control antigens was lower than the PTM antigens in all cohorts. Although South African RA patients had higher anti-lysine and anti-arginine reactivity as compared to RA patients from other cohorts, their reactivity to the corresponding PTM antigen was also higher, leading to a high prevalence of PTM-specific reactivity.

Despite the differences in cohort-specific cut-offs, the higher levels in the FN and SA RA patients also translated into a higher prevalence of several AMPA in these cohorts (**Figure 1** and **Table 1**). Most notably, anti-CarP- and anti-AcVim-antibodies were more prevalent in FN and SA patients.

The level-differences between the cohorts remained when levels were compared only in patients that were positive for AMPA (**Supplementary Figure 2**).

Anti-CCP2 IgG was retested in these ACPA-positive patients to determine levels. Retest positivity for anti-CCP2 IgG was 93% on average (**Figure 1D**) with the most discrepancy found in patients first tested by anti-CCP3 test (not shown). When analyses were performed only in the patients who retested positive for anti-CCP2 IgG (as a sensitivity analysis), the results did not differ.

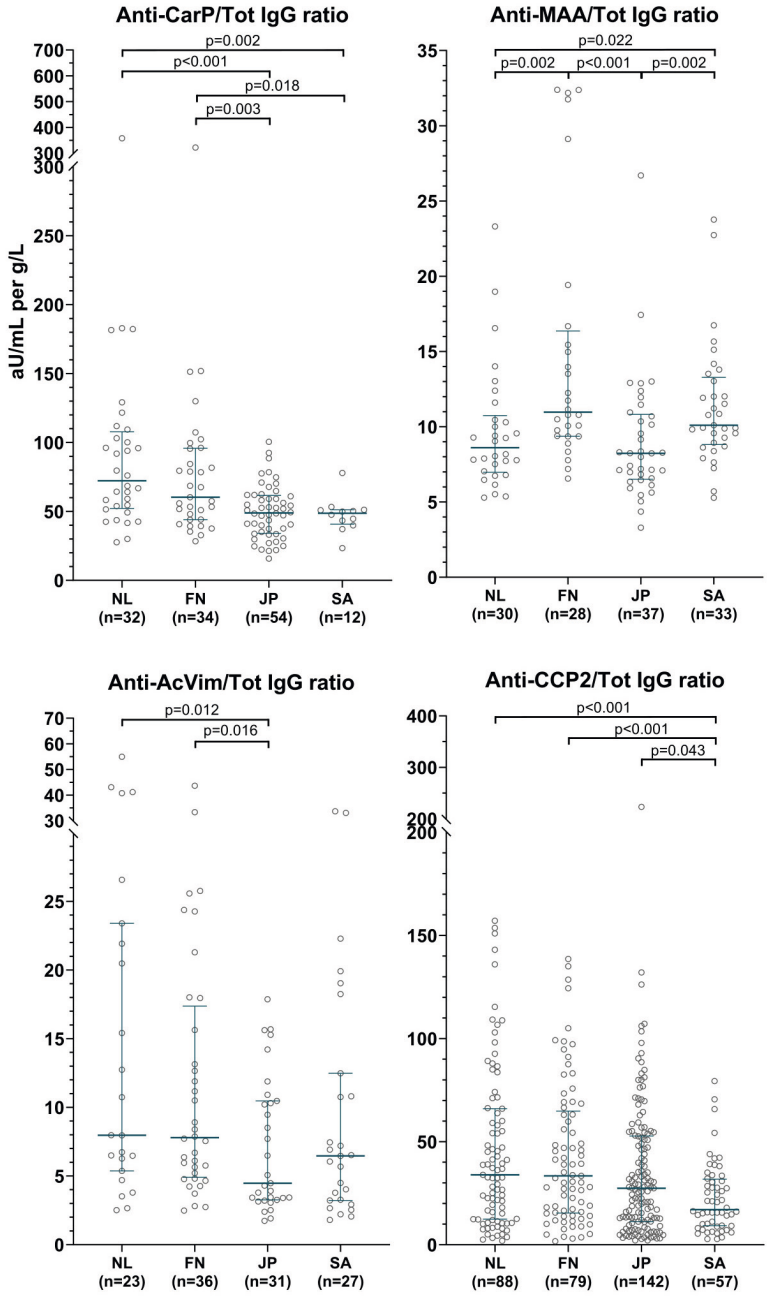
AMPA levels in relation to total IgG

Various studies suggest that differences in total IgG levels may follow ethnographic lines (18-20). To account for the possibility that the AMPA differences correspond to total IgG differences, we also measured total IgG. Levels of total IgG to some extent resembled AMPA levels in that SA patients had the highest levels of both (**Figure 1**). Next, we calculated ratios of AMPA-levels divided by IgG-levels (**Figure 2**).

Although some of these ratios still differed significantly between cohorts, overall differences were now less striking, suggesting that autoantibody-level differences may largely correspond to cohort-specific differences in total IgG.

When patients with levels above the highest standard of an ELISA were removed from the analysis, this did not greatly change the results (**Supplementary Figure 3**).

Figure 2: *AMPA to IgG ratios in geographically and ethnically diverse RA populations. Ratios of levels in arbitrary units (aU/mL) for four AMPAs, per grams per liter (g/L) total IgG in the serum of four RA populations. Ratios are only shown in patients that were positive for the AMPA. Lines indicate median and interquartile range; p-values correspond to Mann–Whitney U tests. The range of aU/mL per g/L is not directly comparable between AMPAs.*



Association of AMPA positivity and smoking, HLA DRB1*03, and SE

Next, we aimed to delineate whether the presence of a certain AMPA might be associated with the known risk factors for autoantibody-positive RA (smoking and HLA risk alleles). To avoid finding spurious associations, we limited our analyses to HLA SE and DRB1*03 alleles. Since the levels of AMPA and the cut-offs differed per cohort (as described above), all association analyses were performed based on dichotomous seropositive versus seronegative autoantibody results, rather than autoantibody levels. The results are displayed in **Figures 3-5**.

Regarding smoking, there was no association with anti-CarP or anti-MAA. However, there appeared to be a positive association with smoking and anti-AcVim in NL and JP, although this did not achieve statistical significance.

For HLA DRB1*03, which was not present in JP and only rarely in FN, there were several positive albeit non-significant associations with the different AMPA. This was found for anti-CarP in NL, (which has been reported before in ACPA-negative patients (21)), and for anti-MAA in several cohorts and anti-AcVim in NL.

With regard to the HLA SE alleles, there was a positive association with anti-CarP in NL. For the other cohorts, there were no associations with anti-CarP, anti-MAA or anti-AcVim in these ACPA-positive patients.

Figure 3: *Association of the different AMPA with smoking in the four populations. The association with smoking was corrected for gender. Please note that in South Africa, correction for gender had a large effect on odds ratios because very few women smoke.*

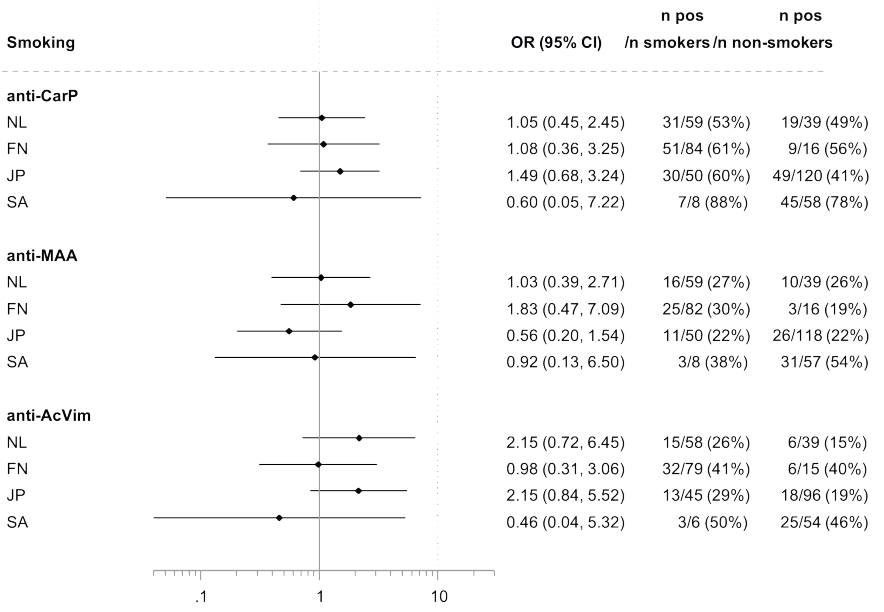


Figure 4: *Associations of the different AMPA with the presence of HLA DRB1*03 alleles. Genetic associations were not corrected for gender*

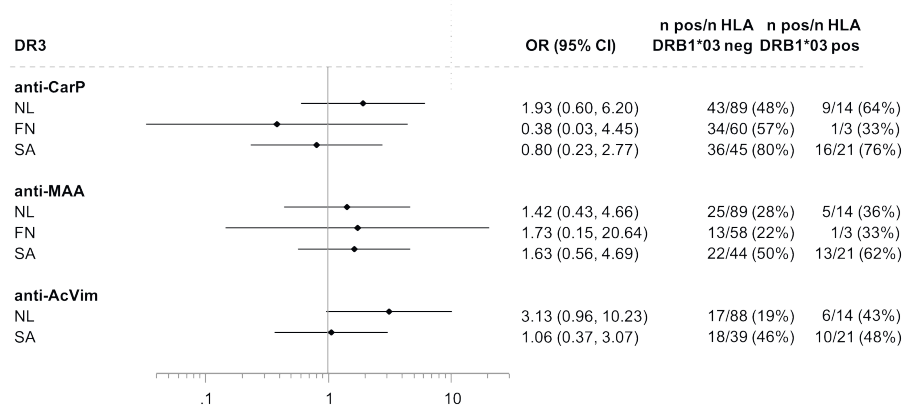
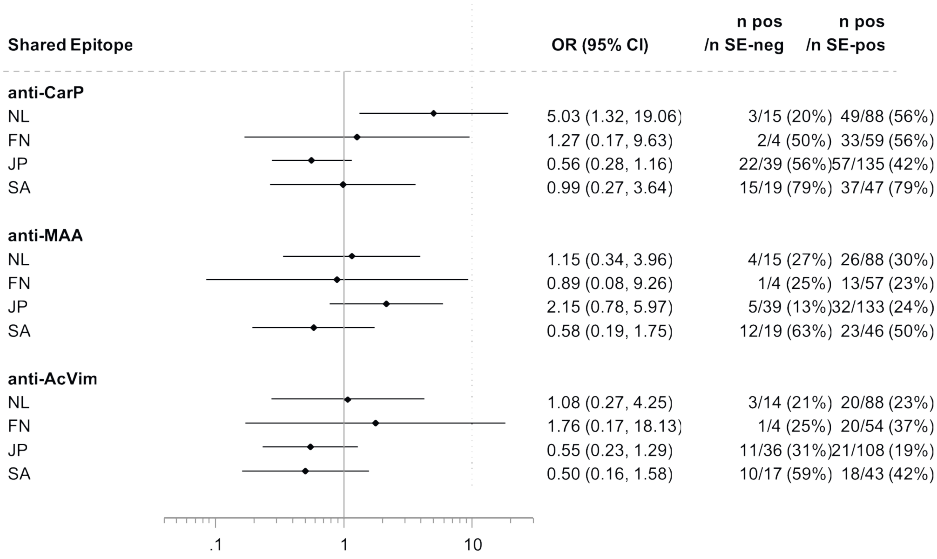


Figure 5: *Associations of the different AMPA with the presence of shared epitope alleles. Genetic associations were not corrected for gender*



DISCUSSION

In light of the recent discovery of several different RA-associated autoantibodies directed against post-translational modifications (AMPA), we determined three major novel AMPA in four geographically and ethnically diverse cohorts of ACPA-positive RA patients, to elucidate whether there might be population-specific risk factors involved in their development. We found differences between cohorts in the prevalence and median levels of anti-CarP, anti-MAA, and anti-AcVim. However, this diversity

corresponded to a large extent to cohort-specific variation in total IgG. Finally, there were no associations between AMPA and the classic risk factors HLA SE, HLA DRB1*03, or smoking that were consistent across all cohorts.

Our results are in line with a limited number of previous publications investigating the prevalence of ACPA and anti-CarP in different ethnic populations and geographic locations. A study from Malaysia investigating RA patients of three different Asian backgrounds (Malay, Chinese, and Indian) reported similar frequencies of ACPA among these patients and an association with HLA SE alleles (22). Likewise, studies from Africa have also found relatively similar ACPA prevalences (16, 23). Regarding the presence of anti-CarP, the previously reported estimates in the same Indigenous North American population and in a similar Japanese cohort are fairly in line with our observations (24, 25). However, to the best of our knowledge, our current study is the first to measure AAPA and anti-MAA in RA patients living outside Europe or the USA and to provide a direct comparison of AMPA measured with the same methods in 4 ethnically diverse RA populations from 4 different continents. Our study leads to new important insights regarding the higher seroprevalence of autoantibodies in populations characterized by high total IgG levels. This is a very relevant finding for future studies focusing on autoantibodies in African populations, which are generally underrepresented in medical research.

The higher AMPA and IgG levels found in black South African RA patients may be due to several independent factors. First, there are multiple publications describing higher IgG levels in people of African descent although the mechanisms underlying this intriguing observation remain unclear (19, 20). While environmental factors such as diet or parasitic infections have been suggested, higher IgG levels in black West Africans residing in Britain for several years have also pointed towards genetic factors (26). Furthermore, treatment of RA is known to be associated with a decrease in AMPA levels (27), and in contrast to the other cohorts, the South African patients were largely treatment-naïve at the moment of serum sampling.

We found no consistent associations of smoking and SE with AMPA positivity in the investigated cohorts. At the most, there was a positive associative trend for smoking and AAPA in NL and JP, but not in the others. This lack of association could have been due to the limited size of the cohorts, although the effect sizes generally did not indicate that greater power would have resulted in significant findings. The positive association of SE with anti-CarP in NL is surprising considering that this has not been found before in analyses of larger datasets (28, 29). This raises the question of whether the fact that the current analysis was performed in ACPA-positive patients only might have resulted in a spurious finding in one single cohort in this case.

Overall, our finding that the various AMPA could be detected in substantial amounts in RA patients of 4 different ethnicities on different continents implies that population-

specific ethnic or geographical risk factors do not seem to play a major role in shaping the AMPA response once ACPA have developed. Contrary to the example of fogo sevalgem in which endemic black flies are a specific factor influencing autoantibody reactivity, this kind of effect does not appear to exist within autoantibody-positive RA. This indicates that the development of a broad AMPA response to different PTMs represents a final common outcome of different pathways leading to the development of ACPA and eventually RA. This hypothesis is in line with murine studies which have shown that immunization with a protein containing one particular PTM can induce cross-reactive AMPA against other PTMs as well (30). However, cross-reactivity between different AMPA cannot be the sole explanation for our findings, considering that (1) both AAPA and anti-CarP-responses have been found to be only partly cross-reactive with citrullinated antigens (3, 31) and (2) anti-MAA-antibodies have been described to display no cross-reactivity at all (32). Thus, it appears likely that other factors, apart from cross-reactivity, also play a role in shaping the AMPA response, and these processes, possibly involving somatic hypermutation of B cell receptors, or survival signals for AMPA-specific B cells, follow a common path in patients with different ethnic and geographical backgrounds.

The main limitation of this study is the fact that we had access only to ACPA-positive RA patients. This precludes any conclusions about the prevalence of each AMPA in the entire RA population consisting of both seropositive and seronegative patients. However, because non-ACPA AMPA occur largely in ACPA-positive RA, this selection does maximize our ability to identify AMPA-positive patients and compare AMPA levels. In addition, the presence of ACPA in all patients does make the comparison between cohorts more straightforward, since differences in ACPA presence would have been a major confounding factor. However, in light of the strong association between HLA risk factors and ACPA, the results regarding genetic associations should be interpreted with extreme caution. Another limitation is the differences in cohort demographics. We attempted to account for these differences by correcting our smoking analyses for gender, which most closely determined smoking habits. Disease duration also differed, which can be seen as a proxy for treatment effects; however, correction for this made some models statistically nonconvergent and did not change the results of the others. Data on clinical characteristics were limited, making it impossible to correlate observed differences in autoantibodies with disease activity, DMARD exposure, and clinical outcomes. Since information on other (environmental) exposures like previous infections, air pollution, or comorbidities was unavailable, we limited our analyses to the communal risk factors which were known for each cohort: smoking and HLA DRB1 alleles. A further limitation is the relatively small sample sizes in each cohort.

Strengths of our study include its innovative design in measuring several different AMPA in 4 different ethnic and geographical cohorts. This leads to relevant insights regarding differences not only in the levels of autoantibodies, but also of total IgG; a fact that has

thus far attracted little attention in the rheumatology autoantibody literature. Another strength of the study is the fact that all AMPA determinations were performed in the same manner. All cohorts were analyzed in the same center on the same day and were represented on each ELISA plate, and the cut-offs were determined using cohort- and ethnicity-matched healthy controls. All these measures serve to maximize comparability between cohorts, which is not a trivial issue as illustrated by the fact that our data show clear differences in calculated cut-offs, thereby underlining the importance of using locally matched controls in determining cut-offs.

CONCLUSION

In these geographically and ethnically diverse ACPA-positive RA populations, AMPA against other post-translational modifications were consistently present, with higher levels in the South African patients. However, total IgG levels were also higher in South African patients, and after correcting for this, differences were less striking. This suggests that in ACPA-positive patients with different genetic and environmental exposures, the development of a broad AMPA response to different PTMs may represent a final common outcome of autoimmune pathophysiology.

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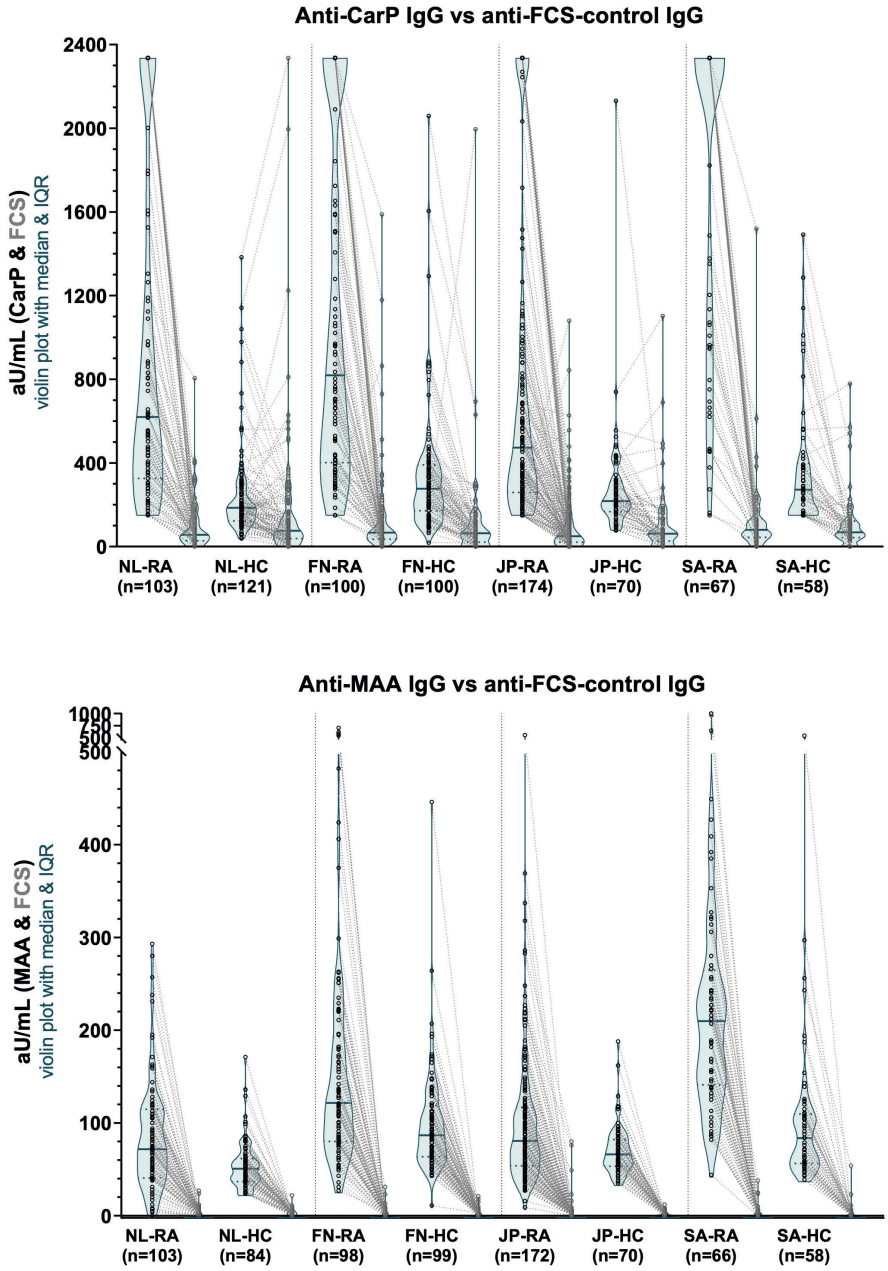
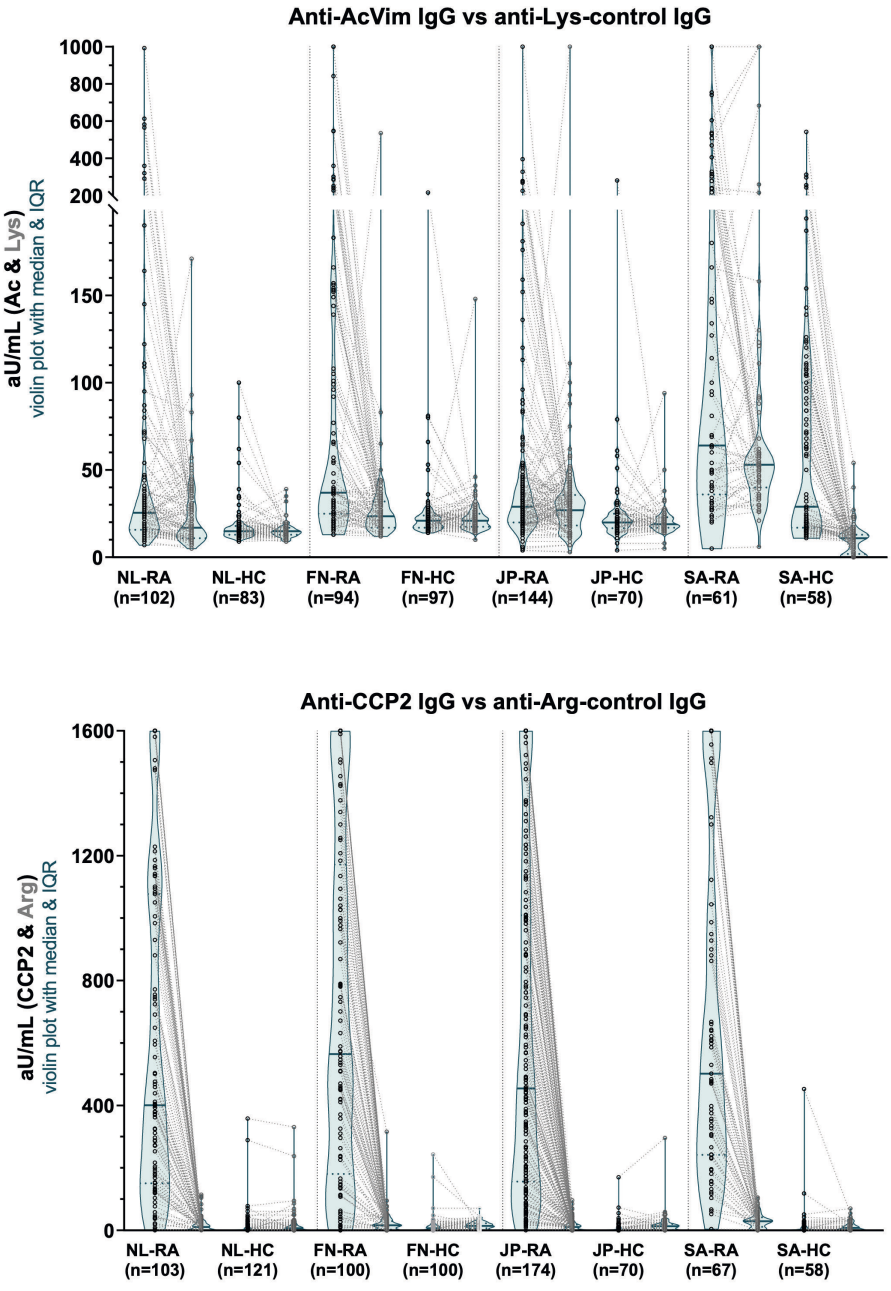
SUPPLEMENTARY FIGURES

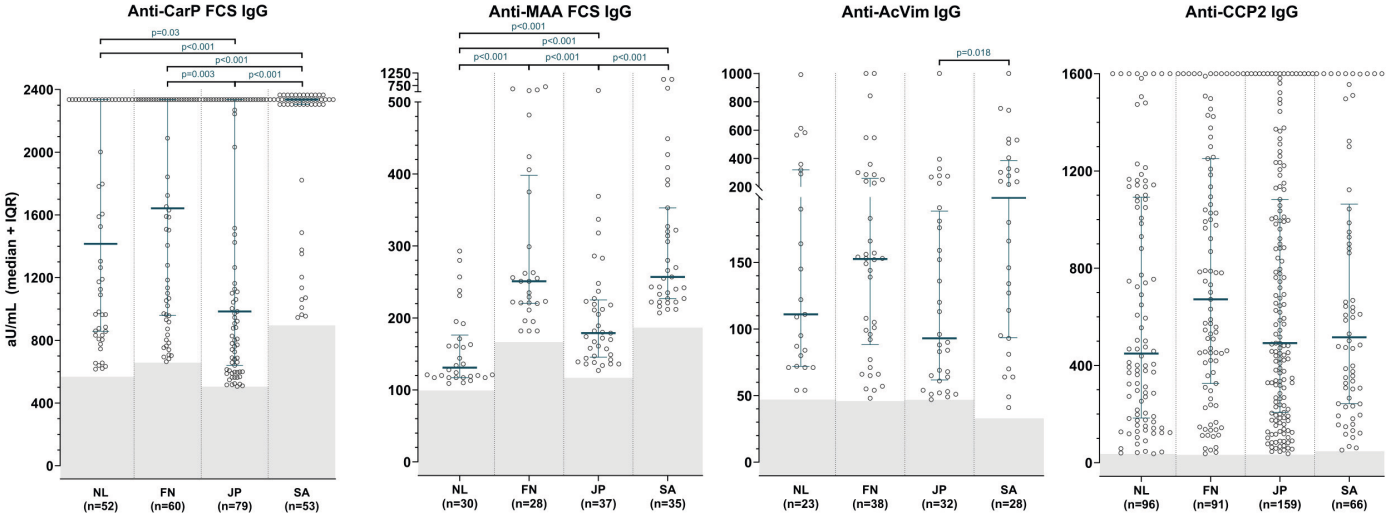
Supplementary Figure 1: AMPA and control peptide reactivity in RA and HCs





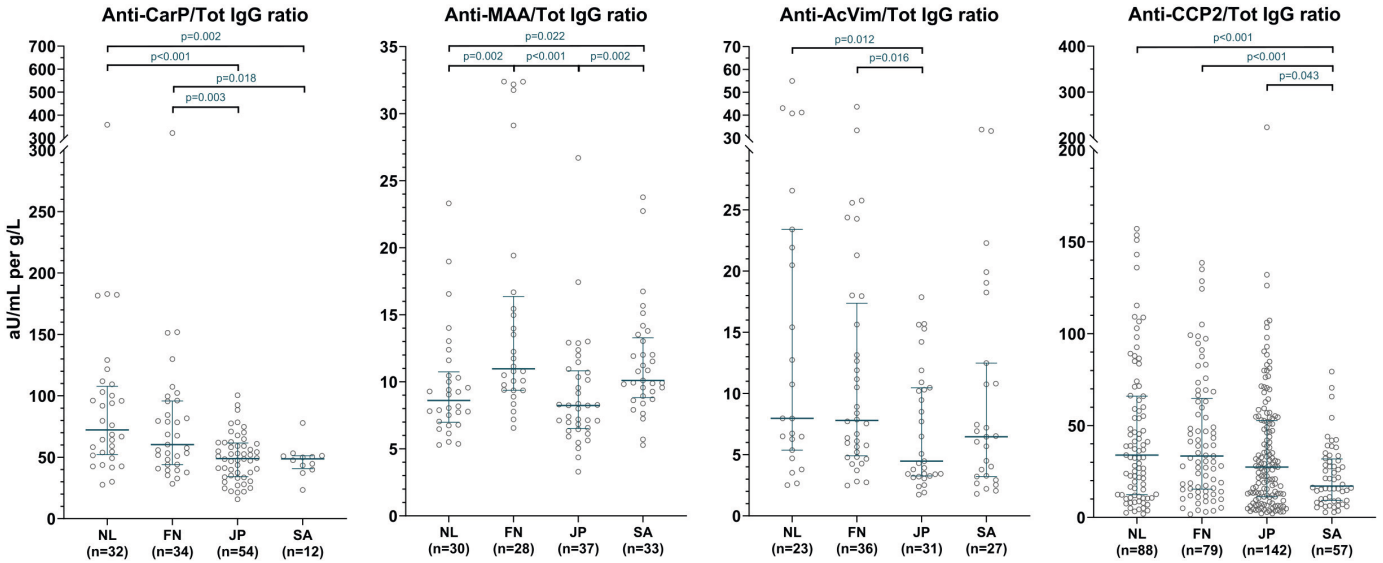
Levels in arbitrary units (aU/mL) for four AMPAs in the serum of four ethnically diverse RA populations and their ethnicity-matched healthy controls (HC). Grey dots indicate reactivity to the control peptide that was tested in tandem with the AMPA (which are shown with black dots). Blue violin plots display the median, interquartile range, and distribution of levels.NL: Netherlands, FN: First Nation, JP: Japan, SA: South Africa, RA: RA patients, HC: healthy controls.

Supplementary Figure 2: Autoantibody levels in patients seropositive for each AMPAs in ethnically diverse RA populations



Levels in arbitrary units (aU/mL) for four AMPAs in the serum of four ethnically diverse RA populations. Patients clustered at the maximum were above the highest standard of the ELISA. Grey shading indicates the cohort-specific cut-off (see Supplementary Figure 2 for data regarding cut-off determination in healthy controls and reactivity to the control peptide). Lines indicate median and interquartile range and p-values correspond to Mann-Whitney U tests.

Supplementary Figure 3: Autoantibody levels in patients seropositive for each AMPAs in ethnically diverse RA populations



Ratios of levels in arbitrary units (aU/mL) for four AMPAs per grams per liter (g/L) total IgG in the serum of four RA populations. Ratios are only shown in patients that were positive for the AMPA and that were also not above the highest standard. Lines indicate median and interquartile range; p-values correspond to Mann-Whitney U tests. The range of aU/mL per g/L are not directly comparable between AMPAs.

CHAPTER 3

Baseline autoantibody profile in rheumatoid arthritis is associated with early treatment response but not long-term outcomes

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ABSTRACT

Background

The autoantibody profile of seropositive rheumatoid arthritis (RA) is very diverse and consists of various isotypes and antibodies to multiple post-translational modifications. It is yet unknown whether this varying breadth of the autoantibody profile is associated with treatment outcomes. Therefore, we investigated whether the composition of the autoantibody profile in RA, as a marker of the underlying immunopathology, influences initial and long-term treatment outcomes.

Methods

In serum from 399 seropositive patients with RA in the IMPROVED study, drawn at baseline and at the moment of drug tapering, we measured IgG, IgM, and IgA isotypes for anti-cyclic citrullinated peptide-2 and anti-carbamylated protein antibodies, IgM and IgA rheumatoid factor, and reactivity against four citrullinated and two acetylated peptides (anti-modified protein antibodies (AMPAs)). We investigated the effect of the breadth of the autoantibody profile on (1) change in disease activity score (DAS)44 between 0 and 4 months, (2) initial drug-free remission (DFR, drug-free DAS44 < 1.6) achieved between 1 and 2 years of follow up, and (3) long-term sustained DFR until last follow up.

Results

Patients with a broad autoantibody profile at baseline had a significantly better early treatment response: ΔDAS 0–4 months of 1–2, 3–4, and 5–6 vs 7–8 isotypes, -1.5 ($p < 0.001$), -1.7 ($p = 0.03$), and -1.8 ($p = 0.04$) vs -2.2. Similar results were observed for AMPA number. However, patients with a broad baseline autoantibody profile achieved less initial DFR. For long-term sustained DFR there was no longer an association with the breadth of the autoantibody response. When assessing autoantibodies at the moment of tapering, similar trends were observed.

Conclusions

A broad baseline autoantibody profile is associated with a better early treatment response. The breadth of the baseline autoantibody profile, reflecting a break in tolerance against several different autoantigens and extensive isotype switching, may indicate a more active humoral autoimmunity, which could make the underlying disease processes initially more suppressible by medication. The lack of association with long-term sustained DFR suggests that the relevance of the baseline autoantibody profile diminishes over time.

Trial registration

ISRCTN11916566. Registered on 7 November 2006. EudraCT, 2006- 06186-16. Registered on 16 July 2007.

BACKGROUND

Patients with rheumatoid arthritis (RA), a chronic autoimmune disease primarily affecting the joints, harbour autoantibodies recognizing several post-translationally modified peptides. The most well-characterised of these are anti-citrullinated peptide 2 (anti-CCP2) antibodies and rheumatoid factor (RF) that are present in approximately 60% of patients. Anti-CCP2 and RF-positive patients have a worse long-term prognosis and are less likely to achieve drug-free remission (DFR)(1-5). Whether they also differ in early treatment response is controversial (5-8).

However, considering only these two autoantibodies may be oversimplifying a complex picture. Novel RA-associated autoantibody systems such as anti-carbamylated (anti-CarP) (9, 10) and anti-acetylated protein antibodies (11) continue to be identified. Moreover, the autoantibody profile is very diverse, with antibodies targeting variable numbers of different peptides with the same post-translational modification, and with marked heterogeneity in isotype usage (12-14). This diversity in the breadth of the autoantibody profile most likely reflects the break of tolerance to multiple autoantigens and the maturity of the humoral autoimmune response (15-17).

It is currently unknown to what extent the breadth of the autoantibody profile influences treatment outcomes. In RA, early initiation of disease-modifying anti-rheumatic drugs (DMARDs) and treat-to-target strategies have improved clinical remission rates (18, 19) and in some patients tapering and withdrawal of DMARDs can be attempted, but not all patients successfully become symptom-free or drug-free. There is a growing need to understand the mechanisms that set apart patients that do achieve early clinical remission or long-term sustained DFR (the closest approximation of disease curative available) (3, 20, 21).

Since autoantibodies are linked to both RA pathophysiology and treatment outcomes, they offer a unique perspective to shed light on the pathophysiological mechanisms underlying RA chronicity. Given the varying composition of the RA autoantibody profile (with its diversity in autoantigen recognition and extensive isotype switching), it appears plausible that the breadth of this profile could be associated with treatment outcomes. No studies to date have investigated the effect of composition of the baseline autoantibody profile on early response to conventional DMARD therapy or long-term DFR. Furthermore, it is also unclear whether the breadth of the profile present at baseline or at the moment of drug-tapering (or both) is more indicative the ability of a patient to reach and maintain DFR. To fill these niches in knowledge, we investigated whether outcomes such as early treatment response to DMARDs and DFR are associated with the breadth of the autoantibody profile at baseline in seropositive patients with RA and at the moment of drug-tapering.

