Exploring seropositive rheumatoid arthritis: from immunological depths to clinical course

Derksen, V.F.A.M.

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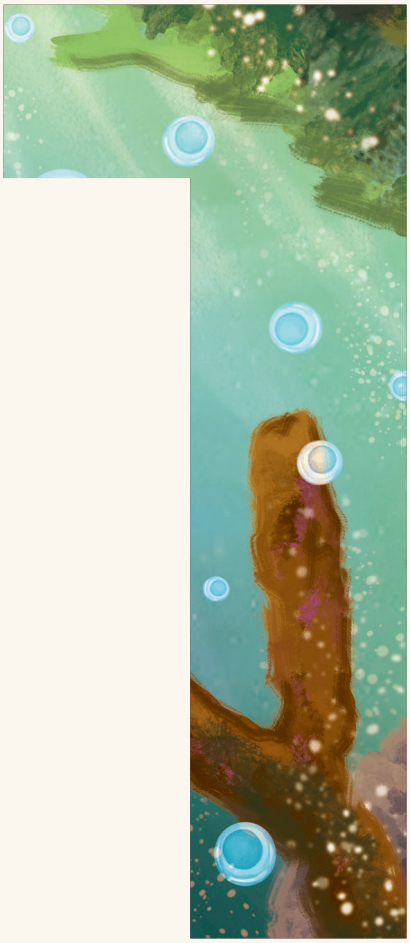
Table of contents



General introduction

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Rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic autoimmune disease affecting approximately 0.25-1% of the general population. The disease is characterized by joint inflammation, mainly in the small joints of the hands and feet, and can also lead to extra-articular manifestations, such as interstitial lung disease and premature cardiovascular morbidity. RA is a heterogeneous disease that encompasses several disease subsets with differences in underlying pathophysiology and disease outcome. RA patients can be divided based on the presence of autoantibodies (1). Positivity for anti-modified protein antibodies (AMPA), directed against post-translationally modified proteins (PTMs), is a hallmark of seropositive rheumatoid arthritis. The most well-known and clinically important AMPA are anti-citrullinated protein antibodies (ACPA) (2), while other AMPA are directed against carbamylated proteins (anti-CarP) (3) or against acetylated proteins (AAPA) (4). Rheumatoid factor (RF) is another, less specific, autoantibody that can be found in seropositive RA. AMPA are commonly seen as markers for underlying autoimmune pathophysiology. However, the pathophysiological processes leading to the break of tolerance against post-translational modified proteins, autoantibody expansion and eventually arthritis remain largely unclear (5). Insights in these processes are of significant importance as they could provide opportunities for novel targeted treatment strategies for RA patients and possibly even prevention of clinical disease in the future.

Anti-Modified Protein Antibodies

ACPA, directed against citrullinated proteins, were the first AMPA to be discovered. Citrullination is an irreversible posttranslational modification of the amino acid arginine (figure 1), mediated by peptidyl arginine deiminases enzymes (PADs) in a calcium-dependent manner. It is a physiological process regulating various cellular mechanisms (6). Thus, citrullination is present in all humans, yet for unknown reasons, tolerance to citrullinated proteins is lost in RA patients. Approximately 50-75% of RA patients are positive for anti-citrullinated protein antibodies (7-9), while ACPA can detected in approximately 1% of healthy individuals (8, 10). ACPA are routinely determined in clinical practice since they are highly specific for RA and are part of the latest RA-classification criteria (11). Cyclic citrullinated peptide (CCP), an artificial antigen, is often used in clinical tests to detect the presence of ACPA. However, ACPA can be reactive to an array of citrullinated self- and foreign proteins (12, 13).

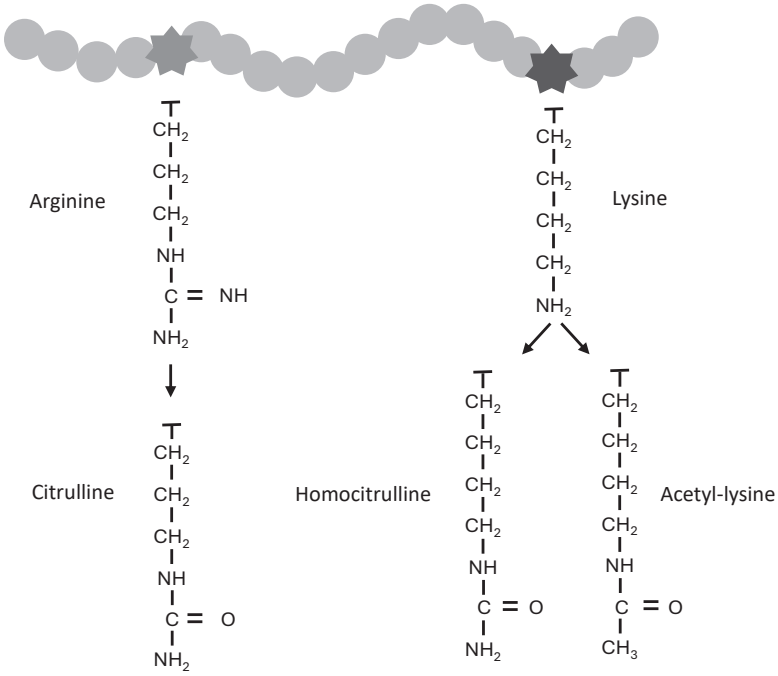

Figure 1: The structure of the different post-translational modifications

Antibodies against carbamylated proteins (anti-CarP) are another AMPA that can be found in RA patients. Carbamylation is a chemical reaction, mediated by cyanide, in which a lysine is converted into a homocitrulline (figure 1). Certain conditions, for example renal disease, smoking and inflammation, can increase cyanide levels, which is in chemical equilibrium with urea, and can thus raise the amount of carbamylation (14). Similar to citrullination, an increased amount of carbamylated proteins alone does not seem to be sufficient to break tolerance and induce autoimmunity. Only 12% of patients with renal disease are positive for anti-CarP antibodies compared to approximately 44% of RA patients (15). The molecular structures of homocitrulline and citrulline are very alike (figure 1), but homocitrulline residues are located at different positions in the protein and have different neighbouring amino acids. ACPA and anti-CarP are thus distinct autoantibody classes, and although anti-CarP are mainly present in ACPA-positive RA patients, they can also be detected in 8%-14% of ACPA-negative patients (3, 16).

Furthermore, anti-acetylated protein antibodies (AAPA) of the IgG isotype have been detected in approximately 40% of RA patients, mainly in the ACPA-positive subset (4). AAPA recognize proteins containing acetyl-lysines, formed out of lysine residues by a reversible acetylation process (figure 1). This process is mediated by enzymes in

eukaryotes, but can occur non-enzymatically in the presence of acetyl-CoA in bacteria (17, 18). N-terminal acetylation, an irreversible enzymatical process occurring at the N-terminus of the polypeptide, does not seem to be recognized by AAPA. Interestingly, AAPA IgM can be found in both ACPA-positive and ACPA-negative RA patients and also in healthy donors (19). This might indicate that AAPA IgM, in contrast to other AMPA, is part of the regular antibody repertoire. One of the current hypotheses is that AAPA IgM might target components of bacterial commensals and can thus contribute to intestinal homeostasis. Therefore, the origin of AAPA IgM might provide useful new insights in pathophysiological mechanisms leading to an AAPA IgG response and possibly even the break of tolerance against other PTMs in RA patients.

ACPA, anti-CarP and AAPA can be seen as spectrum of autoantibodies and can collectively be called anti-modified protein antibodies (AMPA). RA patients tend to be positive for multiple AMPA simultaneously. Polyclonal antibody isolations from sera of RA patients show significant cross-reactivity to other AMPA, for example antibody fractions isolated using citrullinated peptides, can also bind to carbamylated and acetylated proteins (20). This cross-reactivity was even seen on a monoclonal level. Also B cells with anti-citrullinated protein receptors can be activated by stimulation with carbamylated or acetylated antigens, next to citrullinated proteins (12). Mouse studies show that immunization with a single type of modified protein can lead to antibody responses against several different PTMs (20). This indicates that the AMPA response in RA patients could originate from a common B-cell response that can diversify into multiple distinct AMPA responses based on the exposure to different PTM-containing antigens. To date, it is unknown which antigens might lead to the initial break of tolerance against modified proteins. This might even vary between patients, for example due to exposure to different environmental factors.

Antibodies against malondialdehyde-acetaldehyde (MAA)-adducts are also found in RA. These adducts are formed via several modifications out of malondialdehyde (MDA), a product of lipid peroxidation. Anti-MAA are also found in ACPA-negative RA patients and patients suffering from other rheumatic diseases, like osteoarthritis and systemic lupus erythematosus (21). Thus, anti-MAA are not specific for (seropositive) RA. Furthermore, unlike AAPA and anti-CarP, anti-MAA do not cross-react with ACPA (22), indicating that this response is not closely related to the other AMPA.

Rheumatoid factor

Not ACPA, but rheumatoid factor (RF) was the first autoantibody described in RA. RF are directed against the Fc-part of human IgG. The presence of RF was included in the 1987 ACR classification criteria for RA, despite its suboptimal specificity, and it is

still included in the 2010 ACR/EULAR classification criteria used today (11). Different isotypes of RF can be detected in RA patients, of which RF IgM is regularly measured in the clinic (23). The prevalence of RF IgM in RA patients can be as high as 70%, while RF positivity in the general population ranges from 1.3 to 4% (24) and increases with age up to 20% in adults above 85 years (25, 26). Rheumatoid factor can also be found in other rheumatic diseases and transient RF responses can be seen during bacterial, viral and parasitic infections (24). In these conditions RF might contribute to host defence by the formation of larger immune complexes, leading to the clearance of these immune complexes. Furthermore, RF B cells can function as antigen presenting cells (27). Class switching and higher titres of RF seem to be indicative for RA. The mechanisms behind this prime humoral autoimmune process, for example regarding T-cell help, are to date still unknown.

Risk factors for AMPA positivity and RA

Several environmental and genetic factors that increase the susceptibility for RA have been identified, and different risk factors seem to exert their effect at different points during disease development. Twin studies have shown that genetic variation accounts for approximately 60% of the risk of RA development (28). The HLA-DRB1*01,*04 and *10 alleles are the strongest genetic risk factor for development of ACPA-positive RA (29). Most HLA-DRB1 alleles associated with RA share an identical amino acid sequence in the peptide binding groove, which has been termed the shared epitope (SE) (30). Interestingly, HLA-SE alleles do not seem to be associated with ACPA positivity as such, but rather with the development of RA in ACPA-positive individuals (31). The similarity in sequence has led to the hypothesis that all predisposing HLA-molecules containing the SE-sequence might present specific “arthritogenic” peptides, which could lead to a joint-specific autoimmune reaction. However, the exact peptides bound by HLA-SE molecules in vivo have not been identified to date. In contrast, anti-CarP-positive, ACPA-negative RA does not seems to be associated with HLA-SE, but with another HLA type, HLA B*08 (32). Besides the HLA region, multiple single nucleotide polymorphisms (SNP) are associated with rheumatoid arthritis, for example in the PTPN22 gene (33).

Smoking is a major environmental risk factor for the development of seropositive RA and is associated with the concurrent presence of multiple RA-associated antibodies (34). Smoking induces many changes in the airway epithelium, including higher expression of the PAD2 enzyme increasing the degree of citrullination in the lung (35), and raised protein carbamylation and acetylation levels (36, 37). Besides smoking, exposure to silica dust and other inhalants leads to a higher risk of developing RA (38, 39).

Mucosal inflammation and the AMPA response

Inhalation of toxic substances such as cigarette smoke appears to induce airway inflammation and leads to the increased local presence of post-translational modified proteins, which might contribute to the break of tolerance against PTMs and thus autoantibody formation (40). This hypothesis is supported by the finding that bronchial alveolar lavage fluid of ACPA-positive RA patients contained a higher percentage of class-switched B cells, some of which indeed being citrulline reactive (41). Other studies showed that ACPA can be present in both sputum and bronchoalveolar fluid of RA patients (42, 43). ACPA could even be detected in sputum of first-degree relatives of RA patients who did not have RA themselves, and did not have detectable ACPA in serum. Moreover, the presence of ACPA is sputum was associated with higher levels of neutrophil extracellular traps (NETs) (42). NETs are composed of highly condensed chromatin and are expelled by neutrophils to trap and kill pathogens. This process is mediated by peptidylarginine deiminase 4 (PAD4) (44). In the process of NETosis, abundant amounts of citrullinated autoantigens, such as citrullinated histones, are exposed in the context of a local pro-inflammatory environment (45), which might be a potential trigger for autoantibody formation. Taken together, these findings suggest a role of mucosal airway inflammation and local mucosal antibody production in the evolution of the ACPA response. Whether other AMPA can also be found in sputum of RA patients and their first-degree relatives is unknown.

During the recent COVID-19 pandemic, there was widespread exposure to the new Severe Acute Respiratory Syndrome CoronaVirus 2 (SARS-CoV-2) in the population. This virus could lead to severe mucosal inflammation, mainly in the lungs. Like other types of pulmonal inflammation described above, it is hypothesised that COVID-19 could also contribute to ACPA induction. Multiple studies have reported the presence of autoantibodies in patients with COVID-19, such as anti-cardiolipin, anti-β2 glycoprotein I and anti-nuclear antibodies (46, 47). Furthermore, flaring of rheumatoid arthritis (RA) after SARS-Cov-2 infection has also been described (48). Therefore, the COVID-19 epidemic provides new opportunities to investigate the link between local mucosal inflammation, autoantibody production and the development of RA.

The lungs are not the only mucosal sites which may be involved in RA development. The epidemiologic association between RA and periodontitis was already described years ago (49). One of the hypotheses is that Porphyromonas gingivalis, a major pathogen in periodontitis, is involved in RA pathogenesis since this bacterium possesses a PAD enzyme and can thus citrullinate proteins. However, Porphyromonas gingivalis PAD (PPAD) citrullinates proteins at the C-terminus, while human PAD enzymes citrullinate arginine resides within the protein (49). In vivo reactivity against these C-terminal citrullinated proteins produced by PPAD has not been shown, which makes PPAD a less likely (sole)

explanation for the association between RA and periodontitis (50). However, another pathogen involved in periodontitis, Aggregatibacter actinomycetemcomitans, might lead to increased citrullination via another pathway. This bacterium produces pore-forming leukotoxin A (LtxA), which binds to β2 integrin (CD18) on neutrophils, leading to an influx of extracellular calcium and hypercitrullination of intracellular proteins by the neutrophil's own calcium-dependent PAD enzymes. In one study, the presence of anti-LtxA antibodies was significantly associated with ACPA positivity (51), but these results could not be replicated (52). More recently, broader oral microbiome disturbances have been described in RA patients and people at risk of developing RA (53, 54), indicating the association between periodontitis and RA might not be mediated by a single bacterium. RA patients with ongoing periodontitis can experience repeated bacteraemia with a range of oral bacteria. These bacteria can be citrullinated and these citrullinated bacterial epitopes could be recognized by ACPA (55), providing an interesting potential link between a physiological anti-bacterial response and the AMPA response. The presence of ACPA in saliva of a subset of (ACPA-positive) RA patients further supports this hypothesis (56).

The largest mucosal surface is the intestinal tract, where extensive and continuous interaction between gut bacteria and the immune system takes place. Intestinal microbiome dysbiosis has been described in RA patients and individuals at risk of RA (53, 57-60). RA patients could even be distinguished from healthy controls based on alterations in the gut microbiome, for example regarding clostridium, lactobacillus and bifidobacteria species (53). However, due to the diversity and natural variations in the gut microbiome, it is difficult to interpret the specific differences found in number and type of bacteria in RA patients and the consequences thereof on the local metabolome and homeostasis. Recent work showed that circulating plasmablasts in individuals at risk of RA can bind both RA-associated citrullinated autoantigens and bacteria in faeces (61), which suggests that certain micro-organisms in the intestine might be able to induce or augment an ACPA response. One of the bacteria which is currently being investigated in more detail in this context is Prevotella copri, as increased T-cell and B-cell reactivity against this bacterium was found in RA (62-64). Interestingly, transfer of the faecal microbiome from ACPA-positive individuals without arthritis could aggravate arthritis in a mice model of RA. A decreased intestinal barrier function was found after microbiome transfer in these mice (60).

These data suggest that disbalance of intestinal mucosal homeostasis might augment inflammation in RA and could possibly even contribute to the development of an AMPA response, for example via a mechanism called molecular mimicry. This phenomenon might arise when some (post-translationally modified) bacterial components closely resemble human autoantigens and physiological anti-bacterial responses lead to antibodies

reactive to both bacterial- and self-proteins. So far, most research on bacterial triggers for autoantibody formation in RA focused on ACPA. However, AAPA might provide an interesting new angle concerning the potential of microbial components to serve as a trigger for autoimmunity as bacteria have the ability to acetylate self-proteins (17, 18). Acetylated bacterial proteins can in fact be recognized by AMPA and immunisation with these proteins can induce a cross-reactive AMPA response in mice (65). Anti-carbamylated protein responses may also have an intestinal origin, since carbamylation has been shown to occur in the human gastrointestinal tract (66). B cells activated in the gut seem to adhere efficiently to endothelial venules in both the intestines and the synovium, suggesting these cells can enter both tissues (67). Whether ACPA and other AMPA are indeed locally produced in the intestinal tract remains unclear. An alternative hypothesis states that specific microorganisms might breach the mucosal barrier and may populate the synovium, leading to a local inflammatory response in the joints and thus causing arthritis. The detection of genetic material of Prevotella copri and other bacteria in synovial fluid of RA patients supports this idea (63, 68).

Characteristics of mucosal IgA

The humoral response at mucosal sites mostly consists of antibodies of the IgA isotype, while the largest part of the serum antibody pool consists of IgG (69). Most mammals only have a single IgA molecule, but humans, together with chimpanzees, gorillas and gibbons, have two IgA subclasses: IgA1 and IgA2 (figure 2) (70). IgA2 molecules lack a thirteen amino acid long segment present in the hinge region of IgA1. The extended hinge region of IgA1 makes it more susceptible to proteases, which are produced by bacterial pathogens and commensals colonizing the mucosa. The longer hinge may make IgA1 better equipped than IgA2 to interact simultaneously with two antigens separated by a considerable distance, which might lead to better recognition of repeated antigenic structures on the surface of certain pathogens (71). Furthermore, IgA2 might have a more pro-inflammatory effect on neutrophil and macrophages, possibly due to glycosylation differences influencing Fc-receptor binding (72).

In serum, over 90% of total IgA is IgA1, whereas the IgA subclasses are more balanced at mucosal sites, with exact ratios depending on the location (73). In the colon, IgA2 is the dominant subclass. The local IgA subclass distribution may be the result of subclass-specific clonal expansion induced by certain types of antigens. Mucosal antibodies to lipopolysaccharide (LPS) are generally IgA2, whereas protein antigens lead to IgA1 predominant responses (74). However, most commensals are dually targeted by IgA1 and IgA2 and isolated gut memory B cells and plasma cells of both subclasses are largely clonally related (75, 76). To date, the exact molecular events underlying preferential IgA1 or IgA2 class-switching remain unclear.

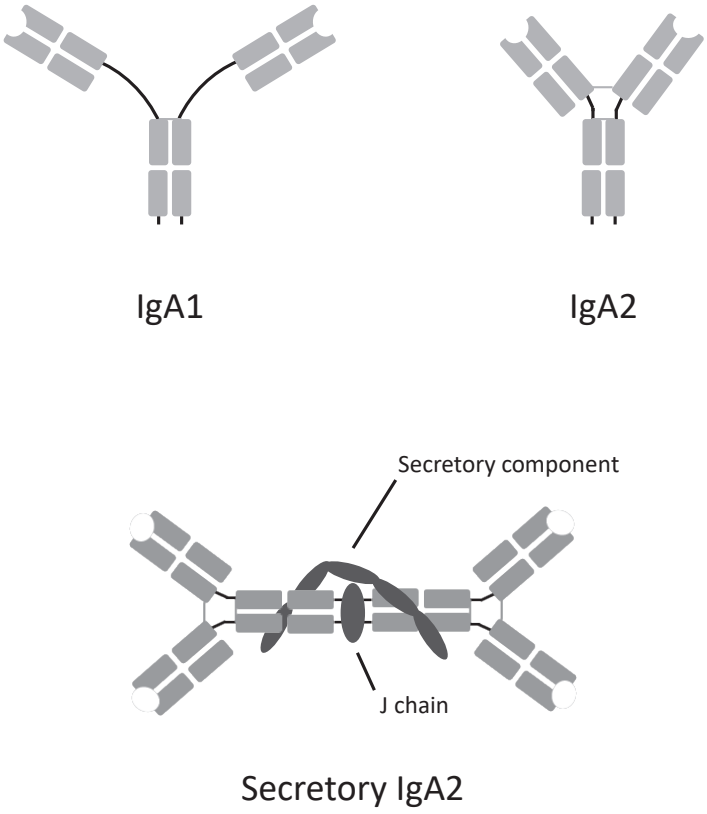

Figure 2: Schematic structure of the IgA subclasses IgA1 and IgA2 and of secretory IgA(2)

Surprisingly, in serum of RA patients ACPA IgA is more often of the IgA2 subclass (up to 80% of all ACPA IgA) compared to total IgA (72). Although the precise mechanisms leading to this IgA subclass distribution in ACPA is unclear, it might indicate involvement of the intestinal immune system, the site with the highest IgA2:IgA1 ratio in the body (73). The IgA-subclass-ratio of other AMPA has not been investigated.

Another feature that differs between serum IgA and IgA secreted at mucosal sites, is the degree of antibody dimerization. In serum, IgA is mainly present in monomeric form, but only dimeric IgA can be actively transport to the mucosal lumen. Dimeric IgA consists of two monomers connected by disulfide bonds and linked by a J-chain, an additional polypeptide which is also found in pentameric IgM (figure 2). Antibodies are actively transported across the epithelium by the poly-Ig receptor, which binds the antibody at the position of the J-chain at the basolateral surface of the epithelium. The antibody is transported by transcytosis and at the apical surface the poly-Ig receptor is cleaved, releasing the antibody in the mucosal lumen. However, a small fragment of

the poly-Ig receptor, called the secretory component, remains bound to the antibody. The complex of dimeric IgA and secretory component is named secretory IgA (figure 2) (77). As many mucosal surfaces are colonized with a variety of micro-organisms, secretory IgA, equipped with four antigen binding sites, has an important function in maintaining mucosal homeostasis by binding micro-organisms and thus preventing them from entering host cells.

Levels of both total secretory IgA and IgM were found to be elevated in the serum of RA patients (78). Furthermore, ACPA with secretory component attached can be found in the circulation of seropositive RA patients and the presence of these secretory ACPA is associated with smoking (79). This suggests mucosal inflammation might be involved the pathophysiology of the AMPA response. However, most of the secretory ACPA seems to be of the IgM isotype instead of IgA, whereas dominance of secretory IgA would be expected based on the mucosal origin hypothesis (80).

The development of the AMPA response over time

Autoimmunity against post-translationally modified proteins can already be detected years before the onset of RA. The autoantibody response develops gradually over time, a process that is mostly studied in ACPA (81-84). Prior to the onset of arthritis, the levels of ACPA IgG rise, additional isotypes become detectable (85, 86) and the number of epitopes recognized by the ACPA response expands (87, 88). Although increased isotype switching is observed, no apparent avidity maturation is seen over time. Under normal circumstances, activated B cells migrate to germinal centres where they undergo both class-switch recombination and somatic hypermutation in the V-region of the heavy-chain and light-chain genes. During class-switch recombination the IgM/IgD B-cell receptor (BCR) is replaced by a different isotype, while somatic hypermutations will in some clones lead to higher affinity of the BCR for the antigen. These high affinity clones have a survival advantage and will be selected, a process called affinity maturation. These selected high-affinity B cells further differentiate into memory B cells or plasma cells (89). However, ACPA and anti-CarP responses have overall low avidity, despite the extensive isotype switching observed. This indicates that, in contrast to physiological recall responses, isotype switching and avidity maturation seem to be uncoupled in AMPA (90). Once patients have clinically apparent disease, the ACPA profile and phenotype remains stable over time (88, 91), at least in the current situation where all RA patients receive treatment. The continuous presence of ACPA IgM during the disease course supports the idea that AMPA B cells are continuously activated by the ubiquitous presence of modified proteins (85, 92).

ACPA glycosylation

Another remarkable feature of ACPA is the increased presence of variable domain glycans (VDG) (93). Glycosylation is a posttranslational modification in which a diverse array of glycan types can be attached to the antibody through linkage to the nitrogen atom of an asparagine residue (N-glycans) or through an oxygen atom on serine/threonine residues (O-glycans). N-glycans are only attached to an asparagine in the presence of an N-glycosylation consensus sequence, most often asparagine-X-serine/threonine (N-X-S/T), where "X" can be any amino acid except for proline. O-glycans are present on the hinge regions of human IgA1, IgG3 and IgD (94), whereas N-glycans are more abundant, with the number and location of potential N-glycosylation sites varying per antibody isotype. For example, all IgG contain a conserved N-glycosylation consensus sequence at position 297 in the CH2 domain of the Fc-region (95). The glycan profile differs between IgG-Fc glycans and IgG-VDG glycans (96, 97), and is also shaped by age, gender and inflammatory state. Alterations in the glycosylation profile can lead to differences in secretion, structural stability, binding and effector function of the antibody (95, 96, 98, 99).

In physiological recall responses, about 15-25% of IgG variable regions contain N-glycosylation consensus sequences and they are mainly introduced by somatic hypermutation. The amount of ACPA variable domain glycosylation rises towards the onset of arthritis, with more than 90% of ACPA IgG bearing Fab-glycans at disease presentation (93, 100). It has been suggested that in ACPA the introduction of variable domain glycans is a driver for clonal selection instead of affinity selection, as it has been shown that Fab-glycans enhance B-cell receptor signalling and delay receptor downmodulation after antigenic stimulation (101). This may provide an explanation for the relative low avidity of matured ACPA responses (102, 103). In addition, not only VDG glycans, but also the Fc glycosylation pattern of ACPA changes over time. The levels of core fucosylation rise, while galactosylation decreases towards disease onset, which could contribute to a more pro-inflammatory ACPA profile (104). The impact of the Fc-glycosylation profile on ACPA IgG B-cell receptor functioning is unclear.

From autoimmunity to disease

To date, the mechanisms underlying the transition from AMPA positivity to the development of arthritis and RA is unclear. The fact that ACPA can be detected years before disease onset infers that the sole presence of ACPA does not directly lead to RA. Furthermore, not all AMPA-positive patients progress towards clinical disease (105, 106) and removal of autoantibodies with plasma exchange does not improve disease activity (107). It is speculated that multiple "hits" might be necessary for the development of arthritis, for example intermittent mechanical joint trauma in the

context of seropositivity. The fact that HLA-SE is strongly associated with the transition from autoimmunity to autoimmune disease, suggests that T cells are involved in this process (31). It is hypothesised that antigens presented by HLA-SE might activate T cells and these activated T cells provide the necessary help to AMPA B cells leading to expansion and evolution of the AMPA response. Whether these T cells are autoreactive or targeting foreign antigens is unknown.

In the presence of this established circulating AMPA response, a local pro-inflammatory microenvironment in the joint might facilitate the pathogenic potential of AMPA resulting in sustained inflammation, which would not occur in homeostatic conditions. AMPA can exert pro-inflammation effects via various mechanisms, which have been mainly studied in ACPA. For example, immune complexes containing ACPA and citrullinated fibrinogen are able to stimulate tumor necrosis factor (TNF) secretion via stimulation of Fcγ-receptors on macrophages in vitro, a process which was enhanced in the presence of RF (108, 109). ACPA also have the ability to recruit complement via both the classical and alternative pathway (110). Complement levels are reduced in synovial fluid of RA patients, while complement cleavage products are increased, indicating local complement activation. Furthermore, ACPA can also stimulate NETosis, potentially leading to a positive feedback loop of ongoing inflammation as NETosis exposes citrullinated autoantigens, a binding target for ACPA (45).

Not only antibodies, but also (antigen-specific) B cells seem to play an important role in arthritis development, given the observations that B cell depletion with rituximab is an effective treatment for RA (111) and can even delay onset of arthritis in seropositive arthralgia patients (112). Circulating ACPA B cells in RA patients have a strong pro-inflammatory phenotype, expressing T cell-stimulatory ligands and showing a phenotype consistent with increased cell proliferation (113).

Nonetheless, there is accumulating evidence that some ACPA might have a beneficial effect on arthritis in mice models. Some monoclonal ACPA from RA patients protected mice from antibody-induced arthritis (114, 115). It is hypothesized that protective ACPA can reduce instead of increase NETosis (116). A beneficial effect of ACPA immune complexes has also been proposed. Studies have shown that these immune complexes can reduce osteoclastogenesis by binding Fcγ-receptor 2B on macrophages and promoting IL- 10 secretion by these cells (117). Not all studies support this theory, as there are also data that passive transfer of ACPA can worsen pre-existent synovitis in collagen induced arthritis (CIA) mouse models (118). So far, no support for a protective effect of AMPA was found in RA patients. Given the lack of a direct pathogenic effect of AMPA, it remains a possibility that AMPA might be a by-effect of the inflammatory process in RA patients, perhaps in an

effort of the immune system to dampen this unwanted auto-inflammation, rather than the driving force of the inflammation. However, given the changes in AMPA phenotype over time and the relation of phenotypic changes and disease onset, it seems plausible that AMPA with different phenotypic characteristics might have diverging effects and can either amplify or dampen local inflammation.

Although AMPA are a hallmark of seropositive RA, the clinical presentation of AMPA-positive and AMPA-negative RA patients is remarkably similar. Therefore, there might be a final common inflammatory pathway in RA development, independent of autoantibody status, in which activation of the innate and the adaptive immune system leads to an influx of leukocytes into the normally sparsely populated synovial compartment via the local expression of adhesion molecules and chemokines (119). The proinflammatory T cells, cytokines and immune complexes present could stimulate macrophages and fibroblast-like synoviocytes to proliferate and to produce even more proinflammatory mediators, leading to synovial hyperplasia, the formation of an invasive pannus and eventually destruction of joint cartilage and bone (120-122). Bone damage is mediated by the upregulated expression of RANK ligand by activated fibroblasts, T cells, B cells and macrophages leading to increased osteoclastogenesis (123-127). Blockage of proinflammatory cytokines, for example by monoclonal antibodies against tumour necrosis factor- alpha (TNF-α), is a successful treatment strategy in both seropositive and seronegative RA, highlighting the key role of TNF in orchestrating synovitis in both RA patient subgroups.

The effect of AMPA on RA phenotype and clinical disease course

RA patients usually present with gradual onset of joint pain, stiffness and swelling, which typically develops into a symmetrical polyarthritis with involvement of periarticular soft tissue. RA mainly affects hands, feet and wrists, yet any synovial joint can be involved. In general, ACPA-positive and ACPA-negative early RA patients cannot be distinguished based on clinical phenotype (11). Whether the conjoint presence of multiple autoantibodies is associated with more pronounced humoral inflammation and leads to differences in clinical phenotype within seropositive RA patients, is an open question. Given the similarities at disease onset, first line treatment is in principle the same for both seropositive and seronegative RA. A conventional synthetic disease-modifying antirheumatic drug (DMARD), usually methotrexate, is given in combination with glucocorticoids. However, AMPA-positive RA patients have a less favourable long-term prognosis compared to seronegative patients, which is reflected in choices regarding treatment escalation. In case of insufficient treatment response, addition of a biological DMARD, such as anti-TNF or a targeted synthetic DMARD, such as Janus kinase (JAK) inhibitors, is more readily recommended in ACPA-positive RA patients instead of opting for other conventional synthetic DMARDS (128).

Disease outcomes in RA are mainly associated with ACPA positivity. The concurrent presence of other AMPA in ACPA-positive patients does not influence prognosis (129). Patients who are ACPA-positive suffer from more joint destruction over time and are less likely to achieve sustained drug-free remission compared to seronegative RA patients (130-133). However, in ACPA-negative patients, positivity for anti-CarP did associate with more radiographic progression compared to seronegative patients (134). Specific ACPA features might be linked to disease severity. For example, patients whose circulating ACPA IgA consisted for a relatively large portion out of IgA2, were reported to have higher disease activity scores (72). Unlike some other autoantibody-associated diseases, changes in autoantibody levels during treatment or even seroconversion are not related to long-term outcomes in RA (135, 136). The inability to reach sustained drug-free remission in ACPA-positive RA patients might be explained by the finding that autoreactive B cells keep actively proliferating despite treatment. Even in patients with clinical disease remission, circulating ACPA B cells displayed a phenotype consistent with recent activation. Clinical remission thus does not seem to equal immunological remission in these patients (113).

