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The characterization of anti-carbamylated protein antibodies in rheumatic diseases : isotype usage, avidity and molecular composition

Delft, M.A.M. van

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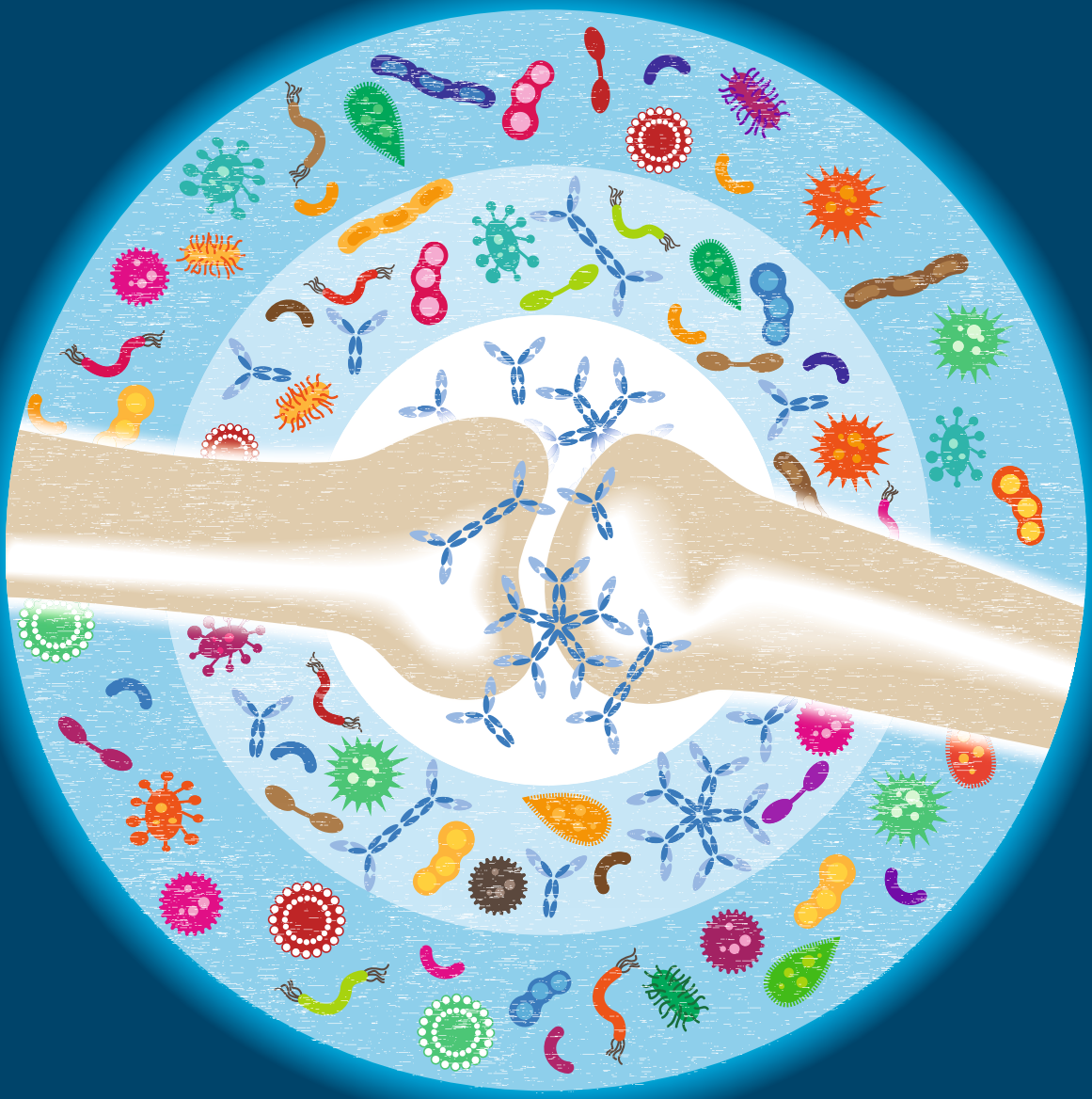

Myrthe van Delft

THE CHARACTERIZATION OF ANTI-CARBAMYLATED PROTEIN ANTIBODIES IN RHEUMATIC DISEASES

ISOTYPE USAGE, AVIDITY AND MOLECULAR COMPOSITION

Myrthe Alexandra Maria van Delft

The characterization of anti-carbamylated protein antibodies in rheumatic diseases

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The characterization of anti-carbamylated protein antibodies in rheumatic diseases

isotype usage, avidity and molecular composition

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Promotores
Prof. Dr. R.E.M. Toes
Prof. Dr. T.W.J. Huizinga

Co-Promotor
Dr. L.A. Trouw

Promotiecommissie
Prof. Dr. A.H.M. van der Helm
Prof. Dr. C. van Kooten
Dr. T. Rispens (Sanquin, Amsterdam)
Dr. J.H.W. Leusen (Utrecht Universiteit, Utrecht)

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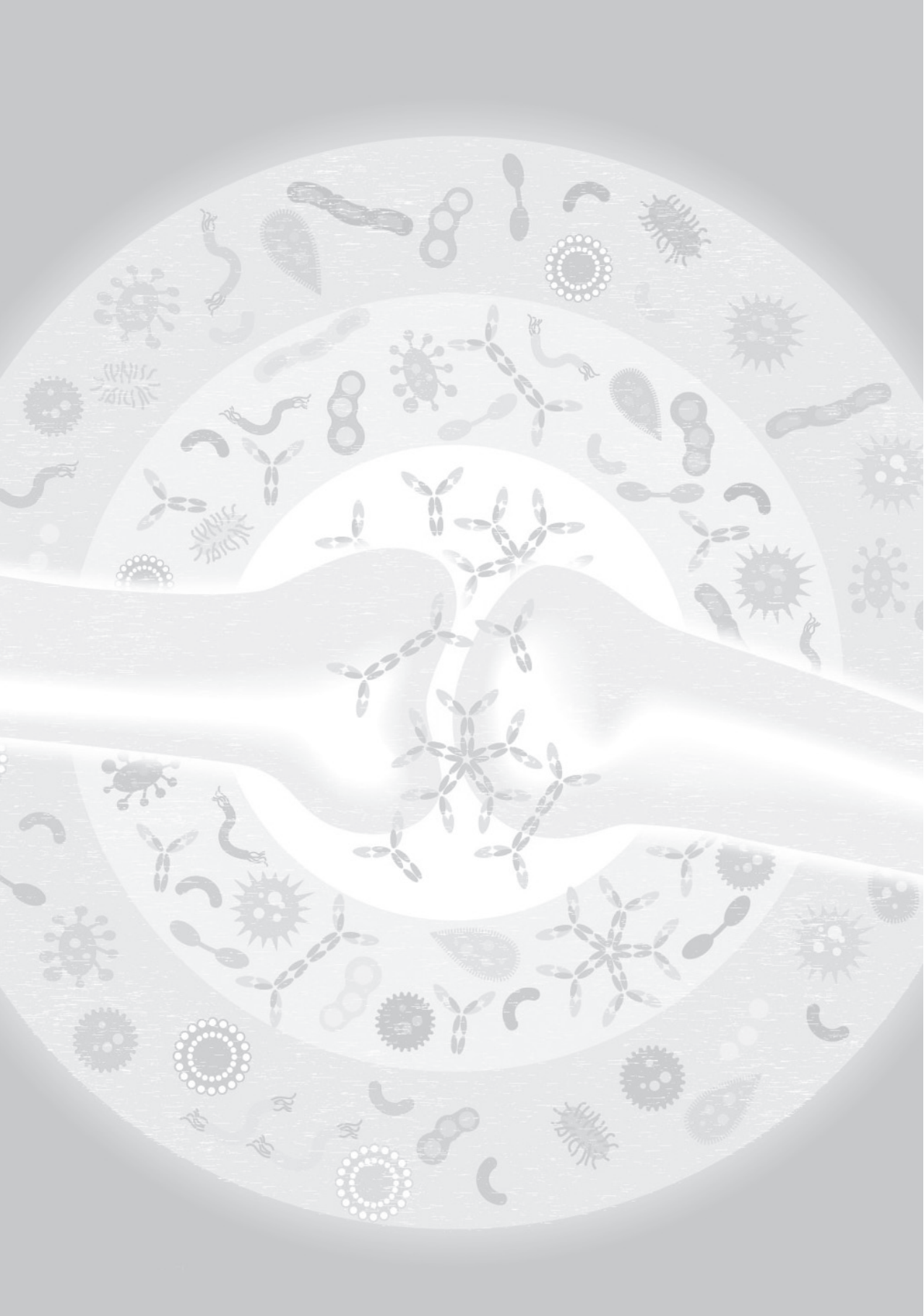

CHAPTER

Introduction

Part of this introduction is submitted as a review

The scope of this thesis is to describe the immunological characteristics, molecular composition and possible site of origin of anti-Carbamylated Protein (anti-CarP) antibodies in rheumatoid arthritis (RA). Furthermore, the presence of anti-CarP antibodies in relation to bone erosions on diseases other than RA is described. Therefore, this thesis is divided in three parts.
Part 1: Characterization of the anti-CarP antibody response in rheumatoid arthritis
Part 2: Possibilities for a mucosal origin of autoantibodies in rheumatoid arthritis
Part 3: Anti-CarP antibodies in other rheumatic diseases that can be characterized by bone erosions

Rheumatic diseases
Rheumatic diseases are diseases characterized by inflammation of connecting and supporting parts of the body. Mostly joints are affected, but tendons, ligaments, bones, muscles and even organs can be affected as well. Some well-known rheumatic diseases in which bone erosion can be observed are Rheumatoid Arthritis (RA, table 1A), Osteoarthritis (OA, table 1B), Systemic Lupus Erythematosus (SLE, table 1C) and Ankylosing Spondylitis (AS). Each of these diseases is associated with various genetic and/or environmental risk factors.

Rheumatoid arthritis
Rheumatoid arthritis (RA) is a common systemic autoimmune disease principally affecting synovial joints. RA is characterized by immune cell infiltration in the joint [1, 2]. Around 0.5-1% of the world population is affected [1, 3]. The incidence of RA is higher in women than in men and increases in elderly people [1, 3]. Although all joints can be affected, preferably the joints of hands, feet and knees are affected by the disease [4, 5]. Main symptoms of RA are pain, joint swelling and stiffness and possibly cartilage and bone degradation which can result in loss of joint function [4, 5]. Next to the joints, also other organs can be affected, like blood vessels, kidneys, heart, lungs and liver. Likewise, RA can cause fatigue, malaise and weight loss [5].

Since 2010, the 2010 American College for Rheumatology/European League Against Rheumatism classification criteria for rheumatoid arthritis (2010 ACR/EULAR criteria) are used for the classification of RA [2]. This classification system includes scores for joints involvement, serology (autoantibodies),

acute phase reactants (inflammatory markers) and symptom duration (Table 1A).

TABLE 1. Classification criteria for rheumatoid arthritis [2], osteoarthritic [6-8] and systemic lupus erythematosus [9, 10]

A.

2010 ACR/EULAR classification criteria RA

Swollen/tender joints (0-5)	**Symptom duration (0-1)**
0 1 large joint	0 < 6 wk
1 2-10 large joints	1 ≥ 6 wk
2 1-3 small joints	**Acute phase Reactants (0-1)**
3 4-10 small joints	0 Normal CRP and normal ESR
5 >10 joints (≥small joint)	1 Abnormal CRP or abnormal ESR
	Serology (0-3)
score > 6 "definite"RA	0 Negative RF and ACPA
	2 low-positive RF or ACPA
	3 High positive RF or ACPA

B.

ACR diagnostic criteria for osteoarthritis of the Hip, Hand and Knee

Hip	**Hand**	**Knee**
Hip pain + ≥2 of:	Hand pain, aching, or stiffness + ≥3 of:	Knee pain + ≥1 of:
* ESR < 20mm/ hour		* Age >50 years
* Radiographic femoral or acetabular osteophytes	* Hard tissue enlargement of ≥2 of 10 selected joints	* Stiffness <30minutes
* Radiographic joint space narrowing	* Hard tissue enlargement of ≥2 DIP joints	* Crepitus (crackling of joints) + osteophytes (small, abnormal bony outgrouwth, or spur)
	* <3 swollen MCP joints	
	* Deformity of ≥ of 10 selected joints	

C.

ACR and SLICC classification criteria of SLE

ACR criteria	SLICC criteria
4 of the 11 criteria over a lifetime:	4 needed with at least 1 clinical + 1 immunological criteria
* Malar rash	*Clinical criteria*
* Discoid rash	* Acute cutaneous lupus
* Photosensitivity	* Chronic cutaneous lupus
* Oral ulcers	* Oral/nasal ulcers
* Non-erosive arthritis	* Non-scarring alopecia
* Serositis: Pleuritis or pericarditis	* Inflammatory arthritis (≥2 joints)
* Renal: Proteinuria >0.5g/day or >3+ or cellular casts	* Serositis
* Neurologic disorders: Seizures/ psychosis	* Renal: 24h usine protein >500mg or red blood cell casts
* Haematological: haemolytic anaemia or leukopenia or thrombocytopenia	* Neurologic: seizures, psychosis, mononeuritis multiplex, myelitis, peripheral or cranal neuropathy, cerebritis
* Immunological: anti-dsDNA or anti-Sm or APLA or LA	* Haemolytic anaemia
* Positive ANA	* Leukopenia (<4000/mm3 at least once) or lymphopenia (<1000/mm3 at least once)
	* Thrombocytopenia (<100,000/ mm3) at least once
	Immunological criteria
	* ANA
	* anti-dsDNA
	* anti-Sm
	* APLA Abs
	* low complement: low C3, low C4, low CH50
	* Direct Coombs test in the absence of haemolytic anaemia

Autoantibodies in rheumatoid arthritis

Several antibody systems have been identified based on the antigens that these antibodies bind to. These autoantibodies can predominantly be detected in serum and synovial fluid (SF) of RA patients [11]. They may form immune complexes in the joints, leading to the attraction of immune cells through e.g. complement activation or direct activation of immune cells resulting in the secretion of chemokines and cytokines [12, 13] which can contribute to chronic inflammation and bone destruction.

Well-known autoantibodies in RA and currently used as biomarkers for diagnostics are rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPA) [2]. RFs are antibodies that recognize the Fc-tail of immunoglobulin (Ig)-Gs, while ACPAs recognize the post-translational modification (PTM) citrullination. Citrullination is the conversion of an arginine into a citrulline by an enzymatic reaction with peptidylarginine deiminase (PAD) (Figure 1a). ACPA and RF uses a broad spectrum of isotypes, including IgM, IgG and IgA [14-17]. The sensitivity of RF and ACPAs are similar for RA patients (sensitivity ACPA IgG ~67% and RF IgM ~69%), however ACPAs are more specific for RA compared to RF (specificity of ACPA IgG ~95% and RF IgM ~85%) when compared to healthy controls (HC) [18]. The presence of autoantibodies is predictive for the development of RA in undifferentiated arthritis patients [14, 19-23] as well as the development of a more severe disease outcome with more joint erosions over time [15, 24-26]. However, not all RA patients are autoantibody positive (based on ACPA IgG and RF IgM), approximately 30% is not [18, 27, 28]. Based on this positivity, normally two clinical subsets of RA are described, namely sero-positive- and sero-negative RA. Both clinical subsets were investigated in this thesis.

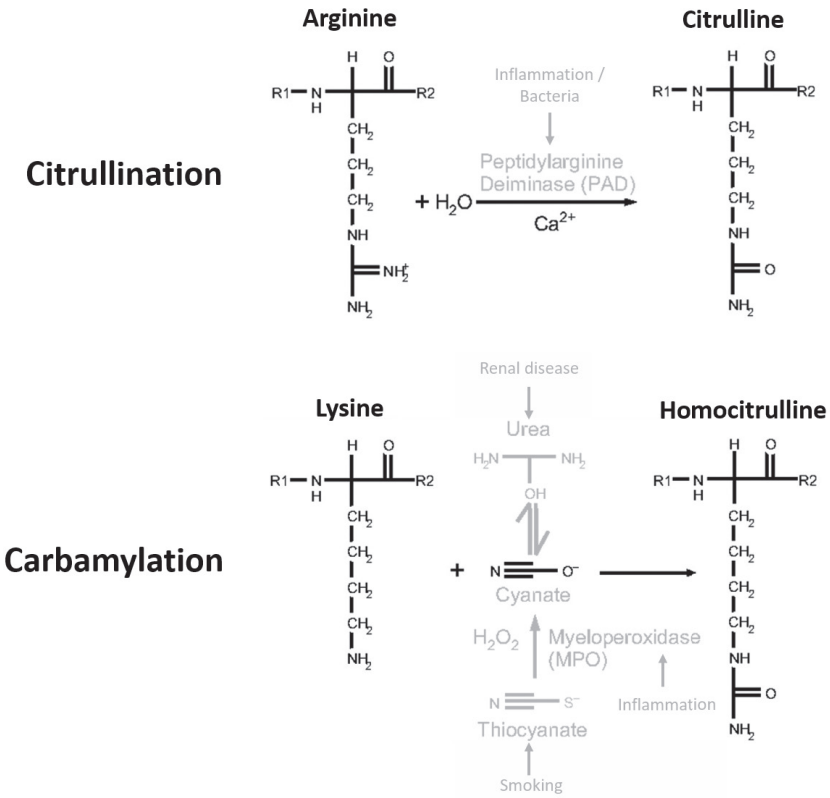

FIGURE 1. Citrullination and Carbamylation pathways. Citrullination is the conversion of an arginine into a citrulline by an enzymatic reaction with peptidylarginine deiminase (PAD) (A). PAD can be released by neutrophils or originate from bacteria. Carbamylation is the conversion of a lysine into homocitrulline by a chemical reaction with cyanate (B). Various conditions can lead to an elevated cyanate level, such as renal disease, inflammation and smoking. Figure adapted from [31].

Pathways for the induction of protein carbamylation

Several other autoantibodies recognizing PTM have been identified in RA patients [11]. One of these autoantibodies target carbamylated proteins and these autoantibodies are called anti-Carbamylated Protein (anti-CarP) antibodies [29]. Carbamylation is the conversion of lysines into homocitrullines by a chemical reaction with cyanate (Figure 1b) [30, 31]. Cyanate is present in fluids of all individuals in equilibrium with urea. Because of this low concentration of cyanate, small amounts of carbamylated proteins can be detected in healthy persons [32, 33] (Verheul et al. submitted). In patients with renal diseases, the blood urea levels

are increased resulting in an elevated carbamylation level [31, 34-36]. In addition to renal dysfunction, other factors can influence the amount of Carbamylation like inflammation, smoking and the inhalation of cyanate. The enhanced carbamylation during inflammation depends on myeloperoxidase (MPO) which can convert thiocyanate into cyanate [37, 38]. MPO is mainly stored in neutrophils, however during inflammation it can be released resulting in increased carbamylation during inflammation. The inhaled cyanate can be derived from different sources, such as cooking, tobacco usage (smoking) and biomass/coal burning.

Anti-carbamylated protein antibodies in rheumatoid arthritis

Citrullination and carbamylation are two rather similar PTMs, yet they represent two different antibody families in RA as, although both autoantibodies are often seen together, also ACPA and anti-CarP single positive RA-patients are present [11, 29, 39]. The presence of anti-CarP antibodies has been analysed in various cohorts of RA patients [27, 29, 39-47] and other (clinical) conditions (diseases and HC) [48-51]. A higher prevalence of anti-CarP antibodies is shown in RA patients compared to controls and other rheumatic diseases [27, 29, 40, 43, 45-47, 52]. Several observations implicate a role for anti-CarP directed immunity in the pathogenesis of RA as anti-CarP antibodies are present years before disease onset and have a gradual increase just before disease onset [42, 43, 53]. Moreover, the presence of anti-CarP antibodies is predictive for the progression to RA in arthralgia patients [54] as well as with increased joint destruction, especially in ACPA neg RA patients, overtime [29, 43-46].

Overall, these data indicate that anti-CarP antibodies contribute to the disease pathogenesis of RA, although little was known about the precise constitution of the anti-CarP antibody response and whether the response type plays a role in disease development. Moreover, the possible origin of anti-CarP antibodies as well as of ACPAs and RFs is unknown.

Risk factors for the development of RA

Genetic risk factors

Genetic factors contribute for about 60% of the risk for developing RA [55, 56]. As mentioned, two different RA subsets are described, sero-positive and -negative RA. The most important risk factor for ACPA-positive RA are the human leukocyte antigen (HLA) class II molecule HLA-DRB1-shared epitope

(SE) alleles [57]. The risk is 4 times higher in persons carrying a single SE allele and 8 times higher in those carrying two SE alleles compared to SE negative individuals [58]. However, HLA-DR alleles can also be protective in case of HLA-DRB1*13 [59]. Further, many non-HLA loci have a genetic contribution to the development of ACPA-positive RA. The best known in this is protein tyrosine phosphatase N22 (PTPN22) [60-64].

There are also genetic risk factors that contribute to the development of ACPA-negative RA. In ACPA-negative disease more HLA-DR3 alleles are found compared to controls [65, 66]. Interestingly, HLA-DR3 is associated with ACPA-negative anti-CarP-positive RA [39, 67]. However, overall it is unknown whether the HLA-gene itself or other genes linked to this locus predispose to the development of the disease.

Environmental risk factors
Besides genetic risk factors, environmental risk factors have a role in increasing the risk for developing RA as well. The best studied one is smoking, which has a dose dependent association with the susceptibility of developing RA, especially with the combined presence of ACPA and SE [68, 69]. For anti-CarP, no association has been found with smoking or the association is gone after correction for ACPA [27, 39]. Moreover, anti-CarP antibody levels are not increased in heavy smokers [48]. Other environmental risk factors are infectious agents, hormones (various roles after menopause or during/after pregnancy, although data are not uniform) and diet [3, 70, 71].

B cell response
Various immune cells are detected in the synovium and synovial fluid of RA joints [72, 73]. It is thought that B cells play an important role in the disease development and progression.

This because the presence of autoantibodies is associated with the development and severity of RA (as explained before). Moreover, positive results are observed for Rituximab treatment in RA patients [74-77]. Rituximab binds CD20 which is a marker specifically expressed on B cells, except plasma cells. Rituximab treatment depletes these CD20 positive B cells in RA patients, thereby depleting precursors of the autoantibody producing plasma cells, however the exact mechanism for the positive results of Rituximab treatment in RA remains unclear.

During a `normal´ protein directed B cell response, activated B cells will enter the germinal center, where they start to proliferate and undergo, upon receiving T-cell help, somatic hypermutation resulting in isotype switching and avidity maturation. These processes are expected to occur side by side.

Isotype switching

In humans, several immunoglobulin (Ig) isotypes or subclasses are known; IgD, IgM, IgG (1-4), IgA (1-2) and IgE. During a primary immune response (first antigen encounter) IgM will be secreted by activated B cells [78]. During the subsequent maturation of an immune response, under influence of various cytokine stimulations, isotype switching occurs. This is the conversion of the constant domain of a certain immunoglobulin into another by deleting some genetic material. Isotype switching leads to an increased diversity of the antibody response. During T cell dependent antigen responses, switching towards IgG is typically associated with a large decline or disappearance of IgM-responses [78]. Different isotypes (IgM, IgG or IgA) and IgG-subclasses (IgG1, IgG2, IgG3 or IgG4) vary in the capacity to trigger immune effector mechanisms. For instance IgM and IgG3 are the most potent complement activators [12]. Likewise, the various IgG-subclasses have different affinities to interact with activating or inhibitory Fc receptors [79]. Moreover, IgG_4 is the IgG-subclass with the unique biological property to undergo `Fab-arm exchange', resulting in the possibility to recognize two different epitopes. Further, serum IgA is a more pro-inflammatory activator of immune cells compared to IgG (Heineke et al., Abstract ECI-2019).

Avidity maturation

Where affinity describes the strength of a single interaction between an epitope and antibody's antigen binding site, antibody avidity is defined as the overall binding strength of polyclonal (multivalent) antibodies to its multivalent antigens. Activated B cells, who entered the germinal center and underwent proliferation and somatic hypermutation, will try to bind their antigen on follicular dendritic cells. Those B cells that bind their antigen with higher avidity, will outcompete other B cells and thereby they attract more survival and proliferation signals. Due to this process, the avidity of immune responses increases over time and typically low avidity antibodies will disappear from the circulation.

While substantial information is available on the avidity maturation of antibody responses against recall antigens [80-82], less information is available on the avidity maturation of autoantibody responses. However, it has been described that the avidity of IgG and IgA autoantibodies (high, moderate and low) associates with different clinical outcomes in several diseases, like low avidity anti-transglutaminase antibodies are associated with a more severe disease in coeliac patients [83]. Moreover, the avidity of ACPAs (IgG) and anti-transglutaminase (IgA) antibodies are low compared to the recall antibodies anti-tetanus toxoid (IgG) antibodies and anti-*E.coli* (IgA) antibodies [84, 85]. This indicates that although these B cells underwent isotype switching towards IgG and IgA, and apparently attract sufficient signals for survival and proliferation, no or little avidity maturation took place.

Glycosylation of immunoglobulins

All immunoglobulins (IgD, IgM, IgG, IgA and IgE) are glycosylated proteins. IgGs contain a conserved N-linked glycosylation site in the Fc-part [86] which is crucial for maintaining the conformation of the Fc tail and plays a role in immune effector functions like the interaction with Fc-receptors, lectins and activation of complement [87-91]. Next to Fc-linked N-glycans, IgG molecules in human serum can harbour N-linked glycans in the variable (V)-domain [92] which influences immune functions, yet there role is less defined [93-96]. Recently, our department discovered that ACPAs from RA patients are highly glycosylated in the V-domain [97]. Before proteins can undergo N-linked glycosylation, they need to express a consensus sequence (N-X-S/T, where X ≠P). These consensus glycan sites are typically not germ line encoded in the Ig V-domain but should be introduced by somatic hypermutation [97]. Recently our group observed that ACPA-IgGs are highly mutated and able to introduce glycan sites during somatic hypermutation [98]. Whether the anti-CarP antibody response can introduce glycan sites as well is yet unknown.

The mucosal origin hypothesis in Rheumatoid Arthritis

Several lines of evidence obtained in recent years indicate a role for mucosal surfaces in the development of auto-immune responses associated with (the development of) RA. Various studies show the presence of RA-associated autoantibodies at several mucosal sites, like the presence of ACPA and RFs in the lung and sputum and ACPA production at the periodontium, intestine and cervical-vaginal sites (reviewed in [99]). Recently, it is reported

that the proportion of IgA plasma blasts is increased in peripheral blood of individuals at risk for developing RA who are autoantibody positive [100]. This suggest that part of the RA-associated autoantibodies develop from mucosal immune responses and may play a role in early disease pathogenesis. Moreover, dysbiosis has been observed in microbiome studies of the faecal, lung and periodontal microbiota of patients with early or new-onset RA (reviewed in [99]), also pointing towards a role of the mucosa in the development of RA. In this thesis we investigated whether autoantibodies in RA could be of mucosal origin.

Mucosal immune response

IgA is the most prominent antibody present at mucosal surfaces and it is the antibody isotype that is produced in the largest quantity in the human body [101, 102]. In addition, it is produced as a dimeric molecule, dimeric IgA (dIgA) at mucosal surfaces [101, 103]. dIgA is locally produced by plasma cells and includes a joining-chain (J-chain) [101, 103]. The J-chain is important for the IgA transportation through epithelial cells by the polymeric Ig receptors (pIgR) and secretion at the luminal site. Part of the pIgR remains attached to the antibody at the luminal site and is known as the secretory component (SC) providing secretory-IgA (SIgA). This protects and stabilizes the IgA in the hostile environment of e.g. the gut. IgA, present at mucosal sites, can prevent the invasion of bacteria by neutralization in the lamina propria, in infected epithelial cells and in the lumen [103-105]. Apparently, secretory antibodies can be detected in serum [106], yet the mechanism how secretory antibodies end up in serum remains unknown.

Outline of this thesis

The scope of this thesis is to describe the immunological characteristics and molecular composition of anti-CarP antibodies in rheumatoid arthritis, as well as the presence of anti-CarP antibodies in relation to bone erosions on diseases other than RA. This thesis is subdivided in three parts.

Part 1: Characterization of the anti-CarP antibody response in rheumatoid arthritis

Both ACPA and anti-CarP antibodies are present in RA. For ACPA it is known that they have a broad isotype and IgG-subclass usage. The presence of more ACPA isotypes associates with a more severe disease course overtime. In ***chapter 2*** we describe the examination of the anti-CarP antibody response

in RA by means of isotype and IgG-subclass usage in baseline serum samples of RA-patients with long-term follow-up data [107]. Moreover, the association of specific isotypes and IgG-subclasses with radiographic progression over time and whether a broader anti-CarP IgG-subclass response associates with higher levels of inflammatory markers was studied.

Based on our previous observations that the ACPA response has a low avidity and our interest in understanding the biology of the anti-CarP antibody response, we studied the avidity of the anti-CarP antibody response (described in ***chapter 3***). Baseline serum samples of RA patients with long-term follow-up data [107] as well as samples from patients in the phase before diagnosis [22, 43, 108] were used to examine the anti-CarP avidity.

In ***chapter 4*** we describe research on the question whether anti-CarP IgG antibodies can have variable (V)-domain glycans, as ACPAs are highly glycosylated in their V-domain [97].

Overall it seems that the ACPA and the anti-CarP antibody responses are two different types of autoantibody reactions, yet both responses could be cross reactive, why and how is unknown.

Part 2: Possibilities for a mucosal origin of autoantibodies in rheumatoid arthritis
Several lines of evidence obtained in the recent years indicate a role for mucosal surfaces in the development of auto-immune responses associated with RA. Therefore, we studied the molecular composition of RA associated IgA autoantibodies, which is described in ***chapter 5***. The size/molecular weight spectrum of these autoantibodies was analysed. In addition, the cross reactivity of anti-CarP- and ACPA IgA was investigated.

The molecular weight-difference of anti-CarP- and RF- IgA made us curious to whether this could be explained by secretory component attached to it and whether the mucosa could then be a possible origin for RA associated autoantibodies. In ***chapter 6*** the determination of secretory (SC)-anti-CarP, SC-ACPA and SC-RF in RA-patients is described. Further, the identification of the isotypes used by these secretory autoantibodies was examined and described.

Part 3: Anti-CarP antibodies in other rheumatic diseases that can be characterized by bone erosions

Because anti-CarP antibodies and ACPAs are also present in other clinical conditions than RA [51], we (in collaboration) investigated their presence and association with bone erosions in two well defined cohorts of Systemic Lupus Erythematosus (SLE). The results of this study are described in ***chapter 7***. Due to this observation and because of our knowledge that the presence of autoantibodies in RA is associated with more bone erosions, we hypothesized that autoantibodies, like anti-CarP and ACPA, are present in hand osteoarthritis (HOA) patients and associate with erosive HOA. Therefor the presence of autoantibodies was studied in the HOSTAS (hand osteoarthritis in secondary care) cohort of the LUMC and their association with erosive disease was determined. This is described in ***chapter 8***.

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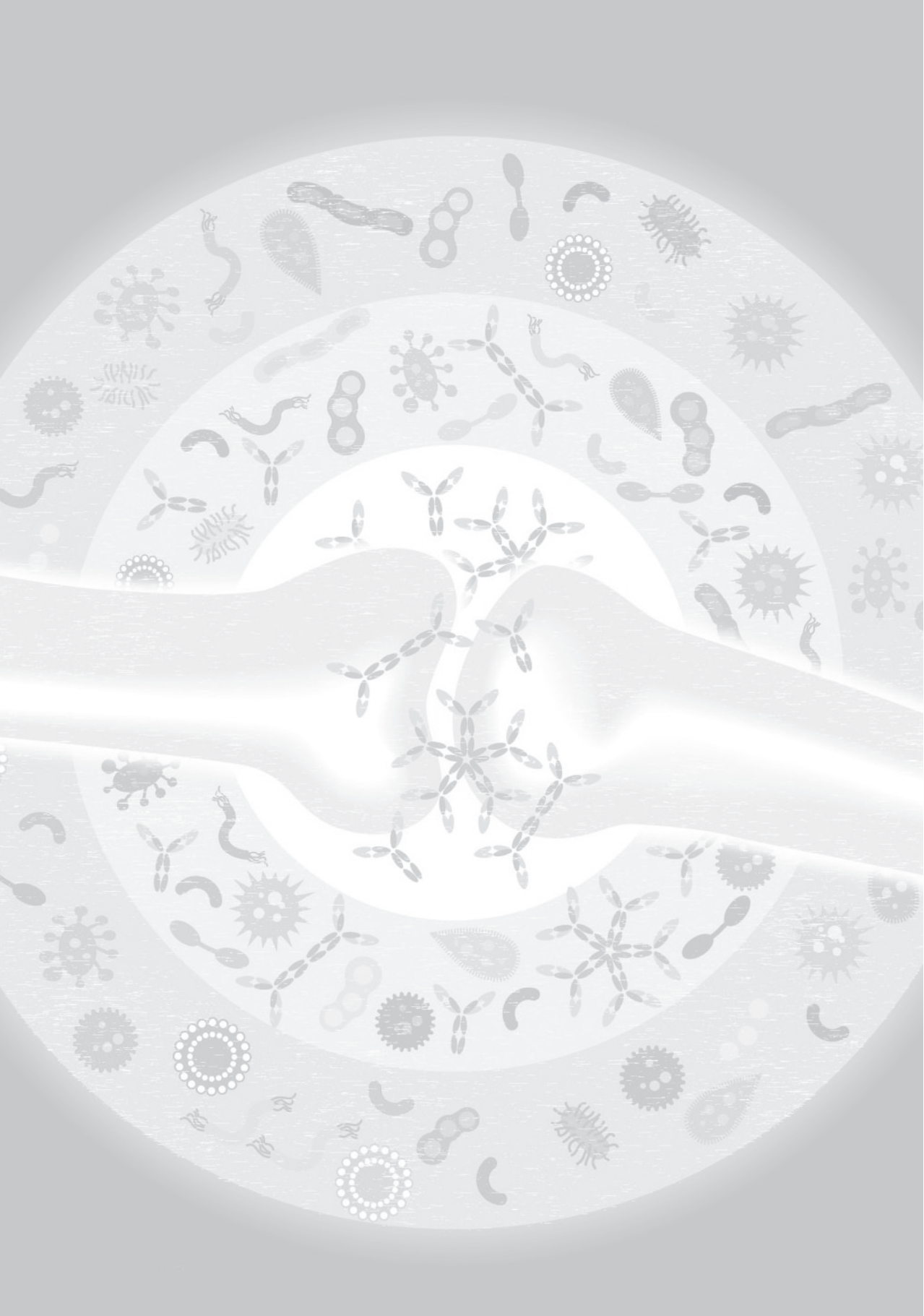
PART

Characterization of the anti-CarP antibody response in rheumatoid arthritis



CHAPTER 2

The Isotype and IgG-Subclass Distribution of Anti-Carbamylated Protein Antibodies in Rheumatoid Arthritis Patients

Myrthe A.M. van Delft
Marije K. Verheul
Leonie E. Burgers
Veerle F.A.M. Derksen
Annette H.M. van der Helm-van Mil
Diane van der Woude
Tom W.J. Huizinga
René E.M. Toes
Leendert A. Trouw

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ABSTRACT

Background: In rheumatoid arthritis (RA) anti-carbamylated protein (anti-CarP) antibodies have recently been described to occur in around 45% of the patients and to have prognostic and diagnostic properties. At present, the breadth and molecular make-up of the anti-CarP antibody response is ill defined. To understand the anti-CarP antibody immune response and potential immune effector mechanisms it can recruit, we determined the anti-CarP antibody isotype and IgG-subclass usage in RA-patients.

Methods: Anti-CarP antibody IgM, IgA, IgG or IgG-subclasses were detected by ELISA in sera of 373 unselected RA-patients and 196 healthy controls. An additional 114 ACPA and anti-CarP IgG double-positive patients were selected to study the concomitant presence of both antibody systems.

Results: Anti-CarP IgG was present in around 45% of the patients and comprised all anti-CarP IgG-subclasses. Particularly the presence of anti-CarP IgG1 associates with radiological damage. Anti-CarP IgM was detected in 16% of RA-patients, even in anti-CarP IgG positive individuals, and is indicative of an actively ongoing immune response. Around 45% of the patients were positive for IgA which included ACPA-positive but also 24% of the ACPA-negative cases. In ACPA and anti-CarP double-positive patients, the distribution and number of isotypes and IgG-subclasses was similar for both autoantibodies at the group level, but substantial variation was observed within individual patient samples.

Conclusions: In RA the anti-CarP antibody response uses a broad spectrum of isotypes and seems to be an actively ongoing immune reaction. Furthermore, the anti-CarP and ACPA autoantibody responses seems to be differentially regulated.

BACKGROUND

Rheumatoid arthritis (RA) is a systemic autoimmune disease principally affecting synovial joints [1, 2]. Autoantibodies can be detected in serum and synovial fluid (SF) of RA patients and may form immune complexes in the joints, leading to the attraction of immune cells through e.g. complement activation [3, 4] which can contribute to chronic inflammation and bone destruction.

Several autoantibodies have been described in RA. Well-known biomarkers that are currently used in clinic for the diagnosis of RA are rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPA) [5]. More recently, anti-carbamylated protein (anti-CarP) antibodies, which target carbamylated proteins, have been detected in RA [6]. Carbamylation is a post translational modification of proteins in which lysines are converted to homocitrullines by a chemical reaction with cyanate [7, 8].

Currently, several studies have shown an increase in the prevalence of anti-CarP antibodies in RA patients [6, 9-11] and like ACPA, also the presence of anti-CarP antibodies associates with increased joint destruction [6, 9, 10, 12]. Moreover, anti-CarP antibodies are already present in individuals before disease onset [9, 13, 14] and the presence of these antibodies is predictive for the progression to RA in arthralgia patients [15]. Overall, these data indicate that anti-CarP antibodies might play a role in the disease pathogenesis of RA, although little is known about the precise constitution of the anti-CarP antibody response or how the make-up of this response may or may not play a role in disease development.

In humans, several immunoglobulin (Ig) isotypes are known. During a primary immune response (first antigen encounter) activated B cells will secrete IgM. During the subsequent maturation of an immune response, isotype switching occurs leading to an augmented diversity of the antibody response. In case of T cell dependent antigen responses, switching towards IgG is typically associated with a large decline or disappearance of IgM-responses [16]. Different isotypes (IgM, IgG or IgA) and IgG-subclasses (IgG1, IgG2, IgG3 or IgG4) differ in the capacity to recruit immune effector mechanisms. For instance IgM and IgG3 are the most potent complement activators [3]. Likewise, the various IgG-subclasses have different affinities to interact with activating or inhibitory Fc receptors [17]. The isotype usage of

the ACPA response has been well studied showing a broad usage of different isotypes by ACPA in RA patients [18-20]. ACPA-IgM and ACPA-IgA are mainly confined to ACPA-IgG positive patients [18]. Furthermore, an expanded isotype usage of the ACPA response in ACPA-IgG positive RA is associated with more severe radiographic damage [19]. At present, such information is not available for the anti-CarP antibody response.