METHODS

Study design

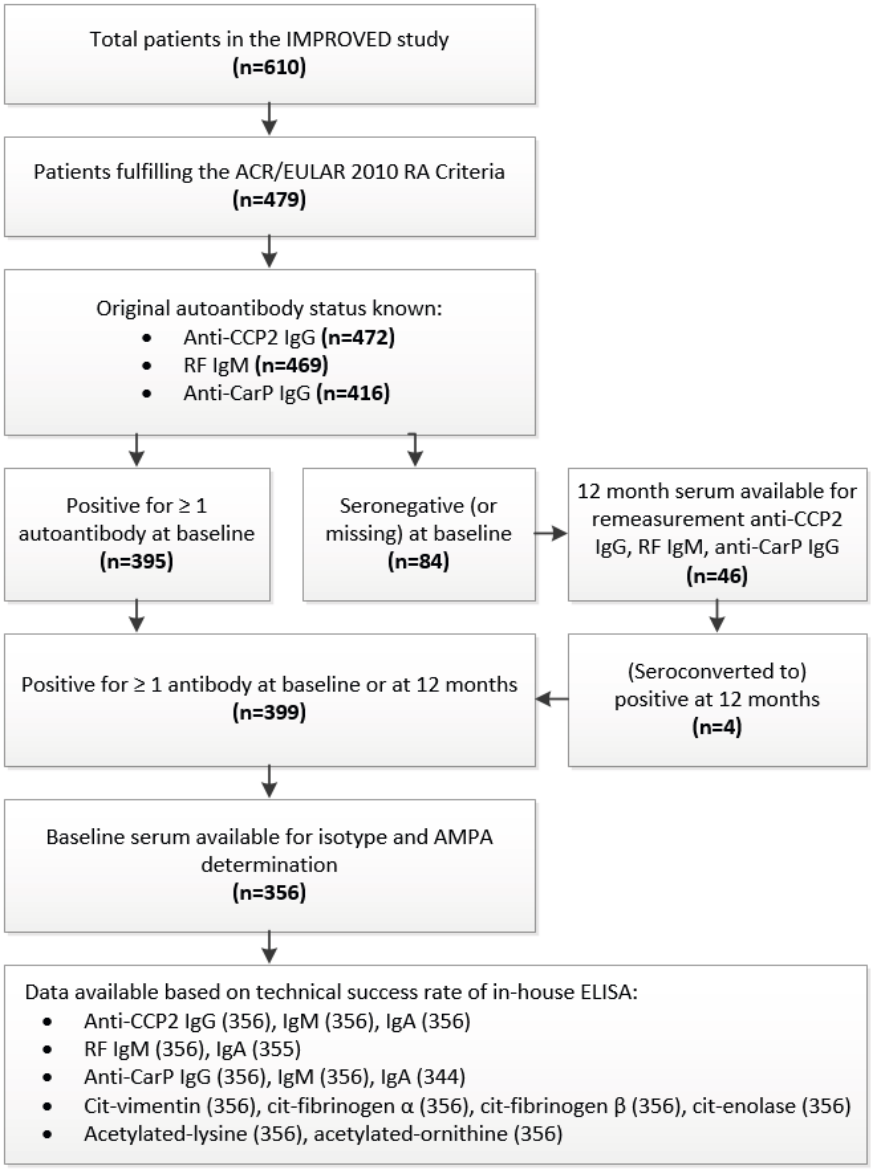
The Induction therapy with Methotrexate and Prednisone in Rheumatoid Or Very Early arthritic Disease (IMPROVED) study is a multicentre, randomized controlled trial that enrolled 610 patients with early (< 2 years) untreated RA or undifferentiated arthritis. It was aimed at change in the disease activity score-remission (DAS44 < 1.6), and for those achieving remission, aimed at drug-free remission (DFR), with treatment adjusted every 4 months according to whether treatment targets had been reached. Initial treatment comprised methotrexate (MTX) and high-dose prednisone, followed by either tapering of medication or randomization to one of two treatment arms: MTX, prednisone, hydroxychloroquine, and sulphasalazine combination (multi-DMARD arm) or MTX and adalimumab combination as described previously (2). According to the protocol, patients tapered and discontinued methotrexate at 8 months if they achieved early remission, allowing them to become drug-free and remain so until the DAS increased to > 1.6.

Patient selection and outcomes

All 479 patients fulfilling the 2010 American College of Rheumatology (ACR)/European League Against Rheumatism (EULAR) RA criteria were selected. Of these patients, those seropositive at baseline by routine clinical testing for anti-CCP2 IgG or RF IgM, or by our in-house assay for anti-CarP IgG (10), were selected (n = 395; see **Figure 1** for detailed selection algorithm). If patients were fully seronegative at baseline, we measured anti-CCP2 IgG, RF IgM, and anti-CarP IgG in serum collected after 1 year of follow up to include any patients that seroconverted to positive, yielding 399 seropositive patients, of whom 356 had baseline, untreated serum available and 209 had 8-month treated serum available for further serological measurements as described subsequently (2).

Main outcomes we investigated were initial DAS change from baseline to 4 months (ΔDAS 0–4 months) and DFR. DAS change from baseline to 4 months occurred under treatment with MTX and high-dose prednisone. DFR was defined as the ability to discontinue medication and remain in remission for (at least) 1 year after achieving DAS44 < 1.6. We differentiated between initial DFR and long-term sustained DFR. Initial DFR was defined as DFR between 1 and 2 years of the study, which due to the protocol could only be achieved by patients that were in early DAS remission at 4 months after tapering prednisone and MTX. Long-term sustained DFR was defined as DFR of at least 1 year duration until the last follow up in all patients (including those who were randomized to the multi-DMARD or adalimumab treatment arm), which is the closest approximation of disease cure currently available for RA. Due to the protocol design, the group of patients that could achieve initial DFR was smaller than (i.e. a subgroup of) all patients who could achieve long-term sustained DFR.

Figure 1: Extended patient selection algorithm.



ACR, American College of Rheumatology; EULAR, European League Against Rheumatism; RA, rheumatoid arthritis; anti-CCP2, anti-citrullinated protein 2; RF, rheumatoid factor; anti-CarP, anti-carbamylated protein antibodies; AMPA, anti-modified protein antibodies; Cit, citrullinated

Serological measurements

Enzyme-linked immunosorbent assay (ELISA) was used essentially as described previously to measure anti-CCP2 IgG, IgM, and IgA(22), RF IgM and IgA(12), and anti-CarP IgG, IgM, and IgA (10, 23) in baseline serum from untreated patients. We also conducted fine-specificity ELISA for IgG directed against four citrullinated peptides: citrullinated-vimentin 59-74, citrullinated-fibrinogen β 36-52 and α 27-43, and citrullinated-enolase 5-20 (24). Last, ELISA for anti-acetylated lysine and anti-acetylated ornithine IgG (Orgentec Diagnostika GmbH, Germany) was performed as previously described (11).

Absorbance was converted to arbitrary units per millilitre (aU/mL) using a standard curve of pooled, serially diluted highly positive patient serum. Samples were considered positive if they fell above the cutoff of the mean aU/mL value plus two standard deviations in serum samples from 76 healthy controls from the Leiden area, run in tandem with the samples for each ELISA. Because antibodies may be aspecifically directed to the unmodified variant of the peptide/protein of interest, we applied a specificity criterion to each ELISA. For anti-CCP2 IgA and IgM, the difference between the citrullinated and unmodified optical density (OD) had to be more than 0.1; for anti-CarP and anti-acetylated peptide ELISAs, the difference (aU/mL) between the modified and unmodified signal had to be above the cut-off. Since previous experiments revealed minimal aspecific signals for the citrullinated fine-specificity ELISA, no specificity criterion was applied.

The technical success rate of the ELISA was at least 96% (**Figure 1**). There was good agreement in positivity between the original baseline measurement performed routinely for anti-CCP-antibodies and RF at inclusion and the in-house baseline re-measurement (**Additional file 1: Figure S1**).The first and second in-house measurement of anti-CarP IgG showed fair agreement (**Additional file 1: Figure S1**). Positivity for the various isotypes measured largely overlapped (**Additional file 1: Figure S2**).

Statistical analysis

We constructed categories reflecting the breadth of the antibody response that consisted of the sum of positive antibody tests. First, the number of isotypes present; both in total (anti-CCP2 IgG, IgM, IgA; RF IgM, IgA; anti-CarP IgG, IgM, IgA; range 1-8) and per family (anti-CCP2 and anti-CarP range 1-3; RF range 1–2). Second, the number of IgG anti-modified peptide responses, both in total (anti-CCP2 IgG, anti-CarP IgG, anti-citrullinated-vimentin 59-74 IgG, anti-citrullinated-fibrinogen β 36-52 IgG, and α 27-43 IgG, anti-citrullinated-enolase 5-20 IgG, anti-acetylated-lysine IgG, anti-acetylated-ornithine IgG; range 1–8) and per modification (citrullinated peptides range 1–4; acetylated peptides range 1–2). Differences between categories were calculated using analysis of variance (ANOVA) for continuous outcomes (DAS and ΔDAS 0–4 months), adjusted for age, gender, and smoking (ever/never), and baseline body mass index and Health Assessment Questionnaire (HAQ) score, which were independent predictors

of early remission in the IMPROVED study (5). Binary logistic regression was used for categorical outcomes, adjusted as above, with the analyses of initial DFR additionally adjusted for baseline DAS and the analyses of long-term sustained DFR additionally adjusted for baseline DAS and treatment arm. Holmes-Bonferroni methods were used to correct the alpha level for multiple testing, assuming the same number of hypotheses as pairwise comparisons made. All reported p values are derived from the analysis models following correction; only p values that remained significant after correction for multiple testing are reported in the figures.

RESULTS

Antibody positivity at baseline and 8 months

At baseline in the full cohort, 68% (323/472) of patients were anti-CCP2 IgG positive, 70% (330/469) were RF IgM positive, and 39% (162/416) were anti-CarP IgG positive. Within the patients that were positive for at least one of these autoantibodies at baseline or at 1 year (n = 399), we (re)measured anti-CCP2, RF, and anti-CarP isotypes and anti-citrullinated and anti-acetylated peptide antibodies in baseline serum and in 8-month serum. Since we selected patients based on baseline seropositivity of anti-CCP2 IgG, RF IgM, or anti-CarP IgG, the high rates of positivity for these antibodies at baseline and 8 months are to be expected (**Table 1**). The lower rates of antibody positivity at 8 months compared to baseline are largely due to seroconversion from positive to negative in this time period.

Initial change in DAS

We first analysed the association between the patients' baseline autoantibody profiles and initial treatment response. As shown in **Figure 2A**, seropositive patients (defined by the presence of anti-CCP2 IgG and/or RF IgM and/or anti-CarP IgG in the original baseline measurement) had a lower DAS at baseline than triple-negative patients. This was most likely due to the ACR/EULAR2010 RA criteria selection we used; seropositive patients require fewer other clinical items to fulfil the criteria and thus have a lower DAS at baseline than seronegative patients. Notably, despite these differences in absolute DAS, the initial change in DAS from baseline to 4 months was equal between seropositive and seronegative patients (**Figure 2B** and **Additional file 1: Figure S3A**), also after correction for relevant covariates.

Strikingly, within the seropositive patients, initial DAS response in patients with many isotypes was more pronounced than in those with few isotypes (ΔDAS 0–4 months of 7–8 isotypes vs 1–2, 3–4, and 5–6 isotypes, respectively: -2.2 vs -1.5 ($p < 0.001$), -1.7 ($p = 0.003$), and -1.8 ($p = 0.001$)) (**Figure 2C**, **D** and **Additional file 1: Figure S3B**). This pattern remained when analysing the number of isotypes present separately for each antibody family: those with more isotypes had better initial DAS response than those with fewer isotypes, and were statistically significant (after correction for multiple testing) for the RF and anti-CarP families (**Figure 2G**).

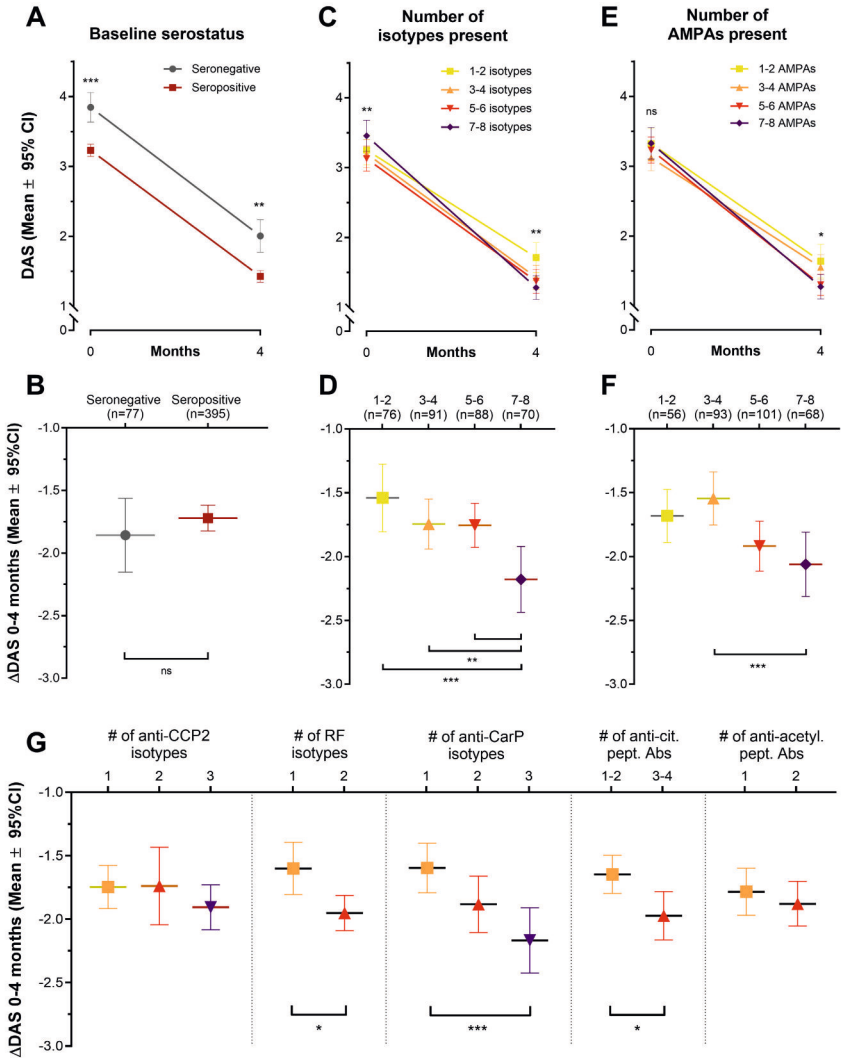
Table 1: Baseline characteristics and antibody positivity.

	Baseline (N = 356)	8 months (N = 209)
RA (2010 criteria), n (%)	356 (100%)	-
Female sex, n (%)	243 (68%)	-
Age, mean years (SD)	51.2 (13.2)	-
Symptom duration (weeks), median (IQR)	18 (9-35)[a]	-
Ever smokers	165 (47%)[a]	-
DAS, mean ± SD	3.3 (0.9)	-
Anti-CCP2 IgG, n (%)	292 (82%)	168 (80%)
Anti-CCP2 IgM, n (%)	146 (41%)	62 (30%)
Anti-CCP2 IgA, n (%)	150 (42%)	58 (28%)
RF IgM, n (%)	267 (75%)	121 (58%)
RF IgA, n (%)	212 (60%)[a]	84 (40%)[a]
Anti-CarP IgG, n (%)	175 (49%)	64 (31%)
Anti-CarP IgM, n (%)	141 (40%)	35 (17%)
Anti-CarP IgA, n (%)	109 (32%)[a]	23 (11%)
Anti-cetyl-Lysine IgG, n (%)	130 (37%)	67 (32%)
Anti-Acetyl-Ornithine IgG, n (%)	252 (71%)	132 (63%)
Anti-Cit-Vim IgG, n (%)	208 (58%)	100 (48%)
Anti-Cit-Fib α IgG, n (%)	101 (28%)	29 (14%)
Anti-Cit-Fib β IgG, n (%)	213 (60%)	105 (50%)
Anti-Cit-Eno IgG, n (%)	115 (32%)	58 (28%)
Number of isotypes, median (IQR)	4 (2–6)[a]	3 (1–4)[a]
Number of AMPAs, median (IQR)	4 (2–-6)	4 (2–5)

Vim vimentin, Fib fibrinogen, Eno enolase, IQR interquartile range, Lys lysine, Orn ornithine, SD standard deviation

aSome missing values. See Figure 1 for number of data available on individual antibody measurements. Available data for symptom duration and smoking, n = 355; for anti-CarP IgA, n = 344 at baseline; for number of isotypes n = 343 at baseline; n = 208 at 8 months

Figure 2: *Disease activity score (DAS) (mean +/- 95% confidence intervals) over 4 months of treatment and mean initial change in DAS from baseline to 4 months (ΔDAS 0–4 months), separated by baseline serological status and breadth of autoantibody response.*



a, b DAS over time and ΔDAS 0–4 months separated by baseline autoantibody seropositivity based on anti-citrullinated protein 2 (anti-CCP2) IgG, rheumatoid factor (RF) IgM, or anti-carbamylated protein (anti-CarP) IgG positivity. Based on availability of antibody data, the total number of patients included in a and b is 472. c, d Within baseline seropositive patients, DAS over time and ΔDAS 0–4 months separated by the total number of isotypes present (anti-CCP2 IgG, IgM, IgA; RF IgM, IgA; and anti-CarP IgG, IgM, IgA). Due to the technical success rate of isotype measurements, and some seropositive patients testing negative upon re-measurement (see Additional file 1: Figure S1), the total number of patients included in c and d is 325. e, f Within patients seropositive at baseline, DAS over time and ΔDAS 0–4 months separated by the total number of anti-modified peptide antibodies (AMPAs) present (anti-CCP2 IgG, anti-CarP IgG, anti-citrullinated-vimentin

*59-74 IgG, anti-citrullinated-fibrinogen β 36-52 IgG and α 27-43 IgG, anti-citrullinated-enolase 5-20 IgG, anti-acetylated-lysine IgG, and anti-acetylated-ornithine IgG). Additional file 1: Figure S1), the total number of patients included in e and f is 318. g Within baseline seropositive patients, ΔDAS 0–4 months separated by the number of isotypes present per antibody family and for the number of antibodies present to citrullinated or acetylated peptides. Reported p values are adjusted for multiple testing using Holmes-Bonferroni methods. ns, not significant (p ≥ 0.05); *p < 0.05; **p < 0.01, ***p < 0.001. Anti-Abs,Abs, anti-acetylated peptide antibodies.*

There was the same dose-dependent association between breadth of the autoantibody profile and DAS decline when analysing the overall number of AMPAs present. Initial DAS response in seropositive patients with many AMPAs was better than in those with few AMPAs, although this was not always statistically significant after correction for multiple testing: ΔDAS 0–4 months of 7–8 AMPAs vs 1–2, 3–4, and 5–6 AMPAs, respectively: -2.1 vs -1.7 (p = 0.016), -1.5 (p < 0.001), and -1.9 (p =0.22) (**Figure 2e, f** and **Additional file 1: Figure S3C**). This pattern was also present when analysing the number of antibodies present per post-translational modification, and was significant for citrullinated peptides: ΔDAS 0–4 months of 3–4 vs 1–2 citrullinated peptides was -2.0 vs -1.6 (p = 0.01) (**Figure 2g**). No single isotype or antibody was disproportionally associated with a better initial DAS response (**Additional file 1: Figure S4A**).

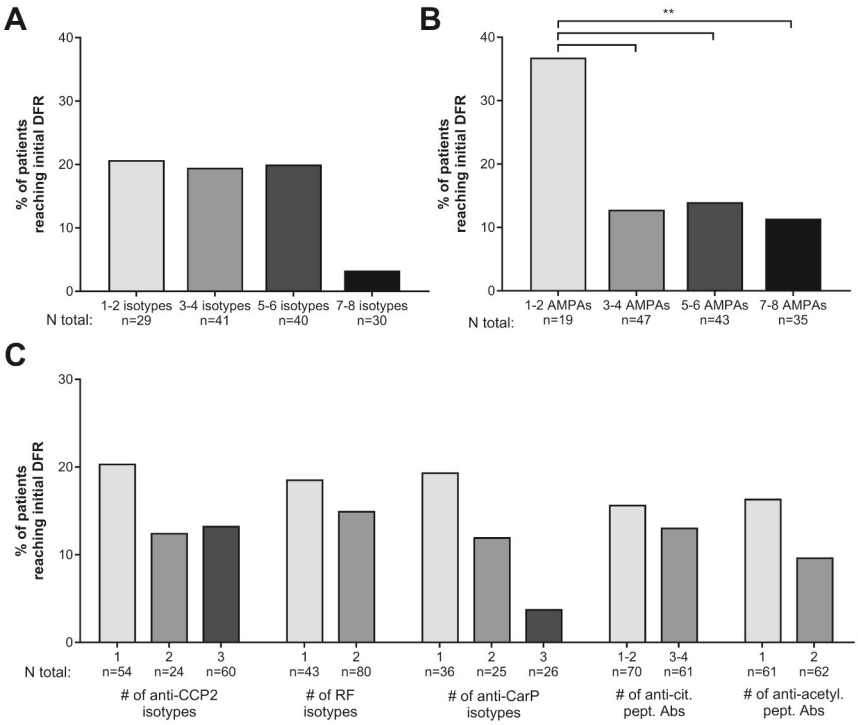
Initial successful drug discontinuation

To investigate whether the autoantibody profile at baseline or at the moment of tapering was also relevant for more long-term treatment outcomes, we next examined whether the autoantibody response is associated with ability to discontinue medication and remain in remission for one year after reaching early remission (initial DFR), independently of factors also associated with this outcome (see "Statistical analysis"). In line with previous findings (2), patients with RA who were positive for anti-CCP2 IgG and/or RF IgM were less likely than their negative counterparts to reach initial DFR, although this difference was not significant: 17% of anti-CCP2 IgG positive versus 20% of negative patients (p = 0.14) and 16% of RF IgM positive versus 19% of negative patients (p = 0.43). Anti-CarP IgG positive patients were also less likely to reach initial DFR than negative patients (11% vs 22%; p = 0.03). Since we selected patients on ACR/EULAR2010 RA criteria, thereby enriching for seropositivity, the differences between anti-CCP2 IgG, RF IgM, and anti-CarP positive and negative patients found here were less pronounced than previously reported in the entire IMPROVED study population because in the current study patients negative for one of these antibodies were by definition positive for another (2).

Interestingly, while a broad baseline autoantibody response was favourable for initial DAS response, it was unfavourable for the chance of achieving initial DFR (**Figure 3**). Within patients seropositive for anti-CCP2 IgG, RF IgM, or anti-CarP IgG at baseline, there was a non-significant trend indicating that patients with more isotypes achieve less initial DFR (1–2, 3–4, and 5–6 isotypes vs 7–8 isotypes, respectively: 21% (p = 0.07), 20% (p = 0.13), and 20% (p = 0.10) vs 3%) (**Figure 3a**). Patients with more AMPAs

also achieved significantly less initial DFR (1–2 AMPAs vs 3–4, 5–6, and 7–8 AMPAs, respectively: 37% vs 13% (p = 0.004), 14% (p = 0.007), and 11% (p = 0.005) (**Figure 3B**).

Figure 3: *Association between baseline autoantibody profile and initial drug-free remission (DFR) in patients seropositive for anti-citrullinated protein 2 (anti-CCP2) IgG, rheumatoid factor (RF) IgM, or anti-carbamylated protein (anti-CarP) IgG at baseline that had serum available for re-measurement (n = 155).*



*Pairwise comparisons between each group were not significant after multiple testing (see text). a Percentage of patients with the specified number of isotypes present reaching initial DFR. The composite number of isotypes consists of the positivity count for anti-CCP2 IgG, IgM, IgA; RF IgM, IgA; and anti-CarP IgG, IgM, IgA. Due to the technical success rate of isotype measurements, and some seropositive patients testing negative upon re-measurement (see Additional file 1: Figure S1), the total number of patients included in a is 140. b Percentage of patients with the specified number of anti-modified protein antibodies (AMPAs) present reaching initial DFR. The composite number of AMPAs consists of the positivity count for anti-CCP2 IgG, anti-CarP IgG, anti-citrullinated-vimentin 59-74 IgG, anti-citrullinated-fibrinogen β 36-52 IgG, α 27-43 IgG, anti-citrullinated-enolase 5-20 IgG, anti-acetylated-lysine IgG, and anti-acetylated-ornithine IgG. c Percentage of patients with the specified number of antibodies present reaching initial DFR. Abs, anti-citrullinated Abs, anti-acetylated peptide antibodies. Reported p values are adjusted for multiple testing using Holmes-Bonferroni methods. ns, not significant ($p \geq 0.05$); *$p < 0.05$; **$p < 0.01$, ***$p < 0.001$*

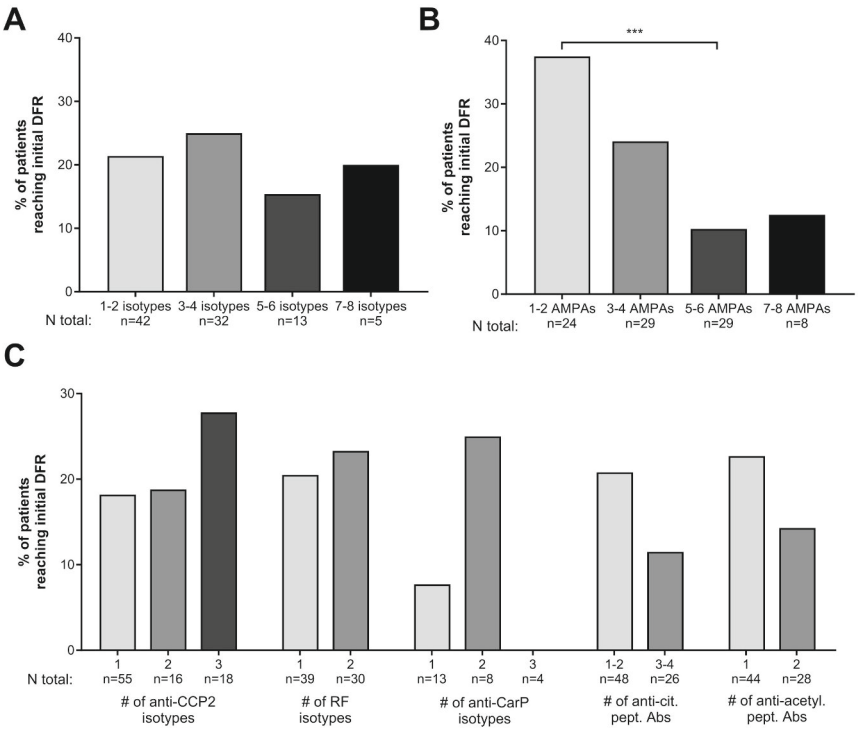
This trend remained when examining the number of isotypes present separately for each antibody family and the number of antibodies present against citrullinated/acetylated peptides (**Figure 3C**). The presence of an anti-CCP2 IgM and/or IgA isotype within patients positive for anti-CCP2 IgG did not decrease the chance of initial DFR, nor did the presence of RF IgA in patients positive for conventional RF IgM (data not shown). Presence of a specific isotype or antibody did not confer increased or decreased chance of reaching initial DFR (**Additional file 1: Figure S4B**).

We investigated whether the autoantibody profile at the moment of tapering had a similar association with initial DFR as the baseline profile. Patients received tapered methotrexate at 8 months if they achieved early remission, allowing them to reach initial DFR between 12 months and 2 years. Seropositive patients with more isotypes at 8 months (i.e. the moment of tapering) tended to achieve less initial DFR than those with few isotypes, but this effect was not as clear as observed in relation to the baseline profile (**Figure 4A**). Patients with more AMPAs at 8 months achieved slightly less initial DFR (1–2 AMPAs vs 3–4, 5–6, and 7–8 AMPAs, respectively: 38% vs 24% ($p = 0.025$), 10% ($p = 0.004$), and 13% ($p = 0.023$) (**Figure 4B**)), but only the comparison of 1–2 AMPAs with 5–6 AMPAs remained significant after correction for multiple testing. When examining the antibody families separately, there was no clear pattern indicating that more isotypes or reactivity against citrullinated/acetylated peptides was associated with less initial DFR (**Figure 4C**).

Long-term sustained DFR

Finally, we wished to determine whether the baseline autoantibody profile was associated with the most favourable long-term outcome of long-term sustained DFR. Fifty-seven percent of patients that had initial DFR also achieved long-term sustained DFR, defined as at least 1 year of DFR lasting until the last follow up, an outcome approximating disease cure. For patients that were not in early remission at 4 months and therefore could not achieve initial DFR, it was still possible to taper medication at a later stage and reach long-term sustained DFR. In the full RA cohort, baseline anti-CCP2 IgG positive patients reached this outcome significantly less often than their negative counterparts (10% vs 26% ($p < 0.001$)); RF IgM and anti-CarP IgG positive patients followed a similar trend (14% vs 19% ($p = 0.05$).

Figure 4: *Association between 8-month autoantibody profile and initial drug-free remission (DFR) in patients seropositive for anti-citrullinated protein 2 (anti-CCP2) IgG, rheumatoid factor (RF) IgM, or anti-carbamylated protein (anti-CarP) IgG at baseline, who had serum available for re-measurement at 8 months (n = 103).*

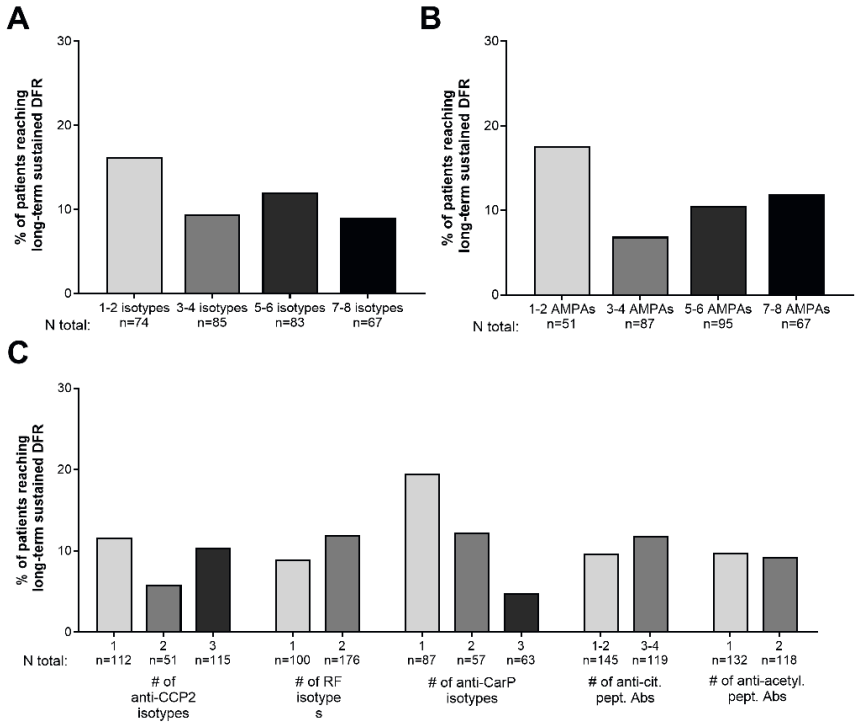


*Pairwise comparisons between each group were not significant after multiple testing (see text). a Percentage of patients with the specified number of isotypes present reaching initial DFR. The composite number of isotypes consists of the positivity count for anti-CCP2 IgG, IgM, IgA; RF IgM, IgA; and anti-CarP IgG, IgM, IgA. b Percentage of patients with the specified number of anti-modified protein antibodies (AMPAs) present reaching initial DFR. The composite number of AMPAs consists of the positivity count for anti-CCP2 IgG, anti-CarP IgG, anti-citrullinated-vimentin 59-74 IgG, anti-citrullinated-fibrinogen β 36-52 IgG and α 27-43 IgG, anti-citrullinated-enolase 5-20 IgG, anti-acetylated-lysine IgG, and anti-acetylated-ornithine IgG. c Percentage of patients with the specified number of antibodies present reaching initial DFR. Abs, anti-citrullinatedAbs, anti-acetylated peptide antibodies. Reported p values are adjusted for multiple testing using Holmes-Bonferroni methods. ns, not significant (p ≥ 0.05); *p < 0.05; **p < 0.01, ***p < 0.001*

In contrast to the previous results on initial DFR (broad baseline autoantibody response: decreased chance of initial DFR), there was no difference in rates of long-term sustained DFR between seropositive patients with many isotypes versus few isotypes, or between patients with many AMPAs versus few AMPAs (**Figure 5a, b**). Furthermore, when separately assessing the number of isotypes in a family and the number of antibodies present against citrullinated/acetylated peptides, there were also no differences in long-term sustained DFR rates (**Figure 5c**). Only anti-CarP isotypes showed a trend

that was no longer present after correction for multiple testing: one isotype versus two isotypes and three isotypes, respectively: 20% vs 12% (p = 0.49) and 5% (p = 0.02). The presence of an anti-CCP2 IgM and/or IgA isotype within patients positive for anti-CCP2 IgG did not decrease the chance of long-term sustained DFR, nor did the presence of a RF IgA in patients positive for conventional RF IgM (data not shown). Last, positivity to a specific isotype or antibody did not confer increased or decreased chances of reaching long-term sustained DFR (**Additional file 1: Figure S4C**).

Figure 5: *Association of baseline autoantibody profile with long-term sustained drug-free remission (DFR) in patients seropositive for anti-citrullinated protein 2 (anti-CCP2) IgG, rheumatoid factor (RF) IgM, or anti-carbamylated protein (anti-CarP) IgG at baseline (n = 336).*



Pairwise comparisons between each group were not significant after multiple testing (see text). a Percentage of patients with the specified number of isotypes present reaching long-term sustained DFR. The composite number of isotypes consists of the positivity count for anti-CCP2 IgG, IgM, IgA; RF IgM, IgA; and anti-CarP IgG, IgM, IgA. Due to the technical success rate of isotype measurements, and some seropositive patients testing negative upon re-measurement (see Additional file 1: Figure S1), the total number of patients included in a is 309. b Percentage of patients with the specified number of anti-modified protein antibodies (AMPAs) present reaching long-term sustained DFR. The composite number of AMPAs consists of the positivity count for anti-CCP2 IgG, anti-CarP IgG, anti-citrullinated-vimentin 59-74 IgG, anti-citrullinated-fibrinogen β 36-52 IgG and α 27-43 IgG, anti-citrullinated-enolase 5-20 IgG, anti-acetylated-lysine IgG, and anti-acetylated-ornithine IgG. c Percentage of patients with the specified number of antibodies present reaching long-term sustained DFR. Abs, anti-citrullinatedAbs, anti-acetylated peptide antibodies

It is conceivable that patients who achieved early remission had different chances of reaching long-term sustained DFR than the full IMPROVED population examined here, and that results would have been different for the patients who achieved early remission. To investigate this, we performed sensitivity analysis of the association between baseline antibody profile and long-term sustained DFR in only the patients who achieved early remission. The results were the same in this group as in the whole cohort (**Additional file 1: Figure S5**).

DISCUSSION

The present study explored the link between the humoral autoimmune response and clinical outcomes by investigating whether the breadth of patients with RA with a seropositive autoantibody profile was associated with early and late treatment outcomes. We were able to show, for the first time, that the number of autoantibodies at baseline was independently and dose-dependently associated with a greater decrease in the DAS after 4 months of conventional DMARD therapy. Conversely, a broad autoantibody profile at baseline was associated with a smaller chance of achieving DFR at early stages of attempted drug tapering (initial DFR), but not later in the treatment regimen, where long-term sustained DFR was unrelated to the breadth of the autoimmune response. We also found that reassessing the autoantibody profile at the moment of drug-tapering does not provide additional information about the chance of successfully discontinuing medication to that provided by the baseline profile.

We examined three primary outcomes: initial DAS response, initial DFR, and long-term sustained DFR. Little is known about the relationship between initial DAS response and autoantibody profile in RA. Although some studies suggest that seropositive patients with RA with a high level or large number of autoantibodies have a better response to B cell-depleting therapy (25, 26), this is the first study that shows that the magnitude of seropositivity is favourable for DAS response under conventional synthetic DMARD therapy as well.

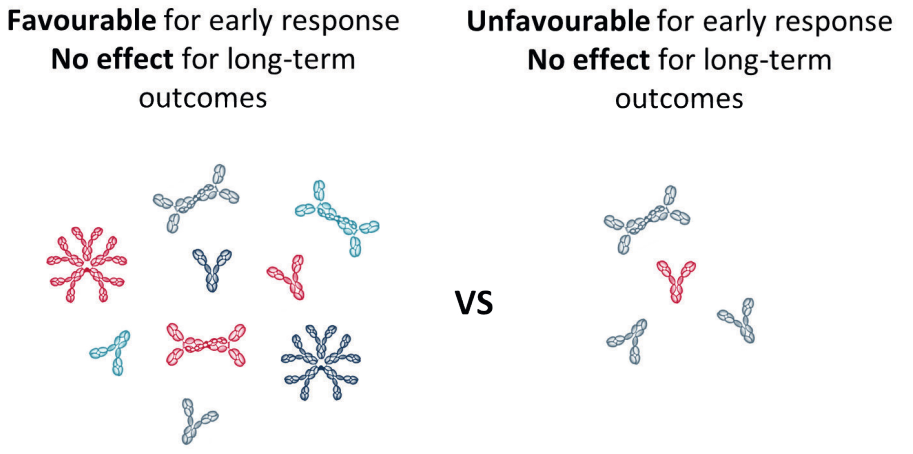
As for the second outcome, initial DFR, Figueiredo et al. recently showed that patients with a broad pattern of AMPA response are at high risk of disease relapse in the first year after DMARD tapering (27). Although the trend we found for initial DFR was significant for the number of AMPAs, our findings do not fully support Figueiredo's observation that a broad autoantibody profile is unfavourable for DFR because we did not identify a dose-dependent effect. The most likely reason is that we used a different, more stringent outcome (i.e. maintaining DFR for a full year) and that we only measured seropositive patients. As such, we had no patients with zero antibodies at baseline, whereas Figueiredo et al. did have such patients, and the contrast with patients with more antibodies was less striking. We also investigated whether the autoantibody profile at the moment of drug tapering (8 months in the IMPROVED study) instead of at baseline

determines the chance of successfully discontinuing medication without disease flare. We found that a broad profile at this moment was not associated with initial DFR. These findings are relevant as they indicate that characterising the autoantibody profile at the moment of tapering does not yield additional information over baseline.