Cardiovascular complications of RA

RA does not only affect joints, since RA patients can develop various other manifestations, including subcutaneous nodules, vasculitis or (interstitial) lung disease. Seropositive patients are at greater risk for these so-called extra-articular manifestations compared to seronegative individuals. In general, the frequency of these complications has decreased over time, most likely due to improved treatment options after the introduction of biological DMARDs (122). Mortality rates have normalized in ACPA-negative RA patients, but to date life expectancy is still shortened in ACPA-positive RA (137), mainly due to cardiovascular disease (138, 139). RA and cardiovascular disease have various shared risk factors, such as smoking and obesity (34, 140), yet this does not fully explain the increased cardiovascular mortality rates in RA patients. Chronic inflammatory processes in these patients could augment the development of atherosclerosis, via mechanisms leading to oxidative stress and endothelial dysfunction (139, 141, 142). Multiple studies reported an association between ACPA positivity and higher cardiovascular mortality (143-146), although results are contradictory (147-150). Autoantibodies can contribute to the chronic inflammation in RA and might in this way promote the development of atherosclerosis (45, 108), but there are also indications that ACPA may directly enhance the atherosclerotic process. Citrullinated proteins are present in atherosclerotic plaque tissue and can be bound by circulating ACPA (151), further stimulating plaque development. Moreover, via FcγRIIa-dependent activation of platelets, ACPA might exert prothrombotic effects (152).

The hypothesis that ACPA play a direct role in cardiovascular disease development is further supported by two studies reporting the presence of ACPA in approximately 10% of patients with coronary artery disease (CAD) without concomitant RA (153, 154). This rate of seropositivity is much higher than expected in the general (non-RA) population. In addition, in one of the studies ACPA were independently associated with poor cardiovascular outcome and significantly increased mortality (153). Importantly, none of these ACPA-positive patients developed RA after long-term follow-up. However, study sizes were too small to draw definitive conclusions. Taken together, it remains unclear whether the increased mortality in seropositive RA patients is mainly due to increased chronic inflammation or whether there is a direct autoantibody-mediated effect, either specific for ACPA or amplified by the concurrent presence of multiple AMPA.

Scope of this thesis

The aim of this thesis is to gain more insight in the pathophysiological processes underlying the development of the AMPA response and to study the effect of AMPA on clinical presentation and disease outcome in rheumatoid arthritis patients. Several chapters specifically focus on the role of the mucosal immune system in the development of autoantibodies against post-translational modified proteins. Furthermore, the breadth of the AMPA response, instead of the sole presence of ACPA, is investigated in this thesis both in relation to pathophysiology as well as clinical outcomes.

ACPA have increased variable domain glycosylation, affecting not only antibody binding and half-life time, but also influencing B-cell receptor functioning. Whether Fc glycosylation also impacts ACPA IgG B-cell receptor function, is studied in **chapter 2**. Using a germinal-center derived ACPA-reactive B-cell line, we explored the effect of conserved IgG-Fc N-linked glycans on membrane expression, antigen binding and other BCR functions.

The second part of this thesis takes a closer look on the potential role of the mucosal compartment in the development of the AMPA response. **Chapter 3** explores the IgA subclasses distribution of circulating ACPA and RF, which could be an indicator of a mucosal origin of these autoantibodies. Given the fact that IgA2 has been ascribed pro-inflammatory properties, we also analysed the association between total and antigen-specific IgA subclass levels and inflammation in RA patients. In **chapter 4,** the mucosal presence of ACPA, anti-CarP, AAPA and RF is studied using unique paired faeces, saliva and serum samples of RA patients and healthy volunteers collected in the MUCOSA study. Using enzyme-linked immunosorbent assays (ELISA), the autoantibody profile

in these different secretions was compared. Results were further substantiated in the Swedish IntestRA study, by analysing ileal wash samples collected using colonoscopy, together with paired saliva and serum samples of RA patients, healthy donors and patients with inflammatory bowel disease. Faeces data were replicated using samples for the Dutch Plants for Joints trial. The hypothesis that a mucosal infection might be a trigger for ACPA development and the onset of rheumatoid arthritis is investigated in **chapter 5** using data collected in the COVID-19 pandemic. During the pandemic the population was widely exposed to a new respiratory virus, SARS-CoV-2, which could induce severe inflammation in the mucosa of the respiratory tract. Examining the development of ACPA and their specific characteristics during COVID-19 disease, provides the opportunity to study the relation between a mucosal infection, the break of tolerance against post-transitionally modified proteins and transition to RA.

Once autoimmunity and eventually autoimmune disease has been established, AMPA can influence clinical presentation and disease outcomes in RA, which is further explored in **part 3** of this thesis. **Chapter 6** looks beyond the effect of ACPA seropositivity and investigates the association between the breadth of the AMPA response and rheumatoid arthritis phenotype at presentation. The autoantibody profile in relation to treatment response is studied in **chapter 7**, using data and material collected in the IMPROVED trial. A range of AMPA was measured in sera of seropositive early RA patients at baseline and during follow-up. The association between the breadth of the autoantibody profile and three specific outcomes was investigated, being treatment response in the first four months after start of therapy, reaching initial drug-free remission after drug tapering and achievement of sustained drug free remission. Long-term effects of ACPA on disease outcome are explored in **chapter 8**. In this chapter the association between ACPA and coronary artery disease is not only investigated in two RA cohorts, the EAC and BARFOT, but also in two cohorts of coronary artery disease patients without RA, the German LURIC study and the Danish CLARICOR trial, as earlier studies reported ACPA positivity in a subset of CAD patients without RA. Specific attention was paid to the role of chronic inflammation in the association between ACPA and increased mortality in RA patients. This approach provides a unique opportunity to study the effect of ACPA in different (inflammatory) contexts.

The findings presented in this thesis are summarized and discussed in **chapter 9**, the general discussion, starting at the immunological depths of B cell receptor glycosylation, the role of mucosal immunity in the development of the AMPA response before arriving to the clinical phenotype and disease course of AMPA-positive RA-patients.

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Part 1

The effect of Fc glycosylation
on ACPA IgG-B-cell receptor function

N-linked Fc glycosylation is not required for IgG-B-cell receptor function in a GC-derived B-cell line

Theresa Kissel[1,#], Veerle F.A.M. Derksen[1,#], Arthur E.H. Bentlage[2], Carolien Koeleman[3], Lise Hafkenscheid[1], Diane van der Woude[1], Manfred Wuhrer[3], Gestur Vidarsson[2], Rene E.M. Toes[1]

[1] Department of Rheumatology, Leiden University Medical Center, The Netherlands
[2] Department of Experimental Immunohematology, Sanquin Research and Landsteiner Laboratory, Amsterdam University Medical Center, The Netherlands
[3] Center for Proteomics and Metabolomics, Leiden University Medical Center, The Netherlands

[#]These authors contributed equally

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Abstract

IgG secreted by B cells carry asparagine N(297)-linked glycans in the fragment crystallizable (Fc) region. Changes in Fc glycosylation are related to health or disease and are functionally relevant, as IgG without Fc glycans cannot bind to Fcɣ receptors or complement factors. However, it is currently unknown whether ɣ-heavy chain (ɣHC) glycans also influence the function of membrane-bound IgG-B-cell receptors (BCR) and thus the outcome of the B-cell immune response. Here, we show in a germinal center (GC)-derived human B-cell line that ɣHC glycans do not affect membrane expression of IgG-BCRs. Furthermore, antigen binding or other BCR-facilitated mechanisms appear unaffected, including BCR downmodulation or BCR-mediated signalling. As expected, secreted IgG lacking Fc glycosylation is unable to carry out effector functions. Together, these observations indicate that IgG-Fc glycosylation serves as a mechanism to control the effector functions of antibodies, but does not regulate the activation of IgG-switched B cells, as its absence had no apparent impact on BCR function.

Introduction

Antibodies of the immunoglobulin G (IgG) isotype are crucial for immune-mediated protection against many pathogens. An important mechanism of IgG-mediated protection is the ability of IgG to bind to Fcγ receptors (FcγR), facilitating the activation of FcγR-bearing cells. In addition, IgG can mediate complement activation, leading, for example, to opsonization or killing of pathogens and the release of chemoattractants, which is important for the recruitment of (innate) immune cells to the site of infection. IgG are produced by plasmablasts and plasma cells which differentiate from B cells that recognize antigens via their B-cell antigen receptor (BCR) (1). Similar to the BCR, IgG are glycoproteins (2) that carry glycans linked to a conserved asparagine (N)-glycosylation consensus sequence (N-S-T) at position 297 in the CH_2 domain of the γ heavy chain (γHC). These N-linked glycans, often referred to as Fc glycans, are conserved between IgG subclasses. Only human IgG3 molecules carry additional glycans, which are linked to serine or threonine residues in the hinge region, so called O-linked glycans (3). Although the N-linked glycosylation of human BCRs is ill- defined, the Fc glycans expressed by IgG antibodies are well defined. Structurally, they are mainly di-antennary (A2) complex-type glycans bearing a core fucose, varying amounts of terminal galactoses and, to a lesser extent, bisecting *N*-acetylglucosamines (attached to the core β-mannose) and terminal sialic acids (4, 5).

To date, it has been extensively studied and shown that IgG Fc glycan composition is highly variable and changes with age, sex, health and disease (4, 6, 7). Specific IgG Fc glycosylation patterns lacking terminal galactoses have been identified, for example, in rheumatic (e.g. rheumatoid arthritis, systemic lupus erythematosus and ANCA-associated vasculitis) and other inflammatory diseases and are clearly associated with inflammation and disease activity (8). In addition, alterations in Fc glycosylation have recently been highlighted in studies of anti-spike protein IgG responses in patients with COVID-19, showing highly dynamic glycosylation patterns including low core fucosylation that correlates with disease severity (9-11). Reduced core fucosylation of IgG has also been reported for alloimmune responses against cellular blood groups and for responses against glycoproteins of HIV and dengue viruses, leading to the hypothesis that membrane-embedded antigens induce a specific afucosylated B-cell response, followed by secretion of afucosylated IgG (9).

Functionally, Fc glycans have a substantial impact on the structure of soluble IgG molecules and are essential for the recruitment of effector functions such as complement activation and binding to FcγRs. The core fucose of N(297)-linked glycans on IgG has been shown to sterically collide with the fucose of N(162)-linked glycans on FcγRIII,

thereby modulating antibody-dependent cellular cytotoxicity (ADCC) (12). This explains the potent immune responses of afucosylated IgG described above, as they are able to efficiently bind and activate immune cells carrying FcγRIII (9). At the functional level, changes in Fc galactosylation have been shown to enhance complement activation, as hypergalactosylated IgG Fc domains have a higher potential for hexamerization, allowing more efficient interaction with the initiator of the classical complement pathway C1q, thereby enhancing complement dependent cytotoxicity (CDC) (13, 14).

Fc glycosylation is thus essential for the function of IgG secreted by B cells, but the role of γHC carbohydrates in BCR function remains unclear. Since B-cell development, survival and activation depends on the expression of functional BCRs (15), it is important to investigate whether Fc glycans influence their structure and function. The IgG-BCR consists of an immunoglobulin (Ig)-like structure that resembles the structure of secreted IgG molecules, but is membrane-bound (mIgG) and thus extended by long transmembrane helices. In addition, the IgG-BCR forms a non-covalent complex with the signal-transducing heterodimer Igα/Igβ (CD79α and CD79β) at a 1:1 stoichiometry (16, 17). After antigen engagement, the intracellular immunoreceptor tyrosine-based activation motifs (ITAM) in the Igα/Igβ subunits get phosphorylated by the kinase Lyn, which then triggers a cascade of signalling events, resulting in B-cell activation, differentiation and antibody production (18).

That glycosylation can alter the function of cellular immune receptors has already been shown for e.g. CD22 (19, 20), MHC-II (21), T-cell receptors (22) and IgM-BCRs. In particular, a study using mouse pre-B-cell lines has shown that N(46)-linked glycans in the CH_1 domain of μHC are required for IgM pre-BCR formation and function (23). However, in contrast to IgG-BCRs, membrane-bound IgM (mIgM) BCRs are heavily glycosylated and contain a total of four conserved N-glycosylation sites. In addition, there are notable differences in the assembly and positioning of IgG- and IgM-BCRs on the cell surface. Compared with mIgM, mIgG contain cytoplasmic domains of considerable length, including signal-amplifying peptide motifs, e.g., the immunoglobulin tail tyrosine (ITT) motif, which plays a key role in IgG-B cell activation (24). In addition, recent cryo-EM studies have shown that the Fc domain of mIgM is located closer to the plasma membrane compared to that of mIgG (16, 25). These alterations could result in different roles of N-glycosylation for different BCR isotypes. For IgG-BCRs, a study knocking out fucosyltransferase (Fut8) in B cells suggests that glycosylation is important for IgG-BCR function (26), although it is unclear whether the observed effects are due to fucosylation changes in the BCR or by prevention of core fucose incorporation on N-linked glycans of other cellular proteins (27).

Given the importance of γHC glycosylation for IgG antibody function, we have now developed tools to investigate the effects of glycosylation on BCRs by generating a γHC glycan-site mutant in human germinal center (GC)-derived B cells from Burkitt Lymphoma (Ramos). Intriguingly, we show that Fc glycosylation has no effect on IgG-BCR function, including antigen recognition, signal transduction, BCR downmodulation and concomitant antigen uptake as well as antibody secretion. Our results indicate that γHC glycans do not regulate BCR-mediated humoral immune responses, but have evolved to direct the effector functions of antibodies produced by these B cells.

Results

Lack of γHC glycosylation has no effect on IgG-BCR surface expression.

We generated human B-cell lines from Burkitt Lymphoma (Ramos) expressing IgG-BCRs in the presence or absence of γHC glycosylation. To this end, Ramos B cells were knocked-out (KO) for their endogenous IgM and IgD BCR and the activation-induced cytidine deaminase (AID) enzyme (referred to as MDL-AID KO), as previously described (28, 29). B-cell lines were generated by transducing the BCR-negative MDL-AID KO cell line with N(297)-γHC or mutated Q(297)-γHC IgG-BCR sequences and the transduction marker GFP (Figure 1a). The BCR sequences used were obtained from single-cell sorted human B cells isolated from patients with the autoimmune disease rheumatoid arthritis as previously described (28). Two BCRs were directed towards citrullinated antigens (2G9 and 3F3), while a third BCR was directed against tetanus toxoid (D2). We next compared the surface expression of the IgG-BCRs in the presence or absence of their Fc glycans (FcG). All three GFP+ B-cell lines (2G9, 3F3 and D2) were able to stably express membrane-bound IgG (mIgG) BCRs in the absence of FcG over a time period of 20 days (Figure 1b-d*;* Supplementary Figure 1a-c). Next, we captured IgG-BCRs and identified the expected apparent molecular weight, indicating a structurally intact BCR (Figure 1e). The FcG-negative (-) mIgG displayed a smaller size compared to the FcG-positive (+) mIgG, as expected due to the absence of two glycans in the γHC.

Since few data are available on glycans expressed by human BCRs, we aimed to analyse the Fc glycans after IgG-BCR capture and tryptic digestion by liquid chromatography coupled with mass spectrometry (LC-MS). As expected, no Fc peptide glycans could be detected for the FcG- B-cell lines (Figure 2a, c). The glycans expressed in the non-mutated γHC of the IgG-BCRs were mainly di-antennary (A2) complex-type glycans containing a core fucose, 77% (2G9 IgG-BCR), 88% (3F3 IgG-BCR) or 78% (D2 IgG-BCR) galactosylation, 47% (2G9 IgG-BCR), 52% (3F3 IgG-BCR) or 46% (D2 IgG-BCR) bisecting N-acetylglucosamines and 16%, (2G9 IgG-BCR), 21% (3F3 IgG-BCR) or 19% (D2 IgG-BCR)

terminal sialic acids (mainly S0 or S1 glycans) (Figure 2a-c; Supplementary Figure 2a, b). The expression of complex-type N(297)-glycans on mIgG was confirmed by cell surface biotinylation and subsequent Western blot analysis of biotinylated IgG treated with EndoH (cleaving only high-mannose structures) or PNGaseF (cleaving all N-glycan structures) (Supplementary Figure 2c). γHC N-glycans on mIgG could only be cleaved after PNGaseF treatment, as indicated by a size-shift, supporting the absence of high-mannose glycans on these mIgG.

Together, these results indicate that γHC glycosylation has no effect on IgG-BCR assembly, cellular trafficking and its membrane expression.

IgG-BCR Fc glycans do not impact antigen binding or BCR downmodulation.

To test whether γHC glycosylation of IgG-BCRs affects binding to antigens, we determined the binding of the three FcG+ and FcG- mIgG B-cell lines to their respective antigens by flow cytometry. The two B-cell lines (2G9 and 3F3) directed against citrullinated antigens [cyclic citrullinated peptide 2 (CCP2)] showed binding to the citrulline but not to the unmodified arginine-containing control peptide (CArgP2) (Figure 3a). The D2 B-cell line showed binding to the tetanus toxoid (TT) antigen, as expected. No binding was observed for the MDL-AID KO control cell line, which lacks IgG-BCRs. The absence of Fc glycosylation had no effect on binding of the IgG-BCRs to their respective antigen (Figure 3a), also at lower antigen concentrations (Figure 3b). Next, we performed a flow-based assay to investigate the downmodulation of IgG-BCRs after (antigenic) stimulation and to determine the effects of FcG on BCR internalization and concomitant antigen uptake. To this end, B-cell lines were stimulated with citrullinated-peptide (CCP2)-tetramers or TT-tetramers on ice, followed by incubation at 37°C for several minutes to allow uptake of the antigen-bound IgG-BCR complex. Subsequently, the expression of the remaining BCRs on the B-cell surface was detected (Supplementary Figure 3). Our data show no effect of FcG on IgG-BCR and concurrent antigen uptake after 5, 15 or 30 min incubation at 37°C (Figure 3c). No BCR downmodulation was observed after stimulation with the arginine control peptide (CArgP2). Similarly, when using TT, we observed no effect of FcG on TT-specific IgG-BCR uptake when using TT. To substantiate these data, we also employed conventional techniques to stimulate and cross-link the BCR using Fab'2 directed against the kappa light chain constant domain to achieve robust downmodulation of the BCR. Again, FcG showed no effect on IgG-BCR internalization (Figure 3c). These data demonstrate that γHC glycosylation is not required for antigen-bound IgG-BCR uptake.

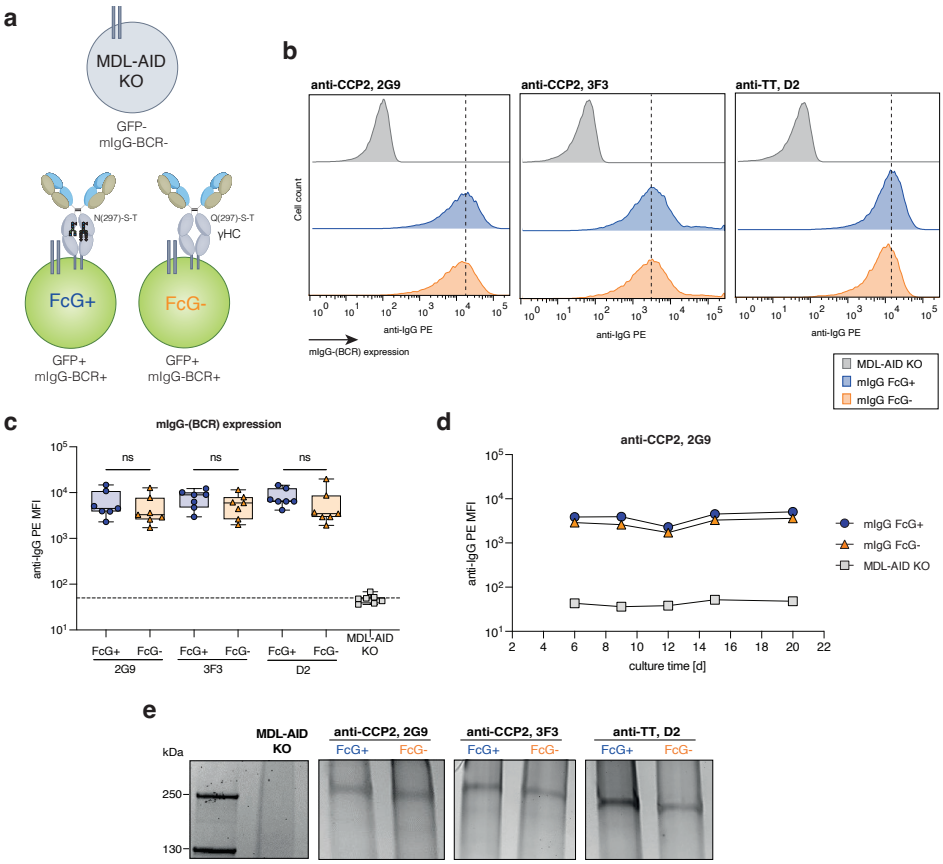


Figure 1: **Membrane-bound IgG (mIgG) [B-cell receptor (BCR)] expression in the presence and absence of Fc glycans (FcG).** a Schematic representation of generated Burkitt Lymphoma (Ramos) B-cell lines. B cells knocked out for their endogenous BCR and AID (MDL-AID KO), and B cells transduced with GFP and mIgG-BCR sequences including the conserved glycosylation site N(297)-S-T or mutant Q(297)-S-T to remove Fc glycans (FcG). b Flow histograms and c bar graphs of mIgG-BCR expression in the presence (FcG+) or absence of Fc glycans (FcG-). No BCR surface expression on MDL-AID KO cell line. Box plots show median, interquartile values, range and all individual data points. n=7 biologically independent experiments. ns (not significant) $p>0.05$ (two-sided unpaired t-tests). d mIgG-(BCR) expression of anti-CCP2 (2G9) FcG+ and FcG- and MDL-AID KO B cells over culture time (14 days). e SDS-PAGE of captured mIgG from FcG+ and FcG- B cell lines, two anti-CCP2 (2G9 and 3F3) and one anti-TT (D2), and MDL-AID KO control cells. Representative results of 2 biological replicates are shown. Source data are provided as a Source Data file.

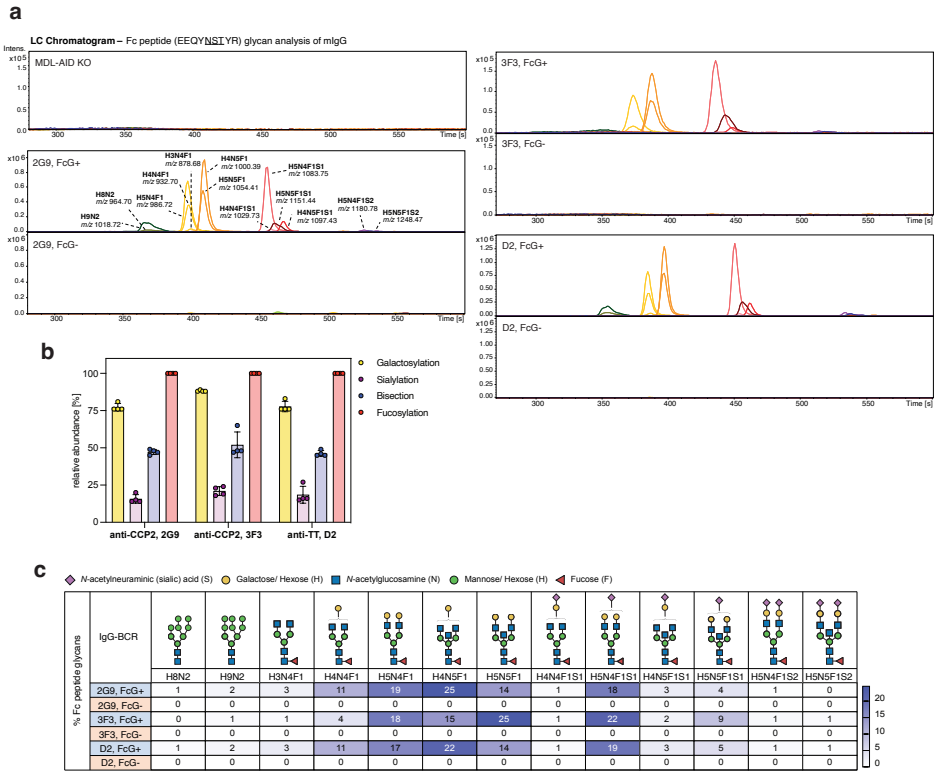


Figure 2: Fc peptide glycan analysis of mIgG-BCRs. a LC-MS Fc peptide glycan analysis of captured FcG+ and FcG- mIgG of all B-cell lines (2G9, 3F3 and D2). LC chromatograms of glycan peaks (with annotation and m/z) are shown. b Percentage of galactosylation, sialylation, bisection and fucosylation on mIgG ɣHC glycans (FcG+ B-cell lines). Bar graphs show mean, standard error and individual data points. n=4 biologically independent experiments. c Percentage of individual ɣHC glycan traits expressed on 2G9, 3F3 and D2 mIgG-BCRs. Glycan traits are schematically depicted. Heat map shows n=4 biologically independent experiments. Source data are provided as a Source Data file.

H = Hexose, N = N-acetylglucosamine, F = Fucose, S = sialic acid.

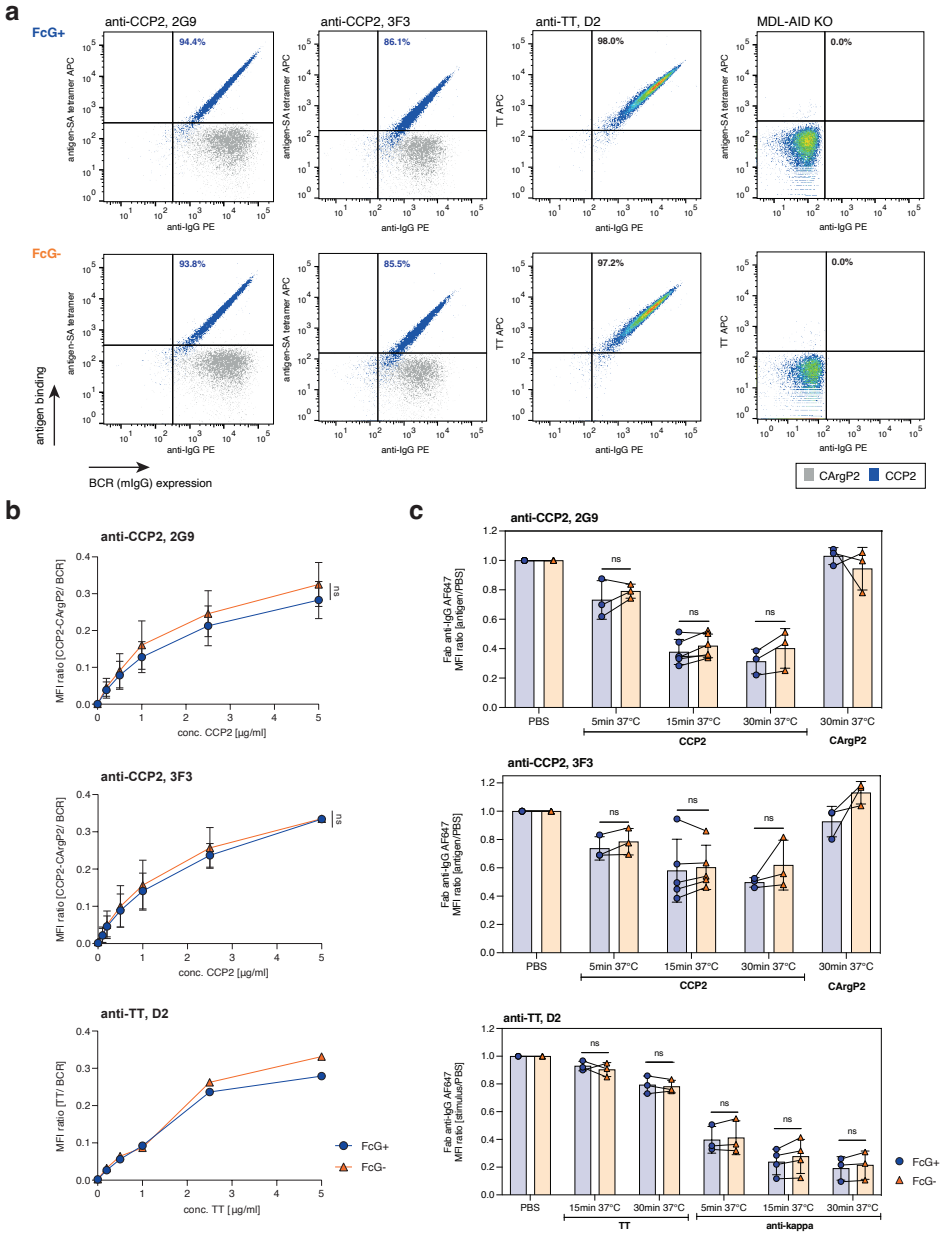
IgG-BCR Fc glycosylation has no effect on BCR signal transduction.

Based on the close proximity of the mIgG N(297)-linked glycans and the transmembrane Igα/Igβ signalling complex (16), we hypothesized that FcG might affect signal transduction of IgG-BCRs. We investigated activation of the IgG-BCR signalling complex by measuring phosphorylation of Syk kinase (Supplementary Figure 4a) and calcium flux (Supplementary Figure 4c) following BCR stimulation. BCRs were stimulated either with their respective antigen (CCP2 and TT) or Fab'2 antibodies directed against the constant domain of mIgG. All B-cell lines showed increased phosphorylation of

Syk (pSyk) after stimulation compared to the PBS treatment control (Figure 4a, b; Supplementary Figure 4b). We did not detect any effect of γHC glycosylation on the activation of the IgG-BCR complex, as indicated by similar median fluorescence pSyk intensities between the FcG- and FcG+ B-cell lines (Figure 4a, b; Supplementary Figure 4b). Similar pSyk expression was also observed after stimulating with titrated amounts of anti-IgG or antigen or when stimulating for different time points, further indicating that γHC glycosylation does not affect Syk-mediated BCR signalling or feedback signalling mechanisms (Figure 4c, d; Supplementary Figure 4d). Consistent with these results, calcium flux peak levels (Figure 5,a, b) and kinetics (Figure 5c; Supplementary Figure 4e) were also similar between FcG- and FcG+ mIgG B-cell lines while no calcium flux was observed after stimulation with PBS (Figure 5a).

Thus, the results are consistent with the data on BCR downmodulation and indicate that N(297)-linked glycans in the Fc domain of mIgG do not affect the function of the BCR:Igα/Igβ signalling complex.