Various genetic, serologic and imaging biomarkers have been identified to be associated with radiographic progression in RA [21, 22]. Also baseline erythrocyte sedimentation rate (ESR) or C-reactive protein (CRP) levels have been reported to associate with radiographic progression, however these results are not always consistent [22]. However, the presence of more auto-antibodies at disease onset associates with increased ESR and CRP-levels [23].

For better understanding of the anti-CarP antibody response, we determined the presence of anti-CarP antibody isotypes and IgG-subclasses in baseline serum samples of RA patients with long-term follow-up data in the Leiden Early Arthritis Clinic (EAC) [24]. We show that the anti-CarP antibody response uses a broad spectrum of anti-CarP antibody isotypes and IgG-subclasses. Furthermore, the presence of anti-CarP IgG1 associates with more radiographic progression over time and a broader anti-CarP IgG-subclass response associates with higher levels of inflammatory markers.

PATIENTS AND METHODS

Patients and control sera

The sera analysed were from 373 RA patients (age 57.2±15.8 and 68.1% female, 58.2% CCP2-IgG positive, Table 1) and 196 healthy controls (HC) (age 44.1±13.8 and 50% female). Anti-CarP antibody IgG data was already available [6] and used in these analysis. Furthermore, as a replication cohort 114 RA patients positive for both ACPA and anti-CarP IgG (double-positive) were tested for all ACPA and anti-CarP antibody isotypes and IgG-subclasses (age 54.5±12.01, 63.2% female, 100% CCP2-IgG positive, Table 1). The RA patients were participating in the Leiden EAC cohort [24] and included between 1993 and 2014. Baseline serum samples of these patients were analysed. Healthy control samples were acquired from persons living in the Leiden region as described before [6]. Most of the RA samples and HC were stored at -20°C and some at -80°C, however, previous experiments showed

no differences in anti-CarP or ACPA levels and positivity when samples were stored in different ways or after freeze-thawing. Informed consent was obtained for all individuals and all protocols were approved by the ethic committee of the LUMC.

TABLE 1. Baseline patient characteristics of the two RA cohorts

	RA 1 (n=373)	**RA 2 (n=114)**
Female, n (%)	254 (68.1)	72 (63.2)
RF-IgM positivity, n (%)	225 (60.3)	109 (95.6)
CCP2 IgG positivity, n (%)	217 (58.2)	114 (100)
Anti-CarP IgG positivity, n (%)	182 (49.2)	114 (100)
"ever"smokers , n (%)	160 (42.9)	75 (65.8)
Age, mean (std)	57.2 (15.8)	54.5 (12.0)

RA 1= discovery cohort, RA 2= replication cohort

Measurement of anti-CarP antibody and ACPA isotypes and IgG-sub-classes

Anti-CarP antibody isotypes and IgG-subclasses were measured by ELISA as described previously [6] with some adaptations. 10µg/ml carbamylated fetal calf serum (CaFCS) or non-modified FCS was coated on plates and after blocking with PBS/1% BSA, serum samples were incubated overnight at 4°C.

ACPA isotypes and IgG-subclasses were measured as described previously [18, 19] with some differences. Briefly, 1µg/ml CCP2-cittruline or CCP2-arginine was coated on plates and serum samples were incubated for 1hour at 37°C. The CCP2 peptides were ordered at the peptide facility of the department of immunohematology and blood transfusion at the LUMC and are provided and produced by Dr. J.W. Drijfhout.

Bound human IgM and IgA was detected using horseradish peroxidase (HRP) -conjugated goat-anti-human (GAH) -IgM (Invitrogen, 627520) or GAH-IgA (Invitrogen, 627420), respectively. For the detection of bound IgG1 and IgG4, HRP- conjugated mouse-anti-human (MAH) -IgG1 (Life Technologies, A10648, Clone HP6069) or MAH–IgG4 (Life Technologies, A10654, Clone HP6025) antibodies were used followed by HRP- conjugated goat-anti-mouse (GAM) -Ig (DAKO, P0447). Bound human IgG2 or IgG3 was detected using MAH-IgG2 (Nordic MUbio, Clone HP6014) or MAH–IgG3 (Nordic MUbio, Clone HP6080) continued with HRP- conjugated GAM-Ig (DAKO, P0447) and rabbit-anti-

goat (RAG) -Ig (DAKO, P0449). After the final washings, ABTS (2,2'-azino-bis 3-ethylbenzothiazoline-6-sulphonic acid) was added to visualize the HRP enzyme activity and the absorbance was measured at 415nm. To determine the cut-off for a positive response of the anti-CarP antibody isotypes, mean plus two times the standard deviation was calculated of the specific anti-CarP response in HC [6]. For anti-CarP IgG-subclasses the cut-off was set at the 97th percentile of the specific anti-CarP response in HC, as this was equal to what was found using mean plus two times the standard deviation of total anti-CarP IgG. The upper limits of the anti-CarP antibody ELISAs with the standards used in these experiments are shown in additional file 1. For ACPA, the cut-off for positivity was defined as the mean plus two times the standard deviation of the measured ACPA response in HC [19].

Statistical analysis

Statistical analysis was performed using statistical package for the social sciences (SPSS) version 23 (IBM). In order to determine differences in antibody levels between patients and HC a Mann-Whitney U test was carried out. The Pearson Chi-Square test was used to determine differences in positivity between RA patients and HC. To investigate whether there are correlations, Spearman Rank tests were performed. P values below 0.05 were considered statistically significant.

In 373 RA patients the association between anti-CarP antibody isotype or IgG-subclass positivity and radiographic progression, as assessed by the Sharp-van der Heijde Score [25], was analysed as described before [6, 24, 26]. As repeated radiographs were taken at yearly intervals, we have used a multivariate normal regression analysis for longitudinal data. Adjustments for treatment strategy, age, sex, baseline ESR, baseline CRP-level and symptom duration had been made. Stratified analysis for ACPA and RF-IgM was performed in 115 RA patients. P values below 0.05 were considered statistically significant.

Risk factors for the presence of anti-CarP antibody isotypes and IgG-subclasses were investigated by logistic regression analysis. The relation between positivity for anti-CarP antibody isotypes or IgG-subclass and CRP-levels and ESR were assessed using the Mann-Whitney U test. The analysis of the IgG-subclasses was performed within the anti-CarP IgG positive group. Furthermore, the association between the total number of isotypes or IgG-

subclasses present and risk factors or clinical parameters were investigated by ordinal regression analysis. To correct for multiple testing the Holm-Bonferroni method was applied on independent tests. Since the increasing presence of anti-CCP2 antibody in anti-CarP antibody positive patients could influence the findings, stratification for anti-CCP2 antibody positivity was performed.

RESULTS

The anti-CarP antibody response uses a wide spectrum of isotypes and IgG-subclasses

Anti-CarP antibody isotypes and IgG-subclasses were measured in baseline serum samples of RA patients (n=373) and in serum samples of healthy controls (n=196). In healthy controls, the mean plus two times standard deviation was set as cut-off, which resulted in positivity for the anti-CarP antibody IgM, IgG and IgA of 4.1%, 3.0% (results from Shi et al. [6]) and 5.1%, respectively, in the control population. At this cut-off we observed that 16.4% of the RA patients were positive for IgM anti-CarP antibodies, while 49.2% and 40.8% were positive for IgG (part of the results from Shi et al. [6]) and IgA (Figure 1A). Furthermore, reactivity for all anti-CarP IgG-subclasses was analysed in healthy controls and RA patients. The results of these analysis show that 50.7% of the RA patients are positive for anti-CarP IgG1, 27.1% for IgG2, 8.0% for IgG3 and 27.3% for IgG4 (Figure 1B). When comparing levels or %-positivity between healthy controls and RA patients, significant differences ($p<0.001$ for all analyses) were observed for all anti-CarP antibody isotypes and IgG-subclasses analysed.

Overall, these data indicate that the anti-CarP antibody response is characterized by wide usage of anti-CarP antibody isotypes and IgG-subclasses in baseline serum samples of RA patients.

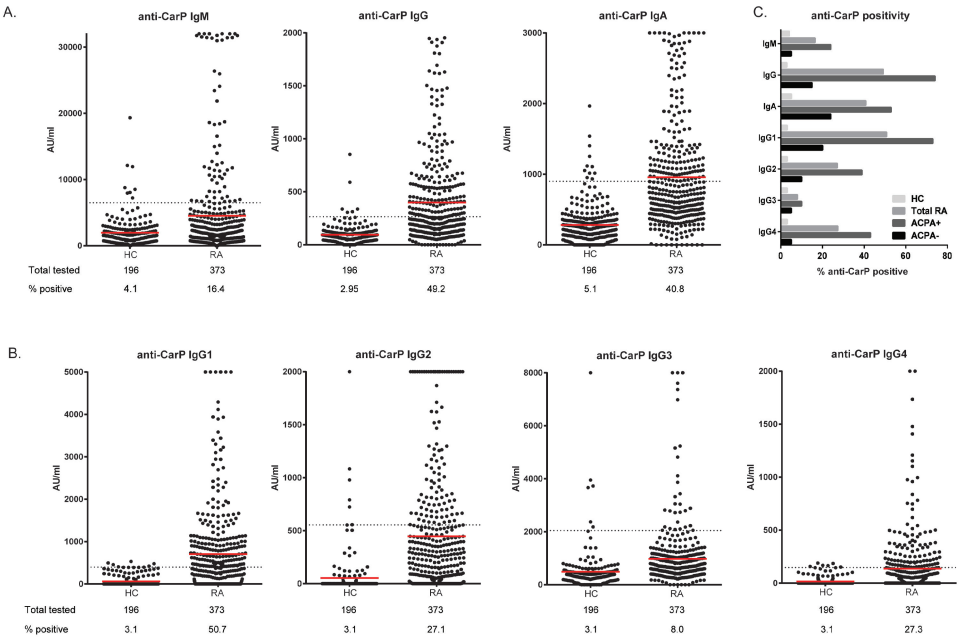

FIGURE 1. Anti-CarP antibody isotypes and IgG-subclasses are present in RA sera. ELISAs were performed to detect anti-CarP antibody isotypes (A) and IgG-subclasses (B) in sera of 196 HC and 373 RA patients. The mean plus two times the standard deviation in HC was established as the cut-off for the anti-CarP antibody isotypes. 97th percentile in HC was used as cut-off for the IgG-subclasses. The dotted line represents the cut-off. The specific anti-CarP reactivity is depicted in arbitrary units per millilitre. The number of samples tested and the percentage positivity is shown below the graphs. Percentage positivity of anti-CarP antibody isotypes and IgG-subclasses in total (grey bars, n=373), ACPA IgG positive(dark grey bars, n=217), ACPA IgG negative (black bars, n=156) RA patients and HC (light grey bars, n=196)(C). HC; healthy controls, RA; rheumatoid arthritis, ACPA; anti-citrullinated protein antibodies, anti-CarP antibody; anti-carbamylated protein antibody, AU/ml; arbitrary units per millilitre.

Presence of anti-CarP antibody isotypes and IgG-subclasses in ACPA positive and negative disease

The anti-CarP antibodies of the IgG isotype are found in approximately 15% of ACPA negative RA patients but are mainly detected in ACPA positive RA [6]. We aimed to investigate the anti-CarP antibody isotype and IgG-subclass distribution in ACPA positive and negative RA. Therefore, the presence of anti-CarP antibody isotypes and IgG-subclasses was determined in the ACPA positive and negative group. Interestingly, all anti-CarP antibody isotypes and IgG-subclasses could be detected in ACPA negative disease (5, 15 and 24% positive for the anti-CarP antibody IgM, IgG and IgA and 20, 10, 5 and 5% positive for anti-CarP antibody IgG1, IgG2, IgG3 and IgG4, respectively

(Figure 1C). However, no significant differences were found for anti-CarP antibody IgM, IgG3 and IgG4 between RA patients and HC at this group size. For ACPA positive RA, a higher proportion of patients were positive for anti-CarP antibodies with 24, 74 and 53% positive for the IgM, IgG and IgA and 73, 39, 10 and 43% positive for IgG1, IgG2, IgG3 and IgG4, respectively (Figure 1C). Significant differences were found for all anti-CarP antibody isotypes and IgG-subclasses between RA patients and HC.

Thus, together, all anti-CarP antibody isotypes and IgG-subclasses analysed can be detected in sera of RA-patients with the highest number of patients positive for anti-CarP antibodies in ACPA positive disease.

The numbers of anti-CarP antibody isotypes and IgG-subclasses are related to anti-CarP levels

Because a wide repertoire of anti-CarP antibody subtypes was identified, we next determined how many different anti-CarP antibody isotypes or IgG-subclasses are present in sera of RA patients. Therefore, the number of anti-CarP antibody isotypes and IgG-subclasses present in RA patients was investigated. Of all tested RA patients, 36% were negative for all anti-CarP antibody isotypes (Figure 2A). Of the anti-CarP antibody positive patients, most expressed one isotype (32%), whereas 21% and 11% expressed two or three isotypes (Figure 2A). Furthermore, anti-CarP antibody IgG-subclasses were detected in 60% of the RA patients; 28% expressed one IgG-subclass, 15% two subclasses, 12% three subclasses and 5% expressed all four subclasses (Figure 2B). For healthy controls, 88% tested negative for all isotypes analysed, 11% was positive for one isotype, 1% for two isotypes and none of the healthy controls were positive for all three isotypes (Figure 2A). Similar percentages of positivity were found for the number of IgG-subclasses in healthy controls (Figure 2B). These data indicate that the anti-CarP antibody response is not uniform and that considerable variation can exist between anti-CarP antibody positive patients with respect to the depth of the anti-CarP antibody response.

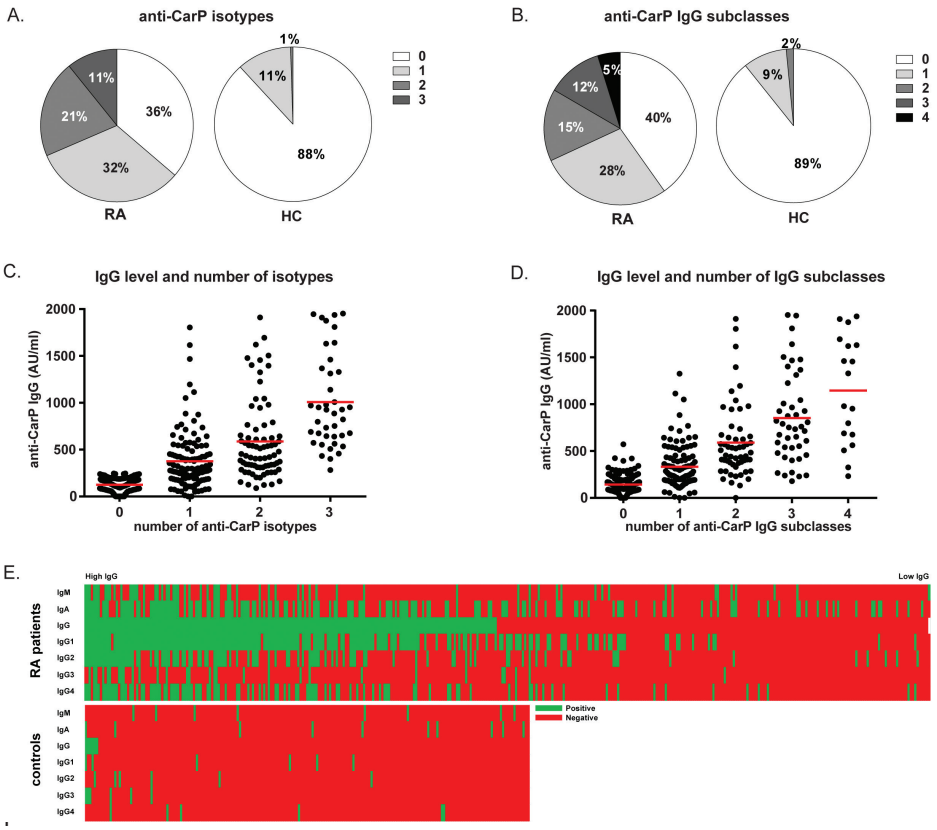

FIGURE 2. The number of anti-CarP antibody isotypes and IgG-subclasses differs between RA patients and is level dependent. Anti-CarP antibody isotypes and IgG-subclasses were measured by ELISA in 373 RA patients and 196 HC. Pie charts show the percentage of RA patients and HC negative or positive for one, two or three anti-CarP antibody isotypes (A) and negative or positive for one, two, three or four anti-CarP IgG-subclasses (B). An increase in level of anti-CarP antibody IgG associates with an increase in number of anti-CarP antibody isotypes (C) and IgG-subclasses (D) in RA patients. Red lines depict means. Heat maps show the presence of anti-CarP antibody isotypes and IgG-subclasses in RA and HC, ranked according to anti-CarP antibody IgG levels (E). Green and red mark positive and negative sera, respectively. HC; healthy controls, RA; rheumatoid arthritis, anti-CarP antibody; anti-carbamylated protein antibody, AU/ml; arbitrary units per millilitre.

In addition, we investigated whether a correlation could be observed between the anti-CarP antibody IgG level and the number of anti-CarP antibody isotypes or IgG-subclasses present in RA patients. As depicted in figure 2C, the number of anti-CarP antibody IgG in RA patients was associated with anti-CarP IgG level (correlation coefficient is 0.748, $p<0.001$),

as was the number of anti-CarP IgG-subclasses (correlation coefficient 0.75, $p<0.001$) (Figure 2D).

An overview of anti-CarP antibody isotype and IgG-subclass expression by RA patients is depicted in a heatmap, ranked according to anti-CarP IgG antibody level (Figure 2E). Interestingly, some of the RA patients tested positive for anti-CarP IgM and/or IgA, although they were negative for the anti-CarP antibody IgG.

Altogether, these data indicate that the anti-CarP antibody response differs between individual RA patients and that higher anti-CarP antibody levels are associated with the presence of more anti-CarP antibody isotypes and IgG-subclasses.

Distribution of anti-CarP antibody and ACPA isotypes and IgG-subclasses

To investigate whether the anti-CarP antibody and ACPA response display a similar usage of isotypes and IgG-subclasses, the presence of anti-CarP isotypes and IgG-subclasses was studied within anti-CarP antibody and ACPA IgG double positive RA patients. We observed a similar distribution of several isotypes and subclasses, although for others a different distribution was noted for both autoantibody responses at the population level (Figure 3A and 3B and additional file 2). Approximately half of the patients tested positive for ACPA IgM or IgA. Similar results were observed for the anti-CarP IgM and IgA antibody. When investigating IgG-subclasses, almost 80% of the patients were positive for IgG1 ACPA or IgG1 anti-CarP antibody and around 40% of the patients showed positivity for ACPA or anti-CarP antibody IgG3. Interestingly, a considerable difference between both autoantibody reactivities was seen for IgG2, as 5% of the patients tested positive for ACPA IgG2, whereas 70% of the patients were positive for anti-CarP antibody IgG2. For IgG4, 28% of the patients were positive for IgG4 ACPA and 56% of the patients for IgG4 anti-CarP antibody. As depicted in figure 3B, patients positive for a particular isotype or IgG-subclass group, could test positive for both auto-antibodies in the same isotype/IgG-subclass, but also discordance is observed frequently (figure 3B). Furthermore, a weak correlation was observed between the anti-CarP antibody and ACPA response for each isotype or IgG-subclass. In the same sample set we observed a more pronounced correlation between anti-CarP IgM and RF-IgM

as well as ACPA IgM and RF-IgM (Additional file 3). We next investigated the correlation between anti-CarP and ACPA in an unselected group of RA patients (also including ACPA / anti-CarP negative patients) to further confirm these findings. In agreement with the data presented above, we again observed weak correlations for anti-CarP and ACPA (Additional file 4). When we examined the number of ACPA and anti-CarP antibody isotypes or IgG-subclasses present in the patient population, similar results were observed for both antibody responses. However, almost half of the ACPA IgG positive patients were positive for 3 isotypes, which was less for anti-CarP antibody positive patients (Figure 3C). Furthermore, approximately half of the ACPA IgG-positive patients tested positive for only 1 ACPA IgG-subclass, whereas this was the case in only 14% of anti-CarP IgG-positive patients (Figure 3D). No association was found between the number of ACPA isotypes/IgG-subclasses and anti-CarP antibody isotypes/IgG-subclasses used by the respective auto-antibody responses (data not shown).

Although, at the population level the usage of most isotypes/IgG-subclasses was similar for both auto-antibody reactivities, it was different for other isotypes/IgG-subclasses. The latter was most prominent within individual patients as they could be single or double positive for the anti-CarP and/or ACPA isotype/IgG-subclass. Together, these data indicate that the expression of these two autoantibody responses is differentially regulated.

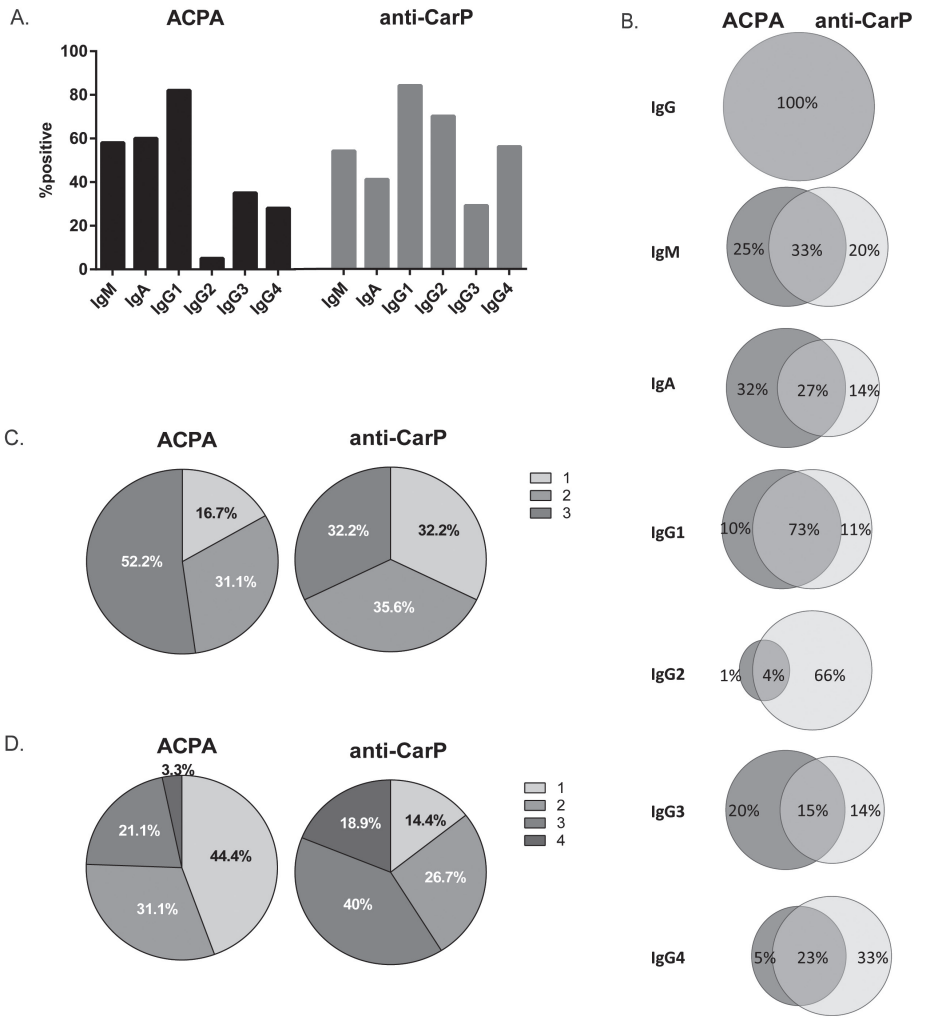

FIGURE 3. Distribution of anti-CarP and ACPA isotypes and IgG-subclasses. Percentage positivity of ACPA (black bars) or anti-CarP antibody (grey bars) IgM, IgA and IgG-subclasses in ACPA and anti-CarP antibody IgG double positive RA patients (n=114) (A). Percentage single or double positive for ACPA (grey) and anti-CarP antibody (light grey) isotypes and IgG-subclasses in IgG double positive RA patients (B). Circles are not on scale. Number of anti-CarP antibody and ACPA isotypes (C) and IgG-subclasses (D) in anti-CarP antibody and ACPA IgG double positive RA patients and at least positive for one IgG-subclass (n=90). RA; rheumatoid arthritis, ACPA; anti-citrullinated protein antibodies, anti-CarP antibody; anti-carbamylated protein antibody.

Anti-CarP IgG1 antibodies are associated with more severe radiological damage, also in ACPA and RF negative RA

Previous data have shown that the presence of anti-CarP IgG antibodies are associated with more severe radiological progression, also after correction for ACPA and RF/ stratification in ACPA positive and ACPA negative RA [6, 9, 10, 12]. Likewise, a similar trend has been observed for ACPA negative patients positive for anti-CarP IgA [6]. To analyse whether the presence of anti-CarP IgM, IgA and anti-CarP IgG-subclasses are also predictive for a more severe disease course, we compared the extent of joint damage over time, measured with the Sharp-van der Heijde method, between anti-CarP antibody positive and negative patients (Figure 4). The calculated β indicates the rate of joint destruction after 7 years (y) compared to the reference group after adjustments for treatment strategy, age, sex, baseline ESR, baseline CRP and symptom duration [24, 26]. Anti-CarP IgG1 positive patients displayed more joint destruction over 7 years than anti-CarP IgG1 negative patients without correction for ACPA and RF (β=1.30, 95% confidence interval (CI) 1.06-1.59, p=0.012) (Figure 4A). Stratified analyses were performed for ACPA alone (corrected for RF, data not shown) and ACPA and RF together. Importantly, this analysis revealed that the presence of anti-CarP IgG1 in ACPA and RF negative RA associates with more severe joint destruction over 7 years (β=1.88, 95% CI 1.21-2.92, p=0.005) (Figure 4B).

Next to anti-CarP IgG1, anti-CarP IgG4 antibody positivity was found to be associated with more joint damage over 7 y as well (β=1.24, 95% CI 1.00-1.53, p=0.045), however this correlation was lost after correction for ACPA (p=0.39) or after stratification for ACPA status (p=0.28). Furthermore, no significant associations with joint damage were found for anti-CarP IgG2, IgG3, IgM and IgA antibodies. Moreover, no associations with severity were found with a broader anti-CarP antibody isotype or IgG-subclass usage.

Taken together, these data indicate that the detection of anti-CarP IgG1 at baseline is predictive for a more severe disease course in ACPA negative (data not shown) and ACPA and RF negative RA (Figure 4B).

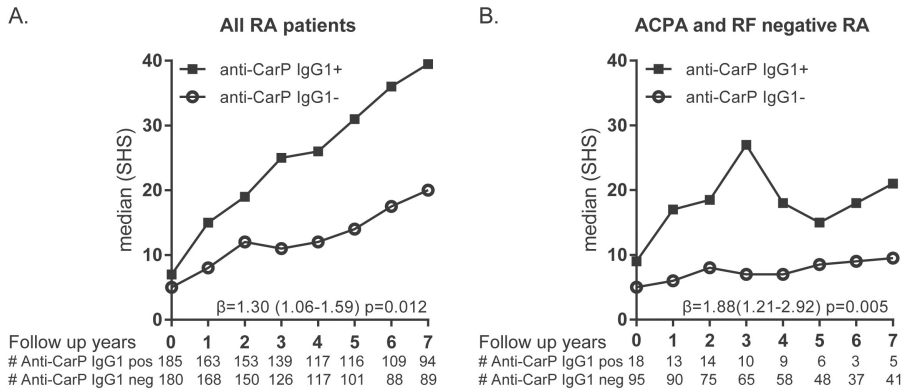

FIGURE 4. Anti-CarP IgG1 is associated with more severe radiological progression. The extent and rate of joint destruction were analysed in all RA patients or separately for ACPA negative and ACPA positive RA and within the ACPA negative RA patients also for RF negative and positive. The severity of joint damage is depicted as median Sharp-van der Heijde score (SHS) on the y-axis and the follow-up years on the x-axis for anti-CarP antibody IgG1 positive and negative patients in all RA patients analysed (A) and for ACPA and RF negative RA patients (B). β and p values are derived from the analysis model as described in Methods and Results. RA; rheumatoid arthritis, anti-CarP antibody; anti-carbamylated protein antibody, RF; rheumatoid factor, β; times increased damage after 7 years corrected for treatment strategy, age and sex.

Broader IgG-subclass usage associates with higher erythrocyte sedimentation rate and higher C-reactive protein levels.

To study the relation between anti-CarP antibody isotypes or IgG-subclasses and baseline characteristics, we next analysed the association between the number of anti-CarP isotypes or IgG-subclasses with ESR or CRP-level at onset using ordinal regression analyses. This showed an association between the number of anti-CarP IgG-subclasses with increased ESR and CRP-level (Table 2), especially in ACPA positive disease (p=0.001 for both, remained significant after Holm-Bonferroni correction, data not shown). When analysing the anti-CarP IgG-subclasses separately in the anti-CarP total IgG positive group, the association with increased ESR and CRP is mostly manifested between the anti-CarP antibody IgG4 positive and negative patients (p<0.001 for ESR and p=0.003 for CRP), also after Holm-Bonferroni correction (Table 3 and additional file 5) and after stratification in

the ACPA positive group (data not shown). To summarize, these data indicate that a broader anti-CarP IgG-subclass response associates with increased ESR and CRP-level in ACPA positive disease.

TABLE 2. Associations for the number of anti-CarP antibody isotypes or IgG-subclasses with risk factors and baseline characteristics

Number of Isotypes	**0 (n=135)**	**1 (n=120)**	**2 (n=77)**	**3 (n=40)**	**Ordinal OR (95% CI)**	***p***
Smoking (ever), n (%)	54 (42.9)	48 (43.6)	35 (48.6)	23 (60.5)	1.38 (0.94-2.03)	0.099
SE positivity, n (%)	79 (60.3)	74 (63.2)	57 (75.0)	33 (86.8)	1.91 (1.27-2.87)	0.002*
ESR, mm/hour M ± SD	41.0 ± 25.6	40.2 ± 25.1	43.5 ± 27.7	47.8 ± 26.8	1.01 (1.00-1.01)	0.206
CRP, mg/liter M ± SD	29.0 ± 32.2	32.3 ± 36.4	29.9 ± 30.3	50.8 ± 47.0	1.01 (1.00-1.01)	0.017‡

Number of IgG-subclasses	**0 (n=150)**	**1 (n=104)**	**2 (n=57)**	**3 (n=44)**	**4 (n=18)**	**Ordinal OR (95% CI)**	***p***
Smoking (ever), n (%)	58 (41.1)	42 (42.4)	30 (58.8)	27 (65.9)	3 (20.0)	1.47 (1.00-2.16)	0.051‡
SE positivity, n (%)	89 (60.5)	65 (64.4)	46 (80.7)	31 (75.6)	13 (76.5)	**1.79 (1.19-2.69)**	**0.005***
ESR, mm/hour M ± SD	37.9 ± 23.2	37.7 ± 24.3	50.4 ± 31.1	52.3 ± 28.5	48.8 ± 21.6	**1.01 (1.01-1.02)**	**<0.001***
CRP, mg/liter M ± SD	30.5 ± 32.9	28.1 ± 33.9	30.5 ± 34.8	45.4 ± 41.5	50.2 ± 42.9	**1.01 (1.00-1.01)**	**0.020**

Ordinal regression analysis for the number of isotypes present and risk factors (smoking, SE positivity) or baseline parameters (ESR, CRP-level)
Bold means significant difference, * remained significant after Holm-bonferroni correction, ‡Test of parallel lines significant
Holm-bonferroni correction was performed for independent analyses in whole cohort
SE, shared epitope; OR, odds ratio; CI, confidence interval; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; M, mean; SD, standard deviation

TABLE 3. Associations of anti-CarP antibody isotypes and IgG-subclasses with baseline characteristics

Isotypes	**IgG- (n=190)**	**IgG+ (n=182)**	***p***	**IgA- (n=221)**	**IgA+ (n=152)**	***p***	**IgM- (n=312)**	**IgM+ (n=61)**	***p***
ESR, mm/hour	41.2 ± 25.3	42.9 ± 26.8	0.676	40.4 ±	44.2 ±	0.157	41.2 ±	45.7 ±	0.216
M ± SD	37.0 (33.3)	37.0 (44.5)		25.7	26.4		25.8	26.8	
Median (IQR)				34.5	39.0		37.0	44.0	
				(33.0)	(43.0)		(35.0)	(45.0)	
CRP, mg/liter	32.0 ± 35.7	33.3 ± 35.4	0.694	28.9 ±	38.0 ±	0.039	29.9 ±	46.0 ±	0.092
M ± SD	19.0 (34.0)	19.5 (36.3)		33.3	38.0		31.8	48.3	
Median (IQR)				17.0	23.0		18.0	23.5	
				(28.8)	(48.0)		(31.0)	(59.8)	

IgG-subclasses	**IgG1- (n=33)**	**IgG1+ (n=149)**	***p***	**IgG2- (n=100)**	**IgG2+ (n=82)**	***p***	**IgG3- (n=157)**	**IgG3+ (n=25)**	***p***	**IgG4- (n=98)**	**IgG4+ (n=84)**	***p***
ESR, mm/hour	36.8 ± 24.2	44.1 ± 27.3	0.176	40.4 ±	45.7 ±	0.156	42.1 ±	47.0 ±	0.323	35.3 ±	51.4 ± 28.3	<0.001*
M ± SD	32.0 (26.0)	37.0 (46.0)		26.3	27.4		26.9	26.6		23.2	49.0 (45.0)	
Median (IQR)				34.0	44.5		34.5	51.0		30.0		
				(39.0)	(46.3)		(45.0)	(45.0)		(29.3)		
CRP, mg/liter	27.5 ± 26.1	34.7 ± 37.2	0.637	29.0 ±	38.7 ±	0.023	31.1 ±	47.5 ±	0.107	25.3 ±	42.1 ± 41.1	0.003*
M ± SD	20.0 (30.0)	19.5 (39.3)		33.1	37.7		33.4	44.8		27.2	28.0 (52.0)	
Median (IQR)				17.5	26.0		18.0	32.0		16.0		
				(31.8)	(41.0)		(33.5)	(58.5)		(28.0)		

Mann-Whitney U test to calculate P-values for baseline parameters (ESR and CRP-level)
IgG-subclass analysis was performed in anti-CarP IgG positive patients
Bold means significant difference, *remained significant after Holm-Bonferroni correction
Holm-bonferroni correction was performed for independent analyses in whole cohort
ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; M, mean; SD, standard deviation

DISCUSSION

In this study, we analysed the presence and levels of anti-CarP antibody isotypes and IgG-subclasses in patients with RA. The results indicate that the anti-CarP antibody response uses a broad spectrum of isotypes and IgG-subclasses in RA patients. The presence of anti-CarP antibody IgM together with other isotypes might indicate that there is an ongoing immune response which is continuously reactivated by IgM producing B-cells. This is because IgM antibodies have a short half-life of a few days [27] and in the presence of T cell help, switching towards IgG is typically associated with the disappearance of the IgM-responses[16].

In the overall RA group (n=373) as well as in the ACPA negative subgroup, a higher percentage of RA patients is tested positive for anti-CarP IgG1 than for total anti-CarP IgG. This apparent discrepancy can likely be explained best by the sensitivities of these different assays in combination with the cut-off's used to define a positive response. For example, the total IgG anti-CarP ELISA measures the presence of all for IgG subclasses, whereas the IgG-subclass ELISA detects only one of them. The cut-offs for each of these assays were calculated separately, based on the measurement in healthy controls. Therefore, patients could be tested positive when measuring only IgG1 and not when analysed for total anti-CarP IgG ("sum" of IgG1, IgG2, IgG3, IgG4).

A limitation of the study is that HC and RA patients are not completely age and sex matched. The HC group was used to set the cut-off for anti-CarP and anti-CCP positivity and it was considered more important to use a control group that is reflecting the overall population of the area in which the disease population is living. Importantly, we do not have indications that the presence of anti-CarP antibody and ACPA in HC is associated with gender, age or smoking.

To study whether there was a similar distribution of ACPA and anti-CarP antibody isotype usage in IgG double positive RA patients, 114 double positive RA patients were tested. We observed that the distribution of isotype and IgG-subclass usage and the number of isotypes and IgG-subclasses expressed is similar for both autoantibodies at the population level. However, within individual patients this pattern could be different for anti-CarP and ACPA. This is of interest as it indicates that it is not merely

a reflection of a cross-reactive antibody. These observations are further strengthened by the absence of a clear correlation between the anti-CarP and ACPA isotypes and IgG-subclasses; a correlation that was found present for the presence of anti-CarP IgM or ACPA IgM, respectively, and RF-IgM. Previously we analysed cross-reactivity between ACPA- and anti-CarP antibodies in a dedicated set-up and observed both cross-reactive and non-cross-reactive antibody recognition profiles [28].

In previous studies, it has been shown that RA patients positive for anti-CarP antibody IgG display more radiological damage over time compared to anti-CarP antibody IgG negative patients, also when corrected for ACPA and RF [6, 12]. This study emphasizes this notion as also the presence of anti-CarP IgG1 antibodies is associated with more joint damage, even after correction and stratification for ACPA and RF. However, measuring one of the IgG-subclasses did not out-perform the measurement of total IgG as a predictor for joint damage. Furthermore, no added value was observed for measuring anti-CarP antibody IgG1 instead of total IgG. The observation that none of the other IgG-subclasses or isotypes provided significant associations with severity could be because the number of individuals positive for the other IgG-subclasses and isotypes is lower which results in a decreased statistical power to detect an association. Although we do find an association between anti-CarP IgG1 positivity and severity using correction for possible confounders (age, sex, treatment, baseline ESR, baseline CRP and symptom duration) and after stratification for ACPA and RF, it is difficult to separate out all possible confounders. Likewise, subdividing the group in all possible subgroups will certainly decrease sample size, leading to a further loss in power.

When investigating the number of anti-CarP antibody isotypes or IgG-subclasses used, no clear associations were found with joint damage. However, a broader anti-CarP IgG-subclass usage was associated with an increase in inflammatory markers (higher ESR and CRP-level) at baseline which was mostly manifested in anti-CarP IgG4. The percentage of ACPA positive patients was similar in the groups that expressed more than one anti-CarP IgG-subclass. However, the ACPA levels are higher in patients with more anti-CarP antibody IgG-subclasses. This could be explained by the fact that the number of anti-CarP antibody IgG-subclasses associates with

higher anti-CarP antibody levels and higher anti-CarP antibody levels are associated with higher ACPA and RF levels and vice versa.

In the current study we have analysed the presence of anti-CarP antibody isotypes and IgG-subclasses in patients diagnosed with early RA. It would be interestingly to study the pre-RA phase as well to see how the distribution of anti-CarP antibody isotypes and IgG-subclasses develops over time.