Last, we found that the ability to achieve the third outcome, long-term sustained DFR (at least 1 year of DFR until the last follow up), was independent of the breadth of the baseline autoantibody profile. Instead, baseline seropositivity for anti-CCP2 IgG was the only relevant factor associated with inability to achieve long-term sustained DFR, which is similar to other publications describing presence of anti-CCP2 IgG and RF IgM as a poor prognosticator of long-term drug-free remission (1-5).

Together, these results indicate that the breadth of the autoantibody response in seropositive patients is relevant for early treatment response, somewhat relevant for early attempted drug tapering, but irrelevant for later outcomes (**Figure 6**). The presence of multiple antibodies at baseline may indicate an active, ongoing autoimmune response against various post-translationally modified proteins and antigenic targets present in RA, and reflects extensive isotype switching. It is likely that such active immune responses are more susceptible to suppression by methotrexate and prednisone in the initial stages, as evidenced by the stronger early DAS improvement.

Figure 6: *Summary of results. Colours of the antibodies indicate diversity in antigenic targets, and structures indicate diversity in the isotype usage. A broad baseline profile (left) is favourable for early response, but has no association with long-term outcomes like drug-free remission, as compared to a less broad baseline profile (right). The association with the breadth of the baseline profile diminishes with time*



The association between the breadth of the autoantibody profile and disease outcomes diminished in magnitude as the outcome investigated became further removed from baseline. This implies that the breadth of baseline autoantibody profile is mostly relevant for short-term outcomes, and has implications for the mechanisms underlying disease chronicity. It has recently been shown that memory B-cells expressing anti-citrullinated peptide antibodies (ACPA) persist in the circulation (28, 29), despite conventional DMARD use and remission of synovial disease (30). These data indicate the persistent presence of a population of auto-reactive B cells that is not affected by therapy. Perhaps the best indicator of this long-lived autoimmunity that accounts for the inability to become drug-free in the long run is presence of this persistent B cell population (the presence of which can be best measured by the anti-CCP IgG test), rather than the recognition of multiple modified antigens or the presence of multiple autoantibody isotypes at baseline. This would explain why anti-CCP2 IgG positivity (firmly established in the literature) but not more antibodies (as in this study) is a poor prognostic factor for DFR.

Another explanation why the breadth of the autoantibody response is only important for early outcomes (i.e. DAS and initial DFR) could be that the autoantibody profile changes during treatment preceding late attempted drug tapering. Indeed, this seemed to be the case as some seroconversion happened between baseline and 8 months. However, considering that the profile at the moment of tapering did not yield more information than the baseline profile for the outcome initial DFR, it does not appear very likely that characterising the profile at even later time points would have yielded more information on later outcomes (i.e. long-term sustained DFR). Furthermore, changes in antibody profile were not the focus of the current investigation.

This study has a few limitations. We chose not to correct for baseline DAS in the case of ΔDAS 0–4 months because doing so may lead to biased results when the explicit outcome of interest is change from baseline (31). We also performed in-depth serotyping only in patients positive for anti-CCP2 IgG, RF IgM, and/or anti-CarP IgG, so it is possible that we missed some patients who harboured a certain isotype or fine-specificity. However, it has been shown that the occurrence of IgA and IgM anti-CCP2 and responses to citrullinated and acetylated peptides are largely confined to the anti-CCP2 IgG positive subset. No data are available for anti-CarP isotypes, but it seems likely that our broad definition of seropositivity would have captured most anti-CarP isotypes as well, especially since anti-CarP is known to co-occur with anti-CCP2 IgG or RF IgM (32).

Strengths of the current study include that, to the best of our knowledge, it is the broadest autoantibody profile investigation in RA to date (eight isotypes and six fine specificities within four autoantibody systems), in a cohort with an exceptionally long follow up. Furthermore, it is the first study that investigates the relationship between

the number of autoantibodies and early response to conventional DMARD therapy. The associations we identified cannot be explained by differences in treatment or in demographic characteristics, as we adjusted all analyses for these. Last, we characterized the antibody profile both at baseline and at the moment of attempted drug-tapering, something that, to our knowledge, has not been investigated thus far.

CONCLUSIONS

This large study shows that seropositive patients with RA with a broader autoantibody profile at baseline initially respond better to treatment and have a slightly worse chance of achieving DFR at early stages, but that the magnitude of seropositivity does not affect the ability to taper off medication and remain in remission later in disease. In early stages of disease, a broad autoantibody profile may reflect active humoral immunity, which could make the underlying disease processes initially more suppressible by medication. The importance of the baseline autoantibody profile for treatment outcomes diminishes over time.

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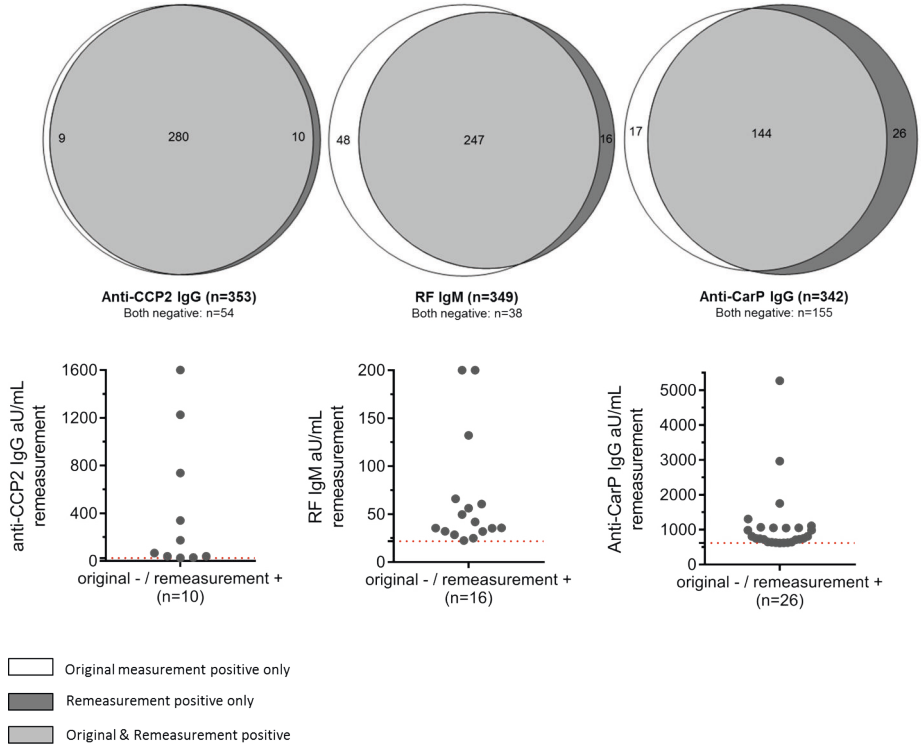
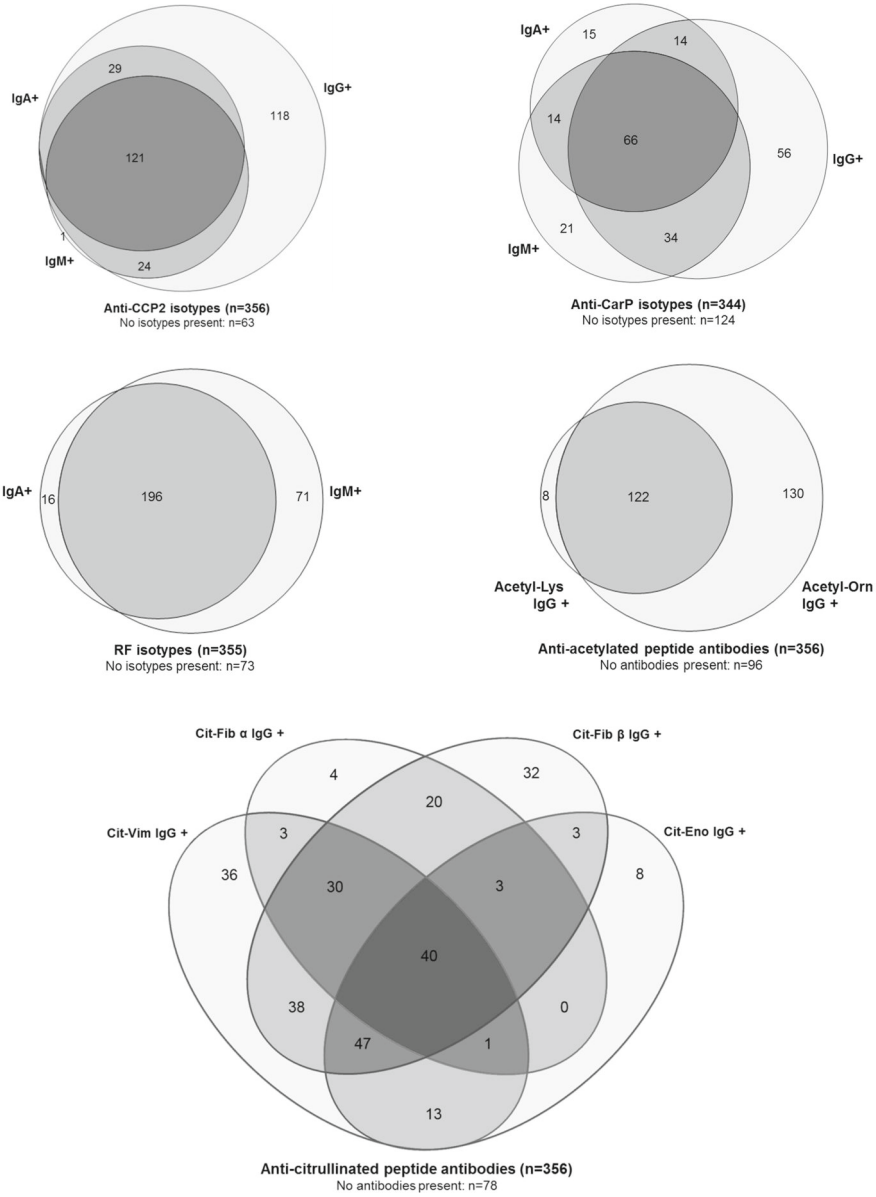
SUPPLEMENTARY FIGURES

Supplementary Figure S 1: *Agreement between previously determined antibody status and remeasurement by ELISA (n=356).* A) *Proportional Venn diagrams displaying agreement between positivity in the previously determined antibody measurement (for anti-CCP2 IgG and RF IgM, by commercial testing; for anti-CarP, in-house ELISA) and the remeasurement (all by in-house ELISA). All samples positive for any antibody were remeasured for the presence of all antibodies. According to Cohen's κ, there was moderate to good agreement between measurements: anti-CCP2 IgG κ=0.82 (p<0.001); RF IgM κ=0.44 (p<0.001); anti-CarP IgG κ=0.75 (p<0.001). The number of patients included in each Venn diagram may be less than 356 due to missing values in the previously determined measurement.* B) *Dotplots representing the arbitrary units (aU/mL) of each antibody for the remeasurement by in-house ELISA within the subset of patients that was previously determined to be negative. Levels upon remeasurement are generally low, suggesting that the discrepancy in positivity between previosly determined and remeasurement may be due to cut-off or inter-test variation. The three patients with high levels upon remeasurement in all three ELISAs are not the same patients.*

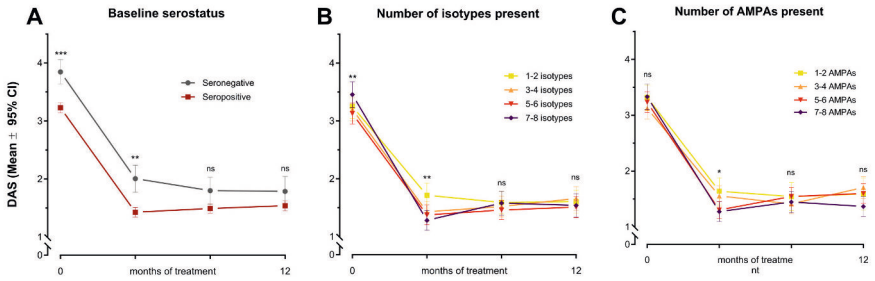


Supplementary Figure S 2: *Overlap of isotypes and antibodies at baseline upon remeasurement by ELISA. Two- and three-way Venn diagrams are proportional to the number of patients indicated in overlap. There was strong overlap of anti-CCP2 IgG positivity and reactivity to citrullinated peptides. Of 292 anti-CCP2 IgG positive patients, only 8,2% had no reactivity to any citrullinated peptide. Conversely, of 64 anti-CCP2 IgG negative patients, 15,6% harboured reactivity to at least one citrullinated peptide.*



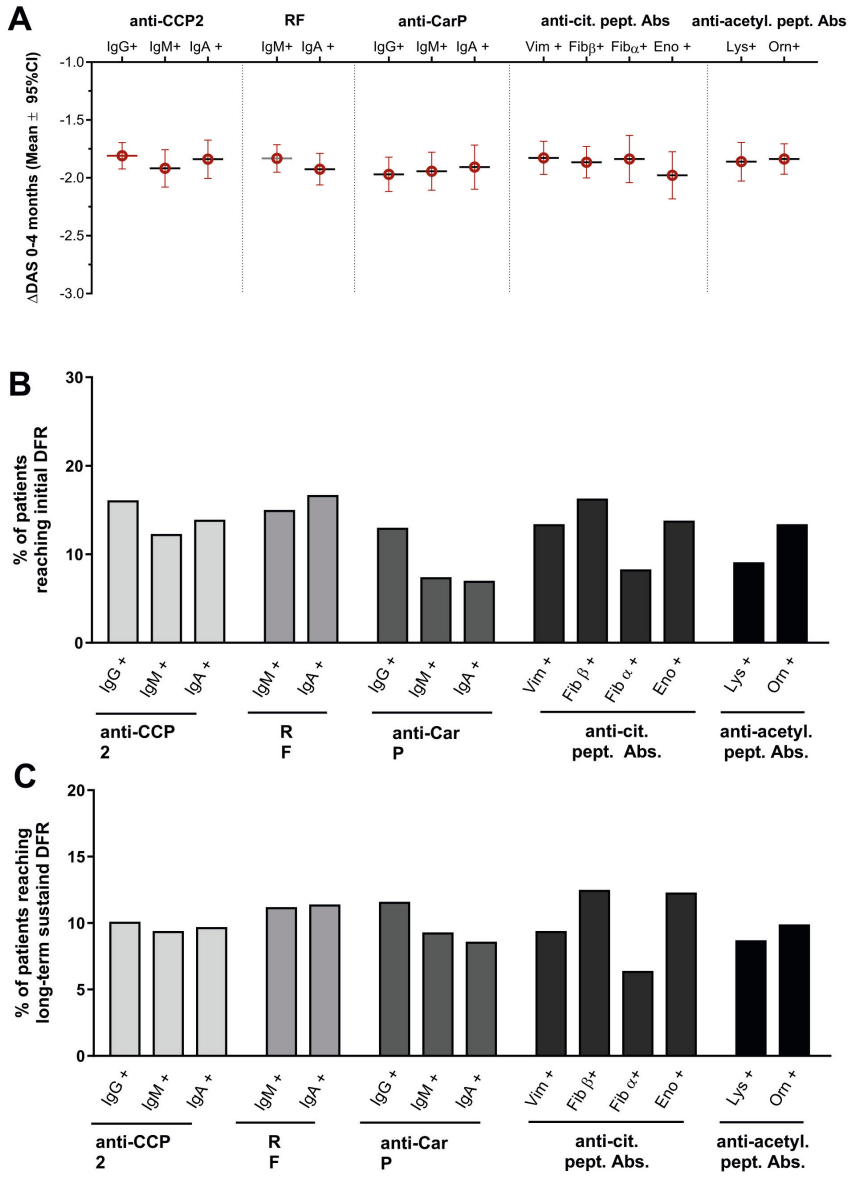
Cit = citrullinated. Vim = vimentin; Fib = fibrinogen; Eno = enolase; Lys = lysine; Orn = ornithine. Acetyl = acetylated. Lys = lysine; Orn=ornithine.

Supplementary Figure S 3: *DAS (mean +/- 95% confidence intervals) over first year of treatment (4 month intervals), separated by baseline serological status and breadth of autoantibody response. Adjusted for age, gender, smoking, body mass index, baseline Health Assessment Questionnaire and baseline DAS. A) DAS over time separated for baseline autoantibody seropositivity based on anti-CCP2 IgG, RF IgM, or anti-CarP IgG positivity. B) Within baseline seropositive patients, DAS over time separated for the total number of isotypes present (anti-CCP2 IgG, IgM, IgA; RF IgM, IgA; anti-CarP IgG, IgM, IgA). C) Within baseline seropositive patients, DAS over time separated for the total number of anti-modified peptide antibodies present (anti-CCP2 IgG, anti-CarP IgG, citrullinated-vimentin 59-74, citrullinated-fibrinogen β 36-52 and α 27-43, citrullinated-enolase 5-20, acetylated-lysine, acetylated-ornithine). Thirty-eight patients were RF IgM positive but had no AMPAs (not shown).*



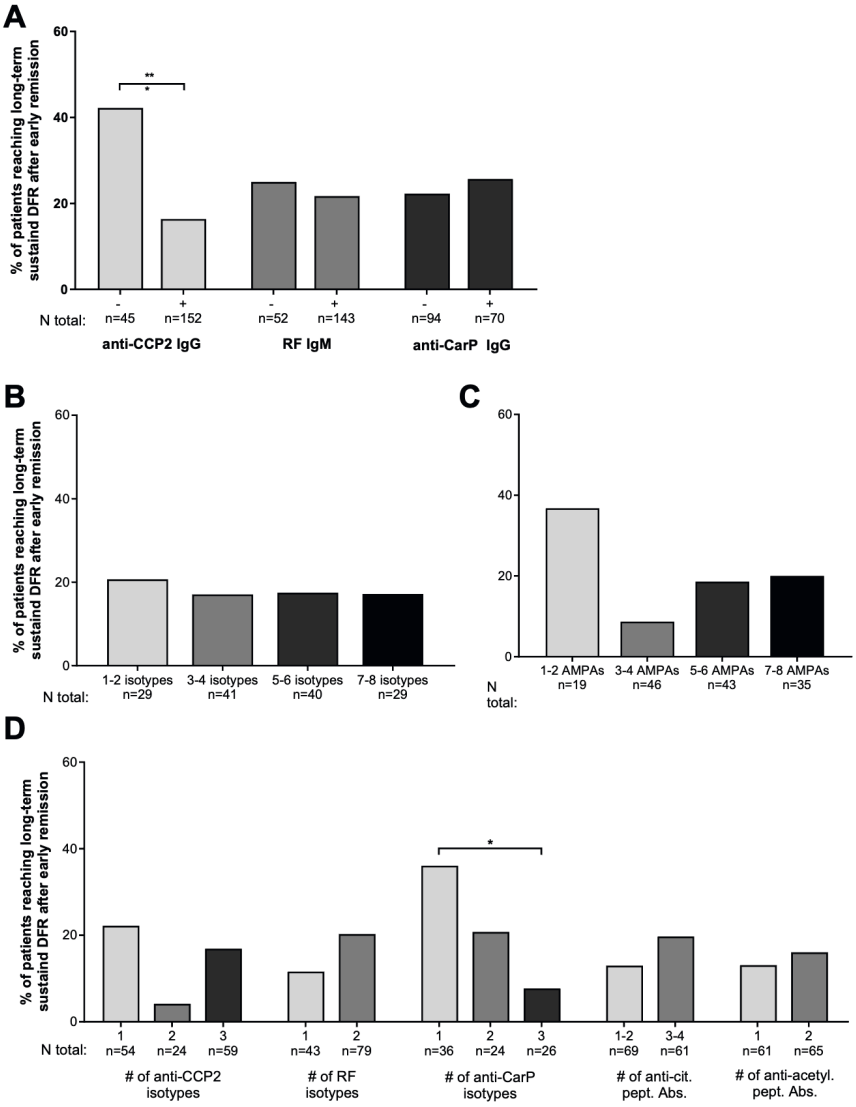
*Reported P-values are adjusted for multiple testing using Holmes-Bonferroni methods. ns: not significant (p≥0.05); *: p<0.05; **: p<0.01, ***: p<0.001*

Supplementary Figure S 4: *Initial change in DAS and DFR outcomes within patients positive for the indicated individual antibody. No statistical testing was possible as patient groups overlap (see Supplementary Figure S 2).* **A)** *Initial change in DAS (mean +/- 95% confidence intervals) from baseline to 4 months within patients positive for anti-CCP2 IgG, RF IgM, or anti-CarP IgG.* **B)** *Percentage of patients positive for the specified antibody that reached initial DFR.* **C)** *Percentage of patients positive for the specified antibody that reached long-term sustained DFR.*



Anti-cit. pept. Abs = anti-citrullinated peptide antibodies; Anti-acetyl. pept. Abs = anti-acetylated peptide antibodies; Vim = vimentin; Fib = fibrinogen; Eno = enolase; Lys = lysine; Orn = ornithine.

Supplementary Figure S 5: *Association of baseline autoantibody profile with long-term sustained drug-free remission within patients that reached early remission and had outcome data available (A; n=199) and in only patients seropositive for anti-CCP2 IgG, RF IgM, or anti-CarP IgG (B-D; n=154). Adjusted for age, gender, smoking, body mass index, baseline Health Assessment Questionnaire and baseline DAS.*



A) Percentage of anti-CCP2 IgG, RF IgM, or anti-CarP IgG positive and negative patients reaching long-term sustained DFR after early remission. B) Within baseline seropositive patients, percentage of patients with the specified number of isotypes present reaching long-term sustained DFR after early remission. The composite number of isotypes consists of the positivity count for: anti-CCP2 IgG, IgM, IgA; RF IgM, IgA; anti-CarP IgG, IgM, IgA. Due to the technical success rate of isotype measurements and some seropositive patients testing negative upon re-measurement

(see Supplementary Figure S2), the total number of patients included in S6B is 139. C) Within baseline seropositive patients, percentage of patients with the specified number of AMPAs present reaching long-term sustained DFR after early remission. The composite number of AMPAs consists of the positivity count for: anti-CCP2 IgG, anti-CarP IgG, anti-citrullinated-vimentin 59-74 IgG, anti-citrullinated-fibrinogen β 36-52 IgG and α 27-43 IgG, anti-citrullinated-enolase 5-20 IgG, anti-acetylated-lysine IgG, anti-acetylated-ornithine IgG. Eleven patients were RF IgM positive but had no AMPA antibodies (not shown). D) Within baseline seropositive patients, percentage of patients with the specified number of antibodies present reaching long-term sustained DFR after early remission.
*Reported P-values are adjusted for multiple testing using Holmes-Bonferroni methods. ns: not significant (p≥0.05); *: p<0.05; **: p<0.01, ***: p<0.001*
Anti-cit. pept. Abs = anti-citrullinated peptide antibodies; Anti-acetyl. pept. Abs = anti-acetylated peptide antibodies

CHAPTER 4

In RA, becoming seronegative over the first year of treatment does not translate to better chances of drug-free remission

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LETTER

In rheumatoid arthritis (RA), it is becoming common to attempt to taper or stop medication, aiming for sustained drug-free remission (SDFR). Autoantibody seropositivity is a poor prognostic factor for this treatment goal. However, autoantibody levels may change and patients may become seronegative, sometimes termed 'immunological remission'(1). Understanding how often this occurs and whether it is favourable for achieving SDFR is important to determine whether becoming seronegative is a meaningful prognostic marker for drug tapering decisions. Furthermore, it will elucidate pathways that lead to long-term resolution of the pathophysiology underlying RA.

To that end, we investigated the relationship between seroconversion and SDFR. In baseline and 1-year serum of 381 patients with seropositive RA, we measured 14 RA-associated autoantibodies by ELISA(2): anti-CCP2 IgG, IgM and IgA; rheumatoid factor IgM and IgA; anti-CarP IgG, IgM and IgA; anti-acetylated lysine vimentin IgG and anti-acetylated ornithine vimentin IgG (Orgentec Diagnostika , Germany); and anti-citrullinated vimentin 59–74 IgG, anti-citrullinated fibrinogen β 36–52 and α 27–43 IgG, anti-citrullinated enolase 5–20 IgG. Patients originated from the IMPROVED study (3), a randomised controlled treat-to-target trial of early (<2 years) untreated RA, steered at disease activity score remission (DAS44<1.6) and DFR, with initial treatment of methotrexate and high-dose prednisone. We investigated whether becoming seronegative over the first year of treatment improves chances of long-term SDFR, defined as remission lasting at least 1 year, starting at any time point and held until the last moment of that individual's follow-up (maximum 5 years).

The prevalence of seroconversion from positive to negative between 0 and 12 months varied substantially depending on the autoantibody from 2% (anti-CCP2 IgG) to 66% (anti-CarP IgA) (**Figure 1**), occurring mostly in low-positive patients (**supplementary table S1**). Demographic and clinical characteristics at baseline and 1 year in patients with seroconversion versus those without did not show marked differences (**supplementary table S1**). Of the 359 patients who had outcome data available, 48 (13.4%) achieved long-term SDFR. Intriguingly, seroconversion from positive to negative was not associated with a greater chance of achieving long-term SDFR for any of the 14 antibodies tested (**figure 1A**). To investigate whether these findings were influenced by low-positive patients whose autoantibody levels merely fluctuated around the cut-off, a sensitivity analysis was conducted including only patients whose baseline autoantibody levels were above the median, with similar results (**supplementary figure S1**). Of the 170 seropositive patients with complete antibody data at 0 and 12 months, only six (3.5%) seroconverted to completely seronegative by 12 months; 33% (2/6) of these completely seroconverted patients achieved long-term SDFR, compared with 11.6% (19/164) of patients who were positive for at least one antibody (p=0.11). Patients who seroconverted to negative for a larger number of autoantibodies did not achieve long-

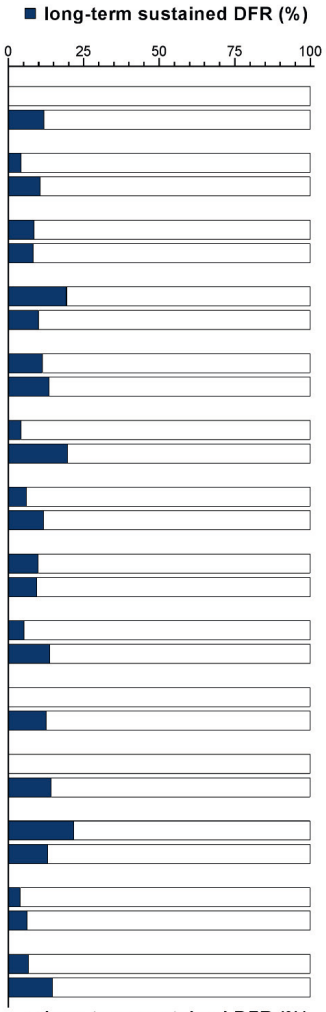
term SDFR more often than those who seroconverted for fewer (**FIGURE 1B**). Relative changes in autoantibody levels between 0 and 12 months did not differ between patients with or without long-term SDFR (data not shown).

The clinical significance of seroconversion in RA and especially its relationship with long-term SDFR, an approximation of disease 'cure' of RA, is a topic of major interest F. Previous studies found no association of seroconversion with remission or radiographic damage (4, 5). We here investigated the association between seroconversion and the most favourable long-term outcome of RA, SDFR, and found no association. Thus, it appears that seroconversion (as measured by current standards) does not identify a group of patients in whom the underlying immunopathology has been favourably modulated, that is, patients in true immunological remission, and is not superior to signals of low inflammatory load (e.g. by DAS (6)) for predicting successful drug tapering. Future studies are needed to identify whether other immunological parameters such as the numbers or phenotype of circulating autoreactive B or T cells might be a better reflection of disease persistence and markers for immunological remission.

Figure 1: (A*) Number and percentage of baseline seropositive patients seroconverting ('Pos to Neg') or non-converting ('Stable pos') between 0 and 12 months are listed on the left, and the percentage of each subset subsequently reaching long-term sustained drug-free remission (DFR) is graphically depicted on the right. (B) Number and percentage of baseline seropositive patients reaching long-term sustained DFR, categorised by the amount of antibody reactivities that were lost (i.e. composite of positive-to-negative seroconversion) between 0 and 12 months. Anti-acetyl pept Abs, anti-acetylated peptide antibodies; Anti-cit pept Abs, anti-citrullinated peptide antibodies; RF, rheumatoid factor*

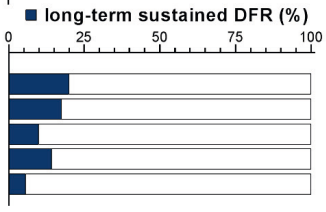
A. Seroconversion per antibody

		N	%
Anti-CCP2	**IgG**	167	
	Pos to neg	4	2%
	Stable pos	163	98%
	IgM	80	
	Pos to neg	23	29%
	Stable pos	57	71%
	IgA	84	
	Pos to neg	24	29%
	Stable pos	60	71%
RF	**IgM**	145	
	Pos to neg	31	21%
	Stable pos	114	79%
	IgA	112	
	Pos to neg	35	31%
	Stable pos	77	69%
Anti-CarP	**IgG**	93	
	Pos to neg	47	51%
	Stable pos	46	49%
	IgM	69	
	Pos to neg	34	49%
	Stable pos	35	51%
	IgA	61	
	Pos to neg	40	66%
	Stable pos	21	34%
Anti-acetyl. pept. Abs	**Lysine IgG**	78	
	Pos to neg	19	24%
	Stable pos	59	76%
	Ornithine IgG	145	
	Pos to neg	15	10%
	Stable pos	130	90%
Anti-cit. pept. Abs	**Vimentin IgG**	118	
	Pos to neg	25	21%
	Stable pos	93	79%
	Fibrinogen α IgG	58	
	Pos to neg	26	45%
	Stable pos	32	55%
	Fibrinogen β IgG	118	
	Pos to neg	23	19%
	Stable pos	95	81%
	Enolase IgG	69	
	Pos to neg	15	22%
	Stable pos	54	78%



B. Change in number of antibodies

		N	%
Change from baseline	Gain of ≥1	30	17%
	No change	42	23%
	Loss of 1	30	17%
	Loss of 2-3	42	23%
	Loss of ≥4	37	20%



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SUPPLEMENTARY FIGURES

Supplementary Table S1 (part 1 of 3): Demographic and clinical differences at baseline and 1 year, between patients that did not seroconvert ("Stable Pos") and those that seroconverted between 0-12 months ("Pos-Neg"). Bold typeface indicates significance after correction for multiple testing by Holmes-Bonferroni methods for 14 tests (14 autoantibodies).

	anti-CCP2 IgG (range: 0-1600 aU/mL)			anti-CCP2 IgM (range: 0-1400 aU/mL)			anti-CCP2 IgA (range: 0-1160 aU/mL)			RF IgM (range: 0-200 aU/mL)			RF IgA (range: 0-200 aU/mL)		
	Stable Pos	Pos-Neg		Stable Pos	Pos-Neg		Stable Pos	Pos-Neg		Stable Pos	Pos-Neg		Stable Pos	Pos-Neg	
N total	163	4	p	57	23	p	60	24	p	114	31	p	77	35	p
Levels Baseline, med (IQR)	**561 (230-1,226)**	**40 (33-81)**	0.00	**538 (381-990)**	**203 (115-273)**	0.00	**269 (102-657)**	**72 (44-156)**	0.00	**117 (76-200)**	**56 (31-81)**	0.00	**200 (94-200)**	**29 (21-50)**	0.00
Age in years, mean ± SD	49 ± 12	56 ± 5	0.27	52 ± 13	52 ± 12	0.97	50 ± 12	53 ± 12	0.38	50 ± 13	53 ± 11	0.27	51 ± 11	52 ± 13	0.61
Symptom duration (weeks), med (IQR)	**23 (10-38)**	**6 (4-7)**	**0.01**	**25 (9-42)**	**21 (11-32)**	**0.58**	**27 (10-48)**	**22 (6-28)**	**0.06**	**22 (10-36)**	**18 (9-30)**	**0.27**	**22 (11-42)**	**20 (8-32)**	**0.25**
BMI (kg/m2), mean ± SD	26 ± 4	25 ± 6	0.66	26 ± 4	26 ± 5	0.82	26 ± 4	27 ± 5	0.66	25 ± 4	26 ± 4	0.32	26 ± 4	27 ± 5	0.08
Female gender (%)	**112 (69%)**	**3 (75%)**	**0.79**	**38 (67%)**	**16 (70%)**	**0.80**	**37 (62%)**	**17 (71%)**	**0.43**	**78 (68%)**	**21 (68%)**	**0.94**	**46 (60%)**	**22 (63%)**	**0.75**
Ever smoker (%)	83 (51%)	0 (0%)	0.04	31 (54%)	12 (52%)	0.86	38 (64%)	13 (54%)	0.38	59 (52%)	10 (32%)	0.05	56 (74%)	12 (34%)	**0.00**
Achieved early remission (%)	**115 (71%)**	**1 (25%)**	**0.05**	**40 (70%)**	**17 (74%)**	**0.74**	**43 (72%)**	**14 (58%)**	**0.24**	**79 (69%)**	**17 (55%)**	**0.13**	**56 (73%)**	**24 (69%)**	**0.65**
RAI index Baseline, med (IQR)	6 (4-9)	8 (4-11)	0.57	6 (4-8)	6 (4-10)	0.82	6 (3-8)	6 (4-10)	0.80	6 (4-9)	7 (6-12)	0.03	6 (4-8)	8 (4-11)	0.23

Supplementary Table S1 (part 1 of 3): Demographic and clinical differences at baseline and 1 year, between patients that did not seroconvert ("Stable Pos") and those that seroconverted between 0-12 months ("Pos-Neg"). Bold typeface indicates significance after correction for multiple testing by Holmes-Bonferroni methods for 14 tests (14 autoantibodies). (continued)

	anti-CCP2 IgG (range: 0-1600 aU/mL)			anti-CCP2 IgM (range: 0-1400 aU/mL)			anti-CCP2 IgA (range: 0-1160 aU/mL)			RF IgM (range: 0-200 aU/mL)			RF IgA (range: 0-200 aU/mL)		
	Stable Pos	Pos-Neg		Stable Pos	Pos-Neg		Stable Pos	Pos-Neg		Stable Pos	Pos-Neg		Stable Pos	Pos-Neg	
N total	163	4	p	57	23	p	60	24	p	114	31	p	77	35	p
RAI index 1 year, med (IQR)	**1 (0-3)**	**4 (0-8)**	**0.60**	**1 (0-3)**	**1 (0-2)**	**0.35**	**1 (0-3)**	**2 (0-3)**	**0.86**	**1 (0-4)**	**1 (0-4)**	**1.00**	**1 (0-4)**	**0 (0-2)**	**0.08**
SJC Baseline, med (IQR)	5 (3-10)	6 (2-10)	0.78	5 (3-9)	5 (4-13)	0.56	5 (3-7)	6 (3-13)	0.57	5 (3-10)	5 (3-11)	0.43	5 (3-9)	7 (5-12)	0.05
SJC 1 year, med (IQR)	**0 (0-1)**	**1 (0-5)**	**0.52**	**0 (0-1)**	**0 (0-0)**	**0.38**	**0 (0-1)**	**0 (0-2)**	**0.46**	**0 (0-1)**	**0 (0-2)**	**0.81**	**0 (0-2)**	**0 (0-2)**	**0.82**
ESR (mm/h) Baseline, med (IQR)	26 (14-36)	45 (30-56)	0.09	30 (18-42)	24 (11-42)	0.34	29 (17-42)	26 (9-47)	0.54	29 (17-36)	28 (11-53)	0.94	29 (14-37)	28 (19-50)	0.50
ESR (mm/h) 1 year, med (IQR)	**9 (4-19)**	**9 (4-58)**	**0.91**	**11 (6-19)**	**6 (2-12)**	**0.02**	**11 (6-19)**	**9 (4-25)**	**0.50**	**11 (5-24)**	**9 (2-15)**	**0.14**	**11 (5-25)**	**9 (2-14)**	**0.08**
VAS (mm) Baseline, mean ± SD	43 ± 24	45 ± 17	0.84	42 ± 25	41 ± 26	0.91	42 ± 25	48 ± 24	0.35	42 ± 24	48 ± 21	0.24	43 ± 25	47 ± 24	0.47
VAS (mm) 1 year, mean ± SD	**24 ± 23**	**38 ± 26**	**0.22**	**25 ± 24**	**19 ± 20**	**0.33**	**25 ± 25**	**16 ± 15**	**0.07**	**24 ± 23**	**22 ± 19**	**0.65**	**26 ± 23**	**19 ± 21**	**0.11**
HAQ Baseline, mean ± SD	1.1 ± 0.7	1.2 ± 0.5	0.83	1 ± 0.7	1 ± 0.6	0.91	0.9 ± 0.7	1.3 ± 0.8	0.04	1 ± 0.7	1.5 ± 0.7	**0.00**	1.1 ± 0.6	1.3 ± 0.8	0.14
HAQ 1 year, mean ± SD	**0.5 ± 0.5**	**0.5 ± 0.7**	**0.93**	**0.5 ± 0.6**	**0.3 ± 0.4**	**0.19**	**0.5 ± 0.6**	**0.4 ± 0.5**	**0.79**	**0.5 ± 0.6**	**0.6 ± 0.6**	**0.58**	**0.6 ± 0.6**	**0.4 ± 0.5**	**0.15**
CRP Baseline, med (IQR)	11 (4-27)	22 (2-50)	0.94	14 (4-29)	9 (4-39)	0.73	12 (4-24)	11 (5-41)	0.43	11 (4-23)	16 (5-53)	0.22	11 (4-28)	11 (3-31)	0.93

Supplementary Table S1 (part 1 of 3): Demographic and clinical differences at baseline and 1 year, between patients that did not seroconvert ("Stable Pos") and those that seroconverted between 0-12 months ("Pos-Neg"). Bold typeface indicates significance after correction for multiple testing by Holmes-Bonferroni methods for 14 tests (14 autoantibodies). (continued)

	anti-CCP2 IgG (range: 0-1600 aU/mL)			anti-CCP2 IgM (range: 0-1400 aU/mL)			anti-CCP2 IgA (range: 0-1160 aU/mL)			RF IgM (range: 0-200 aU/mL)			RF IgA (range: 0-200 aU/mL)		
	Stable Pos	Pos-Neg		Stable Pos	Pos-Neg		Stable Pos	Pos-Neg		Stable Pos	Pos-Neg		Stable Pos	Pos-Neg	
N total	163	4	p	57	23	p	60	24	p	114	31	p	77	35	p
CRP 1 year, med (IQR)	**4 (3-8)**	**4 (3-17)**	**0.95**	**4 (3-7)**	**3 (3-6)**	**0.19**	**3 (3-7)**	**6 (3-11)**	**0.33**	**4 (3-8)**	**3 (3-8)**	**0.10**	**4 (3-8)**	**3 (3-5)**	**0.01**
DAS Baseline, mean ± SD	3.2 ± 0.9	3.4 ± 0.6	0.70	3.3 ± 1.1	3.3 ± 0.9	0.97	3.2 ± 1	3.2 ± 1	0.96	3.2 ± 0.9	3.5 ± 1	0.15	3.2 ± 1	3.5 ± 0.8	0.18
DAS 1 year, mean ± SD	**1.5 ± 0.9**	**1.9 ± 1.5**	**0.42**	**1.6 ± 0.9**	**1.3 ± 0.8**	**0.16**	**1.6 ± 0.9**	**1.5 ± 0.8**	**0.66**	**1.6 ± 0.9**	**1.5 ± 1**	**0.62**	**1.7 ± 1**	**1.3 ± 0.8**	**0.04**
Total SHS Baseline, med (IQR)	0.0 (0.0-0.5)	0.0 (0.0-4.5)	0.87	0.0 (0.0-2.0)	0.0 (0.0-0.0)	0.04	0.0 (0.0-0.9)	0.0 (0.0-1.5)	0.83	0.0 (0.0-0.6)	0.0 (0.0-0.6)	0.99	0.0 (0.0-2.0)	0.0 (0.0-0.0)	0.04
Total SHS 1 year, med (IQR)	**0.0 (0.0-1.4)**	**0.0 (0.0-4.5)**	**0.94**	**0.0 (0.0-2.0)**	**0.0 (0.0-0.0)**	**0.03**	**0.0 (0.0-2.0)**	**0.0 (0.0-0.8)**	**0.20**	**0.0 (0.0-1.9)**	**0.0 (0.0-1.1)**	**0.74**	**0.0 (0.0-2.0)**	**0.0 (0.0-0.0)**	**0.03**

P-values are based on t-tests, Mann Whitney tests, or Chi-squared tests for comparisons of means, medians, and frequencies, respectively. SD: standard deviation. Med: median. IQR: interquartile range. BMI: Body mass index. RAI: Richtie Articular Index. SJC: Swollen joint count. ESR: Erythrocyte sedimentation rate. VAS: Visual analogue scale. HAQ: Health assessment questionnaire. CRP: C-reactive protein. DAS: Disease activity score. SHS: Sharp-van de Heijde score. Acetyl: acetylated. Cit: citrullinated.