Figure 3: mIgG-BCR FcG do not affect antigen binding and subsequent BCR downmodulation. a Gating strategy to assess BCR (mIgG) expression and antigen binding of human Ramos B-cell lines. Binding to the cyclic-citrullinated peptide 2 (CCP2, blue) and the unmodified arginine control peptide (CArgP2, grey) was assessed for 2G9 and 3F3 (overlay dot plot is shown) and binding to tetanus toxoid (TT) for the D2 B-cell line. No antigen binding was observed for the MDL-AID KO B cells that lack BCRs. b Antigen binding titration curves of 2G9, 3F3 and D2 BCRs to their respective antigens (CCP2-CArgP2 or TT) in a concentration range of 0 - 5 µg/ml. The median fluorescence intensity (MFI) relative to the BCR expression is shown. Points show mean and standard error. n=3 biologically independent experiments (2G9 and 3F3) or n=2 biologically independent experiments (D2). ns (not significant) $p>0.05$ (two-sided multiple paired t-tests). c Downmodulation of 2G9, 3F3 and D2 mIgG-BCRs after PBS, antigen or anti-kappa stimulation and incubation at 37oC for 5, 15 and 30 min. The MFI of the remaining mIgG-BCRs detected using Fab anti-IgG is depicted (ratio between MFI of stimulated and PBS treated cells). Bar graphs show mean, standard error and paired individual data points. n=3 biologically independent experiments (5min and 30min CCP2/ CArgP2/ TT or anti-kappa stim. and 15min TT stim.), n=4 biologically independent experiments (15min anti-kappa stim.), n=5 biologically independent experiments (15min CCP2 stim. of 3F3) or n=6 biologically independent experiments (15min CCP2 stim. of 2G9). ns $p>0.05$ (two-sided paired t-tests). Source data are provided as a Source Data file. ▸


a
FcG+
FcG-
anti-CCP2, 2G9
anti-CCP2, 3F3
anti-TT, D2
MDL-AID KO
94.4%
86.1%
98.0%
0.0%
93.8%
85.5%
97.2%
0.0%
antigen-SA tetramer APC
TT APC
anti-IgG PE
antigen binding
BCR (mIgG) expression
CArgP2
CCP2
b
anti-CCP2, 2G9
anti-CCP2, 3F3
anti-TT, D2
MFI ratio [CCP2-CArgP2/ BCR]
MFI ratio [TT/ BCR]
conc. CCP2 [µg/ml]
conc. TT [µg/ml]
ns
FcG+
FcG-
c
anti-CCP2, 2G9
anti-CCP2, 3F3
anti-TT, D2
Fab anti-IgG AF647
MFI ratio [antigen/PBS]
MFI ratio [stimulus/PBS]
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FcG-

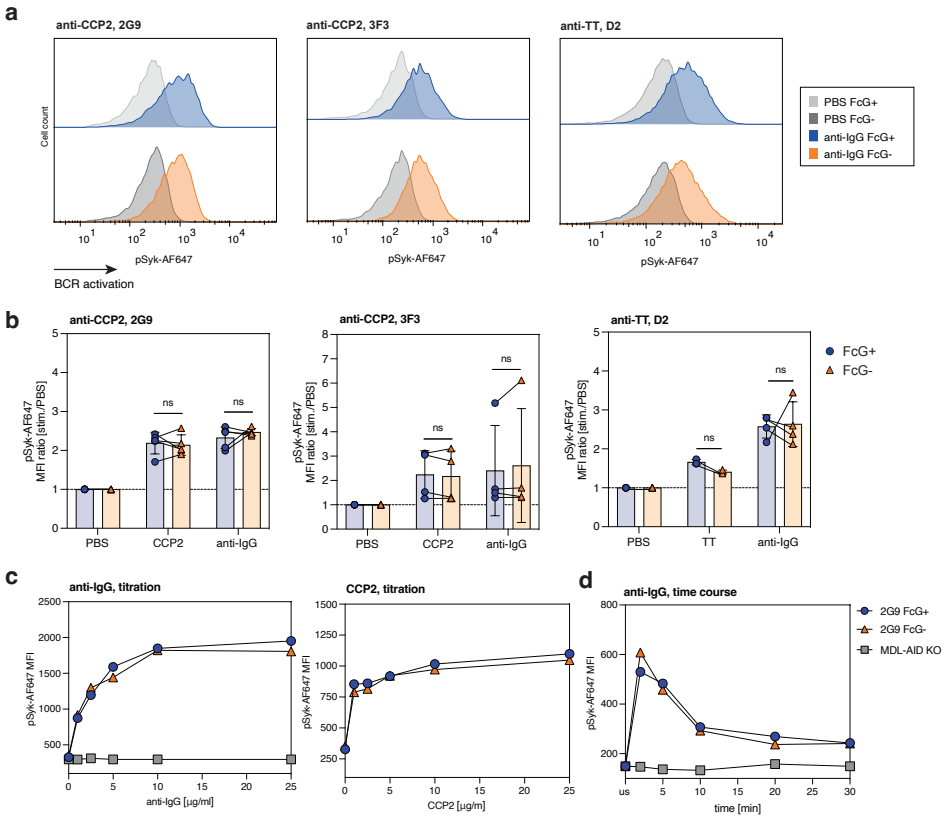

Figure 4: No influence of mIgG FcG on BCR signal transduction. a Flow histograms showing phosphorylation of Syk (pSyk) of 2G9, 3F3 and D2 FcG+ and FcG- B cells after 5 min PBS and anti-IgG stimulation (5 µg/ml). b pSyk MFI values of 2G9, 3F3 and D2 FcG+ and FcG- B cells after 5 min stimulation. Ratio towards PBS unstimulated cells is depicted. Bar graphs show mean, standard error and paired individual data points. n=3 biologically independent experiments (5 µg/ml TT stim. of D2), n=4 biologically independent experiments (5 µg/ml CCP2 stim. of 3F3 and 5 µg/ml anti-IgG stim of 3F3/ D2) or n=5 biologically independent experiments (C5 µg/ml CP2 and 5 µg/ml anti-IgG stim. of 2G9). ns (not significant) p>0.05 (two-sided paired t-tests). c pSyk MFI values of 2G9 FcG+ and FcG- B cells after 5 min stimulation with 0 – 25 µg/ml anti-IgG and CCP2. Representative results of 2 biological replicates are shown. d pSyk MFI values of 2G9 FcG+ and FcG- B cells unstimulated (us) and after 5 – 30 min stimulation with 5 µg/ml anti-IgG. MDL-AID KO cells are shown as control. Representative results of 2 biological replicates are shown. Source data are provided as a Source Data file.

Human B-cell lines can produce IgG in the absence of γHC glycosylation.

To determine whether Fc glycosylation affects IgG secretion by B cells, we next transduced the MDL-AID KO Ramos B cells with N(297)-γHC or mutant Q(297)-γHC IgG sequences in the absence of the transmembrane domain (Figure 6a). We then

screened the supernatant of Ramos B cells for the presence of FcG- or FcG+ 2G9 IgG with an antigen-specific (CCP2) IgG ELISA. The transduced Ramos B cells were able to secrete an average of ~550ng/ml of 2G9 IgG, while the non-transduced MDL-AID KO cells did not. We could not detect an effect of FcG on 2G9 antibody secretion (Figure 6b). Furthermore, the secreted antibodies had the expected apparent molecular weight as determined by gel electrophoresis, with a marginal size shift between the FcG- and FcG+ variants, due to the absence of two γHC glycans (Figure 6c). We confirmed the absence of the γHC glycans in the FcG- secreted IgG (sIgG) by Fc-peptide glycan analysis and LC-MS (Figure 6d, e). Comparable glycan profiles were obtained for the FcG+ sIgG and mIgG (Supplementary Figure 2d). Analogous to 2G9 mIgG, the Fc glycans on 2G9 sIgG were mainly S0 or S1 complex-type glycans with a core fucose. 2G9 sIgG had similar galactosylation (79%), slightly lower bisection (39%) and terminal sialic acid (12%) levels than 2G9 mIgG (Figure 6f).

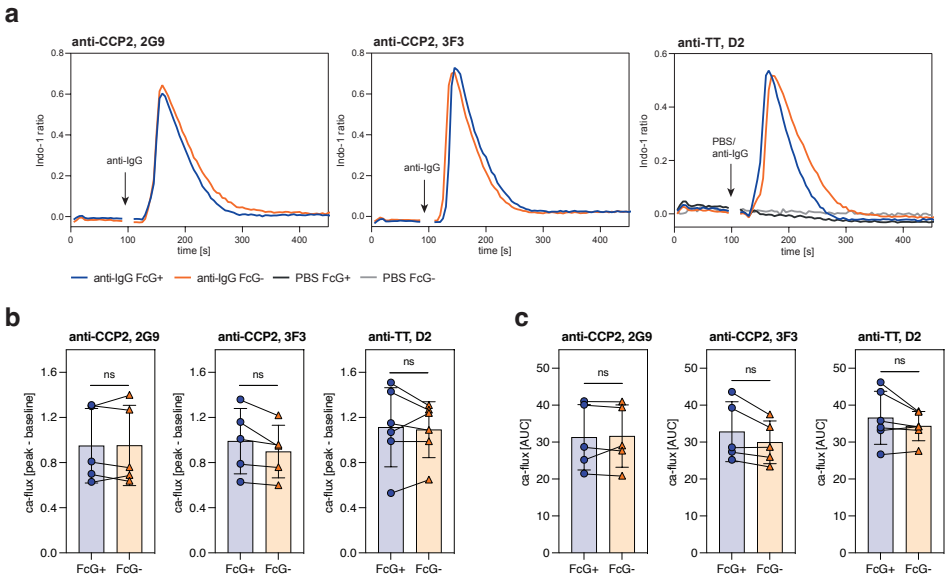

Figure 5: mIgG FcG do not affect BCR-mediated calcium flux. a Calcium flux (Calcium-bound Indo-1/ unbound Indo-1) histograms of 2G9, 3F3 and D2 FcG+ and FcG- B cells after anti-IgG stimulation. PBS treatment control is shown for D2 cell lines. b Calcium flux (peak – baseline) and c kinetics (area under the curve, AUC) of FcG+ and FcG- B-cell lines. Bar graphs show mean, standard error and paired individual data points. n=5 biologically independent experiments (2G9 and 3F3) or n=6 biologically independent experiments (D2). ns (not significant) p>0.05 (two-sided paired t-tests). Source data are provided as a Source Data file.

These data demonstrate that human B cells are capable of secreting IgG in the absence of γHC glycosylation and that sIgG and mIgG express similar complex-type A2 glycans at N(297) characterized by core fucosylation, terminal galactosylation, various amounts of bisection and the presence of one sialylated antennae. The data also indicate that cellular trafficking is not affected by FcG and that sIgG and mIgG follow similar biosynthesis pathways.

Secreted IgG are unable to recruit effector functions in the absence of Fc glycans.
Our results suggest that FcG are not required for mIgG-BCR function. To rule out the possibility that the IgG used in this study are unusual in their biological properties, we next wanted to confirm that their ability to recruit effector functions depends on the presence of FcG.

First, we tested the sIgG for their ability to bind human FcγR using surface plasmon resonance (SPR). As expected, FcG- sIgG were unable to bind to human FcγRII/ IIIa and b and showed only low binding to the high-affinity FcγRI (K_D=250 nM) (Figure 7a, b). In contrast, the FcG+ sIgG showed binding to all assessed FcγRs. Affinities could be calculated for the medium-affinity receptors FcγRIIa 131H (K_D=433 nM), FcγRIIa 131R (K_D=507 nM), FcγRIIIa 158F (K_D=573 nM), FcγRIIIa 158V (K_D=362 nM) and for the high-affinity FcγRI (K_D=12 nM) (Figure 7b), whereas no affinity calculation could be performed for binding to the low-affininity FcγRIIb and IIIb NA2 (Supplementary Figure 5). In addition to FcγR binding, we determined the effect of FcG on activation of the classical complement pathway using a previously described and established complement ELISA (30). Both sIgG were able to bind to the antigen (CCP2)-coated ELISA plate, indicating the production of intact IgG molecules and supporting the finding that FcG do not affect antigen binding (Figure 7c). However, we could only observe the binding of C1q and subsequent C4 and C3c deposition to the antigen-bound IgG that expressed γHC glycans (Figure 7d). The Q(297)-γHC mutant showed complement deposition ELISA signal intensities that were comparable to signals observed in the absence of exogenous complement (normal human serum, NHS) (Figure 7d).

In summary, our results show that γHC glycosylation is crucial for sIgG to exert its effector functions, such as binding to FcγRs and activation of the classical complement cascade.

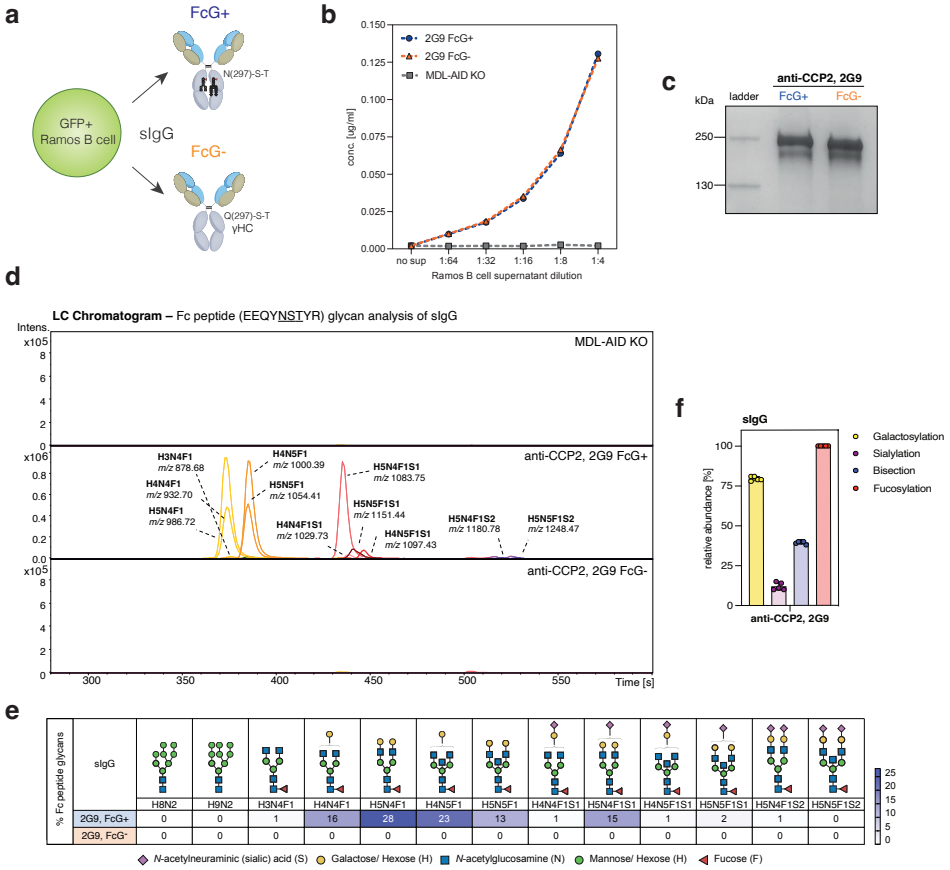

Figure 6: B cell IgG secretion in the presence and absence of γHC glycosylation. a Schematic representation of Ramos B cells secreting IgG with either N(297)-S-T or mutant Q(297)-S-T γHCs. b 2G9 IgG FcG+ and FcG- secreted by Ramos B cells detected using an antigen-specific (CCP2) IgG ELISA. No anti-CCP2 IgG was detected in B-cell supernatant of MDL-AID KO cells that don't secrete IgG. Mean and standard error of 2 technical replicates (one experiment) are shown. c SDS-PAGE of Ramos B cell secreted 2G9 IgG with and without FcG. Representative results of 3 biological replicates are shown. d LC-MS Fc peptide glycan analysis of captured FcG+ and FcG- 2G9 sIgG. LC chromatograms of glycan peaks (with annotation and m/z) are shown. e Percentage of γHC glycan traits expressed on 2G9 sIgG. Glycan traits are schematically depicted. Heat map shows mean of 4 biological replicates. f Percentage of galactosylation, sialylation, bisection and fucosylation on mIgG γHC glycans (FcG+ B-cell lines). Bar graphs show mean, standard error and individual data points. n=5 biologically independent experiments. Source data are provided as a Source Data file.

H = Hexose, N = N-acetylglucosamine, F = Fucose, S = sialic acid

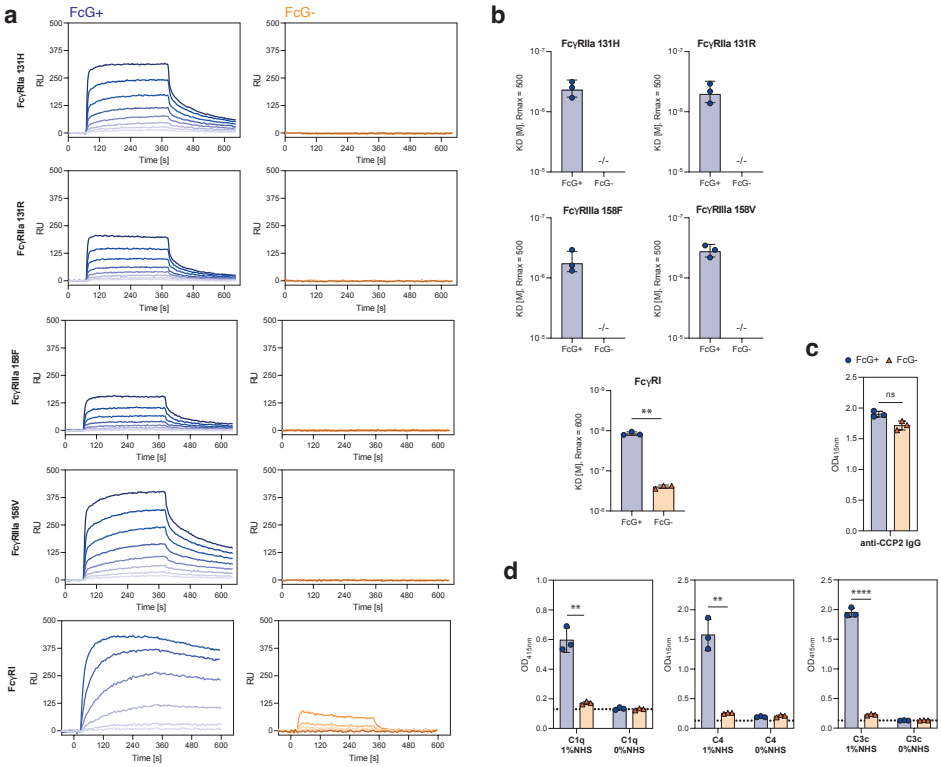

Figure 7: Impact of IgG FcG on the recruitment of effector functions. a Representative SPR sensorgrams of 2G9 sIgG FcG+ and FcG- binding to human FcγRI and II/ IIIa. Association and dissociation are represented as response units [RU] over time [s]. b KD values of binding of FcG+ and FcG- 2G9 sIgG to human FcγRI and II/ IIIa as determined by SPR. Bar graphs show mean, standard error and individual data points. n=3 biologically independent experiments. ** p=0.0036 (two-sided paired t-test). c Detection of intact antigen-specific (CCP2) IgG and d antigen-specific classical complement activation of FcG+ and FcG- 2G9 sIgG determined by ELISA. Deposition of complement components C1q, C4 and C3c was detected in the presence or absence of exogenous complement (normal human serum, NHS). Bar graphs show mean, standard error and individual data points. n=3 technical replicates of one experiment. ns (not significant) p>0.05, ** p=0.001 (C4), ** p=0.0009 (C1q), **** p=0.000002 (C3c) (two-sided unpaired t-tests). Source data are provided as a Source Data file.

Discussion

Here we have shown that while γHC glycosylation is indispensable for secreted IgG to recruit effector functions, it is paradoxically not required for the function of membrane-bound IgG-BCRs. Interestingly, no effect of FcG on a stable BCR surface expression and B-cell activation was observed, although the N(297)-linked glycans on mIgG are in close proximity to the ITAM-bearing Igα/ Igβ signalling complex and are involved in shaping the 3D-structure of BCRs (16). BCR expression was measured over a 20-day period to exclude the possibility that fluctuations in mIgG surface expression influenced subsequent functional responses. Furthermore, we have shown that neither antigen binding nor subsequent BCR antigen uptake is affected by γHC glycosylation and that human B cells secrete aglycosylated antibodies in a similar manner as their glycosylated counterparts. However, when secreted IgG lack FcG, they can no longer recruit effector functions.

A previous study reported that core fucosylation of mIgG is required for BCR function (26). However, it is conceivable that the experimental design used by the authors is not suitable to address this critical question, as core fucosylation was not studied in a BCR-specific manner, rather ubiquitously for all N-linked glycans. It is therefore likely that an aberrant B cell N-glycome affects B-cell function rather than BCR Fc glycosylation as described.

To investigate the effects of IgG-BCR Fc glycans on B-cell function, we made use of the Burkitt lymphoma B-cell line (Ramos) and knocked-out the N(297)-linked glycosylation site on mIgG. Ramos cells are human GC-derived B cells that express co-receptors in close proximity to the BCR that can regulate B-cell fate, such as CD19, CD20 and the sialic-acid binding immunoglobulin-like lectin CD22 (Siglec-2) (31, 32). We hypothesized that FcG could affect the co-localization of these regulatory receptors with the BCR and thus influence B-cell activation. For example, there is evidence suggesting that sialic acids expressed on IgM-BCR N-glycans interact with the lectin CD22 to regulate downstream signalling events (33). It is therefore tempting to speculate that also sialylated IgG-BCR N-glycans interact with CD22. However, we show here that cross-linking between the IgG-BCR and CD22 via glycan-lectin interactions is unlikely, as we did not observe any effect of FcG knock-outs on BCR signalling events. This could likely be explained by the differences in mIgG- vs mIgM-BCR assembly and positioning (16, 24, 25). In addition, the long cytoplasmic tail of mIgG, which amplifies signalling independent of the Igα/ Igβ signalling complex (24), could potentially limit the effect of γHC glycans on B-cell activation.

Nevertheless, we cannot exclude the possibility of interactions with other lectins expressed on the B cell surface (in *cis*) or on surrounding immune cells (in *trans*) that are not present in our B-cell culture system. However, we consider *trans* interactions less likely because FcG on IgG-BCRs are "hidden" between the two ɣHCs and are therefore less accessible to *trans*-lectins than, for example, glycans expressed in the variable domains. In addition, similar to IgM-BCRs(34), IgG-BCR Fc glycans could modulate the spatial organization of the BCR relative to other co-receptors through interactions with secreted lectins, e.g. galectin-9, thereby influencing B-cell activation. The possible effect of Fc glycans on the spatial distribution of IgG-BCRs could potentially also apply to core fucosylation, thereby explaining some of the in vivo results described previously (26). Thus, although our data do not support a direct influence of Fc glycans, including core fucosylation, on BCR signal transduction in the B cell lines studied here, they may affect mIgG-BCR function of primary B cells (e.g., resting memory B cells), especially when studied in the context of other immune cells. However, testing this would be a major technical challenge in the human system, as it is difficult to obtain and maintain antigen-specific human memory B cells in sufficient numbers to knock-out Fc glycan sites and perform functional experiments.

In addition, our study sheds light on the structure of ɣHC glycans expressed on BCRs, which was currently, to the best of our knowledge, unknown. We report that the conserved FcG on Ramos IgG-BCRs are mainly A2 complex-type glycans carrying core fucosylation, 77-88% galactosylation, 16–21% sialylation and 46–52% bisection. We observed a similar glycosylation profile for IgG secreted by human Ramos B cells, suggesting that mIgG and sIgG follow similar biosynthesis routes and that antibody sialylation occurs B cell intrinsically, as has also been evidenced by others (35). Nonetheless, since antibody secreting cells (i.e. plasma blasts and plasma cells) belong to different B-cell subsets compared to B cells expressing membrane-bound IgG (naïve and memory B cells), it is also conceivable that Fc glycan compositions differ between IgG-BCRs and secreted IgG (27). As the mIgG Fc glycan profile was obtained after B cell lysis, a minor fraction of high-mannose glycans was observed. This is probably best explained by co-capturing of IgG-BCRs in the endoplasmic reticulum (ER) or *cis*-Golgi, where proteins express glycans that still need to be processed by glycosidases and glycosyltransferases during transfer to the *medial*- and *trans*-Golgi (36). After capturing of surface biotinylated IgG-BCRs no apparent high-mannose glycans could be detected. In addition, no mannose-rich glycans were detected in the Fc domain of sIgG, as all intact immunoglobulins secreted by B cells passaged the entire Golgi network.

In summary, we report that ɣHC glycosylation has no effect on the stable expression of functional IgG-BCRs on the surface of human B cells, implying that this conserved glycan modification plays no role in heavy and light chain assembly or the interaction with the signalling subunits Igα and Igβ. Similarly, BCRs show no difference in antigen binding, subsequent uptake of BCR-antigen complexes, BCR signalling or the secretion of intact IgG in the absence or presence of ɣHC glycans. As expected, we show that the absence of Fc glycosylation results in IgG that cannot mediate ADCC or CDC. These results indicate that ɣHC glycosylation likely evolved as a feature to control the effector functions of secreted IgG, but not as a mechanism to determine B-cell fate.

Methods

Cell lines and cell culture – Human Burkitt Lymphoma (Ramos) B-cell transfectants (anti-CCP2 2G9, anti-CCP2 3F3 and anti-TT D2) were generated as described earlier (28). In brief, IgG-BCR sequences were obtained from single B cells of ACPA+ patients with RA. Labelled CCP2/ CArgP2-streptavidin tetramers or tetanus toxoid were used for antigen-specific B-cell isolation (37). BCR sequencing was performed from cDNA of single sorted B cells using ARTISAN-PCR as described earlier (38). VH and VL were together with the IGHG1*03 or the IGKC constant domain (Uniprot), the Kozak sequence and the IGHV1-18*01 leader sequence codon-optimized and ordered from GeneArt (Life Technologies). Full-length LC and ɣHC were cloned into the pMIG-IRES-GFP-2AP vector backbone in the presence or absence of the IGHG1 transmembrane domain using the In-Fusion HD Cloning Kit (Clontech). For the generation of FcG- variants, the ɣHC N(297)-linked glycosylation site was mutated into Q(297)-S-T using Site Directed Mutagenesis PCR (NEB). Inserts were verified by Sanger sequencing performed on Applied Biosystems 96-capillary (ABI3730) systems (LGTC facility, Macrogen). Retroviral transductions were performed as described earlier (29). Briefly, Phoenix-ECO (ATCC; CRL-3212™) cells were transfected with PolyJet DNA transfection reagent (SignaGen Laboratories). Retrovirus containing supernatants were collected 72 hours post-transfection and used for the transduction of GFP- MDL-AID (IGHM, IGHD, IGLC and AID) KO Ramos B cells carrying *slc7a1* (generated by Dr He, University Freiburg)(28). B-cell lines were cultured in RPMI1640/GlutaMAX™/10% fetal calf serum (FCS)/10 mM Hepes medium (Thermo Fisher Scientific) with penicillin/ streptomycin (100 U/ml; P/S) (Lonza). To compare GFP+ FcG+ and GFP+ FcG- B-cells in functional assays, B-cell lines were sorted by identical GFP and mIgG BCR expression using 1 µg/ml AF647 NHS ester (Thermo Fisher; A2006) labelled Fab fragment goat anti-human IgG (Jackson ImmunoResearch; 109-007-003) on a CytoFlex SRT cell sorter (Beckman Coulter).

To analyse the ability of FcG+ and FcG- IgG1 antibodies to bind FcγRs and activate the complement system, we produced both variants of 2G9 in Freestyle 293-F cells (Gibco) (28, 39). Therefore, LC and γHC or Q(297)-mutant γHC sequences were cloned into pcDNA3.1 (+) expression vectors using the In-Fusion HD Cloning Kit (Clontech) as mentioned above.

Flow cytometry – Membrane-bound IgG (BCR) expression of transduced FcG+ and FcG- 2G9, 3F3 and D2 GFP+ B-cell lines and the non-transduced MDL-AID KO GFP- B-cell line was analysed using flow cytometry. B cells were stained with 0.5 µg/ml goat anti-human IgG-Fc phycoerythrin (PE) (eBioscience™; 12-4998-82; lot: 2481260) in staining solution (PBS/0.5%BSA/ 0.02% NaN3) on ice for 30 min. To analyse BCR expression over time, rainbow beads (Spherotech; RFP-30-5A) were used for assay standardization. To determine antigen binding, B cells were stained with Allophycocyanin (APC)-labelled CCP2/CArgP2-streptavidin tetramers (0 to 5 µg/ml)(37) or APC-TT (0 to 5 µg/ml) on ice for 30 min. For anti-CCP2 B-cell lines binding to the CArgP2 negative-control antigen was subtracted.

BCR downmodulation after stimulation was assessed as described earlier (39). In short, 0.2 million B cells were incubated for 30 min on ice followed by 15 min stimulation at 4°C either with control PBS, CCP2-/CArgP2-streptavidin tetramers (5 µg/ml), TT-streptavidin tetramers (1.5 µg/ml) or 1 µg/ml goat F(ab')2 anti-human kappa (SouthernBiotech; 2062-01) in PBS/2% FCS. Stimulated B cells were incubated for 0, 5, 15 or 30 min at 37°C to allow BCR-antigen uptake. The surface remaining IgG-BCRs were stained with AF647 NHS (*N*-hydroxysuccinimide) ester (Thermo Fisher Scientific; A20006) labelled Fab goat anti-human IgG (Jackson ImmunoResearch; 109-007-003) diluted 1:2000 in staining solution.

IgG-BCR activation was analysed by the intracellular expression of phosphorylated Syk (pSyk) after antigen (0 to 25 µg/ml) or anti-IgG (0 to 25 µg/ml) stimulation as described earlier (39). Briefly, 0.3 million B cells were stimulated with CCP2-streptavidin tetramer, TT-streptavidin tetramer or goat anti-human IgG F(ab')2 (Jackson ImmunoResearch; 109-006-097; lot: 164786) at 37°C in stimulation medium (RPMI/100 U/ml P/S/GlutaMAX™/10 mM Hepes/1% FCS). The amount of the stimulus is given in the respective figure legend. Afterward, cells were fixed (BioLegend Fixation Buffer; 420801) and permeabilized (True-Phos™ Perm Buffer; 425401). After washing, the intracellular expression of phosphorylated Syk was determined with a mouse anti-human pSyk(Y319)-AF647 mAb (17A/P-ZAP70; BD; 557817; lot: 9165873) diluted 1:10 in staining solution. The rate of pSyk expression was calculated as the median fluorescence intensity (MFI) ratio between stimulated and unstimulated cells. Gating was based on stimulated MDL-AID KO control B cells. Stained cells were analysed on a BD LSR-II flow cytometry instrument. Data were analysed with FlowJo_V10.

Calcium flux measurement – B-cell activation was determined by calcium release after anti-IgG stimulation as described earlier (39). In brief, 1 million B cells were stained in 200 µl calcium- indicator loading dye medium containing 2 µM Indo-1 AM (Abcam; ab142778) and 0.05% pluronic acid (Molecular Probes; P6866) in stimulation medium (RPMI1640/100 U/ml P/S/GlutaMAX™/10 mM Hepes/2%F°S) for 35 min at 37°C in the dark. After washing, B cells were incubated with 500 µl stimulation medium plus 2 mM calcium on ice and in the dark until usage. 15 min before the flow analysis, B cells were prewarmed at 37°C to decrease baseline activation upon measurement. The analysis was performed on a Cytek Aurora 5L instrument including a UV laser and acquiring 500 cells/s at a high speed. After ~1.5 min of baseline measurement, PBS or 20 µg/ml goat anti-human IgG F(ab')2 (final concentration) (Jackson ImmunoResearch; 109-006-097; lot: 164786) was added and mixed adequately. The measurement continued for another 6 min until the signal reached baseline again. Calcium flux was measured as the ratio of calcium-bound Indo-1 to unbound Indo-1. Calcium flux (peak-baseline signal) and kinetics (AUC) were analysed. Data were analysed with FlowJo_V10.

Isolation and gel electrophoresis of IgG-BCR and secreted IgG – To capture IgG-BCRs, 20 million Ramos B cells were washed with PBS, to remove FCS from cell culture medium, and lysed in 8 ml PBS + 1% Triton-X100 for 60 min at 37°C. IgG-BCRs were captured from cell-lysis supernatants using 20 µl CaptureSelect™ FcXL Affinity Matrix (Thermo Fisher Scientific) and an overnight incubation at 4°C. B cell secreted IgG were captured from 12 ml Ramos B cell supernatant (cultured at a density of 2 million cells/ml) by a 4°C overnight incubation with 20 µl CaptureSelect™ FcXL Affinity Matrix (Thermo Fisher Scientific). Laemmli sample buffer (4×) (Bio Rad) was added to the IgG/FcXL bead slurry, boiled for 5 min at 95°C, and loaded on a 4 to 15% SDS gel (Bio Rad). Proteins were detected with Coomassie Brilliant Blue G-250 Dye (Thermo Fisher Scientific) or the SilverQuest™ Silver Staining Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. The size was determined using the PageRuler™ Plus Prestained Protein Ladder (Thermo Fisher Scientific).

Surface biotinylation and membrane-IgG glycan analysis by Western blot – Ramos cell surface biotinylation was performed according to the manufacturer's instructions of the Cell-Surface Protein Biotinylation and Isolation Kit (Pierce; A44390). In brief, cells were biotinylated using Sulfo-NHS-SS-Biotin for 30 min at RT. After cell lysis, biotinylated IgG were captured using NeutrAvidin™ Agarose and eluted in elution buffer containing 10 mM DTT for sample reduction. For further sample denaturation 2% SDS was added and the samples were incubated for 30 min at 60 °C. Reduced biotinylated IgG (~10ng) were treated with 2U EndoH (Roche) in 50 mM sodium acetate buffer pH 5.5 or 2U PNGaseF (Roche) in 1:1 5X PBS/4% NP-40 over night at 37 °C. The presence

of high-mannose (EndoH treatment) or other (PNGaseF treatment) N-glycans on the captured IgG was identified via a size-shift by Western blot analysis. Therefore, lysates were mixed with 4X Laemmli-buffer (BioRad), incubated for 5 min at 95 °C and loaded on a 4% to 15% SDS-polyacrylamide gel (BioRad). Immunoblotting was performed on a Nitrocellulose membrane (BioRad). Blots were incubated in 3% skim milk powder/ PBS/ 0.05% Tween (PTE) for 2h at RT. Following washing with PBS/ 0.05% Tween (PT), blots were incubated at 4 °C overnight with goat anti-human IgG (Invitrogen; 31410) diluted 1:1000 in PTE. Bound antibodies were visualized using enhanced chemiluminescence (GE Healthcare; RPN-2109). Readout was performed on a BioRad Chemidoc Touch Imaging system.