CONCLUSION

In conclusion, the anti-CarP antibody response uses a broad spectrum of isotypes and IgG-subclasses, indicating an ongoing immune response and ample possibilities to employ several effector mechanisms. Furthermore, the anti-CarP and ACPA autoantibody responses seems to be differentially regulated.

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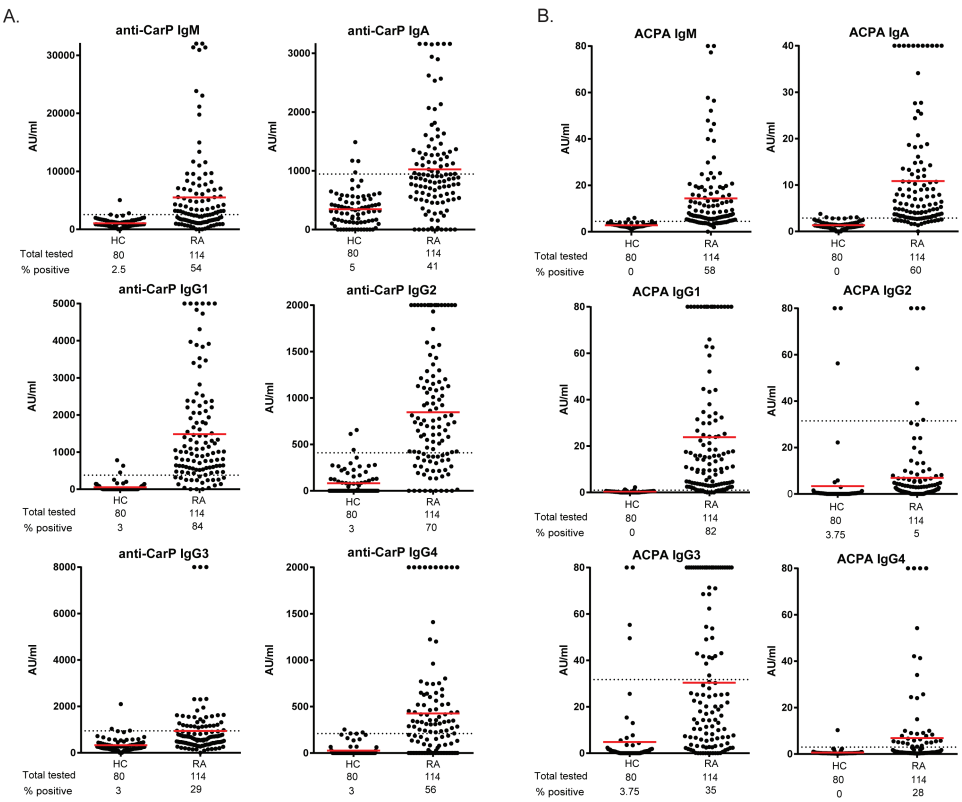
ADDITIONAL FILES

ADDITIONAL FILE 1. Upper limit of the anti-CarP antibody ELISAs

Anti-CarP Isotype/ IgG-subclass	Upper limit (AU/ml)
IgM	32,000
IgG	2,000
IgA	3,160
IgG1	10,200
IgG2	2,000
IgG3	8,000
IgG4	2,000

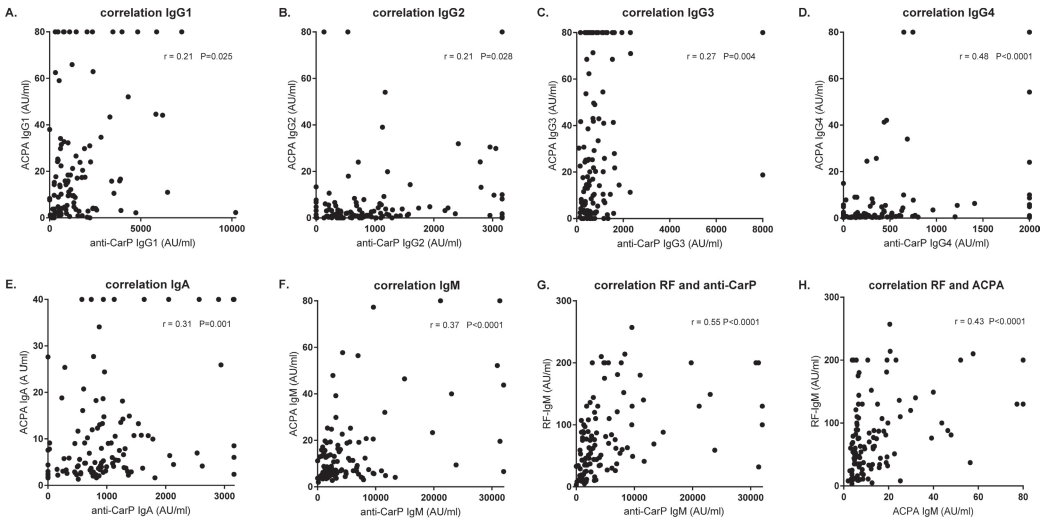
ADDITIONAL FILE 2. Presence of anti-CarP antibody and ACPA isotypes and IgG-subclasses in IgG double positive patients

ELISAs were performed to detect anti-CarP antibody and ACPA isotypes and IgG-subclasses in sera of 80 HC and 114 RA patients. The mean plus two times the standard deviation in HC was established as the cut-off for the anti-CarP antibody isotypes and for the ACPA isotypes and IgG-subclasses. 97th percentile in HC was used as cut-off for the anti-CarP antibody IgG-subclasses. Dotted line represents cut-off. The specific anti-CarP antibody reactivity, FCS reactivity subtracted from the CaFCS reactivity, is depicted in AU/ml (A). For ACPA, the reactivity for CCP2-cittruline is depicted in AU/ml (B) and the % positivity was corrected for reactivity against CCP2-arginine. The amount of samples tested and the percentage positivity is shown below the graphs. HC; healthy controls, RA; rheumatoid arthritis, ACPA; anti-citrullinated protein antibodies, anti-CarP antibody ; anti-carbamylated protein antibody, FCS; foetal calf serum, CaFCS; carbamylated foetal calf serum, AU/ml; arbitrary units per millilitre.



ADDITIONAL FILE 3. Correlation of anti-CarP antibody and ACPA IgM, IgA and IgG-subclasses and RF-IgM with anti-CarP antibody and ACPA IgM in IgG double positive RA

ELISAs were performed to detect anti-CarP antibody and ACPA isotypes and IgG-subclasses in sera of 114 RA patients. Levels of anti-CarP antibodies and ACPAs were plotted against each other, each IgG-subclass and isotype separately (A-F). As internal control anti-CarP IgM and ACPA IgM were plotted against RF-IgM (G, H). Spearman Rank tests were performed to investigate correlations. HC; healthy controls, RA; rheumatoid arthritis, ACPA; anti-citrullinated protein antibodies, anti-CarP antibody; anti-carbamylated protein antibody, RF; rheumatoid factor, AU/ml; arbitrary units per millilitre.

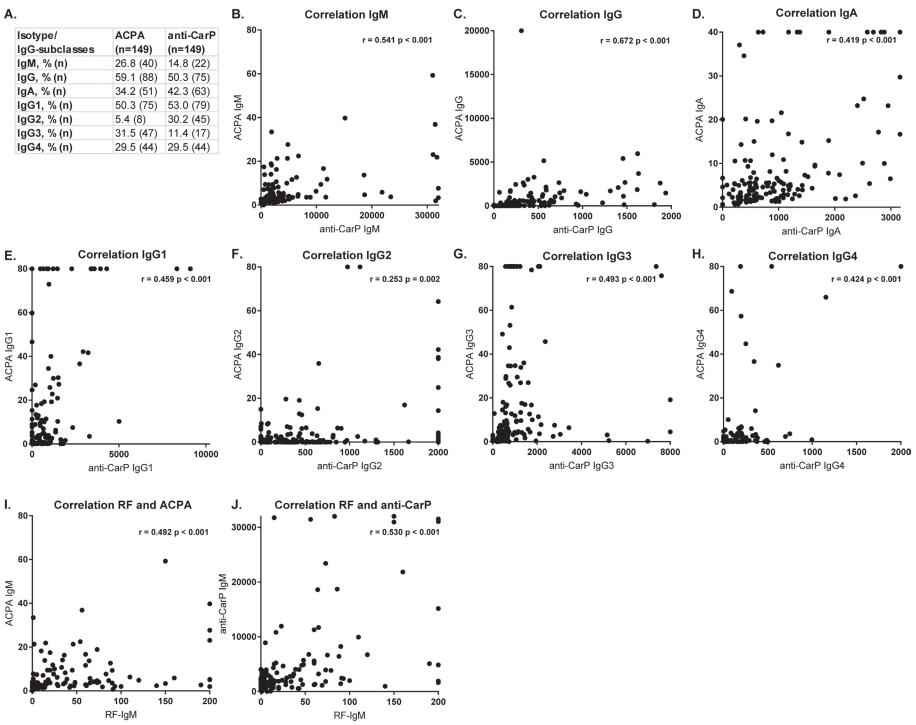


ADDITIONAL FILE 4. Presence and correlation of anti-CarP antibody and ACPA IgM, IgG, IgA and IgG-subclasses and correlations of RF-IgM with anti-CarP antibody and ACPA IgM in an unselected group of RA patients

ELISAs were performed to detect anti-CarP antibody and ACPA isotypes and IgG-subclasses in sera of 149 unselected RA patients. ACPA and anti-CarP antibody isotype and IgG-subclass positivity, percentage and numbers (A). Levels of anti-CarP antibodies and ACPAs were plotted against each other, each isotype and IgG-subclass separately (B-H). As internal control anti-CarP IgM and ACPA IgM were plotted against RF-IgM (I, J). Spearman Rank tests were performed to investigate correlations. HC; healthy controls, RA; rheumatoid arthritis, ACPA; anti-citrullinated protein antibodies, anti-CarP antibody; anti-carbamylated protein antibody, RF; rheumatoid factor, AU/ml; arbitrary units per millilitre.

A.

Isotype/ IgG-subclasses	ACPA (n=149)	anti-CarP (n=149)
IgM, % (n)	26.8 (40)	14.8 (22)
IgG, % (n)	59.1 (88)	50.3 (75)
IgA, % (n)	34.2 (51)	42.3 (63)
IgG1, % (n)	50.3 (75)	53.0 (79)
IgG2, % (n)	5.4 (8)	30.2 (45)
IgG3, % (n)	31.5 (47)	11.4 (17)
IgG4, % (n)	29.5 (44)	29.5 (44)

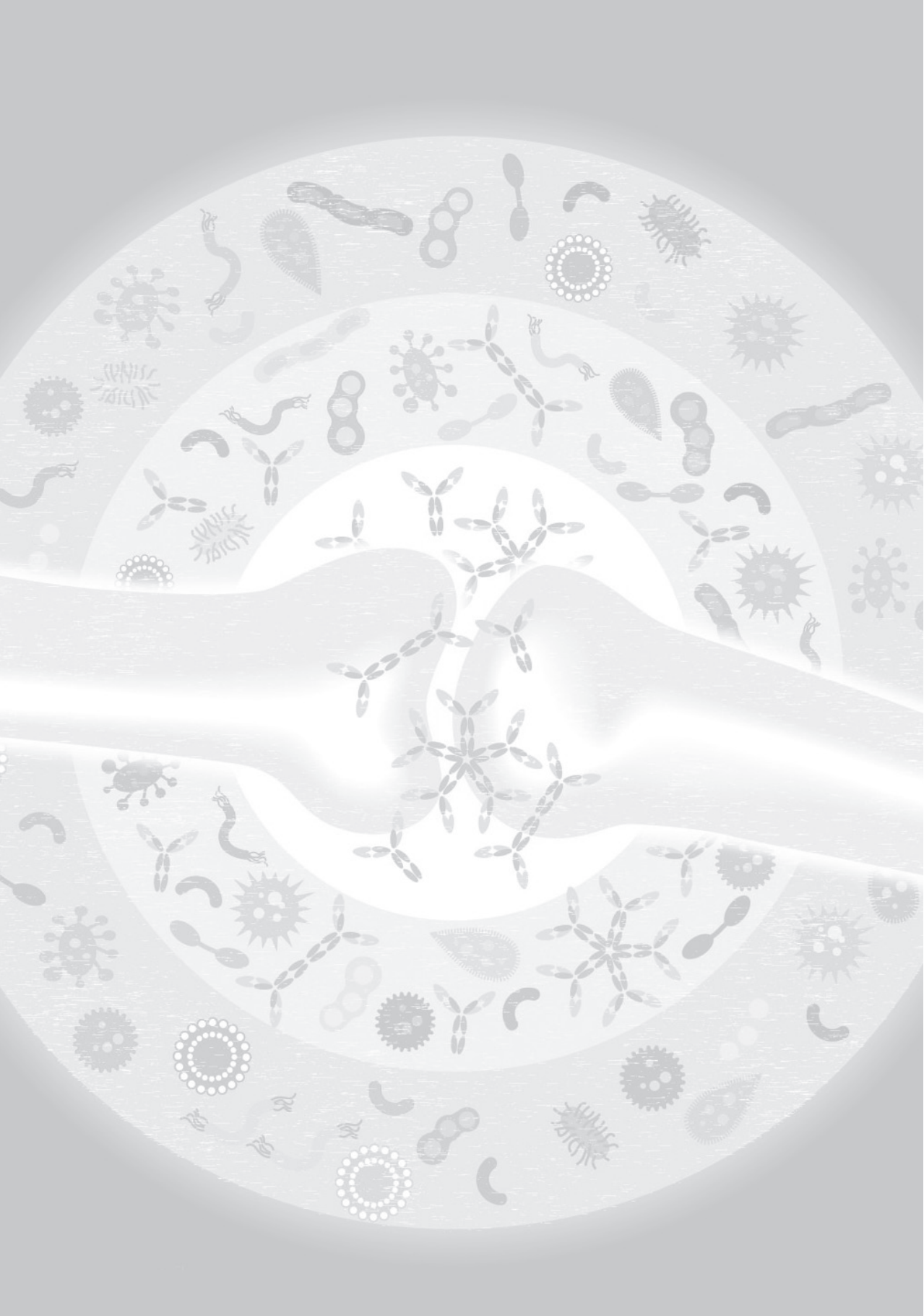


ADDITIONAL FILE 5. Associations of anti-CarP antibody isotypes and IgG subclasses with risk factors.

Isotypes	**IgG- (n=190)**	**IgG + (n=184)**	**OR (95% CI)**	**p**	**IgA- (n=221)**	**IgA+ (n=152)**	**OR (95% CI)**	**p**	**IgM- (n=312)**	**IgM+ (n=61)**	**OR (95% CI)**	**p**
Smoking (ever) n (%)	73 (41.5)	89 (51.7)	1.51 (0.99-2.31)	0.055	88 (42.9)	72 (50.7)	1.37 (0.89-2.10)	0.153	132 (45.5)	28 (49.1)	1.16 (0.66-2.04)	0.618
SE positivity,n (%)	108 (58.1)	137 (77.0)	2.41 (1.53-3.80)	<0.001*	138 (64.5)	106 (71.1)	1.36 (0.86-2.13)	0.185	197 (64.8)	47 (79.7)	2.13 (1.08-4.18)	0.029

IgG subclasses	**IgG1- (n=33)**	**IgG1+ (n=149)**	**OR (95% CI)**	**p**	**IgG2- (n=100)**	**IgG2+ (n=82)**	**OR (95% CI)**	**p**	**IgG3- (n=157)**	**IgG3+ (n=25)**	**OR (95% CI)**	**p**	**IgG4- (n=98)**	**IgG4+ (n=84)**	**OR (95% CI)**	**p**
Smoking (ever)n (%)	14 (43.8)	73 (52.9)	1.44 (0.67-3.13)	0.352	48 (49.5)	39 (53.4)	1.17 (0.64-2.15)	0.611	80 (54.1)	7 (31.8)	0.40 (0.15-1.03)	0.057	43 (46.7)	44 (56.4)	1.48 (0.80-2.71)	0.209
SE positivity,n (%)	23 (71.9)	112 (77.8)	1.37 (0.58-3.25)	0.476	73 (74.5)	62 (79.5)	1.33 (0.65-2.71)	0.437	117 (76.5)	18 (78.3)	1.11 (0.38-3.19)	0.850	74 (77.1)	61 (76.3)	0.95 0.47-1.92)	0.896

Logistic regression univariate for risk factor analysis (smoking and SE positivity) IgG subclass analysis in anti-CarP IgG positive patients Bold means significant difference, *remained significant after Holm-Bonferroni correction Holm-bonferroni correction separately for independent analyses in whole cohort SE, shared epitope; OR, odds ratio; CI, confidence interva



CHAPTER 3

The anti-CarP antibody response is of overall low avidity despite extensive isotype switching

Myrthe A.M. van Delft
Marije K. Verheul
Leonie E. Burgers
Solbritt Rantapää-Dahlqvist
Annette H.M. van der Helm-van Mil
Tom W.J. Huizinga
René E.M. Toes
Leendert A. Trouw

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ABSTRACT

Objective: To better understand the contribution of autoantibodies in RA and the biology of their responses, we evaluated the avidity of the anti-CarP antibody response.

Methods: The avidity of anti-CarP antibody, anti-citrullinated protein antibody (ACPA) and anti-Tetanus Toxoid (TT) IgG were determined using elution assays. Anti-CarP IgG avidity was measured in sera of 107 RA-patients, 15 paired synovial fluid and serum samples and 8 serially sampled sera before and after disease onset.

Results: The avidity of anti-CarP IgG is low compared to the avidity of anti-TT IgG present in the same sera. Likewise, although less pronounced, anti-CarP also displayed a lower avidity as compared to the avidity of ACPA IgG. No difference in anti-CarP IgG avidity is observed between ACPA positive or ACPA negative patients. Anti-CarP IgG avidity is higher in anti-CarP IgM-negative compared to IgM-positive individuals. Furthermore, the anti-CarP avidity in serum is higher than in synovial fluid. Using samples of individuals that over time developed RA we observed no anti-CarP avidity maturation in the years before disease onset. In contrast to ACPA avidity, the anti-CarP avidity is not associated with severity of joint destruction.

Conclusions: The anti-CarP response is of overall low avidity, even lower than the ACPA IgG avidity and does not show apparent avidity maturation before or around disease onset. Overall, isotype switch and avidity maturation seem to be uncoupled as isotype switch occurs without avidity maturation, pointing towards a commonality in the regulation of both autoantibody responses as opposed to the pathways governing recall responses.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic autoimmune disease mainly affecting synovial joints [1, 2]. Several autoantibodies have been identified in serum and synovial fluid (SF) of RA patients [3]. These may form immune complexes in the joints, leading to the attraction of immune cells through e.g. complement activation [4, 5] which can contribute to chronic inflammation and bone destruction.

Well-known autoantibodies that are currently used in the clinic for the diagnosis of RA are rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPA)[2]. More recently, anti-carbamylated protein (anti-CarP) antibodies have been detected in RA [6]. These antibodies detect carbamylated proteins which are post-translationally modified proteins wherein lysines are converted to homocitrullines by a chemical reaction with cyanate [7, 8].

Various studies have shown a higher prevalence of anti-CarP antibodies in RA patients compared to controls [6, 9-14]. Several observations implicate a role for anti-CarP directed immunity in the pathogenesis of RA as anti-CarP antibodies; are present years before disease onset with a gradual increase before disease onset [9, 15, 16], are associated with the development of RA in arthralgia patients [17] and associate with increased joint destruction in RA [6, 9, 10, 12, 18]. Sera of RA patients can be positive for both anti-CarP antibody IgG and IgM [19], which is of interest as switching towards IgG is typically associated with a large decline or disappearance of IgM-responses in case of conventional T cell dependent antigen responses [20].

During a B cell response, somatic hypermutation, affinity maturation and isotype switching occurs in the germinal center. For somatic hypermutation, activated B cells will enter the germinal center and start to proliferate and undergo hypermutation upon receiving T-cell help. Studies using model antigens have shown that various B cell clones will compete for the antigen on follicular dendritic cells. Antibody avidity is defined as the overall binding strength of polyclonal (multivalent) antibodies to its multivalent antigens. Those B cells expressing the surface immunoglobulin that will bind with higher avidity, will outcompete other B cells because they attract more signals necessary for B cell survival and proliferation. Due to this process,

the avidity of the immune response increases over time and low avidity B cells will typically disappear from the circulation.

While substantial information is available on the avidity maturation of antibody responses against recall antigens [21-23], less information is present on avidity maturation of autoantibody responses. However, it is described that the avidity of autoantibodies (high, moderate and low) associates with different clinical outcomes in several diseases [24]. Interestingly, in celiac disease the avidity of autoantibodies targeting transglutaminase is reported to be much lower than the avidity of anti-*E coli* antibodies present in the same sera [25]. In addition, previous data of our group showed that the average avidity of ACPAs is much lower than the avidity of antibodies to recall antigens, even when many isotypes are used and levels are high [26]. These data indicate that although these B cells underwent isotype switching, and apparently attract sufficient signals for survival and proliferation, no or little avidity maturation was taking place. In a 'normal' B cell response; somatic hypermutation, isotype switching and avidity maturation are expected to occur side by side. ACPA can be detected many years prior to disease onset [27, 28] and during these years there is (limited) avidity maturation for the ACPA response [29]. Interestingly the patients with the lowest ACPA avidity experienced the most pronounced joint destruction [30]. A low avidity does not mean that these antibodies are non-pathogenic as low avidity antibodies have for example enhanced capacity to penetrate deeper into tissue [31], and an enhanced capacity to activate complement [30]. Citrullination and carbamylation are two rather similar post-translational modifications, and although antibodies against both modifications are often seen together in RA, they represent two different antibody families as also ACPA or anti-CarP single positive patients are present and cross-reactivity towards the two modifications is incomplete [3].

Based on these previous observations and our interest in understanding the biology of the anti-CarP antibody response, we have studied the avidity of the anti-CarP antibody response in detail using baseline serum samples of RA patients with long-term follow-up data in the Leiden Early Arthritis Clinic (EAC) cohort [32] as well as samples from patients in the phase before diagnosis [9, 28, 33]. We show here that the anti-CarP antibody avidity is low, even lower than the ACPA avidity and that anti-CarP antibody IgG and IgM

positive patients have a lower anti-CarP IgG avidity compared to anti-CarP antibody IgG positive and IgM negative patients.

METHODS

Patients and control sera

Sera of 107 RA patients (average age 55.8 and 64.5% female) were analysed for the anti-CarP antibody IgG avidity. As control, sera of 86 RA patients (average age 47.8 and 59.3% female) and next to this 34 age and sex matched RA patients with healthy controls (HC) (average age 45 and 61.7% female) were analysed for anti-tetanus toxoid (TT) IgG avidity as recall antigen. Anti-CarP antibody [6, 19] and ACPA status were already available [19, 34] and used in these analysis. Furthermore, radiographs taken at yearly intervals were available for almost all tested RA patients. Baseline sera of RA patients participating in the EAC cohort [32] were analysed. RA patients were included between 1993 and 2003 and inclusion required a symptom duration <2 years [32]. Healthy control samples were acquired from persons living in the Leiden region as described before [6]. Informed consent was obtained for all individuals and all protocols were approved by the ethics committee of the LUMC.

Paired serum and synovial fluid (SF) samples of 29 RA patients were analysed for anti-CarP antibody IgG avidity. Samples were kindly provided by RA patients and informed consent was signed.

Sera of 8 anti-CarP antibody IgG positive RA patients, sampled before and after symptom onset, were analysed for anti-CarP antibody IgG avidity maturation. These individuals were participating in the Medical Biobank of Northern Sweden or the Mamography screening project [9, 28, 33]. Informed consent was obtained for all individuals when donating blood and all protocols were approved by the Regional Ethics Committee at the University Hospital of Umea, Sweden. Samples were also collected from these individuals when they were diagnosed with RA later at the Early Arthritis Clinic.

Measurement of anti-CarP antibody and anti-Tetanus Toxoid IgG avidity

To determine the avidity of anti-CarP antibody IgG, ACPA IgG and anti-TT IgG, elution ELISA assays were used [26, 35]. For anti-CarP antibody the

appropriate serum dilution was determined by performing a titration using the anti-CarP IgG ELISA [6]. For ACPA and anti-TT IgG, this was done as previous described [26]. The serum dilution at which the response was in the linear part of the curve with an absorbance value around 1.5 at 415nm was selected as optimal. The minimal dilution we used in the avidity assay was 1:12.5.

Protein carbamylation and citrullination and verification of the modification were done as before [6, 19, 36, 37]. To determine the anti-CarP antibody and ACPA IgG avidity, in house coated CCP2 (1µg/ml) [19], citrullinated foetal calf serum (10µg/ml) (Cit-FCS, Bodinco), carbamylated foetal calf serum (10µg/ml) (Ca-FCS, Bodinco) or Carbamylated alpha-1-antitripsin (10µg/ml) (Ca-A1AT, Lee biosolutions) plates were used and incubated with the appropriate serum dilutions. After washing, the wells were incubated with increasing concentrations of chaotropic agent sodiumthiocyanate (NaSCN;Sigma Aldrich) of 0.25, 0.5, 1, 2, 3, 5M, for 15min at room temperature (RT). After washing the bound antibodies were detected using HRP-labelled rabbit anti human IgG (Dako P0214). The amount of antibodies bound to the plate with and without elution by NaSCN were determined using a standard curve.

The percentage restbinding, defined as the ratio of the amount remaining antibodies to an antigen at a certain molarity NaSCN to the amount of bound antibodies in the absence of NaSCN, was calculated [26, 38]. The relative avidity index (AI) is defined as the ratio of the amount remaining antibodies to an antigen at 1M NaSCN to the amount of bound antibodies in the absence of NaSCN, expressed as percentage [26, 38].

The anti-TT IgG avidity was determined using an in-house ELISA as previously described [26, 35].

Statistical analysis

Statistical analysis was performed using statistical package for the social sciences (SPSS) version 23 (IBM). In order to determine differences in antibody avidity between anti-CarP antibody IgG and anti-TT IgG in RA patients, anti-TT IgG avidity in RA patients and HC or anti-CarP IgM/IgA positive and negative RA patients, Mann-Whitney U tests were carried out. In order to investigate whether there are correlations between anti-CarP levels and AI, between anti-CarP AI and ACPA AI and between anti-CarP AI

in SF and serum, Spearman Rank tests were performed. To study whether the presence of more anti-CarP isotypes associates with antibody avidity, Kruskal-Wallis tests were performed. Wilcoxon signed ranks test were performed to investigate differences in paired samples. P values below 0.05 were considered statistically significant.

In 107 RA patients, divided in quartiles of 27 patients based on the AI of anti-CarP IgG, the association between anti-CarP antibody IgG avidity and radiographic progression, as assessed by the Sharp-van der Heijde Score [39], was analysed as described before [6, 19, 32, 40]. As repeated radiographs were taken at yearly intervals, we have used a multivariate normal regression analysis for longitudinal data. Adjustments for treatment strategy, age and sex had been made. P values below 0.05 were considered statistically significant.

RESULTS

The avidity of anti-CarP is low compared to the avidity of tetanus toxoid

Anti-CarP antibodies can be detected using several antigens in ELISA [6, 36]. Here we have analysed the avidity of anti-CarP antibodies based on Ca-FCS and Ca-A1AT detection. The anti-CarP IgG avidity was compared to the IgG avidity directed against the recall antigen TT. Using increasing concentrations of chaotropic salt in the elution assays we observed a low avidity for antibodies against carbamylated proteins and as expected a high avidity for antibodies directed against the recall antigen TT (Figure 1A). Within the same patients, similar results were found using Ca-FCS or Ca-A1AT as an antigen (Figure 1A). As there were no major differences in the avidity of anti-CarP antibodies as detected by Ca-A1AT or Ca-FCS, we decided to use Ca-FCS as the antigen to study the anti-CarP avidity in a larger cohort.

Analysing 107 RA patients positive for anti-CarP IgG antibodies revealed that the avidity of anti-CarP IgG antibodies is generally low (median AI 21.1%). In contrast the IgG avidity against the recall antigen TT was considerably higher (median AI 99.6%) (Figure 1B). We tested whether patients with higher levels of anti-CarP antibodies, as a sign of a more pronounced anti-CarP response, would also have a higher avidity. However, we observed no correlation between the levels and avidity of anti-CarP antibody IgG (Figure 1C) and patients with a high level do also display a low avidity. We verified whether

RA patients were actually capable of mounting a proper avidity response by comparing the anti-TT avidity of patients to the anti-TT avidity of healthy controls and observed no difference (data not shown). To summarize, anti-CarP IgG antibodies present in sera of RA patients are of low avidity as compared to the avidity of anti-TT IgG, irrespective of anti-CarP levels.

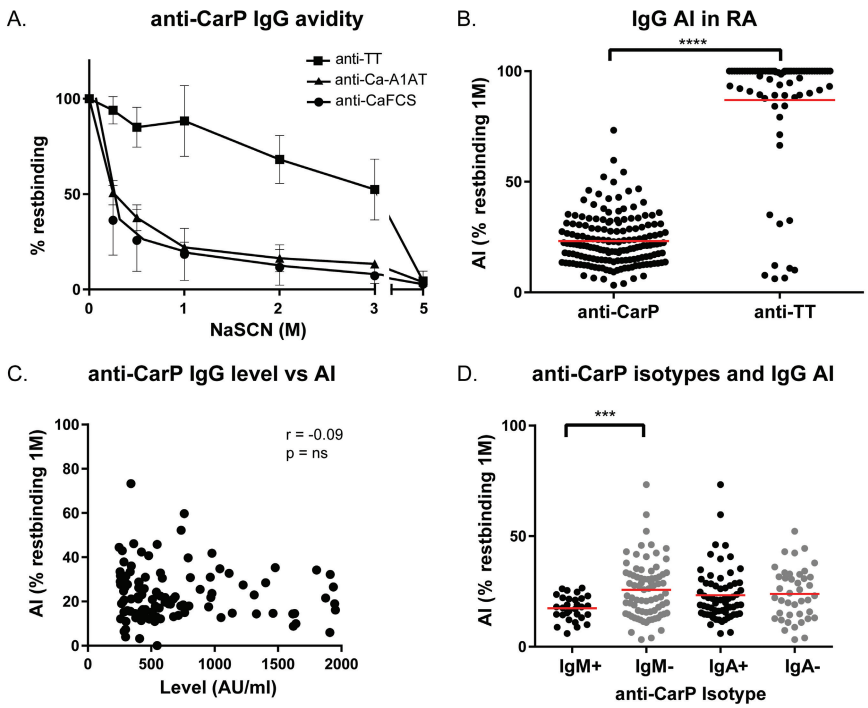

FIGURE 1. The anti-CarP IgG avidity is low and lower in anti-CarP IgM positive RA. Elution ELISA assays were performed to test the anti-CarP antibody IgG avidity on Ca-FCS and Ca-A1AT and TT IgG avidity in sera of 4 RA patients. The percentage restbinding at various molarities of NaSCN is depicted (A). anti-CarP and recall IgG avidity was tested in RA patients (n=107) using Ca-FCS as antigen for anti-CarP IgG and TT as recall antigen (B). The anti-CarP antibody IgG avidity doesn't correlate with anti-CarP antibody IgG levels (AU/ml) (C). The anti-CarP IgG avidity was investigated between anti-CarP IgM or IgA positive and negative patients. The anti-CarP antibody IgG avidity is lower in anti-CarP antibody IgM positive patients (IgM+ n=30, IgM- n=77, IgA+ n=65, IgA- n=41) (D). The avidity index (AI) is depicted as % restbinding at 1M NaSCN (B-D). anti-CarP antibody; anti-carbamylated protein antibody, Ca-FCS; carbamylated fetal calf serum, Ca-A1AT; carbamylated alpha-1-antitripsin, TT; tetanus toxoid, RA; rheumatoid arthritis, NaSCN; sodiumthiocynate, AU/ml; arbitrary units per millilitre, AI; avidity index. Mann-Whitney test (B, D) *p=0.05-0.002, **p=0.002-0.0002, *** p=0.0002-0.0001, **** p< 0.0001, Spearman correlation (C).

Lower anti-CarP IgG avidity in anti-CarP IgM positive RA patients

The anti-CarP antibody response comprises several isotypes and a substantial proportion of the patients is double positive for the anti-CarP antibody IgG and IgM [19]. This is interesting as switching towards IgG is typically associated with disappearance of IgM-responses and the appearance of high avidity IgG antibodies in case of T cell dependent antigen responses [20]. Therefore, we hypothesized that RA patients positive for anti-CarP antibody IgG and IgM have a more actively ongoing, immature, anti-CarP antibody response. In such a scenario, the anti-CarP IgG antibody avidity is conceivably lower in the anti-CarP IgM positive group compared to the anti-CarP IgM negative group. To test this hypothesis, the RA patients analysed were subdivided in an anti-CarP antibody IgM positive and negative group. RA patients positive for anti-CarP IgM showed a slight but significant lower anti-CarP IgG avidity (median AI 17.7%) compared to the anti-CarP IgM negative patients (median AI 24.2%) (Figure 1D). This effect was not found between anti-CarP antibody IgA positive or negative patients, indicating that it is specific for IgM. Furthermore, there was no difference in disease duration between the anti-CarP antibody IgM positive (median 24.1 weeks) and negative patients (median 23.4 weeks) indicating that this is not a reflection of a shorter disease process.

Importantly, the anti-CarP IgG avidity is similar in IgM depleted or non-depleted serum suggesting that the presence of IgM is not interfering with the IgG affinity measurement (Supplementary figure 1).

Overall, these data indicate that a less differentiated antibody response, still including IgM, associates with lower anti-CarP IgG avidity.

Anti-CarP IgG avidity is lower than the avidity of ACPA

Previous data from our group indicates that also the ACPA IgG avidity is low in RA patients [26]. To determine whether the anti-CarP antibody and ACPA IgG avidity is similar, an initial group of 4 patients double positive for ACPA and anti-CarP antibodies were tested for the avidity of the anti-CarP-, ACPA- and anti-TT IgG- response using several concentrations of chaotropic salt in the elution assay. We observed that in almost all patients analysed the avidity of anti-CarP antibodies was lower as compared to ACPA and anti-TT. Data of one representative patient is depicted in figure 2A. Next an additional 18 patients, double positive for anti-CarP and ACPA IgG, were analysed for the anti-CarP, ACPA and anti-TT IgG avidity using 1M NaSCN. The results of these analyses show that both anti-CarP antibody and ACPA

IgG are of low avidity compared to the avidity of anti-TT IgG (p<0.001 for anti-CarP and p=0.0014 for ACPA) (Figure 2B). However, interestingly, the anti-CarP IgG avidity was even lower than the ACPA IgG avidity. Moreover, no obvious correlation is found between the avidity of anti-CarP IgG and ACPA IgG (Figure 2C) and we could not detect a difference in anti-CarP IgG avidity between patients positive or negative for ACPA (n=107) (Figure 2D). To summarize, the avidity of anti-CarP is lower compared to ACPA and antibody avidities are not associated with each other.

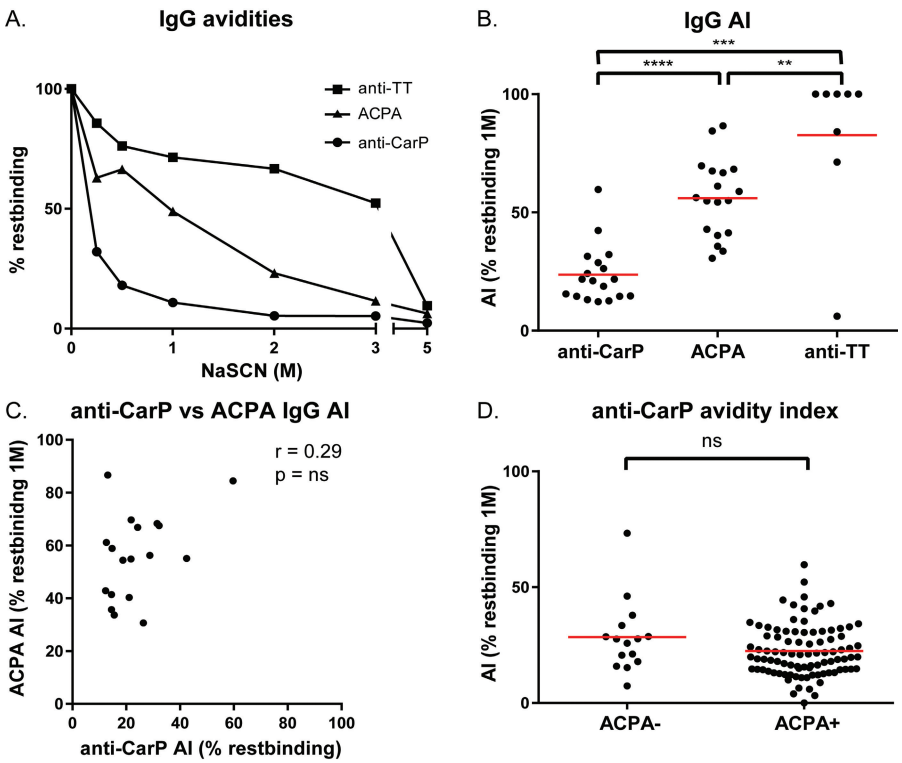

FIGURE 2. The anti-CarP avidity is lower than the ACPA avidity and similar in ACPA positive/negative RA. An initial group of 4 RA patients was tested in titration for the anti-CarP antibody, ACPA and anti-TT IgG avidity. One representative patient is shown (A). Anti-CarP antibody, ACPA and anti-TT IgG avidity in 18 double positive RA patients (B). The anti-CarP antibody and ACPA IgG avidity do not correlate (C) and there is no difference in anti-CarP antibody IgG avidity between patients positive or negative for ACPA (n=107) (D). The avidity index (AI) is depicted as % restbinding at 1M NaSCN. anti-CarP antibody; anti-carbamylated protein antibody, TT; tetanus toxoid, RA; rheumatoid arthritis, ACPA; anti citrullinated protein antibody, AI; avidity index. Mann-Whitney test (B, D) *p=0.05-0.002, **p=0.002-0.0002, *** p=0.0002-0.0001, **** p< 0.0001, Spearman correlation (C).

Anti-CarP avidity is slightly higher in serum compared to synovial fluid

To study whether anti-CarP antibodies present in the SF of inflamed joints may have a different avidity than the anti-CarP antibodies in the circulation, we compared the anti-CarP IgG avidity in SF to the avidity of anti-CarP in paired sera that were collected at the same time point as the SF. These analyses revealed a slightly, but consistently, lower anti-CarP IgG avidity in SF (Figure 3A), with the anti-CarP IgG avidity in SF correlating to the anti-CarP IgG avidity in sera (correlation coefficient 0.6089, p<0.001) (Figure 3B).