Supplementary Table S1 (part 2 of 3): Demographic and clinical differences at baseline and 1 year, between patients that did not seroconvert ("Stable Pos") and those that seroconverted between 0-12 months ("Pos-Neg"). Bold typeface indicates significance after correction for multiple testing by Holmes-Bonferroni methods for 14 tests (14 autoantibodies).

	anti-CarP IgG (range: 0-5272 aU/mL)			anti-CarP IgM (range: 0-3650 aU/mL)			anti-CarP IgA (range: 0-3100 aU/mL)			Anti-acetyl-lysine IgG (range: 0-1000 aU/mL)			Anti-acetyl-ornithine IgG (range: 0-1000 aU/mL)		
	Stable Pos	Pos-Neg		Stable Pos	Pos-Neg		Stable Pos	Pos-Neg		Stable Pos	Pos-Neg		Stable Pos	Pos-Neg	
N total	46	47	p	35	34	p	21	40	p	59	19	p	130	15	p
Levels Baseline, med (IQR)	**1,579 (1069-2,846)**	**1,123 (822-1,463)**	0.00	**3,650 (2375-3,650)**	**2,843 (2221-3,568)**	0.04	**2,478 (1591-2,912)**	**1,128 (955-1,479)**	0.00	**167 (75-532)**	**60 (50-74)**	0.00	**484 (180-1,000)**	**47 (40-74)**	0.00
Age in years, mean ± SD	48 ± 14	54 ± 10	0.04	52 ± 13	50 ± 14	0.65	52 ± 9	53 ± 13	0.90	49 ± 13	52 ± 12	0.51	49 ± 13	53 ± 10	0.19
Symptom duration (weeks), med (IQR)	**19 (8-32)**	**23 (9-38)**	**0.47**	**26 (10-56)**	**19 (7-29)**	**0.11**	**32 (19-63)**	**18 (8-28)**	**0.02**	**25 (8-36)**	**27 (18-44)**	**0.26**	**25 (10-44)**	**24 (6-32)**	**0.79**
BMI (kg/m2), mean ± SD	26 ± 4	25 ± 3	0.28	26 ± 4	26 ± 4	0.80	26 ± 4	26 ± 4	0.75	26 ± 4	26 ± 4	0.99	25 ± 4	27 ± 5	0.10
Female gender (%)	**23 (50%)**	**36 (77%)**	**0.01**	**20 (57%)**	**27 (79%)**	**0.05**	**12 (57%)**	**30 (75%)**	**0.15**	**40 (68%)**	**14 (74%)**	**0.63**	**83 (64%)**	**14 (93%)**	**0.02**
Ever smoker (%)	25 (54%)	25 (54%)	1.00	22 (63%)	16 (47%)	0.19	15 (71%)	24 (60%)	0.38	27 (47%)	10 (53%)	0.65	71 (55%)	5 (33%)	0.11
Achieved early remission (%)	**31 (67%)**	**29 (62%)**	**0.57**	**22 (63%)**	**21 (62%)**	**0.93**	**12 (57%)**	**27 (68%)**	**0.42**	**43 (73%)**	**12 (63%)**	**0.42**	**95 (73%)**	**6 (40%)**	**0.01**
RAI index Baseline, med (IQR)	6 (4-10)	7 (4-10)	0.54	7 (4-10)	7 (4-11)	0.76	7 (4-9)	6 (4-9)	0.91	6 (4-10)	6 (4-9)	0.71	6 (4-9)	10 (3-16)	0.14
RAI index 1 year, med (IQR)	**1 (0-2)**	**1 (0-3)**	**0.83**	**2 (0-3)**	**0 (0-3)**	**0.10**	**2 (0-3)**	**1 (0-3)**	**0.51**	**0 (0-2)**	**0 (0-2)**	**0.87**	**1 (0-2)**	**4 (1-7)**	**0.01**
SJC Baseline, med (IQR)	6 (2-13)	7 (4-12)	0.27	6 (4-14)	6 (3-14)	0.63	6 (4-11)	6 (3-12)	0.84	6 (3-12)	7 (5-11)	0.25	5 (3-10)	4 (2-15)	0.82

Supplementary Table S1 (part 2 of 3): Demographic and clinical differences at baseline and 1 year, between patients that did not seroconvert ("Stable Pos") and those that seroconverted between 0-12 months ("Pos-Neg"). Bold typeface indicates significance after correction for multiple testing by Holmes-Bonferroni methods for 14 tests (14 autoantibodies). (continued)

	anti-CarP IgG (range: 0-5272 aU/mL)			anti-CarP IgM (range: 0-3650 aU/mL)			anti-CarP IgA (range: 0-3100 aU/mL)			Anti-acetyl-lysine IgG (range: 0-1000 aU/mL)			Anti-acetyl-ornithine IgG (range: 0-1000 aU/mL)		
	Stable Pos	Pos-Neg		Stable Pos	Pos-Neg		Stable Pos	Pos-Neg		Stable Pos	Pos-Neg		Stable Pos	Pos-Neg	
N total	46	47	p	35	34	p	21	40	p	59	19	p	130	15	p
SJC 1 year, med (IQR)	**0 (0-1)**	**0 (0-1)**	**0.42**	**0 (0-2)**	**0 (0-1)**	**0.53**	**1 (0-2)**	**0 (0-1)**	**0.08**	**0 (0-0)**	**0 (0-1)**	**0.40**	**0 (0-1)**	**0 (0-1)**	**0.76**
ESR (mm/h) Baseline, med (IQR)	25 (17-36)	29 (19-41)	0.49	29 (17-38)	29 (18-50)	0.75	32 (19-56)	29 (14-36)	0.35	26 (17-36)	33 (18-42)	0.24	26 (15-36)	28 (6-48)	0.66
ESR (mm/h) 1 year, med (IQR)	**10 (5-19)**	**9 (5-14)**	**0.44**	**11 (6-19)**	**9 (5-19)**	**0.80**	**14 (6-25)**	**9 (5-19)**	**0.19**	**9 (5-14)**	**9 (5-27)**	**0.54**	**9 (5-19)**	**9 (2-27)**	**0.92**
VAS (mm) Baseline, mean ± SD	50 ± 23	43 ± 24	0.17	42 ± 23	44 ± 26	0.78	43 ± 23	41 ± 25	0.80	43 ± 25	46 ± 28	0.71	44 ± 25	41 ± 20	0.71
VAS (mm) 1 year, mean ± SD	**22 ± 22**	**21 ± 19**	**0.89**	**24 ± 23**	**22 ± 23**	**0.74**	**30 ± 25**	**23 ± 22**	**0.30**	**24 ± 23**	**19 ± 25**	**0.44**	**22 ± 21**	**30 ± 21**	**0.16**
HAQ Baseline, mean ± SD	1.1 ± 0.6	1.1 ± 0.7	0.81	1 ± 0.6	1.2 ± 0.8	0.29	1.2 ± 0.5	1.2 ± 0.8	0.87	1.1 ± 0.7	1.3 ± 0.7	0.16	1.1 ± 0.7	1.4 ± 0.9	0.09
HAQ 1 year, mean ± SD	**0.4 ± 0.5**	**0.5 ± 0.5**	**0.47**	**0.6 ± 0.6**	**0.4 ± 0.5**	**0.15**	**0.6 ± 0.5**	**0.4 ± 0.6**	**0.21**	**0.5 ± 0.6**	**0.4 ± 0.6**	**0.66**	**0.5 ± 0.5**	**0.8 ± 0.7**	**0.06**
CRP Baseline, med (IQR)	13 (5-27)	11 (4-34)	0.78	13 (5-27)	11 (3-37)	0.88	17 (6-26)	13 (4-28)	0.85	11 (3-26)	16 (3-37)	0.62	11 (4-26)	7 (3-28)	0.64
CRP 1 year, med (IQR)	**5 (3-8)**	**3 (3-7)**	**0.82**	**4 (3-8)**	**3 (3-7)**	**0.64**	**3 (3-10)**	**3 (3-7)**	**0.69**	**3 (3-7)**	**3 (3-7)**	**0.63**	**4 (3-7)**	**4 (3-9)**	**0.39**
DAS Baseline, mean ± SD	3.4 ± 0.9	3.4 ± 1.1	0.77	3.4 ± 0.9	3.5 ± 1.1	0.73	3.4 ± 0.7	3.3 ± 1	0.80	3.2 ± 0.9	3.4 ± 0.9	0.42	3.2 ± 1	3.4 ± 1.2	0.52

Supplementary Table S1 (part 2 of 3): Demographic and clinical differences at baseline and 1 year, between patients that did not seroconvert ("Stable Pos") and those that seroconverted between 0-12 months ("Pos-Neg"). Bold typeface indicates significance after correction for multiple testing by Holmes-Bonferroni methods for 14 tests (14 autoantibodies). (continued)

	anti-CarP IgG (range: 0-5272 aU/mL)			anti-CarP IgM (range: 0-3650 aU/mL)			anti-CarP IgA (range: 0-3100 aU/mL)			Anti-acetyl-lysine IgG (range: 0-1000 aU/mL)			Anti-acetyl-ornithine IgG (range: 0-1000 aU/mL)		
	Stable Pos	Pos-Neg		Stable Pos	Pos-Neg		Stable Pos	Pos-Neg		Stable Pos	Pos-Neg		Stable Pos	Pos-Neg	
N total	46	47	p	35	34	p	21	40	p	59	19	p	130	15	p
DAS 1 year, mean ± SD	**1.5 ± 0.8**	**1.5 ± 0.9**	**0.97**	**1.7 ± 0.9**	**1.4 ± 0.9**	**0.26**	**1.7 ± 0.7**	**1.4 ± 1**	**0.37**	**1.4 ± 0.9**	**1.4 ± 1**	**0.97**	**1.4 ± 0.9**	**2 ± 1**	**0.03**
Total SHS Baseline, med (IQR)	0.0 (0.0-0.8)	0.0 (0.0-1.0)	0.76	0.0 (0.0-2.0)	0.0 (0.0-0.5)	0.27	0.0 (0.0-0.1)	0.0 (0.0-2.0)	0.45	0.0 (0.0-2.0)	0.0 (0.0-0.0)	0.08	0.0 (0.0-0.5)	0.0 (0.0-0.0)	0.13
Total SHS 1 year, med (IQR)	**0.0 (0.0-2.0)**	**0.0 (0.0-2.0)**	**0.88**	**0.0 (0.0-2.0)**	**0.0 (0.0-1.3)**	**0.34**	**0.0 (0.0-0.9)**	**0.0 (0.0-2.0)**	**0.45**	**0.0 (0.0-2.0)**	**0.0 (0.0-0.0)**	**0.04**	**0.0 (0.0-1.0)**	**0.0 (0.0-0.0)**	**0.24**

P-values are based on t-tests, Mann Whitney tests, or Chi-squared tests for comparisons of means, medians, and frequencies, respectively. SD: standard deviation. Med: median. IQR: interquartile range. BMI: Body mass index. RAI: Richtie Articular Index. SJC: Swollen joint count. ESR: Erythrocyte sedimentation rate. VAS: Visual analogue scale. HAQ: Health assessment questionnaire. CRP: C-reactive protein. DAS: Disease activity score. SHS: Sharp-van de Heijde score. Acetyl: acetylated. Cit: citrullinated.

Supplementary Table S1 (part 3 of 3): Demographic and clinical differences at baseline and 1 year, between patients that did not seroconvert ("Stable Pos") and those that seroconverted between 0-12 months ("Pos-Neg"). Bold typeface indicates significance after correction for multiple testing by Holmes-Bonferroni methods for 14 tests (14 autoantibodies).

	Cit-Vimentin IgG (range: 0-10,000 aU/mL)			Cit-Fibrinogen α IgG (range: 0-25,000 aU/mL)			Cit-Fibrinogen β IgG (range: 0-100,000 aU/mL)			Cit-Enolase IgG (range: 0-70,000 aU/mL)		
	Stable Pos	Pos-Neg		Stable Pos	Pos-Neg		Stable Pos	Pos-Neg		Stable Pos	Pos-Neg	
N total	93	25	p	32	26	p	95	23	p	54	15	p
Levels Baseline, med (IQR)	4,003 (2305-8,871)	1,671 (800-3,120)	**0.00**	6,357 (2871-14,730)	2,952 (1763-4,077)	**0.00**	25,874 (12578-57,366)	6,741 (3549-8,961)	**0.00**	32,972 (14634-70,000)	8,777 (6401-11,380)	**0.00**
Age in years, mean ± SD	48 ± 13	50 ± 9	0.56	50 ± 13	51 ± 12	0.79	50 ± 12	49 ± 14	0.85	51 ± 13	50 ± 10	0.88
Symptom duration (weeks), med (IQR)	26 (9-46)	23 (15-32)	0.80	27 (9-55)	23 (10-31)	0.14	26 (11-47)	20 (10-28)	0.21	26 (10-41)	32 (10-56)	0.31
BMI (kg/m2), mean ± SD	26 ± 4	27 ± 6	0.44	26 ± 4	26 ± 5	0.88	26 ± 4	25 ± 4	0.10	26 ± 4	24 ± 3	0.07
Female gender (%)	53 (57%)	20 (80%)	0.04	25 (78%)	18 (69%)	0.44	62 (65%)	18 (78%)	0.23	32 (59%)	8 (53%)	0.68
Ever smoker (%)	50 (54%)	14 (56%)	0.88	14 (44%)	15 (58%)	0.29	49 (52%)	10 (43%)	0.46	34 (64%)	9 (60%)	0.77
Achieved early remission (%)	63 (68%)	18 (72%)	0.68	23 (72%)	16 (62%)	0.40	68 (72%)	17 (74%)	0.82	33 (61%)	12 (80%)	0.17
RAI index Baseline, med (IQR)	6 (4-10)	6 (4-10)	0.75	6 (4-8)	6 (3-8)	0.86	6 (4-8)	8 (5-11)	0.12	6 (4-9)	5 (3-10)	0.79
RAI index 1 year, med (IQR)	1 (0-3)	1 (0-4)	0.58	1 (0-5)	1 (0-3)	0.75	1 (0-3)	0 (0-2)	0.13	2 (0-3)	1 (0-3)	0.67
SJC Baseline, med (IQR)	6 (3-10)	5 (3-12)	0.96	4 (2-7)	5 (3-13)	0.33	4 (3-8)	8 (4-12)	0.06	6 (3-14)	7 (4-12)	0.65

Supplementary Table S1 (part 3 of 3): Demographic and clinical differences at baseline and 1 year, between patients that did not seroconvert ("Stable Pos") and those that seroconverted between 0-12 months ("Pos-Neg"). Bold typeface indicates significance after correction for multiple testing by Holmes-Bonferroni methods for 14 tests (14 autoantibodies). (continued)

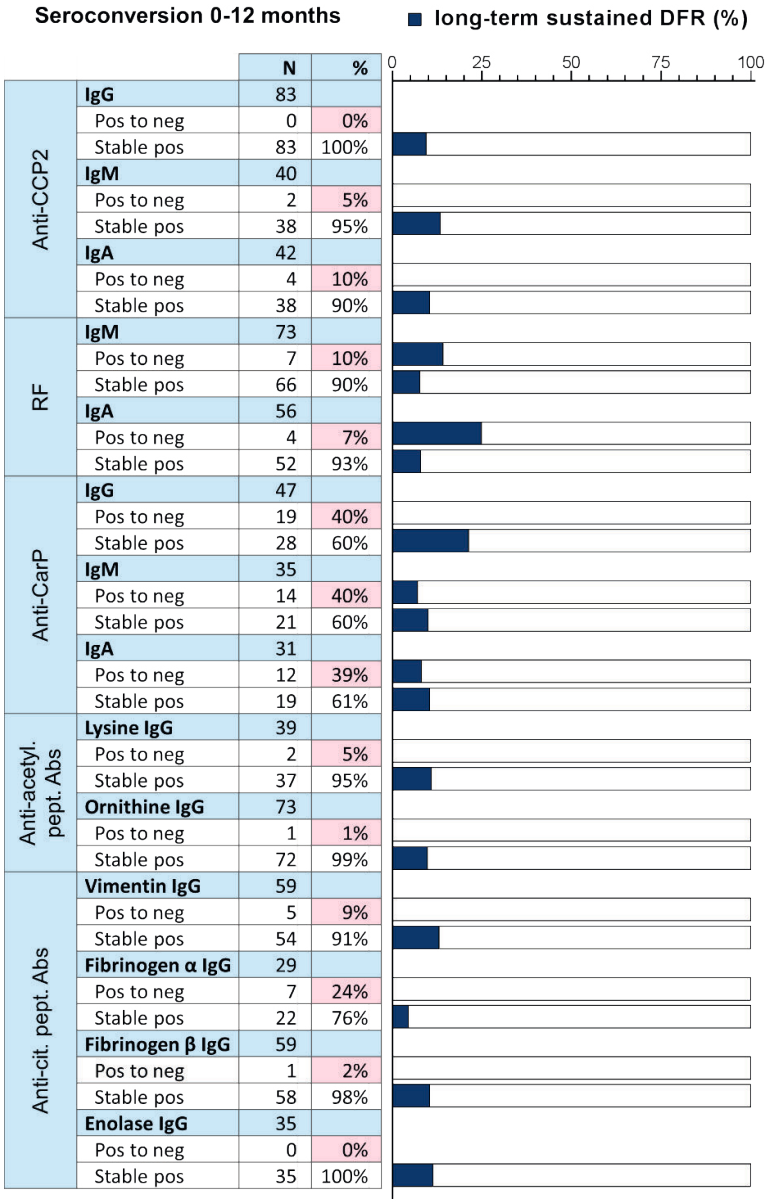
	Cit-Vimentin IgG (range: 0-10,000 aU/mL)			Cit-Fibrinogen α IgG (range: 0-25,000 aU/mL)			Cit-Fibrinogen β IgG (range: 0-100,000 aU/mL)			Cit-Enolase IgG (range: 0-70,000 aU/mL)		
	Stable Pos	Pos-Neg		Stable Pos	Pos-Neg		Stable Pos	Pos-Neg		Stable Pos	Pos-Neg	
N total	93	25	p	32	26	p	95	23	p	54	15	p
SJC 1 year, med (IQR)	0 (0-1)	0 (0-1)	0.65	0 (0-1)	0 (0-1)	0.43	0 (0-1)	0 (0-0)	0.26	0 (0-1)	0 (0-4)	0.48
ESR (mm/h) Baseline, med (IQR)	25 (11-37)	28 (14-55)	0.43	31 (19-38)	31 (13-44)	0.90	26 (14-38)	30 (19-36)	0.57	28 (17-44)	32 (11-55)	0.94
ESR (mm/h) 1 year, med (IQR)	9 (5-18)	9 (3-20)	0.89	11 (6-29)	11 (4-17)	0.43	11 (5-18)	6 (2-14)	0.31	11 (6-25)	7 (2-14)	0.20
VAS (mm) Baseline, mean ± SD	44 ± 25	52 ± 24	0.18	39 ± 22	43 ± 25	0.51	43 ± 23	41 ± 28	0.71	49 ± 23	44 ± 30	0.44
VAS (mm) 1 year, mean ± SD	23 ± 23	25 ± 20	0.74	24 ± 24	24 ± 19	0.98	22 ± 21	21 ± 20	0.73	23 ± 22	28 ± 25	0.45
HAQ Baseline, mean ± SD	1.1 ± 0.7	1.2 ± 0.6	0.63	1 ± 0.7	1.2 ± 0.6	0.33	1 ± 0.7	1.3 ± 0.5	0.05	1.1 ± 0.7	1.2 ± 0.8	0.93
HAQ 1 year, mean ± SD	0.5 ± 0.6	0.5 ± 0.6	0.99	0.5 ± 0.6	0.6 ± 0.4	0.60	0.5 ± 0.5	0.3 ± 0.4	0.17	0.5 ± 0.5	0.5 ± 0.6	0.93
CRP Baseline, med (IQR)	13 (4-28)	12 (4-34)	0.81	12 (3-26)	17 (5-28)	0.32	13 (4-28)	13 (8-21)	0.99	14 (4-43)	21 (9-35)	0.56
CRP 1 year, med (IQR)	4 (3-7)	3 (3-8)	0.51	4 (3-8)	3 (3-7)	0.48	3 (3-7)	4 (3-7)	1.00	3 (3-7)	3 (3-7)	0.95
DAS Baseline, mean ± SD	3.3 ± 1	3.4 ± 1	0.51	3.1 ± 0.8	3.3 ± 1.2	0.57	3.2 ± 1	3.3 ± 0.7	0.41	3.4 ± 1	3.3 ± 1.2	0.70

Supplementary Table S1 (part 3 of 3): Demographic and clinical differences at baseline and 1 year, between patients that did not seroconvert ("Stable Pos") and those that seroconverted between 0-12 months ("Pos-Neg"). Bold typeface indicates significance after correction for multiple testing by Holmes-Bonferroni methods for 14 tests (14 autoantibodies). (continued)

	Cit-Vimentin IgG (range: 0-10,000 aU/mL)			Cit-Fibrinogen α IgG (range: 0-25,000 aU/mL)			Cit-Fibrinogen β IgG (range: 0-100,000 aU/mL)			Cit-Enolase IgG (range: 0-70,000 aU/mL)		
	Stable Pos	Pos-Neg		Stable Pos	Pos-Neg		Stable Pos	Pos-Neg		Stable Pos	Pos-Neg	
N total	93	25	p	32	26	p	95	23	p	54	15	p
DAS 1 year, mean ± SD	1.5 ± 0.9	1.5 ± 0.9	0.78	1.7 ± 1	1.4 ± 0.7	0.31	1.5 ± 0.9	1.2 ± 0.8	0.16	1.6 ± 0.9	1.5 ± 1.1	0.61
Total SHS Baseline, med (IQR)	0.0 (0.0-0.4)	0.0 (0.0-0.0)	0.61	0.0 (0.0-0.4)	0.0 (0.0-2.0)	0.24	0.0 (0.0-0.5)	0.0 (0.0-0.0)	0.16	0.0 (0.0-0.8)	0.5 (0.0-2.6)	0.16
Total SHS 1 year, med (IQR)	0.0 (0.0-0.5)	0.0 (0.0-0.5)	0.93	0.0 (0.0-1.1)	0.0 (0.0-3.5)	0.31	0.0 (0.0-1.5)	0.0 (0.0-0.1)	0.38	0.0 (0.0-1.0)	0.8 (0.0-3.0)	0.12

P-values are based on t-tests, Mann Whitney tests, or Chi-squared tests for comparisons of means, medians, and frequencies, respectively. SD: standard deviation. Med: median. IQR: interquartile range. BMI: Body mass index. RAI: Richtie Articular Index. SJC: Swollen joint count. ESR: Erythrocyte sedimentation rate. VAS: Visual analogue scale. HAQ: Health assessment questionnaire. CRP: C-reactive protein. DAS: Disease activity score. SHS: Sharp-van de Heijde score. Acetyl: acetylated. Cit: citrullinated.

Supplementary Figure S1: Sensitivity analysis within patients with baseline antibody levels above the median for each antibody. Number and percentage of baseline patients seroconverting ("Pos to Neg") or non-converting ("Stable pos") between 0-12 months are listed on the left, and the percentage of each subset subsequently reaching long-term sustained DFR is graphically depicted on the right. Anti-acetyl. pept. Abs = anti-acetylated peptide antibodies. Anti-cit. pept. Abs = anti-citrullinated peptide antibodies.



		N	%
Anti-CCP2	**IgG**	83	
	Pos to neg	0	0%
	Stable pos	83	100%
	IgM	40	
	Pos to neg	2	5%
	Stable pos	38	95%
	IgA	42	
	Pos to neg	4	10%
	Stable pos	38	90%
RF	**IgM**	73	
	Pos to neg	7	10%
	Stable pos	66	90%
	IgA	56	
	Pos to neg	4	7%
	Stable pos	52	93%
Anti-CarP	**IgG**	47	
	Pos to neg	19	40%
	Stable pos	28	60%
	IgM	35	
	Pos to neg	14	40%
	Stable pos	21	60%
	IgA	31	
	Pos to neg	12	39%
	Stable pos	19	61%
Anti-acetyl. pept. Abs	**Lysine IgG**	39	
	Pos to neg	2	5%
	Stable pos	37	95%
	Ornithine IgG	73	
	Pos to neg	1	1%
	Stable pos	72	99%
Anti-cit. pept. Abs	**Vimentin IgG**	59	
	Pos to neg	5	9%
	Stable pos	54	91%
	Fibrinogen α IgG	29	
	Pos to neg	7	24%
	Stable pos	22	76%
	Fibrinogen β IgG	59	
	Pos to neg	1	2%
	Stable pos	58	98%
	Enolase IgG	35	
	Pos to neg	0	0%
	Stable pos	35	100%

CHAPTER 5

In rheumatoid arthritis, changes in autoantibody levels reflect intensity of immunosuppression, not subsequent treatment response

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ABSTRACT

Background

Rheumatoid arthritis (RA) is characterized by the presence of autoantibodies like rheumatoid factor (RF), anti-cyclic citrullinated peptide-2 (anti-CCP2), and anti-carbamylated protein (anti-CarP) antibodies. It is currently unclear whether changes in autoantibody levels are associated with disease activity/treatment outcomes and whether they are modified by treatment intensity. Therefore, we determined longitudinal changes in RA-autoantibody levels, the association between these changes and activity score (DAS) and treatment outcomes, and the effect of intensity of immunosuppressive treatment on levels.

Methods

In 381 seropositive RA patients from the IMPROVED study, we measured IgG, IgM, and IgA of anti-CCP2 and anti-CarP; IgM and IgA of RF; and IgG against four citrullinated and two acetylated peptides at 4-month intervals over the first year of treatment. Following initial prednisone and methotrexate (MTX), treatment was changed every 4 months aiming for DAS < 1.6. We investigated changes in autoantibody levels following treatment escalation versus tapering, and the association of levels with DAS over time, EULAR response, and drug-free remission (DFR) ≥ 1 year.

Results

For all 14 autoantibodies, levels decreased from 0 to 4 months and then rose until 12 months. Following treatment escalation, autoantibody levels dropped markedly, while they rose following tapering: RF IgM levels, a representative autoantibody, dropped 10% after restarting prednisone and rose 15% aU/mL after tapering MTX ($p < 0.0001$). There was no association between autoantibody levels and DAS over time or EULAR response. Greater relative changes between 0 and 12 months did not predict DFR (0–12-month relative change RF IgM, − 39% for no DFR (n = 126) and − 16% for DFR (n = 18)).

Conclusions

Changes in RA-autoantibody levels are not associated with DAS or long-term treatment response, but reflect intensity of immunosuppression. This suggests that autoantibody levels are modifiable by current therapies, but that modifying levels is in itself of limited clinical relevance.

Trial registration

ISRCTN11916566. Registered on 7 November 2006

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory joint disease characterized by the presence of autoantibodies. Rheumatoid factor (RF) and anti-cyclic citrullinated peptide-2 (anti-CCP2) are the most well-known of these, but other autoantibody systems such as anti-carbamylated protein (anti-CarP) and anti-acetylated peptide antibodies have also been identified (1, 2). Autoantibody-positive patients have a worse prognosis, more radiographic damage, and a lower chance of achieving drug-free remission (3, 4).

Serum concentrations of these autoantibodies may change over time. Given the link between autoantibodies and disease severity and the value of measuring autoantibodies in other autoimmune diseases, these serological changes in RA may hold promise as an accessible biomarker for the future disease course. A substantial drop in autoantibody levels may, for example, be hypothesized to precede successful drug-free remission. However, studies documenting the relationship between fluctuations in autoantibodies and disease activity have been conflicting (5-7). Importantly, most of these studies did not account for factors like intensity of immunosuppressive treatment, which likely influences both level changes and disease activity. It is unknown whether changes in autoantibody levels reflect immunosuppressive therapy, or whether changes are indicative of future disease course. Furthermore, most studies investigated a limited number of autoantibodies and did not take “newer” autoantibodies into account, such as anti-CarP, which has been described to be associated with disease activity (8).

Because of this, the clinical implications of changes in autoantibody levels remain unclear, but are potentially relevant for two reasons. First, if autoantibody levels are a marker of future disease activity, it may be useful to measure pre-treatment values or monitor level changes over time. Second, understanding the changes in autoantibody levels and their association with both immunosuppression and disease activity might shed new light on mechanisms underlying the B cell autoimmune response in RA and its role in disease persistence. To that end, we longitudinally characterized changes in RA-associated autoantibody levels over time and investigated whether levels are affected by intensity of immunosuppressive treatment, how they associate with disease activity over time, and whether level changes associate with both short-term and long-term treatment outcomes.

METHODS

Study design, patient selection, and outcomes

The Induction therapy with Methotrexate and Prednisone in Rheumatoid Or Very Early arthritic Disease (IMPROVED) study is a multicenter, randomized controlled trial that enrolled 610 patients with early (< 2 years) untreated RA or undifferentiated arthritis. It was steered at disease activity score-remission (DAS44 < 1.6) and for those achieving remission, at drug-free remission (DFR), with treatment adjusted every 4 months according to whether treatment targets had been reached. Initial treatment comprised methotrexate (MTX) and high-dose prednisone (4).

Subjects selected for this study were all 381 patients fulfilling the 2010 ACR/EULAR RA criteria with serum available at least once within the first year and seropositive by routine clinical testing for anti-CCP2 IgG, RF IgM, or our in-house assay for anti-CarP IgG at baseline or at 1 year (details described in (9)). Clinical outcomes investigated were DAS, health assessment questionnaire (HAQ), EULAR response at 4 and 12 months, and long-term sustained DFR. Long-term sustained DFR was defined as disease-modifying anti-rheumatic drug (DMARD)-free remission lasting at least 1 year, starting at any time point and continuing until the last moment of that individual's follow-up (maximum of 5 years follow-up). Radiographic progression at 1 and 5 years was assessed using the Sharp/van der Heijde Score (SHS), as previously described (4).

Serological measurements

Enzyme-linked immunosorbent assays (ELISAs) were used as described previously (9) to measure at 4-month intervals over the first year of treatment: anti-CCP2 IgG, IgM, and IgA; RF IgM and IgA; anti-CarP IgG, IgM, and IgA; anti-citrullinated-vimentin 59–74 IgG, anti-citrullinated-fibrinogen β 36–52 and α 27–43 IgG, and anti-citrullinated-enolase 5–20 IgG (all in-house assays); and anti-acetylated lysine vimentin IgG and anti-acetylated ornithine vimentin IgG (Orgentec Diagnostika GmbH, Germany) (1). Samples were considered positive if they fell above a cutoff of the mean arbitrary units (aU) per milliliter plus two standard deviations of 76 sera of healthy controls from the Leiden area.

Composites reflecting the number of autoantibodies present at every time point were constructed: the number of isotypes: anti-CCP2 IgG, IgM, IgA; RF IgM and IgA; anti-CarP IgG, IgM, IgA (range 1–8), and the number of IgG anti-modified peptide antibodies (AMPAs): anti-CCP2, anti-CarP, and the antibodies against citrullinated and acetylated peptides described above (range 1–8).

Statistical analysis

Longitudinal, repeated measures data (autoantibodies, DAS, HAQ) were modeled using generalized estimating equations (GEE), which allow missing data in the outcome. A model with a Toeplitz (m-dependent) correlation structure and a standard Gaussian distribution was chosen (akin to linear regression). For the number of autoantibodies over time (count data), a negative binomial model was specified.

With repeated measurements of the autoantibody levels/number as the dependent variable, we investigated by GEE whether a certain treatment decision (4 months, no change versus escalation of treatment; 8 months: tapering versus escalation of treatment) was associated with a subsequent change in autoantibody levels/number. An interaction term of treatment decision * time was used to assess whether changes in the autoantibody levels over time were different in patients that tapered/did not change versus those that escalated immunosuppressive therapy. For comparison purposes, a normalization of the different measurement units was applied to the final model estimates (which were in aU/mL) by dividing them by the maximum of the autoantibody's range.