In gel IgG Fc peptide glycan analysis by LC-MS – The IgG-BCR protein bands were extracted from the SDS gel, to exclude glycosylated contaminants, and transferred to 1.5 ml Eppendorf safe-lock microcentrifuge tubes (Merck). Gel pieces were destained according to the SilverQuest™ Silver Staining Kit (Thermo Fisher Scientific), washed with 25 mM ammonium bicarbonate (ABC), followed by 100% ACN and, for protein reduction, incubated for 30 min at 56°C in 10 mM dithiothreitol (DTT)/25 mM ABC. After reduction, gel pieces were washed with 100% ACN and, for alkylation, incubated for 30 min at RT in the dark in 55 mM iodoacetamide/25 mM ABC to block reactive cysteine groups. After washing with 25 mM ABC and 100% ACN, gel bands were dried in a centrifugal vacuum concentrator for 5 min. In gel proteins were digested by adding 16.5 µg/ml sequencing grade modified trypsin (Promega; V5111) in 25 mM ABC, followed by overnight incubation at 37°C. Trypsinised IgG (1-2 µl) were separated on an Ultimate 3000 UHPLC system (Dionex/ Thermo Fisher Scientific, Breda, The Netherlands) coupled to a MaXis Impact HD quadrupole-TOF mass spectrometer (MS) (MaXis HD, Bruker Daltonics, Bremen Germany) equipped with a CaptiveSpray NanoBooster source (Bruker Daltonics, Bremen, Germany) (40, 41). Briefly, trypsinised samples were extracted by a C18 trap column (Acclaim PepMap 100; 100 µm by 2 cm, particle size of 5 µm, pore size 100 Å; Dionex/Thermo Fisher Scientific) and washed for 2 min with 15 µl/min of 0.1% formic acid (FA)/ 1% ACN. Samples were separated on a C18 analytical liquid chromatography (LC) column (Acclaim PepMap 100; 75 µm by 15 cm, particle size of 3 µm, pore size of 100 Å; Dionex/Thermo Fisher Scientific), and elution was performed at a flow rate of 700 nl/min with buffer A (0.1% FA (v/v)) and buffer B (95% ACN/ 0.1% FA (v/v)). A gradient of 1% to 70% buffer B in 70 min was applied (t=0 min, B=1%; t=5 min, B=1%; t=30 min, B=50%; t=31 min, B=70%; t=35 min, B=70%; t=36 min, B=1%; t=70 min, B=1%). The CaptiveSpray NanoBooster was operated with ACN-enriched gas (0.2 bar) and dry gas (3 liters/min) at 180°C and a capillary voltage of 1150 V. Mass spectra were acquired within a mass range of m/z 550 to 1800. Data were collected using Compass 1.9 for OTOF version 4.0.15.3248 (Bruker

Daltonik GmbH). Data were assessed using DataAnalysis (Bruker Daltonics) and the calculated masses of IgG1 Fc glycan peptides after tryptic digestion. Data processing, including peak integration, was performed using LaCyTools v1.1.0 (https://github.com/Tarskin/LaCyTools). Quality control (QC) was based on signal-to-noise (S/N) ratio above nine, a mass accuracy of +/-20, and an isotopic peak quality of 0.2 (42). The degree of galactosylation (G), sialylation (S), fucosylation (F) and the frequency of bisecting N-acetylglucosamine (GlcNAc, N) were calculated as described earlier (41).

Enzyme-linked immunosorbent assay (ELISA) – To analyse the amount of functional secreted IgG by Ramos B cells, an antigen-specific IgG ELISA was performed. For this purpose, the B cell supernatant was diluted (from 1:4 to 1:64) in PBS/ 0.05%Tween/ 1% BSA (PBT) and added overnight at 4°C on streptavidin plates (Microcoat; 65001) coated with biotinylated CCP2 (1 µg/ml). Deposition of anti-CCP2 IgG to the ELISA plate was detected with an HRP-conjugated rabbit-anti-human IgG secondary detection antibody (DAKO; P0214; lot: 20036015; 1:3000). The ELISA was developed with ABTS and H_2O_2, and the absorbance was read at 415 nm.

To assess classical complement activation of the secreted anti-CCP2 IgG, complement ELISA were performed as described earlier (30). Briefly, IgG were added on CCP2-coated plates (4 µg/ml) and anti-CCP2 IgG deposition detected as described above. To assess complement deposition, 0% or 1% complement-active normal human serum (NHS) diluted in GVB++ (veronal buffered saline (VBS) containing 0.5 mM MgCl2, 2 mM CaCl2, 0.05% Tween 20, and 0.1% gelatin [pH7.5]) were added to the anti-CCP2 IgG coated ELISA plates for 1 hour at 37°C. Complement deposition was determined with rabbit anti-C1q (DAKO; A0136; 1:1000), goat anti-C4 (QUIDEL; A305; 1:1000) or rabbit anti-C3c (DAKO; A0062; 1:1000) secondary antibodies, added in PBT for 1 hour at 37°C. Binding was detected with HRP-labelled goat anti-rabbit (DAKO; P0448; 1:3000) or rabbit anti-goat (DAKO; P0449; 1:3000) detection antibodies, each diluted in PBT at 37°C for 1 hour. ELISA read out was performed using ABTS and H_2O_2 and an absorbance at 415 nm.

SPR measurements – SPRs measurements were performed on an IBIS MX96 (IBIS technologies) as described earlier (43). In brief, biotinylated hFcγRs were spotted using a Continuous Flow Microspotter (Wasatch Microfluidics) onto a single SensEye G-streptavidin sensor (Ssens; 1-08-04-008). The hFcγRs were spotted in dilutions ranging from 10 nM to 0.3nM for hFcγRIIa 131H, hFcγRIIa 131R and hFcγRIIβ, from 30 nM to 1nM for hFcγRIIIa 158F and hFcγRIIIβ NA2, and from 100 nM to 3nM for hFcγRIIIa 158V in in PBS + 0.075% Tween-80 (VWR; M126–100 ml), pH 7.4. Biotinylated anti-His mIgG1 (GenScript; A00613) was spotted in duplicate and 3-fold dilution, ranging from 30 nM to 1 nM. Subsequently, 50 nM His-tagged hFcγRI was loaded onto the sensor. IgG

were then injected over the IBIS at 2-fold dilution series starting at 0.49 nM until 1000 nM in PBS + 0.075% Tween-80. Regeneration after every sample was performed with 10 nM Glycine-HCl, pH 2.4. Dissociation constant (K_D) was calculated by equilibrium fitting to R_{max}=500. Association and dissociation curves of His-tagged hFcγRI were subtracted before calculation of IgG binding affinity using SPRINT 1.9.4.4 software (IBIS technologies). Analysis and calculation of all binding data was performed with Scrubber software version 2 (Biologic Software) and Excel.

Statistics and Reproducibility – Statistical analyses were performed using GraphPad Prism. (Multiple) paired or unpaired two-sided *t* tests were performed as indicated in the respective figure legends. Number of biologically independent replicates (B cells or antibodies were harvested and processed in individual experiments on different days) or technical replicates (samples were processed in the same experiment) are indicated in the respective figure legends. No statistical method was used to predetermine sample size. No data were excluded from the analyses. The experiments were not randomized. The Investigators were not blinded to allocation during experiments and outcome assessment.

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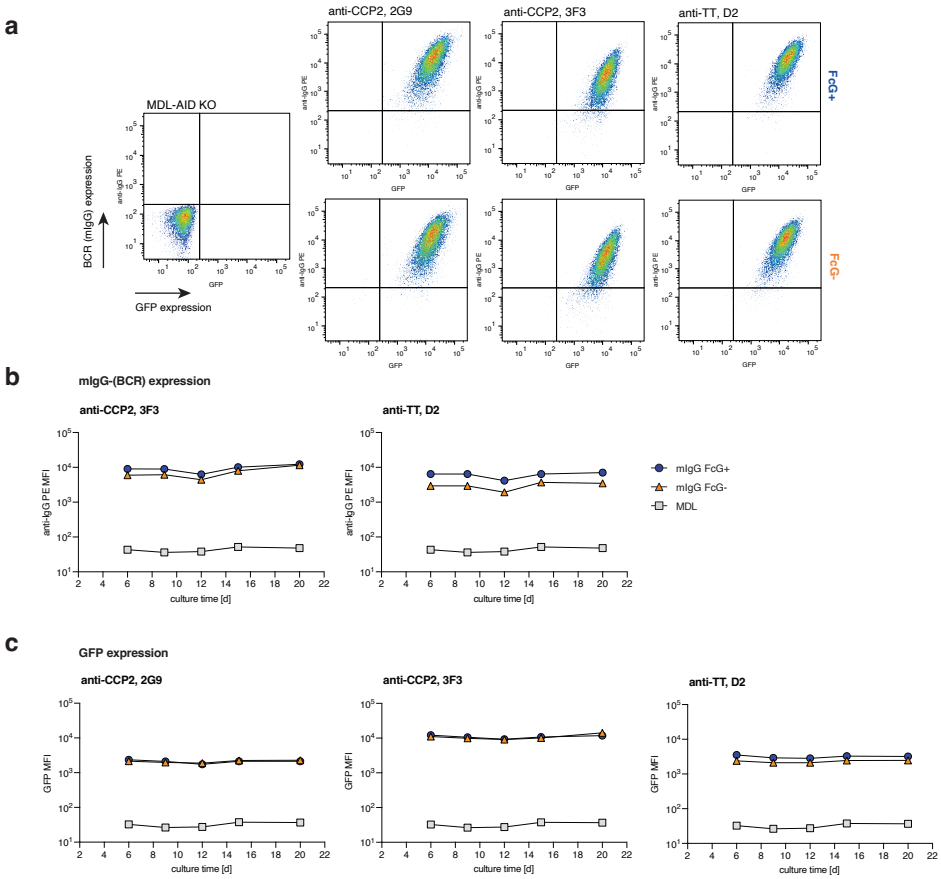
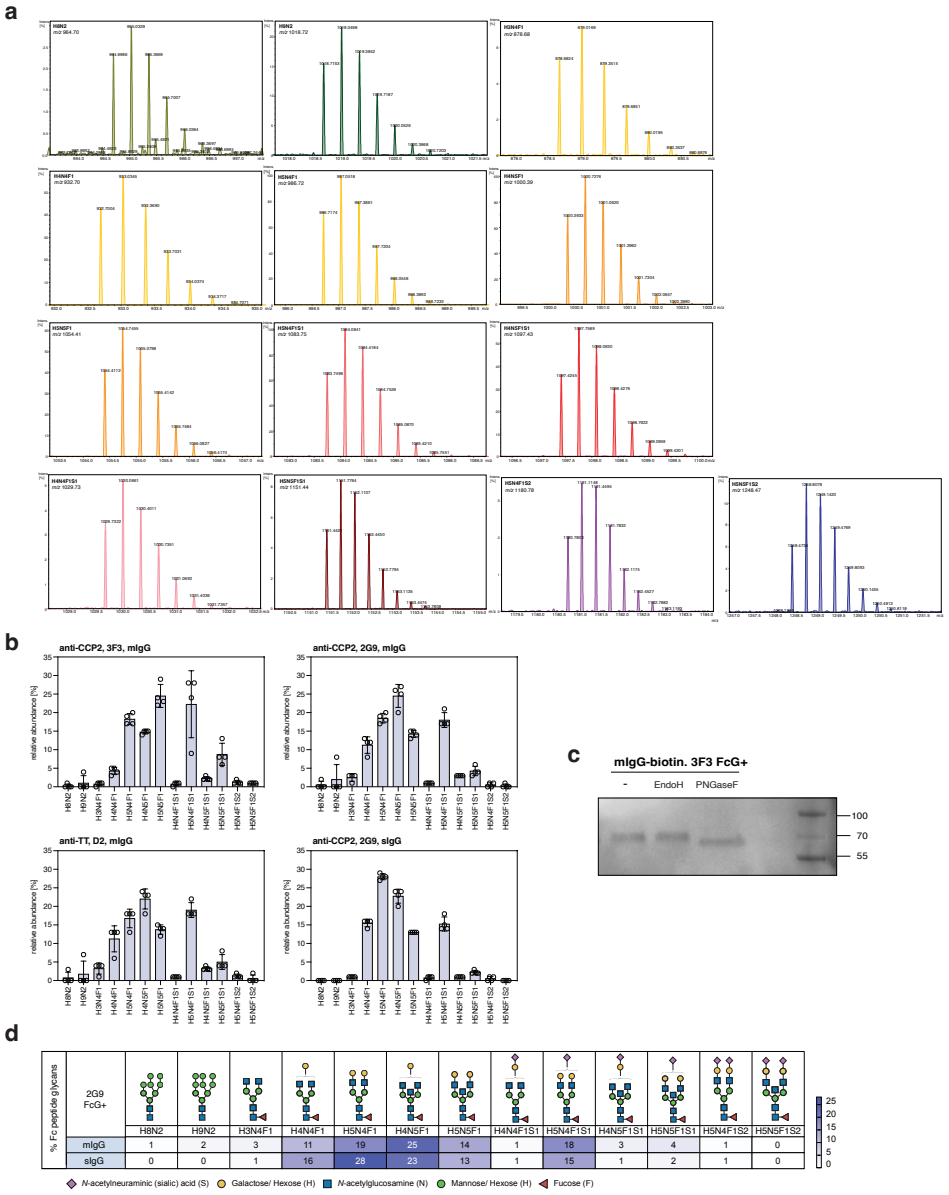
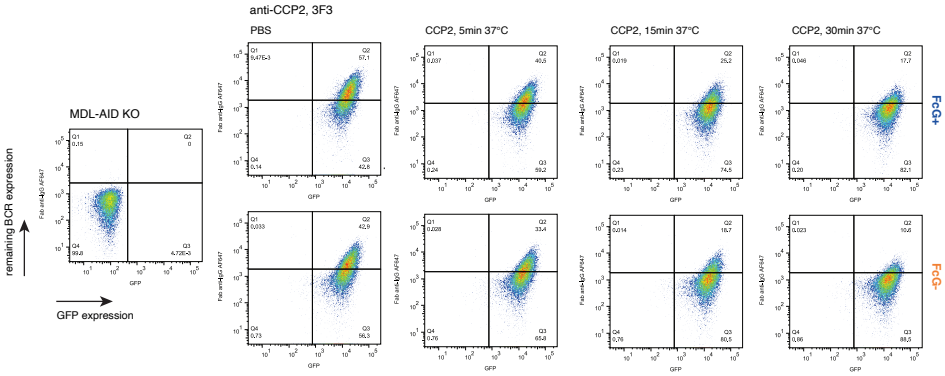
Supplementary information



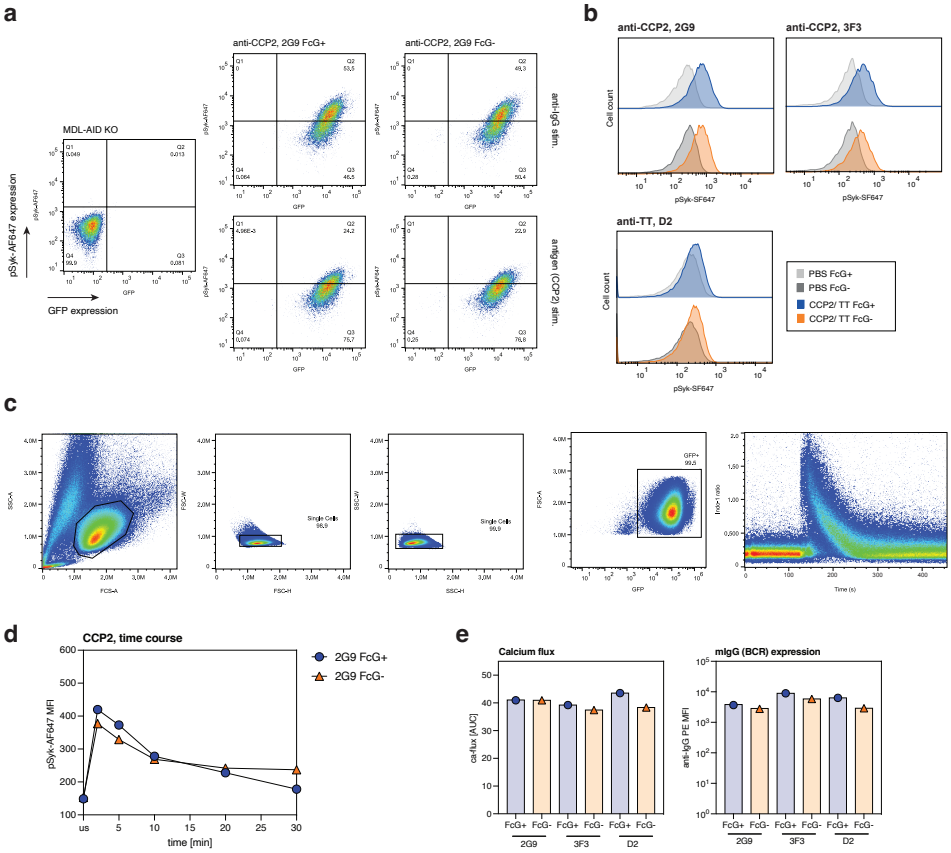
Supplementary Figure 1: BCR and GFP expression of B-cell lines. a Gating strategy to determine GFP and mIgG-BCR expression of transduced FcG+ and FcG- 2G9, 3F3 and D2 Ramos B cell lines. b mIgG-(BCR) and c GFP expression of anti-CCP2 (3F3) and anti-TT (D2) FcG+ and FcG- and MDL-AID KO B cells over culture time (20 days). Source data are provided as a Source Data file.



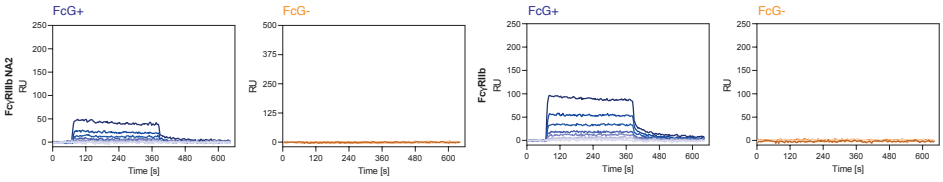
Supplementary Figure 2: Fc peptide glycan analysis. a Exemplary MS spectrum view of LC glycan peptide peaks shown and annotated in Figure 2a. b Percentage of individual γHC glycan traits expressed on 2G9, 3F3 and D2 mIgG-BCRs and 2G9 sIgG. Bar graphs show mean, standard error and individual data points of 4 biological replicates. c Western blot analysis of FcG+ γHC after capturing of biotinylated mIgG (3F3) and EndoH (cleaving high mannose N-glycans) or PNGaseF (cleaving all N-glycans) treatment. Representative results of 2 biological replicates are shown. d Percentage of individual γHC glycan traits expressed on 2G9 mIgG-BCRs vs sIgG. Glycan traits are schematically depicted. Heat map shows mean of 4 biological replicates. Source data are provided as a Source Data file.



Supplementary Figure 3: IgG-BCR downmodulation after (antigenic) stimulation. Gating strategy to determine IgG-BCR downmodulation after activation (PBS or CCP2) in the presence and absence of FcG. Shown are the MDL-AID KO negative control and the anti-CCP2, 3F3 B-cell line.



Supplementary Figure 4: Activation of mIgG-BCRs in the presence and absence of FcG. a Gating strategy to determine the pSyk expression after activation (anti-IgG or antigen) in the presence and absence of FcG. Shown are the MDL-AID KO negative control and the anti-CCP2, 2G9 B-cell line. b Flow histograms of pSyk expression of 2G9, 3F3 and D2 FcG+ and FcG- B cells after PBS treatment and antigenic stimulation. c Gating strategy to determine the calcium flux after activation (anti-IgG) in the presence and absence of FcG. Shown is the anti-CCP2, 2G9 FcG+ B-cell line. d pSyk MFI values of 2G9 FcG+ and FcG- B cells unstimulated (us) and after 5 – 30 min stimulation with 5 µg/ml CCP2. Representative results of 2 biological replicates are shown. e Calcium flux (AUC) and mIgG-BCR expression of FcG+ and FcG- B cell lines (2G9, 3F3 and D2). Measurements were performed within the same experiment. Source data are provided as a Source Data file



Supplementary Figure 5: Impact of IgG FcG on binding to FcγRII/IIIb. Representative SPR sensorgrams of 2G9 sIgG FcG+ and FcG- binding to human FcγRII/IIIb. Association and dissociation are represented as response units [RU] over time [s]. Source data are provided as a Source Data file.

Part 2

The role of mucosal immunity in the development of the AMPA response in RA

In rheumatoid arthritis patients, total IgA1 and IgA2 levels are elevated: Implications for the mucosal origin hypothesis

Veerle F.A.M. Derksen[1], Cornelia F. Allaart[1], Annette H.M. Van der Helm-Van Mil[1,2], Tom W.J. Huizinga[1], René E.M. Toes[1], Diane van der Woude[1]

[1] Department of Rheumatology, Leiden University Medical Center, Leiden, the Netherlands
[2] Department of Rheumatology, Erasmus Medical Center, Rotterdam, the Netherlands

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Abstract

Objectives Mucosal initiated immune responses may be involved in the pathophysiology of rheumatoid arthritis (RA). The most abundant immunoglobulin at mucosal surfaces is IgA, of which two subclasses exist: IgA1 and IgA2. IgA2 is mainly present at mucosal sites and has been ascribed pro-inflammatory properties. As IgA subclasses might provide insights into mucosal involvement and pro-inflammatory mechanisms, we investigated IgA responses in sera of RA patients.

Methods In two cohorts of RA patients, the EAC and IMPROVED, total IgA1 and IgA2 were measured by ELISA. Furthermore, IgA subclass levels of rheumatoid factor (RF) and anti-citrullinated protein antibodies (anti-CCP2) were determined. The association of these IgA subclass levels with CRP and smoking was investigated.

Results Total IgA1 and IgA2 were increased in RA patients compared to healthy donors in both cohorts. This increase was more pronounced in seropositive RA versus seronegative RA. For RF and anti-CCP2, both IgA1 and IgA2 could be detected. No strong associations were found between IgA subclasses (total, RF and anti-CCP2) and CRP. In smoking RA patients, a trend towards a selective increase in total IgA2 and RF IgA1 and IgA2 was observed.

Conclusion RA patients have raised IgA1 and IgA2 levels. No shift towards IgA2 was observed, indicating that the increase in total IgA is not due to translocation of mucosal IgA into the bloodstream. However, mucosal inflammation might play are role, given the association between smoking and total IgA2 levels. Despite its pro-inflammatory properties, IgA2 does not associate strongly with pro-inflammatory markers in RA patients.

Introduction

IgA is the most abundant class of immunoglobulin at mucosal sites and it has an important function in maintaining intestinal homeostasis. Recently, mucosal immune responses have gained increasing attention for their potential role in the pathophysiology of (seropositive) rheumatoid arthritis (RA) (1). Therefore, it is worthwhile to study the IgA response in more detail in RA patients.

Several findings suggest mucosal involvement in the disease mechanisms of seropositive RA. Smoking is a well-known risk factor for the development of RA (1), implying that processes occurring at the pulmonary mucosa could play a role in disease pathogenesis. Furthermore, ACPA have been detected in sputum and saliva of seropositive RA patients, suggesting local production of autoantibodies (2, 3). The gut, another vast mucosal site, might also be important and dysbiosis of the gut microbiome has been described in RA (4). It has been hypothesized that dysbiosis of the microbiome could lead to local inflammation, loss of barrier function and possibly even bacterial translocation. Given that IgA plays an important role in mucosal immune responses, the characteristics of the IgA response might provide more insight into local inflammatory processes and thus possible mucosal origins of RA.

Humans harbor 2 IgA subclasses: IgA1 and IgA2 (5). The biggest differences between IgA1 and IgA2 are the structure of the hinge region and their distribution at different sites. In serum, over 90% of total IgA is IgA1, whereas the IgA subclasses are more balanced at mucosal sites, with exact ratios depending on the location (5). Furthermore, a recent report described a pro-inflammatory effect of IgA2 on neutrophils and macrophages, while this was not found for IgA1 (6). Thus IgA1 and IgA2 not only differ in structure and localization, but may also recruit different effector functions. Moreover, the same study reported total IgA subclass levels to be lower in RA patients compared to healthy controls, and most strikingly, that ACPA IgA in serum is more often of the IgA2 subclass (up to 80% of all ACPA IgA) compared to total IgA. A higher proportion of ACPA IgA2 was also positively correlated with disease activity score (DAS) (6) and ACPA IgA2 levels were weakly correlated with the severity of flares during DMARD tapering (7). These findings suggest that ACPA IgA may have a mucosal origin, compatible with the high amount of IgA2, and that it might translocate from the mucosa into the bloodstream, leading to an elevated ACPA IgA2 percentage in serum. Furthermore, the pro-inflammatory properties of ACPA IgA2 might contribute to disease processes in RA.

Thus, studying IgA subclasses might provide more insight into both the origin as well as the potential pro-inflammatory pathophysiological mechanisms in RA. We hypothesized that mucosal inflammation in RA patients might result in elevated IgA2 levels, which could contribute to the ongoing pro-inflammatory processes in RA. To investigate these hypotheses, we set out to examine IgA subclass levels of total and autoantibody-specific IgA in sera of RA, as well as their link with inflammation and smoking, as a proxy for mucosal inflammation.

Patients and Methods

Stored sera from 2 cohorts of early RA patients were used, the IMPROVED study and the Leiden Early Arthritis Clinic (EAC), of which details are described elsewhere (8, 9). All patients fulfilled either the 2010 (IMPROVED) or the 1987 (EAC) ACR criteria for RA and gave written informed consent. Samples were selected based on previous autoantibody measurements (8, 9). In this study, seropositivity was defined as positivity for anti-CCP2 IgG and/or RF IgM. Of the IMPROVED study, baseline samples from 125 seropositive RA patients were included, most of whom previously tested positive for anti-CCP2 IgA and RF IgA, as well as baseline sera of 68 seropositive RA patients who were negative for RF IgA and anti-CCP2 IgA, and 56 RA patients who were seronegative for both anti-CCP2 IgG and RF IgM. To investigate the generalizability of the findings, samples of a second cohort, the EAC, were used. This included sera of 95 seropositive, mostly anti-CCP2 IgA positive, and 64 seronegative RA patients collected at the one-year visit. Sera of 60 healthy donors, not matched for age or sex, were taken along as control.

Total, RF and anti-CCP2 IgA subclasses were measured with in-house enzyme-linked immunosorbent assays (ELISA) (Supplementary data S1 for details). To test whether RF IgA could influence the readout of the anti-CCP2 IgA subclass ELISA's by binding to anti-CCP2 IgG, sera of anti-CCP2 IgG-positive/IgA-negative, RF IgA-negative patients were mixed 1:1 with sera of anti-CCP2 IgG-negative/IgA-negative, RF IgA-positive patients or with control sera before addition to the plate. Moreover, part of the samples were tested side-by-side before and after IgG depletion on anti-CCP2 IgA2 ELISA (Supplementary data S1). As RF was measured in arbitrary units and not in exact amounts, no percentage RF IgA2 of total RF could be calculated. Instead the ratio RF IgA1 / RF IgA2 was used.

Mann-Whitney U tests were used to compare IgA-levels between groups. Multivariate linear regression was performed to analyze total IgA subclass levels in RA patients versus healthy controls corrected for known confounders age and gender (10), using the IgA subclass levels (^{10}log transformed due to skewness of the data) as dependent

variable. To assess the relationship between IgA subclass levels and C-reactive protein (CRP), DAS, health assessment questionnaire score (HAQ) and BMI, spearman's rank correlation coefficient were calculated, due to the presence of outliers. Patients with missing CRP values (IMPROVED n=5, EAC n=20) or smoking status (IMPROVED n=1) were excluded from analyses involving CRP and smoking respectively. For smoking analysis a multivariate linear regression model including the possible confounders age, gender and CRP was used, with the log-transformed IgA subclass levels as dependent variable. For analysis regarding CRP, similar models were performed for each IgA subclass separately (included as independent variable), together with age, gender and smoking as confounders, using the log-transformed CRP levels as dependent variable. The associations between RF IgA subclass levels and smoking or CRP were analyzed within patients who tested positive for both RF IgA1 and IgA2. Analyses regarding anti-CCP2 IgA1 levels were performed within the anti-CCP2 IgA1-positive group only. The Holm-Bonferroni method was applied to correct for multiple testing.

Results

Total IgA subclasses in RA

In seropositive RA IMPROVED patients, both total IgA1 (tIgA1, $p<0.001$) and total IgA2 (tIgA2, $p<0.001$) levels were strikingly elevated compared to healthy donors (Figure 1A-B). Similar results were found using linear regression including age and gender as potential confounders (Supplementary table S1). Both IgA subclasses were raised to the same extent, since the percentage of IgA2 (%tIgA2, $p=0.18$) did not differ between seropositive patients and healthy donors (Figure 1C). In seronegative IMPROVED patients total IgA1, total IgA2 and the %tIgA2 were not raised compared to healthy controls (Figure 1A-B, Supplementary table S1). To investigate if the raise in total IgA levels in seropositive RA was due to the presence of IgA autoantibodies, seropositive RA patients who were negative for RF IgA and anti-CCP2 IgA were tested. In those patients, total IgA1 ($p<0.001$) and IgA2 levels (tIgA2 $p<0.001$) were also elevated (Supplementary figure S1). This indicates that the elevation in total IgA in seropositive RA is not solely caused by the presence of IgA autoantibodies.

To investigate the generalizability of these findings, we also performed these measurements on sera of patients included in the EAC. While IMRPOVED-sera were collected at baseline, these EAC-sera were collected at the patients' one-year visit. A complete overview of difference between the cohorts can be found in Table 1. Also in the EAC, total IgA1 and IgA2 levels were raised in seropositive RA patients compared to healthy donors, although total IgA2 was just not significant after multiple testing correction (Figure 1D-E) (tIgA1

p<0.001, tIgA2 p=0.02). Increased total IgA1 levels were now also observed in seronegative RA patients compared to healthy donors (tIgA1 p<0.001, tIgA2 p=0.06). Linear regression with correction for age and gender yielded similar results (Supplementary table S1). IgA subclass levels did not differ between seropositive and seronegative EAC patients (Figure 1D-E) (tIgA1 p=0.13, tIgA2 p=0.70). Thus, both total IgA1 and IgA2 levels are raised in seropositive RA compared to healthy controls and might also be higher in seronegative RA patients with longer disease duration.

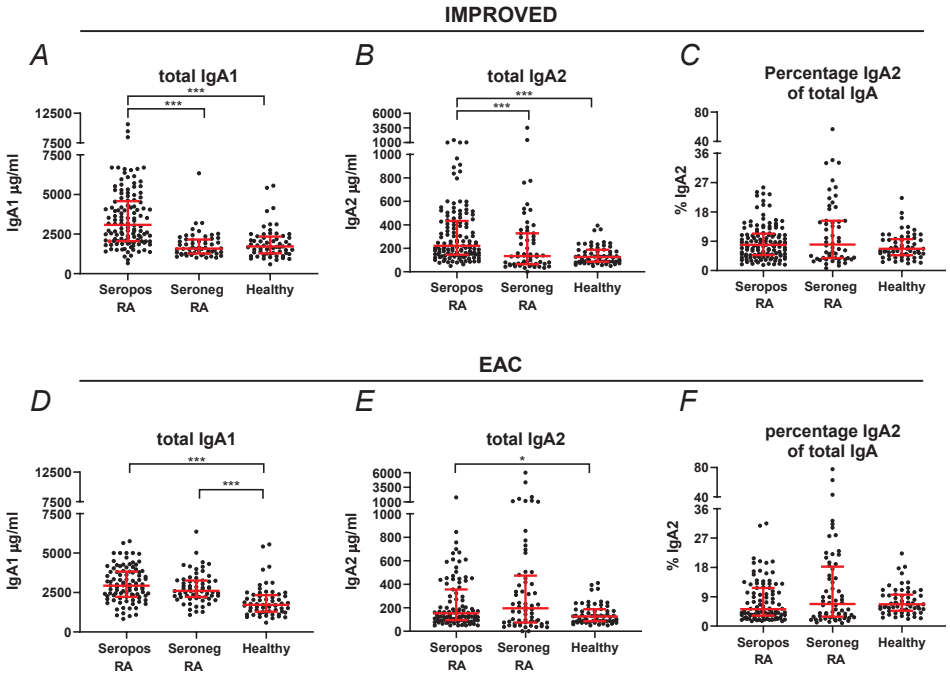


Figure 1: Total IgA1, total IgA2 and percentage IgA2 of total IgA in seropositive and seronegative RA patients compared to healthy controls. Mann–Whitney U tests to compare IgA levels between seropositive or seronegative RA patients and healthy donors. Red bars: median and interquartile range. *p<0.05, **p<0.01, ***p<0.001, no brackets shown when p>0.05. Not all samples were measured in the same experiment.

RF and anti-CCP2 IgA1 and IgA2

Next, we established assays to measure RF and anti-CCP2 IgA subclasses. For RF, both IgA subclasses could be readily detected in the seropositive IMPROVED RA patients with 77% positivity for RF IgA1 and 70% for RF IgA2 (Figure 2A-B). Replication in the EAC showed 85% positivity for RF IgA1 and 60% RF IgA2 in seropositive RA (Figure 2C-D).

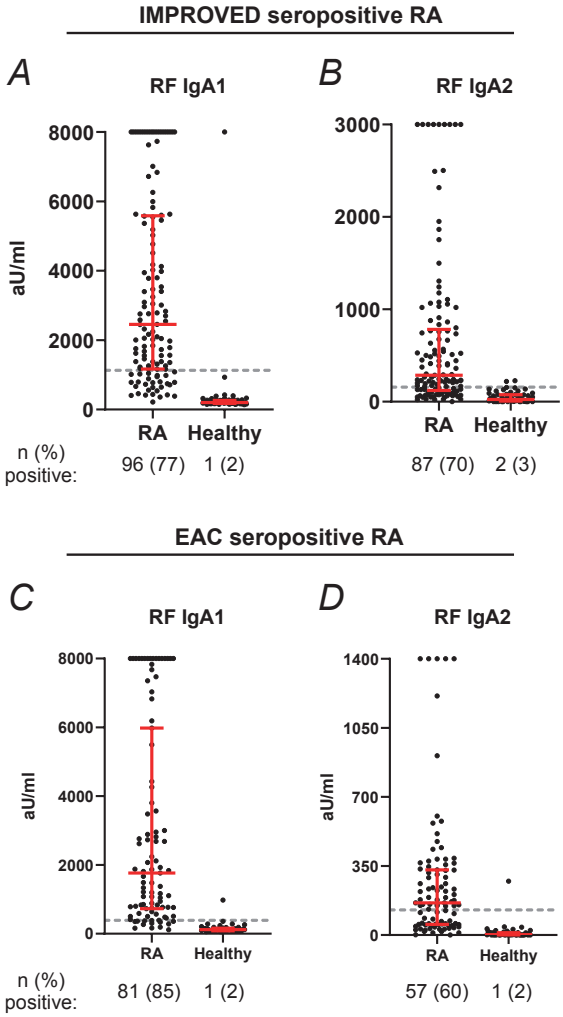


Figure 2: RF IgA subclasses in seropositive RA patients. A RF IgA1 levels and B RF IgA2 levels in IMPROVED seropositive RA patients, C RF IgA1 levels and D RF IgA2 levels in EAC seropositive RA patients. Gray dashed line represents cut-off. Red bars: median and interquartile range. Arbitrary units cannot be directly compared between subclasses.