Blocking experiments were performed to investigate whether the difference in avidity could be explained by the presence of carbamylated antigens, to which the high avidity anti-CarP IgG might have bound. Paired serum was pre-incubated with or without Ca-FCS or FCS and the avidity was analysed. However, no difference in avidity was observed between the different conditions (data not shown).

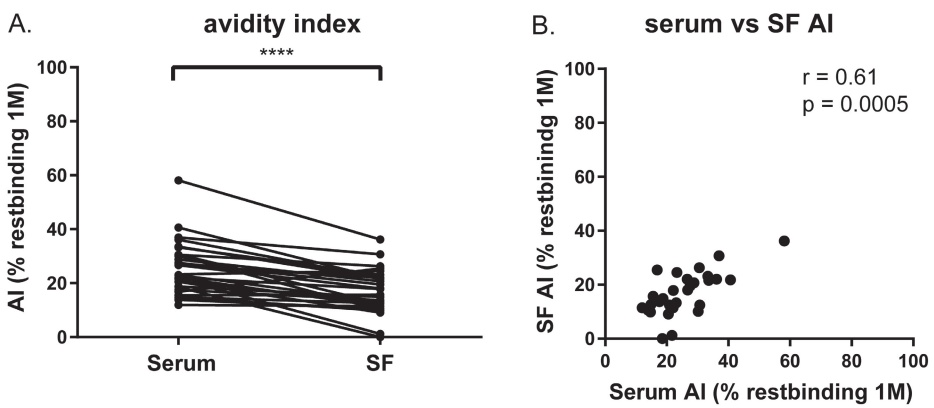

FIGURE 3. Anti-CarP IgG avidity is slightly lower in synovial fluid compared to serum. To investigate whether the anti-CarP antibody IgG avidity differs between serum and SF, SF and paired sera samples of 29 RA patients were tested (A). The anti-CarP antibody IgG avidity in SF correlates the avidity in serum (B). The avidity index (AI) is depicted as % restbinding at 1M NaSCN. Anti-CarP antibody; anti-carbamylated protein antibody, SF; synovial fluid, RA; rheumatoid arthritis, AI; avidity index. Wilcoxon signed ranks test (A) *p=0.05-0.002, **p=0.002-0.0002, *** p=0.0002-0.0001, **** p< 0.0001, Spearman correlation (B).

No evidence for anti-CarP avidity maturation before disease onset

RA-associated autoantibodies, including anti-CarP antibodies can be detected many years prior to disease onset [9, 16] and for ACPA we have shown previously that there is (limited) avidity maturation taking place during this 'pre-RA' period [29]. As we observed that RA patients display a certain range of low to moderately high anti-CarP IgG avidity at baseline, this might indicate that a certain degree of avidity maturation has occurred in a proportion of the anti-CarP antibody IgG positive patients. Therefore we next wished to study the avidity maturation in longitudinal samples of early RA patients collected before and after the symptom onset [9, 33]. The samples were available over a period of 15 years. Overall, we observed no anti-CarP IgG avidity increase during the period before symptom onset (Figure 4).

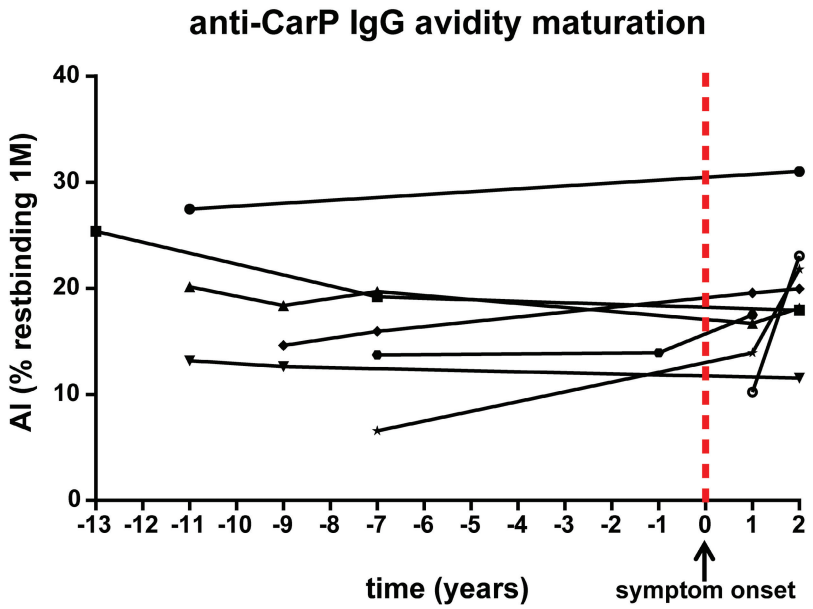

FIGURE 4. No evidence for anti-CarP avidity maturation before symptom onset. To investigate the anti-CarP antibody IgG avidity maturation, 8 anti-CarP antibody IgG positive RA patients, sampled before and after symptom onset, were analysed. No anti-CarP antibody IgG avidity was detected before symptom onset. The avidity index (AI) is depicted as % restbinding at 1M NaSCN. anti-CarP antibody; anti-carbamylated protein antibody, RA; rheumatoid arthritis, AI; avidity index.

Anti-CarP IgG avidity is not associated with more severe joint destruction

Previous data of our group showed that the presence of anti-CarP IgG at baseline associates with a more severe joint destruction over time [6]. Furthermore, we have shown that low avidity ACPA IgG at baseline associates with more radiological damage over time[30]. Next to these findings in RA, it has also been shown that antibody avidity associates with different clinical outcomes in other autoimmune diseases [41-43]. Therefore we investigated whether the high or low anti-CarP IgG avidity associates with more severe joint destruction over time. However, when we compared the joint destruction and anti-CarP avidity, we observed no difference in severity at baseline or over time between the different anti-CarP avidity quartiles (Figure 5).

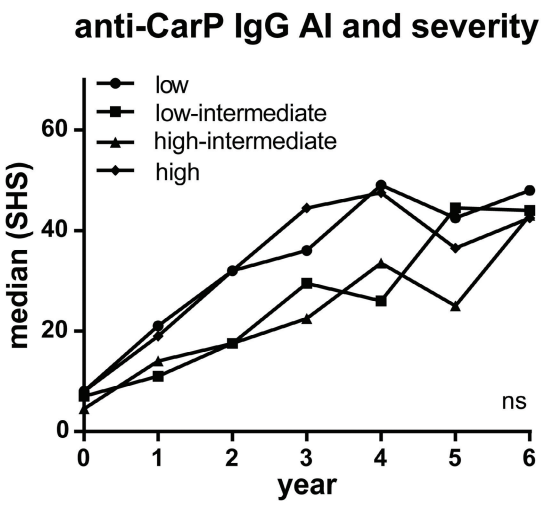

FIGURE 5. Anti-CarP avidity is not associated with more radiological damage. The extent and rate of joint destruction were analysed in all RA patients. RA patients were subdivided in anti-CarP IgG avidity quartiles with equal numbers of patients in each quartile. The severity of joint damage is depicted as median Sharp-van der Heijde score (SHS) on the y-axis and the follow-up years on the x-axis. RA; rheumatoid arthritis, anti-CarP antibody; anti-carbamylated protein antibody, AI; avidity index.

DISCUSSION

In this study we observed that the anti-CarP IgG avidity is low, in both serum and SF, compared to the avidity of the recall antibody against TT. Furthermore, the anti-CarP IgG avidity seems to be even lower than the ACPA IgG avidity and no anti-CarP avidity maturation has been observed. These data indicate that the regulation of the anti-CarP immune response is different from the regulation of recall responses and the ACPA response.

Although substantial information is available on the avidity (maturation) of antibody responses against recall antigens [24], less information is present on avidity (maturation) of autoantibody responses. It is known that autoantibodies have different avidities (high-, moderate- or low) in autoimmune diseases and different autoantibody avidities associates with a more or less severe disease course (reviewed in [24]). In a few studies, the autoantibody avidity is compared to the avidity of recall antibodies [25, 26] and these studies revealed a low avidity of autoantibodies compared to recall antibodies. We also found in this study a low avidity for anti-CarP antibodies compared to antibodies against TT. This suggests that autoantibodies are overall of low avidity compared to recall antibodies and within this avidity range the avidity of autoantibodies associates with various clinical outcomes. Importantly, a low avidity of autoantibodies does not indicate that these antibodies are not relevant, as we know that the presence of both ACPA and anti-CarP is associated with e.g. disease development [17] and with severity of the disease [6, 9, 10, 12, 18]. It does however imply that the mechanisms driving the production of protective vaccine antigens is very different from the mechanisms that lead to the production of autoantibodies.

Interestingly in this study is the lower anti-CarP IgG avidity in patients positive for anti-CarP antibody IgM. This cannot easily be explained by competing influences for binding of anti-CarP IgM with IgG, as in IgM depleted serum the anti-CarP IgG avidity is similar to total serum. This suggests limited competition for binding between anti-CarP IgM and IgG. In addition, in the context of ACPA avidity, we have previously compared whether the ACPA IgG avidity as measured in serum would be different from that of purified IgG. In ACPA IgG and IgM double positive patients we observed no differences in ACPA IgG avidity in total serum or purified IgG [26]. This indicates that the presence of IgM does not impact on the measured IgG avidity. As a possible explanation for the concomitant presence of anti-CarP IgM with

low avidity anti-CarP IgG we consider it conceivable that these patients display a less mature response, as anti-CarP IgG single positive or anti-CarP IgG and IgA double positive patients have overall higher anti-CarP IgG avidities (data not shown). Importantly, there is no difference in symptom duration between anti-CarP IgM positive and negative patients from the EAC. Indicating that anti-CarP IgG avidity is not related to time but rather to maturation of the anti-CarP response. Whether patients with a less mature response at baseline will undergo this maturation later during the disease progression is unknown.

Furthermore, we found that in baseline serum samples of ACPA and anti-CarP IgG double positive patients, the ACPA IgG avidity is higher than the anti-CarP IgG avidity. This might suggest that the ACPA response is differently regulated than the anti-CarP response.

Moreover, despite isotype switching before disease onset [19], no evidence for anti-CarP IgG avidity maturation was observed before symptom onset; however some patients might have a minimal avidity increase after symptom onset. All together, these data suggest that the isotype switch and avidity maturation in the anti-CarP B cell response are uncoupled. This uncoupling could be explained by various non-excluding mechanisms. For example, it might be due to a difference in additional stimulation of the B cells; such as the degree of innate or T cell help (reviewed in [44]) and/or the abundance of its antigen. It could be that low avidity antibodies are a marker for chronic antigen overload and chronic antibody responses. Another option could be that anti-CarP antibodies and ACPA cross react with another antigen, which is currently unknown, to which the antibodies might have a more “normal” response.

In this study we also investigated whether the anti-CarP antibody avidity associates with joint damage over time. The presence of anti-CarP antibodies, especially in the ACPA negative stratum, is associated with a more severe disease course [6, 19]. When investigating anti-CarP avidity, we did not observe an association with joint damage. This is different from our previous observations regarding ACPA where low avidity ACPA associates with more radiological damage at baseline and over time [30]. Furthermore, low avidity ACPA was a better complement activator compared to high avidity ACPA [30]. Therefor ACPA avidity might be of clinical relevance for

the determination of RA patients at risk for a more severe disease course. Moreover, low avidity antibodies might be better in tissue penetration, as shown for anti-tumour antibodies [31], and possibly resulting in immune activation at a deeper location. For ACPA we observed that especially the patients with very low avidity ACPA presented with severe radiological damage [30]. Also anti-CarP antibodies are associated with radiological damage [6] and since the avidity of the anti-CarP response is as low as the lowest avidities for ACPA it is conceivable that this explains why for anti-CarP we do not observe an association between the avidity and severity of joint destruction.

To conclude, the anti-CarP IgG avidity is low compared to the avidity against the recall antigen TT and points to a different regulation of anti-CarP antibody responses as compared to anti-TT responses. This is also indicated by the uncoupling of avidity maturation despite extensive isotype switching.

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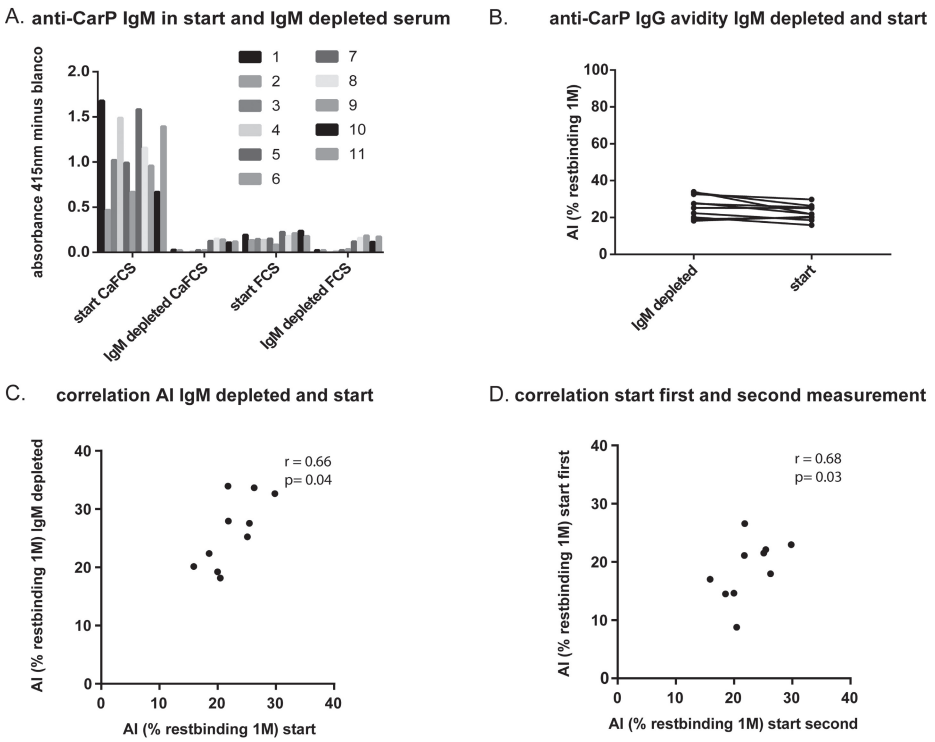
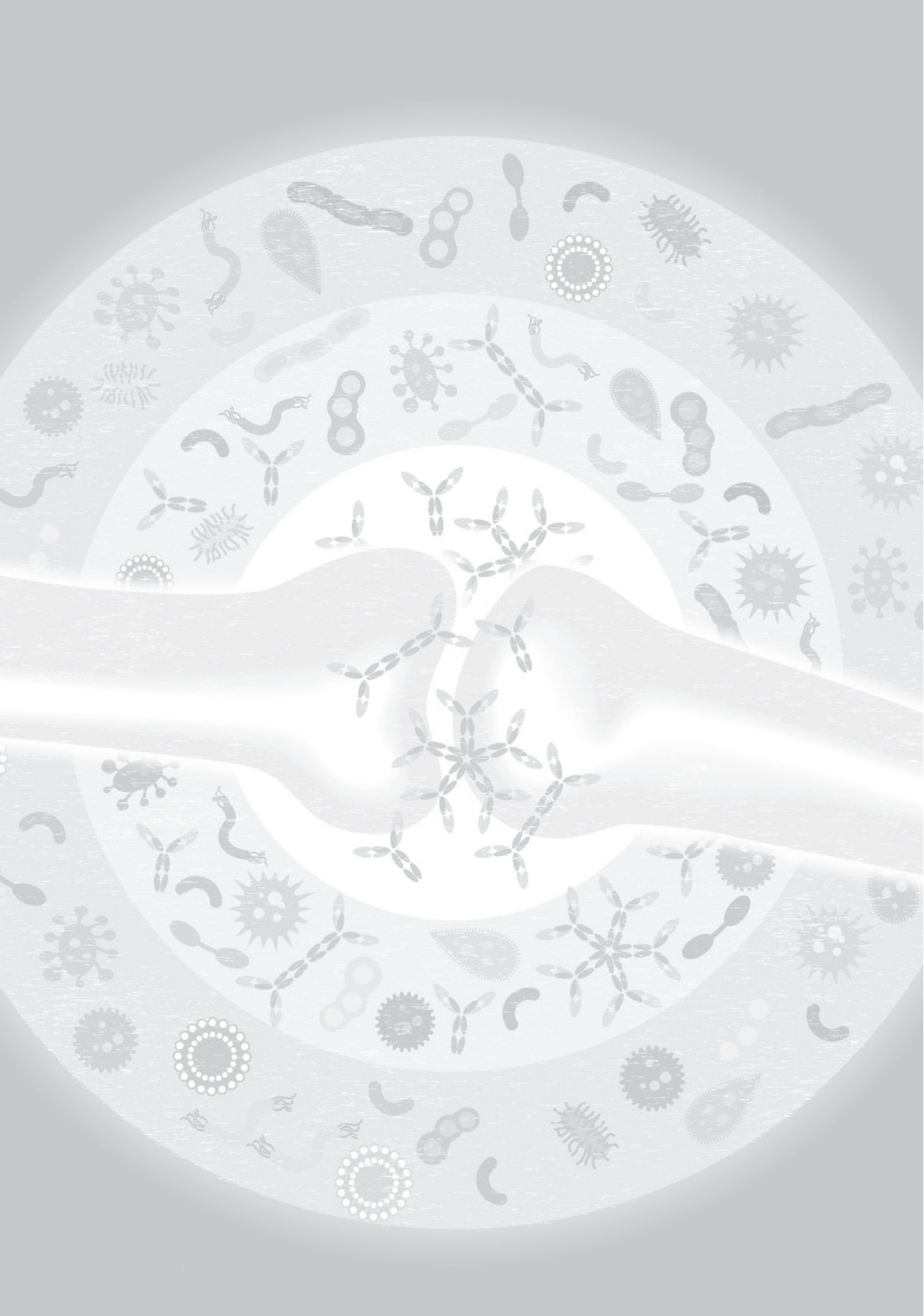
SUPPLEMENTARY MATERIAL

SUPPLEMENTARY FIGURE 1. Anti-CarP IgG avidity in start and IgM depleted serum. To investigate whether the presence of anti-CarP IgM is influencing the anti-CarP IgG avidity measurement, sera of RA patients (n=11) was IgM depleted (A). The anti-CarP IgG avidity was similar in IgM depleted and start serum (B) and correlate well (C). The anti-CarP IgG avidity in this second measurement correlates with the avidity measured in the big cohort (first measurement) (D). The avidity index (AI) is depicted as % restbinding at 1M NaSCN. Anti-CarP antibody; anti-carbamylated protein antibody, RA; rheumatoid arthritis, AI; avidity index. Spearman correlation (C,D).





CHAPTER 4

ACPA and anti-CarP-antibody responses are differentially regulated as indicated by differences in size and glycosylation

Myrthe A.M. van Delft
Marije K. Verheul
Nivine E.W.N. Levarht
Theresa Kissel
Lise Hafkenscheid
Albert Bondt
René E.M. Toes
Leendert A. Trouw

Manuscript in preparation

Immunoglobulins are glycoproteins that contain *N*-linked glycans. IgGs contain a conserved *N*-linked glycosylation site located in the Fc region [1] which is crucial for Fc-Receptor, lectin and complement mediated effector functions [2-5]. Next to Fc-linked *N*-glycans, IgG molecules can also contain *N*-linked glycans in the variable domain (V-domain) [6]. Also, these glycans have been postulated to influence immune functions, but their role is less defined [7-9]. Recently, we discovered that IgG anti-citrullinated protein antibodies (ACPA) from rheumatoid arthritis (RA) patients are highly glycosylated in the V-domain as they harbour bi-antennary, highly-sialylated N-linked glycans [10, 11]. To undergo *N*-linked glycosylation, proteins need to express the *N*-linked glycosylation consensus sequence (N-X-S/T, where X ≠P) that is most often not germline encoded in the V-domain but introduced by somatic hypermutation [10]. Indeed, sequencing ACPA V-domains revealed that ACPA contain abundant glycosylation-sites in the V—domain that had been introduced by somatic hypermutation [12]. Anti-Carbamylated Protein (anti-CarP) antibodies occur frequently in RA patients and recognize a homologous structure as ACPA (e.g. homocitrulline versus citrulline) [13]. Nonetheless, despite cross-reactivity in some but not in other patients, they seem to represent a separate class of autoantibodies in RA, as they are also detected in ACPA-negative disease [13-15].

At present, it is unknown whether anti-CarP antibodies also display enhanced V-domain glycosylation or whether this feature is mainly confined to ACPA. Therefore, we investigated the presence of glycans on anti-CarP antibodies and investigated whether they have an increased content of sialylated glycans compared to control-IgG. First size exclusion chromatography was performed with sera from 11 RA patients, either single positive for anti-CarP antibodies or double positive for both anti-CarP and ACPA [10]. Next, total- and antigen-specific IgG ELISAs were performed on the fractions [10, 14]. We observed that anti-CarP antibodies of ACPA-negative patients nearly always migrated in the same pattern as total IgG (Fig 1a). The anti-CarP IgG reactivity in ACPA-positive patients was occasionally observed to migrate at a higher molecular size as compared to total IgG (Fig1b), while ACPA consistently show the size shift as previously reported [10]. These data indicate that anti-CarP antibodies can display an increased size, although less often compared to ACPA. Similar results were observed when testing for anti-CarP IgG1 in anti-CarP IgG single and anti-CarP and ACPA IgG double positive patients, excluding that a difference in subclass usage may be

responsible for differences in size (suppl. Fig 1a, b). These data could suggest that anti-CarP antibodies are less V-domain glycosylated.

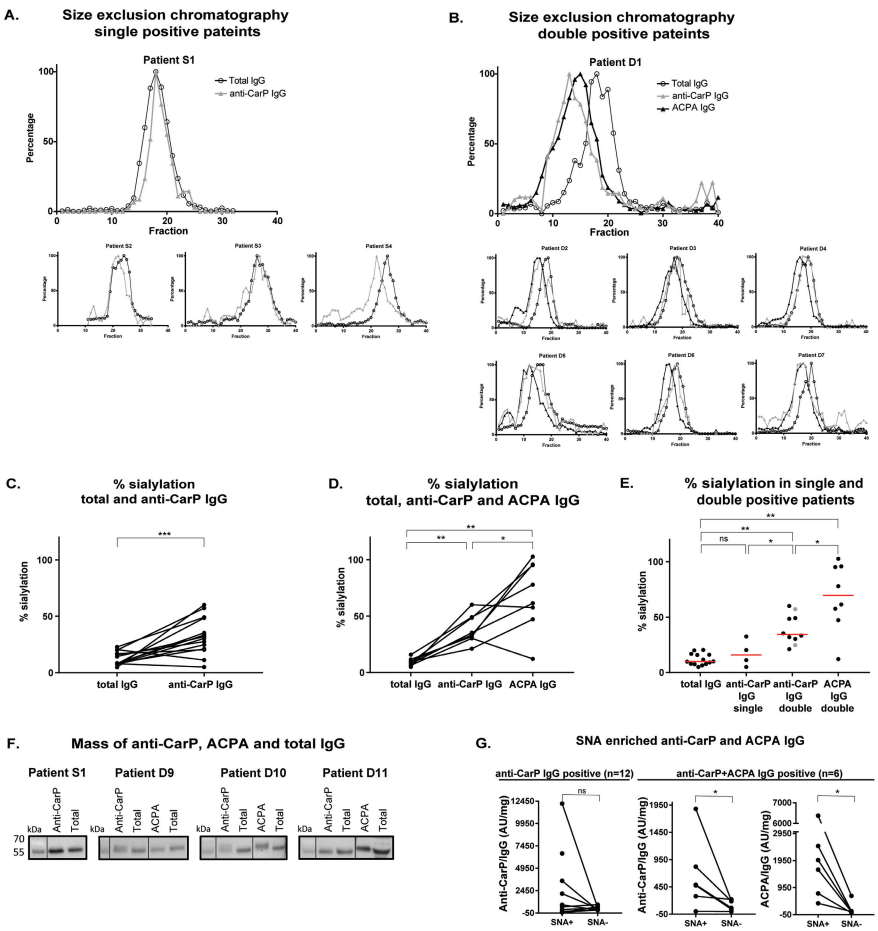


FIGURE 1. Size and glycosylation analysis of anti-CarP IgG antibodies.
Presence of anti-CarP (grey triangle), ACPA (black triangle) and total IgG (white dots) antibody reactivity in size exclusion chromatography fractions of anti-CarP IgG single positive RA patients (A) and anti-CarP and ACPA IgG double positive RA patients (B). Lower fraction number indicates larger size. HPLC analysis for the percentage sialylated glycans of isolated anti-CarP IgG antibodies (median=31.2%) and total IgG (median=8.4%) of single and double positive RA patients (n=14) (C). HPLC analysis for the percentage sialylated glycans of paired total IgG (median=9.5%), isolated anti-CarP IgG (median=34.4%) and isolated ACPA IgG (median=69.7%) of double positive RA patients (n=8) (D). Total overview of the HPLC analysis for the percentage sialylated glycans of total IgG (median=10%), anti-CarP IgG in single (median=15.8%) and double (median=34.4%)

positive RA patients and ACPA IgG in double (median=69.7%) positive RA patients (E). Western blot for anti-CarP, ACPA and total Ig isolated from an anti-CarP single positive RA patient (patient S1, representative of n=2) and anti-CarP and ACPA double positive RA patients (patient D9-11, representatives of n=8 showing an anti-CarP shift only in n=1, anti-CarP and ACPA shift in n=4, ACPA shift only in n=3), stained for total IgG (F). Ratio of anti-CarP IgG (AU) or ACPA IgG (AU) reactivity per total IgG (mg) in the SNA-positive and SNA-negative fraction after SNA affinity chromatography in anti-CarP single (n=12) and anti-CarP and ACPA double (n=6) positive RA patients (G). Anti-CarP; Anti-Carbamylated Protein, ACPA; Anti-Citrullinated Protein Antibody, HPLC; high performance liquid chromatography, RA; Rheumatoid Arthritis, Ig; immunoglobulin, SNA; *Sambucus Nigra* Lectin. Wilcoxon signed rank tests (paired samples) or Mann Whitney U (single vs double positives) test, *p=0.05-0.01, **p=0.01-0.001.

Next, anti-CarP antibodies were isolated from sera of 14 RA patients (4 single and 10 double positives) using affinity chromatography [10]. From 8 of these double positive patients ACPAs were isolated as well [10]. To further verify the presence of the IgG size difference of isolated anti-CarP antibodies and ACPA, western blot analysis of purified antibodies was performed. An increase in size was absent for anti-CarP IgG in single positive patients, however it was occasionally present in double positive patients (fig 1f). This increase in size was observed for ACPA IgG in all positive patients, except for one patient (fig 1f). The same results were obtained when investigating anti-CarP and ACPA IgG1 (suppl. Fig 1c).

Using high performance liquid chromatography (HPLC) the presence of glycans was investigated as described before [11, 16]. Overall, a slight increase in sialylated glycans was observed for anti-CarP IgG (fig 1c,d,e). For anti-CarP single positive samples, no increase in sialylated glycans was observed and only a minor increase in the percentage of sialylated glycans was detected for anti-CarP IgG in double positive RA patients (fig 1e). When investigating the amount of sialylated glycans present for anti-CarP and ACPA of double positive RA patients, the percentage glycosylation of anti-CarP IgG was considerably lower compared to what was found for ACPA IgG (fig 1d, e). These data points towards a lower percentage of V-domain glycosylation of anti-CarP antibodies compared to ACPA, because IgG glycans of the V-domain are typically highly sialylated [6, 11].

Yet we do not exactly know the purity of the isolated anti-CarP IgG antibodies. Therefore, to further confirm these results, antibodies of 12 anti-CarP single

positive and 6 anti-CarP and ACPA double positive RA patients were isolated using a *Sambucus nigra* Agglutinin (SNA) affinity chromatography column that specifically binds to sialic acid residues (of the IgG V-domain region). Subsequently the presence of anti-CarP IgG antibodies in the SNA-positive and negative fractions was determined. In most, 8 out of 12, of the anti-CarP single positive patients no enrichment for anti-CarP in the SNA positive fraction was observed (fig 1g). An increase in ACPA reactivity was detected in the SNA-positive fraction of all double positive RA patients, while an increase in anti-CarP reactivity was not detected in the SNA-positive fraction of all RA patients (fig 1g). This suggests that anti-CarP antibodies probably do not carry sialylated glycans in the V-domain of these patients.

Overall, we conclude from these data that anti-CarP antibodies could carry sialylated glycans in the V-domain, although they carry these glycans much less frequently or to a much lesser extent as compared to ACPA. The observed higher occurrence of anti-CarP harbouring V-domain glycans in double positive RA patients may potentially be due to some cross-reactivity between anti-CarP antibodies and ACPAs. These data point towards a different regulation of these autoantibody responses.

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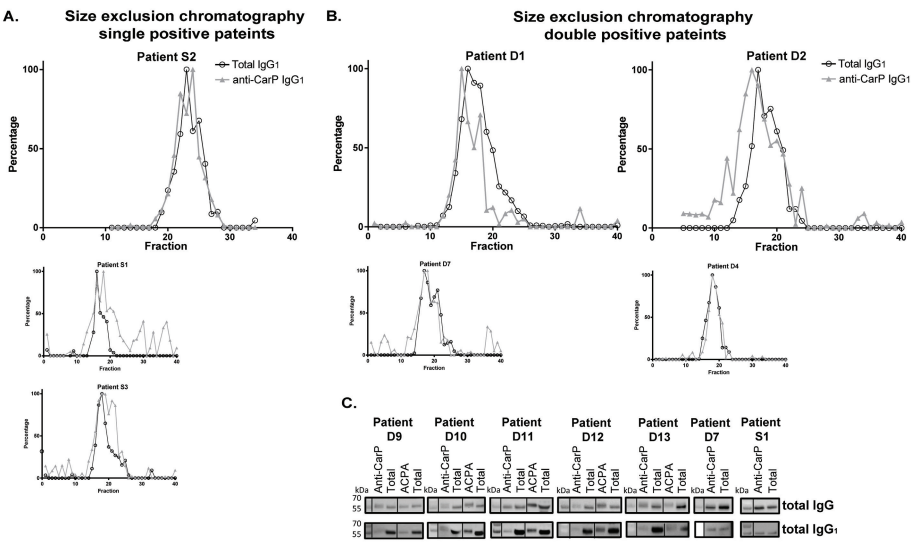
SUPPLEMENTARY MATERIAL

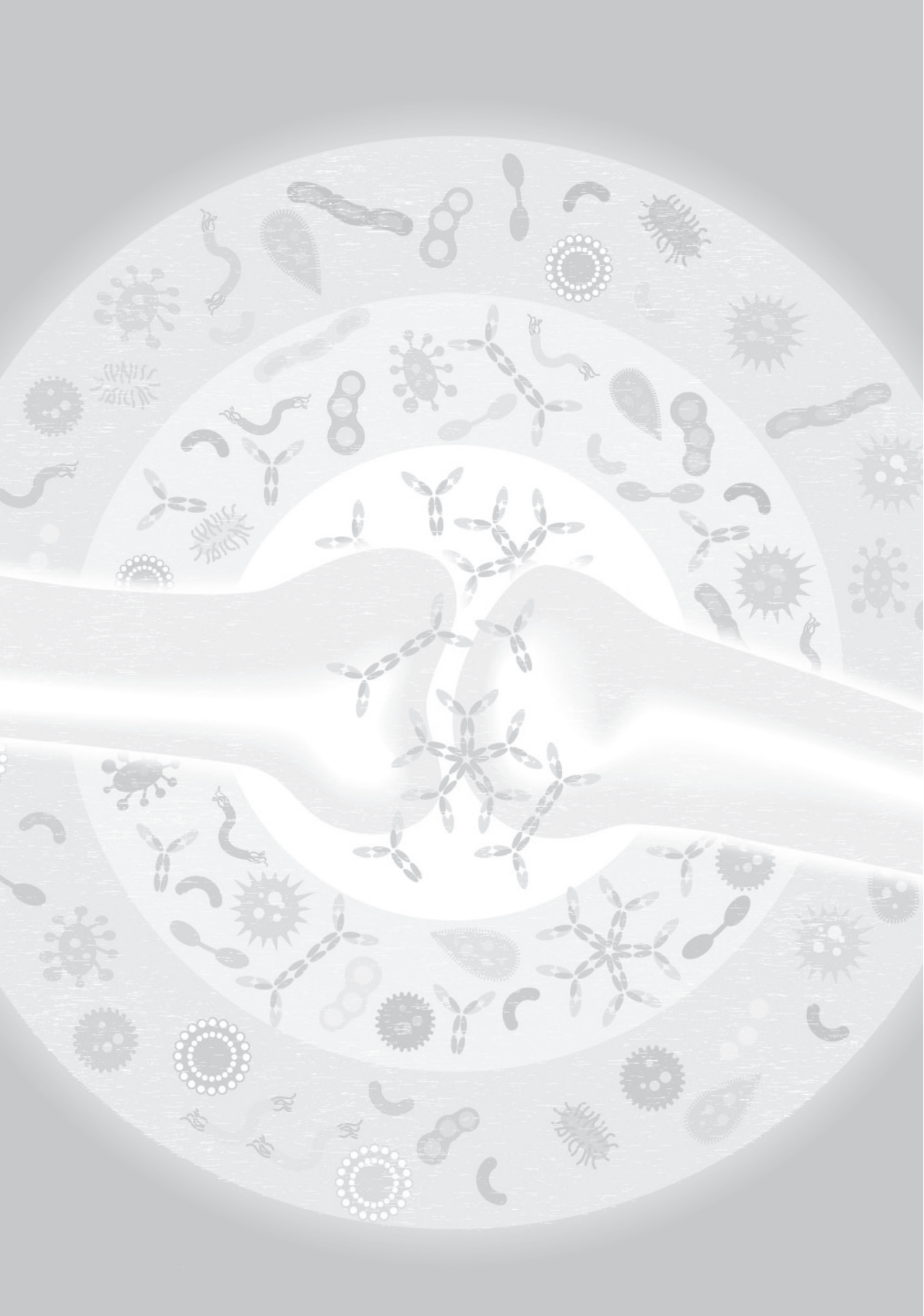
Supplementary figure 1. Size analysis of anti-CarP IgG_1 antibodies.

Presence of anti-CarP (grey triangle) and total (white dots) IgG_1 antibody reactivity in size exclusion chromatography fractions of anti-CarP IgG single positive RA patients (A) and anti-CarP and ACPA IgG double positive RA patients (B). Western blot for anti-CarP, ACPA and total Ig isolated from an anti-CarP single positive RA patient (patient S1) and anti-CarP and ACPA double positive RA patients (patient D9-13), stained for total IgG and IgG_1 (C).



PART 2

Possibilities for a mucosal origin of autoantibodies in rheumatoid arthritis



CHAPTER 5

Differential composition of ACPA and anti-CarP IgA point to differential regulation and origins

Myrthe A.M. van Delft
Nivine E.W.N. Levarht
Diane van der Woude
René E.M. Toes
Leendert A. Trouw

Manuscript in preparation

ABSTRACT

Background: The presence of autoantibodies targeting post-translationally modified proteins, such as citrullinated (ACPA) and carbamylated (anti-CarP antibodies) proteins, are a hallmark of RA. As these autoantibody responses target homologous structures (citrulline and homocitrulline), ACPA- and anti-CarP- IgG responses are partially cross-reactive in some- but not in other patients. Both responses use a broad spectrum of isotypes, including IgM, IgG and IgA. To better understand the nature of these prominent autoantibodies, we investigated the molecular composition of both ACPA and anti-CarP antibody IgA. Moreover, the degree of cross-reactivity of these IgA autoantibodies was investigated.

Methods: Sera of 13 RA-patients, positive for anti-CarP/ACPA/rheumatoid factor (RF) IgA, were fractionated using size exclusion chromatography and tested by ELISA for anti-CarP, ACPA, RF-IgA as well as total IgM, IgA and IgG. As control anti-*E.coli* and anti-Tetanus Toxoid (TT) IgA were analysed. Inhibition studies were performed to investigate the cross-reactivity between anti-CarP and ACPA.

Results: Our results show that anti-CarP antibody, RF and anti-*E.coli* IgA are predominantly present as polymeric-IgA, whereas ACPA and anti-TT IgA are mostly present as monomeric-IgA. These data are intriguing as they indicate that the anti-CarP and ACPA IgA responses are differently regulated and potentially of different origin. Approximately 70% of the anti-CarP IgA reactivity cannot be inhibited by citrullinated antigens and vice versa for ACPA IgA by carbamylated antigens, indicating that these autoantibodies are directed against different antigens.

Conclusion: Our data indicate that the anti-CarP- and ACPA-autoantibody responses are differently regulated and may have a different origin, with possibly a (more pronounced) mucosal associated origin for anti-CarP antibodies.

BACKGROUND

Rheumatoid arthritis (RA) is a chronic autoimmune disease mainly affecting synovial joints [1, 2]. Several autoantibodies have been identified in serum and synovial fluid (SF) of RA-patients [3]. These may form immune complexes in the joints, leading to the attraction of immune cells through e.g. complement activation [4, 5] which can contribute to chronic inflammation and bone destruction.

Well known autoantibodies in RA are rheumatoid factor (RF), anti-citrullinated protein antibodies (ACPA) and anti-carbamylated protein (anti-CarP) antibodies [3]. RF recognize the Fc-tail of immunoglobulin (Ig) G whereas the latter two target post-translationally modified (PTM) proteins. Citrullinated proteins are PTM proteins wherein arginines are converted into citrullines by an enzymatic reaction with peptidyl arginine deiminase (PAD) [6, 7]. Carbamylated proteins are PTM proteins wherein lysines are converted to homocitrullines by a chemical reaction with cyanate [6, 7]. Citrulline and homocitrulline are homologous structures that differ only in one CH_2-group. Although citrulline and homocitrulline (the result of carbamylation) are structurally highly related and although cross-reactive responses to citrulline and homocitrulline have been noted at the poly- and monoclonal antibody-level, ACPA- and anti-CarP-antibodies are thought to represent two different antibody families as ACPA and anti-CarP single positive patients are present as well [3, 8-10]. Both autoantibody responses use a broad spectrum of isotypes, including IgM, IgG and IgA [10-12].

IgA is different from other Ig, as it comprises monomeric and dimeric (polymeric) forms. Monomeric IgA is mainly produced by bone marrow plasma cells [13-17]. At mucosal surfaces, IgA is the most prominent antibody present and is produced as a dimeric molecule, dimeric IgA (dIgA) [15-18], containing a joining-chain (J-chain) [19]. dIgA can be transported through epithelial cells by the polymeric Ig receptor (pIgR) and secreted at the luminal site [19]. Part of the pIgR remains attached to the antibody known as the secretory component (SC) providing secretory-IgA. SC protects and stabilizes the IgA in the “hostile” environment of e.g. the gut. Nevertheless, secretory (auto-)antibodies can be detected in serum, but the precise mechanism how this occurs remains unknown [20-22].