The association between autoantibody levels and DAS over time was investigated using GEE, with DAS as the outcome. The same was conducted for HAQ over time. Ordinal, logistic, and linear regression was used to investigate the association of relative changes in autoantibody levels/absolute changes in number of autoantibodies with EULAR response, long-term sustained DFR, and SHS radiographic progression scores, respectively.

All models were adjusted for gender and age; clinical outcome analyses were adjusted for treatment decisions. Other covariates (i.e., disease duration, smoking, body mass index (BMI), baseline HAQ/DAS) were only included in final models if they were univariably associated with the outcomes of interest ($p < 0.1$). Holmes-Bonferroni methods were used to correct all analyses for multiple testing, assuming the same number of tests as autoantibodies investigated (14 tests).

RESULTS

Autoantibody levels decrease upon initiation and escalation of immunosuppressive treatment

For all 14 autoantibodies, median levels decreased significantly between baseline and 4 months when prednisone and MTX were initiated, and then stabilized or steadily increased until 12 months, while DAS plummeted between 0 and 4 months and stayed low between 4 and 12 months (**Figure 1; Supplementary Figure S1** for all autoantibodies).

Due to its design, the IMPROVED study can be used to investigate whether autoantibody levels might decrease not only upon treatment initiation, but also upon decisions regarding intensity of immunosuppression, by examining changes in autoantibodies after treatment was either tapered or escalated. We first looked at the moment in the IMPROVED study when one would expect the largest differences: at 8 months, patients that had achieved DAS-remission tapered MTX monotherapy to drug-free, while those not in remission escalated therapy by restarting prednisone (next to MTX). It was found that autoantibody levels rose between 8 and 12 months following the decision to taper MTX to drug-free and dropped if prednisone was restarted **(Figure 2)**. This finding was significant for 12/14 autoantibodies.

Another treatment decision was the one made at 4 months: patients that achieved remission at 4 months continued MTX monotherapy, while those that did not were randomized to one of the two treatment arms, where therapy was escalated by an addition of multiple DMARDs (prednisone, hydroxychloroquine, and sulfasalazine; arm 1) or adalimumab (arm 2). Levels consistently rose between 4 and 8 months during continued treatment with MTX while they either dropped or stayed relatively stable following treatment escalation (**Supplementary Figure S2**). At this moment, the difference in treatment intensity was less pronounced than at 8 months (i.e., no patients tapered to drug-free), as were the differences in autoantibody level changes.

The total autoantibody number changed in a manner similar to the changes in levels, both at treatment initiation (**Supplementary Figure S1**) and for the decision at 4 and at 8 months (not shown).

This indicates that autoantibody levels are responsive to immunosuppression and likely change upon decisions to escalate or taper therapy. The next question was whether these autoantibody changes are associated with treatment outcomes, and thus whether the changes in autoantibody levels in response to treatment might be clinically relevant to monitor over time.

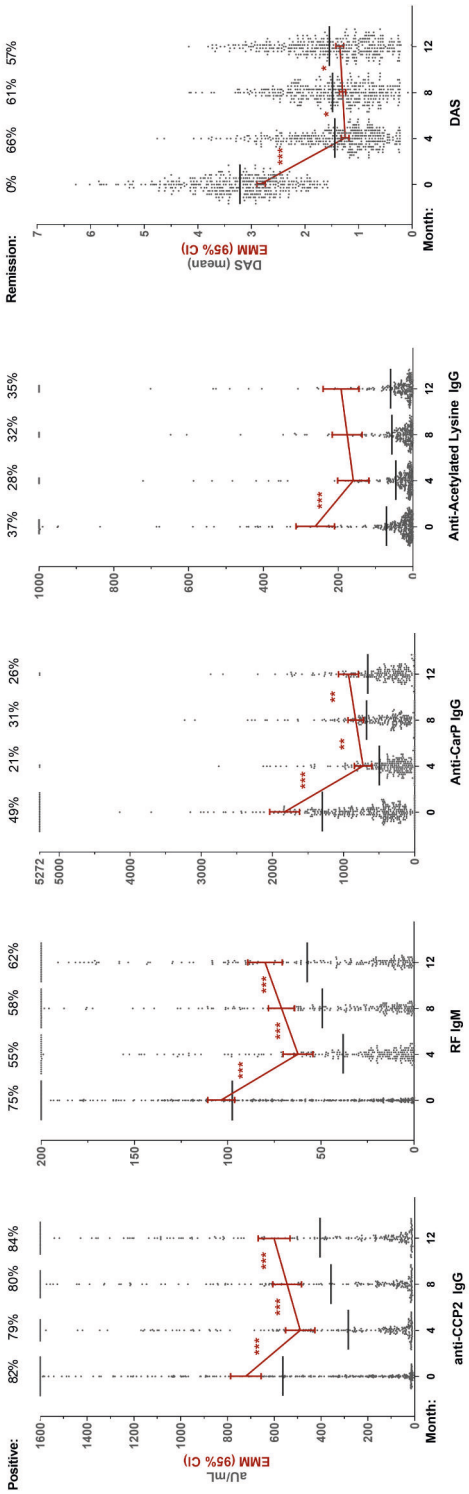
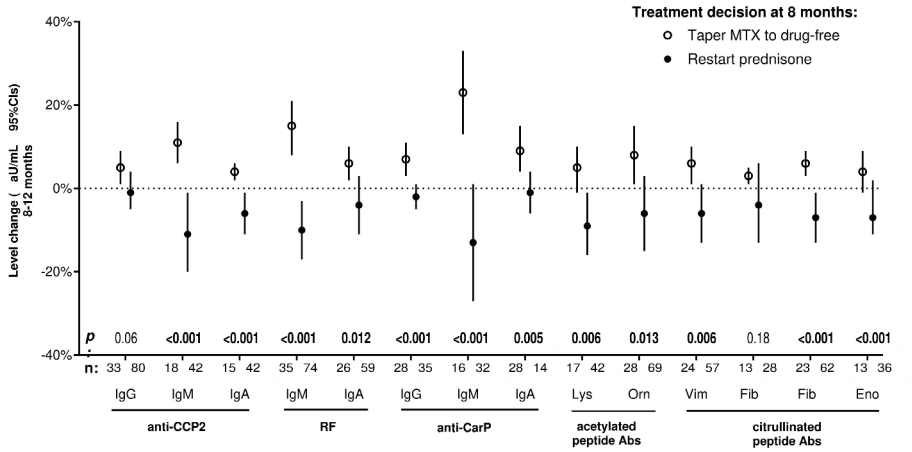


FIGURE 1 Autoantibody levels initially decrease and then steadily rise over time, paralleled by disease activity.

*Levels in arbitrary units (aU/mL) of four representative autoantibodies and DAS over the first year of treatment as measured in the serum of seropositive RA patients (for levels: N 0 months = 356; N 4 months = 225; N 8 months = 209; N 12 months = 212; for DAS: N 0 months = 381; N 4 months = 374; N 8 months = 361; N 12 months = 357). Patients clustered at the maximum were above the highest standard of the ELISA. Black lines indicate median level in arbitrary units per milliliter or mean DAS. Red lines indicate estimated marginal mean (EMM) arbitrary units per milliliter or DAS with 95% confidence intervals (calculated by GEE). p values (asterisk) refer to the change between two time points. *p < 0.05, **p < 0.01, ***p < 0.001*

Figure 2 Autoantibody levels change following treatment decisions.



*Change in autoantibody levels (calculated by GEE) following treatment decision at 8 months, within patients that were positive for that autoantibody at least once over the first year. Depicted regression coefficients (β in aU/mL, with 95% CIs) are of the predictor time from a GEE model stratified for the treatment decision, and thus indicate autoantibody level changes between 8 and 12 months for that treatment decision group. Coefficients were normalized for comparison purposes by dividing by the maximum arbitrary units per milliliter of the ELISA range of each autoantibody. Models were adjusted for age, gender, smoking status, and disease duration. Bold typeface of p values calculated by GEE (interaction term treatment*time) indicates significance after Holmes-Bonferroni correction for multiple testing (14 tests)*

Autoantibody levels are not longitudinally associated with DAS

Given the way both autoantibody levels and DAS decreased upon treatment, we addressed the question whether the two are longitudinally and independently associated (**Table 1**). The GEE models reported in **Table 1** are congruent with both a cross-sectional and a longitudinal interpretation. First, a patient that is 1 unit (aU/mL) higher in an autoantibody level is expected, at that moment, to have a higher DAS of the indicated magnitude (e.g., 0.011 DAS units per 100 aU/mL anti-CCP2 IgG). Second, a patient that increases 1 unit in an autoantibody (for any given time interval) is expected to have an increase in DAS of the indicated magnitude for that same time interval. Although almost all associations of autoantibodies and DAS changes were significant (confidence intervals do not cross zero), the magnitude of association was miniscule and far from clinically relevant. We conclude that there is no relevant association between autoantibody level changes and DAS changes.

Autoantibody levels and HAQ were generally not significantly associated, and if they were (significant for only 3/14 autoantibodies), the magnitude of association was similarly minute as found for DAS (not shown).

Table 1: Association of DAS over time with autoantibody levels over time.

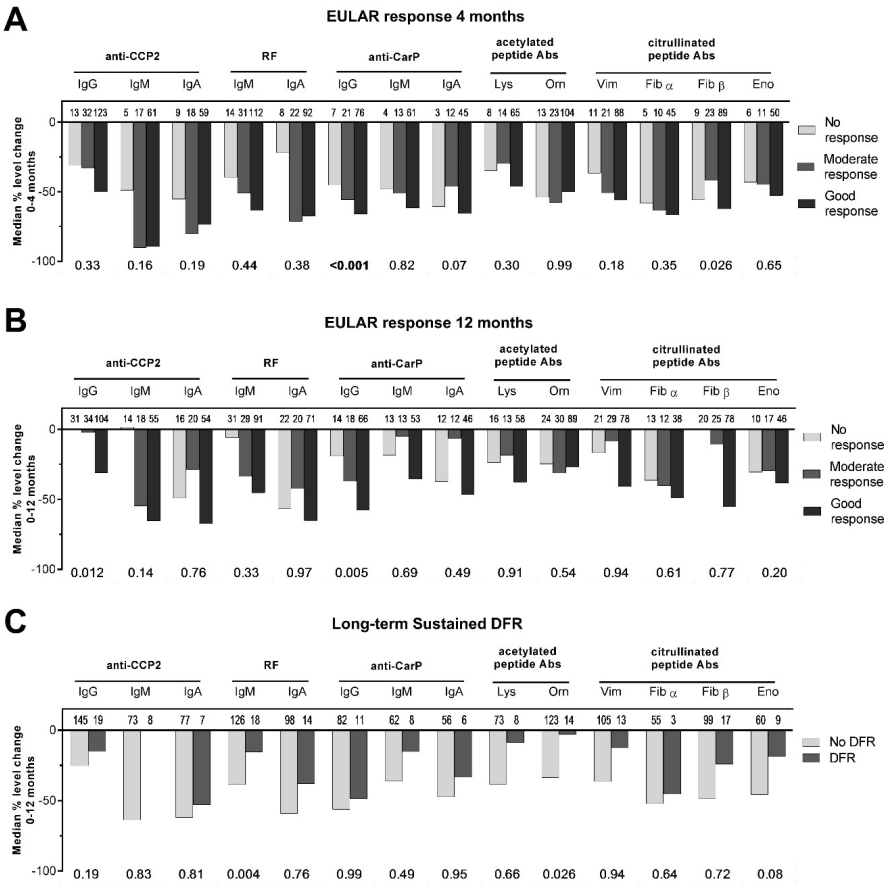
Outcome: DAS over time	n	β (95% CI) in DAS units per aU/mL
anti-CCP2 IgG (0-1600)	259	$1.1x10^{-4}$ ($9.3x10^{-6}$ to $2.0x10^{-4}$)
anti-CCP2 IgM (0-1400)	129	$3.5x10^{-4}$ ($1.4x10^{-4}$ to $5.7x10^{-4}$)
anti-CCP2 IgA (0-1160)	128	$3.6x10^{-4}$ ($3.8x10^{-5}$ to $6.7x10^{-4}$)
RF IgM (0-200)	245	$1.8x10^{-3}$ ($8.2x10^{-4}$ to $2.8x10^{-3}$)
RF IgA (0-200)	188	$7.9x10^{-4}$ ($-8.9x10^{-5}$ to $1.7x10^{-3}$)
Anti-CarP IgG (0-5272)	149	$1.1x10^{-4}$ ($4.1x10^{-5}$ to $1.7x10^{-4}$)
Anti-CarP IgM (0-3650)	128	$1.7x10^{-4}$ ($8.9x10^{-5}$ to $2.6x10^{-4}$)
Anti-CarP IgA (0-3100)	99	$2.7x10^{-4}$ ($1.6x10^{-4}$ to $3.8x10^{-4}$)
Anti-acetyl-lysine IgG (0-1000)	128	$2.5x10^{-4}$ ($2.1x10^{-5}$ to $4.7x10^{-4}$)
Anti-acetyl-ornithine IgG (0-1000)	226	$2.0x10^{-4}$ ($2.4x10^{-5}$ to $3.8x10^{-4}$)
Cit-Vim IgG (0-10,000)	188	$3.7x10^{-5}$ ($1.2x10^{-5}$ to $6.1x10^{-5}$)
Cit-Fib α IgG (0-25,000)	88	$1.6x10^{-6}$ ($-2.0x10^{-5}$ to $2.3x10^{-5}$)
Cit-Fib β IgG (0-100,000)	191	$4.2x10^{-6}$ ($2.2x10^{-6}$ to $6.2x10^{-6}$)
Cit-Eno IgG (0-70,000)	105	$2.9x10^{-6}$ ($-8.8x10^{-7}$ to $6.8x10^{-6}$)

Generalized estimating equation of the continuous outcome DAS over first year of treatment, within patients that were positive for that autoantibody at least once over the first year. Models were adjusted for age, gender, baseline HAQ, time, randomization arm at 4 months, and treatment decision at 8 months. Values behind autoantibody names indicate range (in aU/mL).

Changes in autoantibody levels are not associated with EULAR response

Most patients (264/381; 70%) had a good EULAR response at 4 months. Patients that achieved good/moderate EULAR response at 4 months had somewhat higher baseline autoantibody levels, but this small difference was not significant (data not shown). Patients that achieved good/moderate EULAR response at 4 months (or at 12 months) did not have significantly greater relative decreases in autoantibody levels between 0 and 4 months (or between 0 and 12 months, respectively) than patients with no response (**Figure 3A-B**). Changes in composites of the number of isotypes or AMPAs were also not associated with EULAR response (not shown).

Figure 3: Changes in autoantibody levels do not associate with treatment outcomes.



Relative change (%) in autoantibody levels (raw data) preceding a EULAR response at 4 months, b EULAR response at 12 months, and c long-term sustained DFR. Bold typeface of p values below graphs, calculated by ordinal (a, b) and logistic (c) regression, indicates significance after Holmes-Bonferroni correction for multiple testing (14 tests). Besides age and gender, models in a are adjusted for baseline DAS and BMI, in b are additionally adjusted for randomization arm at 4 months and treatment decision at 8 months, and in c are adjusted for baseline DAS, disease duration, and treatment decisions as per b

Changes in autoantibody levels are not associated with long-term outcomes like sustained DFR or radiographic progression

The next outcome we wished to analyze was long-term sustained DFR, which is the closest approximation of RA cure currently available. Interestingly, relative changes in autoantibody levels or number of isotypes or AMPAs between 0 and 12 months did not significantly differ between patients that did and did not achieve DFR (**Figure 3C;**

composites not shown). There was also no association between autoantibody levels at 8 months and ability to achieve DFR at 12 months (not shown). This indicates that autoantibody level changes over the first year of treatment are not informative for the ability to become drug-free.

In patients with radiographs available, 19% (67/360) had radiographic progression (≥ 0.5-point change from baseline) at 1 year, and 52% (152/294) had progression at 5 years. There was no association between baseline autoantibody levels or relative changes in autoantibody levels (0–12 months) and radiographic progression points at either 1 or 5 years (not shown).

DISCUSSION

In RA, it is unknown whether changes in autoantibody levels are associated with disease activity and treatment outcomes, or whether levels are modified by intensity of immunosuppressive therapy. Most studies have shown that RF (IgM, IgA, and IgG) levels decrease after treatment initiation with different DMARD classes (5-7, 10, 11), while anti-CCP2 (IgG) levels decrease only marginally, rebound after decreasing, or do not decrease at all (5-7, 10-13). Our results support this notion, as multiple forms and combinations of immunosuppressive medication in the IMPROVED led to reduction of autoantibody levels. Our results also showed that RF IgM decreases somewhat more than anti-CCP2 IgG, in line with previous reports (7).

Although the diagnostic and prognostic value of testing for autoantibody positivity in RA is well-established, reports are conflicting regarding the potential association of level fluctuations with disease activity in seropositive patients (5-7). In the current study, there was no relevant association between autoantibody changes and disease activity. Moreover, we also found no association with functional status, treatment response, or long-term outcomes such as DFR and radiographic progression. Instead, autoantibody level changes seem to be largely a reflection of immunosuppressive therapy, rather than an indication of disease-specific clinically-relevant processes. Therefore, their monitoring over time seems of limited value.

Autoantibody stability in different autoimmune diseases varies substantially, with some autoantibodies fluctuating with flares of disease, while others remain stable. These differences may be due to differences in longevity and place of residence of the autoantibody-producing cells. In RA, the synovial compartment appears to function as an inflammatory niche that promotes long-term survival of anti-citrullinated protein antibody (ACPA)-producing plasma cells (14). ACPA-producing plasmablasts also home to the bone marrow and differentiate to long-lived plasma cells there (14, 15). As RA disease activity subsides with anti-inflammatory treatment, the survival niches in inflamed joints may be eliminated and plasma cells residing there could be displaced

and die (16). Meanwhile, it appears plausible that bone marrow plasma cells remain unaffected by resolution of peripheral inflammation and continue to stably secrete RA-associated autoantibodies. This could explain why circulating autoantibodies show an initial decrease when treatment is initiated or intensified (elimination of joint survival niches) but are never fully eradicated (persistence of bone marrow niches), even in the absence of clinical symptoms. Whether the surviving bone marrow B cells/plasma cells contribute to the chronic pathogenic cascade remains to be determined. It is possible that such contributions may occur via pathways independent of autoantibody production, such as antigen presentation to T cells, cytokine secretion, or other immunoregulatory mechanisms. There may also exist autoantibody-specific differences in survival niches that plasma cells utilize that might explain why some autoantibodies change quite substantially upon immunosuppressive treatment while others remain more stable.

The current findings have some limitations. First, we chose not to further dilute serum samples above the highest standard of the ELISA, precluding the detection of level changes in patients with very high concentrations. However, sensitivity analyses excluding these patients yielded the same conclusions (not shown). Second, it is possible that the autoantibody level decreases seen between 0 and 4 months in all patients and between 8 and 12 months for those with treatment intensification are primarily due to the effect of prednisone, as prednisone has been shown to decrease total circulating immunoglobulin levels, especially IgG (17). The design of this study did not allow us to investigate whether this was the case. Thirdly, we do not have serological data in the timeframe between 12 months and the long-term outcomes investigated. It is possible that antibody changes closer to the outcome are more relevant than those over the first year of treatment. Fourth, only the effect of conventional DMARDs and anti-TNF has been investigated. It may be that DMARDs with other modes of action, such as rituximab, have different effects on autoantibody levels. It is also possible that the same agents applied during a disease state may associate with different level changes than demonstrated here, as our results only apply to early RA. Finally, we recognize that due to the limited radiographic progression that occurred in the IMPROVED study, we lacked sensitivity for finding a relationship with autoantibody levels.

Strengths of this study include the extensive array of autoantibodies measured and the longitudinal nature of the analyses, which allowed analysis of absolute changes in 14 different autoantibodies spanning four autoantibody families in 4-month intervals over the first year of treatment while accounting for missing serum. The nature of the IMPROVED trial and its long follow-up also allowed us to investigate multiple short-term and long-term outcomes not previously linked to autoantibody changes, as well as the effect of immunosuppression on level changes.

CONCLUSIONS

We conclude that in early RA, changes in autoantibody levels do not associate DAS over time or with treatment response. Instead, autoantibody level changes seem to be a reflection of treatment intensity. Together, these results suggest that autoantibody levels change over time and are modifiable by commonly used DMARDs, but that autoantibody level changes are in itself of limited clinical relevance and not useful to monitor over time.

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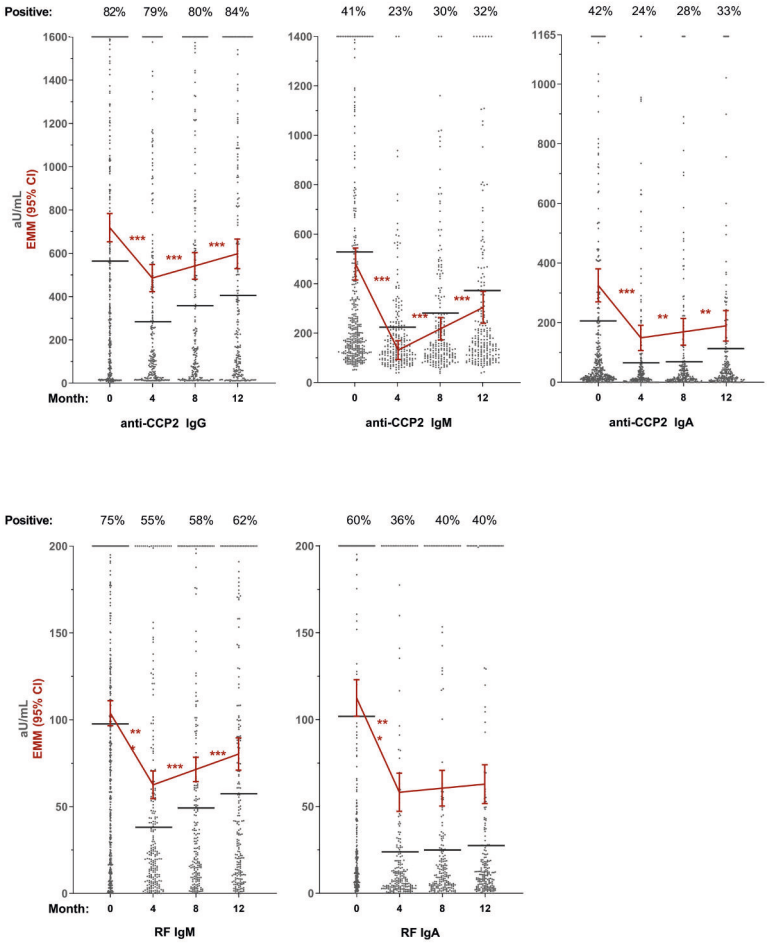
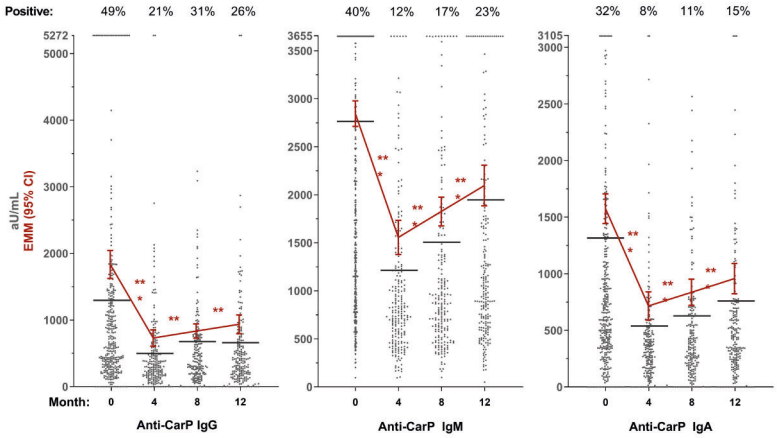
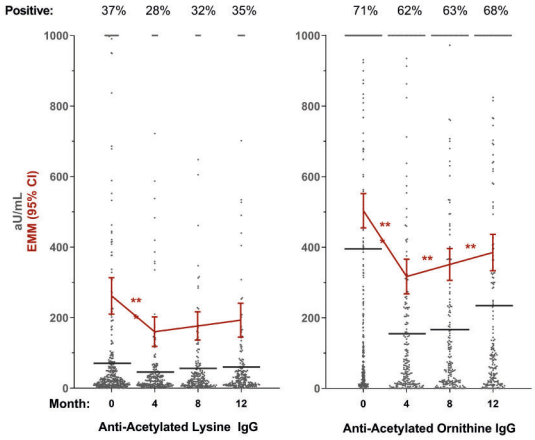
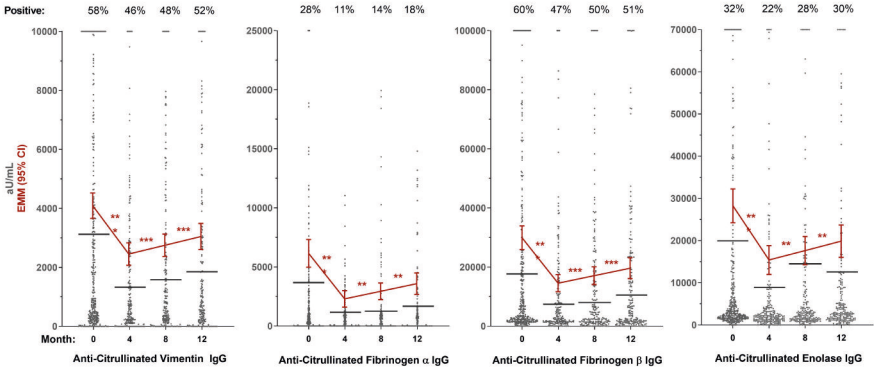
SUPPLEMENTARY FIGURES

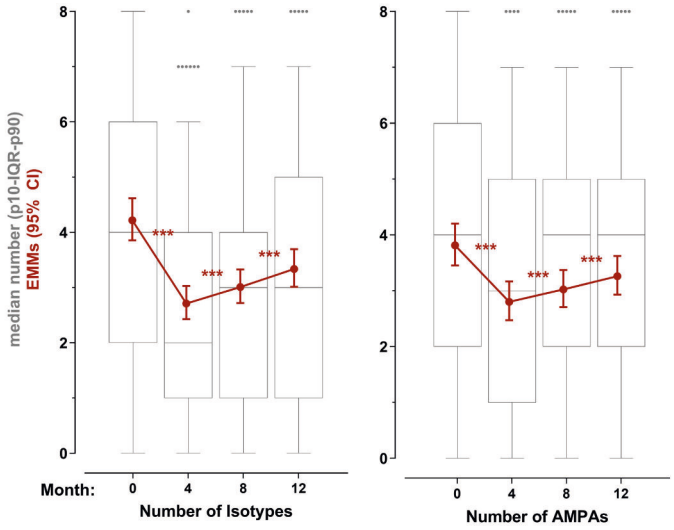
FIGURE S1: Levels (aU/mL), number of autoantibodies, and DAS over 1st year of treatment. *Autoantibody data is from serum of seropositive RA patients by ELISA (N 0 months=356; N 4 months=225; N 8 months=209; N 12 months=212). For levels, patients clustered at the maximum were above the highest standard of the ELISA. Black and red lines respectively indicate median aU/mL and estimated marginal mean (EMM) aU/mL with 95% confidence intervals (calculated by GEE) in patients that were positive for that autoantibody at least once. Box plots indicate median, interquartile range, and 10th and 90th percentiles. P-values (asterisk) refer to the level change between two time-points. *p<0.05 **p<0.01 ***p<0.001 Number of isotypes based on anti-CCP2 IgG, IgM, IgA; RF IgM, IgA; and anti-CarP IgG, IgM, IgA; number of AMPAs based on anti-CCP2 IgG, anti-CarP IgG, anti-citrullinated-vimentin 59-74 IgG, anti-citrullinated-fibrinogen β 36-52 IgG and α 27-43 IgG, anti-citrullinated-enolase 5-20 IgG, anti-acetylated-lysine IgG, and anti-acetylated-ornithine IgG.*




Positive:
49%
21%
31%
26%
40%
12%
17%
23%
32%
8%
11%
15%
aU/mL
EMM (95% CI)
Month:
0
4
8
12
Anti-CarP IgG
Anti-CarP IgM
Anti-CarP IgA


Positive:
37%
28%
32%
35%
71%
62%
63%
68%
aU/mL
EMM (95% CI)
Month:
0
4
8
12
Anti-Acetylated Lysine IgG
Anti-Acetylated Ornithine IgG


Positive:
58%
46%
48%
52%
28%
11%
14%
18%
60%
47%
50%
51%
32%
22%
28%
30%
aU/mL
EMM (95% CI)
Month:
0
4
8
12
Anti-Citrullinated Vimentin IgG
Anti-Citrullinated Fibrinogen α IgG
Anti-Citrullinated Fibrinogen β IgG
Anti-Citrullinated Enolase IgG


median number (p10-IQR-p90)
EMMs (95% CI)
Month:
0
4
8
12
Number of Isotypes
Number of AMPAs

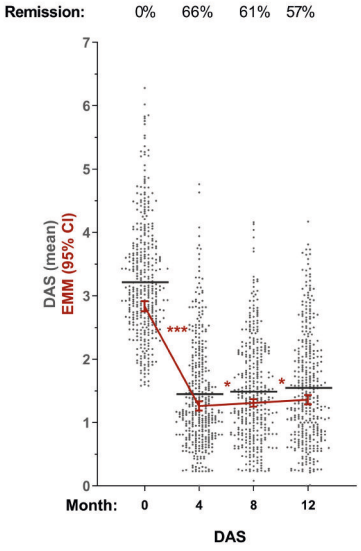
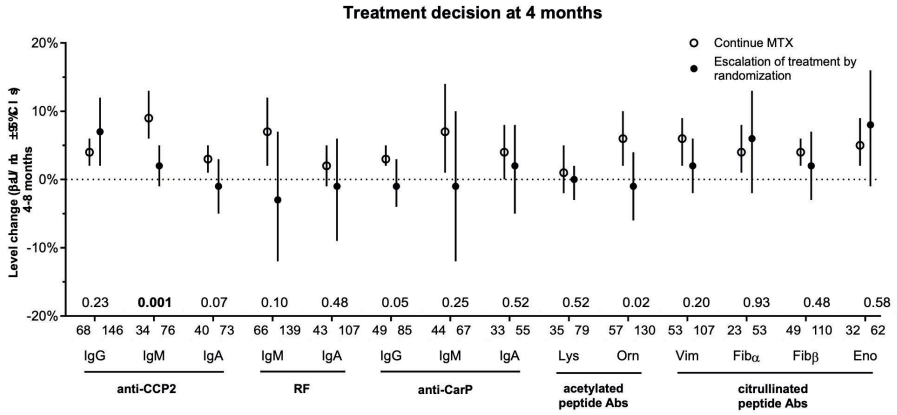

Remission:
0%
66%
61%
57%
DAS (mean)
EMM (95% CI)
Month:
DAS

Figure S2: Autoantibody levels change following treatment decision at 4 months.



*Change in autoantibody levels (calculated by GEE) following treatment decision at 4 months, within patients that were positive for that autoantibody at least once over the 1st year. Escalation of treatment by randomization comprised addition of prednisone, hydroxychloroquine, and sulfasalazine (Arm 1) or of adalimumab (Arm 2) to MTX monotherapy. Depicted regression coefficients (β in aU/mL, with 95% CIs) are of the predictor time from a GEE-model stratified for the treatment decision, and thus indicate autoantibody level changes between 8-12 months for that treatment decision group. Coefficients were normalized for comparison purposes by dividing by the maximum aU/mL of the ELISA range. GEEs were adjusted for age, gender, smoking status, and disease duration. Bold typeface of p-values calculated by GEE (interaction term treatment*time) indicates significance after Holmes-Bonferroni correction for multiple testing (14 tests).*

CHAPTER 6

Circulating calprotectin (S100A8/A9) is higher in rheumatoid arthritis patients that relapse within 12 months of tapering anti-rheumatic drugs

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ABSTRACT

Objective

To investigate whether calprotectin (S100A8/A9 or MRP8/14), an inflammatory complex released by monocytes, could indicate residual subclinical inflammation in rheumatoid arthritis (RA) patients who are in stable remission on disease-modifying anti-rheumatic drugs (DMARDs) and serve as a marker for disease flare after DMARD tapering.

Methods

We used data from two trials. Patients from the IMPROVED study had early (< 2 years) RA, and when they achieved disease activity score remission (DAS44 < 1.6), they stopped methotrexate to attempt drug-free remission. Patients from the RETRO study had established RA in stable remission (DAS28 < 2.6) and either tapered by 50% or stopped (biological or conventional) DMARDs. Circulating calprotectin at the tapering time point was determined by ELISA, and its predictive value for flare (loss of remission) within 12 months of DMARD tapering/stopping was determined.

Results

In both IMPROVED (n = 104) and RETRO (n = 57), patients that flared within 12 months had higher calprotectin at the moment of DMARD tapering/stopping. Twofold higher calprotectin at the moment of DMARD tapering/stopping was associated with an increased risk (odds ratio) of flare of 1.07 (95% CI 0.98–1.18, p = 0.14) in the IMPROVED and 3.62 (95% CI 1.76–7.46, p < 0.001) in the RETRO. Correcting for clinical predictors of flare (DAS at study inclusion, anti-CCP2 positivity, gender) did not change these estimates. The area under the receiver operating curve of calprotectin levels for predicting flare within 12 months was 0.63 (95% CIs 0.51–0.76) in the IMPROVED study and 0.80 (95% CIs 0.69 to 0.92) in the RETRO study.

Conclusion

Circulating calprotectin levels in RA patients in remission on DMARDs are higher in patients that will flare upon DMARD tapering/stopping. Since the differences between the cohorts precluded definitive conclusions, more research is needed to determine whether calprotectin has prognostic value in predicting flare after attempting drug tapering in RA.

Trial registration

IMPROVED, ISRCTN11916566. RETRO, 2009-015740-42.

INTRODUCTION

In rheumatoid arthritis (RA), improvements in the control of inflammation by conventional synthetic and biologic disease-modifying antirheumatic drugs (DMARDs) are slowly shifting therapeutic goals from preventing damage by suppressing disease activity to tapering DMARDs in patients in stable remission to achieve drug-free remission (DFR). However, DFR will not be reached by all RA patients in remission, and the disease flare that can occur after unsuccessful DMARD tapering can increase disease burden that could have been avoided if the DMARDs had not been interrupted. Hence, identifying factors associated with increased risk of disease flare after tapering DMARDs would aid clinical decision-making.

Unfortunately, this endeavor has proven difficult. Most studies agree that seropositivity [both rheumatoid factor (RF) and anti-cyclic citrullinated protein antibodies (ACPA)], shared epitope presence, high disease activity at baseline, and long symptom duration are risk factors for flaring upon DMARD tapering (1, 2). Residual subclinical inflammation has also been proposed to be a major predictor of flare, prompting multiple studies to identify biomarkers sensitive in detecting residual inflammation at the moment of tapering, including multibiomarker disease activity scores (3) and inflammation on imaging (1). However, these markers are limited by variable associations and lack of replication.

More recently, circulating calprotectin has gained traction as a promising biomarker for RA disease activity. Calprotectin, also called S100A8/A9 or myeloid-related protein (MRP) 8/14, is a heterodimeric complex of the S100 family constitutively expressed in leukocytes. During infectious and inflammatory events, calprotectin acts as alarmin and amplifies inflammation by binding to receptors for advanced glycation end-products and Toll-like receptor 4. Traditionally measured in feces in inflammatory bowel disease diagnostics, calprotectin differs from hepatocyte-dependent acute-phase reactants like C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR) because it is locally released by leukocytes at the sites of inflammation (4). In arthritis, activated granulocytes and tissue-resident macrophages in inflamed joints may contribute to calprotectin release. Various studies indicate that calprotectin actively promotes inflammation during arthritis(5, 6), is elevated in the synovial fluid and blood of RA patients, correlates consistently with markers of RA disease activity, is associated with ultrasound-detected inflammation, predicts future structural damage, and may have value in monitoring early response to biological DMARDs(7, 8). Furthermore, in juvenile idiopathic arthritis (JIA), a high level of calprotectin correlated with a high risk of relapse after etanercept(9) and methotrexate(10) discontinuation. The same was true in systemic-onset JIA after anakinra discontinuation (11).

All of these findings point to the intimate link between calprotectin, neutrophil activation, and inflammation in arthritis. If calprotectin indeed indicates residual subclinical

inflammation in RA patients who are in stable remission on DMARDs, it could serve as a practical biomarker for future disease flare after DMARD tapering and facilitate risk stratification to predict whether DMARD tapering will be successful. Such data could aid in clinical decision-making and replace the trial-and-error strategies by which DFR is currently—and infrequently—achieved. To that end, we investigated in two independent cohorts whether circulating calprotectin in patients who are in remission on DMARDs is associated with risk of disease flare after DMARD tapering and discontinuation.