Table 1: Characteristics of the different patients subsets

	IMPROVED seropositive* RA	IMPROVED seronegative RA	IMPROVED seropositive, RF IgA-/CCP2 IgA- RA	EAC seropositive* RA	EAC seronegative RA	Healthy donors
Number	125	56	68	95	64	60
Time sampling	Baseline	Baseline	Baseline	1 -year visit	1 -year visit	NA
Age, yearsm, Mean ± SD	53.3 ± 12.4	54.3 ± 15.1	47.1 ± 14.7	59.1 ± 15.3	59.1 ± 16.8	44.7 ± 14.4
Female, n (%)	80 (64)	39 (70)	48 (71)	64 (67)#	41 (64)#	35 (58)
Ever smoking, n (%)	76 (61)	23 (42)	18 (27)	50 (53)#	22 (35)#	Unknown
CRP, mg/L Mean ± SD	24.5 ± 34.1 (n=120)	22.2 ± 32.3 (n=55)	25.3 ± 33.0 (n=64)	26.3 ± 37.7 (n=75)	11.0 ± 21.8 (n=55)	NA
DAS, Mean ± SD	3.29 ± 0.91	3.70 ± 0.85	3.20 ± 0.83	Unknown	Unknown	NA
RF IgM positivity, n (%)	112 (92)	0 (0)	56 (85)	75 (79)#	0 (0)#	NA
RF IgA positivity, n (%)	106 (85)	NA	0 (0)	Unknown	NA	NA
Anti-CCP2 IgG positivity, n (%)	118 (95)	0 (0)	38 (57)	95 (100)#	0 (0)#	NA
Anti-CCP2 IgA positivity, n (%)	102 (82)	NA	0 (0)	63 (67)#	NA	NA
Total IgA1, µg/ml Median (IQR)	3082 (2057-4579)	1581 (1273-2155)	2913 (1968-3912)	2936 (2229-3828)	2605 (2215-3259)	1720 (1280-2338)
Total IgA2, µg/ml Median (IQR)	221 (145-433)	134 (65-328)	197 (139-400)	153 (95-356)	196 (74-473)	127 (86-188)
% IgA2 total IgA Median (IQR)	7.9 (4.7-11.4)	8.0 (3.8-15.3)	6.0 (4.5-9.2)	5.2 (3.2-11.7)	6.7 (2.9-18.3)	6.7 (4.8-9.6)

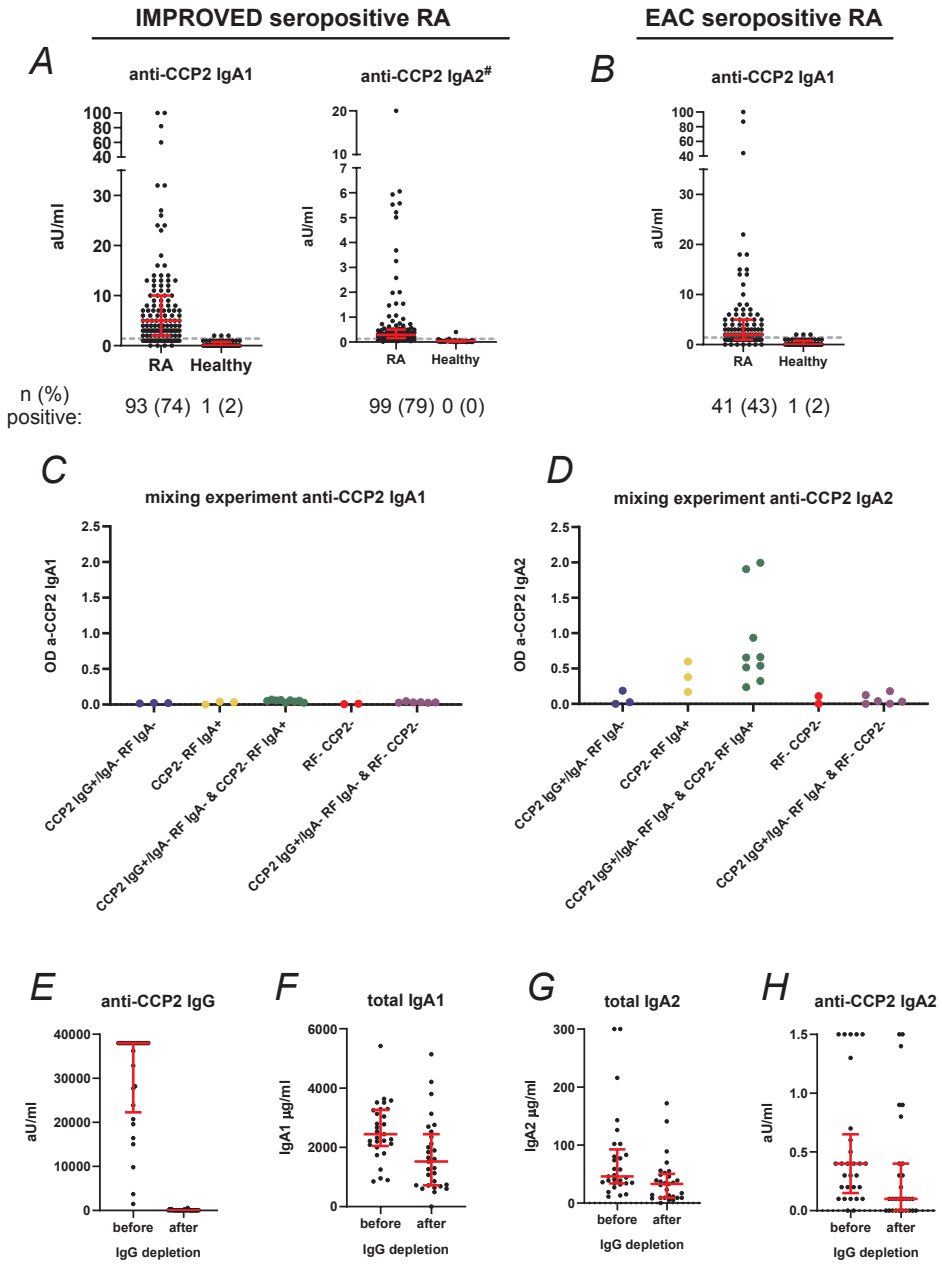
*Enriched for ACPA IgA positivity #Information collected at baseline

Anti-CCP2 IgA1 was present in 74% of seropositive patients in the IMPROVED (Figure 3A). In the EAC 43% of seropositive RA patients tested positive for anti-CCP2 IgA1 (Figure 3B), which is partly due to the lower amount of anti-CCP2 IgA-positive patients within the seropositive group in the EAC compared to IMPROVED. Where anti-CCP2 IgA1 could be readily detected, the detection of anti-CCP2 IgA2 proved challenging. The ELISA protocol needed to be modified extensively to obtain decent signals for anti-CCP2 IgA2 (Supplementary data S1). However, with these adaptations, including the use of less diluted serum and incubation of serum overnight, we eventually managed to obtain sufficient readouts (Figure 3A). During quality controls of this new protocol, mixing experiments showed no effect of RF on the outcome of the anti-CCP2 IgA1 ELISA (Figure 3C). However using the anti-CCP2 IgA2 protocol, RF IgA2 could bind to anti-CCP2 IgG and give a false positive anti-CCP2 IgA2 signal (Figure 3D). Therefore, we concluded that anti-CCP2 IgA2 could not be reliably detected in sera containing RF IgA2. To further assess the impact of this RF IgA interference on anti-CCP2 IgA2, IgG depletion was performed in a subset of patients and anti-CCP2 IgA2 measurements were compared before and after IgG depletion. As expected anti-CCP2 IgG was undetectable after IgG depletion (Figure 3E), whereas only a slight non-specific IgA loss was observed after the procedure (Figure 3F-G). Anti-CCP2 IgA2 could still be detected after IgG depletion in part of the seropositive RA samples (Figure 3H). Moreover, anti-CCP2 IgA2 could also be detected in some RF-negative patients, indicating that not the entire signal could be attributed to RF. In conclusion, anti-CCP2 IgA2 can be present in part of seropositive RA patients, but technical difficulties posed by RF IgA interference prohibited precise determination of anti-CCP2 IgA2 levels. Therefore, no further analyses using anti-CCP2 IgA2 levels were performed.

IgA subclasses and inflammation

To investigate whether total and antigen-specific IgA subclass levels in seropositive RA are associated with inflammation, correlations with CRP were examined. In the IMPROVED, no association was observed for either total or antigen-specific IgA1 or IgA2 and CRP in univariate analysis (Figure 4A). Also no correlations were seen for %tIgA2 and RF IgA subclass ratio (supplementary figure S2A). To correct for confounders, analyses were adjusted for age, gender and smoking. After this correction, a small difference was seen for RF IgA2 (p=0.007), but this did not remain significant after multiple testing correction (Supplementary table S2). In seropositive EAC patients, spearman correlation yielded significant results for CRP and total IgA1 (r_s=0.352, p=0.002) and RF IgA2 (r_s=0.385, p=0.007) (Figure 4B, supplementary figure S2B). However, no clear pattern was visible in the scatterplots and the strength of the correlation was limited. Similarly, in multivariate analyses, small, but significant associations were found between CRP and total IgA1 and RF IgA2 (supplementary table S2). In the IMPROVED cohort, similar analyses with DAS, HAQ, BMI and the presence of erosions were performed. No significant associations with

total or antigen-specific IgA subclasses was observed for these parameters, after correction for multiple testing (Supplementary figure S3). In conclusion, although there were some correlations between CRP and IgA antibodies of both subclasses, especially regarding RF IgA2-levels, these associations were not very strong in both cohorts.



◂ **Figure 3: Anti-CCP2 IgA subclasses in seropositive RA.** A Anti-CCP2 IgA1 and IgA2 subclass measurements in seropositive IMPROVED RA patients and healthy controls. Number and percentage of patients above the cut-off is indicated. Gray dashed line represents cut-off. Red bars: median and interquartile range. #Results might be influenced by RF IgA2 binding B Anti-CCP2 IgA1 in seropositive EAC RA patients C-D Mixing experiment for anti-CCP2 IgA1 and IgA2 ELISA to investigate whether RF IgA binding could influence the read-out. For anti-CCP2 IgA1 no RF interference is observed, for the anti-CCP2 IgA2 ELISA results could be influence by RF IgA2 binding, as the combination of anti-CCP2 IgG+ IgA- RF IgA- serum with anti-CCP2- RF IgA+ serum can give high OD values E-H Measurements before and after IgG depletion in a selection of IMRPOVED seropositive RA patients. After IgG depletion the anti-CCP2 IgA2 signal remains clearly visible in part of the samples, while all anti-CCP2 IgG is depleted. The procedure led to some aspecific loss of total IgA1 and total IgA2.

IgA subclasses and smoking

As IgA is the dominant antibody at mucosal surfaces, mucosal inflammation, for example caused by long-term smoking, might lead to a more prominent IgA response and higher IgA serum levels. To investigate this hypothesis, levels of total, RF and anti-CCP2 IgA subclasses were compared between smoking and non-smoking RA patients. In seropositive IMPROVED patients, smokers had significantly increased serum levels of total IgA2 (p=0.004), as well as higher RF IgA1 levels (p=0.004) (Figure 5A). Percentage IgA2 of total IgA (p=0.02) and RF IgA2 (p=0.04) were not significant after multiple testing correction. However, the ratio RF IgA1 / RF IgA2 was similar in smokers versus non-smokers (Supplementary figure S4A), indicating that both RF subclasses are elevated in smokers, even though this was not significant for RF IgA2-levels. Interestingly, levels of total IgA1 and anti-CCP2 IgA1 were not elevated in smokers. Similar results were found in multivariate analyses corrected for age, gender and CRP (Supplementary table S3). In the EAC seropositive samples, RF IgA1 levels appeared to be higher in smokers, but this was not statistically significant (p=0.36) (Figure 5B). Smoking was also not associated with other total or antigen-specific subclass levels (Figure 5B, supplementary figure S4B and table S3). Similar to the pattern observed in seropositive RA, in seronegative IMPROVED patients total IgA2 levels were also selectively increased in smokers (tIgA2 p=0.004, %tIgA2 p=0.002) (Supplementary figure S4C, table S3), while no difference was observed between smoking versus non-smoking seronegative EAC patients after 1 year of treatment (Supplementary figure S4D, table S3). In conclusion, smoking, a proxy for mucosal inflammation, might lead to a selective increase in serum total IgA2 levels and RF IgA levels at disease onset, but does not seem to affect serum total IgA1 and anti-CCP2 IgA1 in RA patients.

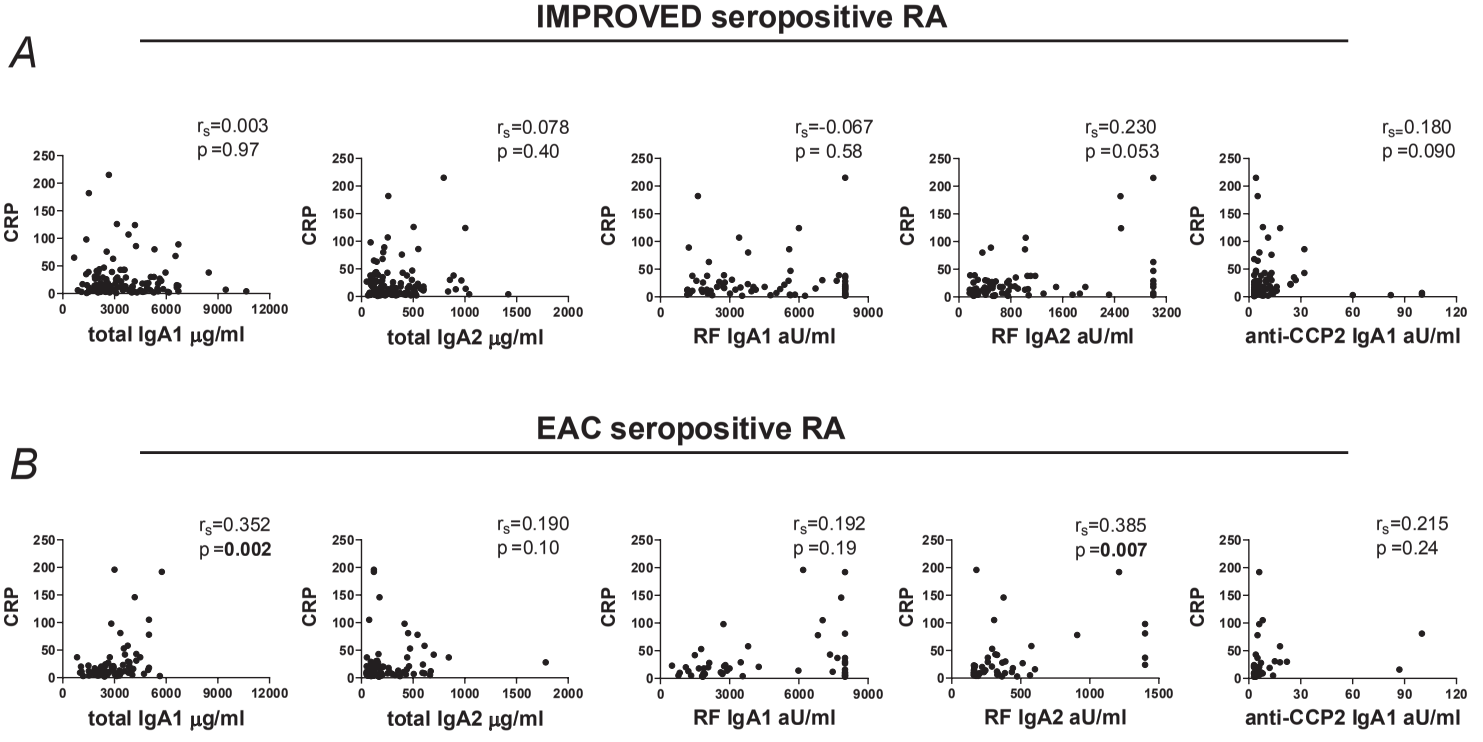


Figure 4: IgA subclass levels and CRP levels in seropositive RA. Correlation between IgA subclass levels and CRP levels in A IMPROVED seropositive RA patients and in B EAC seropositive patients, calculated using Spearman's rank correlation coefficient (r_s). In RF IgA subclass analyses, only patients positive for both RF IgA1 and RF IgA2 are included. For anti-CCP2 IgA1 analysis only anti-CCP2 IgA1 positive patients are included. Of note, RF and anti-CCP2 IgA subclass levels were not titrated.

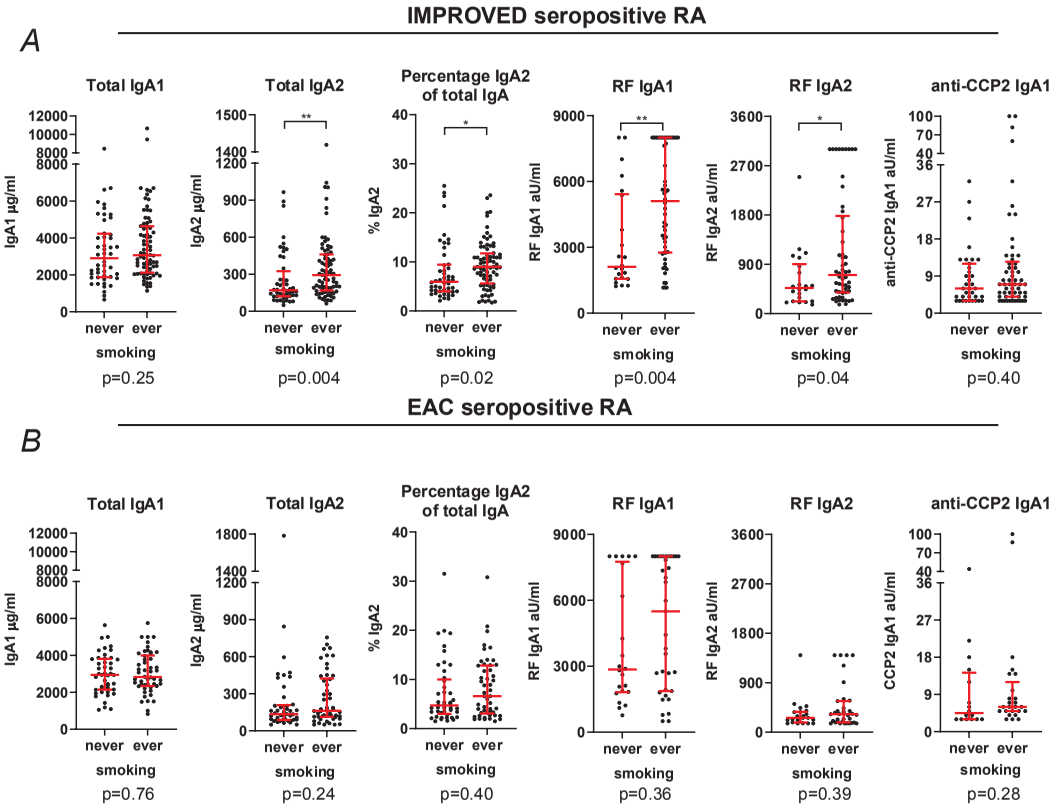


Figure 5: IgA subclass levels and smoking in seropositive RA. IgA subclass levels in ever- versus never-smoking seropositive RA patients in A IMPROVED and B EAC, analyzed using Mann–Whitney U tests. Red bars: median and interquartile range. *p<0.05, **p<0.01, ***p<0.001, #not significant after correction for multiple testing. In RF IgA subclass analyses, only patients positive for both RF IgA1 and RF IgA2 are included. For anti-CCP2 IgA1 analysis only anti-CCP2 IgA1 positive patients are included. Of note, RF and anti-CCP2 IgA subclass levels were not titrated.

Discussion

Since studying IgA subclasses can provide insight in both mucosal involvement as well as potential pro-inflammatory pathophysiological processes, we explored the IgA subclass distribution of total IgA as well as RF- and anti-CCP2-specific IgA in RA. Strikingly, total IgA1 and IgA2 levels were increased in seropositive RA patients in both the IMPROVED and EAC cohort. This was much less pronounced for seronegative patients, in whom only total IgA1 in the EAC was significant. Furthermore, both RF and anti-CCP2 IgA1 and IgA2 were detectable in a subset of seropositive RA patients. However, technical difficulties posed by RF IgA interference prohibited precise determination of anti-CCP2 IgA2 levels.

Several observations, for example the detection of RF and ACPA in sputum and saliva of seropositive RA patients, indicate that the mucosal immune system might be involved in the pathophysiology of RA (1-3, 11). Both IgA subclasses have an important function in mucosal immune responses, but the relative amount of IgA2 is increased at mucosal sites (12). A previous study suggested that also in serum of RA patients the relative amount of IgA2 is increased (6). Based on these findings, one might hypothesize that in RA patients IgA2 can translocate from (inflamed) mucosal sites, where it is highly abundant, into the circulation, leading to an elevated percentage of IgA2 in serum. However, we found that both IgA1 and IgA2 levels were elevated in seropositive RA patients. The percentage of IgA2 in serum was not increased in RA patients when compared to healthy donors. Thus, these data do not support the notion that direct translocation of mucosal IgA(2) is one of the main mechanisms leading to the elevated IgA subclass levels in RA.

Nonetheless, chronic mucosal inflammation might still be involved in the hyperproduction of IgA subclasses in RA patients. The link between mucosal immune responses and serum immunoglobulins is currently not completely understood. Research in celiac disease showed that mucosal and serum IgA are related, but produced by different plasma cells (13). Therefore, one might hypothesize that it is possible that the initial mucosal response is predominantly of the IgA2 subclass, while the related serum response is predominantly IgA1. This means the elevated IgA1 and IgA2 serum levels in RA could still be the result of increased mucosal IgA responses, most likely not by direct translocation of mucosal IgA, but potentially via the generation of specific plasma cell populations that contribute to the serum antibody pool. On the other hand, various studies have described that IgG and IgM can also be elevated in RA (14-17). This suggests the elevated IgA subclass levels could also be part of a general immunoglobulin (Ig) hyperproduction in RA patients, for example due to aspecific B

cell hyperreactivity in the context of systemic inflammation. Another possibility is that the hyperglobulinemia reflects intrinsic B cell alterations in RA patients, which could be in line with the important role that B cells play in the pathophysiology of RA (18-20).

To investigate whether chronic mucosal inflammation might play a role in the elevated IgA subclass levels in RA patients, we used smoking status as a proxy for mucosal inflammation, since smoking is known to cause chronic pulmonary inflammation (21). Intriguingly, smoking was associated with a selective increase in total IgA2 levels in serum of RA patients in the IMPROVED. Also RF IgA subclass levels tended to be increased in smoking RA patients in IMRPOVED, whereas anti-CCP2 IgA1 levels were not. This is interesting, as both RF and anti-CCP2 total IgA have been detected in sputum of RA patients, suggesting they are produced locally in the lungs (2). However, our findings suggest that smoking might have a larger influence on the production of RF IgA than on anti-CCP2 IgA. This is in line with the observations that smoking is associated with RF IgM positivity rather than the presence of ACPA IgG (22, 23). Of note, we could not replicate these associations with smoking in the EAC, where autoantibody levels were overall lower than in the IMPROVED, possibly due to the immunosuppressive treatment that most patients had received (9). In conclusion, our data suggest that chronic mucosal inflammation may be one of the mechanisms playing a role in the elevated RF IgA and total IgA2 levels in RA, although smoking status does not explain the full extent of the increase in total IgA subclasses in RA patients.

Although the presence of IgA2 in humans was described decades ago, novel findings regarding pro-inflammatory effector functions of IgA2 were recently described (6). However, in RA patients we did not observe an association between total IgA2 levels and two important markers of inflammation, CRP and DAS. On the other hand, a significant correlation between total IgA1 levels and CRP was seen in the EAC. Furthermore, significant associations between CRP and RF IgA2-levelswere found, although the effect was small and not significant after correction for multiple testing correction in the IMPROVED. Higher anti-CCP2 IgA1 levels were not associated with lower inflammation in our study, in contrast to a weak correlation between low anti-CCP2 IgA1 and high DAS described before (6). A recent study also describes that anti-CCP2 IgA2 levels decline in ongoing remission, although, based on our data regarding RF interference, this effect might be mediated by a decline in RF IgA levels (7, 9). Taken together, our findings do not appear to support an essential role for IgA2 in the ongoing pro-inflammatory processes in RA.

One of the limitations of our study is the use of in-house ELISA's. Technical difficulties posed by RF IgA interference prohibited precise determination of anti-CCP2 IgA2 levels. To obtain decent signals in the ELISA, serum was diluted less and incubated overnight

instead of 1 hour, which might have provided RF IgA with the chance to bind anti-CCP2 IgG. As a result the anti-CCP2 IgA1/IgA2 ratio could not be calculated. This precluded our attempts to replicate the findings that the ACPA IgA response is shifted towards IgA2 (6). It is unclear whether possible interference of RF IgA2 was investigated in other studies. However, based on the fact that anti-CCP2 IgA1 was readily detectable whereas anti-CCP2 IgA2 was not, it seemed unlikely that a majority of total ACPA IgA was of the IgA2 subclass. On the contrary, both RF IgA1 and IgA2 were readily detectable, in line with previous studies (11).

Furthermore, we repeated the measurements on an independent cohort to investigate the generalizability of our findings. The results of the in-house IgA subclass ELISA's were largely reproducible between the two different cohorts. Another strength is that many anti-CCP2 IgA positive patients were included and detailed information regarding smoking status and inflammatory markers was available. Although our findings are in contrast with a previous study on IgA subclasses in RA (which found both IgA1 and IgA2 to be lower in RA patients) (6), multiple other studies have described raised total IgA levels in RA (14-16, 24-27).

In conclusion, seropositive RA patients have raised total IgA1 and IgA2 levels and can also harbor RF and ACPA IgA subclasses. Since no shift towards the IgA2 subclass was observed, the increase in total IgA levels appears not to be due to translocation of mucosal IgA over mucosal barriers into the bloodstream. However, chronic mucosal inflammation might be one of the mechanisms involved in the raise in IgA(2) levels in RA, given the association between smoking and total IgA2 levels. Despite the pro-inflammatory properties of IgA2, our data does not seem to support a large role of IgA2 in chronic inflammatory processes in RA patients.

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Supplementary information

Supplementary data S1: Methodology

ELISAs

For total IgA1 and IgA2, detection Nunc maxisorp plates (Thermo scientific; 430341) were coated with 10 µg/ml goat anti-human-IgA-Fc (Bethyl; A80-102A) and blocked with PBS/1%bovine serum/50 mM Tris. Sera were diluted 1:6000 for IgA1 and 1:2000 and 1:6000 for IgA2 in PBS/1%BSA/0.05% Tween/50 mM Tris and incubated together with a serial dilution of a commercial standard (Nordic Mubio, NOR-04) on the same plate for 1 hour at room temperature. Detection antibodies used for detecting IgA subclasses in all the different assays are described below. If samples were above the linear range of the standard, serial titrations of the sera were performed. The percentage IgA2 of total IgA was calculated by dividing total IgA2 levels by the sum of IgA1 and IgA2 levels times 100. For RF IgA1 and IgA2 ELISA, Nunc maxisorp plates were incubated overnight at room temperature with 10 µg/ml human IgG (Jackson Immunoresearch;009-000-003) and blocked for 1 hour at 37°C with PBS/1%BSA. Samples were diluted 1:25 and 1:50 for IgA1 and 1:10 for IgA2 in PBS/1%BSA/0.05% Tween and incubated for 1 hour at 37°C. A pooled serum standard was used to calculate arbitrary units. For CCP2 IgA1 and IgA2 measurements, biotinylated CCP2 (patent EP2071335) and a biotinylated control peptide containing arginine instead of citrulline (CargP2) were coated 1 µg/ml in PBS/0.1%BSA on pre-coated streptavidin plates (for IgA1 standard capacity -Microcoat 604500; for IgA2 High capacity Microcoat 604501) and incubated 1 hour at room temperature (RT) for IgA1 and overnight at 4°C for IgA2. The patent protected CCP2 and CargP2 were provided by Dr. J. W. Drijfhout (Dept. of IHB, LUMC). For IgA1, sera were diluted 1:50 in PBS/0.05% Tween/ 1% BSA and incubated for 1 hour at 37°C. For IgA2, plates were first blocked with PBS/2%BSA for 6 hours at 4°C on ice and thereafter serum was added in a 1:12.5 dilution and incubated overnight at 4°C on ice. For both anti-CCP2 IgA1 and IgA2 ELISA's the same standard of pooled isolated anti-CCP2 was used.

IgA subclasses were detected using either mouse anti-human IgA1 (Nordic MUbio, 6688) with a subsequent incubation of goat anti-mouse Ig-HRP (DAKO p0447) or with mouse anti-human IgA2-HRP (Southern biotech, 9140-05), each incubated for 1 hour at 37°C. All ELISA's are visualized with ABTS/H_2O_2. Between each incubation step the plates were washed with PBS/0.05% Tween 20.

Anti-CCP2 IgG ELISA was performed similar to the anti-CCP2 IgA1 ELISA, but with rabbit anti-human IgG-HRP (DAKO, P0214) for detection. For the anti-CCP2 IgA ELISA's, blanks were subtracted for both the citrullinated and the unmodified arginine control peptide separately. A signal was considered specific if the OD on the citrullinated

peptide was at least 0.1 higher compared to the arginine control and above the cut-off. Cut-offs for RF and anti-CCP2 were calculated using the mean plus 2 times standard deviation (SD) of healthy donors. RF and anti-CCP2 IgA subclasses levels above or below the linear part of the standard were not titrated.

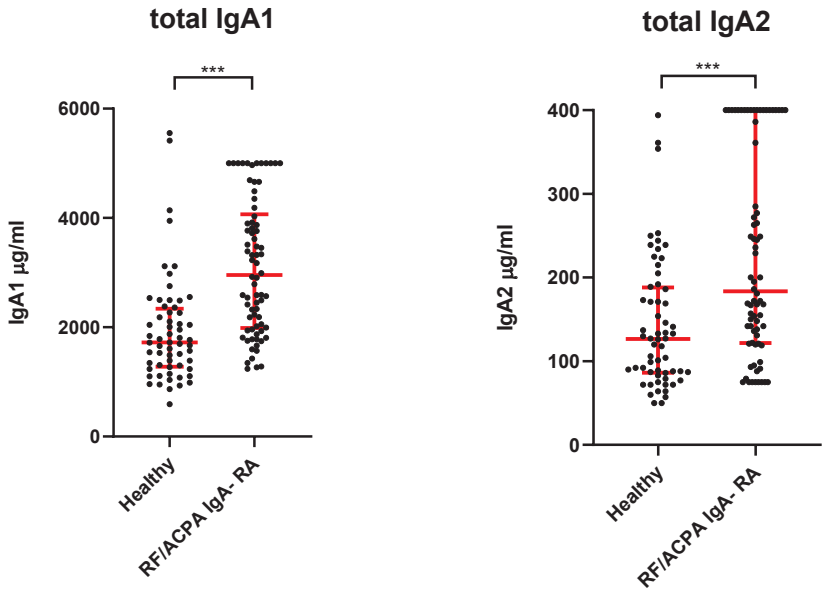
IgG depletion

To deplete IgG from the serum samples, protein G Agarose beads (Thermofisher 20397) were diluted 1:3 in PBS and pipetted 168 µl/well in a 10 µm filter plate (Orochem; OF1100). After washing, 70 µl 1:5 diluted serum was added to each well and the plate was incubated on a shaker at 900 rpm for 1 hour. Flow-through was collected after centrifugation for 1min at 350g. After regeneration of the beads with 100ul/well 0.1M glycine pH 3 and washing, the cycle was repeated using the flow-through. The flow-through of the second cycle was analysed on ELISA in a final dilution similar to the dilution of the non-depleted serum.