Previously, it has been shown that RF-IgA is predominantly present in its polymeric form in all tested fluids; serum, synovial fluid and saliva of RA-patients [23]. To better understand the nature of the anti-PTM autoantibodies ACPA and anti-CarP, we investigated the composition of both IgA ACPA and anti-CarP antibody responses. Moreover, the degree of cross-reactivity to citrulline and homocitrulline for anti-CarP- and ACPA-IgA was investigated.

PATIENTS AND METHODS

Patients and control sera

Sera of 13 RA-patients were collected at the outpatient clinic of the rheumatology department at the Leiden University Medical Center (LUMC). Patients were analysed for the presence of anti-CarP, ACPA and RF IgA. Next to these, 3 healthy control sera were analysed as well. This to compare the total IgM, IgA and IgG spectra of RA-patients and HC. Informed consent was obtained for all individuals and all protocols were approved by the ethic committee of the LUMC.

Size exclusion chromatography and ELISAs

Size exclusion chromatography was performed using the ÄKTA equipped with a Hiload Superdex 200 (GE Healthcare). Fractions were analysed by ELISA to detect total IgM, total IgA and total IgG (Bethyl laboratories) according to manufacturer's protocol. Next to this, anti-CarP IgA, anti-CCP2 (ACPA) IgA and RF-IgA was measured in the fractions. The anti-CarP and ACPA ELISAs were performed using in house ELISAs as described previously [10]. RF-IgA was measured using an in-house ELISA similar to the one described for RF-IgM [24], but using horseradish peroxidase (HRP) conjugated goat-anti-human (GAH)-IgA (Novex A18781) as detection antibody.

Moreover, as control, anti-Cit-FCS (ACPA) IgA, anti-tetanus toxoid (TT) IgA and anti-*E.coli* IgA were measured. Briefly, anti-Cit-FCS IgA was measured using the same protocol as for anti-CarP IgA but with a coating of 10ug/ml (Cit-)FCS. For anti-TT IgA 1.5Lf/ml TT (merck millipore 582231) was coated and afterwards blocked with 1% BSA in coating buffer. Bound human IgA was detected using HRP-conjugated GAH-IgA (Novex A18781). Anti-*E.coli* IgA was measured as described previously and using HRP-conjugated GAH-IgA (Novex A18781) [25]. For all ELISAs, after the final washings, ABTS (2,2'-azino-

bis 3-ethylbenzothiazoline-6-sulphonic acid) was added to visualize the HRP enzyme activity and the absorbance was measured at 415nm.

The fraction with the highest absorbance was set as 100 percent for each ELISA separately. Based on this absorbance percentage, the percentage of specific antibody reactivity for all measured fractions was calculated. The fractions were divided, based on the fraction in the middle of the two total IgA peaks, in dimeric (polymeric) vs monomeric IgA fractions. To calculate the percentage dimeric (polymeric) IgA (total and antigen specific), the area under the curve was used for all IgA and polymeric IgA.

Inhibition studies of anti-CarP and ACPA IgA

Inhibition studies for Ca-FCS, Cit-FCS and native FCS were performed to investigate the cross-reactivity of anti-CarP and ACPA IgA. Briefly, sera or fractions were preincubated for 1hour (h) at room temperature with 100µg/ml Ca-FCS, Cit-FCS or their non-modified versions before adding serum samples to the anti-CarP (based on Ca-FCS) and ACPA (based on Cit-FCS) ELISA plates. The ELISAs were performed as described before. The AU/ml were analysed and the percentage inhibition by Ca-FCS or Cit-FCS to non-modified FCS was calculated.

Western blot for IgA and J-chain

To investigate the presence and type of IgA, 20-40 ng IgA was loaded to 4-15% mini protean TGX precast protein gels (Biorad). For studies aiming to visualize the J-chain, between 2-4µg IgA was loaded. Proteins were blotted on mini 0.2µm PVDF Trans-blot Turbo (Biorad) and washed with milliQ (mQ). Afterwards blots were blocked with PBS/0.05% tween (Sigma)/3% skim milk powder (Sigma), at 4 degrees overnight (O/N) for IgA and 2h at RT for J-chain. The IgA blots were stained for 1h at RT with 10,000x diluted rabbit-anti-human-IgA (DAKO A0262) in PBS/0.05% tween/3% skim milk powder and afterwards for 1h at RT with 5,000x diluted HRP conjugated goat-anti-rabbit-Ig (DAKO P0448) in PBS/0.05% tween/3% skim milk powder. J-chain blots were stained O/N at 4 degrees with 4,000x diluted goat-anti-human-J-chain (Nordic MUbio batch 3208) in PBS/0.05% tween/3% skim milk powder and afterwards for 1h at RT with 5,000x diluted HRP conjugated rabbit-anti-goat-Ig (DAKO P0449) in PBS/0.05% tween/3% skim milk powder. Blots were developed using ECL (GE healthcare).

Statistical analysis

Statistical analysis was performed using GraphPad Prism 7.02. Wilcoxon signed ranks test were performed to investigate differences in percentage polymeric IgA. P values below 0.05 were considered statistically significant.

RESULTS

Anti-CarP IgA is predominantly present in the high molecular weight fractions

To investigate the size-distribution of antibodies, size exclusion chromatography was performed of sera obtained from RA-patients and healthy control subjects. Fractions were subsequently analysed by ELISAs and WB. The pattern of chromatography profiles for total IgA, M and G are similar for HC and RA-patients, with total IgM reactivity detectable in the early, high molecular weight (HMW), fractions (figure 1a, b). For total IgA two peaks are detectable, a smaller peak in the early, HMW, fractions and a larger second peak in the later, lower molecular weight (LMW), fraction range. The first peak represents polymeric (dimeric) IgA and the latter peak represents monomeric IgA [23, 26, 27]. Total IgG is the smallest Ig analysed and reactivity is present in the late, lowest molecular weight, fractions.

To confirm that polymeric IgA is present in the HMW- peak, we separated some fractions on SDS page gels followed by western blot and stained for J-chain and total IgA. HMW (polymeric) IgA including J-chain is only detectable in the fractions of the HMW- IgA peak whereas LMW (monomeric) IgA is predominantly detected in the fractions of the LMW IgA fractions (figure 1e). These results confirm the presence of J-chain positive polymeric IgA in HMW fractions. Next, we determined the presence of anti-CarP- and ACPA IgA in the size-exclusion fractions obtained. Unexpectedly, anti-CarP IgA reactivity is detected in the HMW fractions whereas ACPA IgA reactivity was predominantly found in the LMW fractions. These results indicate that anti-CarP IgA is mainly present in the polymeric IgA form, in contrast to ACPA IgA (figure 1c). As mainly polymeric IgA rather than monomeric IgA has been associated with mucosal immune responses [14, 15, 18], we next analyzed the size-distribution of IgA derived from immune responses typically associated with mucosa. For this reason, we determined the profile of anti-*E.coli* IgA in the chromatogram-fractions. As expected, anti-*E.coli* IgA is predominantly detected in the HMW fractions containing polymeric IgA (figure 1d). Likewise, investigating anti-TT IgA, a response most likely derived

from intramuscular vaccination, revealed that anti-TT IgA is mostly present in the LMW fractions containing monomeric IgA (figure 1d).

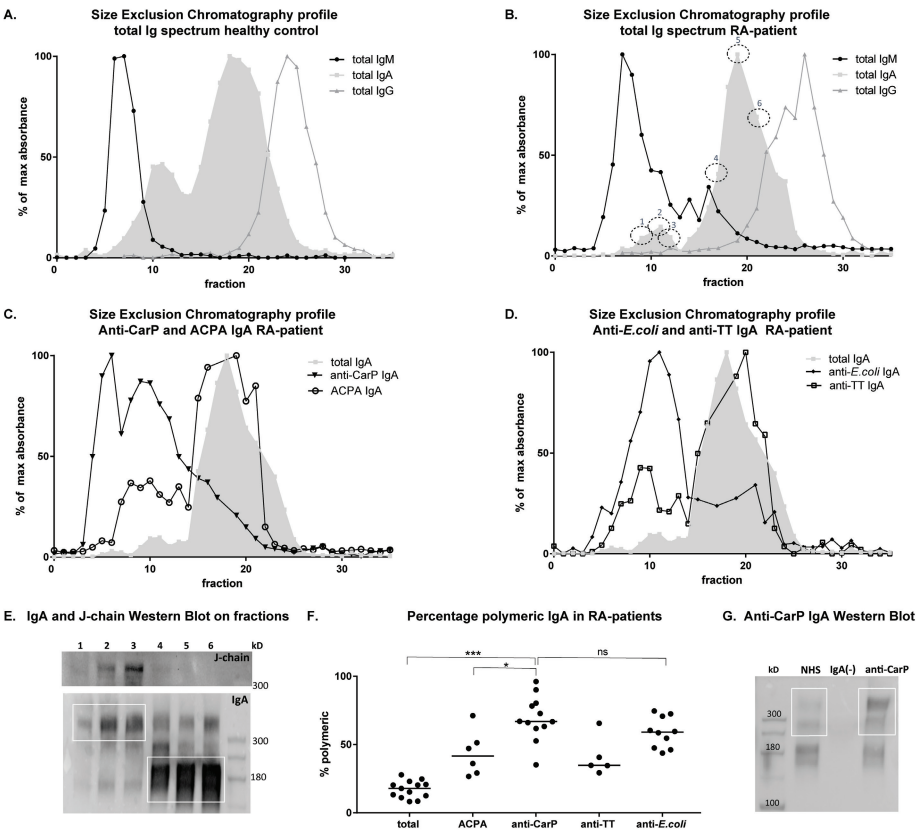


FIGURE 1. Anti-CarP IgA is present in a more polymeric form compared to ACPA and total IgA. Total IgM, total IgA and total IgG were measured in size exclusion chromatography fractions of 2 HC and 13 RA-patients. One representative figure is shown for a HC (a) and an RA-patient (b). Anti-CarP IgA, ACPA IgA, anti-*E.coli* IgA and anti-TT IgA were measured in the same size exclusion chromatography fractions as total IgA for 13 RA-patients. One representative figure is shown for the autoantibodies anti-CarP and ACPA IgA (c) and for the control antibodies anti-*E.coli* and anti-TT IgA (d). Western blot analysis for J-chain and total IgA was performed in three polymeric and three monomeric size exclusion chromatography fractions of total IgA (b,e). For all RA patients (if positive for that antibody) the percentage polymeric total IgA, anti-CarP IgA, ACPA IgA, anti-*E.coli* IgA and anti-TT IgA was calculated. The total overview is shown (f). Isolated anti-CarP showed also an increased IgA mass on western blot (g). Ig; immunoglobulin, HC; healthy controls, RA; rheumatoid arthritis, anti-CarP; anti-carbamylated protein, ACPA; anti-citrullinated protein antibody, anti-TT; anti-tetanus toxoid. Wilcoxon signed rank tests ns; p>0.05, *; p=0.05-0.01, **; p=0.01-0.001, ***; p<0.001

Subsequently, we separated sera from an additional 12 patients by size exclusion chromatography to investigate anti-CarP, ACPA, anti-*E.coli* and anti-TT IgAs. The percentage IgA present in the HMW-fractions was calculated. An increase in the percentage of polymeric anti-CarP IgA compared to total and ACPA IgA was observed (figure 1f). A similar percentage as seen for anti-CarP IgA was detected for anti-*E.coli* IgA (figure 1f). When investigating ACPA IgA using cit-FCS instead of CCP2 as read-out, a similar ACPA IgA profile was obtained (data not shown).

Together these data indicate that anti-CarP IgA is different from ACPA IgA and similar to mucosal type of immune responses, like anti-*E.coli*. To confirm the high molecular weight of anti-CarP IgA, purified anti-CarP antibodies were loaded on western blot and IgA was detected (figure 1g). The results obtained show an increase in the amount of HMW anti-CarP IgA compared to normal total IgA as well.

Anti-CarP and ACPA IgA are partially cross-reactive, indicating that these autoantibodies are directed against different antigens

Because of the differences in the anti-CarP and ACPA IgA spectra, we wished to address the question to which extent anti-CarP and ACPA IgA are cross-reactive. Previously, we have shown for IgG ACPA and anti-CarP antibody responses that they are partially cross-reactive [8, 28]. Therefore, inhibition studies were performed. Samples were preincubated with Ca-FCS, Cit-FCS or their native counterparts followed by anti-CarP and ACPA IgA ELISAs. Around 70% of the anti-CarP antibody IgA response cannot be inhibited by Cit-FCS and vice versa for ACPA (figure 2a,b). For IgG, similar results as previously published were observed. Around 70% of the anti-CarP IgG response cannot be inhibited by Cit-FCS and almost 95% of the ACPA IgG response is not inhibited by Ca-FCS (figure 2c,d).

Overall, these data indicate that anti-CarP and ACPA IgA are only partially cross-reactive, indicating that both responses recognize different antigens and are differently regulated.

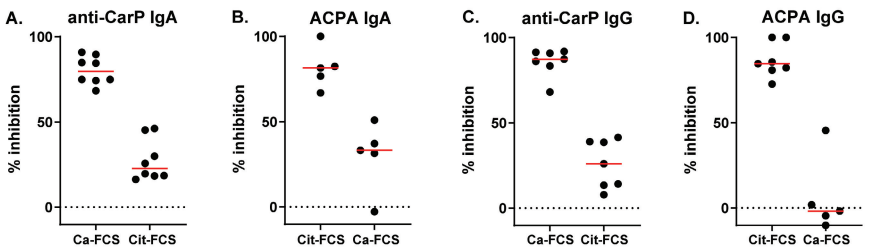


FIGURE 2. Anti-CarP IgA antibodies can only be partially inhibited by citrullinated antigens.
Inhibition studies using Ca-FCS and Cit-FCS were performed to investigate whether anti-CarP and ACPA IgA and IgG were cross-reactive or recognized different antigens. About 70% of the anti-CarP IgA was not inhibited by Cit-FCS (a) and vice versa for ACPA IgA (b). When testing IgG, about 70% of anti-CarP IgG was not inhibited by Cit-FCS (c), whereas almost 95% of ACPA IgG was not inhibited by Ca-FCS (d). Ca-FCS; carbamylated FCS, Cit-FCS; citrullinated FCS, anti-CarP; anti-carbamylated protein, ACPA; anti-citrullinated protein antibody, Ig; immunoglobulin

DISCUSSION

In this study, we analysed the size-distribution and cross-reactivity of anti-CarP and ACPA IgA in RA-patients. Interestingly, the results indicate that anti-CarP IgA is present in a more polymeric (HMW) form, whereas ACPA IgA is predominantly present in a monomeric (LMW) form. This size shift for anti-CarP IgA cannot, most likely, be explained by the presence of secretory component to anti-CarP IgA, as secretory anti-CarP and ACPA are both predominantly detected in the IgM isotype [20]. Therefore, these data suggest a different regulation for the anti-CarP antibody and ACPA response in RA-patients. Moreover, anti-CarP antibodies and ACPAs seem to be directed against different antigens as their IgA and IgG antibodies are only partially cross-reactive [8, 28].

Overall these data might indicate that these autoantibody responses are of different origin, with probably a mucosal associated origin for anti-CarP antibodies, as polymeric IgA is typically the most prominent antibody produced at mucosal surfaces [14, 15, 19]. To confirm that HMW IgA is polymeric IgA, it is important to detect the J-chain, as typically polymeric IgA includes a J-chain[15]. At present, we could not confirm whether HWM anti-CarP IgA antibodies also contained the J-chain as setting up an anti-

CarP J-chain ELISA turned out to be technically challenging. Both IgM and IgA include the J-chain and therefore anti-CarP IgM and IgA will be detected. Likewise, we were able to detect anti-CarP IgA including the J-chain by WB (and mass-spec), the technical limitations of several purification and elution rounds made the analysis of J-chain on such purified fractions unreliable. The best way to study whether anti-CarP IgA is produced including the J-chain seem to be the examination of anti-CarP B cells. After single cell isolation of anti-CarP (IgA) B cells, the presence of J-chain on mRNA and protein level can be studied. Moreover, it is interesting to investigate whether polymeric IgA including J-chain can be secreted by these isolated B cells.

The observation that anti-CarP IgA is predominantly present as HMW is in line with the hypothesis that the anti-CarP IgA response is of mucosal origin. Therefore, it is interesting to examine whether the microbiome can be recognized by isolated polyclonal anti-CarP and/or ACPA IgA. Moreover, it is interesting to know whether carbamylated or citrullinated proteins are detectable in mucosal tissue; like in faeces, lung and periodontal tissue or fluid. Some carbamylation and citrullination has been detected in lung tissue of (ex-)smokers [29], however most of the experiments were performed using the 'Senshu' method, which cannot discriminate between carbamylation and citrullination [28, 30]. A recent paper showed the presence of carbamylated and citrullinated proteins in mild and moderate periodontitis tissue [31]. Yet, a non-specific anti-citrullinated protein antibody (anti-peptidyl-citrulline, Millipore clone F95) was used, which recognizes carbamylated proteins as well [30]. Mass-spec analysis will be important to study the presence of carbamylated or citrullinated proteins in (mucosal) tissue [30].

Another possibility is that the anti-CarP IgA response is a reflection of an active immune response. Eijgenraam et al. showed that after an intranasally (mucosal) and subcutaneously (nonmucosal) immunization, predominantly an antigen specific HMW, polymeric, IgA response was detectable in serum [26]. The *E.Coli* IgA response is detectable as HMW IgA in serum as well. As *E.Coli* is (in most individuals) permanently present in the microbiome of the intestine, the immune reaction to *E.Coli* can be seen as an active response inducing polymeric IgA. Moreover, RF IgA is detected as HMW IgA in RA patients [23], whereas in a few HC it was detected as LMW IgA (data not shown), probably also due to an active immune reaction in RA patients.

Overall, we show that the molecular weight of anti-CarP and ACPA IgA is different in RA-patients, with a more polymeric appearance for anti-CarP IgA. This suggests a different regulation for the anti-CarP antibody and ACPA response. Together our results indicate that these autoantibody responses are of different origin, with probably a mucosal associated origin of anti-CarP antibodies.

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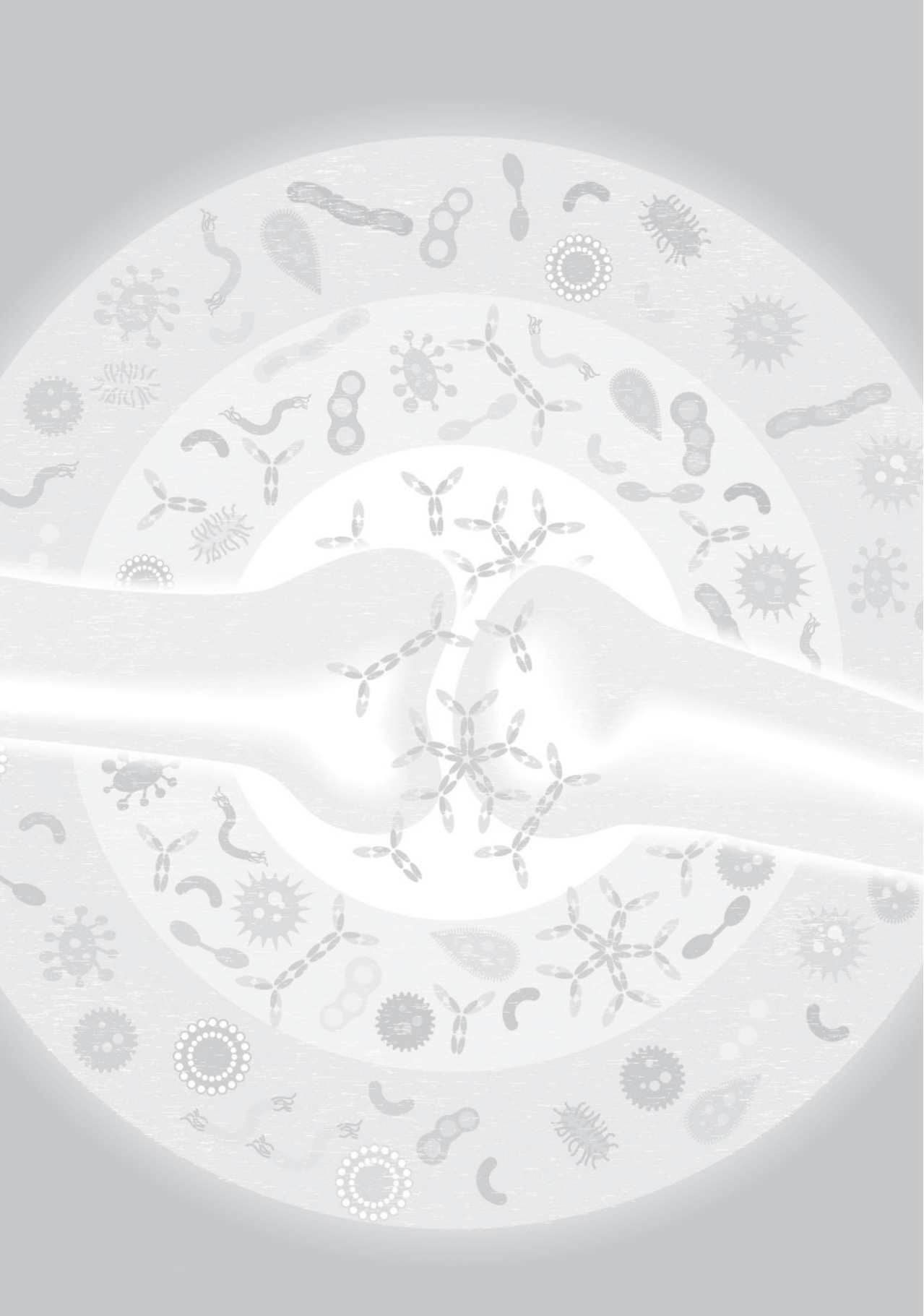

CHAPTER 6

The secretory form of rheumatoid arthritis associated auto-antibodies in serum are mainly of the IgM isotype, suggesting a continuous reactivation of auto-antibody responses at mucosal surfaces

Myrthe A.M. van Delft
Diane van der Woude
René E.M. Toes
Leendert A. Trouw

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Several lines of evidence obtained in recent years indicate a role of mucosal surfaces in the development of auto-immune responses associated with rheumatoid arthritis (RA). Therefore, more attention is going to the influence of the microbiome in RA development. Secretory-antibodies are typically produced at mucosal surfaces and transported through epithelial cells for secretion at the luminal site. Importantly, secretory-antibodies comprise both immunoglobulin-M (IgM) and immunoglobulin-A (IgA) and both isotypes can harbour a J-chain that binds to the polymeric Ig receptor (pIgR) [1]. Following transport through epithelial cells, the antibodies are enzymatically released at the luminal site by cleavage of the pIgR leaving a fragment of the receptor, the secretory-component (SC) bound to the immunoglobulin. SC-containing antibodies can however also be detected systemically in the circulation, although the mechanism by which these SC-containing antibodies arise in serum is still unclear [2]. Interestingly, antigen-specific secretory-antibodies are present in serum after mucosal immunization [3], indicating that the presence of secretory-antibodies may provide information about their origin; the mucosa. Well known auto-antibodies in RA are rheumatoid factor (RF), ACPA (against citrullinated proteins) and anti-CarP (against carbamylated proteins) [4]. Secretory-RF (SC-RF) and secretory-ACPA (SC-ACPA) have been detected in RA-sera [5, 6], indicating a mucosal origin. Although it is often postulated that SC-containing auto-antibodies are of the IgA isotype, this is unknown as both IgM and IgA can harbour SC. Nonetheless, it is important to define the isotype of SC-containing auto-antibodies as this provides relevant information on the type of B-cell (re)activated at mucosal sites.

Therefore, we determined whether secretory-anti-CarP (SC-anti-CarP), SC-ACPA and SC-RF are present in RA-patients and identified the isotypes used. SC-anti-CarP, SC-ACPA, SC-RF, SC-total-IgA and SC-total-IgM as well as total-IgA and -IgM were measured by ELISA in 363 RA-patients of the Leiden Early Arthritis Cohort (EAC) and 207 Healthy Controls (HC) [7, 8]. Groups were compared using non-parametric tests. Sera of 9 RA-patients were depleted for IgM or IgA after which SC-anti-CarP, SC-ACPA and SC-RF were determined.

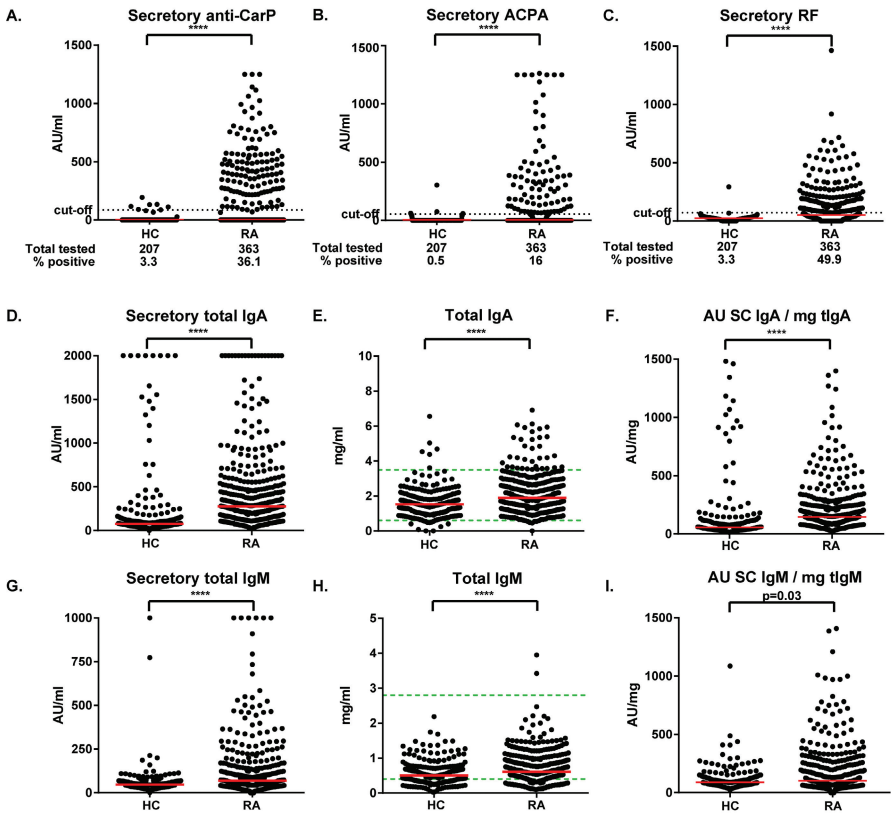

FIGURE 1. Secretory anti-CarP, ACPA and RF are increased in rheumatoid arthritis patients. ELISAs were performed to detect secretory anti-CarP antibodies (A), secretory ACPA (B) and secretory RF (C) in sera of 207 HC and 363 RA-patients. Next to this total secretory IgA (D), total-IgA (E), AU secretory IgA per mg total-IgA (F), total secretory IgM (G), total-IgM (H) and AU secretory IgM per mg total-IgM (I) were measured in all HC and RA-patients. Plates were coated with Ca-FCS (A), CCP2 (B) and human IgG (C) and after serum incubation bound antibodies were detected using goat-anti-human secretory component (Nordic Mubio). For the detection of total secretory IgA and IgM, plates were coated with mouse-anti-human secretory component (SPM217) and after serum incubation bound antibodies were detected using goat-anti-human IgM (Millipore) or IgA (Novex). Total-IgA and -IgM was measured using the Bethyl kit (manufactures protocol). The 97th percentile in HC was used as cut-off for the presence of secretory auto-antibodies. The dotted line represents the cut-off and the red line the median. The green lines represent the "normal" range for total-IgA and -IgM in serum. The specific secretory antibody reactivity is depicted in arbitrary units per millilitre and total-IgA and -IgM in milligram per millilitre. Please note, as levels are depicted in AU, no direct comparisons in absolute antibody-levels can be made. The number of samples tested and the percentage positivity is shown below the graphs. Mann-Whitney U tests were carried out to determine differences in antibody levels between RA-patients and HC. The Pearson chi-squared test was used to determine differences in positivity between RA-patients and HC. HC; healthy controls, RA; rheumatoid arthritis, anti-CarP antibody; anti-carbamylated protein antibody, ACPA; anti-citrullinated protein antibodies, RF; rheumatoid factor, AU/ml; arbitrary units per millilitre, mg/ml; milligram per millilitre. Mann-Whitney U test *p=0.05-0.002, **p=0.002-0.0002, *** p=0.0002-0.0001, **** p< 0.0001

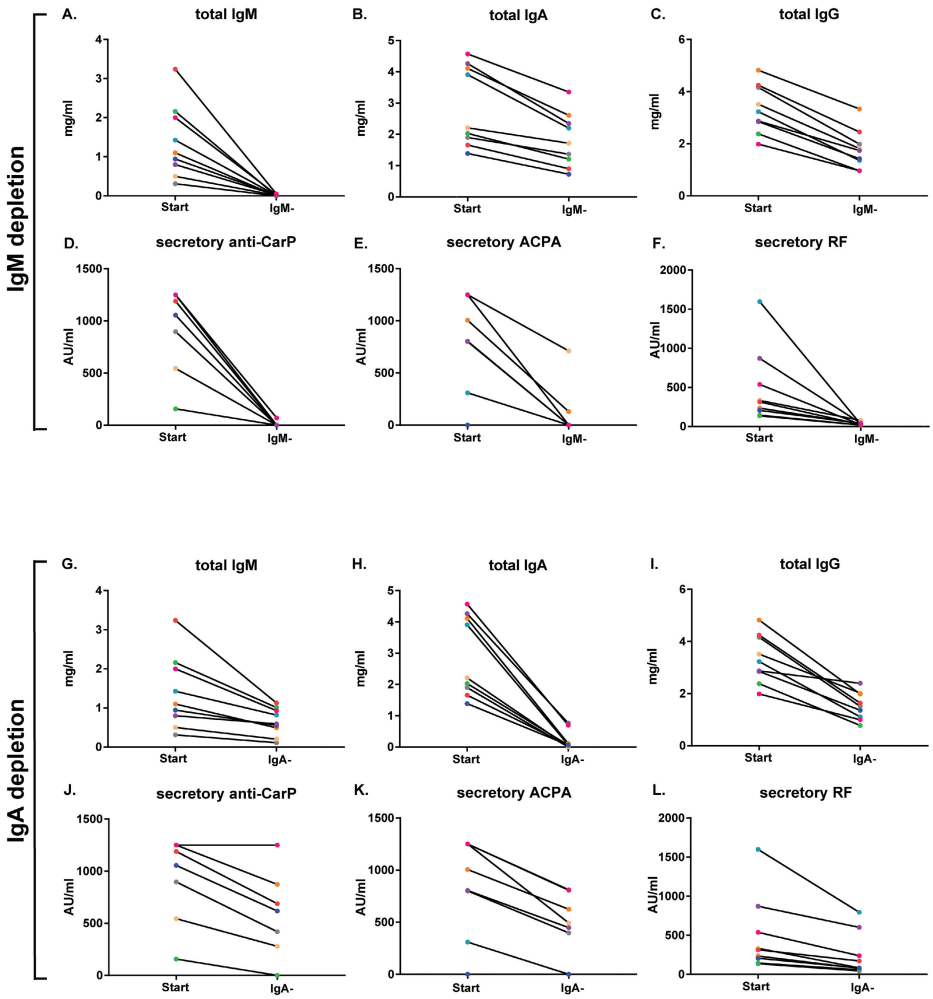

FIGURE 2. Secretory anti-CarP, ACPA and RF are predominantly of the IgM isotype. Serum samples of 9 RA-patients were depleted for IgM (A-F) or IgA (G-L) and the presence of secretory anti-CarP antibodies, secretory ACPA and secretory RF (D-F, J-L) as well as total-IgM, -IgA and -IgG (A-C, G-I) was analysed in the start and depleted material. Plates were coated with CaFCS (D,J), CCP2 (E,K) and human IgG (F,L) and after serum incubation bound antibodies were detected using goat-anti-human secretory component (Nordic Mubio). Total-IgM, -IgA and -IgG was measured using the Bethyl kit (manufactures protocol). Every colour represents a patient. anti-CarP antibody; anti-carbamylated protein antibody, ACPA; anti-citrullinated protein antibodies, RF; rheumatoid factor, AU/ml; arbitrary units per millilitre, mg/ml; milligram per millilitre.

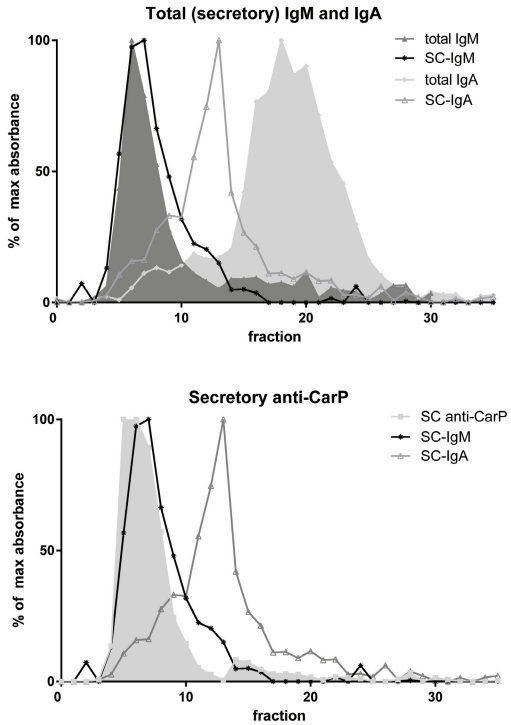

FIGURE 3. Secretory anti-CarP is detectable in the secretory total IgM positive size exclusion fractions. Size exclusion chromatography was performed on sera from 12 RA-patients. 4 of these patients turned out to be secretory anti-CarP positive whereas all were negative for secretory ACPA. Total IgM, total IgA, total secretory IgM, total secretory IgA and secretory anti-CarP ELISAs were performed. Secretory total IgM (SC-IgM) is detected in the total IgM fractions and secretory total IgA (SC-IgA) in the polymeric total IgA fractions (a). Secretory anti-CarP (SC anti-CarP) is predominantly detected in the secretory IgM positive fractions (b). RA; rheumatoid arthritis, anti-CarP antibody; anti-carbamylated protein antibody.

The prevalence and levels of SC-anti-CarP, SC-ACPA and SC-RF are increased in RA-patients compared to HC (prevalence: $p<0.0001$, levels: $p<0.0001$) (fig. 1a-c). Levels of SC-total-IgA and SC-total-IgM as well as total-IgA and total-IgM were increased in RA-patients compared to HC ($p<0.0001$ for all) (fig.1d,e,g,h). The increase of SC-total-IgM appeared RA-specific as no increase was observed in disease controls (fig.S1). Intriguingly and unexpectedly, we observed that the SC-auto-antibodies were predominantly present as IgM and not of the IgA isotype as mostly assumed (fig. 2). IgM and IgA depletion was checked beforehand on ELISA (fig. 2) and western blot (fig. S2). Indeed, the presence of SC-containing auto-antibodies correlates best

with SC-total-IgM (fig.S3), also pointing towards their presence in the IgM isotype. When performing SC-total-IgM, SC-total-IgA and SC-anti-CarP ELISAs on size exclusion chromatography fractions of SC-anti-CarP positive RA-patients, SC-anti-CarP was detectable in the same fractions as SC-total-IgM, also pointing towards the presence of SC-anti-CarP predominantly in the IgM isotype (fig. 3). Unfortunately none of these RA-patients tested positive for SC-ACPA. The increased representation of SC-IgM auto-antibodies is not a general feature of SC-immunoglobulins present in RA, but likely more specific for auto-antibodies as correction for total levels of IgM normalized the observed increase in SC-total-IgM (fig. 1f,i). Importantly, the avidity of anti-CarP IgA and IgM, ACPA IgA and IgM and RF IgA and IgM are similar (fig. S4), indicating that the observed differences are not explained by "technical issues" related to the sensitivity of the methods used to detect SC-IgA vs SC-IgM auto-antibodies.

Overall, SC-anti-CarP, SC-ACPA and SC-RF are present in- and specific for RA and consist predominantly of the IgM isotype. These findings are of importance as this indicate that especially autoreactive IgM-expressing B-cells represent the most prominent B-cell subset that is reactivated at mucosal areas, possibly (re)activating and keeping the immune response ongoing. This (re)activation could involve both naïve and pre-existing memory IgM B-cells or even (gut) IgM+ plasma cells directed against commensals [9] , possibly pointing to a role of the microbiome in steering RA-specific auto-immune responses.