METHODS

Study design, patient population, and outcomes

We used data from two multicenter randomized controlled trials: the IMPROVED study (an acronym for Induction therapy with MTX and Prednisone in Rheumatoid Or Very Early arthritic Disease) and the RETRO study (an acronym for Study of Reduction of Therapy in Patients with Rheumatoid Arthritis in Ongoing Remission). The IMPROVED study (12) enrolled 610 patients with early (< 2 years) untreated RA or undifferentiated arthritis and was steered at disease activity score remission (DAS44 < 1.6). In patients achieving remission, DMARDs were stopped, aiming for drug-free remission (DFR), with treatment adjusted every 4 months according to whether remission was maintained. Initial treatment comprised MTX and high-dose prednisone. Subjects selected for this study were all 104 patients who (1) fulfilled the 2010 ACR/EULAR RA criteria, (2) achieved remission at 4 months and stopped MTX at 8 months, and (3) had serum available for calprotectin testing at 8 months.

The RETRO study(13) enrolled patients with established RA who had been in stable remission (DAS28-ESR ≤ 2.6) for at least 6 months on conventional and/or biological DMARD treatment regimen. Patients were randomized to three treatment arms: (1) continue DMARDs at full dose for 12 months, (2) taper current dose by 50% for the next 12 months, or (3) reduce current dose by 50% for the first 6 months before entirely stopping all DMARDs. All patients in the taper arm (arm 2, n = 33) or stop arm (arm 3, n = 24) were included for this analysis.

Autoantibody status of the patients from both studies was determined by routine clinical testing for anti-CCP2 (IgG) and RF (IgM). The main outcome for both studies was flare within 12 months of tapering/stopping DMARDs, defined in the IMPROVED as a DAS44 ≥ 1.6 determined at 4-month intervals and in the RETRO as DAS28-ESR > 2.6 determined at 3-month intervals.

Calprotectin measurements

Patient serum was collected when patients were in remission and stored in − 80 °C until use. In the IMPROVED, circulating calprotectin was measured at baseline and 8 months (month of MTX stopping) using a fecal Calprotectin Extended Range kit adapted for

serum (QUANTA Lite®, Inova Diagnostics, research use only for serum). In the RETRO, calprotectin was measured in baseline samples, which was at study inclusion directly preceding DMARD tapering, before any tapering or stopping changes were made to existing DMARDs, using an in-house sandwich ELISA developed by Johannes Roth (Muenster) for predicting flares in juvenile idiopathic arthritis as previously described (11). Absorbance was converted to nanograms per milliliter using a standard curve of serial dilutions of a known concentration.

Statistical analysis

Calprotectin levels (in ng/mL) were compared between the groups using Mann-Whitney U tests, and correlations with inflammatory parameters were explored cross-sectionally using Spearman tests. Univariable and multivariable binary logistic regression was performed with disease flare within 12 months as the outcome. Calprotectin levels were log2-transformed and included as a continuous covariate. Area under the receiver operating characteristic curves (AUCs), test characteristics, and predictive values of calprotectin levels for flare were calculated, and the optimum cut-off was determined using the Liu index(14).

Additionally, logistic regression models were used to evaluate whether the addition of calprotectin to a clinical predictor model for flare significantly improved prediction of flare within 12 months. Clinical predictors of flare with a p value of ≤ 0.1 in univariable models were considered for the multivariable models to discover clinical predictors common to both cohorts, as well as specific to each cohort (as a sensitivity analysis, see Supplementary Table S1A-B). Areas under the ROC curve were calculated from the predicted values of the model with and without calprotectin, and compared using a Chi-square-based test for equality (15). All analyses were conducted separately for each cohort, using Stata SE 14.1.

RESULTS

In patients in remission, calprotectin is not associated with inflammatory parameters or autoantibody status

The characteristics of both cohorts are displayed in **Table 1**.

Circulating calprotectin levels, measured at the moment of DMARD tapering (for RETRO) or stop (for IMPROVED), were equal in the IMPROVED and the RETRO (p = 0.51), as well as between the two arms of the RETRO study (p = 0.43). Calprotectin levels were not correlated with acute-phase reactants (ESR, CRP), disease activity scores (DAS), or physical function as measured by the Health Assessment Questionnaire (HAQ) in either cohort, most likely due to the low variability of these parameters in these patients in remission (**Supplementary Figure S1**), and baseline demographics did not differ

between patients with high vs. low calprotectin levels (**Supplementary Table S2**). In baseline serum of the IMPROVED study, when patients had active disease activity (DAS44 ≥ 2.6) and had not yet received DMARD treatment, calprotectin did correlate with these inflammatory parameters (not shown), in a similar magnitude to what has been reported (7).

Table 1 Patient characteristics

	IMPROVED (N=104)	RETRO (N=57)
Age, mean years (SD)	49 (13)	55 (13)
Female, n (%)	67 (64%)	37 (65%)
BMI, mean (SD)	25 (4)	25 (4)
Ever smoker, n (%)	47 (46%)	18 (32%)
Disease duration, median (IQR)	19 (9-36) weeks^	5 (3-10) years
Anti-CCP2 IgG positive, n (%)	85 (83%)	34 (62%)
RF IgM positive, n (%)	79 (79%)	39 (68%)
Current DMARD use†		
Methotrexate	104 (100%)	47 (82%)
Glucocorticoids	0 (0%)	11 (19%)
Other csDMARDS	0 (0%)	6‡ (11%)
Biological DMARDs	0 (0%)	21 (37%)
Etanercept	-	5 (9%)
Adalimumab	-	6 (11%)
Tocilizumab	-	5 (9%)
Golimumab	-	2 (4%)
Certolizumab	-	3 (5%)
DAS44 at tapering/stop moment, mean (SD)	0.9 (0.4)	-
DAS28-ESR at tapering/stop moment, mean (SD)	-	1.8 (0.7)
Disease flare within 12 months	78 (75%)	26 (46%)
Calprotectin ng/mL, median (IQR)	1000 (230-2422)	1000 (650-2000)

Footnote: SD standard deviation, IQR interquartile range, Anti-CCP2 anti-citrullinated protein 2 antibody, RF rheumatoid factor, cs/bDMARD conventional synthetic/biological disease-modifying antirheumatic drugs, DAS disease activity score, ESR erythrocyte sedimentation rate. ^Disease duration is based on the moment of study inclusion; in the IMPROVED study, this excludes the first 4 months of treatment †Categories are not mutually exclusive ‡Patients received sulfasalazine (n = 2), hydroxychloroquine (n = 2), leflunomide (n = 1), or azathioprine (n = 1)

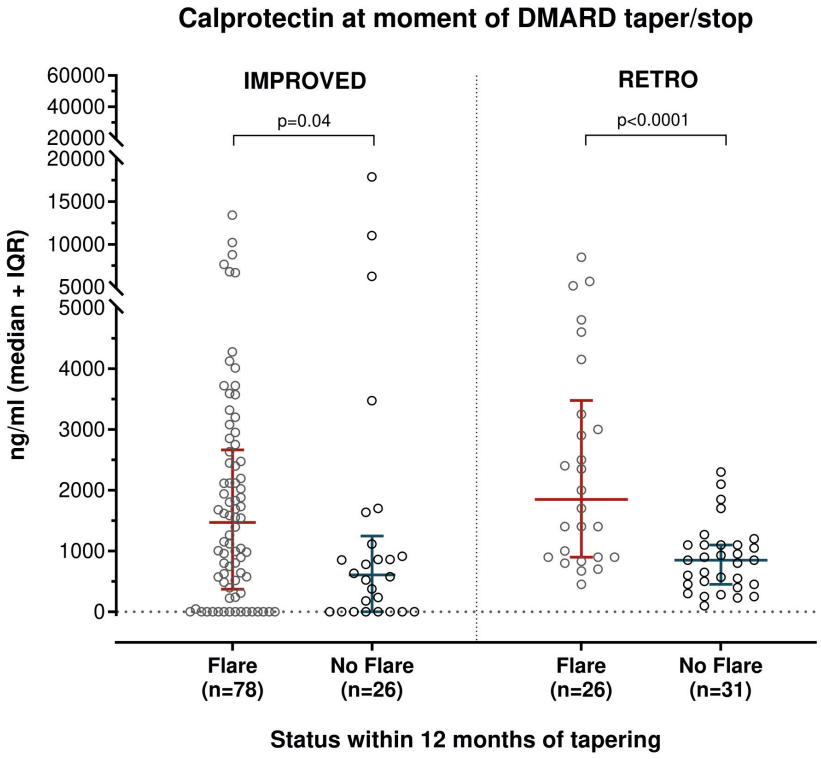
Calprotectin levels were higher in seropositive than seronegative patients, although not significantly: median (IQR) levels in anti-CCP2 IgG-positive vs. anti-CCP2 IgG-negative patients were 1115 (309–2749) ng/mL vs. 764 (0–1636) ng/mL in IMPROVED (p = 0.21)

and 1075 (800–2400) ng/mL vs. 930 (570–1850) ng/mL in RETRO (p = 0.45). In RF IgM-positive vs. RF IgM-negative patients, this was 1115 (237–2448) ng/mL vs. 982 (224–3318) ng/mL in IMPROVED (p = 0.96) and 1050 (550–1700) ng/mL vs. 965 (650–2300) ng/mL in RETRO (p = 0.75).

Calprotectin levels are higher preceding flare than no flare

In the IMPROVED study, disease flare was defined as DAS44 ≥ 1.6 within 12 months of stopping MTX. In the RETRO, flare was defined as DAS28-ESR > 2.6 within 12 months of tapering or stopping (biological or conventional) DMARDs. As indicated by **Figure 1**, calprotectin was significantly higher at the moment of DMARD tapering/stopping in patients that experienced a disease flare within 12 months.

Figure 1: Circulating calprotectin levels (ng/mL) at the moment of DMARD-tapering/stop, separated by whether patients experienced a disease flare within 12 months of tapering. p-values were calculated by Mann-Whitney-U tests

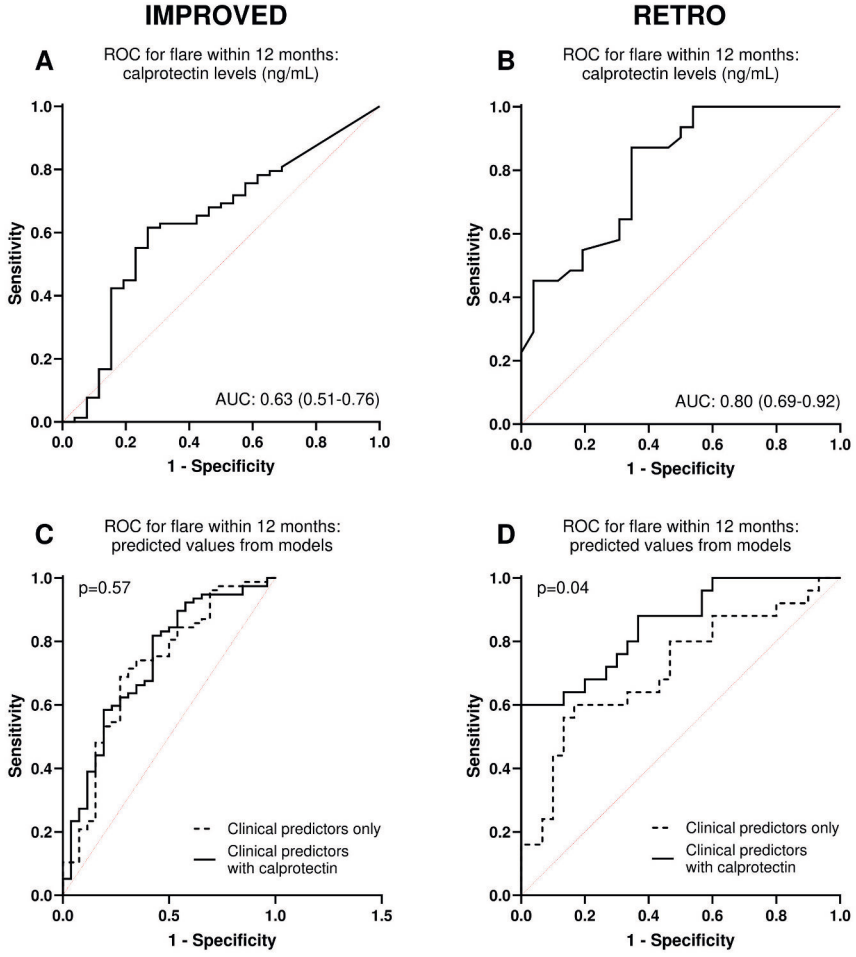


Calprotectin levels are associated with disease flare

In a logistic regression model with log2-transformed calprotectin as a predictor, twofold higher calprotectin at the moment of DMARD tapering/stopping was associated with an increased risk (odds ratio) of flare of 1.07 (95% CI 0.98–1.18, p = 0.14) in the IMPROVED

study and 3.62 (95% CI 1.76–7.46, p < 0.001) in the RETRO study. Correcting for clinical predictors of flare (DAS at study inclusion, anti-CCP2 positivity, gender) resulted in similar estimates: 1.09 (95% CI 0.98–1.21, p = 0.11) in the IMPROVED study and 3.77 (95% CI 1.74–8.18, p < 0.001) in the RETRO study. Correcting for clinical predictors of flare selected for their predictive value specific for each cohort (ESR at tapering moment, gender, anti-CCP2 positivity, and DAS at study inclusion in IMPROVED; SJC and anti-CCP2 positivity in RETRO) resulted in similar estimates (not shown).

Figure 2: *ROC-curves indicating the predictive value of calprotectin and clinical predictors for disease flare within 12 months of tapering in the IMPROVED (A,C) and RETRO (B,D) studies. A,B: circulating calprotectin levels (ng/mL) at the moment of DMARD-tapering/stop; C,D: comparison of models including clinical predictors only (dashed line) and clinical predictors in combination with circulating calprotectin levels (solid line). The p-value is based on the test for equality of AUCs.*



The AUC of calprotectin levels predicting flare in the IMPROVED study was 0.63 (95% CI 0.51–0.76, p = 0.002), indicating modest to poor discriminatory capacity (**Figure 2A**). In the RETRO, calprotectin had a slightly better AUC of 0.80 (95% CI 0.69–0.92, p = 0.0001) for predicting flare within 12 months (**Figure 2B**).

In the IMPROVED, addition of calprotectin levels did not significantly improve a model including only the clinical predictors (DAS as baseline, anti-CCP2 positivity, gender): the clinical predictor model had an AUC of 0.72 (95% CI 0.60–0.84), compared to a model combining calprotectin and clinical predictors that had an AUC of 0.73 (95% CI 0.62–0.85) (p for test of equality of AUCs = 0.57; **Figure 2C**). However, in the RETRO, the addition of calprotectin to a clinical predictor model significantly improved the AUC from 0.71 (95% CI 0.57–0.85) to 0.85 (95% CI 0.75–0.95) (p for the test of equality of AUCs = 0.04; **Figure 2D**). These results were the same when cohort-specific clinical predictors mentioned above were used (not shown).

Test characteristics based on optimal and clinically relevant cutoffs

At an optimal cutoff determined using the Liu index, calprotectin levels above 936 ng/mL in the IMPROVED had the following test characteristics (95% CI) for disease flare within 12 months: 62% (50–72%) sensitivity, 73% (52–88%) specificity, 87% (76–95%) positive predictive value (PPV), and 39% (25–54%) negative predictive value (NPV). In the RETRO, calprotectin levels above 1335 ng/mL had slightly better test characteristics (95% CI): 65% (44–83%) sensitivity, 87% (70–96%) specificity, 81% (58–95%) PPV, and 75% (59–88%) NPV.

Since identifying patients with high flare risk that should not taper DMARDs is particularly relevant, we also examined a practical cutoff that maximized specificity. For the IMPROVED, calprotectin levels above 3571 ng/mL (corresponding to the 85th percentile) were 88% (70–98%) specific for flaring. In the RETRO, calprotectin levels above 2350 ng/mL (corresponding to the 79th percentile) were 100% (89–100%) specific for flaring. Patients classified as at risk for flare in this way (i.e., above this optimal cutoff) did not have higher inflammatory parameters (ESR, CRP, DAS, VAS, or HAQ) than those predicted not to flare (i.e., below the cutoff; not shown). In clinical practice, one could therefore infer from very high calprotectin levels that a patient is likely to flare regardless of other inflammatory parameters and should not taper DMARDs.

DISCUSSION

In this study, we evaluated the role of circulating calprotectin in RA as a predictor for disease flare upon DMARD tapering and discontinuation in two independent cohorts. Higher circulating calprotectin levels were associated with significantly increased risk of flare within 12 months after DMARD tapering in the RETRO study and a small, non-significant effect in the IMPROVED study. In the RETRO study, calprotectin significantly improved the identification of patients that would flare within 12 months over clinical predictors alone.

Calprotectin has been reported to correlate better with clinical disease activity of RA than acute-phase reactants do (16). These findings suggest that calprotectin levels may reflect local inflammatory processes in the joints and therefore make it of particular value in predicting whether inflammation has been abrogated and patients can achieve drug-free remission. Possessing a marker that can accurately indicate residual inflammation in patients in remission would help to optimize therapeutic decision-making by accurately stratifying which patients should and should not attempt to taper DMARDs.

In line with the literature (16), circulating calprotectin levels in our study were variable and sometimes quite high, even in patients in remission, a disease state in which systemic inflammation is, in principle, low. Although we were not able to replicate previous findings regarding the correlation between calprotectin and joint indices due to the lack of variability in joint indices, the fact that calprotectin did not correlate with other inflammatory parameters in these patients in remission suggests that calprotectin may indeed indicate residual inflammation not measured by these parameters, probably because it is highly expressed and released specifically at local sites of inflammation (4).

However, calprotectin's value for predicting disease flare was not consistent in both cohorts. Although the direction of the found effect was the same in both cohorts, the effect sizes we found are quite divergent and only the RETRO study reached consistent statistical significance, possibly due to the differences between the cohorts. One major difference is the use of DAS44 in the IMPROVED and DAS28-ESR in the RETRO, which does not detect affected joints in the feet and thus could miss peripheral disease activity in the feet. It also is conceivable that calprotectin is not sensitive enough to detect residual inflammation when DAS is extremely low, as was the case in the IMPROVED study (DAS44 < 1.6 instead of DAS28-ESR < 2.6 in RETRO). However, a sensitivity analysis restricting the analyses in the IMPROVED cohort only to patients on the higher side of the spectrum (DAS44 ≥ 0.9 at 8 months) did not indicate that calprotectin had more value in patients with slightly higher disease activity (not shown). It is also possible that calprotectin cannot predict minute changes in DAS (e.g., small flares), but a sensitivity analysis in the IMPROVED study in which DAS44 ≥ 2.6 within 12 months was regarded as flare (more comparable to the RETRO definition) did not indicate that calprotectin had greater value in predicting larger DAS44 change-based flares than smaller flares (not shown). Differences in the biological use, disease durations, and remission duration could explain the discrepant results as well. However, stratification on biological use in the RETRO resulted in very small numbers and inconclusive results, and the disease and remission durations between the cohorts were so divergent that stratification on this parameter would not yield fair comparisons.

Some previous studies investigating calprotectin and flares in rheumatic diseases other than RA support calprotectin as a marker for flare after tapering DMARDs (10, 11), but Tweehuysen et al. found no value of calprotectin for predicting successful DMARD

tapering in RA (17). The main difference between our cohorts and the DRESS study (an acronym for Dose REduction Strategy of Subcutaneous TNF inhibitors) they investigated was that all patients in their study tapered biological DMARDs, specifically TNFα inhibitors. It is possible that TNFα inhibitors may directly inhibit the release of calprotectin in vivo and dampen calprotectin's value as a marker of local inflammation, thereby also limiting its ability to predict future disease flare. However, beyond the upregulation of calprotectin by TNFα in murine models (18, 19), an inverse correlation between trough serum TNFα-inhibitor levels and circulating calprotectin (20), a consistent decrease of calprotectin upon TNFα-inhibitor initiation(8), no studies to date have explored this possibility.

Surprisingly, the population of the RETRO study, in which the association of calprotectin with flare was most convincing, is more similar to the DRESS study (long-standing disease, biological use including TNFα inhibitors, higher DAS at tapering) than the IMPROVED population is, even though the latter two cohorts showed little to no association of calprotectin with flare. This makes it very difficult to speculate on the reasons why these three cohorts show such divergent results, as there is clearly no pattern regarding patient characteristics and strength of associations found. Because of this, our results cannot definitively refute or commend calprotectin as a marker for successful DMARD tapering.

A further limitation to consider is that calprotectin was determined using different assays, and although circulating concentrations were converted to nanograms per milliliter using a known concentration curve in both cohorts, small differences in the sensitivity of the assays could remain. Sample handling may also have differed between various centers participating in the studies. However, there was no difference in calprotectin levels between the cohorts on the group level, so the effect of these limitations is most likely minor.

Although our results show modest effects, they are important because they situate calprotectin in a new field of prognostication in RA—that of drug-free remission. Currently, there is no consensus regarding calprotectin's use as a measure of disease activity or a biomarker for treatment response, let alone the possibility of using it as a marker of future disease flare, and more studies are needed to evaluate calprotectin in this role.

CONCLUSION

In conclusion, we found that calprotectin levels are higher in RA patients that will flare upon DMARD tapering. More research is needed to validate calprotectin as a biomarker for flare in RA patients tapering or discontinuing DMARDs.

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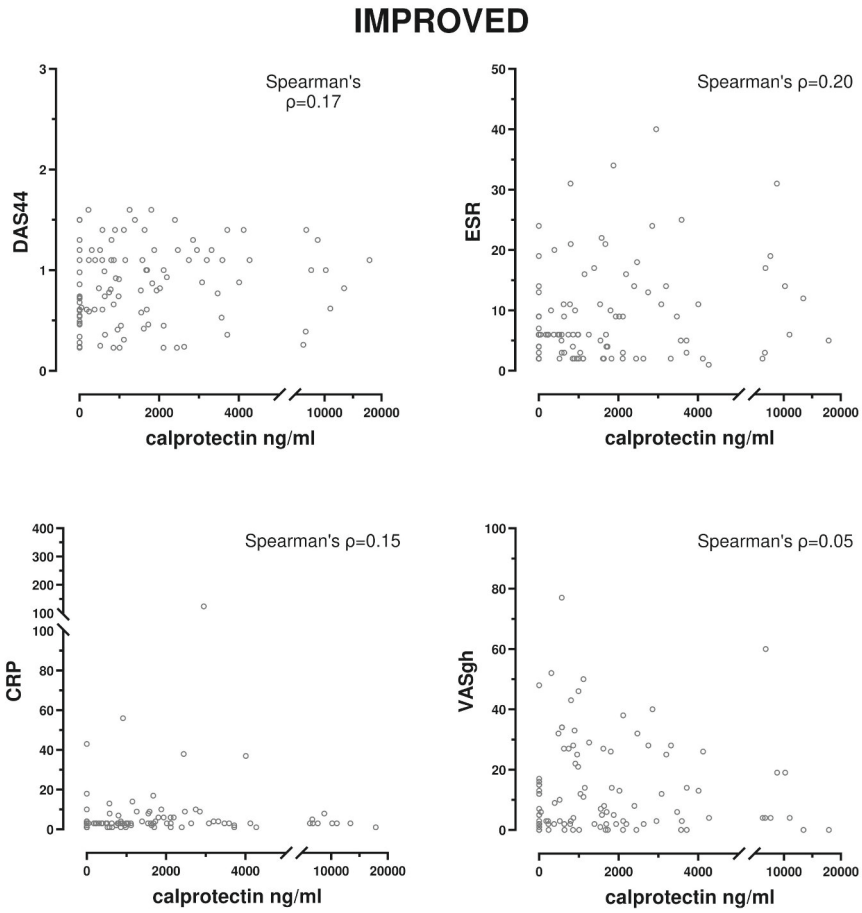
SUPPLEMENTARY FIGURES

Figure S1: *Circulating calprotectin levels (ng/mL) at the moment of DMARD tapering/stop does not correlate with inflammatory parameters in the IMPROVED (left) or the RETRO (right) study. VASgh = VAS global health.*



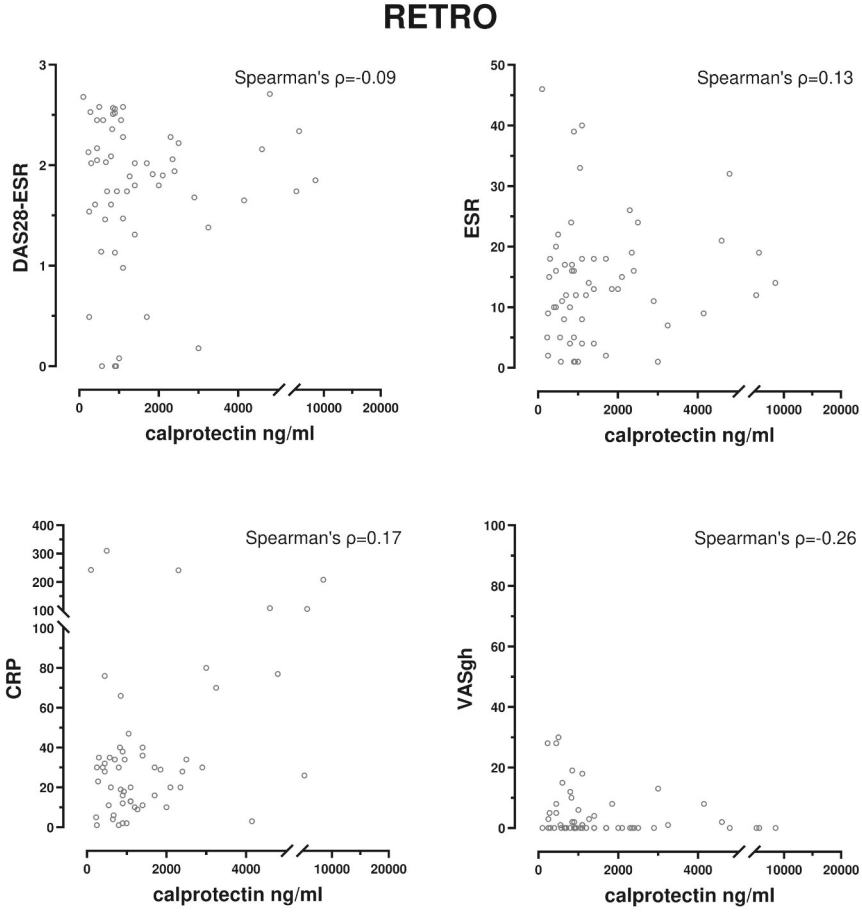

RETRO
Spearman's ρ=-0.09
DAS28-ESR
calprotectin ng/ml
Spearman's ρ=0.13
ESR
calprotectin ng/ml
Spearman's ρ=0.17
CRP
calprotectin ng/ml
Spearman's ρ=-0.26
VASgh
calprotectin ng/ml

Table S1A: Model building for outcome flare in the IMPROVED study. All variables were tested univariably for association with the outcome; only those variables with p≤0.1 were included in multivariable models. Multivariable models were tested separately for activity parameters (top) and for demographic/baseline variables (bottom).

IMPROVED study (n=104)						
Activity parameters at tapering/stop moment	**Flare (n=78)**	**No Flare (n=26)**	**Univariable OR (95% CI)**	**p-value**	**Multivariable OR (95% CI)**	**p-value**
DAS44, mean (SD)	0.9 (0.4)	0.7 (0.4)	3.67 (1.07-12.6)	**0.04**	1.16 (0.21-6.44)	0.87
ESR, median (IQR)	6 (3-14)	6 (2-9)	1.10 (1.01-1.19)	**0.04**	1.10 (1.01-1.20)	**0.04**
CRP, median (IQR)	3 (3-6)	3 (1-3)	1.01 (0.97-1.06)	0.56		
VASgh, median (IQR)	10 (2-26)	4 (2-8)	1.05 (1.00-1.09)	**0.03**	1.03 (0.98-1.08)	0.21
Tender joint count-28, median (IQR), (minimum-maximum)	0 (0-0), (0-4)	0 (0-0), (0-4)	1.02 (0.62-1.70)	0.93		
Swollen joint count-28, median (IQR), (minimum-maximum)	0 (0-0), (0-3)	0 (0-0), (0-1)	1.84 (0.47-7.28)	0.87		
HAQ, median (IQR)	0 (0-0.38)	0 (0-0.13)	33.0 (1.53-711)	**0.03**	18.8 (0.71-498)	0.08

Table S1A: Model building for outcome flare in the IMPROVED study. All variables were tested univariably for association with the outcome; only those variables with p≤0.1 were included in multivariable models. Multivariable models were tested separately for activity parameters (top) and for demographic/baseline variables (bottom). (continued)

Demographic/baseline variables	Flare (n=78)	No Flare (n=26)	Univariable OR (95% CI)	p-value	Multivariable OR (95% CI)	p-value
Age, mean years (SD)	49 (14)	51 (10)	0.99 (0.95-1.02)	0.43		
Female, %	69%	50%	2.25 (0.91-5.57)	**0.08**	3.10 (1.11-8.66)	**0.03**
BMI, mean (SD)	26 (4)	25 (3)	1.06 (0.92-1.22)	0.41*	1.15 (0.97-1.36)	0.11
Ever smoker, %	44%	52%	0.71 (0.29-1.76)	0.46		
Disease duration, weeks median (IQR)	19 (3-39)	18 (11-26)	1.02 (0.99-1.04)	0.14		
Anti-CCP2 IgG positive, %	84%	77%	1.63 (0.54-4.89)	0.39*	3.47 (0.95-12.6)	**0.05**
RF IgM positive, %	80%	75%	1.36 (0.46-4.00)	0.58		
DAS44, mean (SD)	3.1 (0.8)	2.7 (0.9)	2.11 (1.12-3.97)	**0.02**	2.45 (1.24-4.85)	**0.01**
HAQ, median (IQR)	1 (0.63-1.5)	0.75 (0.38-1.13)	1.95 (0.89-4.26)	0.10		

** Anti-CCP2 IgG positivity and BMI were noteworthy at p=0.07 and p=0.03 respectively as a likely predictors for flare in a screening model including all demographic/baseline variables together (not shown), and were therefore included in the subsequent multivariable model.*
Final IMPROVED-specific multivariable model including ESR at tapering moment, gender, anti-CCP2 positivity, and DAS44 at baseline had a pseudo-R2 of 14%. Final model including predictors common to both cohorts (DAS at baseline, anti-CCP2 positivity, gender) had a pseudo R2 of 11% in the IMPROVED study. Choice of these common predictors was based on known predictors of flare reported in literature as well as commonalities in predictors for both cohorts, reported above.

Table S1B: Model building for outcome flare in the RETRO study. All variables were tested univariably for association with the outcome; only those variables with p≤0.1 were included in multivariable models. Multivariable models were tested separately for activity parameters (top) and for demographic/baseline variables (bottom).

RETRO study (n=57)						
Activity parameters at tapering/stop moment	**Flare (n=26)**	**No Flare (n=31)**	**Univariable OR (95% CI)**	**p-value**	**Multivariable OR (95% CI)**	**p-value**
DAS28-ESR at tapering/stop moment, mean (SD)	1.8 (0.7)	1.8 (0.8)	1.02 (0.50-2.11)	0.95		
ESR, median (IQR)	13 (7-19)	13 (8-18)	1.00 (0.94-1.05)	0.87		
CRP, median (IQR)	32 (11-70)	20 (13-34)	0.94 (0.41-2.20)	0.89		
VASgh, median (IQR)	0 (0-5)	0 (0-4)	0.97 (0.90-10.5)	0.47		
Tender joint count-28, median (IQR), (minimum-maximum)	0 (0-0), (0-1)	0 (0-0), (0-1)	0.27 (0.03 -2.58)	0.26		
Swollen joint count-28, median (IQR), (minimum-maximum)	0 (0-0), (0-2)	0 (0-0), (0-1)	6.27 (0.74-53.18)	**0.09**	**n.d.***	

Table S1B: Model building for outcome flare in the RETRO study. All variables were tested univariably for association with the outcome; only those variables with p≤0.1 were included in multivariable models. Multivariable models were tested separately for activity parameters (top) and for demographic/baseline variables (bottom). (continued)

Demographic/baseline variables	Flare (n=26)	No Flare (n=31)	Univariable OR (95% CI)	p-value	Multivariable OR (95% CI)	p-value
Age, mean years (SD)	56 (13)	55 (13)	1.00 (0.96-1.04)	0.98		
Female, %	69%	61%	1.42 (0.47-4.28)	0.62		
BMI, mean (SD)	25 (3)	26 (4)	0.91 (0.79-1.05)	0.22		
Ever smoker, %	31%	32%	0.93 (0.30-2.87)	0.90		
Disease duration, years median (IQR)	8 (3-10)	4 (2-8)	1.04 (0.98-1.12)	0.20		
Anti-CCP2 IgG positive, %	80%	47%	4.57 (1.36-15.40)	**0.01**	**n.d.***	
RF IgM positive, %	73%	65%	1.49 (0.48-4.65)	0.49		
Biological DMARDs (%)	38%	35%	1.14 (0.39-3.34)	0.82		
Randomization arm 3 (Taper) (%)	50%	35%	1.82 (0.63-5.27)	0.27		

** Multivariable model not done (n.d.) as only one variable within each model subsection was significantly associated with the outcome.*
Final RETRO-specific multivariable model including swollen joint count-28 and anti-CCP2 positivity had a pseudo-R2 of 15%. Final model including predictors common to both cohorts (DAS at baseline, anti-CCP2 positivity, gender) had a pseudo R2 of 9% in the RETRO study. Choice of these common predictors was based on known predictors of flare reported in literature as well as commonalities in predictors for both cohorts, reported above.

Table S2: Inflammatory parameters and baseline demographics separated by low/high circulating calprotectin levels (ng/mL) split on its median at the moment of DMARD tapering/stop. P-values are based on t-tests, Mann-Whitney tests, or Chi-square tests for the differences in mean, median, and frequency of reported variables, respectively.

IMPROVED study (n=104)	Low calprotectin (n=52)	High calprotectin (n=52)	p-value
DAS44 at tapering moment, mean (SD)	0.8 (0.4)	0.9 (0.4)	0.22
ESR, median (IQR)	6 (3-9)	8 (3-15)	0.38
CRP, median (IQR)	3 (2-3)	6 (3-8)	**0.03**
VASgh, median (IQR)	6 (2-25)	6 (2-19)	0.73
Tender joint count-28, median (IQR), (minimum-maximum)	0 (0-0), (0-3)	0 (0-0), (0-4)	0.49
Swollen joint count-28, median (IQR), (minimum-maximum)	0 (0-0), (0-1)	0 (0-0), (0-3)	0.53
Age, mean years (SD)	49 (14)	49 (12)	0.95
Female, %	62%	67%	0.54
BMI, mean (SD)	25 (3)	26 (4)	0.09
Ever smoker, %	42%	49%	0.49
Disease duration, weeks median (IQR)	19 (10-30)	19 (9-50)	0.63
Anti-CCP2 IgG positive, %	81%	84%	0.64
RF IgM positive, %	78%	80%	0.73

Table S2: Inflammatory parameters and baseline demographics separated by low/high circulating calprotectin levels (ng/mL) split on its median at the moment of DMARD tapering/stop. P-values are based on t-tests, Mann-Whitney tests, or Chi-square tests for the differences in mean, median, and frequency of reported variables, respectively. (continued)

RETRO study (n=57)	Low calprotectin (n=29)	High calprotectin (n=28)	p-value
DAS28-ESR at tapering/stop moment, mean (SD)	1.7 (0.9)	1.8 (0.6)	0.67
ESR, median (IQR)	11 (5-17)	14 (10-19)	0.16
CRP, median (IQR)	28 (11-35)	29 (13-59)	0.36
VASgh, median (IQR)	1 (0-8)	0 (0-1.5)	0.10
Tender joint count-28, median (IQR), (minimum-maximum)	0 (0-0), (0-1)	0 (0-0), (0-0)	**0.02**
Swollen joint count-28, median (IQR), (minimum-maximum)	0 (0-0), (0-2)	0 (0-0), (0-1)	1.00
Age, mean years (SD)	53 (12)	59 (5)	0.08
Female, %	66%	64%	0.92
BMI, mean (SD)	26 (5)	25 (3)	0.39
Ever smoker, %	38%	25%	0.29
Disease duration, years median (IQR)	5 (3-10)	6 (2-10)	0.72
Anti-CCP2 IgG positive, %	57%	66%	0.47
RF IgM positive, %	66%	71%	0.63
Biological DMARDs (%)	34%	43%	0.52
Randomization arm 3 (Stop) (%)	38%	46%	0.52

CHAPTER 7

Autoantibody Development under Treatment with Immune Checkpoint Inhibitors

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ABSTRACT

Immune-checkpoint inhibitors (ICIs) activate the immune system to assault cancer cells in a manner that is not antigen specific. We hypothesized that tolerance may also be broken to autoantigens, resulting in autoantibody formation, which could be associated with immune-related adverse events (irAEs) and antitumor efficacy. Twenty-three common clinical autoantibodies in pre- and posttreatment sera from 133 ipilimumab-treated melanoma patients were determined, and their development linked to the occurrence of irAEs, best overall response, and survival. Autoantibodies developed in 19.2% (19/99) of patients who were autoantibody-negative pretreatment. A nonsignificant association was observed between development of any autoantibodies and any irAEs [OR, 2.92; 95% confidence interval (CI) 0.85–10.01]. Patients with antithyroid antibodies after ipilimumab had significantly more thyroid dysfunction under subsequent anti–PD-1 therapy: 7/11 (54.6%) patients with antithyroid antibodies after ipilimumab developed thyroid dysfunction under anti–PD1 versus 7/49 (14.3%) patients without antibodies (OR, 9.96; 95% CI, 1.94–51.1). Patients who developed autoantibodies showed a trend for better survival (HR for all-cause death: 0.66; 95% CI, 0.34–1.26) and therapy response (OR, 2.64; 95% CI, 0.85–8.16). We conclude that autoantibodies develop under ipilimumab treatment and could be a potential marker of ICI toxicity and efficacy.