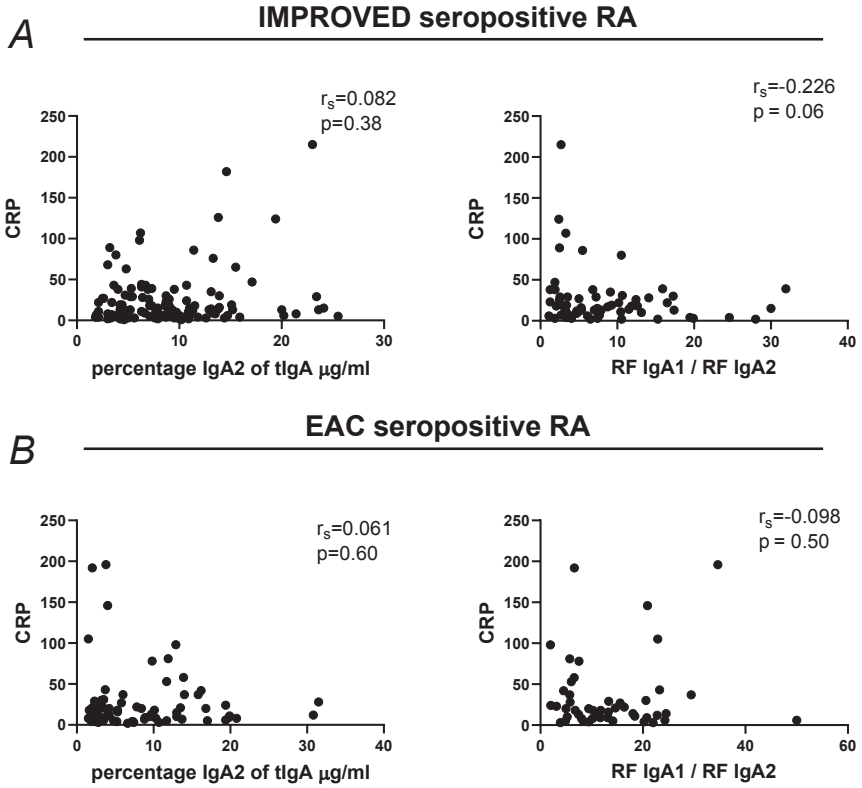
Supplementary table S1: Total IgA subclass levels in seropositive and seronegative RA patients.

	Total IgA1 levels		**Total IgA2 levels**		**Percentage IgA2 of total IgA**	
	B (95% CI)	**p-value**	**B (95% CI)**	**p-value**	**B (95% CI)**	**p-value**
IMPROVED seropositive RA	0.21 (0.15-0.29)	**p<0.001**	0.26 (0.17-0.35)	**p<0.001**	0.04 (-0.04-0.12)	p=0.36
IMPROVED seronegative RA	-0.04 (-0.11-0.03)	p=0.22	0.08 (-0.05-0.22)	p=0.22	0.10 (-0.03-0.23)	p=0.12
EAC seropositive RA	0.17 (0.11-0.24)	**p<0.001**	0.14 (0.03-0.25)	**p=0.01#**	-0.03 (-0.14-0.07)	p=0.53
EAC seronegative RA	0.15 (0.08-0.22)	**p<0.001**	0.22 (0.06-0.38)	**p=0.007#**	0.04 (-0.10-0.18)	p=0.59

The difference in total IgA subclass levels between RA patients and healthy controls, analysed using linear regression for each cohort separately. Due to skewness of the data ^{10}log(total IgA1), ^{10}log(total IgA2) or ^{10}log(percentage total IgA2) were used as dependent variable and age and gender were included as possible confounders. In each row the coefficients and accompanying p-value represent the effect of being an RA patient (compared to a healthy donor (reference category)) on the IgA subclass levels (dependent variable). #not significant after correction for multiple testing.



Supplementary figure S1: Total IgA subclass levels in seropositive RA patients in IMPROVED. Total IgA1 and IgA2 levels in seropositive (RF IgM+ and/or anti-CCP2 IgG+) IMPROVED RA patients, who are negative for RF IgA and anti-CCP2 IgA. Mann–Whitney U tests were used to compare IgA levels between RA patients and healthy donors (total IgA1 $p<0.001$, total IgA2 $p<0.001$). Red bars: median and interquartile range. Not all ELISA measurements were performed on the same day.

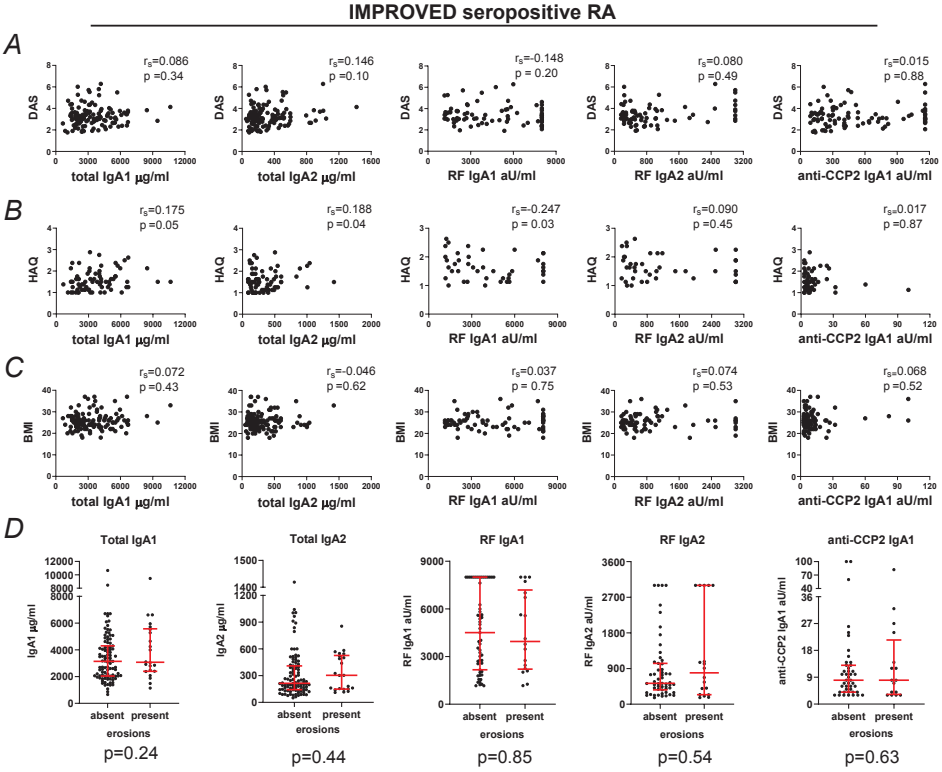


Supplementary figure S2: Correlation between CRP and total/RF IgA1:IgA2 ratio. Correlation between CRP and percentage IgA2 of total IgA and RF IgA1 / RF IgA2 ratio in A IMPROVED seropositive RA patients and in B EAC seropositive RA patients. Correlations are calculated using spearman's rank correlation coefficient (r_s). In RF IgA subclass analyses, only patients positive for both RF IgA1 and RF IgA2 were included. Of note, RF IgA subclass levels were not titrated.

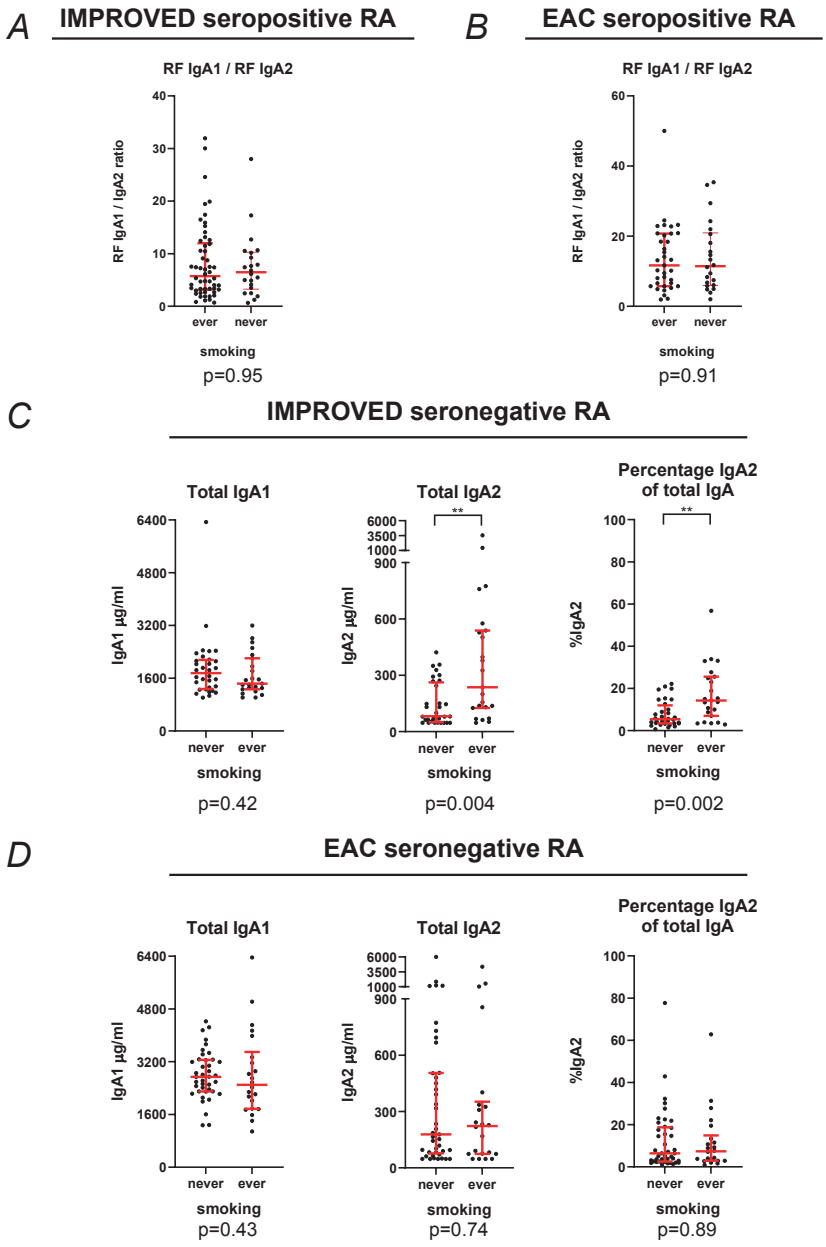
Supplementary table S2: Total and autoantibody-specific IgA subclass levels and CRP in RA.

	Dependent variable: CRP levels			
	IMRPOVED seropositive RA		EAC seropositive RA	
	B (95% CI)	p-value	B (95% CI)	p-value
total IgA1 levels	0.000 (0.000-0.000)	p=0.81	0.000 (0.000-0.000)	**p=0.002**
total IgA2 levels	0.000 (0.000-0.001)	p=0.42	0.000 (0.000-0.001)	p=0.17
percentage IgA2 of total IgA	0.009 (-0.008-0.026)	p=0.29	0.002 (-0.014-0.018)	p=0.79
RF IgA1 levels	0.000 (0.000-0.000)	p=0.61	0.000 (0.000-0.000)	p=0.052
RF IgA2 levels	0.000 (0.000-0.000)	**p=0.007#**	0.001 (0.000-0.001)	**p=0.001**
Anti-CCP2 IgA1levels	-0.002 (-0.008-0.004)	p=0.53	0.003 (-0.005-0.011)	p=0.47
	Dependent variable: CRP levels			
	IMRPOVED seronegative RA		**EAC seronegative RA**	
	B (95% CI)	**p-value**	**B (95% CI)**	**p-value**
total IgA1 levels	0.000 (0.000-0.000)	p=0.67	0.000 (0.000-0.000)	p=0.07
total IgA2 levels	0.000 (0.000-0.000)	p=0.31	0.000 (0.000-0.000)	p=0.58
percentage IgA2 of total IgA	0.008 (-0.006-0.022)	p=0.26	0.003 (-0.006-0.013)	p=0.48

Multivariate linear regression investigating the association between the different IgA subclass levels and CRP in each cohort of RA patients, using log transformed CRP levels as dependent variable. In each row the coefficients and accompanying p-value represent the effect of the specific IgA subclass level (independent variable) on CRP levels (dependent variable) per cohort. Besides IgA subclass levels, also age, gender and smoking were included as independent variables. #not significant after correction for multiple testing.



Supplementary figure S3: IgA subclass levels and disease severity in RA. Correlation between IgA subclass levels and A DAS, B HAQ (all non-significant after correction for multiple testing) and C BMI and D association between the presence of erosions at baseline and IgA subclass levels in IMPROVED seropositive patients. Correlations are calculated using spearman's rank correlation coefficient (r_s). Differences in IgA subclass levels between patients with and without erosions at baseline were tested using Mann-Whitney U tests. Patients with missing values for BMI (n=3), HAQ (n=2) and erosions (n=4) were excluded from the respective analyses. In RF IgA subclass analyses, only patients positive for both RF IgA1 and RF IgA2 were included. In graphs of anti-CCP2 IgA1 only anti-CCP2 IgA1 positive patients are included. Of note, RF and anti-CCP2 IgA subclass levels were not titrated.



Supplementary figure S4: Smoking and IgA subclass ratios in RA. A-B Ratio RF IgA1 / RF IgA2 in ever- versus never-smoking seropositive RA patients in A IMPROVED and B EAC. C-D Total IgA subclass levels in ever- versus never-smoking seronegative RA patients in C IMPROVED and D EAC, analysed using Mann–Whitney U tests. Red bars: median and interquartile range. *p<0.05, **p<0.01, ***p<0.001. In both cohort one seronegative patient had unknown smoking status and was excluded. Of note, RF IgA subclass levels were not titrated.

Supplementary table S3: Smoking and IgA subclass levels in RA patients.

	Total IgA1 levels		Total IgA2 levels		Percentage IgA2 of total IgA	
	B (95% CI)	**p-value**	**B (95% CI)**	**p-value**	**B (95% CI)**	**p-value**
Smoking IMPROVED seropositive RA	0.05 (-0.04-0.13)	p=0.27	0.16 (0.06-0.27)	**p=0.006**	0.10 (0.002-0.20)	**p=0.05#**
Smoking EAC seropositive RA	-0.03 (-0.12-0.05)	p=0.43	-0.002 (-0.18-0.17)	p=0.98	0.03 (-0.14-0.20)	p=0.72
Smoking IMPROVED seronegative RA	-0.04 (-0.13-0.05)	p=0.42	0.34 (0.11-0.58)	**p=0.006**	0.33 (0.11-0.54)	**p=0.004**
Smoking EAC seronegative RA	-0.02 (-0.11-0.08)	p=0.68	-0.16 (-0.49-0.18)	p=0.35	-0.11 (-0.40-0.18)	p=0.46
	RF IgA1 levels		**RF IgA2 levels**		**Anti-CCP2 IgA1 levels**	
	B (95% CI)	**p-value**	**B (95% CI)**	**p-value**	**B (95% CI)**	**p-value**
Smoking IMPROVED seropositive RA	0.21 (0.07-0.35)	**p=0.005**	0.23 (0.04-0.42)	**p=0.02#**	0.13 (-0.03-0.29)	p=0.12
Smoking EAC seropositive RA	0.11 (-0.09-0.32)	p=0.28	0.09 (-0.09-0.26)	p=0.34	0.11 (-0.24-0.45)	p=0.52

Multivariate linear regression investigating the association between smoking and IgA subclass levels in RA patients. The log transformed IgA subclass levels were used as the dependent variable. The coefficients and p-values represent the effect of smoking (independent variable) in each cohort on the levels of the different IgA subclasses (dependent variables). Besides smoking, also potential confounders age, gender and CRP were included as independent variables. Smoking is defined as current or former smoking. #not significant after correction for multiple testing.

Evidence of site-specific mucosal autoantibody secretion in rheumatoid arthritis

Veerle F. A. M. Derksen[1], Klara Martinsson[2], Anouk G. van Mourik[1], Carlijn A. Wagenaar[3, 4], René E. M. Toes[1], Wendy Walrabenstein[3], Daniel Sjöberg[5,6], Dirkjan van Schaardenburg [3,4], Tom W. J. Huizinga[1], Alf Kastbom[2], Anna Svärd[2,5#], Diane van der Woude[1#]

[1] Department of Rheumatology, Leiden University Medical Center, Leiden, The Netherlands
[2] Department of Rheumatology and Department of Biomedical & Clinical Sciences, Linköping University, Linköping, Sweden
[3] Reade Center for Rheumatology and Rehabilitation, Amsterdam Rheumatology and Immunology Center, Amsterdam, The Netherlands
[4] Department of Clinical Immunology and Rheumatology, Amsterdam UMC Location AMC, Amsterdam, The Netherlands
[5] Center for Clinical Research Dalarna, Uppsala University, Uppsala, Sweden
[6] Department of Internal Medicine, Falun Hospital, Falun, Sweden

[#] These authors contributed equally

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Abstract

Objectives Anti-citrullinated protein antibodies (ACPA) have been detected in sputum and saliva, indicating that anti-modified protein antibodies (AMPA) can be produced at mucosal sites in rheumatoid arthritis (RA) patients. However, the body's largest mucosal compartment, the gut, has not yet been examined. We therefore investigated the presence of several AMPA (antibodies against citrullinated (ACPA), carbamylated (anti-CarP) and acetylated (AAPA) proteins) at different mucosal sites, including the intestinal tract.

Methods Paired faecal/ileal wash, saliva and serum samples of RA patients and healthy volunteers were collected in two independent cohorts. Data involving faeces was replicated in a third cohort. In these secretions AMPA were analysed using in-house ELISA with unmodified peptides as control. In faecal samples total IgA and anti-E. coli IgA were measured.

Results ACPA, anti-CarP and AAPA IgA were measurable in saliva of seropositive RA patients (prevalence 9-40%). No AMPA could be detected in faeces. IgA was present since total IgA and anti-E. coli IgA was detectable in faeces of ACPA-positive RA patients and healthy donors. Results were confirmed in another cohort using colonoscopically collected ileal wash samples.

Conclusions Our study shows the presence of ACPA, anti-CarP and AAPA IgA in saliva of ACPA-seropositive RA patients. However, no AMPA could be detected in faeces/ileal wash samples of these patients, although our assays were able to measure other antigen-specific antibodies. These data suggest that mucosal autoantibody secretion may occur in the oral mucosa of RA patients, while no evidence could be found for this process in the lower gastro-intestinal tract.

Introduction

The presence of anti-modified protein antibodies (AMPA) directed against post-translational modified proteins (PTMs) is a hallmark of seropositive rheumatoid arthritis (RA). The most well-known and clinically important AMPA are anti-citrullinated protein antibodies (ACPA), while other AMPA recognize carbamylated proteins (anti-CarP) or acetylated proteins (AAPA). The processes leading to the break of tolerance against PTMs, to the maturation of the AMPA response, and eventually to the development of seropositive RA are not fully understood. One of the hypotheses gaining increased attention is that mucosal surfaces play a role in AMPA formation (1-3). Inflammation at mucosal surfaces, triggered by environmental factors and microbiome-host interactions in combination with the local presence of PTMs, might create conditions in which tolerance to PTMs is broken (1, 2, 4, 5).

There is accumulating evidence that the airway mucosa is involved in seropositive RA. Smoking, along with exposure to silica dust and other inhalants, are a major risk factor for the development of seropositive RA and the concurrent presence of multiple RA-associated antibodies (6-8). It is hypothesized that these environmental factors induce airway inflammation, which can contribute to autoantibody formation (9). However, the lungs are not the only mucosal sites which may be involved in RA development. The epidemiologic association between RA and periodontitis was already reported years ago (10). Oral and intestinal microbiome disturbances have been described in RA patients and people at risk of developing RA (11-14). Novel research shows that RA patients with ongoing periodontitis experience repeated bacteraemia with oral bacteria. Bacteria can be citrullinated and citrullinated bacterial epitopes can be recognized by ACPA. These findings provide an interesting potential link between a physiological anti-bacterial responses and autoimmunity in RA (15). The association between the lung and oral mucosa and RA is further substantiated by the discovery that ACPA can be present in both sputum, bronchoalveolar fluid and saliva of RA patients (16-18). Rheumatoid factor can also be present in saliva of those patients (19).

However, the largest mucosal site in the body, the intestine, has received less attention over the years compared to the lung and mouth. Lately, this has changed with the finding that monoclonals derived from circulating plasmablasts in individuals at risk of RA can bind both RA-associated citrullinated autoantigens and bacteria in faeces (20). These findings suggests that also the intestinal mucosa might be involved in the pathophysiology of RA. The presence of other AMPA (besides ACPA) at mucosal sites has not yet been investigated, though this could provide new information on the development of the AMPA response in RA, especially in the gut. AAPA might provide an interesting angle when it comes to

mucosal microbial exposures as potential trigger for autoimmunity, since various bacterial species use acetylation of self-proteins to regulate cell processes (21, 22). Anti-carbamylated protein responses may also have an intestinal origin, since carbamylation has been shown to occur in the human gastrointestinal tract (23). It therefore appears plausible that anti-CarP and AAPA could be produced at mucosal sites and that local availability of specific post-translational modifications, as a product of microbiome, food constituents and host cell interactions, might diversify and broaden the AMPA response in RA. Nevertheless, whether AMPA are secreted in the intestinal tract is currently unknown.

Hence, we investigated whether ACPA, anti-CarP and AAPA can all be detected in mucosal secretions, with emphasis on material derived from the intestinal tract of RA patients. To this end, we collected paired serum, saliva and faeces of RA patients and healthy donors and tested these samples for the presence of ACPA, anti-CarP and AAPA. Two other independent cohorts were used to replicate our findings.

Patients and Methods

Cohorts

In the Dutch MUCOSA (MUCosal Origin of Serum Autoantibodies in rheumatoid arthritis) study, paired serum, saliva, and faeces samples were collected cross-sectionally from 47 RA patients visiting the outpatient clinic (of whom 36 were ACPA-seropositive) and from 21 healthy controls. Saliva was collected by passive drooling and faeces was collected by participants themselves and immediately frozen. One patient suffered from severe hyposalivation precluding the collection of saliva. Details are provided in supplementary data 1.

To substantiate our findings, we made use of samples from another independent study. The Swedish IntestRA study included 20 ACPA-seropositive RA patients, 10 healthy donors and 9 patients with inflammatory bowel disease (IBD) as additional controls. Serum and saliva samples were collected in a similar fashion. However, to investigate the presence of autoantibodies in the gut, ileal wash fluid was collected via colonoscopy instead. Details are provided in supplementary data 2.

Faeces samples of a third cohort, the Dutch Plants For Joints trial (PFJ) (24, 25), were examined to corroborate the results regarding AMPA in faeces. Baseline faeces samples of 42 ACPA-seropositive RA patients and 10 osteoarthritis patients (OA) as control were investigated. Faeces was collected by participants themselves at home and sent by mail, after where samples were stored at -80°C.

In all three studies, all patients fulfilled the 2010 ACR/EULAR criteria for RA (26) and most had long-standing disease. Throughout this manuscript, ACPA-seropositive refers to ACPA IgG seropositivity. All studies were performed in concordance with the declaration of Helsinki, approved by the relevant local medical ethical committees and all participants provided written informed consent.

Measurements

The presence of AMPA and RF in serum, saliva, and faeces in the MUCOSA study was tested by in-house enzyme-linked immunosorbent assay (ELISA) using peptides containing different modifications on a CCP2 (Cyclic Citrullinated Peptide 2) backbone. Measurements in the PFJ trial were performed in accordance with the protocols used for faeces samples in the MUCOSA. In the IntestRA study, modified commercial anti-CCP assays (CCPlus® Immunoscan, Svar Life Science) were used to measure ACPA IgA in ileal wash and saliva samples. Autoantibody analyses in IntestRA focused on ACPA only. Total IgA levels and anti-E. coli antibodies were also measured in mucosal secretions by ELISA. For anti-E. coli, ELISA plates were coated with E. coli lysates. After blocking and adding undiluted faecal extracts, horseradish peroxidase (HRP)-labelled detection antibodies were used stepwise before visualization with ABTS (supplementary data 1). Furthermore, analysis of inflammatory markers in saliva (total protein and matrix metalloproteinase-8 levels (MMP-8)) and faeces (calprotectin) was performed in the MUCOSA study using ELISA kits (Total MMP-8 ELISA kit R&D systems; DMP800B and Calprotectin ELISA kit Orgentec; ORG580) according to manufacturer's instruction.

Saliva samples were homogenized and centrifugated before use to remove any debris. To be able to detect autoantibodies in faecal matter, protein fractions were prepared by diluting the faeces 1:5 in faeces dilution buffer (phosphate-buffered saline + 0.05M EDTA + 1.66mM phenylmethylsulfonyl fluoride (PMSF) + 0.1mg/ml soybean trypsin inhibitor (Sigma)). Samples were mixed vigorously for 10-20 minutes until homogeneous and spun down. Ileal wash samples were centrifuged and frozen within one hour after collection.

When testing for AMPA positivity, all samples were also measured simultaneously on the unmodified control peptide to investigate whether binding was specific for the post-translational modification. In the MUCOSA and PFJ, a sample was considered AMPA-positive when both of the following criteria were met: 1) the value measured on the modified peptide was higher than the cut-off based on the mean + 2 times the standard deviation (SD) of the signal of healthy controls on that peptide, and 2) the optical density (OD) of the signal measured on the modified peptide was >2 times higher than the OD of the same sample measured on the unmodified peptide. In the IntestRA study OD signals on the arginine control were first subtracted from the ACPA OD values. Thereafter, a cut-off was calculated in a similar fashion.

More information about sample processing and autoantibody detection can be found in supplementary data 1 (MUCOSA and PFJ) and 2 (IntestRA). Mann-Whitney U tests, Chi-square tests or Fisher's exact tests, as appropriate depending on the kind of data, were performed to compare antibody positivity and inflammatory markers between groups.

Results

AMPA in saliva

Mucosal autoantibodies were investigated in three independent cohorts, of which the clinical characteristics are listed in Table 1. First, autoantibodies were measured in serum and saliva of participants in the MUCOSA study. 17% of ACPA-seropositive RA patients in the MUCOSA had detectable ACPA IgA in saliva, while ACPA could not be detected in saliva of ACPA-seronegative patients or healthy donors (Figure 1A) (not significant (ns)). In the Swedish IntestRA study, ACPA IgA was found in saliva of 40% of the ACPA-seropositive patients (Figure 1B), while none of the IBD patients or healthy controls were positive (p=0.03 compared to healthy). However, the presence of AMPA was not limited to ACPA IgA. Also, anti-CarP IgA and AAPA IgA could be detected in saliva of seropositive RA patients in the MUCOSA (Figure 1C-D), although the number of patients positive for salivary autoantibodies was low (9%) for both autoantibodies. RF IgA could be found in saliva 46% of ACPA-seropositive of RA patients (Figure 1E) (p=0.001 compared to healthy) and was present more frequently compared to salivary ACPA IgA.

Notably, when examining reactivity to the modified peptides (citrulline, homocitrulline and acetylated lysine) and unmodified peptides (arginine and lysine respectively) in saliva in more detail, a substantial number of seropositive RA patients, but not seronegative patients or healthy controls, had high reactivity to the modified peptide, but also showed a similarly high degree of reactivity to the unmodified peptide (Figure 2A-C). When the reactivity to the modified and unmodified peptide was similar, antibody binding was not specific for the PTM and samples were considered AMPA negative. The high signal measured on the unmodified peptide in saliva samples is markedly different from serum, where background signals are usually very low (Figure 2D-F). In the IntestRA unmodified peptide signals were more equally distributed among RA patients and controls (Figure 2G) (ns).

Table 1: Patient characteristics of all cohorts at inclusion.

	MUCOSA		IntestRA			Plants For Joints	
	RA	Healthy	RA	Healthy	IBD	RA	OA
	n=47	n=21	n=20	n=10	n=9	n=42	n=10
Age, mean ± SD	59 ± 13	48 ± 16	61 ± 9	62 ± 9	30 ± 8	55 ± 12	62 ± 6
Female, n (%)	37 (79)	13 (62)	15 (75)	7 (70)	6 (67)	36 (86)	10 (100)
Disease duration, years, median (IQR)	14 (8-16)	-	0.7 (0-12)	-	-	6.5 (3-15)	-
Smoking ever, n (%)	26 (55)	6 (29)	12 (60)	4 (44) (n=9)	3 (33)	-	-
ESR, mm/h median (IQR)	9 (2-34)	-	13 (8-20)	6 (4-24)	8 (4-15)	15 (8-32)	-
DAS28, median (IQR)	2.6 (1.7-3.6) (n=45)	-	3.0 (2.0-4.0) (n=10)	-	-	3.9 (3.2-4.5)	-
Serum antibody positivity[a]							
ACPA IgG, n (%)	36 (77)	0 (0)	20 (100)	0 (0)	0 (0)	42 (100)	-
Anti-CarP IgG, n (%)	21 (45)	0 (0)	-	-	-	-	-
AAPA IgG, n (%)	21 (45)	0 (0)	-	-	-	-	-
ACPA IgA, n (%)	18 (38)	0 (0)	20 (100)	0 (0)	0 (0)	-	-
Anti-CarP IgA, n (%)	4 (9)	0 (0)	-	-	-	-	-
AAPA IgA, n (%)	5 (11)	0 (0)	-	-	-	-	-
RF IgM n (%)	34 (72)	3 (14)	-	-	-	-	-
RF IgA, n (%)	21 (45)	1 (5)	-	-	-	-	-

AAPA, anti–acetylated protein antibodies; ACPA, anti–citrullinated protein antibodies; CarP, carbamylated protein antibodies; DAS28, Disease Activity Score in 28 joints; ELISA, enzyme-linked immunosorbent assay; ESR, erythrocyte sedimentation rate; IBD, inflammatory bowel disease; IQR, interquartile range; MUCOSA, MUCosal Origin of Serum Autoantibodies in rheumatoid arthritis; OA, osteoarthritis; RA, rheumatoid arthritis; RF, rheumatoid factor. All serum autoantibody measurements in the MUCOSA study are done with in-house ELISAs. [a]All serum autoantibody measurements in MUCOSA are done with in-house ELISAs.

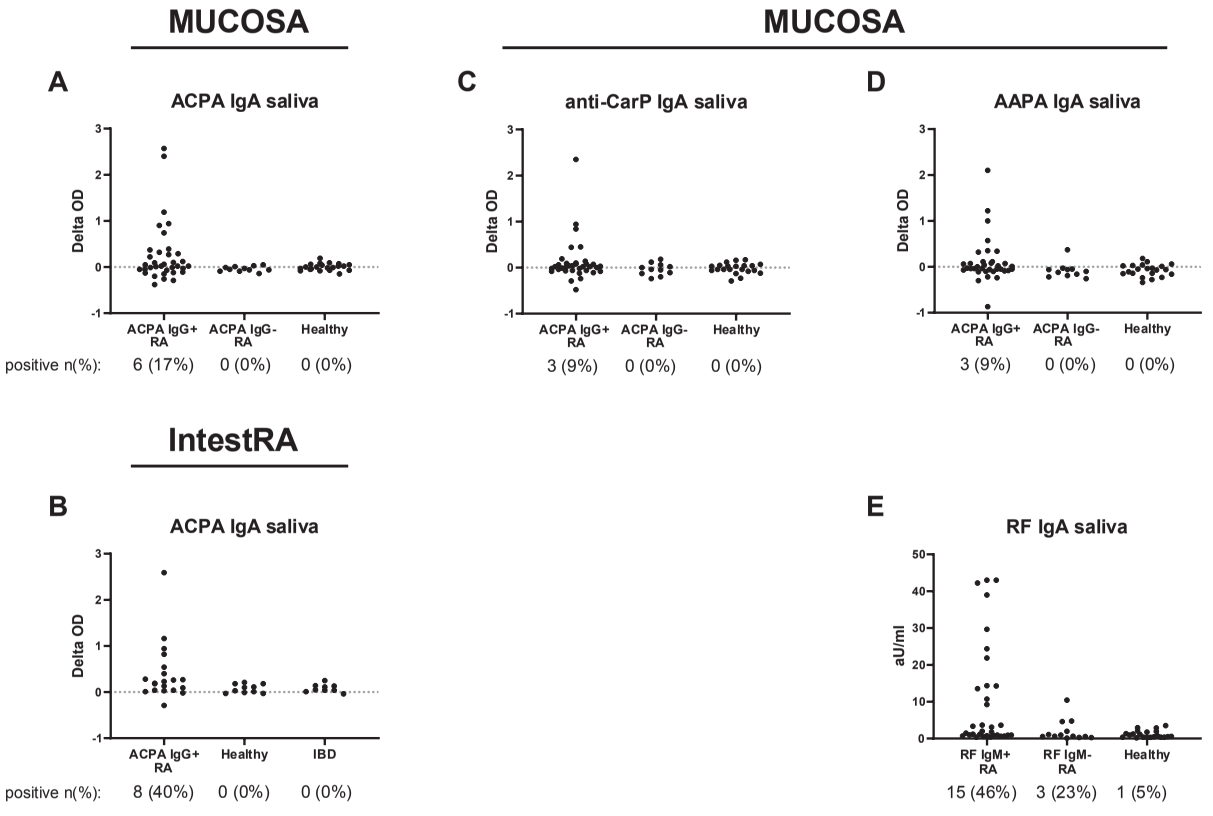

Figure 1: Autoantibody measurements in saliva. A ACPA IgA in saliva in MUCOSA, B ACPA IgA in saliva in IntestRA, C anti-CarP IgA, D AAPA IgA, E RF IgA in saliva in MUCOSA. Delta OD (difference in OD between modified peptide and unmodified peptide) for AMPA and aU/ml (arbitrary units per ml) for RF are depicted. Groups on X-axis are based on diagnosis and seropositivity (graph E uses RF IgM seropositivity to define groups). The number (%) of positive patients for that specific autoantibody is given.