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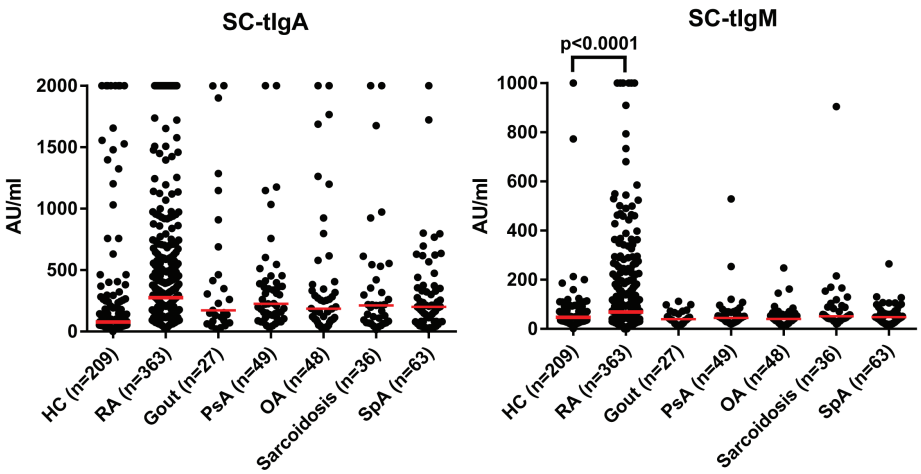
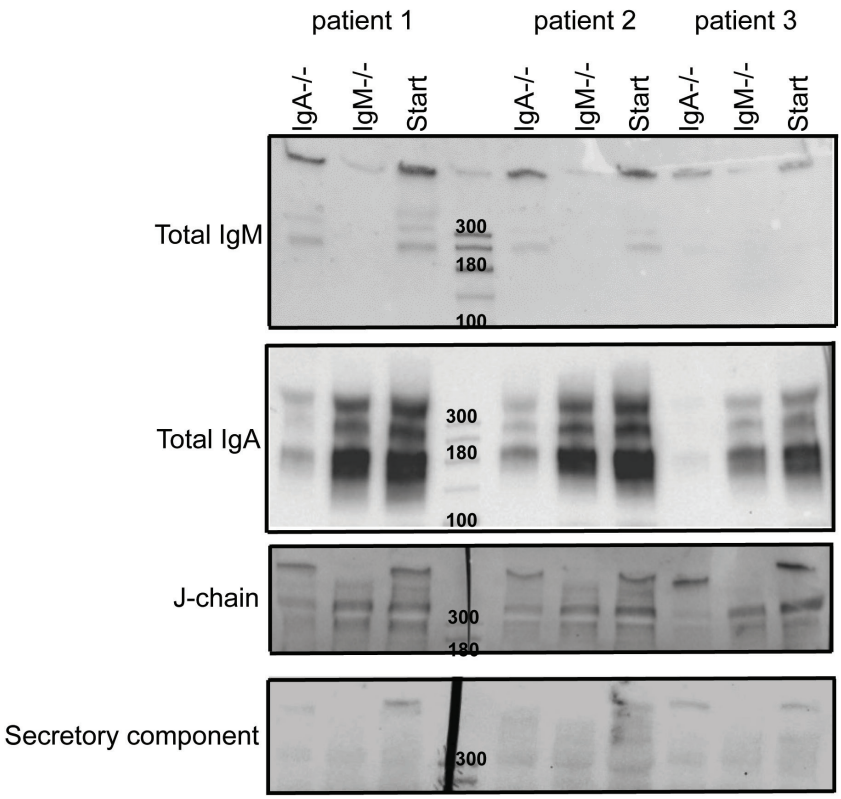
SUPPLEMENTARY MATERIAL

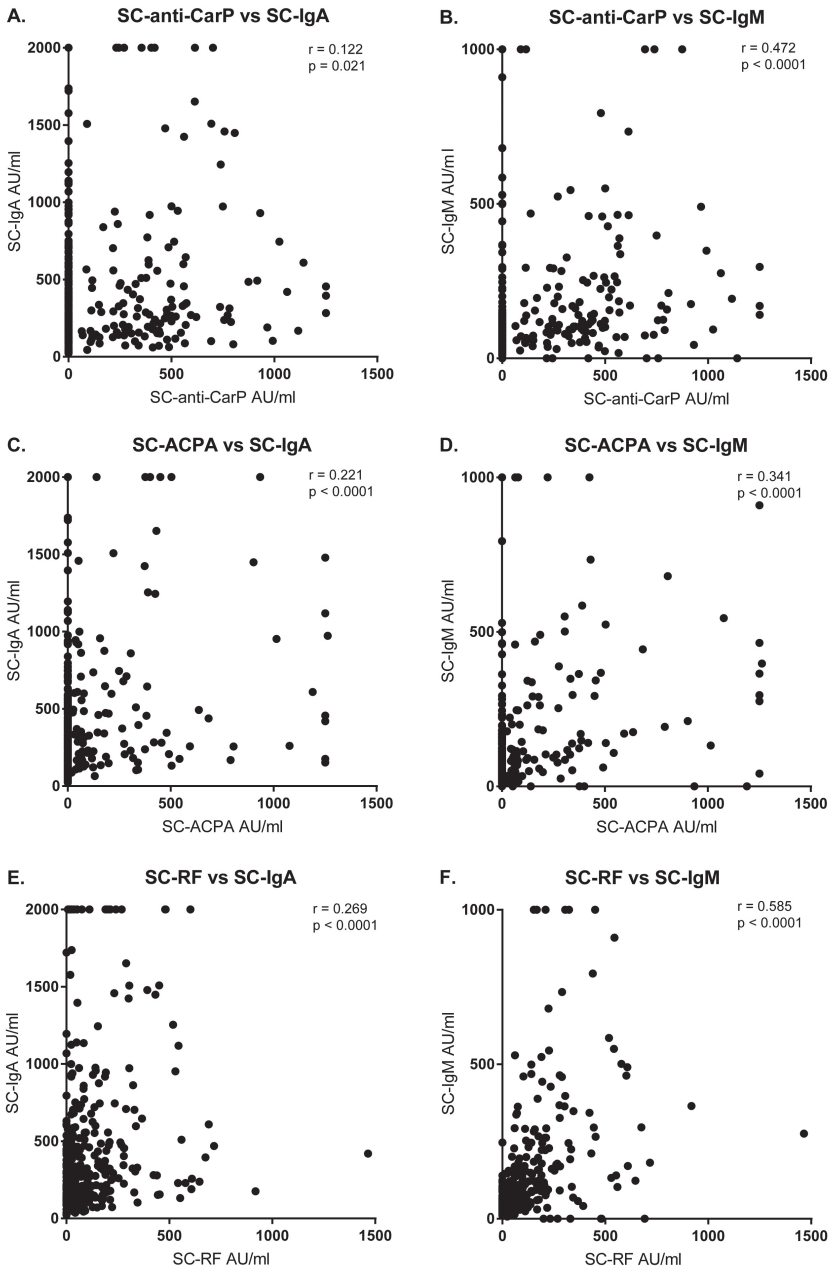
SUPPLEMENTARY FIGURE 1. The increase in secretory total IgM is specific for RA. Secretory total IgM and IgA was analysed in 207 HC, 363 RA-patients, 27 gout-patients, 49 PsA-patients, 48 OA-patients, 36 Sarcoidosis-patients and 63 SpA-patients. Secretory total IgM is only increased in RA-patients compared to HC (Mann Whitney U test p<0.0001). HC; healthy controls, RA; rheumatoid arthritis, PsA; psortiatic arthritits, OA; osteoarthritis, SpA; spondyloarthritis.



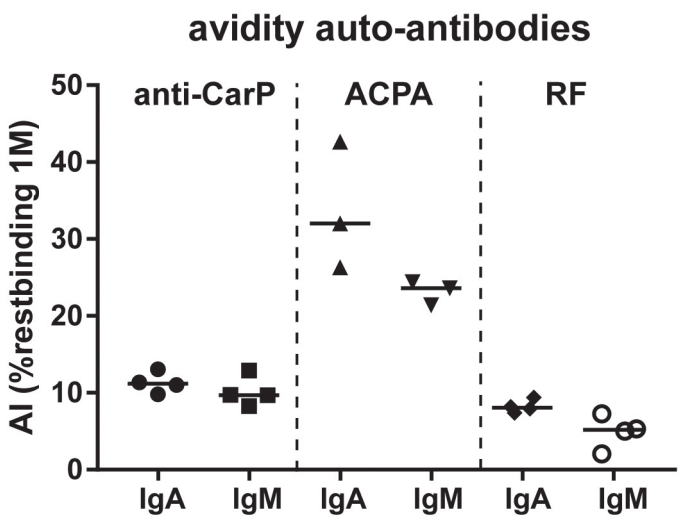
SUPPLEMENTARY FIGURE 2. IgM and IgA depletion check on western blot. To check whether the IgM and IgA depletions went well and whether we were still able to detect J-chain and secretary component in the different samples, total IgA, total IgM, J-chain and secretory component western blots were performed. Representative results of three patients are shown. Patient 3 is an ACPA negative patient. ACPA; anti-citrullinated protein antibody, J-chain; joining chain



SUPPLEMENTARY FIGURE 3. Secretory auto-antibodies have the strongest correlation with secretory IgM. To investigate correlations between secretory auto-antibodies and secretory IgA (A,C,E) or secretory IgM (B,D,E) Spearman Rank tests were carried out on all tested RA-patients. anti-CarP antibody; anti-carbamylated protein antibody, ACPA; anti-citrullinated protein antibody, RF;rheumatoid factor

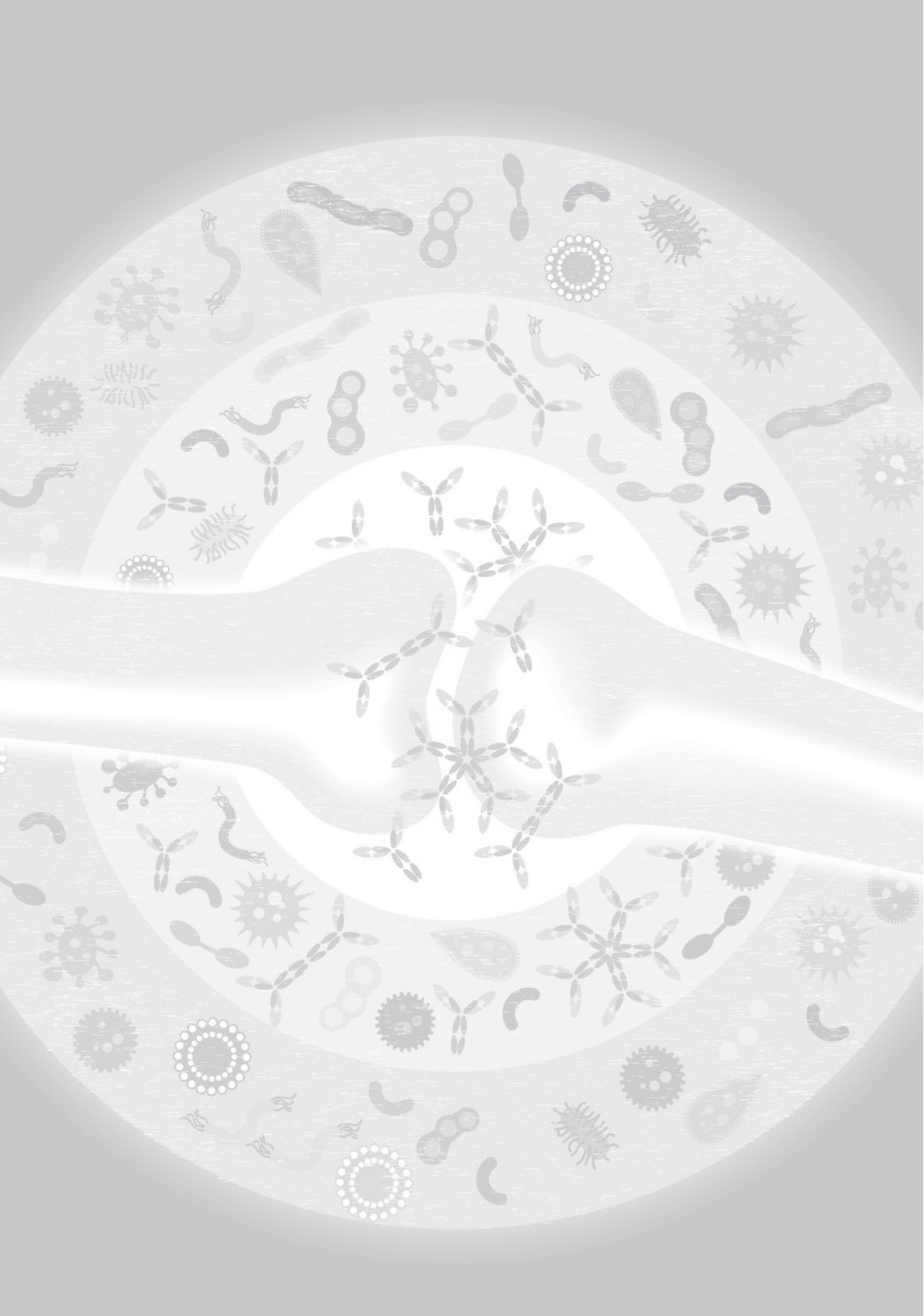


SUPPLEMENTARY FIGURE 4. The anti-CarP, ACPA and RF IgA and IgM avidity are similar. Elution ELISA assays were performed to test the anti-CarP antibody, ACPA and RF IgA and IgM avidity on Ca-FCS, CCP2 and human IgG in sera of 4 RA-patients. The avidity index (AI) is depicted as % restbinding at 1M NaSCN. anti-CarP antibody; anti-carbamylated protein antibody, ACPA; anti-citrullinated protein antibody, RF;rheumatoid factor, Ca-FCS; carbamylated fetal calf serum, CCP; cyclic citrullinated peptide, RA; rheumatoid arthritis, AI; avidity index , NaSCN; sodiumthiocynate



PART 3

Anti-CarP antibodies in other rheumatic diseases that can be characterized by bone erosions



CHAPTER 7

Antibodies against carbamylated proteins and cyclic citrullinated peptides in systemic lupus erythematosus: Results from two well-defined European cohorts

Michael Ziegelasch
Myrthe A.M. van Delft
Philip Wallin
Thomas Skogh
César Magro-Checa
Gerda M. Steup-Beekman
Leendert A. Trouw
Alf Kastbom
Christopher Sjöwall

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ABSTRACT

Background. Articular manifestations are common in systemic lupus erythematosus (SLE) whereas erosive disease is not. Antibodies to 'cyclic citrullinated peptide' (anti-CCP) are citrulline-dependent in rheumatoid arthritis (RA), whereas the opposite is suggested in SLE since reactivity with 'cyclic arginine peptide' (CAP) is typically present. Antibodies targeting carbamylated proteins (anti-CarP) may occur in anti-CCP/rheumatoid factor (RF) negative cases long before clinical RA onset. We analysed these antibody specificities in sera from European SLE patients in relation to phenotypes, smoking habits and imaging data.

Methods. 441 SLE cases from Linköping, Sweden, and Leiden, the Netherlands, were classified according to ACR and/or SLICC criteria. IgG anti-CCP, anti-CAP and anti-CarP were analysed by immunoassays. X-ray data were available from 102 Swedish patients.

Results. 16 Linköping (6.8%) and 11 Leiden patients (5.4%) were anti-CCP positive, of which approximately one third were citrulline-dependent. 40/441 (9.1%) were anti-CarP positive. 33% of the anti-CarP positive cases were identified as anti-CCP positive. No associations were found comparing anti-CCP or anti-CarP with ACR-defined phenotypes, immunologic abnormalities or smoking habits. X-ray-proven erosions were found in 10 patients, and associated significantly with anti-CCP, anti-CarP and RF. Musculoskeletal ultrasonography showed higher scores in anti-CCP positive compared to negative cases.

Conclusions. In the hitherto largest anti-CarP study in SLE, we demonstrate that anti-CarP is more prevalent than anti-CCP and that the overlap is limited. We obtained some evidence that both autoantibodies seem to associate with erosivity. Similar pathogenetic mechanisms as in RA may be relevant in a subgroup of SLE cases with a phenotype dominated by arthritis.

BACKGROUND

In clinical practice, the diagnosis of systemic lupus erythematosus (SLE) is often based on involvement of at least two organ systems combined with a diversity of immunological abnormalities [1]. The presence of antinuclear antibodies (ANA) and reduced levels of circulating complement proteins are typical immunological abnormalities in SLE, and both of these items are included in the most recent proposal of classification criteria [2]. Over the years, several attempts to link specific autoantibodies to certain clinical phenotypes of SLE have been made. For instance, antibodies against double-stranded DNA (dsDNA) and C1q are commonly found in lupus nephritis [3, 4], anti-Ro/SSA antibodies often coincide with lupus-related rash and photosensitivity [5], and anti-phospholipid antibodies are frequently found in SLE patients with thromboembolic events [6].

Detection of antibodies against 'cyclic citrullinated peptide' (anti-CCP) is an important diagnostic and prognostic tool in arthritis, since it is highly specific for rheumatoid arthritis (RA) and predictive of erosive disease. While a positive anti-CCP test in RA is typically citrulline-dependent [7], it has been suggested that anti-CCP in other conditions is generally not, and thus also reacts with the corresponding 'cyclic arginine peptide' (anti-CAP) [8, 9]. During the last years, it has been repeatedly shown that antibodies targeting carbamylated proteins (anti-CarP) may occur in anti-CCP/rheumatoid factor (RF) negative cases [10–12]. Like RF and anti-CCP, also anti-CarP antibodies can be detected many years before the onset of RA [13–15].

The process of carbamylation is mediated by a chemical reaction of cyanate with mainly lysine residues in proteins [16]. Cyanate is present in the body in equilibrium with urea. Inflammation, smoking and renal failure have been reported to increase the non-enzymatic post-translational modification in which cyanate binds to molecules containing primary amine or thiol groups and forms carbamyl groups [16]. Carbamylation of proteins can lead to the loss of tolerance with formation of antibodies directed against carbamylated proteins (anti-CarP antibodies) in susceptible individuals [10–12].

Over the last years, this novel group of autoantibodies has been intensively studied in cases before the onset of clinical RA symptoms, as well as in patients with established RA, in relation to prognostic factors such as anti-CCP/RF and to disease outcomes (*i.e.* radiological progression) [10, 17, 18].

Anti-CarP was also recently reported in primary Sjögren's syndrome, and found to be strongly associated with increased focal lymphocyte infiltration, formation of ectopic germinal centre-like structures, and to the degree of affected salivary gland function [19]. To our knowledge, only two previous small studies have addressed the occurrence of anti-CarP antibodies in SLE. Thus, Scinocca *et al* reported the occurrence of anti-CarP (homocitrullinated fibrinogen) antibodies in 49% of 81 RA patients, but in none among the 37 SLE cases, 37 psoriatic arthritis patients, or 27 healthy controls [20]. In the study by López-Hoyos *et al*, 48% (16/33) of anti-CCP/RF negative patients with elderly-onset RA were judged seropositive regarding anti-CarP compared to 39% (48/124) of patients with polymyalgia rheumatica, and 11% (4/37) of the SLE cases [21].

Although articular involvement is common in SLE (at least 80%) and often constitutes the presenting symptom, it has historically received limited attention [22, 23]. A plausible reason hereto is that arthritis causes significant problems to the patient at disease onset, but often responds rather well to treatment with glucocorticoids, hydroxychloroquine and methotrexate [22]. Development of deformities is occasionally seen, but the vast majority of SLE patients do not develop X-ray proven erosions [22]. Accordingly, several investigators have estimated the frequency of erosive arthritis in SLE to 2–5%, but the finding of erosions on X-ray often complicates the distinction between SLE and RA [22, 24]. As a result, the concept of "rhupus" was introduced to describe patients who simultaneously fulfil classification criteria for both conditions [25, 26]. It is still a matter of debate as to whether this group represents a true overlap of RA and SLE, rather than a subset of SLE [27, 28].

Hitherto, only few studies on lupus arthritis have been published based on imaging modalities other than conventional X-ray. A limited number of studies have evaluated MRI in the assessment of patients with articular involvement in SLE [29–32]. Recently, Ball and colleagues demonstrated that MRI is highly sensitive in identifying erosions, synovitis and bone oedema, independently of anti-CCP and RF antibody status [32]. In SLE, there are some reports on musculoskeletal ultrasonography (US), but so far this modality is seldom used in clinical routine [31, 33].

The primary aim of this study was to describe the presence of citrulline-dependent anti-CCP and anti-CarP antibodies in 441 well-characterized patients within two European SLE cohorts. Secondly, in the Swedish data set, we aimed to investigate the associations between autoantibody status and articular involvement as defined by classification criteria, conventional X-ray data and evaluation with musculoskeletal US.

METHODS

Discovery cohort

236 patients diagnosed with SLE were included in the discovery cohort. All patients took part in the prospective, structured follow-up program "KLURING" (Swedish acronym for *Clinical LUpus Register In Northeastern Gothia*) at the rheumatology outpatient clinic, University Hospital, Linköping, Sweden. The patient material has previously been described in detail [3, 34]. Patients were recruited consecutively and classified as SLE according to the 1982 American College of Rheumatology (ACR) criteria and/or the 2012 Systemic Lupus International Collaborating Clinics classification criteria (SLICC-12) [2, 35]. Most were prevalent cases (83%), but 41 patients (17%) had a newly diagnosed disease at inclusion. Information on smoking habits (current/former/never) was recorded at the time of blood sampling, as well as activity limitations defined according to the validated Swedish version of health assessment questionnaire (HAQ) [36]. Further patient characteristics are summarized in Table 1.

It should be emphasized that the definition of arthritis slightly differs between the ACR-82 and SLICC-12 criteria. Whereas ACR-82 requires "non-erosive arthritis involving ≥2 peripheral joints, characterized by tenderness, swelling, or effusion", the presence of "synovitis involving 2 or more joints, characterized by swelling or effusion OR tenderness in 2 or more joints and at least 30 minutes of morning stiffness" is demanded to meet the SLICC-12 arthritis criterion.

TABLE 1. Clinical and immunological features of the 441 included patients.

	Discovery cohort (n=236)	**Replication cohort** (n=205)	**Mann–Whitney *U* test or Chi-square**
Patient characteristics			*p*-value
Background variables			
Females	88% (207)	89% (183)	N.S.
Age (years, median, range)	54 (19-94)	42 (13-80)	<0.001
Disease duration (years, median, range)	15 (0-52)	8 (0-32)	<0.001
Caucasian ethnicity	93% (219)	69% (142)	<0.001
Conventional radiology available	43% (102)	N.A.	
Erosions on X-ray	4.2% (10)[$]	N.A.	
HAQ score (median, range)	0.13 (0-3)	N.A.	
Ever smoker (former or current)	45% (107)	43% (88)	N.S.
Meeting ACR-82	85% (201)	91% (188)	0.038
Meeting SLICC-12	99% (233)	99% (203)	N.S.
Clinical phenotypes (SLICC-12 definitions on criteria 1 to 11)			
1) Acute cutaneous lupus	45% (106)	53% (108)	N.S.
2) Chronic cutaneous lupus	15% (36)	18% (36)	N.S.
3) Oral ulcers	12% (29)	32% (66)	<0.001
4) Non-scarring alopecia	22% (51)	19% (39)	N.S.
5) Synovitis	76% (180)	70% (143)	N.S.
6) Serositis	37% (87)	23% (48)	0.003
Pleuritis	35% (83)	18% (37)	<0.001
Pericarditis	14% (34)	14% (28)	N.S.
7) Renal	28% (66)	26% (53)	N.S.
8) Neurologic	11% (26)	22% (44)	0.004
Seizures	4.2% (10)	5.4% (11)	N.S.
Psychosis	1.7% (4)	4.4% (9)	N.S.
Mononeuritis multiplex	0.4% (1)	0% (0)	N.S.
Myelitis	0.4% (1)	2.9% (6)	N.S.
Peripheral or cranial neuropathy	5.1% (12)	8.3% (17)	N.S.
Acute confusional state	0.8% (2)	2% (4)	N.S.
9) Hemolytic anemia	4.7% (11)	5.9% (12)	N.S.
10) Leuko- and/or lymphopenia	52% (122)	30% (62)	<0.001

11) Thrombocytopenia	12% (28)	18% (37)	N.S.
Raynaud	26% (61)	40% (82)	0.002
Interstitial lung disease	3.5% (8)	3.4% (7)	N.S.
Immunological features (SLICC-12 definitions on criteria 1 to 6)			
1) Antinuclear antibody (ANA)	100% (236)	100% (205)	N.S.
2) Anti-dsDNA antibody (anti-dsDNA)	50% (118)	56% (115)	N.S.
3) Anti-Smith antibody (anti-Sm)	8.1% (19)	10% (21)	N.S.
4) Antiphospholipid antibody	59% (139)	44% (90)	0.002
Lupus anticoagulant	35% (69)*	32% (65)	N.S.
Anti-cardiolipin antibody	34% (80)	24% (49)	0.027
Anti-□2-glycoprotein I antibody	26% (62)	18% (27)[#]	N.S.
5) Low complement	53% (124)	50% (102)	N.S.
6) Direct Coombs' test	56% (59)[§]	23% (38)[ø]	<0.001
Anti-small nuclear ribonucleoprotein antibody (anti-snRNP)	37% (88)	17% (35)	<0.001
Anti-Ro/Sjögren's syndrome A antibody (SSA)	37% (88)	42% (87)	N.S.
Anti-La/Sjögren's syndrome B antibody (SSB)	28% (66)	13% (27)	<0.001
Anti-cyclic citrulline peptide antibody (anti-CCP)[Ω]	6.8% (16)	5.4% (11)	N.S.
Anti-carbamylated protein antibody (anti-CarP)	9.8% (23)	8.3% (17)	N.S.
Rheumatoid factor[Ω]	25% (26)[§]	15% (31)	0.046

[$] Calculated on 236 patients

* Data available on 195 patients

[#] Data available on 148 patients

[§] Data available on 107 patients

[ø] Data available on 164 patients

[Ω] Not performed with identical assays

N.A. = not applicable

N.S. = not significant

Replication cohort

A total of 205 consecutive SLE patients were included in the replication cohort. All patients were selected from the Leiden NPSLE-clinic, at the rheumatology department, Leiden University Medical Center (LUMC), the Netherlands. The LUMC serves as a national referral centre for SLE patients

suspected of having neuropsychiatric involvement. All patients included were admitted for one day and underwent a complete examination that has previously been described in detail [37]. All patients were classified as SLE according to the 1997 updated ACR-82 and/or the SLICC-12 criteria [2, 38]. Data concerning gender, smoking status and ethnicity were retrospectively recorded. Detailed patient characteristics relevant to the present study are summarized in Table 1.

The following definitions of smoking habits were used in both cohorts [39]. "Former smoker" means to have ever been a regular smoker; occasional smokers are not included. "Ever smoker" constitute the sum of "former smoker" and "current smoker".

Laboratory analyses

In the discovery cohort, IgG-class anti-CCP and anti-CAP antibodies were analysed by enzyme-linked immunosorbent assays (CCPlus and anti-CAP respectively, Euro-Diagnostica, Malmö, Sweden). Levels ≥25 units/mL defined a positive test as suggested by the manufacturer. Citrulline-dependency was defined as a higher antibody level obtained in the anti-CCP test than in the anti-CAP test. RF was detected by nephelometry at the clinical immunology department, University Hospital, Linköping, Sweden.

For the replication cohort, anti-CCP and anti-CAP antibodies were analysed using an in-house CCP2 assay with a defined cut-off of ≥25 units/mL [40]. 193 healthy controls, living in the Leiden area, were included for comparison. Briefly, biotinylated CCP2 citrulline and corresponding arginine control peptide were coupled to streptavidin coated ELISA plates. Serum samples were diluted 1:50 and bound IgG was detected using rabbit anti-human IgG horseradish peroxidase (HRP) (DAKO, Glostrup, Denmark).

IgG anti-CarP antibody analyses for both cohorts were performed in Leiden, using a previously described immunoassay detecting antibodies against carbamylated fetal calf serum proteins [10]. Other laboratory analyses, including IgM RF quantified by fluoroenzyme immunoassay, were performed at the routine diagnostic lab of the Leiden University Medical Center, Leiden, the Netherlands.

Radiology

Through meticulous chart review of each patient, we identified all conventional X-ray examinations of hands, wrists and/or feet performed at the University Hospital, Linköping (discovery cohort). The results were scrutinized by an experienced rheumatologist (MZ) who was blinded to the patients' antibody status. In cases where erosions had been identified by the radiologist, as well as in all cases with indistinct findings, the X-rays were finally evaluated by MZ in order to determine whether erosions were present or not.

Musculoskeletal US

In the discovery cohort, all anti-CCP positive cases plus just as many anti-CCP negative cases matched according to sex, age, disease duration and present prednisolone dose were systematically investigated with musculoskeletal US by an experienced examiner (MZ) blinded to the patients' antibody profiles and conventional radiology results. Characteristics of the 32 patients, representing a subgroup of the discovery cohort, are shown in Table 2.

US examination was performed using LOGIQ-E9 (GE Healthcare, Milwaukee, WI, USA) with a linear scanner 6-15 MHz. All patients were examined with the same settings for both B-mode (grey scale) and power Doppler (PD). The protocol included dorsal assessments of the following 36 joints: bilateral radiocarpal, intercarpal, distal radioulnar, metacarpophalangeal (MCP) I-V, interphalangeal (IP) thumb joints, proximal interphalangeal (PIP II-V), and metatarsophalangeal (MTP) joint I-V. In addition, six tendons (extensor carpi ulnaris in the wrist, tibialis posterior and flexor digitorum longus in the feet) were evaluated. To grade synovitis, we used the scoring system by Szkudlarek *et al* in which synovial hypertrophy is graded 0–3 (0=no thickening, 3=synovial thickening bulging over the tops of the periarticular bones and extension over diaphysis of at least one side) and perfusion 0–3 (0=no Doppler signals in the synovium, 3=confluent Doppler signals in more than one half of the synovium) [41, 42].

TABLE 2. Characteristics of the 32 discovery cohort patients evaluated with musculoskeletal ultrasonography.

	Anti-CCP positive (n=16)	**Anti-CCP negative** (n=16)	***P* value**
Age (years, median)	58	58	N.S.
Disease duration (years, median)	10.5	10.5	N.S.
Females (number of individuals)	14	13	N.S.
Fulfilled ACR-82 criteria (number, median)	4	4	N.S.
Meeting arthritis criterion according to ACR-82 (number)	13	14	N.S.
Meeting nephritis criterion according to ACR-82 (number)	4	2	N.S.
Conventional radiology available (hands/wrists/feet)	16	16	N.S.
Erosions on X-ray (number of individuals)	4	0	N.S.
HAQ (median)	0.5	0.19	N.S.
Ever smoker (number of individuals)	9	3	N.S.
Rheumatoid factor positive (number of individuals)	6	1	N.S.
anti-CarP antibody level (median)	275	102	0.04
anti-CarP positive (number of individuals)	6	1	N.S.
Daily dosage of prednisolone (median)	2.5	2.5	N.S.

Statistics

Potential associations between antibody status and clinical characteristics were tested by Fisher's exact test regarding categorical variables and by Mann-Whitney *U* test for numerical variables. Mann-Whitney *U* or Chi-square tests were used to evaluate differences between the cohorts. Statistical analyses were performed using SPSS v23.

For analyses where we had prior hypotheses, a significance level of 5% was regarded as statistically significant (two-sided *p*-values <0.05). For all other tests performed in a more exploratory manner, the exact *p*-values (if $p<0.05$) are reported as reference.

RESULTS

Comparison between cohorts

As shown in Table 1, the size of the two cohorts was similar, whereas the clinical phenotypes with regard to classification criteria fulfilled showed significant differences in some instances (oral ulcers, serositis, neurological involvement, Raynaud). Patients in the discovery cohort were older, had longer disease duration, and were Caucasians to a significantly higher extent than in the replication cohort. In addition, laboratory criteria such as the presence of leuko-/lymphocytopenia, antiphospholipid antibody, anti-snRNP antibody, anti-La/SSB antibody, RF and direct Coomb's test differed between the cohorts.

Presence of anti-CCP/CAP/CarP antibodies in SLE

In the discovery cohort, 16 patients (6.8%) were anti-CCP positive, 9 (56%) of whom were also anti-CAP positive using Euro-Diagnostica kits. However, only one of the 9 anti-CCP/anti-CAP positive cases showed a higher antibody level for anti-CAP than for anti-CCP in the assays. 4 of the 7 patients with a positive citrulline-dependent anti-CCP test had a history of biopsy-proven lupus nephritis. 23 patients (9.8%) were anti-CarP positive. Only 6 (26%) of the anti-CarP positive cases were identified as anti-CCP positive (Figure 1A).

In the replication cohort, 11 patients (5.4%) were anti-CCP positive of which 8 (73%) were also positive for anti-CAP using in-house assays. In 4 of the 8 anti-CCP/anti-CAP positive cases, a higher optical density signal for anti-CAP than for anti-CCP was achieved. 17 patients (8.3%) were anti-CarP positive of which 2 (12%) were also identified as anti-CCP positive (Figure 1B).

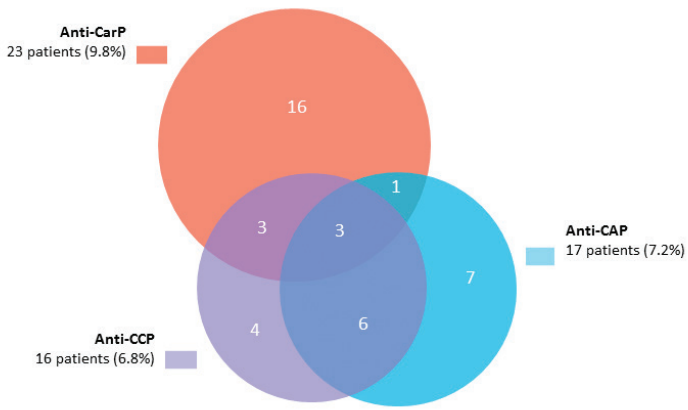
A limited number of samples from the discovery cohort where also analysed with the anti-CCP in-house assay in Leiden. The agreement between the assays was fair (*rho*=0.75; concordance 90%).

Associations of anti-CCP, anti-CarP, and RF with clinical and other laboratory features

In the discovery cohort, neither anti-CCP nor anti-CarP were associated with arthritis by classification judged by physical examination. However, the presence of anti-CarP was associated with neurological involvement as defined by SLICC-12 (p=0.028); this association was particularly driven by the presence of cranial/peripheral neuropathy (p=0.021). On the contrary, anti-

CarP was less common among patients with a positive lupus anticoagulant test (p=0.012). Positive anti-CCP tests were inversely related to anti-La/SSB (p=0.007). Smoking habits and HAQ did not associate with anti-CCP, anti-CarP or RF.

A.



B.

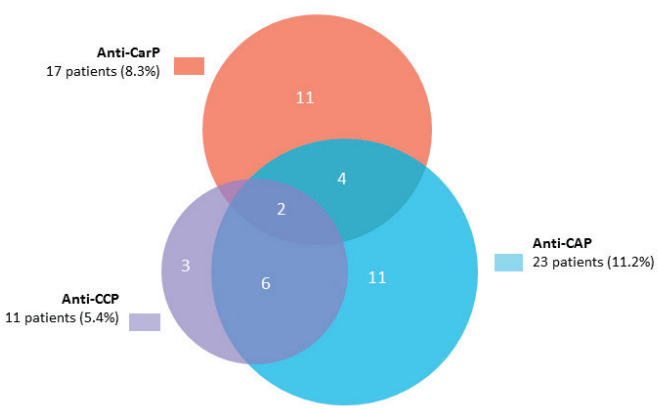


FIGURE 1. Venn diagrams illustrating the distribution of anti-CarP, anti-CCP and anti-CAP positive cases in the (A) discovery cohort (n=236), and in the (B) replication cohort (n=205).

In the replication cohort, the presence of anti-CarP was not significantly associated with neurological involvement as defined by SLICC-12 (p=0.126); and no associations were found with specific neurological manifestations included in SLICC-12. Anti-CarP was not associated with any other SLICC-12 definition or SLE autoantibody. On the contrary, anti-CCP was more common among patients with a positive anti-Sm (p=0.017). No associations were found between anti-CCP positivity and anti-La/SSB. Smoking habits did not associate with anti-CCP, anti-CarP or RF.

Associations of anti-CCP, anti-CarP and RF with radiography

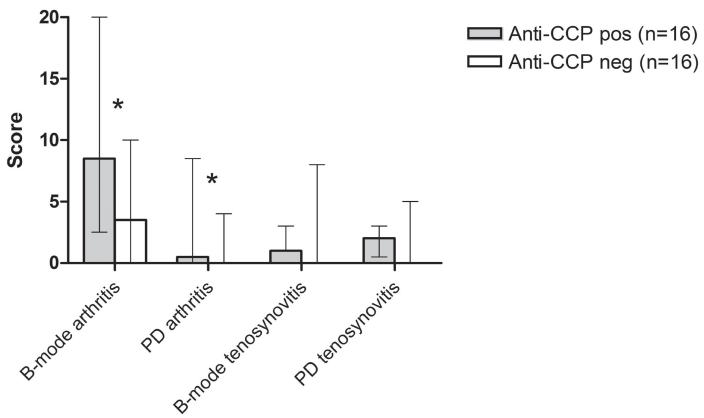
In the Swedish cohort, conventional X-rays of hands, wrists and/or feet were available in 102 cases (43%), and erosions (mostly demonstrated in intercarpal, MCP and MTP joints) were found in 10 patients. This corresponds to 4.2% calculated for the entire Swedish cohort, and to 9.8% calculated strictly for patients with available X-rays. Radiological erosions were significantly associated with a positive test for anti-CCP (p<0.05), anti-CarP (p<0.05), and RF (p<0.05), respectively.

All patients with a positive anti-CCP test were subject to X-ray examination of hands, wrists and feet. Thus, patients with X-rays available were more often anti-CCP positive (16/102; 16%) as compared to patients without X-rays available (0/134; p<0.001). Similarly, 'ever' smokers were significantly overrepresented among patients with available X-rays (55/102; 54%) compared to those without (52/134; 39%; p=0.025). There were no significant differences regarding age, sex or fulfilment of the SLICC-12 arthritis criterion. Neither did the occurrence of anti-CarP, anti-CAP or RF significantly differ according to X-ray data availability.

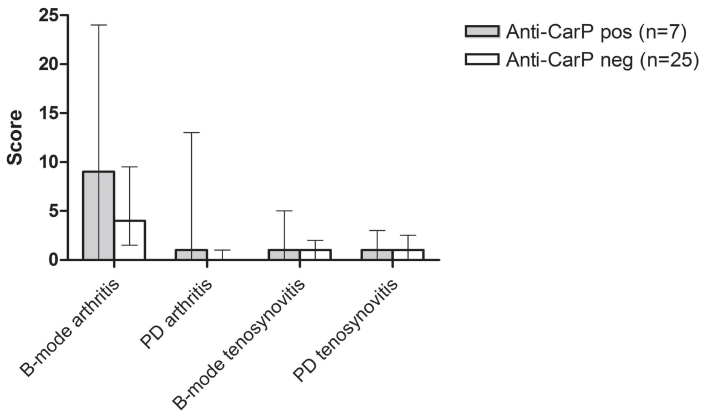
Associations of anti-CCP, anti-CarP and RF with musculoskeletal US

In anti-CCP positive cases, US examination of joints and tendons resulted in significantly higher scores for arthritis, but not for tenosynovitis (Figure 2A). Similar trends were found for anti-CarP and RF, but did not reach statistical significance (Figure 2B–C). Patients with radiological erosions had significantly higher scores for arthritis, but not for tenosynovitis (Figure 2D). Individuals with ongoing prednisolone medication achieved higher scores with regard to arthritis, but not for tenosynovitis (Figure 2E), whereas fulfilment of the SLICC-12 criterion for arthritis did not separate the groups (Figure 2F).

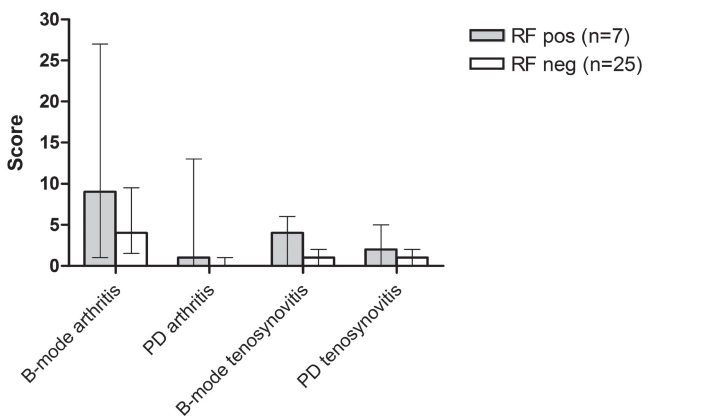
A.


Anti-CCP pos (n=16)
Anti-CCP neg (n=16)
Score
20
15
10
5
0
*
*
B-mode arthritis
PD arthritis
B-mode tenosynovitis
PD tenosynovitis

B.


Anti-CarP pos (n=7)
Anti-CarP neg (n=25)
Score
25
20
15
10
5
0
B-mode arthritis
PD arthritis
B-mode tenosynovitis
PD tenosynovitis

C.