INTRODUCTION

Immune-checkpoint inhibitors (ICIs) have improved the previously dismal prognosis of patients with various types of cancer, but at the cost of immune-related adverse events (irAEs), including arthritis, colitis, hepatitis, and various endocrinopathies (1). ICIs inhibit negative costimulatory signals to T cells, thereby enhancing antitumor T-cell responses (2). Because this mode of action is not antigen-specific, ICIs may also (re) activate otherwise dormant autoreactive T cells. This, in turn, might lead to a break in T-cell tolerance to not only tumor antigens but also autoantigens, resulting in activation of autoreactive B cells and ultimately the formation of autoantibodies. If true, the occurrence of autoantibodies may be associated with more frequent irAEs. Production of autoantibodies may indicate enhanced global immunogenicity, which may, in turn, be associated with better antitumor responses, as has been reported for changes in the T-cell repertoire (3-5). Therefore, we determined if autoimmune disease-associated autoantibodies were formed with ICI treatment and investigated their association with irAEs and clinical outcome.

MATERIALS AND METHODS

Patients and serological measurements

For this analysis, we included 133 patients with late-stage melanoma who were treated with ipilimumab, a CTLA-4 inhibitor, and for whom pre- and posttreatment serum or plasma samples were available. Patients were treated with a maximum of four cycles of ipilimumab 3 mg/kg in an expanded access program or according to the label after approval at the Netherlands Cancer Institute or the Leiden University Medical Center. Patients were included if they were at least 18 years of age and had histologically or cytologically proven irresectable stage IIIc or IV melanoma, with measurable metastatic lesions according to the RECIST 1.1 criteria. Patients were treated with four cycles of intravenous 3 mg/kg ipilimumab every 3 weeks. Sixty-six (49.6%) patients were treated with anti–PD-1 therapy following ipilimumab: either 2 mg/kg intravenous pembrolizumab every 3 weeks or 240 mg intravenous nivolumab every 4 weeks. The study was conducted in accordance with the Declaration of Helsinki after approval by the institutional review boards of both centers. All patients signed informed consent for withdrawal of extra blood samples for biomarker analysis. According to the study protocol, serum or plasma for autoantibody determination was collected before initiation of ipilimumab treatment and 12 weeks after. Pre- and posttreatment serum was snap-frozen and stored at −80°C until autoantibody determination. Due to failed measurements, post-ipilimumab autoantibody status could not be determined in 4 patients (**Supplementary Figure S1**).

Indirect immunofluorescence assays

Autoimmune hepatitis and primary biliary cirrhosis-associated antismooth muscle, antimitochondrial, and anti-liver/kidney microsome (LKM) antibodies were measured by indirect immunofluorescence assay (IFA) using mouse liver/kidney/stomach substrate (Aesku). Antinuclear antibodies (ANA) were determined in all patients by IFA using the HEp-2000 ANA Test System which uses human epithelioid cells stably transfected with the SSA/Ro autoantigen, cultured, and fixed directly on the test wells (Immuno Concepts). Patient serum samples at a dilution of 1:40 were incubated with antigen substrate for 30 minutes at room temperature to allow specific binding of autoantibodies to cell nuclei. After washing with phosphate-buffered saline to remove nonspecifically bound antibodies, the substrate was incubated with an anti-human antibody conjugated to fluorescein. After another washing step, the nuclear staining pattern was read using the international consensus on antinuclear antibody pattern (ICAP; Ref (6)) by two experienced, independent readers trained in ANA-pattern reporting and blinded to time order and patient data of samples. In the case of lack of consensus, a third reader functioned as tiebreaker. All system reagents, conjugates, calibrators, and positive and negative controls were provided by and used according to instructions of the manufacturer (Immuno Concepts). All steps of the IFA were conducted using a Helmed fully automated IFA slide processor (Aesku).

Fluorescence enzyme immunoassays

Anticyclic citrullinated peptide 2 (CCP2) IgG, rheumatoid factor (RF) IgM, antigliadin IgG, and (if ANA was positive by IFA) antibodies to extractable nuclear antigens (ENA) were determined by EliA technique on a Phadia ImmunoCap 250 instrument (Thermo Fisher Scientific). This is a fully automated and high-throughput fluorescence enzyme immunoassay system used for routine diagnostic laboratory testing. The fluorescence signal of measured serum samples is compared with calibrators with known concentrations. For anti-CCP2 IgG, citrullinated synthetic peptides (second-generation antigen) were used as antigen, for RF IgM, aggregated rabbit IgG was used, for antigliadin IgG, synthetic deamidated gliadin peptides were used, and for ENA, a Symphony Well of various antigens was used: human recombinant U1RNP (RNP70, A, C), SS-A/Ro (60 kDa, 52 kDa), SS-B/La, Centromere B, Scl-70 protein, Jo-1 protein, and native purified Sm proteins. Anti-ENA–positive patients were further assayed for the following specific ENA antibodies by EliA (coated antigens in parentheses): anti-SSA (human recombinant SS-A/Ro (60 kDa, 52 kDa) proteins), anti-SSB (human recombinant SS-B/La protein), anti-RNP70 (human recombinant RNP70 protein), anti-U1RNP (human recombinant U1RNP (RNP70, A, C) proteins), anti-Smith (synthetic SmD3 peptide), anti-Jo1 (human recombinant Jo-1 protein), anti-CENP (human recombinant centromere protein B), anti-PMSCL (human recombinant PM-Scl protein), anti-RNAP3 (human recombinant RNA polymerase III protein), anti-Scl70s (human recombinant Scl-70 protein). All system reagents, conjugates, calibrators, and positive and negative controls were used according to the manufacturer's instructions.

Chemiluminescent immunoassays

Antithyroid peroxidase (TPO), antithyroglobulin (TG), and, in ANA-positive patients, anti-dsDNA were determined by noncompetitive chemiluminescent immunoassay (CLIA) using Immulite 2000 (Siemens Healthineers). These assays use a luminescent adamantyl dioxetane phosphate tracer and were performed using reagents provided by the manufacturer according to instructions in the package insert.

Clinical data

Information about demographics, treatment response, survival status, and the occurrence of irAEs was obtained from retrospective review of medical records. irAEs were recorded starting from the first ipilimumab treatment until one year later, death, or the start of different therapy (whichever occurred first), using Common Terminology Criteria for Adverse Events version 4.03: any grade arthralgia/arthritis, colitis, hypophysitis, primary adrenal insufficiency, primary thyroid dysfunction, dermatitis (rash, vitiligo, or psoriasis), uveitis, or grade 3–4 hepatitis. Primary thyroid dysfunction as an irAE during anti–PD-1 treatment (nivolumab or pembrolizumab) following ipilimumab treatment was determined in the same manner. Hematologic and serum parameters necessary for making the above diagnoses were determined at baseline, every 3 months during follow-up, at progressive disease, and according to the treating oncologist's clinical judgment. Three patients had preexisting hypothyroidism. Thyroid dysfunction was registered only as an irAE in these patients if symptoms were aggravated and a new medical intervention was indicated. Two of the four cases of arthralgia/arthritis constituted a flare of preexisting rheumatoid arthritis (RA). Survival was defined as time from start of ipilimumab to death of any cause, recorded between start of first ipilimumab treatment until January 2018, for a median follow-up time of 20.4 months (IQR: 8.8–40.8). Radiologic evaluation (CT or PET/CT scanning) was performed at baseline, week 12, and subsequently every 3 months until progression. Response was scored according to RECIST 1.1 criteria. Best overall response was defined as the best response recorded from start of ipilimumab until date of progression, death, or the start of a different therapy (whichever occurred first). Patients achieving a partial response or complete response were considered responding patients.

Statistical analysis

We used McNemar test for paired data to test whether autoantibody positivity increased post-ipilimumab. Frequencies of irAEs in patients who developed antibodies versus those who did not were compared using Fisher exact tests. To test whether post-ipilimumab autoantibody positivity was associated with (i) the development of any irAEs under ipilimumab, primary thyroid dysfunction under subsequent anti–PD-1 therapy, or better overall response, and (ii) overall survival, binary logistic regression and Cox proportional hazards regression, respectively, were used, and adjusted for age, gender, treating hospital, and number of ipilimumab cycles received. All analyses were conducted using Stata statistical software, Special Edition, release 14.1 (StataCorp LP).

RESULTS

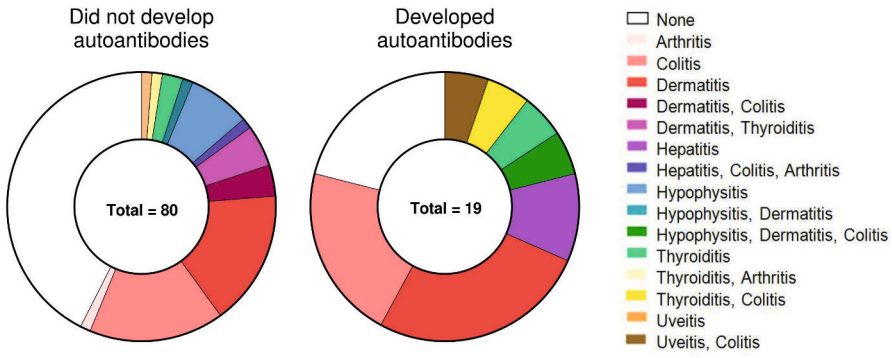
Autoantibody development

Mean age was 59 years [standard deviation (SD): 14], and 62% of patients were male. Of 127 patients with complete pre-ipilimumab autoantibody data, 26 (20%) were positive for any of the autoantibodies before treatment. In total, 29% (36/125) of patients with complete autoantibody data were autoantibody-positive after ipilimumab treatment. Of patients who were fully autoantibody-negative before ipilimumab treatment, 19.2% (19/99) developed any autoantibodies posttreatment ($P < 0.0001$). Predominantly anti-TPO (4.8%, 6/125) and anti-TG (6.0%, 8/132) appeared in patients who were negative for these autoantibodies at baseline ($P = 0.03$ and $P = 0.008$, respectively). For all other autoantibodies, posttreatment positivity did not greatly change (**Supplementary Figure S1**).

Association of autoantibodies with irAEs under ipilimumab

A nonsignificant association was seen between the development of any autoantibody and irAEs: 15/19 (78.9%) patients who developed any autoantibody experienced irAEs compared with 46/80 (57.5%) patients who did not develop autoantibodies [OR, 2.92; 95% confidence interval (CI) 0.85–10.01; **Figure 1**]. When disregarding autoantibody status pre-ipilimumab, patients with autoantibodies posttreatment also experienced more irAEs (**Supplementary Figure S2**). No significant association between pre-ipilimumab autoantibody positivity and irAEs was observed: 8/26 (31%) patients who were autoantibody-positive pre-ipilimumab experienced irAEs compared with 38/100 (37%) patients who were autoantibody-negative pre-ipilimumab (OR, 1.61; 95% CI, 0.62–4.18; $P = 0.33$).

Figure 1. Frequency of irAEs in pre-ipilimumab autoantibody-negative patients who did not develop autoantibodies (left) versus those who developed autoantibodies (right) after ipilimumab treatment.



In a prespecified subgroup analysis, we focused only on the irAEs related to the tested autoantibodies (arthritis/arthralgia, hepatitis, thyroid dysfunction, colitis, adrenal insufficiency, dermatitis, or sicca symptoms). In this analysis, 14/19 (73.7%) patients who developed autoantibodies had irAEs related to the tested antibodies compared with 37/80 (46.3%) patients who did not develop autoantibodies, indicating a significant association between the development of autoantibodies and irAEs (OR, 3.64; 95% CI, 1.13–11.75). However, the appearance of a specific autoantibody did not associate with the occurrence of an irAE in the organ system affected by the disease for which the specific autoantibody has diagnostic value (**Table 1**).

Table 1: Association between autoantibody development and irAEs.

	Converted to positive for…	Stayed negative for…	p
Any irAE / any antibody	15/19 (78.9%)	46/80 (57.5%)	0.12
Any autoantibody-related irAE † / any antibody	14/19 (73.7%)	37/80 (46.3%)	**0.04**
Arthralgia or arthritis / anti-CCP2 or RF	0/3 (0%)	3/121 (2.5%)	1.00
Hepatitis / autoimmune hepatitis antibodies‡	1/8 (12.5%)	4/109 (3.7%)	0.30
Thyroiditis / anti-TPO or anti-TG	2/13 (15.4%)	8/111 (7.2%)	0.28
Colitis / anti-endomysium or anti-gliadin IgG	0/2 (0%)	30/129 (23.3%)	1.00
Adrenal insufficiency / anti-adrenal cortex	0/0 (0%)	0/133 (0%)	N/A
Dermatitis / anti-nuclear antibodies	1/4 (25%)	33/122 (27%)	1.00
Sicca symptoms / anti-nuclear antibodies	0/4 (0%)	1/122 (0.8%)	1.00

In each cell, n/N indicates the number of patients who developed the irAE (n) out of the total number who converted to positive or stayed negative for the indicated antibody (N). p-values are calculated by Fisher's exact test. † arthritis/arthralgia, hepatitis, thyroid dysfunction, colitis, adrenal insufficiency, dermatitis, or sicca symptoms. ‡ anti-smooth muscle, anti-mitochondria, anti-liver-kidney-microsome, or anti-nuclear antibodies.

Association of autoantibodies with thyroid dysfunction under anti–PD-1 therapy

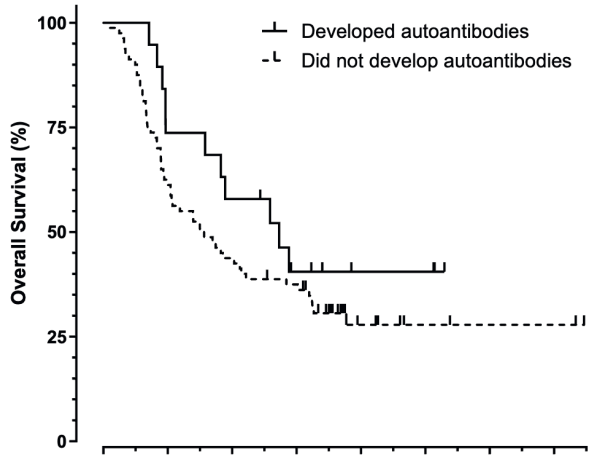
We hypothesized that autoantibody development with ipilimumab treatment might predispose patients to irAEs during subsequent anti–PD-1 therapy. Following progression on ipilimumab treatment and after exclusion of patients who had thyroid dysfunction with ipilimumab (n = 12), 61 (50.4%) patients received anti–PD-1 therapy. In these patients, we found a significant association between the development of thyroid autoantibodies while on ipilimumab and subsequent thyroid dysfunction under PD-1 blockade: 4/9 (44.4%) patients who developed thyroid autoantibodies with ipilimumab and subsequently received anti–PD1-therapy had thyroid dysfunction under anti–PD-1 compared with only 7/48 (14.6%) patients who did not develop autoantibodies (OR, 6.26; 95% CI, 1.07–36.5; P = 0.04). The association between the development of thyroid autoantibodies while on ipilimumab and subsequent thyroid dysfunction under PD-1

blockade was even stronger when autoantibody status pre-ipilimumab was disregarded and all anti–PD-1-treated patients were included in the analysis (n = 60; one patient missing anti-TPO measurement): 7/11 (54.6%) patients who had thyroid autoantibodies after ipilimumab treatment developed thyroid dysfunction under anti–PD-1 compared with 7/49 (14.3%) patients who did not develop autoantibodies (OR, 9.96; 95% CI, 1.94–51.1; P = 0.006).

Association of autoantibody development with survival and response

We next investigated the association of autoantibody development following the initial ipilimumab treatment with survival and response. During the median follow-up time of 20.4 months (IQR, 8.8–40.8), 92 (69%) patients died after a median of 11.2 months (IQR 7.3–21.9), 87 patients (65%) had stable or progressive disease, and 46 patients (35%) achieved complete or partial response. Patients who developed autoantibodies had a minor survival benefit compared with those that stayed autoantibody-negative, although this was not significant (HR for all-cause death: 0.66; 95% CI, 0.34–1.26; P = 0.21; **Figure 2**). There was no significant association between the presence of a specific autoantibody and survival (**Supplementary Table S1**).

Figure 2. Overall survival in patients who were negative for autoantibody at baseline.



Patients who developed autoantibodies (n = 19) were compared with those who did not develop autoantibodies (n = 80). Numbers below the graph indicate the number of patients at risk within each group.

There was also a trend toward an association between development of any autoantibody and treatment responses (OR for response: 2.64; 95% CI, 0.85–8.16; P = 0.09). When assessing specific autoantibodies, only the development of thyroid autoantibodies was significantly associated with treatment response (OR for response: 5.43; 95% CI, 1.38–21.4; P = 0.02).

To determine whether the observed associations between autoantibody development and clinical outcomes were due to an association of irAEs with both entities, we also investigated the association between irAEs and clinical outcomes. No association between occurrence of irAEs and survival or treatment response was found (HR for all-cause death: 1.12; 95% CI, 0.70–1.79], P = 0.64; OR for response: 1.53; 95% CI, 0.68–3.46, P = 0.31). The estimates of the association between autoantibodies and survival and treatment response reported above did not greatly change after adjusting the analyses for occurrence of irAEs: HR for all-cause death: 0.65; 95% CI, 0.34–1.25, P = 0.20; OR for response: 2.50; 95% CI, 0.80–7.84, P = 0.12. All associations of autoantibody status with survival and treatment response were similar when autoantibody status pre-ipilimumab was disregarded and all patients were included in the analysis (**Supplementary Table S2**). Patients who were autoantibody-positive pre-ipilimumab had no survival or response benefit compared with patients who were autoantibody-negative (HR for all-cause death: 1.16; 95% CI, 0.67–2.03, P = 0.59; OR for response: 0.53; 95% CI, 0.19–1.46; P = 0.22).

DISCUSSION

In this study, we found that ipilimumab treatment induced development of autoantibodies in a fifth of melanoma patients. Our analyses revealed a trend for association between autoantibodies and irAEs under ipilimumab, and a much stronger, significant association between ipilimumab-induced thyroid autoantibodies and thyroid dysfunction under subsequent PD-1 blockade. Lastly, we found a minor survival and response benefit in patients who developed autoantibodies, specifically in those who developed thyroid autoantibodies.

We determined the presence autoantibodies both pre- and posttreatment with ICI therapy and linked these data to irAEs and clinical outcomes. Our results expand previous findings regarding the presence thyroid autoantibodies in patients with ICI-induced thyroid dysfunction (7-10) by showing that these autoantibodies also develop in the absence of overt thyroid dysfunction. Antithyroid antibodies are common in populations without overt thyroid disease, associated with or induced by concomitant autoimmune disease (i.e., type 1 diabetes mellitus, RA, and Celiac disease, refs (11-17)), mutations in CTLA-4 (18, 19), upregulation of MHC class II molecules on thyrocytes leading to thyroid antigen presentation to autoreactive cells (20), or as we show here, ipilimumab treatment. Our data also confirm previous studies reporting that patients rarely develop RA autoantibodies (21-24) or autoimmune hepatitis antibodies even in the presence of the related irAE (25-28).

Our findings demonstrated that the development of thyroid autoantibodies predisposes euthyroid ipilimumab-treated patients to subsequent thyroid dysfunction under anti–PD-1 therapy. The association between thyroid autoantibodies and thyroid dysfunction under anti–PD-1 therapy has been described previously (29, 30). Our results confirm that it is clinically useful to monitor patients with preexisting thyroid autoantibodies closely for thyroid dysfunction with anti–PD-1 therapy.

Although several studies report an association between irAEs and clinical outcome under ICIs (31, 32), we did not find such an association in this study. A relationship between immune-related thyroid dysfunction and clinical outcome has been described previously for various types of cancer immunotherapy, including IL2 (33-35), interferon-2α (35-37), and pembrolizumab (29). Some of these studies also found a response or survival benefit under IL2 (33, 34) or interferon-2α (36) for patients who developed thyroid autoantibodies. These findings are in line with our observations that patients developing thyroid autoantibodies with ICI have a better treatment response.

Our results indicate that CTLA-4 inhibition may lead to loss of B-cell self-tolerance. ICIs execute their function in an antigen-independent manner by diversifying the T-cell repertoire against a multitude of tumor antigens (3, 5). In this study, we found that tolerance to nontumor autoantigens is broken as well and that breaking of tolerance may be associated with signs of clinical autoimmunity, under CTLA-4 inhibition as well as subsequent PD-1 blockade. We did not observe that specific autoantibodies induced disease in their related organ systems, but our results lacked the power to test this hypothesis. Breaking of B-cell tolerance and development of autoantibodies was also associated with better treatment response (statistical trend) and a survival benefit (though nonsignificantly). Previous studies have shown that greater expansion of the T-cell repertoire by ICIs is associated with better response (3, 5, 38). We hypothesize that this expansion is paired with T cell–dependent activation of autoreactive B-cells and autoantibody production. If this is the case, autoantibodies may function as a marker for effective ICI-induced immunogenicity, and it is this enhanced immunity (rather than the autoantibodies themselves) which leads to reactions against both clinically favorable (e.g., tumor) and unfavorable (e.g., nontumor/self) tissues. This could explain the link found in this study between autoantibodies and both treatment response and irAEs.

The main limitation of this study is a lack of power due to the limited number of patients. This may explain why some of our findings failed to reach statistical significance. We also had limited clinical data for which to correct our analyses. However, our study tested all patients treated with ipilimumab (not just the subset that developed irAEs) for a broad panel of autoantibodies in a longitudinal manner, facilitating a pre- and posttreatment comparison of autoantibody prevalence.

We conclude that the development of autoantibodies is common with ipilimumab treatment and that autoantibody presence is associated with the development of irAEs and a trend for better overall response and survival. These results indicate a promising avenue for future research in the quest for biomarkers predicting ICI therapy toxicity and efficacy.

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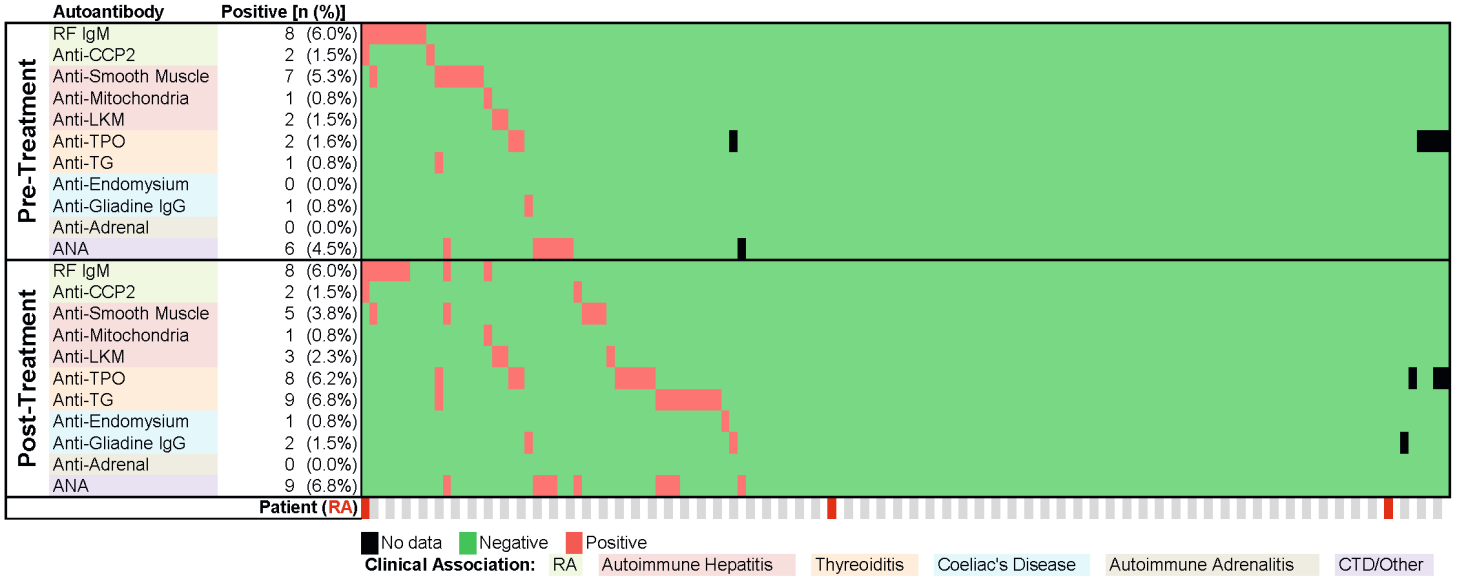
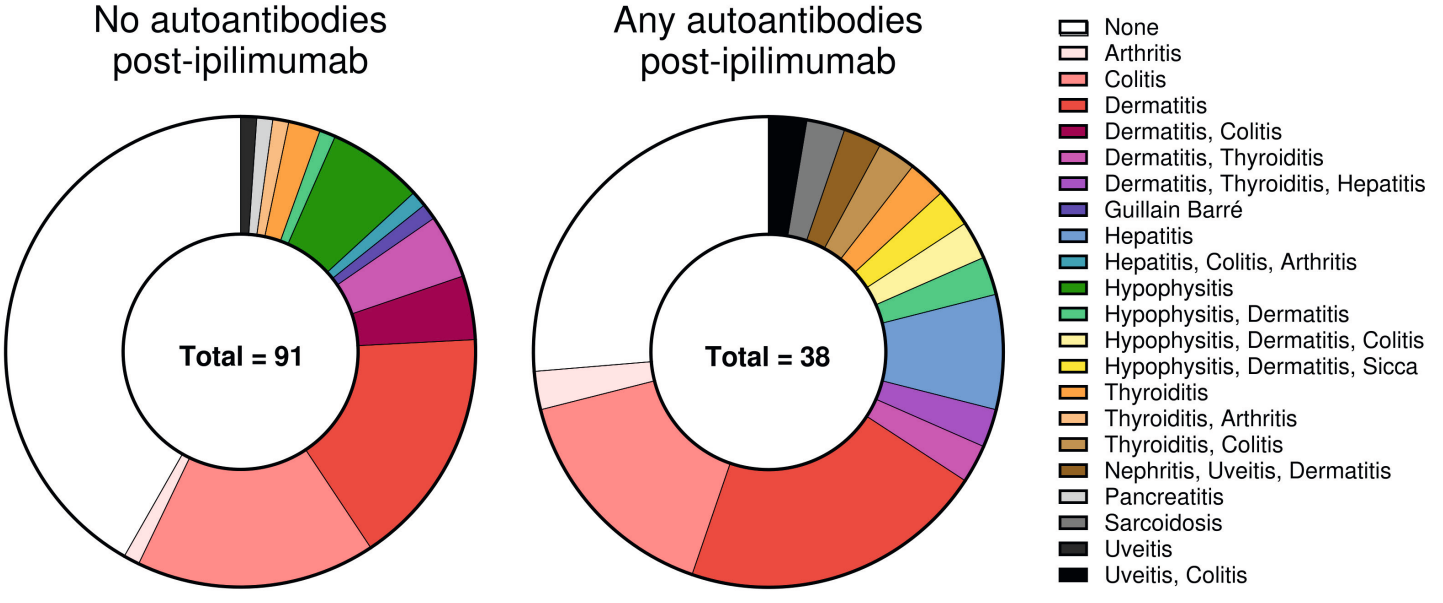
SUPPLEMENTARY FILES

Supplementary Figure S1: Heatmap of antibody positivity pre- and post-ipilimumab treatment. Not shown: all patients were anti-ENA negative at baseline, while at follow-up, two patients became anti-ENA positive, specifically anti-SSA positive. RA: rheumatoid arthritis. CTD: Connective tissue diseases.



Supplementary Figure S2: Frequency of (combinations of) irAEs in patients that had (right) or did not have (left) any autoantibodies post-ipilimumab. OR for irAE development: 2.28 (95% CI: 0.95 to 5.46), p=0.064.



Supplementary Table S1: Association between autoantibody development post-ipilimumab (in pre-ipilimumab negative patients) and all-cause death as calculated by multivariable Cox proportional hazards regression, adjusted for age, gender, treating hospital, and number of ipilimumab cycles received.

Development of…	n positive post-ipi/ N total negative pre-ipi	HR (95% CIs) for all-cause death	p
any autoantibody	19/99	0.66 (0.34 to 1.26)	0.21
anti-CCP2 or RF	3/124	0.68 (0.53 to 1.34)	0.60
autoimmune hepatitis antibodies†	8/117	1.00 (0.43 to 2.40)	0.98
anti-TPO or anti-TG	13/124	0.50 (0.47 to 1.35)	0.10
anti-endomysium or anti-gliadin IgG	2/131	2.67 (0.63 to 11.3)	0.18
anti-adrenal cortex	0/133	N/A	N/A
anti-nuclear antibodies	4/126	0.96 (0.30 to 3.11)	0.95

Ipi: ipilimumab. † anti-smooth muscle, anti-mitochondria, anti-liver-kidney-microsome, or anti-nuclear antibodies.

Supplementary Table S2: Association between autoantibody positivity post-ipilimumab and all-cause death and treatment response. All analyses adjusted for age, gender, treating hospital, and number of ipilimumab cycles received.

Positive post-ipilimumab for..	n positive/ N total	HR (95% CIs) for all-cause death	p	OR (95% CIs) for treatment response	p
any autoantibody	38/129	0.84 (0.52 to 1.36)	0.47	1.60 (0.68 to 3.78)	0.28
anti-CCP2 or RF	9/133	0.95 (0.41 to 2.21)	0.91	1.22 (0.25 to 5.88)	0.80
autoimmune hepatitis antibodies†	17/133	0.80 (0.41 to 1.56)	0.51	1.81 (0.53 to 6.17)	0.34
anti-TPO or anti-TG	16/130	0.57 (0.28 to 1.19)	0.14	3.30 (1.02 to 10.7)	0.05
anti-endomysium or anti-gliadin IgG	3/132	3.35 (1.02 to 11.0)	0.05	0.81 (0.07 to 9.71)	0.87
anti-adrenal cortex	0/133	N/A	N/A	N/A	N/A
anti-nuclear antibodies	9/133	1.25 (0.57 to 2.76)	0.58	2.04 (0.35 to 11.9)	0.43

HR calculated by Cox proportional hazards regression. OR calculated by logistic regression. † anti-smooth muscle, anti-mitochondria, anti-liver-kidney-microsome, or anti-nuclear antibodies.

CHAPTER 8

Discussion

Rheumatoid arthritis (RA) is a chronic autoimmune disease affecting mainly small joints, associated with specific autoantibodies recognizing modified protein epitopes, termed anti-modified protein antibodies (AMPAs). Among these, anti-citrullinated protein antibodies (ACPA) targeting citrullinated epitopes are well-studied. Additionally, other AMPAs recognizing carbamylated, acetylated, and malondialdehyde-acetaldehyde modified proteins have emerged (1-3). Understanding AMPAs, particularly ACPA, has reshaped RA pathophysiology theories, linking them to genetic factors like HLA shared epitope (SE) alleles and environmental factors like smoking (4-6).

Although ACPA-positive RA has been associated with specific genetic and environmental factors, the exact mechanisms triggering autoantibody formation remain unclear. Research on other autoimmune diseases in diverse populations has uncovered unique interactions between genetic susceptibility and environmental triggers, exemplified by fogo selvagem in Amerindians(7), as described in **Chapter 2**. Investigating autoantibody responses across diverse populations and ethnic groups could unveil novel risk factors and disease mechanisms that play a role in the development of the autoantibody response and of the resulting autoimmune disease like RA.

To that end, **Chapter 2** explored the geoepidemiology of AMPAs (anti-CarP, anti-MAA, and anti-AcVim) in four ethnically diverse ACPA-positive RA populations from different continents: The Netherlands, First Nations (Canada), Japan, and South Africa. We observed evident differences in AMPA prevalence and levels across cohorts, in line with previous studies that reported prevalence of anti-CarP in non-Caucasian/non-Western populations (8, 9); no other data yet exists on population differences in anti-MAA or anti-AcVim. However, observed differences largely corresponded to variations in total IgG levels rather than the classic RA risk factors HLA SE, HLA DRB1*03, or smoking. In fact, we found no consistent associations between smoking, HLA SE alleles, and AMPA positivity across cohorts. Later studies also found no association of non-ACPA AMPAs with HLA SE and smoking in a clinically suspect arthralgia cohort (10). In another study, the association with HLA-SE and smoking with various AMPAs showed conflicting results, also when restricting the analysis only to anti-CCP2 positive RA like in our study: a positive association of smoking or HLA-SE was found with some anti-acetylated proteins and with some anti-carbamylated proteins but not with others (11). It is difficult at present to draw conclusions from this, and more research is needed to elucidate whether AMPAs associate with classic RA genetic and environmental risk factors, as is the case for ACPA.

Overall, despite the lack of association, our findings do tell us something interesting: while AMPAs are present in diverse RA populations across the world, population-specific risk factors do not seem to significantly shape their development. This implies that the development of a broad AMPA response, pathognomonic in seropositive RA, is the final common outcome of different pathways involved in disease. Work by our colleagues supports this theory in mice, where immunization with carbamylated proteins induces

development of antibodies recognizing both carbamylated and acetylated (self) proteins, and vice versa for immunization with acetylated proteins (12, 13). Anti-CarP and ACPA responses are also cross-reactive to a certain degree (13, 14), as are the B-cells producing them (15). In this way, different AMPA responses can originate from a common B-cell response that may lead to disease development, regardless of the original PTM or antigen this B-cell clone recognized, which may indeed vary greatly dependent on local exposures to environmental stimuli, microbiotic infections, or genetic susceptibility.

However, other factors must also play a role in shaping the AMPA response because cross-reactivity alone is not enough: 1) both AAPA and anti-CarP-responses are only partly cross-reactive with citrullinated antigens (3, 16) and 2) anti-MAA-antibodies display no cross-reactivity at all (17). There are multiple other processes that have been suggested to play a role in ACPA-producing B-cells surviving the various natural checkpoints that normally eliminate autoreactive clones. One promising mechanism is the selective introduction of V-domain N-glycosylation sites during T-cell aided somatic hypermutation (SHM) of the ACPA-reactive B-cell receptor that, contrary to SHM in other immune responses, apparently does not produce affinity maturation toward citrullinated antigens (18, 19). This may allow autoreactive ACPA B-cells, cross reactive to other PTMs, to escape negative selection by impairing the strength of their binding while still allowing the clones to remain constantly stimulated due to ever-present autoantigens, as demonstrated by presence and persistence of classically short-lived IgM ACPAs (20) and IgM AMPAs (**Chapter 4**).

Alternatively, or additionally, glycosylation of the B-cell receptor may lower the threshold for B-cell activation, preferentially allowing autoreactive clones to survive (21). These processes have so far exclusively been investigated in ACPA-specific B-cells; more research is needed to determine if the same mechanisms could be at play in B-cells reactive to the most recently discovered AMPA targets. Our results suggest that whatever the mechanisms to broad AMPA development, it follows a common path in patients with different ethnic and geographical backgrounds.

Chapters 3-5 extensively explored the AMPA response in RA and its implications for treatment outcomes. The autoantibody profile exhibits great diversity. AMPAs target variable numbers of distinct peptides with identical and divergent post-translational modifications and demonstrate significant heterogeneity in isotype usage (11, 22, 23). This breadth of the autoantibody profile likely arises from the breakdown of tolerance to multiple autoantigens and underscores the maturity of the humoral autoimmune response once RA manifests.

The diversity and breadth of the autoantibody profile raises the question whether this is associated with treatment outcomes. Early initiation of disease-modifying drugs (DMARDs) has improved clinical remission rates (24, 25), but not all patients

achieve sustained drug-free remission (DFR) – the closest approximation of disease cure available (26). Since autoantibody seropositivity is a poor prognostic factor for this treatment goal, we hypothesized that the breadth of the baseline autoantibody profile (**Chapter 3**), seroconversion to autoantibody-negativity (**chapter 4**), and changes in autoantibodies over time (**Chapter 5**) may set apart patients that do achieve early clinical remission or long-term sustained DFR. Additionally, we investigated, as previous publications have done, whether one specific AMPA, a combination of them, and/or a specific pattern, with its changes over time, have any prognostic value.