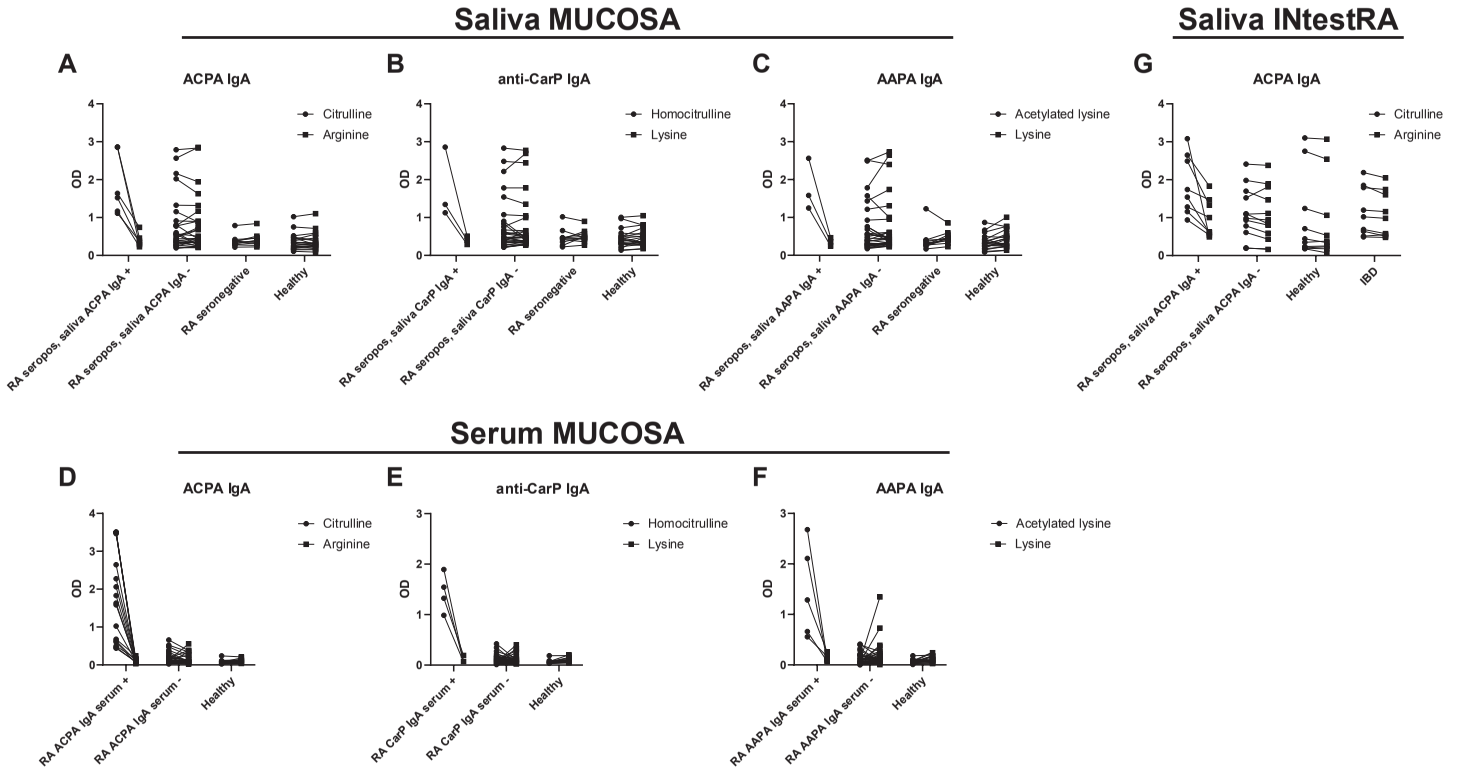

Figure 2: Paired optical density (OD) values on the modified and unmodified peptide for each AMPA. Paired OD values on the modified and unmodified peptide for each AMPA in saliva (A-C) and serum (D-F) of patients and healthy donors in MUCOSA and in saliva (G) of patients and controls in IntestRA. A-C First group of each graph shows all seropositive RA patients who tested positive for that specific AMPA in saliva, while the second group depicts all seropositive RA patients who tested negative for that specific AMPA in saliva. D-F First group shows patients who were positive for that specific AMPA IgA in serum, while the second group shows patients negative for that specific AMPA IgA in serum.

Next, we investigated whether the presence of autoantibodies in saliva was related to the amount of total IgA in these samples, as salivary IgA levels can differ between individuals and over time (27). There was no significant difference in salivary total IgA between seropositive RA patients who were positive for AMPA in their saliva and those who were negative (Supplementary Figure 1). Therefore, it seems that prevalence of IgA AMPA in saliva cannot solely be attributed to differences in salivary total IgA levels, but may rather point to inherent differences between patients.

AMPA profile in saliva and serum

Different types of AMPA in saliva tend to co-occur. Among the 7 saliva AMPA positive patients in the MUCOSA, 2 were triple positive for ACPA, anti-CarP and AAPA and 1 patient was anti-CarP and AAPA double positive (Figure 3). Furthermore, 6 saliva AMPA positive patients were also saliva RF IgA-positive. The presence of AMPA in saliva always coincided with the presence of that specific AMPA in serum, although the isotype could differ (Figure 3). For example, one patient is positive for AAPA IgA in saliva, while AAPA IgG but no AAPA IgA could be detected in serum. Similar findings were made for rheumatoid factor. However, 3 RA patients who tested positive for RF IgA in saliva, tested negative for both RF IgM and IgA in serum. This suggests a local origin and subsequent secretion of autoantibodies rather than leakage from serum antibodies to the saliva. Furthermore, the amount of IgG measured in these saliva samples is on average ± SD, 9 ± 8 μg/ml, a thousand-fold lower than serum IgG (reference levels 7-16 g/L), making contamination of saliva samples with serum autoantibodies in measurable amounts less likely. Thus, the saliva autoantibody profile displays similarity to, but does not necessarily originate from, the serum autoantibody profile.

Saliva AMPA and local inflammation

Next, we investigated whether the presence of salivary autoantibodies was associated with oral inflammation. Total protein content, matrix metalloproteinase-8 levels (MMP-8) and total IgA levels in saliva have been determined as markers of local inflammation (27, 28). In the MUCOSA, RA patients in general had slightly lower total IgA levels in saliva compared to healthy donors, while there was a non-significant trend towards higher MMP-8 and total protein values in RA patients. There were no significant differences in all three salivary inflammatory markers between RA patients who were positive for ACPA in their saliva (median (IQR): total protein 1380 μg/mL (1057-1747), total IgA 343 μg/mL (253-562), MMP-8 123 ng/mL (33-150)) and those who were not (median (IQR): total protein 1411 μg/mL (1040-1621), total IgA 259 μg/mL (169-386), MMP-8 83 ng/mL (26-138)) (Supplementary Figure 2A-C). As the number of ACPA saliva-positive patients is small, salivary inflammatory markers were also compared between ACPA-seropositive RA patients, ACPA-seronegative RA patients and healthy

donors (Supplementary Figure 2D-F), which showed similar results. As smoking directly affects the oral mucosa, the relation between autoantibody positivity in saliva and smoking was examined. No significant relation between smoking and the presence of autoantibodies was seen in saliva (Supplementary Table 1), despite the significant association between RF IgM-seropositivity and smoking in the MUCOSA study (p=0.04) and a similar trend for ACPA-seropositivity (Supplementary Table 1).

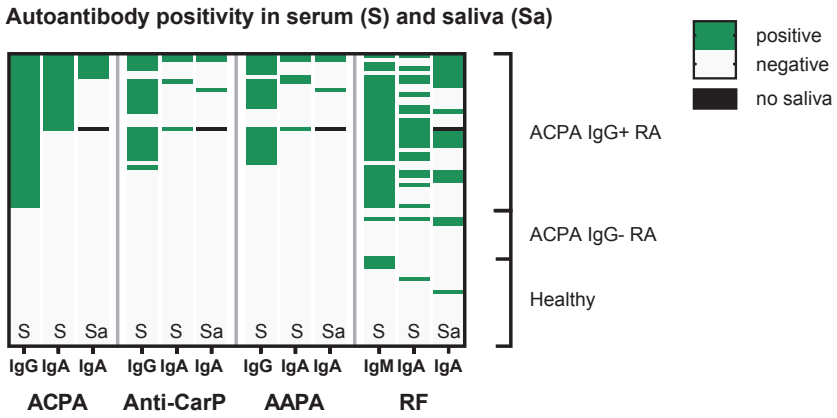

Figure 3: Autoantibody profile in serum (S) and saliva (Sa) in MUCOSA. Positivity for autoantibodies in serum and saliva. Each row depicts a study participant. Black field: no saliva available due to hyposalivation.

AMPA in the intestinal tract

In the MUCOSA, no ACPA, anti-CarP or AAPA were found in faeces samples of RA patients or healthy controls (Figure 4A-C), as there was almost no difference between the signals on the modified and unmodified peptides. Also in the PFJ, no AMPA was found in faeces (Figure 4D-F).

Like in saliva, reactivity to the modified and unmodified peptides was evaluated separately in faeces as well. Multiple ACPA-seropositive RA patients, ACPA-seronegative RA patients and controls showed an OD >1 to the citrullinated peptide in their stool, but also to unmodified arginine-containing peptide, which was thus considered as non-specific binding/AMPA negative (Figure 5A-B). Similar results were found for anti-CarP and AAPA (Figure 5C-F) in both cohorts. The high OD signals on the unmodified peptides in faeces are in contrast to saliva, where only seropositive RA patients showed a high background (Supplementary Figure 3).

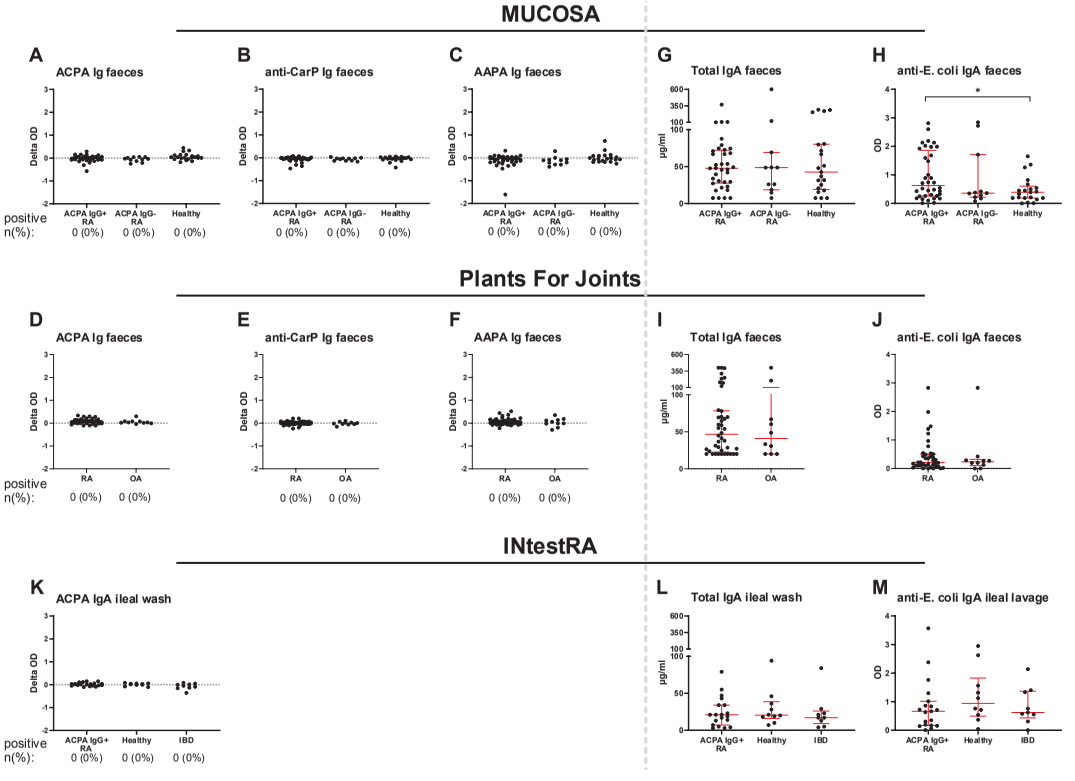

Figure 4: AMPA Ig in faeces and ileal lavage of RA patients. A-C ACPA, anti-CarP and AAPA Ig respectively, in faeces from patients and healthy donors in MUCOSA. Groups on X-axis are based on diagnosis and seropositivity, D-F AMPA Ig in faeces of PFJ, G/I Total IgA levels in µg/ml and H/J OD on the anti-E. coli IgA ELISA in the same faeces samples, K ACPA IgA in ileal lavage samples from IntestRA, L Total IgA levels and M anti-E. coli IgA OD in the same ileal lavage samples. For AMPA, the number (%) of positive patients is given. For AMPA the Y-axis depicts difference in OD (delta OD) between the modified and unmodified peptide. Reds bars show the median and interquartile range. *p<0.05

To determine whether the faecal supernatants contained sufficient amounts of immunoglobulins to fall within the detection range of our ELISAs, total IgA levels were measured. Faecal supernatants in MUCOSA contained a median of 48 μg/ml total IgA, with no significant differences between seropositive RA patients, seronegative RA patients and healthy donors (Figure 4G). Similar results were found in the PFJ (Figure 4I). These total IgA levels are roughly comparable to the amount of total IgA in the diluted saliva samples used for AMPA ELISA. Furthermore, anti-E. coli IgA was used as additional (antigen-specific) control. High anti-E. coli signals could both be detected in RA patients and healthy donors. Interestingly, anti-E. coli reactivity was significantly higher in seropositive RA patients compared to healthy donors in MUCOSA (p=0.04) (Figure 4H), although numbers are small. In the PFJ no significant difference was observed (Figure 4J). These data indicate that the methods used are able to detect the presence of (antigen-specific) antibodies in faeces samples in general, and that the lack of AMPA signal is not due to the absence of total IgA in these samples.

To substantiate our findings regarding the intestines, we also investigated ileal wash samples, collected via colonoscopy in the independent IntestRA cohort. Antibodies in such samples might be less prone to degradation compared to faeces. However, also in the ileal wash samples no ACPA IgA was detected (Figure 4K), while there was total IgA (median 20.5 μg/ml) (Figure 4L) and anti-E. coli IgA (Figure 4M) detectable. ODs for both the modified and unmodified peptides were overall low (Figure 5G). Total IgA levels and anti-E. coli antibody signals were also slightly lower compared to the faeces samples, possibly due to dilution by the lavage fluid instilled in the ileum used to collect these samples.

Calprotectin in faeces of RA patients

Since previous reports have suggested that RA might be characterized by a leaky intestinal barrier, gut dysbiosis and inflammation (3, 11, 29, 30), intestinal inflammation was investigated in the MUCOSA. Calprotectin was measured in faeces of both RA patients and healthy donors by commercial ELISA (Orgentec) (Supplementary Figure 4A). Values above 200 μg/g are considered significantly elevated and reflect active inflammatory intestinal disease. 6/47 RA patients and 1/21 healthy control had calprotectin values above 200 μg/g and an additional 7 patients had slightly elevated calprotectin levels between 100-200 μg/g. However, 5/7 subjects with strongly elevated calprotectin levels and 4/7 subjects with slightly elevated calprotectin levels reported regular NSAID use (Supplementary Figure 4B) (ns, p=0.07). NSAIDS are known to cause gastro-intestinal mucosal damage (31) and it has been reported that 2 weeks of NSAID use in healthy individuals can lead to significantly elevated faecal calprotectin levels (32).

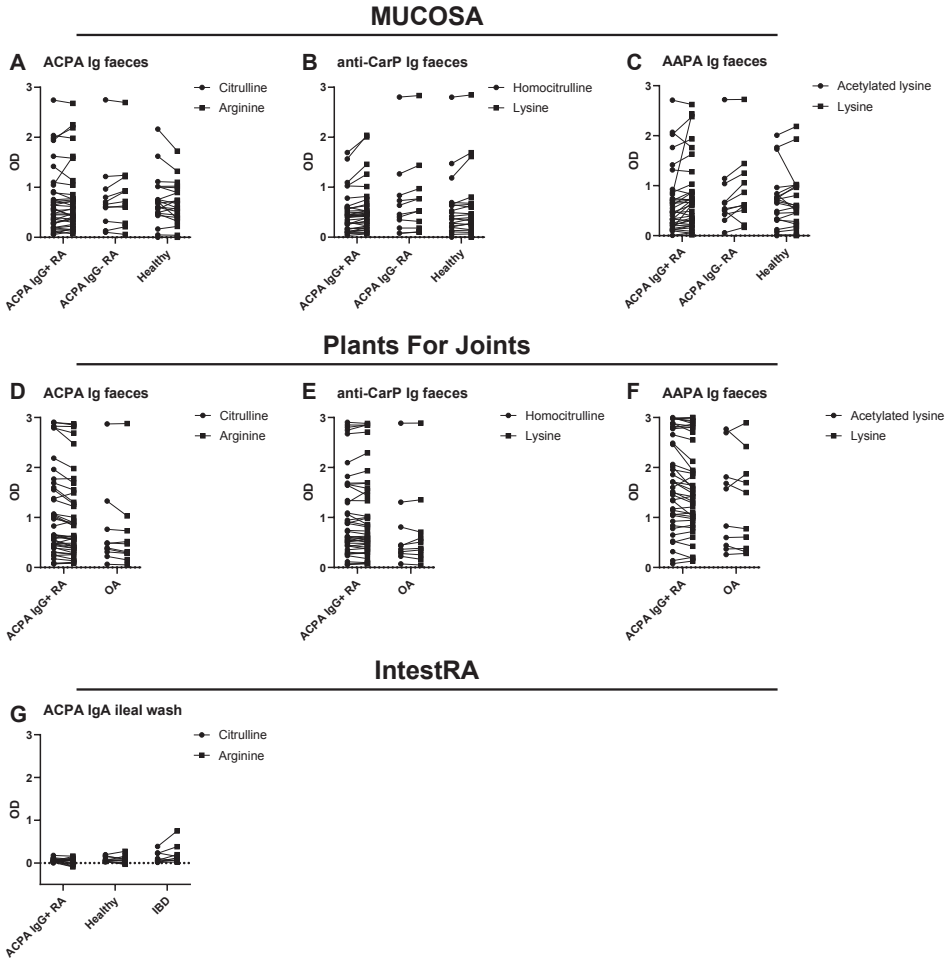

Figure 5: Paired optical density (OD) values on the modified and unmodified peptide for each AMPA Ig in faeces of patients and controls. A, C, E in MUCOSA and B, D, F in PFJ and ACPA IgA in ileal wash in patients and G controls in IntestRA.

Discussion

This study aimed to examine the RA-associated autoantibody profile in various mucosal compartments. In saliva of established seropositive RA patients, we found that ACPA, anti-CarP and AAPA IgA antibodies can all be present, although in modest quantities compared to serum. Differences in the percentage of saliva ACPA positive patients (17-40% in ACPA IgG seropositive RA) between cohorts might reflect the inclusion of only patients with moderate to high ACPA serum levels in

the IntestRA study. When salivary AMPA are present, the breadth of the response is similar to the AMPA serum profile. In contrast, no AMPA were found in faeces of the same RA patients, although the faecal supernatants did contain total IgA and anti-E. coli IgA antibodies. These findings were confirmed in faeces and in ileal lavage samples from two independent cohorts of RA patients. Our findings suggest that secretion of AMPA is limited to certain mucosal sites, with local secretion of all AMPA taking place in the oral cavity, but not to a detectable degree in the lower intestinal tract.

These observations are in line with previous studies showing the presence of salivary ACPA IgA in RA patients (17). ACPA IgA and IgG were also previously found in sputum of seropositive RA patients and first-degree relatives (FDR) of RA patients (16). Part of the FDRs who were positive for ACPA in their sputum, were serum ACPA-negative, indicating that sputum ACPA are produced locally and can precede or occur independently of a serological ACPA response. Our findings provide further evidence that autoantibodies found in mucosal secretions can be secreted locally at mucosal sites, as the AMPA isotype present in saliva was not always measurable in serum. The low amount of IgG present in the saliva samples may further support that both the detected AMPA and RF originated from the mucosal lining of the oral cavity instead of leaking from serum, since leakage would have led to a higher quantity of IgG as the most abundant isotype present in serum. Furthermore, monomeric IgA from serum cannot be actively transported over the mucosal epithelium by the polymeric immunoglobulin receptor, while mucosal derived dimeric IgA can.

These findings raise the question where the initial activation of autoreactive B-cells in RA can take place and which triggers elicit these anti-modified protein responses. Activated B-cells re-enter the tissue where they were activated based on homing marker expression, although there probably is some crossover to other, often anatomically closely related tissues (27). This suggests that the cells secreting AMPA in the oral mucosa, are probably derived from B-cells activated in local lymphoid tissue. Our study shows that the salivary AMPA response not only includes anti-citrullinated protein antibodies, but also antibody responses against carbamylated and acetylated proteins, suggesting the local presence of these antigens. Interestingly, bacteria can acetylate self-proteins (21, 22) and thus might evoke an anti-acetylated bacterial protein response, which could be cross-reactive to acetylated self-proteins. It is hypothesized that via this mechanism the antibody responses against acetylated bacterial content can contribute to diversification and epitope spreading of the AMPA response in RA. Furthermore, in the MUCOSA

seropositive RA patients tended to have a higher reactivity in saliva towards the unmodified peptides, compared to seronegative RA and healthy donors, although this was not as clear in the IntestRA. This higher reactivity towards unmodified peptides in seropositive RA patients could also point to activated humoral immune responses in general in these patients, for example due to decreased barrier function or local inflammation.

Our study did not include a dental examination to determine the presence of periodontitis, inflammation of the gums, which can be caused by bacterial infection. To gather some information on oral inflammation nonetheless, total protein content, MMP-8 levels and total IgA were measured in saliva. No association between these inflammatory markers and ACPA positivity in saliva was seen. This could be explained in several ways: the sensitivity of these markers might be more limited than a dental examination, and gingivitis or periodontitis could have been missed. Alternatively, a true lack of association could suggest that oral production of autoantibodies is independent of simultaneously occurring mucosal inflammation, and would either not require inflammation at all, or could be related to barrier dysfunction and inflammation in the past.

Not only the oral mucosa, but also the gut could represent a large source of citrullinated, carbamylated and acetylated (microbial) proteins. From an immunological point of view, it appears conceivable that a T-cell response against post-translationally modified bacteria (as foreign/non-self) may provide the required T-cell help to activate self-reactive AMPA directed B-cells. Reactivity to intestinal bacteria was found to be a normal property of the human CD4+ T cell repertoire in healthy individuals (33). Moreover, it has been described that monoclonal ACPA derived from individuals at risk for RA can bind bacterial isolates from human faeces (20). Based on these findings, one would have expected to find AMPA in intestinal secretions as well, but we did not find ACPA, anti-CarP or AAPA in faeces or ileal wash samples. This suggests there is no substantial ACPA production in the lower intestinal tract. Anti-E. coli IgA and total IgA were measurable in these intestinal samples, indicating that the methodology is adequate to detect (antigen-specific) antibodies.

Our results suggest that mucosal AMPA production is site specific, with local secretion of AMPA taking place in the oral mucosa, but not substantially in the gut. In addition, earlier studies provide evidence for AMPA responses in the airways (16, 18). This spatial variation might be due to differences in the local micro-environment, such as antigen availability and local inflammatory processes like NETosis (release of Neutrophil Extracellular Traps). However, there are several other reasons why AMPA might not

be detectable in the gut, for example due to strong binding to their antigen, degradation of antibodies by digestive enzymes, or the amount of AMPA being under the detection limit of our assays. The fact that total IgA and anti-E. coli IgA were detectable in faeces and that ACPA were also not present in ileal lavage samples, which might be less prone to degradation, makes it more likely the gut is not a major site of secretion for AMPA. Nevertheless, further research on barrier dysfunction and presence of PTMs in the intestinal tract of seropositive RA patients is warranted, as there are other potential mechanisms via which the intestinal mucosal compartment could contribute to the systemic AMPA response, which are beyond the scope of our study, such as microbiome dysbiosis, decreased intestinal barrier function and trafficking of immune cells primed in the intestine to the systemic circulation. For example, in 1 cohort there was a difference in faecal anti-E. coli reactivity between seropositive RA patients and healthy donors, which could point to increased interactions between gut bacteria and the immune system in RA.

Our study provides new insights in the autoantibody profile at mucosal surfaces, but it comes with some limitations. Most patients had longstanding RA and used various immunosuppressive therapies, which could have influenced the results. Previous studies investigating the effect of anti-rheumatic treatment on serologic AMPA responses have revealed that the presence of AMPA in serum is quite stable under treatment (34), but it is unknown whether mucosal AMPA responses originate from antibody secreting cells with similar (long-lived) characteristics. Positivity for secretory ACPA in serum declined more strongly compared to ACPA IgG after initiation of therapy (35). Furthermore, despite the use of three independent cohorts to verify our findings, the number of patients included is limited. Due the COVID-19 pandemic we were prohibited from collecting paired sputum samples in the MUCOSA, as originally planned. Therefore, whether anti-CarP and AAPA are also present in sputum of RA patients remains unknown.

To the best of our knowledge, our study nonetheless represents the most extensive investigation to date of a large variety of autoantibodies in a most diverse array of bodily fluids. Our results show that ACPA, anti-CarP and AAPA can be secreted locally in the oral mucosa. This suggests local immune responses against post-translational modified proteins, for example in the context of an anti-bacterial response, might contribute to the development and diversification of the AMPA response in RA patients. No support for local AMPA secretion in the lower intestinal tract was found. This study therefore, for the first time, sheds light on one of the possible roles (or potential lack thereof) of the intestinal mucosa in the onset of the AMPA responses in RA.

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Supplementary information

Supplementary data 1: Methodology MUCOSA study and faeces samples Plant For Joints

Patients and sample collection

All patients with a definite diagnosis of rheumatoid arthritis based on the ACR/EULAR 2010 criteria visiting the outpatient clinic of the Leiden University Medical Center (LUMC) were eligible for inclusion, regardless of disease duration or treatment. As a control, healthy donors without any history of inflammatory arthritis were included. All participants were 18 years or older and provided written consent. Individuals with current upper airway infection, presence of oral ulcers, dental treatment within the previous month or known IBD were excluded. Data on smoking and medication use was collected using a digital patient survey. Medication use was verified by status review. Participants were asked to refrain from vigorous exercise for 2 hours prior to saliva collection and refrain from food or beverage (except water), smoking, chewing gum and brushing their teeth for 1 hour prior. Before starting saliva collection, the participants were asked to rinse the mouth with water. Saliva was collected by 'passive drooling': the donor leaning forward allowing saliva to drip into a test tube for 10 to 15 minutes. Saliva samples were incubated at 56°C for 45 minutes as a precautionary measure to prevent spreading of SarS-CoV-2 virus particles. After incubation samples were homogenized and spun down at 7000g for 10 minutes. Supernatants were collected and stored at -80°C until further use. After thawing, saliva samples were spun down for 5 minutes at 5000g directly before use.

Faeces was self-collected at home within 2 weeks of saliva and serum donation. The faeces was immediately stored in a house-hold freezer for a maximum of 2 days, before it was transferred on dry ice to a -80 freezer. Saliva and faeces samples containing macroscopic blood were excluded.

To prepare protein fractions of the faeces sample to use on ELISA, circa 1 gram of faeces was diluted in 5ml/gram faeces dilution buffer (PBS + 0.05M EDTA + 1.66mM PMSF + 0.1mg/ml soybean trypsin inhibitor (sigma)). Glass beads (2mm, Merck) were added, and samples were mixed vigorously for 10-20 minutes until homogeneous and spun down at 2000g for 10 minutes at 4°C. Supernatants were collected and centrifuged for 10 minutes at 10.000g. Supernatants were aliquoted and stored at -20°C until further use. Faeces supernatants were spun down for 5 minutes at 10000g directly before use. The same protocols for preparing the faeces samples as well as for the ELISAs on faecal extracts were used in the Plant For Joints study.

AMPA measurements using ELISA

ELISAs were performed using 384 Well Flat Bottom High Bind Microplates (Corning), unless stated otherwise. For AMPA detection, biotinylated CCP2 (patent EP2071335) or similar peptides where the citrulline was replaced by a homocitrulline (CHcitP2) or acetylated lysine (CAcetylP2) were used. Control peptide containing an arginine (CargP2) instead of citrulline, or a lysine (ClysP2) instead of a homocitrulline or acetylated lysine were coated on the same plate. The peptides including the patent protected CCP2 and CargP2 were provided by Dr. J.W. Drijfhout (Dept. of IHB, LUMC). After each step, ELISA plates were washed with PBS/0.005% Tween 20. All ELISAs are visualized with ABTS/H_2O_2. For ACPA, anti-CarP and AAPA measurements in saliva and faeces a modified version of the serum assay was used. The biotinylated modified or control peptides were coated in a concentration of 1 µg/ml in PBS/0.1%BSA on streptavidin coated plates (microcoat, standard capacity; 604500) for serum or on 384 well microplates pre-incubated overnight at 4°C with 1 µg/ml streptavidin (invitrogen) for saliva/faeces and incubated for 1 hour at room temperature (RT). Serum was diluted 1:50 in PBS/0.05% Tween/ 1% BSA (PBT) (for IgG) or PBS/1%BSA/50 mM TRIS/0.05% Tween, pH 8.0 (PBTT) (for IgA) and incubated for 1 hour at 37°C (ACPA, anti-CarP) or overnight at 4°C (AAPA). Saliva was diluted 1:4 in PBS/0.05% Tween/2%Casein (PTC) and incubated for 2 hours at 37°C. Faeces homogenates were added undiluted and incubated overnight at 4°C on ice. A pooled serum standard was used to calculate arbitrary units, and positive and negative serum controls were included on each plate. After washing, plates were incubated with rabbit anti-human-IgG-HRP (DAKO, P0214) 1:4000 (anti-CarP, AAPA) or 1:8000 (ACPA) in PBT for serum or goat anti-human-IgA-HRP (Invitrogen; A18781) 1:5000 for serum and 1:3000 for saliva in PBTT for 1 hour at 37°C. For faeces, goat anti-human-Ig-HRP (Bethyl) 1:2000 in PBTT was added an incubated for 3.5h at 4°C on ice. Blanks were subtracted and samples were considered AMPA positive when they were above the cut-off and the OD (optical density) of the modified peptide was larger than 2 times the OD on the unmodified peptide. The cut-offs were determined based on the mean plus 2 times the standard deviation of the OD of healthy controls. In case the cut-off was below the linear range of the standard, the lowest point of the linear range was used as cut-off.

Rheumatoid factor ELISA

For RF IgM and IgA ELISA, ELISA plates (Nunc maxisorp plates (VWR) for serum) were incubated overnight at room temperature with 10 µg/ml human IgG for RF IgM (Jackson Immunoresearch; 009-000-003) or 5 µg/ml rabbit IgG for RF IgA (Nordic-MUbio) and blocked for 1 hour at 37°C with PBS/1%BSA (RF IgM) or PBS/2%Casein (RF IgA). Serum samples were diluted 1:100 in PBT for RF IgM and 1:185 in PBTT for RF IgA and incubated for 1 hour at 37°C. Saliva samples were diluted 1:4 in PTC and incubated

for 2 hours at 37°C. A commercial standard (N/T Rheumatology control SL/2, Siemens) was used to calculate arbitrary units. Plates were incubated with goat anti-human-IgM-HRP (Millipore; AP114P) 0,3 µg/ml in PBT or goat anti-human-IgA-HRP 1:5000 in PBTT for 1 hour at 37°C.

Total IgA and total IgG ELISA

For total IgA level measurements in saliva and faeces, ELISA plates were coated with goat anti-human-IgA-Fc (Bethyl; A80-102A) 10 µg/ml in bicarbonate/carbonate coating buffer (pH 9.6). All incubation steps were performed for 1 hour at RT, except blocking with PBT which was done for 30 minutes at RT. Two dilutions were used of both saliva and faeces: saliva was diluted 1:400 and 1:800 and faeces 1:80 or 1:100 and 1:400 in PBTT. A serial dilution of purified secretory IgA (Bio-rad PHP133, 1mg/ml) was used as standard. Goat anti-human-IgA-HRP (Bethyl; A80-102P), diluted to 50 ng/ml in PBTT, was used for IgA detection. Total IgG levels in saliva were measured in a similar fashion, using goat anti-human-IgG-Fc (Bethyl; A80-104) diluted 10 µg/ml in bicarbonate/carbonate coating buffer (pH 9.6) as coating antibody and goat anti-human-IgG-HRP (Bethyl; A80-104P), diluted to 50 ng/ml in PBTT as detection antibody. A serial dilution of human IgG (Jackson Immunoresearch; 009-000-003), starting with 0.25ug/ml was used as standard. Saliva samples were diluted 1:50 and 1:200 in PBTT.