RF pos (n=7)
RF neg (n=25)
Score
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25
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15
10
5
0
B-mode arthritis
PD arthritis
B-mode tenosynovitis
PD tenosynovitis

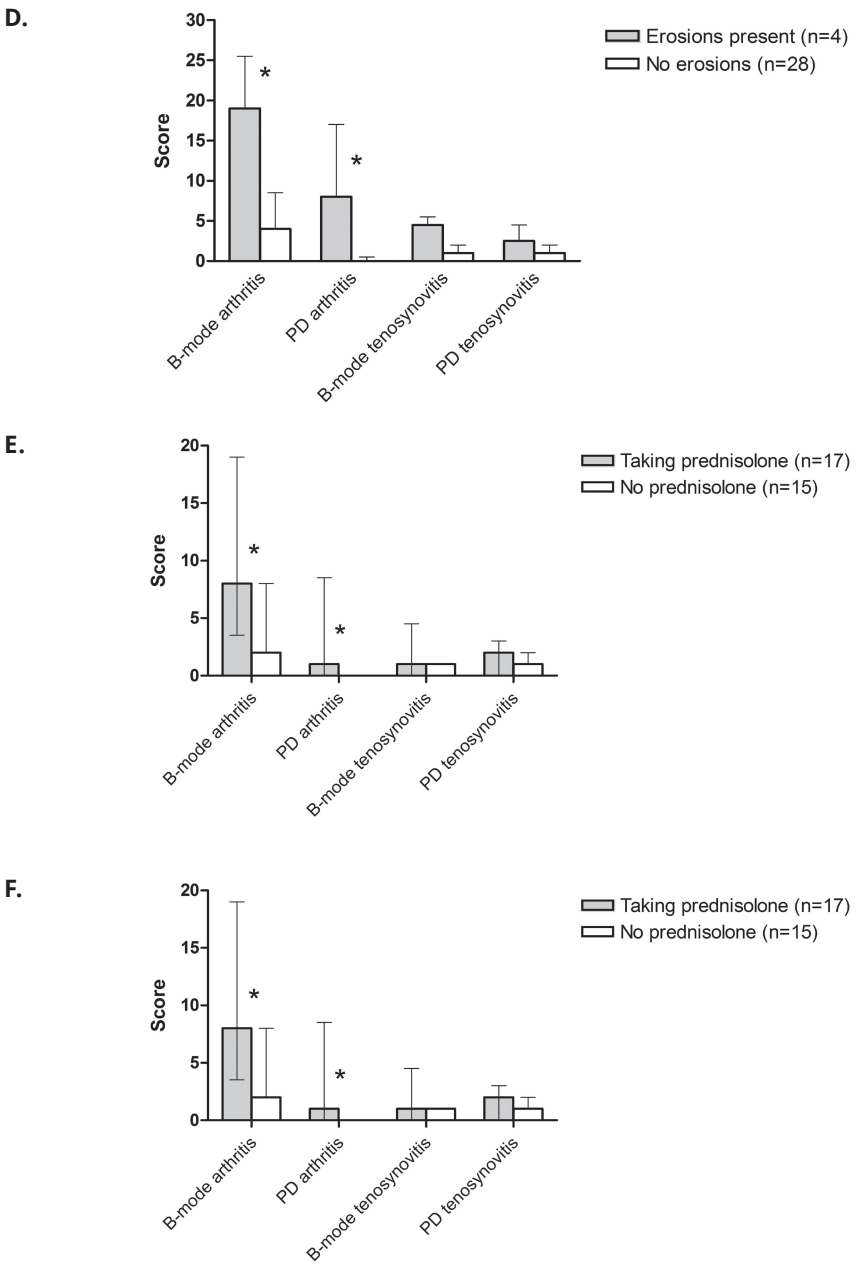

FIGURE 2. Standardized musculoskeletal ultrasonography grading of arthritis and tenosynovitis with grey scale (B-mode) and power Doppler (PD) in 32 patients divided with regard to the presence of: (A) anti-CCP, (B) anti-CarP, (C) rheumatoid factor, (D) X-ray-proven erosions, (E) daily intake of prednisolone, and with regard to (F) the fulfilment of the 1982 ACR classification criterion 5 (arthritis) [35]. Median and interquartile range are illustrated. * p<0.05

Associations of anti-CCP and anti-CarP with the fulfilment of RA classification

Besides meeting the classification criteria for SLE, we evaluated to which extent patients with a positive anti-CCP and/or anti-CarP antibody test simultaneously fulfilled the 2010 ACR/EULAR criteria for RA [43]. As illustrated (Figure 3), the likelihood of meeting RA criteria was higher given a positive anti-CCP, compared to a positive anti-CarP antibody test. However, when taking citrulline-dependency into account for the anti-CCP test this difference was less pronounced, particularly in the discovery cohort.

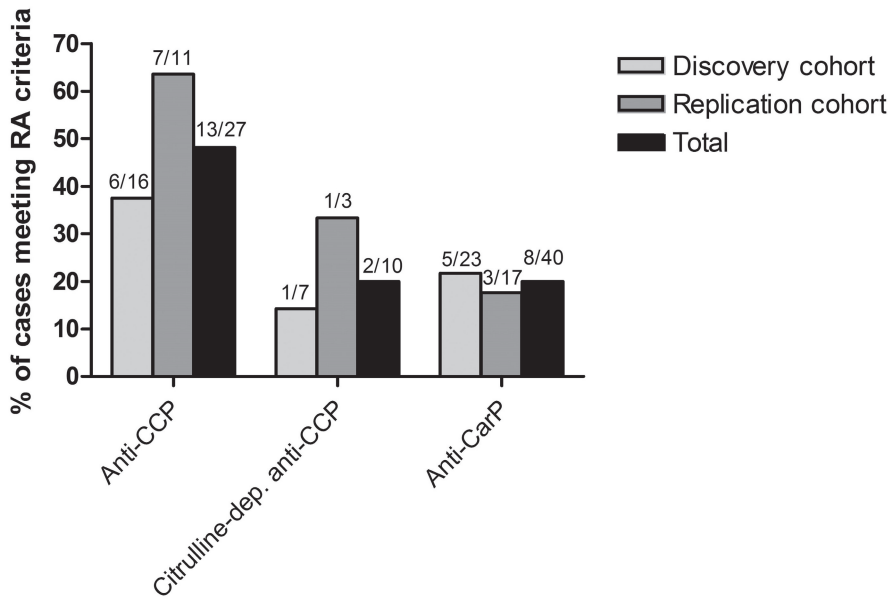

FIGURE 3. The graph illustrates which percentage of patients that - besides being classified as SLE - fulfil the 2010 ACR/EULAR classification criteria for RA [43] provided being: (i) anti-CCP antibody positive, (ii) identified as citrulline-dependent anti-CCP antibody positive (*i.e.* a higher anti-CCP than anti-CAP antibody level), and (iii) anti-CarP antibody positive.

DISCUSSION

This is hitherto the largest evaluation of anti-CarP antibodies in SLE, and the first study on anti-CarP in a European SLE population. Herein, we demonstrate similar frequencies of anti-CarP positive SLE cases in the two cohorts (9.8% *vs.* 8.3%) and that the overlap with anti-CCP antibodies is

limited. Our findings are in line with what has been reported by López-Hoyos *et al*, but clearly higher than observed by Scinocca and co-workers [20, 21]. The latter may be explained by a difference in the antigen used for the detection of anti-CarP antibodies (fibrinogen *vs.* fetal calf serum). Furthermore, we found significant associations between all three RA-associated antibodies (anti-CCP, anti-CarP, RF) and X-ray proven erosions in the Swedish data set. Based on the results, we hypothesize that pathogenetic mechanisms could be similar in RA and in a small group of SLE patients with a clinical phenotype dominated by arthritis [44]. Interestingly though, 60% of the patients with radiology-proven erosions were not identified by any of the antibodies (Figure 1).

Articular manifestations affect a majority of patients with SLE, at least at some time during the disease course (73% in the present study). However, only a minority of SLE patients display an arthritic phenotype and simultaneously meet RA classification [24, 25, 31]. The presence of anti-CCP antibodies is considered highly specific for RA, but can also be found in other conditions, including SLE, where frequencies from 2–17% have been described [9, 32, 45–51]. Whether a true association between a positive anti-CCP test and erosive arthritis in SLE exists or not remains an open question, since several investigators have found this [9, 46–50] whereas others did not [31, 45]. Kakumanu *et al* reported a prevalence of 17% anti-CCP positives among 329 SLE patients but claimed that citrulline-dependent anti-CCP was mainly found in patients with erosive arthritis, which however concerned only 26 cases [9]. Pooled data from the present study indicate that 10 of 27 anti-CCP positive cases (37%) displayed a citrulline-dependent anti-CCP, which corresponds to 2.3% in the whole study population.

As previously mentioned, smoking affects the carbamylation process [16] and smoking is also strongly associated with anti-CCP positive RA, especially in patients with the HLA-DRB1/shared epitope [52]. Thus, we anticipated that smoking would also be overrepresented among anti-CarP and anti-CCP positive SLE patients. To our surprise, we did not detect such associations in this evaluation. In line with our findings, however, recent studies from Stockholm did not reveal any correlation between smoking habits and anti-CCP in SLE [51], and anti-CarP was not significantly associated with smoking in RA patients [11]. Of potential interest regarding these results, the frequency of 'ever' smokers in the discovery and replication cohorts were

similar (approximately 45%), but clearly lower than in two early RA cohorts (TIRA-2 and EIRA-1) from southern and central Sweden, where the frequency approximately reached 60% [52].

Evaluations with musculoskeletal US were performed in 32 SLE patients (whereof 16 were anti-CCP positive) in the discovery cohort, showing significantly higher arthritis scores among anti-CCP positive cases (Figure 2A–C). Previous studies on musculoskeletal US in SLE with relevant comparators are scarce and a considerable strength in the present study is the matched control group (Table 2). Some studies have reported a higher extent of affected tendons in SLE, compared to what we found [31, 53–55]. Interestingly, the effects of corticosteroids on B-mode/PD arthritis scores seemed to be limited (Figure 2E).

We also investigated which RA-associated autoantibody positive SLE cases that simultaneously met the classification for RA. This group could be referred to as "rhupus", although no agreed-upon definition exists for this overlap condition [25–27]. Although anti-citrullinated protein antibodies (ACPA) are indeed included in the 2010 ACR/EULAR criteria for RA, we used this most recent RA classification [43]. We found the "risk" of meeting RA criteria being higher given a positive anti-CCP test as compared to a positive anti-CarP antibody test (52% *vs.* 20%). When taking citrulline-dependency into account for the anti-CCP test, this difference appeared to decrease. We considered using the 1987 ACR RA criteria (which does not include ACPA), but we were unable to retrieve sufficient data to determine fulfilment of this classification setup.

Apart from the statistically significant connection between a positive anti-CarP test and erosive arthritis in our study, the presence of anti-CarP associated significantly with neurological involvement only in the discovery cohort whereas a non-significant trend was found in the replication cohort. This may be due to the low number of patients with this clinical presentation. However, it is tempting to speculate that carbamylation may have an effect on neural tissue. Of relevance to this is the fact that supplementation of sodium cyanate to patients suffering from sickle cell anemia disrupts the equilibrium between urea and cyanate in body fluids resulting in an increased carbamylation and severe polyneuropathy as side-effects [56]. Furthermore, in animal models, cognitive impairment and loss of memory

functions have been described as a result of increased carbamylation [57]. Whether or not anti-CarP antibodies can also be found in patients receiving sodium cyanate is unknown. It should be emphasized that both irreversible neuropathy and cognitive impairment are recorded as SLE damage accrual in the neuropsychiatric domain of the SLICC/ACR damage index [58]. Although renal function also affects the carbamylation process, we did not find any association between anti-CarP and renal involvement. The reason hereto is most likely that renal flares in well-controlled SLE cases are identified early and treated aggressively, resulting in a subsequently very low number of cases meeting end-stage renal disease [59]. In addition, carbamylation alone may not be sufficient for a break of tolerance against carbamylated proteins [60].

Although radiology data were at hand for all cases with a positive anti-CCP test, this was the case only for a minority of patients in the discovery cohort and not at all in the replication cohort, which is a limitation of our study. Furthermore, in the musculoskeletal US evaluations, it is likely that a larger number of evaluated patients with different autoantibody profiles would have enabled statistically significant and meaningful differences with regard to anti-CarP and RF as well. Finally, anti-CCP, anti-CAP and RF were analysed with different assays in the two cohorts. The agreement with anti-CCP was fair, but we cannot exclude that small methodological differences might have influenced the results to some extent.

CONCLUSIONS

In summery, we demonstrate that anti-CarP is more common than anti-CCP in well-characterised SLE cases in two European cohorts, whereas the overlap of these antibody specificities is limited. In the Swedish data set we obtained some evidence that both autoantibodies associate with erosive joint disease.

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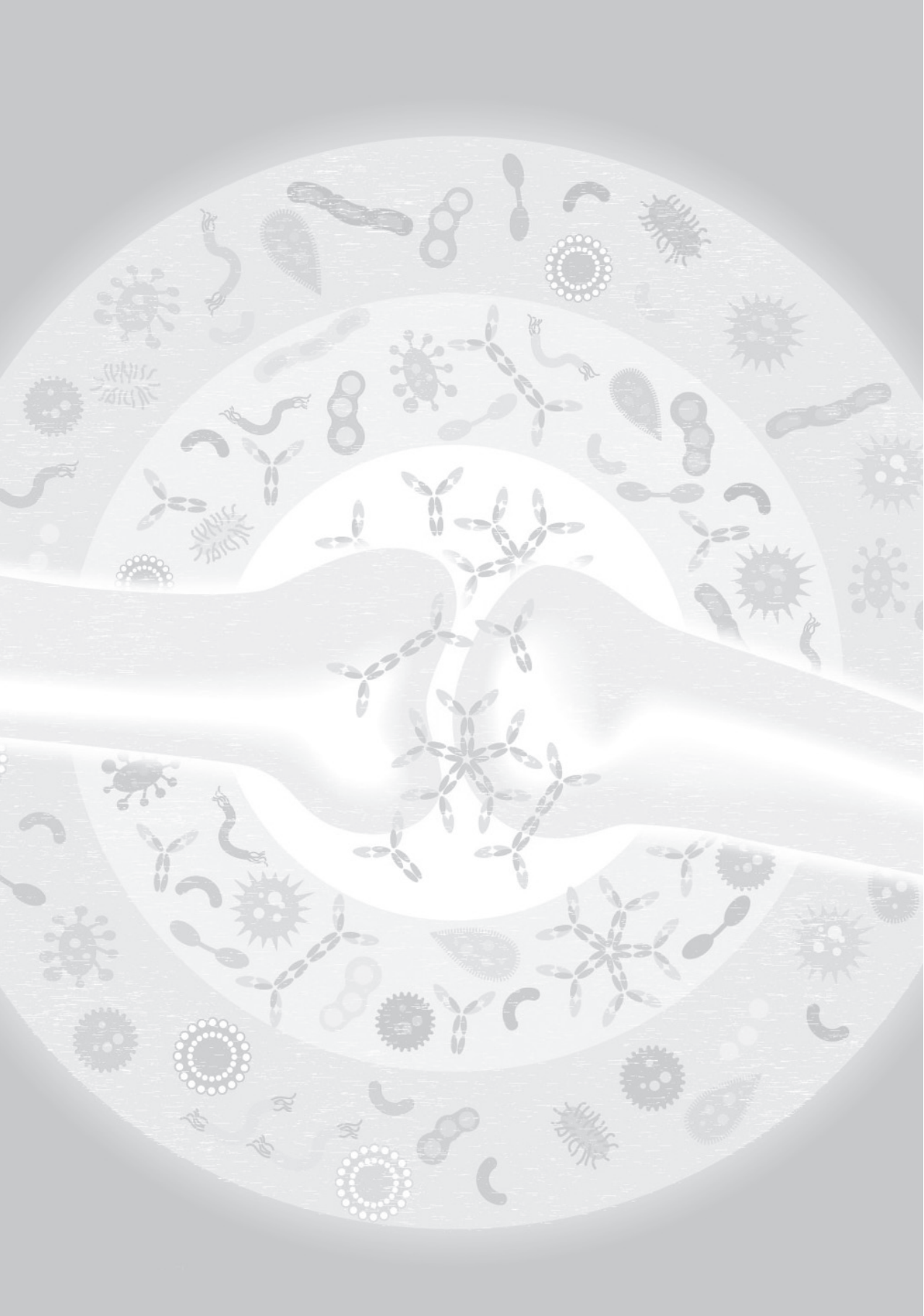

CHAPTER 8

Presence of autoantibodies in erosive hand osteoarthritis and association with clinical presentation

Myrthe A.M. van Delft
Sjoerd van Beest
Margreet Kloppenburg
Leendert A. Trouw
Andreea Ioan-Facsinay

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ABSTRACT

Objective: To investigate whether three rheumatoid arthritis-associated antibodies, Rheumatoid Factor (RF) and antibodies directed against citrullinated (ACPA) or carbamylated proteins (anti-CarP antibody), are present in hand osteoarthritis (HOA) and associate with erosive OA (EOA).

Methods: Anti-CarP IgG was measured by ELISA in baseline sera of HOA patients from 3 cohorts: HOSTAS (n=510, 27.2% EOA), ECHO (n=47) and EHOA (n=23), and in sera of healthy controls (HC; n=196, mean age 44.1 years, 51.0% women). Moreover, ACPA IgG and RF IgM were additionally determined in HOSTAS and HC. The prevalence of autoantibodies was compared between HOA and HC and between erosive and non-erosive HOA. In HOSTAS, hand radiographs were scored (Kellgren-Lawrence, OARSI osteophyte and joint space narrowing) and C-reactive protein (CRP) levels, representing inflammation, were assessed. Groups were compared using non-parametric tests.

Results: The prevalence of anti-CarP was low and not significantly different between the total HOA group and HC (6.6% vs 3.6%, p=0.12). In HOSTAS, the prevalence of all tested autoantibodies was low (anti-CarP 7.1%, ACPA 0.8%, RF 6.1%) and there were no significant differences observed between HOA patients and HC or between erosive and non-erosive HOA. Furthermore, radiographic damage and CRP levels were similar in anti-CarP+ and anti-CarP-, and RF+ and RF- HOSTAS patients.

Conclusions: The prevalence of autoantibodies is similar in HOA patients and healthy controls and these autoantibodies are not associated with erosive disease, structural damage or inflammation in HOA patients, indicating that another mechanism is driving erosive disease.

INTRODUCTION

Osteoarthritis (OA) is a joint disease characterized by cartilage damage (joint space narrowing (JSN)), bone abnormalities (osteophytes, bone marrow lesions) and inflammation (1). One of the most prevalent clinical phenotypes is hand OA (HOA), which can be divided into two subsets namely erosive OA (EOA) and non-erosive HOA (1-3). In EOA central erosions and subchondral destruction are seen, and imaging studies have indicated that in EOA hand joints display more inflammatory signs than in non-erosive HOA (4). Because patients with EOA have a higher clinical burden and socio-economic impact than non-erosive HOA (5-7), it is important to understand the mechanisms involved in development of EOA in order to develop therapeutic interventions.

Autoantibodies directed against post-translationally modified proteins, such as citrullinated (ACPA) and carbamylated (anti-CarP antibodies) proteins, are a hallmark of rheumatoid arthritis (RA) (8, 9). Together with rheumatoid factor (RF) these antibodies can be detected in sera many years before the disease becomes clinically manifest (8). In patients with RA, the presence of anti-CarP antibodies and ACPA is associated with more severe joint damage and inflammation (8, 10). ACPA and anti-CarP antibodies are mostly present in RA patients, but can also be detected in other clinical conditions (11, 12). Interestingly, these antibodies are present in a small subset of SLE patients as well, in which they associate with bone erosions (13). This suggests the possible involvement of autoantibodies in inflammation and joint damage in other conditions than RA.

Therefore, we hypothesized that autoantibodies, like anti-CarP and ACPA, are present in HOA patients and that the presence of these autoantibodies associate with erosive disease.

MATERIALS AND METHODS

Patients and controls

In this study, 196 healthy controls (HC) and 580 primary HOA patients from three different cohorts were included. Consecutive HOA patients visiting the Leiden University Medical Center (LUMC) rheumatology outpatient clinic were included in HOSTAS (n=510) and ECHO (n=47) cohorts. The remaining

patients (n=23) were LUMC participants in the EHOA multicentre study (14-16) (Table 1).

TABLE 1. Baseline characteristics of cohorts

Patient characteristics	HOSTAS (n=510)	EHOA (n=23)	ECHO (n=47)	Controls (n=196)
Age (years)	61.0 ± 8.6	57.1 ± 7.0	63.4 ± 9.2	44.1 ± 13.9
Women	85.7%	73.9%	89.4%	51.0%
BMI (kg/m^2)	27.1 ± 4.7 (n=496)	26.3 ± 4.7	27.4 ± 4.5 (n=45)	23.7 ± 3.0 (n=195)
Ever smoker (former or current)	64.9% (323/498)	65.2%	33.3% (15/45)	34.9% (68/195)
Fulfilling ACR criteria[a]	90.0%	100%	100%	-

mean ± SD, SD=standard deviation, [a]American College of Rheumatology classification criteria for hand OA

The OA patients were included between May 2008 and October 2015. Inclusion and exclusion criteria differed slightly between the cohorts and were described in detail before (14-16). Briefly, patients could participate when primary HOA was diagnosed according to the clinical judgment of the treating rheumatologist, in absence of any other pathological condition that could explain the patient's symptoms (including inflammatory rheumatic diseases, tendinitis, carpal tunnel syndrome, strain, fibromyalgia). In addition, ECHO and EHOA participants had to meet the American College of Rheumatology (ACR) criteria for HOA, and were excluded if they previously tested RF or ACPA positive. EHOA patients also had to have at least one (pre) erosive interphalangeal (IP) joint (i.e. J or E phase) on conventional radiography, according to Verbruggen-Veys (VV) anatomical phases (N-S-J-E-R) (17). Patients with at least one joint in E phase (erosive) or R phase (remodelled) were considered to have EOA. For sensitivity analyses, those with at least one joint in J phase (loss of joint space) were considered to have pre-erosive HOA. Baseline serum samples from all patients were analysed. HC samples were acquired from persons living in the Leiden region, as described before (18). These persons were healthy and without any kind of rheumatic disease. Informed consent was obtained for all individuals and all protocols were approved by the medical ethical committee of the

LUMC. EHOA: EudraCT Number: 2007-003994-18, HOSTAS: CCMO reference: NL26201.058.08, ECHO: CCMO reference: NL17809.058.07

Laboratory analysis of anti-CarP and ACPA (anti-CCP2) IgG, rheumatoid factor IgM and C-reactive protein

In-house ELISAs were used to detect anti-CarP IgG, ACPA IgG and RF-IgM (18). Protein carbamylation was performed and verified as described before (18). Briefly, plates coated with 10µg/ml carbamylated fetal calf serum (CaFCS, Bodinco) or non-modified FCS (Bodinco) were blocked with PBS/1%BSA and subsequently used to detect anti-CarP IgG antibodies in 50 times diluted sera, upon overnight incubation at 4°C. For detection of ACPA IgG, plates were coated with 1µg/ml CCP2-citrulline or CCP2-arginine (produced and provided by Dr. J.W. Drijfhout, IHB, LUMC), for RF-IgM plates were coated with 10µg/ml human IgG (Jackson immune research, 009-000-003) and blocked with PBS/1%BSA. Sera, 50 times diluted for ACPA and 100 times for RF IgM, were incubated for 1 hour at 37°C. Bound human IgG or IgM were detected using matched horseradish peroxidase (HRP) -conjugated secondary antibodies (rabbit-anti-human–IgG/HRP (DAKO P0214) or goat-anti-human–IgM/HRP (Invitrogen, 627520). ABTS (2,2'-azino-bis 3-ethylbenzothiazoline-6-sulphonic acid, Sigma Aldrich, A1888) was added to visualize the HRP enzyme activity and absorbance at 415nm was measured. Levels higher than the mean plus two times the standard deviation (M+2SD) of the specific anti-CarP or RF levels in HC were considered positive. The specific anti-CarP response was calculated by subtracting the response of non-modified FCS from the response of CaFCS. For ACPA IgG the commercially advised cut-off of 25 units was used. Only sera positive for CCP2-citrulline and negative for CCP2-arginine were considered positive.

C-reactive protein (CRP) levels were measured by the Roche Modular P800 (Diamond Diagnostics Inc., Holliston Massachusetts, USA) at the routine diagnostic laboratory of the LUMC.

Radiographs

In HOSTAS, conventional dorsopalmar radiographs of both hands were obtained. The proximal and distal IP joints were scored according to -VVanatomical phases (N-S-J-E-R) (17). Patients with at least one joint in E phase (erosive) or R phase (remodelled) were considered to have EOA, those with at least one joint in J phase (loss of joint space) were considered to have

pre-erosive HOA. Sensitivity analyses were performed using pre-erosive HOA as cut-off for EOA.

All IP joints, metacarpal phalangeal (MCP) joints and first carpometacarpal (CMC-1) joints were scored following the Kellgren-Lawrence (KL) grading scale (0-4, maximum sum score 120) (17). In addition, IP joints, CMC-1 joints and scaphotrapeziotrapezoid (STT) joints were scored for osteophytes and JSN separately using the OARSI atlas (first IP and STT 0-1, other joints 0-3, maximum sum score 58) (17). Scoring was performed by one well-trained reader, blinded for clinical and demographic data. Intra-reader reliability was assessed on a randomly selected sample (n=31) and resulted in high ICCs (EOA-status 0.92, Sum scores: KL 0.95, OARSI osteophytes 0.97, OARSI JSN 0.93).

Statistical analysis

Statistical analyses were performed using statistical package for the social sciences (SPSS) version 23 (IBM). Mann-Whitney U tests were carried out in order to determine differences in anti-CarP antibody levels between total HOA patients and HC, differences in any antibody levels between HOSTAS and HC, and between erosive and non-erosive OA patients within HOSTAS. Moreover, the same statistical test was used to determine differences in radiographic features (KL score, OARSI osteophyte and JSN scores) between anti-CarP IgG or RF-IgM positive and negative HOSTAS patients. Furthermore, the Pearson Chi-Square test was used to determine whether there was a difference in the number of anti-CarP IgG, ACPA IgG and RF-IgM positive subjects between the same groups as described for the antibody levels comparison. The same test was carried out to investigate whether there was a difference in the number of HOSTAS patients with a raised CRP level (>5mg/ml) between anti-CarP IgG or RF-IgM positive and negative patients. P values below 0.05 were considered statistically significant.

RESULTS

Anti-CarP IgG and RF-IgM have a low prevalence in OA patients

Anti-CarP IgG, ACPA IgG and RF-IgM were measured. Prevalences of 3.6% for anti-CarP IgG and 4.1% for RF-IgM were observed in the control population, using M+2SD in HC as cut-off. The prevalence of ACPA IgG was 1.5% in HC, using the commercially available cut-off. Applying the same methods, a prevalence of 6.6% was observed for anti-CarP IgG in the total HOA

population, this was not significantly different from the control population (p=0.12). The level of anti-CarP IgG was also not different between the total HOA group and HC (p= 0.37). In HOSTAS, a prevalence of 7.1% was observed for anti-CarP IgG, 0.8% for ACPA IgG and 6.1% for RF-IgM. These prevalences were not significantly different compared to HC (anti-CarP p=0.08; ACPA p=0.37; RF p=0.30). Furthermore, for the most prevalent autoantibodies, anti-CarP IgG and RF-IgM, antibody levels did not differ between these groups (anti-CarP p=0.67; RF p=0.82). Dot plots for anti-CarP IgG in HC and total HOA is depicted in figure 1A. All tested autoantibodies in HOSTAS are depicted in figure 1B.

Although previously known RF-IgM or ACPA IgG seropositivity was an exclusion criterium in the EHOA and ECHO cohorts, it was not mandatory to test for these antibodies, explaining why we found two of these patients to be RF-IgM positive (1 EHOA and 1 ECHO), however all were ACPA negative (data not shown).

In HOSTAS, three patients developed RA during a follow-up time of 2.0 to 8.4 years: one RF-IgM positive patient was diagnosed with RF+/ACPA- RA after 28 months of follow-up, and two triple-antibody negative patients developed RF+/ACPA- and RF+/ACPA+ RA after 46 and 61months follow-up time, respectively. The antibody levels, clinical and demographic features were not significantly different for the patients who developed RA compared to the remainder of the HOSTAS cohort (data not shown).

Furthermore, only four patients (all HOSTAS) were positive for more than one autoantibody: two for anti-CarP and ACPA, one for anti-CarP and RF, and one for ACPA and RF; none developed RA during a follow-up time of 3.4 to 6.1 years. Merely one anti-CarP (out of 36 positive) and one ACPA (out of 4 positive) positive patient had increased CRP, in contrast to none of the outliers (anti-CarP 2000AU/ml, ACPA 1600AU/ml, RF-IgM 200IU/ml) in all HOA patients. Moreover, these patients did not differ from other HOA patients in terms of demographic features or radiographic damage (data not shown).

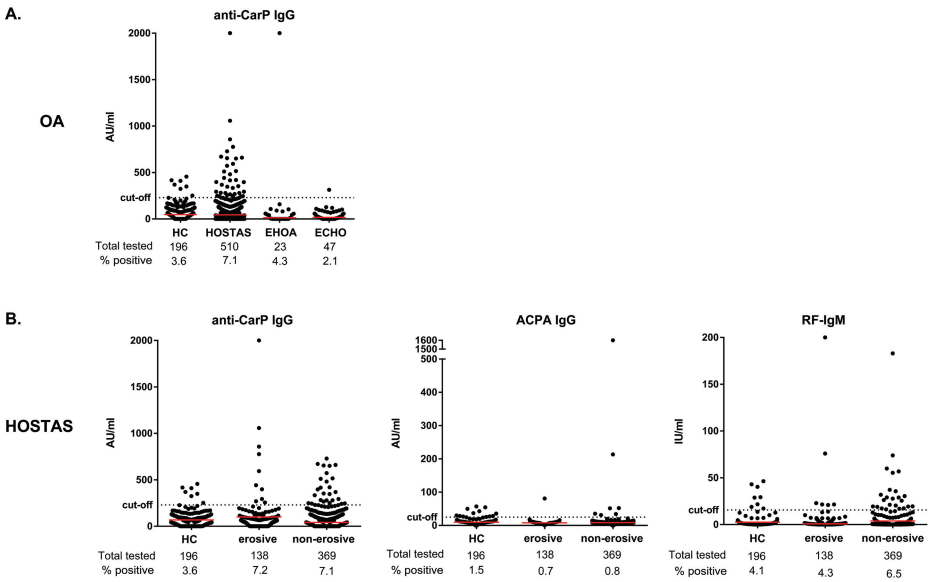

FIGURE 1. Autoantibodies in hand OA patients of HOSTAS, EHOA and ECHO. ELISAs were performed to detect anti-CarP antibody, ACPA and RF in sera of 196 HC and 580 HOA patients (510 HOSTAS, 23 EHOA and 47 ECHO) (A). The HOSTAS group was subdivided in erosive and non-erosive HOA (B). The mean plus two times the standard deviation in HC was established as the cut-off for the anti-CarP antibody and RF. For ACPA a cut-off of 25 AU/ml was used. The red line is the median and the dotted line represents the cut-off. The number of samples tested in each group and the prevalence, corrected for the response on non-modified FCS or CCP2-arginine, is shown below the graphs. Mann Whitney U test for level and Pearson Chi-Square test for prevalence were performed in erosive versus non-erosive OA. HC; healthy controls, HOA; hand osteoarthritis, anti-CarP antibody; anti-carbamylated protein antibody, ACPA; anti-citrullinated protein antibodies, RF; rheumatoid factor, AU/ml; arbitrary units per millilitre, IU/ml; international units per millilitre.

Anti-CarP IgG and RF-IgM are not associated with erosive disease in OA

To investigate whether the presence of autoantibodies was different in EOA compared non-erosive HOA, the largest available cohort, HOSTAS, was used, in which 27.2 percent of the patients (n=138) had EOA. Neither the frequency (anti-CarP IgG p=0.94, RF-IgM p=0.36) nor the levels (anti-CarP IgG p=0.69, RF-IgM p=0.53) of the most prevalent autoantibodies were increased in EOA patients compared to non-erosive HOA (Figure 1B). To explore the possibility that using VV anatomic E and R phase as cut-off for EOA is too conservative, causing patients with early erosive disease to be

miscategorised, we performed the same analyses with the preceding J phase in at least one joint as cut-off for EOA. This more sensitive approach yielded similar results (data not shown).

Furthermore, no associations were found between the presence of anti-CarP or RF-IgM and increased radiographic damage or inflammation (i.e. CRP level >5mg/L) (Table 2).

TABLE 2. Other clinical features of HOSTAS

	anti-Carp + (n=36)	anti-Carp - (n=471)	P-value	RF + (n=30)	RF - (n=477)	P-value
Sum-score Kellgren-Lawrence (0-120)[a] median (IQR)	17.5 (9.5-30.75)	18.0 (8.0-29.0)	0.74	13.5 (8.75-30.5)	18.0 (8.0-30.5)	0.59
Sum-score OARSI osteophytes (0-58)[a] median (IQR)	9.5 (6.0-19.0)	10.0 (4.0-18.0)	0.64	8.0 (4.75-16.25)	10.0 (4.5-18.0)	0.74
Sum-score OARSI joint space narrowing (0-58)[a] median (IQR)	5.5 (3.0-16.75)	7.0 (3.0-17.0)	0.72	6.5 (2.75-18.0)	7.0 (3.0-17.0)	0.98
CRP raised (>5.0 mg/L)[b] % (n/total)	11.5 (3/26)	10.4 (34/326)	0.86	7.7 (2/26)	10.7 (35/326)	0.63

[a]IQR= interquartile range. For 3 patients no hand radiographs were available. Mann-Whitney U test was performed.
[b]percentage above cut-off level of 5.0 mg/L. Only 352 patients had their CRP levels measured within 22 days from baseline. Pearson chi-square test was performed.

DISCUSSION

In this study, we analysed the presence of anti-CarP IgG, ACPA IgG and RF-IgM in patients with HOA. To our knowledge, this is the first time that autoantibodies are measured in such a large group of OA patients. The results indicate that only few HOA patients are positive for autoantibodies and these autoantibodies are not linked to erosive disease or inflammation in OA, which is in contrast to other inflammatory diseases in which bone erosions are present, such as RA and SLE (8, 13). We confirmed earlier results showing limited prevalence of ACPA in HOA (19). Moreover, we detected similar prevalence of RF-IgM in HOA as in a previous (small) study and confirmed that there are no detectable differences in RF-IgM between EOA and non-erosive OA (20). In another previous small study, all EOA patients were negative for RF isotypes (21), supporting the fact that RF does not play a role in EOA.

Myeloperoxidase (MPO) is one of the enzymes involved in carbamylation and the levels of MPO are increased during inflammation (8). Interestingly, HOA patients do have higher serum levels of MPO than HC (22). Moreover, EOA have even higher serum levels of MPO compared to non-erosive EOA, which is in line with the presence of more inflammatory signs in EOA compared to non-erosive HOA (4, 22, 23), as MPO is increased during inflammation. Similarly, RA and SLE patients also have increased serum MPO levels compared to HC (24) and RA patients with more inflammatory signs have higher levels of MPO than less inflammatory RA patients. Although it is currently unknown whether the inflammation associated increased levels of serum MPO are associated with increased carbamylation of proteins (in joints), it is conceivable that increased MPO levels can lead to more carbamylation and potentially break of tolerance towards carbamylated proteins. Previous studies analysed the presence of anti-CarP antibodies in sera of patients suffering from conditions that are known to have an increased degree of carbamylation due to: increased levels of urea (renal failure), increased levels of thyocyanate (smoking) or chronic inflammation (inflammatory bowel disease) (12). Compared to RA patients, the percentage anti-CarP positivity was much lower and, with the exception of renal failure, not different from healthy controls (12). Collectively, the current data and these previous studies indicate that increased protein carbamylation, e.g. via increased levels of MPO, is not sufficient to drive anti-CarP antibody production.

In a case report, carbamylated proteins were shown to be present in both erosive and non-erosive joints in RA (25) and our work also indicates that carbamylated proteins are present in the joints of OA patients (manuscript in preparation). Carbamylated proteins can therefore be present in OA with no apparent breach of tolerance against these antigens. Whether this is due to the type of protein that is carbamylated, its localization, the degree of carbamylation or other factors remains to be investigated in future studies.

A strength of this study is the replication of autoantibody measurements in three different cohorts. Since the HOSTAS cohort comprehends patients in all disease stages, from early to severe, we were able to also study the link between radiographic damage and antibody status.

A limitation of this study is that HC and OA patients are not age and sex matched, as it is difficult to achieve such a large group of HC around the age of 60 years, 80% women and completely free of hand OA. The HC group was used to set the cut-off for anti-CarP positivity and it was considered more important to use a control group that is reflecting the overall population of the area in which the disease population is living. Importantly, we did not observe a relation between the presence of anti-CarP antibody in HC with gender, age or smoking (unpublished data).

The erosiveness in OA and RA are different from each other with mainly central erosions in OA and marginal erosions in RA (26), suggesting different mechanisms for the driving of erosive disease. Our findings also support this hypothesis as we observed just a small subset of HOA patients to be positive for the RA-associated autoantibodies, anti-CarP IgG, ACPA IgG or RF-IgM, and we did not observe an association between the presence of these autoantibodies with erosive disease or inflammation in OA. Altogether, the data suggests that another mechanism, possibly independent of autoantibodies, is driving erosive disease in HOA.