Chapter 3 demonstrated that a broader autoantibody profile at baseline was associated with a greater early response to treatment, parameterized by a greater decrease in DAS after 4 months of conventional DMARD therapy in the IMPROVED trial. Conversely, that same broad profile was linked to a smaller chance of achieving initial DFR during early drug tapering attempts. It did not impact chances of achieving long-term sustained DFR. In fact, baseline seropositivity for anti-CCP2 IgG was the only relevant factor associated with inability to achieve long-term sustained DFR.

Our results show that the breadth of the autoantibody response is mainly relevant for early treatment response. We also found a trend that the number of AMPAs present was inversely related to likelihood of achieving initial DFR. Analyses of the RETRO study by others support this trend: patients with a broad pattern of AMPA response relapse more often in the first year after DMARD tapering (27, 28). Reasons for the difference in effect size in our study lie in different definitions of the outcome and in our lack of a seronegative comparator group. Studies that dichotomize RA into seropositive and seronegative disease have not found an association with autoantibody status and early treatment response, again underlining the importance of specific characteristics of the autoantibody profile, over simple dichotomy (29). The association of seropositivity as a poor prognostic factor for long-term outcomes is well-established in literature, and our results align with that (26).

What our findings suggest is that the presence of multiple antibodies at baseline may be a marker of an active autoimmune response, characterized by reactivity against multiple PTMs, extensive isotype usage, and possibly ongoing activation (23). We propose that patients with this profile may be more susceptible to immunosuppression by methotrexate and prednisone, explaining their better early response. However, the importance of the baseline autoantibody profile diminishes over time, despite autoantibodies staying relatively stable over time (more on this in **Chapter 4**). At later time points closer to tapering, the breadth of the autoantibody profile did not provide additional insights into long-term outcomes.

Baseline anti-CCP2 IgG positivity was the only aspect of the profile to remain significant for long-term DFR. Multiple studies have demonstrated the enduring presence of ACPA

memory B cells in circulation, even in patients in clinical remission under conventional DMARDs (30-32). Additionally, persistent antibody positivity could also be due to bone marrow long-lived plasma cells. It is likely that the clinical anti-CCP-test is good indicator of long-lived autoimmunity and, compared to a broader autoantibody profile, therefor seropositivity for this indicates a subset of patients unable to successfully drug-tapering in the long run.

Chapter 4 builds upon previous findings to explore whether autoantibody levels exhibit changes over time and whether this change confers any prognostic advantage. It examines the concept of 'immunological remission', wherein conversion from seropositive to seronegative status for RA-associated autoantibodies could potentially signify a state of more profound disease remission than the absence of symptoms or synovitis (33). The study determined the frequency and significance of seroconversion for various RA-associated autoantibodies in a cohort aiming for drug-free remission, and the impact of seroconversion on successful drug discontinuation.

In the IMPROVED study, longitudinal measurement of fourteen autoantibodies revealed modest rates of seroconversion to negative, yet this did not correlate with favorable outcomes for sustained drug-free remission (SDFR). Despite efforts to establish stricter criteria for seroconversion, such as complete or multiple autoantibody seroconversion, no association with SDFR was found (supplementary material). We conclude that autoantibody seroconversion does not effectively identify patients in a form of immunological remission wherein underlying autoimmune processes have been quenched. Investigations by colleagues in the Leiden Early Arthritis Clinic cohort found similar results for anti-CCP2 IgG/IgM and RF IgM seroconversion (34). Other studies support this notion on a cellular level: patients in clinical remission, similarly defined as in our study, retain an activated phenotype of ACPA-specific B-cells, suggesting that clinical remission is simply a state of suppressed inflammation in which immunological processes persist (30, 35). Currently, signals of low inflammatory load, such as DAS or ultrasound findings, appear to be better predictors of successful drug tapering than markers of humoral autoimmunity loss.

This study pioneered the attempt to operationalize immunological remission as a measurable entity and assess its relevance to drug tapering success, concluding that seroconversion to negative is not associated with the success of drug discontinuation and is not be a meaningful biomarker in tapering decision-making. However, it does not refute the possibility of the existence of immunological remission in RA. Kristyanto et al. also investigated ACPA-specific B-cells in the pre-disease phase in 'at risk' arthralgia patients, and found that ACPA-expressing memory B-cells are already present and produce phenotypically identical ACPAs as in established RA patients, although the B cells do not yet show an activated phenotype (35). This subset proliferates and activates as patients progress to arthritis, and, as of yet, have not been found to revert to their quiescent state,

even in states of clinical remission. This quiescent state, or other cellular phenotypical states of ACPA B-cell deactivation, may be the true immunological remission that indicates which patients may safely taper DMARDs. The first results of the long-awaited ASCARA trial suggest that indeed, ACPA-expressing B-cells can be modulated by a CTLA-4 agonist (abatacept) towards a deactivated phenotype compatible with immunological remission (36). Whether this form of immunological remission leads to improved clinical outcomes or ability to taper DMARDs remains to be seen.

Chapter 5 further explores the potential of autoantibodies as biomarkers for disease severity and treatment outcomes in RA, focusing this time on changes in levels within seropositive subsets. We had hoped that serological changes in RA might prove to be an easily accessible biomarker for the future disease course, as is the case in other autoimmune diseases. A substantial drop in autoantibody levels could for example be a clinically tangible prognostic tool if it precedes successful drug-free remission. Additionally, such an association and its relationship with immunosuppression and disease activity could shed light on autoimmune B cell response mechanisms in RA. Studies documenting the relationship between fluctuations in autoantibodies and disease activity have been conflicting (37-39) possibly because they did not consider the influence of immunosuppressive treatment on level changes and disease activity. Additionally, **Chapter 4** has shown that seroconversion to negative, which of course requires a change in levels, had little prognostic value. However, seroconversion happened mostly in patients whose antibody levels were already close to the cut-off for positivity. It is possible that robust changes in levels hold more prognostic information that merely fluctuations around a cut-off.

Therefore, we conducted a longitudinal characterization of changes in RA-associated autoantibody levels over time. Our results revealed no significant association between autoantibody changes and disease activity, functional status, treatment response, or long-term outcomes like DFR and radiographic progression. Instead, the observed changes in autoantibody levels appear to primarily reflect the effects of immunosuppressive therapy, rather than indicating disease-specific clinically relevant processes. Consequently, monitoring autoantibody levels over time appears to offer limited value in clinical settings.

However, it does teach us something about RA pathophysiology. Despite both autoantibody levels and disease activity decreasing significantly under anti-inflammatory therapy, RA persists in the long term. We hypothesize that this may reflect differences in longevity and place of residence of the autoantibody-producing cells. It seems plausible that synovial inflammation provides a niche that promotes the survival of ACPA-producing plasma cells (40), which falls away or is eliminated when treatment is initiated or intensified, thereby decreasing autoantibodies. However, the autoantibody-producing plasma cells may never be fully eradicated from their bone marrow niches, even in the absence of clinical symptoms, as previously discussed.

Following in the footsteps of Chapter 5 and of other groups evaluating multi-biomarker disease activity scores and imaging markers, Chapter 6 explored a marker of possible residual subclinical inflammation: circulating calprotectin. Calprotectin, also known as S100A8/A9 or myeloid-related protein (MRP) 8/14, is a heterodimeric soluble protein complex released by leucocytes during infectious and inflammatory conditions. Traditionally used in inflammatory bowel disease diagnostics (as fecal calprotectin measurements), calprotectin differs from conventional acute-phase reactants like C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR) as it is locally released at sites of inflammation (41). In RA, calprotectin correlates with disease activity markers and synovial inflammation, is associated with erosive progression, and may predict response to (especially biological) DMARDs (42, 43). Calprotectin levels show special promise in juvenile idiopathic arthritis, where they correlate with disease activity and relapse risk after DMARD discontinuation (44, 45). If such an association exists in RA, it could serve as a practical biomarker for predicting disease flare after tapering, aiding risk stratification and clinical decision-making.

Chapter 6 evaluated circulating calprotectin's role in predicting flare upon DMARD tapering in two independent cohorts (IMPROVED and RETRO study) and found that higher calprotectin levels were associated with increased flare risk within 12 months post tapering in the RETRO study, and that this improved upon conventional flare prediction using clinical parameters. There was a smaller, non-significant effect in the IMPROVED study, possibly because of differences in disease duration, outcome measures (DAS44 vs DAS28-ESR), definitions of remission stability, and treatment use (prednisone in IMPROVED, biologicals in RETRO). Potentially, calprotectin might not be able to discriminate residual disease activity when the definition of remission is very strict, as was the case in the IMPROVED study (DAS44<1.6, compared to DAS28-ESR<2.6 in the RETRO study); however, sensitivity analyses in the IMPROVED study using RETRO's remission definition did not change the results. Even more puzzling is the fact that a group in Nijmegen has conducted a very similar investigation of calprotectin in DFR in the DRESS study, as has another group in France, in the STRASS study, and found no value of calprotectin for predicting successful anti-TNF tapering (46, 47). The cohorts in these studies are similar to especially the RETRO study regarding patient characteristics and design, including: long-standing disease, concomitant conventional synthetic DMARD use (particularly prednisone), minimal 6 months remission before tapering, previous DMARD use, and current use of TNFα inhibitors. Main differences are in the definition of remission (RETRO: DAS-28-ESR<2.6; DRESS: DAS28-CRP<3.2; STRASS: DAS28<2.6), and in the definition of RA (respectively: EULAR 2010; ACR 1987/ EULAR 2010/clinical diagnosis; ACR 1987). However, all of these disease activity indices have been shown to correlate well(48), and given the disease durations in these cohorts (all long-standing disease), differences in selection criteria are unlikely to explain the disparity in calprotectin results. These inconsistent findings across all cohorts challenge calprotectin's application; it is unclear whether the results in the RETRO study may be a

spurious finding. Further research is needed to examine its utility in clinical practice, for doing so could improve RA management, offering personalized strategies for achieving drug-free remission while minimizing disease flare risks.

This thesis also delved into the pathophysiology of a different type of autoimmunity; namely, iatrogenic autoimmune reactions induced by immune checkpoint inhibitors (ICIs). Ipilimumab, a CTLA-4 inhibitor and the first ICI, caused a paradigm shift in cancer immunotherapy when first introduced in 2011 in the field of metastatic melanoma, and since then, multiple ICIs have improved the previously dismal prognosis of patients with various types of cancer. However, this came at the cost of serious immune-related adverse events (irAEs), described to date in virtually every organ system, including the joints (49). The mechanism of these reactions lie in ICIs inhibition of negative costimulatory signals to T cells which enhance antitumor T-cell responses (50). Because this mode of action is not antigen-specific, ICIs may also (re)activate otherwise dormant autoreactive T cells. We hypothesized that this expansion would be accompanied by T cell–dependent activation of autoreactive B-cells and autoantibody production, which in turn may be associated with more frequent irAEs and with better antitumor responses, as has been reported for changes in the T-cell repertoire (51-53).

In Chapter 7, we observed that ipilimumab treatment induced autoantibody development in a significant portion of melanoma patients. There was a trend suggesting an association between autoantibodies and irAEs under ipilimumab treatment, and a subanalysis in only irAEs related to the development of any of the antibodies measured was significant for this association. Our study was unfortunately underpowered to directly investigate the association between antibody development and the irAE in the organ system for which that antibody has conventional diagnostic use. However, patients who developed autoantibodies, particularly thyroid autoantibodies, showed minor survival and response benefits.

We gave special attention to thyroid autoimmunity in our study. As we found, previous research shows that ipilimumab treatment can induce thyroid autoantibodies, even without overt thyroid dysfunction (54-57). Thyroid autoantibodies are a special case in autoimmunity because they occur commonly in populations without overt thyroid disease or non-thyroid concomitant autoimmune disease (58-64). Additionally, mutations in CTLA-4, a key regulator in autoimmunity (65) and the target of ipilumumab, contributes to the genetic susceptibility to thyroid autoantibodies development, possibly independently of manifest thyroid autoimmunity (66, 67). Lastly, thyrocytes, which are not classically antigen presenting cells, are able to present MHC class II molecules and thyroid antigens in autoimmune thyroiditis in vivo (68), which are recognized by T-cells which in turn activate B-cells to produce autoantibodies.

Interestingly, in our study, the development of thyroid autoantibodies predisposed euthyroid ipilimumab-treated patients to subsequent thyroid dysfunction under anti-PD-1 therapy. Induction of thyroid autoimmunity by anti-PD-1 therapy in patients with pre-existing thyroid autoantibodies has been described previously (69-71), and confirmed recently (72, 73). This probably rests in the vital role of PD-1/PD-L1 axis in maintaining thyroid tolerance (74): in autoimmune thyroiditis, circulating levels of PD-1 positive T-cells are enriched. In response, thyrocytes have increased levels of PD-L1 expression, intent on keeping budding autoimmunity in check, and possibly explaining why autoimmune thyroiditis is generally a slowly progressive illness. In PD-1 inhibition with ICIs, however, this attenuation falls away, and could explain why thyroid antibodies and overt thyroid autoimmunity develops. We suggest that in patients who have been treated with previous CLTA-4 inhibition, this process may be primed and occur more frequently, though future studies are needed to elucidate the mechanism.

In our study, autoantibody development – thyroid autoimmunity in particular – was also associated with (minor) survival and response benefits. These results are in line with another longitudinal study in nivolumab (PD-1 inhibitor) treated lung cancer patients, wherein development of auto-antibodies (anti-nuclear antibodies, anti–extractable nuclear antigen, and/or anti–smooth muscle antibodies) was predictive of irAEs development and survival (75). It is likely that this autoimmunity is a marker for effective ICI-induced immunogenicity, reflecting enhanced immunity that targets both tumors and self-tissues.

Thyroid autoantibody production in particular was most strongly associated with irAEs, survival, and therapy response. One other study noted that thyroid-autoantibodies are associated with overt thyroid dysfunction, and that thyroid irAEs are associated with better survival, but a direct association between autoantibodies and survival is unfortunately not reported(70). Whether there is a specific mechanistic link between anti-thyroid immunity, in the form of autoantibodies or irAEs, and anti-tumor immunity is unknown, and more studies are needed to validate this association.

It is worth noting in the larger context of this thesis that we found that classical rheumatoid arthritis autoantibodies (anti-CCP2 and RF) only developed in 3 out of 133 patients who were seronegative at baseline, and that in these patients, no one developed a rheumatic irAE. Conversely, in patients who did develop a rheumatic irAE, no one developed autoantibodies. A recent meta-analysis of 372 anti-PD1 or anti-CTLA-4 treated patients with various forms of cancer confirms that seropositivity in “rheumatoid arthritis–like” irAEs is rare: 9%, compared to the 70% in classic RA (76). This begs the question: how similar are the mechanisms that lead to induction of ICI rheumatic illness compared to those that lead to RA? Comparisons between RA populations in **Chapter 2** showed striking similarity in the prevalence of AMPAs, despite vastly different genetic and environmental backgrounds, pointing to a common final

pathway. Studies that attempt to discern the immunogenetics of patients that develop rheumatic irAEs or autoantibodies have shown conflicting results. One study showed that HLA SE was enriched in patients with ICI-induced rheumatic irAEs compared with ethnically matched healthy controls, with similar rates of HLA-SE heterozygosity as matched RA controls (77). This is a surprising finding as, in line with our study, very few patients with rheumatic irAEs were seropositive for anti-CCP2, whilst considering the strong association of HLA SE and ACPA in RA, one would perhaps also have expected HLA SE-positive oncology patients with rheumatic irAEs to develop ACPA. A later study was unable to replicate the association of rheumatic irAEs with SE, but was able to show that ACPA in patients with rheumatic irAEs was different from established RA patients, with lower ACPA levels and less epitope usage (78). Currently, there is too little data available to draw conclusions about the interplay of autoantibodies, genetic risk, and immune dysregulation in rheumatic irAEs. However, the importance of this cannot be understated. At its basis, irAEs in ICI treatment can serve as a human model for classic autoimmune disease. Identifying the inciting events and risk factors that lead to autoimmunity could shed light on early stages of pathogenesis of diseases like RA.

CLOSING REMARKS

This chapter provides a summary and discussion of the findings of the studies described in this thesis. First, to better understand the pathogenesis of RA, we investigated the relationship of genetic and environmental risk factors with the AMPA profile in different populations of RA, and found epidemiological evidence for a final common pathway in AMPA formation. We went on to further characterize this AMPA profile over time, and showed that baseline composition, but not changes over time, were relevant for RA prognosis. The outcome of drug-free remission in particular was highlighted as a potential proxy for RA cure, but our results suggest that the holy grail of immunological remission lies beyond changes in serological measurements of AMPAs. Calprotectin's use as clinical decision-making tool in attempting drug-free remission requires further investigation. Lastly, we showed that autoantibodies are formed during treatment with ICIs, and that a subset of these are possibly relevant for treatment outcomes.

Throughout this chapter, I placed results in context of what is known, attempted to address some of the major challenges of research in autoantibodies, and proposed future avenues of research to build upon what we discovered. Although the studies in this thesis provide us with new insights in different aspects of autoantibodies and rheumatoid arthritis, more questions were raised than were answered, as is inherent to research. I hope that this thesis contributes to new understandings of disease mechanisms in RA and autoimmunity, and that these findings may, following expansion by other clinical and basic science studies, translate into new approaches for prognostic evaluation and more targeted treatment of rheumatoid arthritis.

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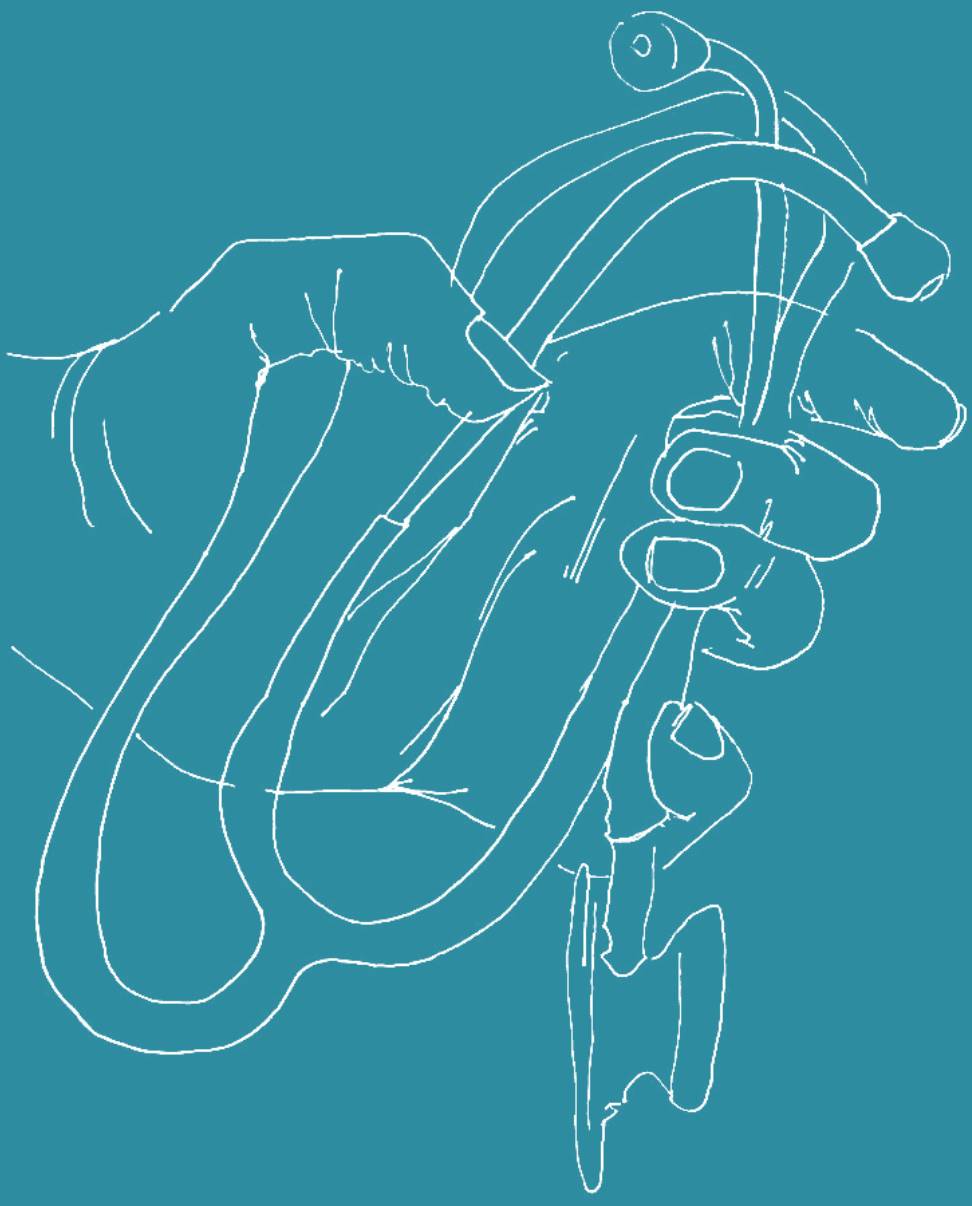

APPENDICES

Nederlandse samenvatting
Curriculum Vitae
List of publications

NEDERLANDSE SAMENVATTING

Reumatoïde artritis (RA) is een ernstige auto-immuunziekte van onbekende etiologie, die symmetrisch de kleine gewrichten van handen en voeten aantast. RA manifesteert zich meestal met klachten zoals gewrichtspijn, zwelling, stijfheid en vermoeidheid, wat een aanzienlijke impact heeft op de kwaliteit van leven en productiviteit van patiënten. Onbehandeld leidt RA tot progressieve gewrichtsschade en invaliditeit.

RA wordt geclassificeerd in seropositieve en seronegatieve RA, op basis van de aanwezigheid of afwezigheid van specifieke autoantilichamen in het serum van patiënten. Seropositieve RA wordt gekenmerkt door de aanwezigheid van reumafactor (RF) en/of antistoffen tegen gecitrullineerde peptiden (*anti-citrullinated protein antibodies*; ACPAs), terwijl seronegatieve RA deze conventionele autoantilichamen mist. Seropositieve RA patiënten hebben een agressievere ziektefenotype, hogere ziekteactiviteit, snellere radiografische progressie en slechtere behandelingsresponsen vergeleken met seronegatieve patiënten.

Autoantilichamen spelen een waarschijnlijk cruciale rol in de pathogenese van RA. RF was het eerste autoantilichaam welke bij RA werd gevonden, en wordt gedetecteerd bij ongeveer 60-70% van de patiënten. RF is opgenomen in zowel de classificatiecriteria van de American College of Rheumatology uit 1987 als de ACR/EULAR-criteria uit 2010 en is geassocieerd met ernstiger gewrichtsschade en extra-articulaire manifestaties. RF richt zich op het Fc-deel van IgG en bestaat uit verschillende isotypen, waarbij IgM het meest voorkomt. RF is betrokken bij de vorming van immuuncomplexen, vooral in de synoviale vloeistof, waar RF de ontsteking kan bevorderen door stimulatie van pro-inflammatoire cytokineproductie.

ACPAs zijn zeer specifieke biomarkers voor RA, gedetecteerd bij ongeveer 70-80% van de patiënten. Ze richten zich op gecitrullineerde eiwitten zoals fibrinogeen, vimentine, α-enolase en collageen type II. De aanwezigheid van ACPAs correleert met ernstiger ziektefenotypes, snellere radiografische progressie en slechtere behandelingsresponsen. ACPAs kunnen al jaren voor het begin van klinische symptomen in het serum van RA-patiënten worden gedetecteerd, wat wijst op hun betrokkenheid bij de preklinische stadia van de ziekteontwikkeling.

Naast RF en ACPA zijn er steeds meer autoantilichamen tegen andere post-translationele modificaties ontdekt, de zogenoemde anti-modified protein antibodies (AMPAs). Antilichamen tegen gecarbamyleerde eiwitten (*anti-carbamylated protein antibodies*, anti-CarP) worden gedetecteerd bij ongeveer 30-40% van de patiënten. Anti-acetylated protein antibodies (AAPA) richten zich op geacetyleerde eiwitten, maar hun klinische betekenis en functionele rol in de ziektepathogenese is onduidelijk. Een nog nieuwere groep antilichamen richt zich op producten gevormd door de reactie van

malondialdehyde (MDA) en acetaldehyde met lysine in eiwitten (*anti-malondialdehyde-acetaldehyde adduct antibodies* anti-MAA).

De ontwikkeling van autoantilichamen wordt beïnvloed door genetische en omgevingsfactoren. De belangrijkste genetische risicofactor is een gedeelde aminozuursequentie (positie 70–74) op HLA-DRB1, bekend als het *shared epitope* (HLA-SE), sterk geassocieerd met ACPA-positieve RA. Omgevingsrisicofactoren, zoals roken en blootstelling aan inhalantia, hebben ook sterkere associaties met ACPA-positieve RA. Beschermende HLA-allelen, zoals HLA-DRB1*13, benadrukken dat genetische predispositie complex en tweezijdig is.

Hoewel RA al duizenden jaren voorkomt en zelfs in skeletresten en 17e-eeuwse Nederlandse schilderkunst is beschreven, bleef de ziekte tot zo'n 50 jaar geleden grotendeels onbehandelbaar. Met de introductie van ziekte-modificerende anti-reumatische middelen (DMARDs), later gevolgd door biologicals en small molecules, is ziekte-remissie nu voor veel patiënten haalbaar. Toch blijft het bereiken van een medicatievrije remissie (*drug-free remission, DFR*) bij slechts 10–20% van de patiënten mogelijk. DFR is pathofysiologisch bijzonder interessant, omdat dit wellicht een toestand van 'echte' immunologische remissie weerspiegelt, waarin de onderliggende auto-immuunprocessen tot rust zijn gebracht. Welke patiënten dit kunnen bereiken en welke immunologische mechanismen hieraan ten grondslag liggen, is echter grotendeels onbekend.

Autoantilichamen spelen hierin mogelijk een sleutelrol. Seropositiviteit is doorgaans geassocieerd met slechtere behandeluitkomsten en meer gewrichtsschade, waardoor er veel interesse bestaat in de prognostische waarde van de samenstelling en eigenschappen van autoantilichamen bij RA. Toch is het nog onduidelijk of veranderingen in autoantilichaamniveaus of -profielen daadwerkelijk iets zeggen over het bereiken van immunologische remissie en DFR. Eerdere studies laten tegenstrijdige resultaten zien, vooral met betrekking tot de relatie tussen fluctuaties in antilichaamniveaus en ziekteactiviteit. Daarnaast is er behoefte aan aanvullende biomarkers, zoals calprotectine, die mogelijk gevoeliger indicatoren zijn van resterende ziekteactiviteit en kunnen helpen bij beslissingen rondom DMARD-afbouw.

Tegelijkertijd hebben recente ontwikkelingen in de oncologie, zoals immuuncheckpoint-remmers (immune checkpoint inhibitors, ICIs), nieuwe vragen opgeroepen. Deze therapieën kunnen auto-immuunbijwerkingen veroorzaken (*immune-related adverse events*, irAEs), die soms sterk lijken op reumatische ziektebeelden. Het bestuderen van autoantilichamen in dit kader kan niet alleen helpen bij het voorspellen van irAEs, maar ook nieuwe inzichten bieden in de rol van autoantilichamen bij het ontstaan van klassieke auto-immuunziekten zoals RA.

De belangrijkste doelstellingen van dit proefschrift waren dan ook:

- Het karakteriseren van de relatie tussen genetische en omgevingsrisicofactoren en het AMPA-profiel in verschillende RA-populaties.
- Het beschrijven van longitudinale veranderingen in kenmerken van het AMPA-profielen en het onderzoeken van hun prognostische waarde voor vroege behandeluitkomsten en DFR.
- Het beoordelen van calprotectine als marker van resterende ontsteking bij patiënten in remissie, en als klinisch hulpmiddel bij het afbouwen van DMARDs.
- Het onderzoeken van autoantilichaamvorming en de relatie met irAEs bij behandeling met ICIs.

Hoofdstuk 2 onderzocht de geo-epidemiologie van AMPAs (anti-CarP, anti-MAA en anti-AcVim) in vier etnisch diverse ACPA-positieve RA-populaties: Nederland, First Nations (Canada), Japan en Zuid-Afrika. We vonden duidelijke verschillen in AMPA-prevalentie en -niveaus tussen de cohorten, maar deze hingen vooral samen met variaties in totale IgG-spiegels en niet met klassieke genetische en omgevingsrisicofactoren zoals HLA-SE of roken. Dit sluit aan bij eerdere studies dat anti-CarP vaker voorkomt in niet-Westerse populaties, terwijl data over anti-MAA en anti-AcVim nauwelijks beschikbaar waren. Onze resultaten suggereren dat de ontwikkeling van een brede AMPA-respons een gemeenschappelijk eindpunt is van verschillende ziekteprocessen, onafhankelijk van de oorspronkelijke post-translationele modificatie of het antigeen dat de B-cel herkende. Dit impliceert dat populatiespecifieke risicofactoren mogelijk minder bepalend zijn voor AMPA-vorming dan gedacht, en dat de brede AMPA-respons een fundamenteel kenmerk is van seropositieve RA.

Hoofdstukken 3–5 onderzochten uitgebreid de diversiteit van het autoantilichaamprofiel in RA en de implicaties hiervan voor behandeluitkomsten. Het profiel bleek zeer breed, met herkenning van verschillende peptiden en modificaties, en met uitgebreid isotypegebruik.

In **hoofdstuk 3** werd aangetoond dat een breder autoantilichaamprofiel bij baseline samenhing met een sterkere vroege behandelrespons in de IMPROVED-studie, waarschijnlijk doordat dit profiel een actieve, maar ook goed onderdrukbare auto-immuunrespons weerspiegelt. Tegelijkertijd ging een breed profiel gepaard met een kleinere kans op vroege DFR tijdens afbouwpogingen. Op de langere termijn was echter alleen anti-CCP2 IgG-seropositiviteit geassocieerd met het niet behalen van DFR, wat aansluit bij de literatuur dat ACPA-memory B-cellen en langlevende plasmacellen therapieresistent zijn. Deze bevindingen impliceren dat autoantilichaamprofielen vooral informatief zijn voor vroege behandelrespons, maar dat anti-CCP2-seropositiviteit de sterkste voorspeller blijft voor ongunstige lange termijnuitkomsten.

Hoofdstuk 4 onderzocht of seroconversie naar autoantilichaam-negativiteit betekenisvol was. Een deel van de patiënten werd seronegatief, maar was dit niet geassocieerd met betere kansen op langdurige DFR. Dit suggereert dat seroconversie geen marker is voor 'immunologische remissie', waarbij de onderliggende auto-immuunprocessen daadwerkelijk tot rust zijn gekomen.

Hoofdstuk 5 richtte zich op veranderingen in autoantilichaamniveaus over de tijd. Niveauveranderingen bleken grotendeels het gevolg van immunosuppressieve therapie en hadden geen voorspellende waarde voor ziekteactiviteit, functionele status, DFR of radiografische progressie. Dit wijst erop dat (veranderingen in) autoantilichaamniveaus weinig directe informatie geven over de immunopathologie die RA in stand houdt, maar eerder de mate van ontstekingsremming weerspiegelen. Het ondersteunt het idee dat de autoantistofproducerende cellen in niches zoals beenmerg grotendeels blijven bestaan, zelfs bij klinische remissie.

Hoofdstuk 6 onderzocht calprotectine als biomarker voor subklinische ontsteking en flare na DMARD-afbouw, in de IMPROVED- en RETRO-studie. In RETRO was een hoger calprotectineniveau geassocieerd met een verhoogd risico op flare binnen 12 maanden, bovenop conventionele klinische parameters. In IMPROVED werd dit verband niet gevonden, mogelijk door verschillen in populatie, definitie van remissie en gebruikte therapieën. Deze discrepanties benadrukken zowel het potentieel van calprotectine als een marker voor residuele ziekteactiviteit, als de huidige onzekerheid over de toepasbaarheid in de klinische praktijk.

Hoofdstuk 7 richtte zich op immuun-gerelateerde bijwerkingen (irAEs) door immuuncheckpointremmers (ICIs). Bij een aanzienlijk deel van de melanoompatiënten die ipilimumab kregen, ontstonden nieuwe autoantilichamen, vooral tegen de schildklier. Deze ontwikkeling ging samen met een iets betere overleving en therapierespons, en bij sommige patiënten met latere schildklierdisfunctie onder PD-1-remming. Dit suggereert dat autoantilichaamvorming een marker kan zijn voor krachtige immuunactivatie die zowel tumoren als zelfweefsels treft. Opvallend was dat klassieke RA-antilichamen (anti-CCP2 en RF) nauwelijks werden geïnduceerd en ook zelden aanwezig waren bij patiënten met reumatische irAEs. Dit wijst erop dat de mechanismen die ten grondslag liggen aan autoantilichaamvorming bij RA anders zijn dan bij reumatische irAEs. Juist deze verschillen maken het onderzoeksveld bijzonder interessant: ICIs bieden een uniek model om te begrijpen hoe auto-immuniteit ontstaat, en kunnen nieuwe inzichten opleveren die zowel bijdragen aan veiligere en effectievere kankerimmunotherapie als aan beter begrip en behandeling van klassieke auto-immuunziekten zoals RA.

Conclusie

Samengevat laten de studies in dit proefschrift zien dat autoantilichaamprofielen belangrijk zijn voor de vroege behandelrespons in RA, maar slechts beperkte waarde

hebben voor het voorspellen van langdurige DFR of het stoppen van onderliggende auto-immuunprocessen. Calprotectine is een veelbelovende marker van subklinische ontsteking, maar de inconsistentie tussen cohorten verhindert voorlopig brede toepassing. Autoantilichaamontwikkeling tijdens ICI-therapie illustreert hoe immuunactivatie zowel gewenste anti-tumorreacties als ongewenste auto-immuniteit kan veroorzaken. Gezamenlijk benadrukken deze bevindingen dat autoantilichamen en biomarkers belangrijke bouwstenen zijn om RA en aanverwante auto-immuunziekten beter te begrijpen, maar dat meer onderzoek nodig is om ze te vertalen naar klinisch bruikbare voorspellers voor gepersonaliseerde behandeling en veilige DMARD-afbouw.

CURRICULUM VITAE

Emma de Moel werd op 13 januari 1991 geboren in Zwolle. Als gevolg van haar vaders werk als luitenant-kolonel bij de NAVO groeide ze grotendeels internationaal op. Elke twee tot vier jaar verhuisde het gezin, waardoor Emma haar basis- en middelbare school doorliep op verschillende internationale scholen in Tiberias, Israël; Napels, Italië; Norfolk, Virginia, Verenigde Staten; en Madrid, Spanje. In 2009 behaalde ze haar diploma aan de American School of Madrid, met een Internationaal Baccalaureaat (Honors), vergelijkbaar met het Nederlandse vwo-niveau.

In datzelfde jaar begon ze aan het bachelorprogramma Liberal Arts and Sciences aan University College Roosevelt in Middelburg, met specialisaties ("majors") in Biomedische en Levenswetenschappen en Engelse literatuur. Hier ontwikkelde ze haar langgekoesterde passie voor creatief schrijven verder. Na het behalen van de hoogste onderscheiding als valedictorian in 2012, koos zij echter voor een vervolgopleiding in de geneeskunde: de master Klinisch Arts-Onderzoeker (A-KO) aan de Universiteit Maastricht.

In 2016 sloot zij haar studie af met een semiartsstage op de afdeling reumatologie in het Maastricht Universitair Medisch Centrum, gevolgd door een onderzoeksstage op de afdeling reumatologie van het Leids Universitair Medisch Centrum (LUMC), onder begeleiding van Diane van der Woude. Hierna trad ze toe tot het LUMC als arts-onderzoeker, waar ze onder supervisie van Diane van der Woude en René Toes werkte aan het onderzoek dat wordt beschreven in dit proefschrift. Haar academische prestaties werden tijdens deze periode erkend met verschillende onderscheidingen, waaronder de prijs voor de beste orale presentatie op het jaarlijkse congres van de Nederlandse Vereniging voor Reumatologie (NVR Najaarsdagen), en de prijs voor de beste poster op de LUMC Research Conference.

In maart 2020 begon ze aan haar opleiding tot reumatoloog in het HagaZiekenhuis in Den Haag. Haar vooropleiding interne geneeskunde begon daar met een vliegende start door de wereldwijde strijd tegen de COVID-19-pandemie. Momenteel volgt zij de vervolgopleiding reumatologie in het LUMC. Emma woont in Leiden met haar partner, Myrthe Bouwhuyzen.

LIST OF PUBLICATIONS

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