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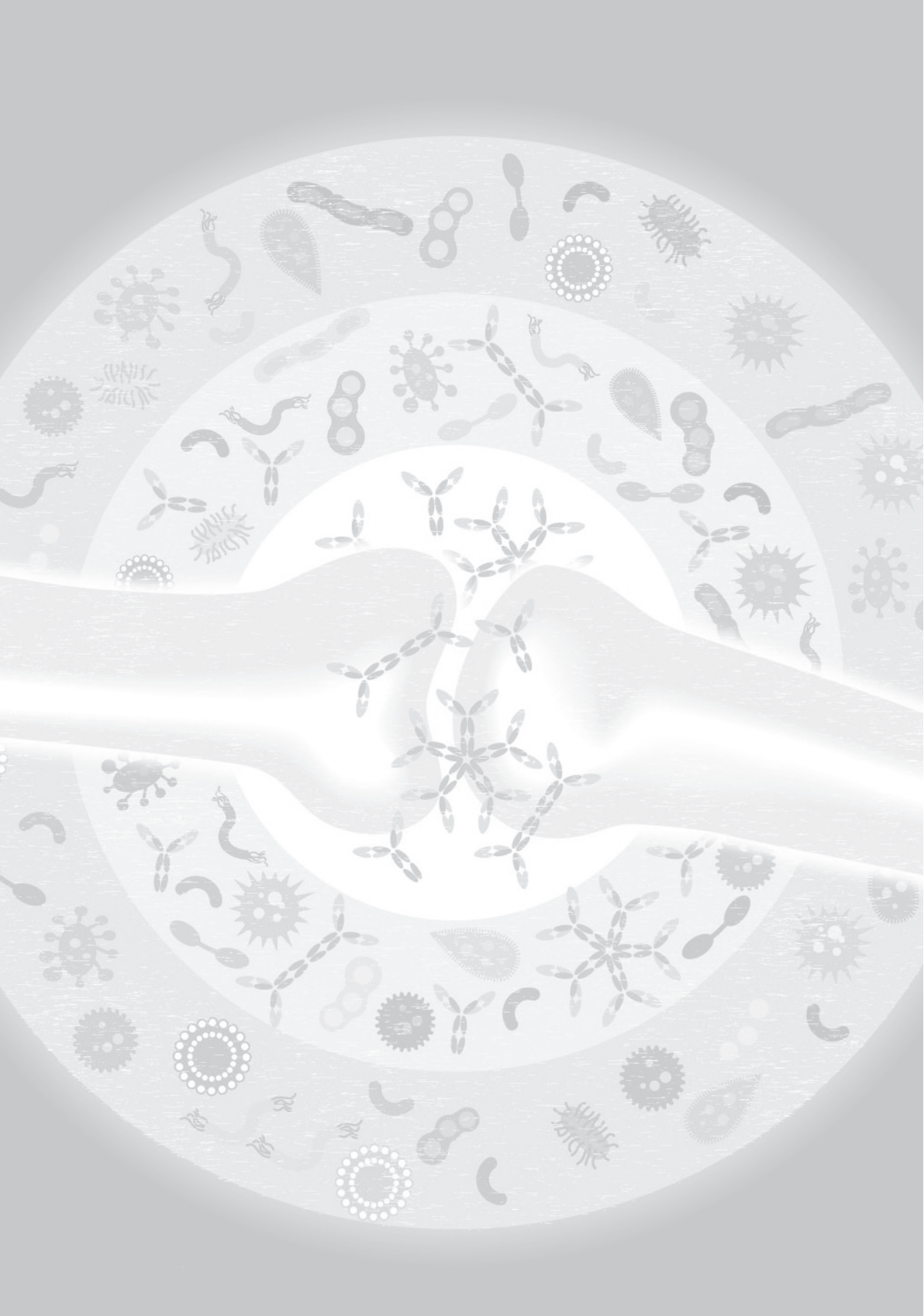

CHAPTER 9

Summary and Discussion

Part of this discussion is submitted as a review

Rheumatoid arthritis (RA) is an autoimmune disease principally affecting synovial joints. Various immunological processes play a role in the development and continuation of the disease and the presence of autoantibodies is a hallmark for the disease. Well known autoantibodies in RA are rheumatoid factor (RF), anti-citrullinated protein antibodies (ACPA) and anti-carbamylated protein (anti-CarP) antibodies [1].

The focus of this thesis was to unravel the anti-CarP antibody response in rheumatic diseases with an emphasize on RA (part 1 and 3). Furthermore, the possible origin of autoantibody responses present in RA was studied (part 2).

Part 1: Characterization of the anti-CarP antibody response in RA

In the first part of this thesis the characterisation of the anti-CarP antibody response in RA-patients is described. To do so the anti-CarP isotype and IgG-subclass usage, the avidity and the presence of variable (V)-domain glycans on anti-CarP IgG was investigated. The results in ***chapter 2*** show that the anti-CarP antibody response uses a broad spectrum of isotypes and IgG-subclasses, including IgM, IgG_{1-4} and IgA On population level we have found that the isotype and IgG-subclass usage are quite similar for anti-CarP antibodies and ACPA, except for IgG_2.Within individual patients , the anti-CarP and ACPA pattern could be different. These data indicate that anti-CarP antibodies and ACPA are not merely a reflection of a cross-reactive antibody, but rather be two types of autoantibody families. This observation is further strengthened by the absence of a clear correlation between the anti-CarP and ACPA isotypes and IgG-subclasses; the only correlation found was for the presence of anti-CarP IgM or ACPA IgM, respectively, and RF-IgM. Surprisingly we observed the presence of anti-CarP IgM in conjunction with anti-CarP IgG and/or IgA. This might indicate an ongoing immune response which is continuously reactivated by (anti-CarP) IgM producing B cells as IgM antibodies normally have a short half-life of a few days [2], and switching towards IgG in the presence of T cell help is typically associated with the disappearance or strong decline in the IgM-responses [3].

Interestingly, as mentioned earlier, we observed a low use of IgG_2 in the ACPA response and there was apparently a high prevalence of IgG_2 in the anti-CarP response. It has been suggested that the presence of IgG_2 is indicative for a T cell independent polysaccharide antigen response (from bacteria) [4].

Whether this is the case for the anti-CarP antibody response is unknown. Future research will be necessary to study whether infections could lead to the induction of anti-CarP antibodies. For example, patients with periodontal disease can be screened for the presence of anti-CarP antibodies before and after they encounter severe periodontitis and chronic inflammation of the gums. Moreover, it would be interesting whether patients who were treated for various infectious disease which could be associated with arthritis, like tuberculosis, rubella, Ebstein Barr Virus and arboviruses[5], developed anti-CarP antibodies.

In this thesis, we focussed on the presence of anti-CarP antibody isotypes and subclasses in baseline RA samples. For future work it would be interesting to investigate the isotype and IgG-subclass usage before disease onset. This will result in more insight in the development of the anti-CarP isotype and IgG-subclass response. Maybe this will give information about an antigen type to which the response is primarily directed.

In ***chapter 3*** the avidity (overall binding strength) of anti-CarP antibodies is described as well as the association of avidities with joint erosions. The avidity of ACPA has been previously investigated by our group showing a lower avidity for ACPA IgG compared to recall responses like tetanus and diphtheria toxoid [6]. The avidity data described in this thesis indicate that the anti-CarP IgG response is of low avidity in both serum and synovial fluid compared to the avidity of the recall antibody against tetanus toxoid. Interestingly, the anti-CarP IgG avidity is even lower than the ACPA IgG avidity in serum. For ACPA, the lowest avidity quartile is associated with more bone damage [7], however, this pattern is not seen for the anti-CarP avidity. Yet, almost all anti-CarP avidity measurements falls within the lowest avidity quartile of ACPA, which probably makes it difficult to detect any differences for anti-CarP. Surprisingly for us was despite the presence of isotype switching for anti-CarP before disease onset, no clear avidity maturation has been observed for anti-CarP IgG in the time period, before disease onset, analysed in this thesis. This suggests that the isotype switch and avidity maturation in the anti-CarP B cell response are uncoupled. The mechanism for this is unknown, but an explanation is that it might be due to a difference in additional stimulation of the B cells; such as the degree of innate or T cell help (reviewed in [8]) and/or the abundance of its antigen. Probably low avidity antibodies are a marker for chronic antigen

overload and chronic antibody responses. Moreover, it could be that anti-CarP antibodies and ACPA cross react with a currently unknown antigen, to which both antibodies might have a more "normal" response. But as long as "the" autoantigen, if any, leading to RA is unknown, it is difficult to study this concept.

As anti-CarP antibodies and ACPAs seem to be two differentially regulated autoantibody responses, by means of isotype and IgG-subclass usage and IgG avidity, described in this thesis, we wondered whether anti-CarP IgGs could be glycosylated in the V-domain. This because for ACPA it is known that the IgG isotype is highly V-domain glycosylated and harbour bi-antennary highly-sialylated N-linked glycans [9, 10]. Our findings regarding the presence of V-domain glycans on anti-CarP IgG and IgG_1 are described in ***chapter 4***. A larger size was only occasionally detected for anti-CarP IgG and IgG_1 by size exclusion chromatography and western blot analysis, suggesting that anti-CarP IgG antibodies could have glycans in their V-domain. Similar results were observed when performing sialylated glycan analysis using High Performance Liquid Chromatography (HPLC) or affinity chromatography using a *Sambucus Nigra* Agglutinin (SNA) column. Overall, these data indicate that anti-CarP IgG antibodies can be V-domain glycosylated, however they are much less glycosylated as compared to ACPA. Importantly, the occurrence of anti-CarP V-domain glycosylation is higher in anti-CarP and ACPA double positive compared to anti-CarP single positive RA-patients. This could be explained by some cross-reactive anti-CarP antibodies and ACPAs. Yet, most of the anti-CarP IgG antibodies bind better to citrullinated antigens than the binding of ACPA IgGs to carbamylated antigens [11] (and chapter 4), this suggests that the percentage glycosylation in the V-domain for ACPA is probably an underestimation as anti-CarP antibodies could be co-isolated using citrullinated antigens as well. Further, ACPAs can be co-isolated using carbamylated antigens, which could result in an overestimation of the percentage glycosylation in the V-domain of anti-CarP. As almost no increased glycosylation has been detected in the few anti-CarP single positive patients, more research is necessary to study whether non cross-reactive anti-CarP antibodies in anti-CarP and ACPA double positive patients have an increase in V-domain glycosylation.

Overall, these data indicate again that the ACPA and the anti-CarP antibody response are differentially regulated.

Part 2: Possibilities for a mucosal origin of autoantibodies in rheumatoid arthritis

The most prominent antibody present at mucosal areas is IgA. It is typically locally produced by plasma cells as a dimeric molecule, dimeric IgA (dIgA) including a joining-chain (J-chain) [12, 13]. Previous data of our department showed that RF IgA is predominantly present in its polymeric form in serum, synovial fluid and saliva of RA-patients [14]. Moreover, both anti-CarP and ACPA IgA are detected in sera and synovial fluid of RA-patients [15-17]. Furthermore, ACPA IgA has been observed in saliva of some RA patients [18]. Hence, we tried to investigate whether autoantibodies in RA could be of mucosal origin, which is described in the second part of this thesis. To better understand the nature of the anti-CarP and ACPA autoantibodies, we investigated the molecular composition of both IgAs (***chapter 5***). To our surprise anti-CarP IgA was predominantly detected in the high molecular weight (HMW), polymeric, IgA fractions, whereas ACPA IgA was predominantly present in the lower molecular weight (LMW), monomeric, IgA fractions. The shift of anti-CarP IgA to the HMW fractions was detected irrespective of ACPA and/or RF positivity. This HMW anti-CarP IgA could be suggestive for a mucosal origin of anti-CarP antibodies, as a similar pattern was observed for anti-*E.coli* IgA.

As anti-CarP IgA could even be detected in the very HMW fractions, we wished to address the question whether anti-CarP IgA had a part of the poly-immunoglobulin-receptor (pIgR) known as secretory component (SC) attached to it. Both IgA and IgM harbour a J-chain that can bind to the pIgR [19]. Normally, upon transfer through epithelial cells, IgM and IgA antibodies are cleaved at the luminal site leaving a fragment of the pIgR, known as SC. Although the mechanism is still unclear, SC containing antibodies can be detected in the circulation [20]. To know whether anti-CarP IgA contain the SC, which might than be an explanation for the HMW, we investigated the presence and isotype usage of secretory anti-CarP, ACPA and RF in RA-patients (***chapter 6***). Unexpectedly the secretory form of anti-CarP was predominantly observed in the IgM isotype and the same results were achieved for ACPA and RF. After specific isolation of anti-CarP IgM and IgA, SC was only detectable in the HMW IgM and not the IgA compartment using mass-spec analysis (data not shown). Therefore, the presence of SC could not be the explanation for the HMW of anti-CarP IgA. However, the presence of SC-containing IgM autoantibodies might indicate that autoreactive IgM B

cells represent the most prominent B cell subset that can be (re)activated at mucosal surfaces and thereby keeping the immune response ongoing. The mechanism how SC-IgM autoantibodies end up in the circulation remains unclear. Possibilities are cleavage at the basolateral site of the epithelium or back leakage into the systemic circulation due to inflammation and damaged epithelium.

Future perspectives for IgA responses in (auto)immunity

Mucosal origin

Although we were able to detect J-chain in isolated anti-CarP IgA antibodies, it is currently unknown if and why anti-CarP IgA is secreted as polymeric IgA containing the J-chain. If this turns out to be true in future research, this might suggest that the anti-CarP antibody response could be of mucosal origin, as in case of a mucosal immune response, polymeric IgA including the J-chain is normally produced by B plasma cells at the lamina propria [12, 21]. To investigate whether the J-chain is present for anti-CarP, it would be interesting to isolate anti-CarP (IgA) B cells and study them for the presence of J-chain on mRNA and protein level. Furthermore, it would be interesting to examine whether these B cells can secrete polymeric IgA.

To investigate the mucosal origin of anti-CarP antibodies (or autoantibodies in RA) in more depth, it is interesting to study the presence of carbamylated and/or citrullinated antigens in mucosal tissue, like gut (faeces), lung and periodontal tissue or fluid. Yet, some carbamylation and citrullination can be detected in lung tissue of (ex-)smokers [22] and in mild and moderate periodontitis tissue [23]. However, most of the experiments on lung tissue were performed using the 'Senshu' method, which cannot discriminate between carbamylation and citrullination [11, 24]. Unfortunately, a non-specific anti-citrullinated protein antibody was used to stain periodontitis tissue [24]. Therefor mass-spec analysis will be important to study the presence of carbamylated or citrullinated proteins in (mucosal) tissue [24]. Moreover, it would be interesting to examine whether the microbiome, and more specifically which species of bacteria, can be recognized by isolated polyclonal anti-CarP and/or ACPA IgA.

Recently, the group of Scheel-Toelner et al. identified an enrichment of Fc receptor like 4 (FcRL4) positive B cells in the RA synovium. FcRL4 positive B cells are normally a marker for mucosal associated B cells [25-27] and FcRL4

acts as a low affinity IgA receptor [28]. Although the most prominent isotype expressed by FcRL4 positive B cells in RA is IgG, IgG is equally expressed over the FcRL4 positive and negative subsets. However, in RA-patients, IgA B cells are increased in the FcRL4 positive B cell subset compared to the FcRL4 negative B cells and some ACPA reactivity was only found in the IgA-FcRL4 positive B cell subset [29, 30]. It would be interesting to know whether these FcRL4 positive IgA B cells consist of anti-CarP B cells as well and whether these B cells, when stimulated, produce (polymeric) anti-CarP IgA. These data might strengthen the link between the mucosa and RA and the possibly mucosal associated origin for autoantibodies like anti-CarP in RA.

Active immune response

Till now, dimeric IgA is predominantly described as specific for mucosal immune responses. However, antigen specific polymeric IgA and secretory antibodies have also been detected in serum after non-mucosal immunisation [31]. Moreover, RF IgA is detected in a polymeric form in sera of RA-patients which can be secreted into the saliva as well [14]. However, this RF IgA pattern was not observed in sera of 2 healthy controls, instead a monomeric RF-IgA pattern was observed (data not shown).

Both polymeric IgM and IgA harbour a J-chain which is of importance to pass the epithelial layer and to be secreted into the mucosal fluid. As at mucosal areas the immune response is continuously activated by the microbiome or pathogens, polymeric IgM and IgA are constantly produced by activated B cells in that area. Nevertheless, predominantly an antigen specific HMW, polymeric, IgA response was detectable in serum after an intranasally (mucosal) and subcutaneously (non-mucosal) immunization [31]. This might indicate that a polymeric IgA response does not in particular point towards a mucosal immune response but potentially points towards an active immune response. Perhaps, polymeric IgA is just a reflection of an active immune response against that specific antigen.

I hypothesize that during a current or active immune response, a B cell receives a specific B cell receptor, toll like receptor and cytokine signal which will activate the B cell for the production of the J-chain. In case of an IgM or IgA B cell, this will result in the production and secretion of J-chain containing polymeric IgM or IgA. Because these polymeric J-chain containing IgM and IgA can bind the pIgR, it can be transported through the epithelial layer and

secreted at the luminal site as SC-IgM and SC-IgA. Hence, polymeric IgM and IgA antibodies of current infections can be secreted in breast milk as well and thereby protecting the offspring for currently available infections. Moreover, these activated B cells might travel to other parts of the body where necessary.

Altogether, to study this hypothesis, it would be interesting to investigate whether the difference in IgA composition is due to the presence of a current or active immune response. A possibility is to examine this feature in vaccination studies before and after giving a first immunization or a booster (e.g. anti-tetanus toxoid vaccination). Another option could be isolating and stimulating naïve or memory B cells *in vitro*, using various stimuli to mimic active mucosal and systemic infections or immune responses, and study the presence of J-chain on mRNA and protein level.

Effector functions on immune cells

In the recent years it has become more clear that IgA and its Fc-alpha receptor have various effector functions depending on the environment and type of activation [32-36]. Moreover, indications about the contribution of IgA complexes to a more severe or worse disease in e.g. RA rises [35, 37]. Recent published [37-40] and unpublished data (Heineke et al. and van Gool et al. Abstract ECI 2018) show the different effector functions of IgG and IgA on neutrophils and monocytes. A more pro-inflammatory response was induced by IgA as shown by the induction of cytokine and chemokine release by both neutrophils (e.g. IL-8, TNF-α) and monocytes (e.g. IL-6, IL-4, TNF-α) and the release of pro-inflammatory lipids by neutrophils (e.g. leukotriene B4) [39]. For RA it is known that anti-CarP antibodies, ACPA and also RF uses a broad spectrum of isotypes [15-17, 41, 42]. Moreover, RF IgA is associated with bone erosions in RA [43, 44] and unpublished data of our group indicate a more severe disease course over time in anti-CarP IgA positive compared to negative RA-patients. Furthermore, anti-CarP and RF IgA seem to be present in a more polymeric form in RA-patients, which does not seem to be the case for ACPA IgA [14](this thesis).

Overall, these data suggest an important role for IgA in RA development and propagation. It will be interesting to study the possible effector functions of anti-CarP and ACPA IgA (and IgG) on e.g. neutrophils and monocytes. Moreover, studying the potential (in)direct effector functions

on chondrocytes and osteoclasts would be interesting to better understand the development of bone erosions in RA. All these cells seem to play an important role in RA pathogenesis and are probably differently affected by anti-CarP and ACPA IgA (and IgG).

Hypothesis for the autoreactive IgA immune response in Rheumatoid Arthritis
Various hits, besides genetic and environmental risk factors, like a trauma, infection or vaccination, might activate the immune response and the production of autoantibodies (anti-CarP antibodies, ACPA and RF) in RA patients. Next to this, carbamylated proteins are detectable in synovium, synovial fluid but mostly in cartilage of RA-patients, OA-patients and HC (Verheul et al. submitted). However, RA-patients can produce antibodies recognizing these carbamylated proteins [17, 45-48]. In addition, neutrophils are the most prominent cell type present in synovial fluid of RA-patients and this influx seems to be continuously reactivated, as weeks after an arthrocentesis neutrophils are detected in the SF again (Unpublished data Brouwers et al.) [49, 50].

Due to the presence, and probably accumulation, of carbamylated proteins in the joint, anti-CarP antibodies might bind to these proteins, thereby forming immune complexes of anti-CarP and RF IgA. Once a neutrophil passed by, this could lead to the attraction of more immune cells, as IgA immune complexes could crosslink the Fc-alpha receptor (FcαR) on neutrophils and subsequently inducing LTB4 (and IL-8) release which lead to the attraction of even more neutrophils [37, 39, 40] (Heineke et al., Abstract ECI 2018). Moreover, activation of neutrophils by immune complexes could also lead to the induction of various other pro-inflammatory responses, like cytokine release, reactive oxygen species (ROS), myeloperoxidase release (MPO) and the formation of neutrophil extracellular traps (NETs) [35, 37] (Heineke et al. ECI 2018). The released MPO by neutrophils might potentially result in an increased carbamylation in the joint.

After an arthrocentesis, synovial fluid is released from the joint, however the synovium is still inflamed, carbamylated antigens are still available and the autoantibodies are still present, resulting in reactivation of the same reaction. Because the antigen is still available, neutrophils will not go into apoptosis, therefore, clearance of apoptotic cells by macrophages, which induces anti-inflammatory signals [51], is not occurring. Joint erosions will

occur, as the body tries to clear the (carbamylated) antigens, probably by simultaneously activation and interplay of various immune cells with other cells like osteoclasts and fibroblasts.

Part 3: anti-CarP antibodies in other rheumatic diseases

In the final part of this thesis the presence of anti-CarP antibodies in other rheumatic diseases than RA, in which patients can develop bone erosions as well, was investigated. In **chapter 7** the presence of anti-CarP antibodies in Systemic Lupus Erythematosus (SLE) is described. These data showed thatanti-CarP antibodies were slightly higher present in SLE patients compared to ACPAs and only a minor overlap of these antibodies was observed. Interestingly, we found that the presence of these autoantibodies, both anti-CarP and ACPA, associates with joint erosions. This might be because overlapping symptoms of RA can be seen in a subgroup of SLE patients which is probably important for the arthritic phenotype in some SLE patients. This overlap feature between SLE and RA is often termed Rhupus. However, it is still a debate whether Rhupus represents a single distinct disease or whether it is an overlap disease [52, 53]. More research is necessary to really understand the pathogenic mechanism of Rhupus and which factors contribute to the onset and progression of the disease. This because the therapy and outcome of Rhupus patients could differ from RA or SLE patients alone.

Another disease characterized by cartilage damage, bone abnormalities and inflammation is osteoarthritis (OA) [54]. The most prevalent clinical phenotype of OA is hand OA (HOA) which can be divided in erosive and non-erosive HOA. Because anti-CarP antibodies and ACPAs are present and associated with bone erosions in some rheumatic disease (RA and SLE) [15, 17, 55], the presence of these autoantibodies and their association with erosive disease in OA was examined (***chapter 8***). Although a few of the HOA-patients were positive for autoantibodies, this positivity was not associated with erosive disease. These data suggest that another mechanism, probably independent of autoantibodies, is driving erosive disease in HOA. Data about the types of erosiveness in OA and RA also support this hypothesis, as in OA mainly central erosions and in RA mainly marginal erosions are detected [56]. Although carbamylated proteins are present in the joints of OA-patients and RA-patients (Verheul at al. submitted), there is no apparent

break of tolerance against these antigens in OA-patients. Future studies are needed to examine the mechanism that drives erosive disease in OA.

Conclusion

From this thesis it can be concluded that anti-CarP antibodies and ACPAs represent two different types of autoantibody families. The responses seem to be differently regulated based on their isotype and IgG-subclass usage, avidity and V-domain glycosylation. Furthermore, they seem to have a different origin, with probably a more mucosal associated origin for anti-CarP antibodies based on their IgA composition.

Moreover, the RA-associated autoantibodies anti-CarP and ACPA are less prevalent in other rheumatic diseases and they are not always associated with bone erosions in these diseases. This indicates that various mechanisms, autoantibody dependent and independent, underly the development of bone erosions.

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PART 4

Addendum

NEDERLANDSE SAMENVATTING

Dit proefschrift

Reumatoïde artritis (RA) is een auto-immuunziekte die vooral in gewrichten voorkomt. Verschillende immuunprocessen spelen een rol bij de ontwikkeling en het verloop van de ziekte. De aanwezigheid van auto-antilichamen is een kenmerk van RA en heeft wellicht een belangrijk immunologisch aspect. De meest bekende auto-antilichamen van RA zijn anti-gecitrullineerde eiwit-antilichamen (ACPA), anti-gecarbamyleerde eiwit-antilichamen (anti-CarP-antilichamen) en reumafactor (RF) [1]. Het doel van dit proefschrift is het ontrafelen van de anti-CarP antilichaamreactie in reumatische ziektes. Hierbij ligt de focus op RA. Aanvullend wordt er gekeken naar de mogelijke oorsprong van auto-antilichamen bij RA.

Deel 1: Karakterisatie van de anti-gecarbamyleerde eiwit-antilichaam reactie in reumatoïde artritis

Het eerste deel van dit proefschrift beschrijft de karakterisatie van de anti-CarP reactie in RA-patiënten. Er wordt gekeken naar: het gebruik van antilichaam isotypes en immunoglobuline (Ig) G-subklassen, de aviditeit (bindingssterkte) van antilichamen én de aanwezigheid van glycanen (suikers) in het variabele domein van anti-CarP IgG. **Hoofdstuk 2** beschrijft dat de anti-CarP-antilichaam reactie een breed spectrum van isotypen en IgG-subklassen gebruikt. Dit gebruik van isotypen en IgG-subklassen is, op IgG_2 na, vrijwel hetzelfde bij anti-CarP-antilichamen en ACPA. Dit geldt overigens alleen op populatieniveau. Als we kijken naar individuele patiënten, kan het gebruik van anti-CarP-antilichamen en ACPA verschillen. Verrassend is dat de anti-CarP reactie redelijk veel IgG_2 gebruikt. Dit is interessant omdat IgG_2 normaal gesproken een indicatie is voor een T-cel onafhankelijke polysacharide antigeen respons [2]. Ook verrassend is dat anti-CarP IgM tegelijkertijd aanwezig kan zijn met anti-CarP IgG en/of IgA. Hierdoor lijkt het erop dat de anti-CarP-antilichaam reactie een doorlopende immuunrespons is die steeds opnieuw wordt geactiveerd door anti-CarP IgM B-cellen. Voor de toekomst is het belangrijk om te onderzoeken wat het isotype en IgG-subklassegebruik is van anti-CarP antilichamen voordat de ziekte zich ontwikkelt. Zo kunnen we te weten komen wanneer welke isotypen en IgG-subklassen gebruikt worden en hoe de respons zich ontwikkelt.

In **Hoofdstuk 3** wordt de aviditeit van anti-CarP IgG beschreven én vergeleken met de aviditeit van antilichamen tegen tetanus toxoïde. Daarbij wordt een lage aviditeit voor anti-CarP antilichamen waargenomen in zowel serum als synoviaal vocht. Bovendien blijkt dat bij serum de aviditeit van anti-CarP IgG lager is dan bij ACPA IgG. Voor ACPA is bekend dat de laagste avide ACPAs geassocieerd zijn met meer botschade [3]. Bij anti-CarP antilichamen kunnen we dit effect niet detecteren. Mogelijk komt dat doordat de totale aviditeit van anti-CarP IgG minstens zo laag is als de laagst avide ACPAs. Normaal vinden isotype wissel en aviditeit maturatie bij een T-cel afhankelijke respons zij aan zij plaats. Ondanks dat er een anti-CarP isotype wissel is voor de start van de ziekte (breed spectrum aan isotypes en IgG-subklassen), is er geen aviditeit maturatie detecteerbaar voor anti-CarP IgG voor ziekte aanvang. Dit suggereert dat de isotype switch en aviditeit maturatie van de anti-CarP B cel respons ontkoppeld zijn.

Zoals eerder gezegd, het verhoogde gebruik van IgG_2 in de anti-CarP antilichaam respons kan suggestief zijn voor een polysacharide antigeen reactie. Hiernaast is ook een gelimiteerde of afwezige affiniteit maturatie beschreven voor deze respons (gereviewd in [4]). Dit type respons wijst vermoedelijk op een meer bacteriële of mucosale reactie van anti-CarP antilichamen. Omdat de anti-CarP en ACPA respons verschillend gereguleerd lijken te zijn, is bestudeerd of anti-CarP IgG ook een verhoogde glycosylatie van het variabele domein heeft. Voor ACPA IgG is het bekend dat het een hoge mate van V-domein glycosylatie heeft [5, 6]. De bevindingen over de aanwezigheid van V-domein glycanen op anti-CarP IgG en IgG_1 zijn beschreven in **hoofdstuk 4**. Uit de analyse van de serum fractionering, op basis van molecuul grootte, en western blot analyse blijkt dat een verschuiving naar een groter molecuulgewicht voor anti-CarP IgG en IgG_1 soms aanwezig is. Dat kan suggereren dat anti-CarP IgG antilichamen soms ook groter zijn en wellicht V-domein glycanen hebben. Deze data is bevestigd met gesialileerde glycaan analyses via High Performance Liquid Chromatografie (HPLC)-analyse en Sambucus nigra agglutinine (SNA) kolommen. Alles bij elkaar laat dit zien dat anti-CarP IgG glycanen kan hebben op zijn V-domein. Al zijn ze lang niet zo hoog geglycosyleerd als ACPA. De meest verhoogde aanwezigheid van V-domein glycanen op anti-CarP IgG zien we als we anti-CarP en ACPA dubbel positieve RA-patiënten vergelijken met anti-CarP singel positieve RA-patiënten. Dit kan verklaard worden door de kruisreactiviteit tussen anti-CarP antilichamen

en ACPAs. Hierdoor zie je wellicht ook wat ACPA glycosylatie in die anti-CarP antilichamen. Meer onderzoek is nodig om te zien of niet-kruisreactieve anti-CarP antilichamen in anti-CarP en ACPA dubbel positieve patiënten een verhoogd V-domein glycosylatie hebben of niet. Maar over het algemeen laat ook deze data zien dat de productie van anti-CarP antilichamen en ACPAs anders gereguleerd is.

Deel 2: Aanwijzingen voor een mucosale oorsprong van auto-antilichamen in Reumatoïde artritis

In het tweede deel van dit proefschrift worden de aanwijzingen voor een mucosale oorsprong van auto-antilichamen in RA besproken. IgA is het meest geproduceerde antilichaam in het menselijk lichaam en het is vooral aanwezig bij mucosale oppervlaktes. Hier wordt het meestal lokaal geproduceerd door plasmacellen als dimeer IgA (dIgA) met een joining-chain (J-chain) [7, 8]. Eerdere data van onze afdeling lieten zien dat RF IgA vooral aanwezig is in de polymere vorm in serum, synoviaal vocht en speeksel van RA-patiënten [9]. Omdat anti-CarP en ACPA IgA allebei voorkomen in sera van RA-patiënten én om de mogelijke respons oorsprong van anti-CarP antilichamen en ACPA beter te leren begrijpen, is de moleculaire compositie van beide soorten IgA bestudeerd in **hoofdstuk 5**. Tot onze verbazing zagen we dat anti-CarP IgA vooral in het hoogmoleculaire gewicht (polymere) IgA fracties aanwezig is. Bij ACPA IgA is dat vooral in het laagmoleculaire gewicht (monomeer) IgA fracties. De verschuiving van anti-CarP IgA in de polymere fracties is zichtbaar ongeacht de ACPA- of RF-positiviteit van de patiënt.

Omdat anti-CarP IgA ook aanwezig is in hoogmoleculaire gewicht fracties, is onderzocht of aan anti-CarP IgA ook een deel van de poly-immunoglobulin-receptor (pIgR) vastzat. Dit stukje pIgR staat dan ook wel bekend als het secretoire component (SC). Polymeer IgA en IgM hebben allebei een J-chain die kan binden aan de pIgR [10]. Normaal worden antilichamen na transport door een epitheelcel aan de luminale kant “afgeknipt” van de pIgR. Hierbij blijven delen van receptoren achter: SC-houdende antilichamen. Deze antilichamen zijn ook aanwezig in de circulatie [11]. Het mechanisme hierachter is nog niet bekend. Om te weten of anti-CarP IgA het SC bevat in de circulatie, is de aanwezigheid en isotype van secretoire anti-CarP, ACPA en RF in RA-patiënten onderzocht in **hoofdstuk 6**. Onverwacht observeerden we dat de secretoire vorm van deze auto-antilichamen vooral aanwezig is als IgM. Dit betekent dat de aanwezigheid van SC geen verklaring kan zijn voor

het hoogmoleculaire gewicht van anti-CarP IgA. Wel geeft de aanwezigheid van SC-houdende auto-antilichamen in het IgM isotype een andere indicatie. Namelijk dat auto-reactieve IgM B-cellen de meest prominente B-cel subset representeren die opnieuw geactiveerd kunnen worden in mucosale gebieden. Op die manier kunnen ze de immuunrespons gaande houden.

Deel 3: anti-CarP-antilichamen in andere reumatische ziektes
In het laatste deel van dit proefschrift is gekeken naar de aanwezigheid van anti-CarP-antilichamen bij reumatische ziektes waar botschade optreedt: Systemische Lupus Erythematosus (SLE) en Osteoartritis (OA). Bij het onderzoek is gekeken of aanwezigheid van auto-antilichamen geassocieerd wordt met botschade in gewrichten. **Hoofdstuk 7** beschrijft de aanwezigheid van anti-CarP antilichamen en ACPA in SLE. Uit de resultaten blijkt dat er bij SLE iets meer anti-CarP antilichamen aanwezig zijn dan ACPAs. De mate waarin anti-CarP antilichamen en ACPAs aanwezig zijn, kan worden geassocieerd met gewrichtsschade. Soms kunnen SLE-patiënten ook de symptomen van RA vertonen. De combinatie van RA en SLE wordt ook wel Rhupus genoemd. Mogelijk geeft de aanwezigheid van auto-antilichamen die geassocieerd zijn met gewrichtsschade een verklaring voor de aanwezigheid van een meer RA-fenotype in een deel van de SLE-patiënten. Om de juiste therapie vast te stellen, is het belangrijk om de pathologische mechanismes en de factoren die bijdragen aan de ontwikkeling en voortgang van Rhupus beter te begrijpen. Dit omdat de therapie en uitkomst van Rhupus-patiënten anders kan zijn dan die van RA- of SLE-patiënten.

Een andere ziekte die gekarakteriseerd wordt door botafwijkingen, kraakbeenschade en ontstekingen is OA. Het meest voorkomende fenotype van OA is Hand Osteoartritis (HOA). Dat wordt opgedeeld in Erosieve Hand Osteoartritis (EOA) en Niet-erosieve Hand OA. **Hoofdstuk 8** beschrijft de aanwezigheid van RA-geassocieerde auto-antilichamen en hun associatie met EOA-patiënten. Een klein deel van de HOA-patiënten is namelijk positief voor deze auto-antilichamen. Echter associeert dit in HOA-patiënten niet met erosieve ziekte. De data suggereert dan ook dat een ander mechanisme de erosieve vorm van de ziekte aandrijft in HOA. Dat mechanisme is mogelijk onafhankelijk van auto-antilichamen. Toekomstige studies zijn nodig om te onderzoeken welk mechanisme de erosieve variant van OA veroorzaakt.

Conclusie

Vanuit dit proefschrift kan geconcludeerd worden dat anti-CarP-antilichamen en ACPAs, ondanks dat ze vaak samen voorkomen, twee verschillende antilichaamfamilies representeren. Deze twee antilichaam reacties lijken verschillend gereguleerd te zijn. Deze conclusie is gebaseerd op isotype gebruik, aviditeit en aanwezigheid van V-domein glycanen. Verder lijkt het erop dat ze een verschillende oorsprong in respons hebben. Mogelijk heeft de anti-CarP-antilichaam reactie, gebaseerd op de vorm van IgA, een meer mucosale oorsprong dan ACPA.

Bovendien blijkt uit dit proefschrift dat de RA-geassocieerde auto-antilichamen anti-CarP en ACPA niet veel voorkomen bij andere reumatische ziektes. Als ze al aanwezig zijn, zijn ze niet altijd geassocieerd met botschade. Dit wijst erop dat verschillende mechanismes, afhankelijk en onafhankelijk van auto-antilichamen, ten grondslag liggen aan de ontwikkeling van botschade.

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CURRICULUM VITAE

Myrthe van Delft werd op 5 november 1989 geboren in Leiden. In 2008 slaagde zij voor haar Atheneum examen op het Sint-Maartens College te Maastricht. Van 2008 tot 2014 studeerde zij Biomedische wetenschappen in het Leids Universitair Medisch Centrum. Tijdens de bacheloropleiding heeft zij stagegelopen op de afdeling Reumatologie in het Leids Universitair Medisch Centrum. Ze behaalde haar bachelor diploma in augustus 2011. Voordat ze aan haar master begon, is ze een half jaar naar Nepal gegaan om als Engels docent te werken op een basisschool in het Dhading District. Ook heeft ze daar meegeholpen met de organisatie van medische hulpprogramma's met lokale health care centers en zijn werknemers.

In september 2012 is zij aan haar research master voor biomedische wetenschappen begonnen aan het Leids Universiteit Medisch Centrum. Tijdens haar master heeft ze stages gelopen op de Urologie afdeling in het Erasmus Medisch Centrum en op de afdeling Klinische Immunologie en Reumatologie in het Amsterdam Medisch Centrum. Ze behaalde haar masterdiploma in oktober 2014 met een specialisatie in immunologie (Immunity, infection and tolerance research track). In januari 2015 begon ze als promovendus op de afdeling Reumatologie aan het onderzoek beschreven in dit proefschrift, onder leiding van Prof. Dr. René Toes, Prof. Dr. Tom Huizinga en Dr. Leendert Trouw.

Momenteel is Myrthe werkzaam als Postdoc in de mucosale immunologie groep onder leiding van Marjolein van Egmond op de afdeling voor moleculaire celbiologie en immunologie in het VU Medisch Centrum.

PORTFOLIO

PhD student: MAM van Delft MSc F
Primary thesis advisor: prof.dr. REM Toes
Other thesis advisor(s): prof.dr. TWJ Huizinga
dr. LA Trouw

Research programme: 20701 Pathophysiology and treatment of rheumatic diseases
Title of Thesis: Immunological characterization of anti-carbamylated protein antibodies in Rheumatoid Arthritis and other diseases

PhD training

Mandatory courses	**Year**
PhD Introductory Meeting (done)	2015
Basic Methods and Reasoning in Biostatistics (exempted)	
BROK Course (done)	2018
Generic/disciplinary courses	
Leiden Institute for Immunology, advanced immunology course	2015
FACS course LUMC	2017
Attended lectures, LUMC presentations, participation in meetings	
IRC lectures	2015-2018
Oral presentation IRC LUMC	2019
Congress attendance and poster or oral presentations	
Poster presentation at EWRR in York	2016
Oral presentation at NVVI/BSI congress	2016
Poster presentation B cell meeting “To B or not to B”	2017
Oral presentation NVVI winterschool	2017
Oral presentation ECI	2018
Oral presentation world immune regulation meeting	2018
Traineeship abroad	
Microbiome FACS DRFZ	2018

Teaching activities

Lecturing, lab assistance, student supervision	Year
Supervising a bachelor student from Life Science and Technology	2016
Supervising bachelor student BW internship	2017
Practical education BW second year	2018

Parameters of esteem

Personal grants	Year
RTcure travel grant	2018
Leids university fund travel grant	2016
EMBO travel grant	2017

LIST OF PUBLICATIONS

ACPA and anti-CarP-antibody responses are differentially regulated as indicated by differences in size and glycosylation.
Myrthe A.M. van Delft, Marije K. Verheul, Nivine E.W.N. Levarht, Theresa Kissel, Lise Hafkenscheid, Albert Bondt, René E.M. Toes, Leendert A. Trouw
Ready to submit

An overview of autoantibodies in Rheumatoid Arthritis.
Myrthe A.M. van Delft, Tom W.J. Huizinga
Submitted

Anti-carbamylated protein antibodies: are they useful for the diagnosis of Rheumatoid arthritis?
Ponchel F, van Delft MAM, Burska A, Duquenne L, Hunt L, Trouw LA; Emery P.
Submitted

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