Total protein and MMP-8 measurement in saliva

For total protein measurements in saliva, Pierce BCA protein Assay kit (Thermoscientific) was used in combination with Nunc maxisorp plates. Saliva samples were diluted 1:2 in PBS before use and measurements were performed conform manufacturer's protocol. Total MMP-8 ELISA kit (R&D systems; DMP800B) was used to determine matrix metalloproteinase 8 levels (MMP-8) in saliva. The kit was used according to manufacturer's instructions. Saliva samples were diluted 1:15 and a log-log standard curve was used to calculate MMP-8 levels.

Anti-E. coli and calprotectin ELISA

Escherichia coli (E. coli) of strain BL21 (kind gift of Can Amaran) were cultured locally manufactured M9 medium (containing KH_2PO_4 (3 g/L), Na_2HPO_4 (6 g/L), NaCl (0.5 g/L), $MgSO_4$ (1 mmol/L), $CaCl_2$ (0.1 mmol/L) and lysed using French press at 10,000 psi. The lysis procedure was performed three times. The solution with lysed bacteria was spun down at 21,000g, 4°C for 60 min, and the supernatant was collected. Concentration was measured with spectrophotometry. ELISA plates were coated with 10µg/ml E. coli lysate overnight at 4°C. After blocking with PBT for 1 hour at 37°C, undiluted faecal extracts and a pooled serum standard were added and incubated overnight at 4°C. Serum samples and purified secretory IgA were taken along as controls. Two HRP-

labelled detection antibodies were used. First, goat anti-human-IgA-HRP was added and after washing, polyclonal rabbit anti-goat Ig-HRP (DAKO, P0449) was used. Both antibodies were diluted 1:1000 in PBTT and incubated for 1 hour at 37°C. Afterwards, signals were visualized with ABTS. Calprotectin in faeces was measured using a commercial calprotectin ELISA kit (kindly provided by Orgentec; ORG580) according to manufacturer's instruction.

Supplementary data 2: Methodology IntestRA study

Study subjects and samples

20 patients with RA (10 with disease duration <1 year and 10 with established RA), 10 patients with Crohn's disease and 10 healthy controls from the County of Dalarna, Sweden, were included between 2016 and 2019 in the IntestRA study. Only RA patients with a moderate or high level of IgG anti-CCP in serum were included, as these were expected to more likely have detectable ACPA in the intestine. Patients with known Crohn's disease with engagement of terminal ileum and a scheduled colonoscopy were recruited consecutively from the endoscopy department's waiting list. Indication for colonoscopy was assessment of disease activity. Persons participating in a screening study for colorectal cancer were asked to also contribute with samples from terminal ileum. None of these patients were diagnosed with colorectal cancer during the procedure.

Serum and saliva samples were collected at the rheumatology clinic. Participants were asked to restrain from eating, drinking other liquids than water, brushing teeth or smoking one hour before saliva sampling. Saliva was collected using passive secretion during 10 min, the sample was kept on ice and centrifuged 5 minutes at 5000 g. Serum samples were centrifuged 5 minutes at 5000g.

A colonoscopy was performed in all patients with at least 5 cm intubation of terminal ileum. 50 mL PBS was first instilled into the intestinal lumen using a catheter through the working channel of the endoscope. Intestinal fluid was then aspirated through the catheter. Ileal wash fluid samples were centrifuged 5 minutes at 5000 g, frozen within one hour after collection and stored at -80°C until further analyses.

IgA ACPA in saliva

Commercially available serum IgG-class anti-cyclic citrullinated peptide (anti-CCP) enzyme-linked immunoassays (ELISA) tests (CCPlus® Immunoscan, Svar Life Science) were modified to analyse IgA ACPA in saliva. All samples were analysed in duplicate. Saliva samples were thawed at room temperature and spun down (11000g at 4°C for 10 minutes) directly before use, to remove non-soluble material. The supernatant was

diluted to a final concentration of 1:20. The secondary antibody, polyclonal rabbit anti-human-IgA-HRP (DakoCytomation) was diluted 1:200. To adjust for non-specific IgA adsorption, all samples were tested against a control peptide (cyclic arginine peptide, CAP, EuroDiagnostica AB). Anti-CCP and anti-CAP analyses were performed in parallel and anti-CAP background levels were subtracted from anti-CCP values (delta optical density measurements).

IgA ACPA in ileal wash

Commercially available serum IgG anti-cyclic citrullinated peptide (anti-CCP) enzyme-linked immunoassays (ELISA) tests (CCPlus® Immunoscan, Svar Life Science) were modified to analyse IgA ACPA in ileal wash. All samples were analysed in duplicate.

Ileal wash samples were thawed at room temperature and diluted to a final concentration of 1:5. Samples were incubated 1 hour at room temperature on the pre-coated 96-well plates. The secondary antibody, polyclonal rabbit anti-human-IgA-HRP (DakoCytomation) was diluted 1:200, added to the wells for 1h at RT. The plate was washed and the substrate Tetramethylbenzidine (TMB, Sigma Aldrich) added. The plate was read at 450nm. A positive control and blanks were included on the plate. Blanks were subtracted. To adjust for non-specific IgA adsorption, samples were tested against a control peptide (cyclic arginine peptide, CAP, EuroDiagnostica AB). Anti-CCP and anti-CAP analyses were performed in parallel and anti-CAP background levels were subtracted from anti-CCP values (delta optical density measurements). Samples were considered ACPA positive when they were above the cut-off determined based on the mean plus 2 times the standard deviation of the OD of healthy controls.

IgG and IgA ACPA in serum

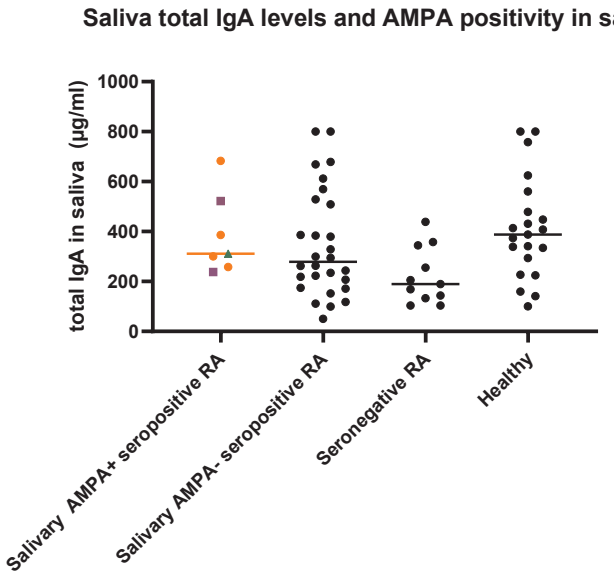
Serum samples were analysed for IgG ACPA according to the manufacturer's instruction (CCPlus® Immunoscan, Svar Life Science), with cut-off set at 25 U/mL. IgA ACPA was analysed using the same pre-coated CCP-plates, adding serum diluted 1:100. The secondary antibody of the kit was replaced with polyclonal rabbit anti-human-IgA-HRP (DakoCytomation) diluted 1:2000. A serum with high level of IgA ACPA was used to create a standard curve and cut-off levels for positive serum tests of IgA ACPA was set to 25 arbitrary units (AU)/mL.

Total IgA in saliva and ileal wash samples

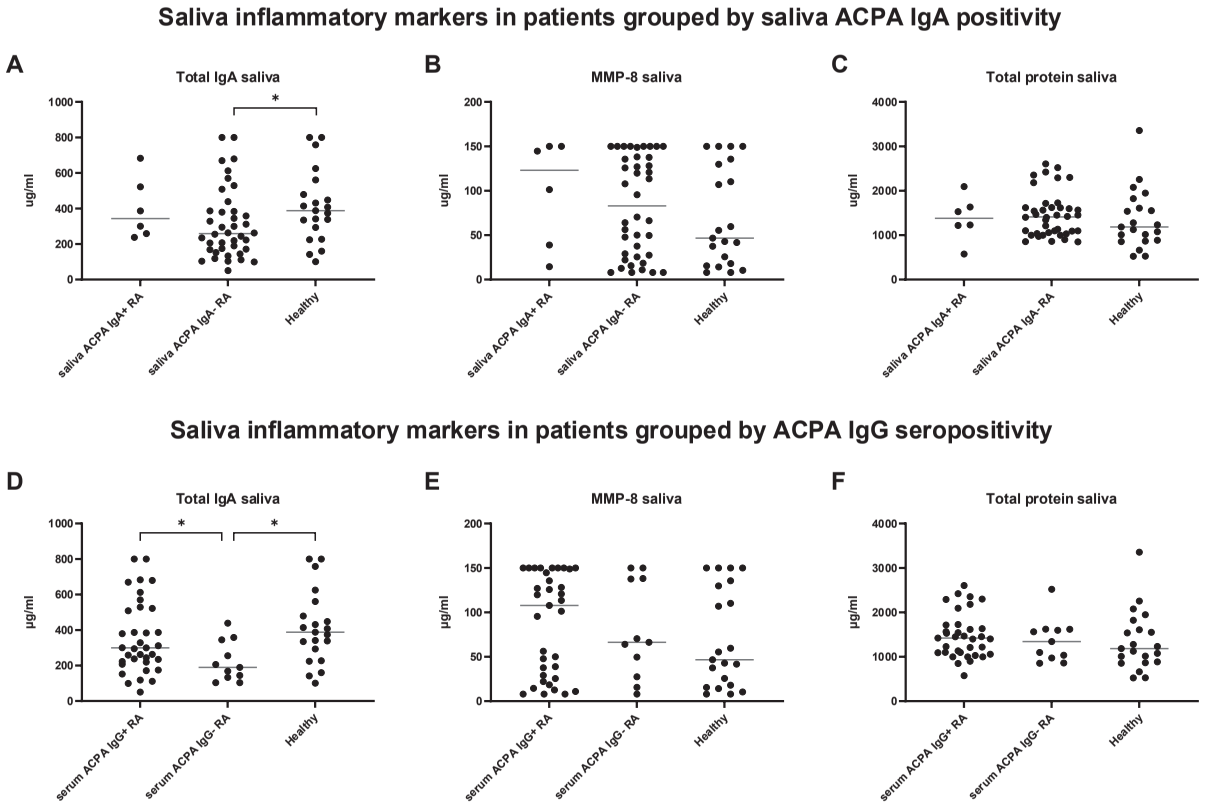
For total IgA level measurements in saliva and ileal wash, the IgA Saliva ELISA (IBL International, DM 59171) was performed according to the manufacturer’s instructions. Saliva was analysed in dilution 1:1000 and Ileal wash in dilutions 1:100 and 1:500.

Anti-E. coli IgA in ileal wash samples

Anti-E. coli IgA in ileal washes samples was measured using the same in-house ELISA as used for the faeces samples with minor modification. ELISA plates were coated with 10µg/ml E. coli lysate overnight at 4°C. After washing and blocking with PBT for 1 hour at RT, ileal washes, diluted 1:5, a positive control and a pooled serum standard were added and incubated overnight at 4°C. The plate was washed and goat anti-human-IgA-HRP (Invitrogen), diluted 1:1000 in PBTT, was added. Following incubation for 1 hour at 37°C the plate was washed and the substrate, TMB, added. The plate was read at 450nm (TECAN).



Supplementary Figure 1: AMPA positivity in saliva related to total IgA levels (µg/ml) in these saliva samples, collected in the MUCOSA study. Patients were divided into groups based on both saliva AMPA positivity and ACPA-seropositivity. The first column shows seropositive RA patients positive for any AMPA in saliva. Colour coding is used to indicate for which AMPA they were positive in saliva; Orange: saliva ACPA single-positive, anti-CarP negative and AAPA negative patients, green: saliva ACPA negative, anti-CarP and AAPA double-positive patient, purple: saliva ACPA, anti-CarP and AAPA triple-positive patients. Error bar shows the median.

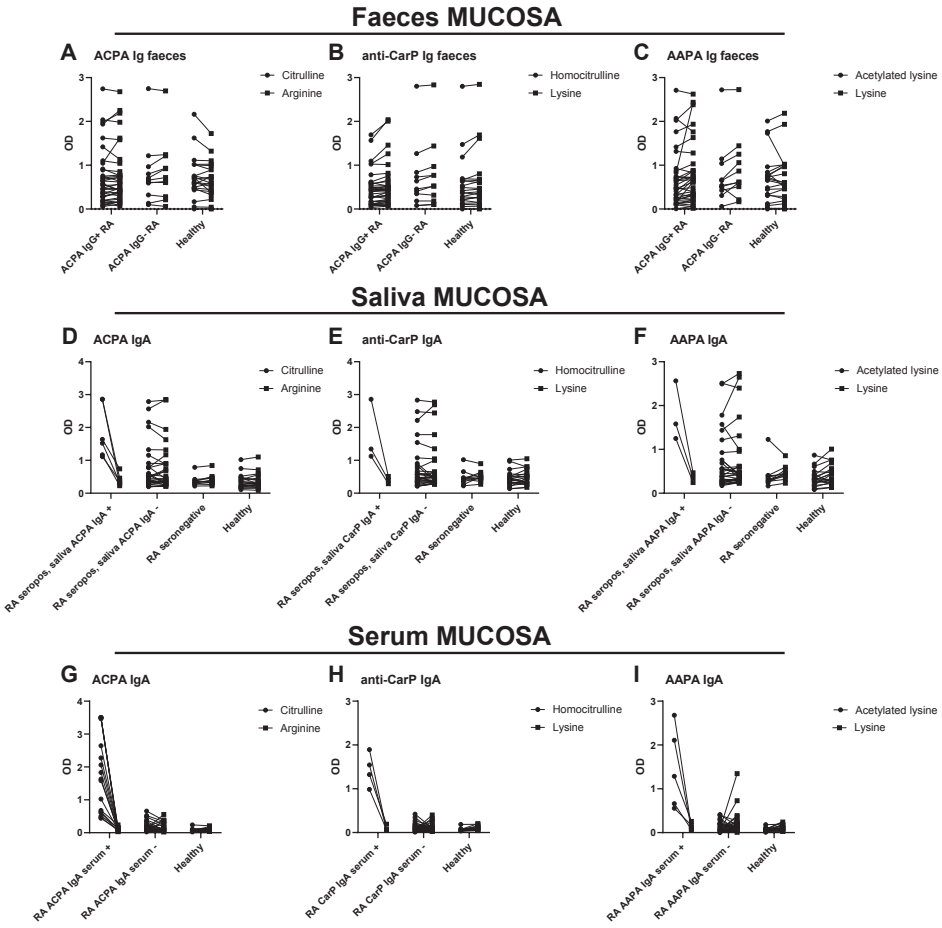


Supplementary figure 2: Inflammatory markers in saliva of patients in the MUCOSA study. A-C Saliva-ACPA IgA positive RA patients, saliva-ACPA IgA negative RA patients and healthy donors are compared. D-F Similar analyses were performed for ACPA seropositive RA patients, seronegative RA patients and healthy donors. The inflammatory markers measured in saliva are total protein levels, metalloproteinase-8 levels (MMP-8) and the amount of total IgA present in saliva. Mann-Whitney U tests were used to compare levels between groups. Bars depict the median, *p<0.05.

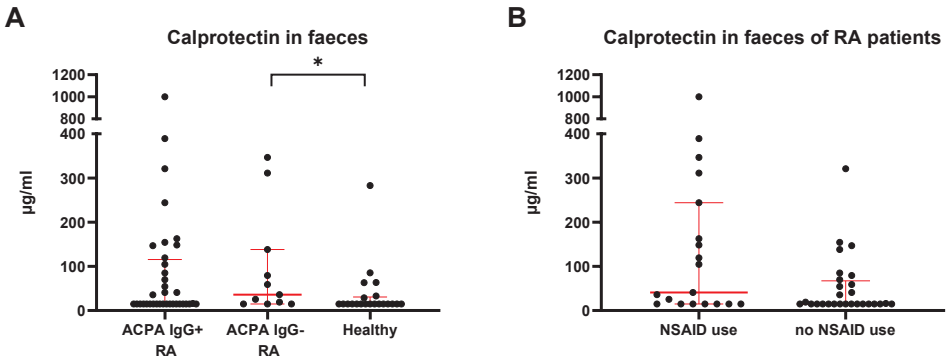
Supplementary table 1: The association between serum and saliva autoantibody positivity and smoking in RA patients in the MUCOSA study.

MUCOSA study	Never Smoked n=21	Ever Smoked n=26	p-value
ACPA IgG serum positive, n (%)	14 (67)	22 (85)	0.15
ACPA IgA serum positive, n (%)	8 (38)	10 (39)	0.98
RF IgM serum positive, n (%)	12 (57)	22 (85)	**0.04**
RF IgA serum positive, n (%)	5 (24)	16 (62)	**0.01**
ACPA IgA saliva positive, n (%)	4 (19)	2 (8) (n=25)	0.39*
RF IgA saliva positive, n (%)	10 (48)	8 (32) (n=25)	0.28

Significance was tested with chi-square test or 2-sided Fisher's exact test (indicated with *). Smoking was defined as ever- vs never-smokers.



Supplementary figure 3: Paired signals on the modified and unmodified peptide for each AMPA in faeces, saliva and serum collected in the MUCOSA study. A-C Faeces: The first group in each figure shows ACPA IgG seropositive RA patients, the middle group shows ACPA IgG seronegative RA patients. D-F Saliva: The first group in each figure shows ACPA IgG seropositive RA patient positive for that AMPA IgA in saliva, the second group shows ACPA IgG seropositive RA patient who are negative for that AMPA IgA in saliva, the third group shows ACPA IgG seronegative RA patients. G-H The first group shows RA patients positive for that specific AMPA IgA in serum, the second shows RA patients negative for that specific AMPA IgA in serum. The last column of all figures includes healthy donors.



Supplementary figure 4: Calprotectin levels in faeces samples collected in the MUCOSA study. A Seropositive RA patients, seronegative RA patients and healthy donors and B RA patients with and without NSAID use are compared. Calprotectin levels are measured by ELISA. Values above 200 µg/g are considered to be significantly elevated. NSAID used was based on the medication list in the electronic medical file and/or self-reported use as indicated in the patient survey. All NSAIDs (no information on dosage or frequency available) except for low dose acetylsalicylic acid as platelet aggregation inhibitor, was included in the NSAID group. Mann-Whitney U tests were used to compare levels between groups. Red bars depict median with interquartile range. *p<0.05

The onset of rheumatoid arthritis after COVID-19 – coincidence or connected?

Veerle F.A.M. Derksen[1], Theresa Kissel[1], Femke B.G. Lamers – Karnebeek[2], Arie E. van der Bijl[3], Annemarie C. Venhuizen[4], Tom W.J. Huizinga[1], René E.M. Toes[1], Anna H.E. Roukens[5], Diane van der Woude[1]

[1] Department of Rheumatology, Leiden University Medical Center, Leiden, the Netherlands
[2] Department of Rheumatology, Hospital Bernhoven, Uden, the Netherlands
[3] Department of Rheumatology, Isala Hospitals, Zwolle, the Netherlands
[4] Department of Rheumatology, Hospital Rivierenland, Tiel, the Netherlands
[5] Department of Infectious Diseases, Leiden University Medical Center, Leiden, the Netherlands

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Letter

COVID-19, caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), can lead to severe inflammation and has been suggested to induce autoimmune phenomena. Multiple studies have reported autoantibodies in patients with COVID-19, particularly anti-cardiolipin, anti-β2 glycoprotein I and anti-nuclear antibodies (1, 2). Anti-citrullinated protein antibodies (ACPA) and flaring of rheumatoid arthritis (RA) after SARS-Cov-2 infection have also been described (1, 3). However, it is unclear how often ACPA occur after COVID-19 and whether they differ from ACPA normally found in RA patients.

We have therefore performed a detailed investigation into ACPA-positivity after COVID-19. To determine the seroprevalence of ACPA after COVID-19, ACPA was measured using routine tests or in-house enzyme-linked immunosorbent assay (ELISA) in 61 patients visiting the post-COVID outpatient clinic of the LUMC 5 weeks after hospitalization. None of the patients tested positive for ACPA, except two patients previously diagnosed with ACPA-positive RA. Thus, we could not observe an increase in ACPA-positivity after COVID-19.

Furthermore, we identified five patients across various Dutch rheumatology clinics presenting with polyarthritis compatible with RA after SARS-CoV-2 infection. To study the impact of COVID-19 on disease presentation, we closely examined their clinical phenotype and autoantibody characteristics (Supplementary table S1). All had suffered from moderate to severe COVID-19. On average, joint complaints started 6.6 weeks after infection, although two patients reported symptoms before infection. 4/5 patients fulfilled the ACR 2010 criteria for RA. Three patients were phenotypically very similar to regular new-onset RA patients. Patient 3 had a history of seronegative RA and had been in DMARD-free remission for 5 years. She flared 6 weeks after SARS-CoV-2 infection. Patient 2 had a remarkably different presentation. He was admitted with acute polyarthritis and high inflammatory markers 6 weeks after COVID-19. Pneumonia with reactive polyarthritis or septic polyarthritis were considered and treatment was started accordingly. ACPA-level was low positive. The patient died unexpectedly after two days and autopsy revealed dilating myocarditis of unclear underlying cause. No causative pathogen, nor compelling evidence of autoimmunity, could be identified.

Previous studies have shown that RA-patients are most often either seronegative or triple-positive for rheumatoid factor, ACPA and anti-carbamylated protein antibodies. ACPA IgM and IgA are most frequently found within patients positive for ACPA IgG (4). Autoantibody measurements on sera of the post-COVID polyarthritis patients using

in-house ELISA's (4), revealed patterns very similar to RA (figure 1A) with two patients being completely seronegative, and three patients positive for a range of autoantibodies at presentation. Sera prior to presentation to the rheumatologist are not available.

A unique feature of ACPA IgG in RA patients is that they harbour glycans not only in their Fc-part, but also in their variable domains (V-domains) (5). We analysed the ACPA IgG V-domain glycosylation profiles of the above-mentioned 3 ACPA-positive post-COVID patients and established RA patients (Supplementary table S1) using UHPLC (5). In all post-COVID samples, the percentage of ACPA V-domain glycosylation was increased compared to total IgG (figure 1B), similar to regular RA. Inflammatory conditions, among which COVID-19, can induces changes in the composition of antibody Fc-glycans (6). A detailed examination of the specific ACPA IgG V-domain glycan traits revealed a significant decrease in bisecting N-Acetylglucosamine containing moieties (G2FBS1, G2FBS2) after COVID-19 (figure 1C), comparable to patterns described for total IgG Fc-glycosylation post-COVID (6). The biological causes and consequences of these glycosylation changes currently remain unclear.

Limitations of this study include the small sample size and limited follow-up duration after COVID-19. Although autoantibody responses can develop rapidly after (SARS-Cov-2) infections, replication in a larger cohort with a longer follow-up would be valuable. Furthermore, part of the samples were measured on in-house instead of commercial tests. However, the characteristics of these assays appear very comparable based on previous experience.

In conclusion, we found that the seroprevalence of ACPA is not increased after COVID-infection and that patients presenting with polyarthritis post-COVID resemble regular RA patients, both regarding clinical phenotype and autoantibody characteristics. Based on these data, it appears that RA post-COVID may be coincidence rather than connected.

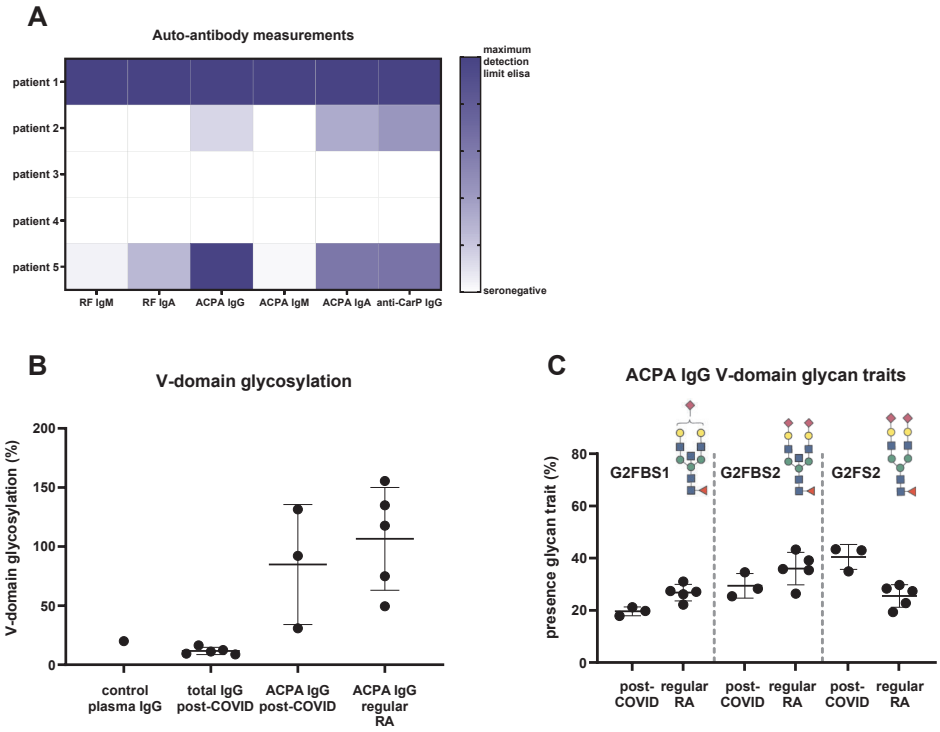

Figure 1: Autoantibody positivity and (auto)antibody glycosylation. A Auto-antibody measurements using in-house ELISA's: Rheumatoid factor (RF), anti-citrullinated protein antibody (ACPA) and anti-carbamylated protein antibody (anti-CarP) isotype levels per patient. White – seronegative, Gradient light to dark blue – low to highest levels, normalized against maximum detection limit ELISA per antibody isotype. B Percentage of variable domain glycosylation (mean, SD). Average value of duplicates plotted. V- domain glycosylation in ACPA IgG post-COVID is significantly increased compared to total IgG ($p<0.05$;Mann-Whitney U test), no significant difference between ACPA IgG V-domain glycosylation post-COVID and in regular RA (disease characteristics in supplementary table S1). C Percentage of specific glycan traits of all ACPA IgG V-domain glycans (mean, SD). Average value of duplicates plotted. Glycan trait G2FS2 without bisecting N-Acetylglucosamine is significantly increased, while G2FBS1, a glycan traits with bisecting N-Acetylglucosamine is significantly decreased post-COVID-19 ($p<0.05$; Mann-Whitney U test). Blue square – N-Acetylglucosamine (B when bisecting), green circle – mannose, red triangle – Fucose (F), yellow circle – galactose (G), purple diamond – sialic acid (S).

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Supplementary information

Supplementary table S1: Clinical characteristics of new-onset RA patients after SARS-CoV-2 infection.

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Regular RA (n=5)
Age, years	67	49	70	67	65	58 (36-67) mean (range)
Gender	Male	Male	Female	Female	Male	2/5 male
Smoking	Never	Never	Former	Never	Current	1 current (n=4)
Hospitalisation due to COVID-19	Yes	Yes (not in acute phase)	Yes	Yes	No	-
ICU admission due to COVID-19	Yes	No	No	No	No	-
Joint symptoms before COVID-19	Yes	No	Yes	No	No	-
Time between positive COVID-19 -test and onset joint symptoms	Already present	6 weeks	6 weeks	14 weeks	3 days	-
ESR (mm/hour)	36#	79#	52#	49#	26#	49 (35-79) mean (range)
CRP (mg/l)	6	449#	44#	168#	6	-
Fulfilment ACR 2010 criteria	Yes	No	Yes	Yes	Yes	Yes (All)
Swollen joint count 28	7	4	10	13	10	15 (2-22) mean (range)
Tender joint count 28	7	4	1	3	8	6 (0-16) mean (range)
Joints affected (small/large/both)	Both	Both	Both	Small	Both	-

Supplementary table S1: Continued

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Regular RA (n=5)
Location affected joints	Both extremities	Both extremities	Both extremities	Both extremities	Upper extremity	-
Symmetric onset	Yes	Yes	Yes	Yes	Unknown	-
DAS28	Unknown	Unknown	Unknown	5.76	4.69	5.12 (2.98-6.49) mean (range)
Additional information	-	acute poly-arthritis 6 weeks after COVID-19, died unexpectedly 2 days later*	Previous diagnosis RA, 5 years DMARD-free remission	History of sarcoidosis	-	Disease duration 9.2 (3-26) years mean (range)

Clinical characteristics of the five patients presenting with polyarthritis compatible with RA after SARS-CoV-2 infection, as compared to patients with established, regular RA whose serum was used for the glycosylation experiments. #elevated ESR/CRP according to local reference values

*Full case description: A 49-year-old male with morbid obesity was admitted with polyarthritis and very high inflammatory markers. He also had petechiae on the lower legs and proteinuria. 6 weeks before he experienced flu-like symptoms, after which he remained extremely fatigued and slightly short of breath. Based on serology these symptoms were most likely caused by COVID-19. The chest X-ray at the time of admission (6 weeks after COVID19-symptoms) showed a consolidation, which could be compatible with pneumonia on the right basal side. The working diagnosis was reactive polyarthritis with an (atypical) pneumonia or a septic polyarthritis with bacteraemia and the patient was treated empirically with ceftriaxone iv. He died unexpectedly after 2 days. Obduction showed a dilating myocarditis as cause of death, but the underlying cause of the myocarditis remains unclear. The nature of the myocardial infiltrate was not typical for viral myocarditis due to the diffuse localization, CD4+ T-cell dominance and many neutrophils. These histopathological findings, especially the presence of neutrophils, are also not compatible with an idiopathic inflammatory myopathy such as polymyositis. The myocardial infiltrate could be caused by focal ischemia under the influence of catecholamines secondary to sepsis. However, no causative pathogen or focus of infection could be identified. The blood and synovial fluid cultures, as well as the SarS-Cov-2 PCR on pulmonary and myocardial tissue were negative. Furthermore, the lung tissue showed oedema, but no signs of viral or bacterial infiltrate. No convincing evidence for an autoimmune disease could be found, but this can also not be completely excluded. Thus the underlying cause of death remains unclear.

Part 3

The AMPA profile in relation to clinical phenotype and disease outcomes in RA patients

Rheumatoid arthritis phenotype at presentation differs depending on the number of autoantibodies present

Veerle F.A.M. Derksen[1], Sofia Ajeganova[1,2], Leendert A. Trouw[1], Annette H.M. van der Helm-van Mil[1], Ingiäld Hafström[2], Tom W.J. Huizinga[1], René E.M. Toes[1], Björn Svensson[3], Diane van der Woude[1]

[1] Department of Rheumatology, Leiden University Medical Center, Leiden, The Netherlands
[2] Rheumatology unit, Department of Medicine, Karolinska Institutet at Karolinska University Hospital Huddinge, Stockholm, Sweden
[3] Department of Clinical Sciences, Section of Rheumatology, Lund University, Lund, Sweden

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Abstract

Objectives In rheumatoid arthritis (RA), seropositive and seronegative disease may be two entities with different underlying pathophysiological mechanisms, long-term outcomes and disease presentations. However, the effect of the conjoint presence of multiple autoantibodies, as proxy of a more pronounced humoral autoimmune response, on clinical phenotype remains unclear. Therefore, this study investigates the association between the number of autoantibodies and initial clinical presentation in two independent cohorts of early RA patients.

Methods Autoantibody status (rheumatoid factor, anti-citrullinated protein antibodies and anti-carbamylated protein antibodies) was determined at baseline in the Leiden Early Arthritis Cohort (EAC, n=828) and the Swedish BARFOT study (Better Anti-rheumatic Farmaco-therapy, n=802). The association between the number of autoantibodies and baseline clinical characteristics was investigated using univariable and multivariable ordinal regression.

Results In both cohorts the following independent associations were found in multivariable analysis: patients with a higher number of RA-associated antibodies were younger, more often smokers, had a longer symptom duration and a higher erythrocyte sedimentation rate at presentation compared to patients with few autoantibodies.

Conclusions The number of autoantibodies, reflecting the breadth of the humoral autoimmune response, is associated with clinical presentation of RA. Pre-disease pathophysiology is thus reflected by the initial clinical phenotype.

Introduction

Approximately 60% of early rheumatoid arthritis (RA) patients are positive for RA-associated autoantibodies like rheumatoid factor (RF), anti-citrullinated protein antibodies (ACPA) and anti-carbamylated protein antibodies (anti-CarP). Anti-CarP is a novel autoantibody system in RA and is associated with the development of RA in arthralgia patients and radiographic damage (1, 2). Autoantibodies may develop years before disease onset and are commonly seen as markers for underlying autoimmune pathophysiology. Thus, clinical phenotype at presentation may be a reflection of pathophysiological mechanisms taking place before disease onset.

Seropositive and seronegative RA differ in terms of risk factors and disease course. Seropositivity, especially ACPA-positivity is associated with risk factors like smoking and HLA shared epitope (SE) alleles (3). Seropositive patients also have a worse disease outcome with more radiological damage over time (4). In contrast, seronegative patients have recently been described to have more joint inflammation at initial presentation (5). Even though novel autoantibodies may be discovered in the future, seropositive and seronegative RA as defined at the moment are likely to differ in underlying pathophysiology and phenotype (5, 6).

However, within the seropositive subset a varying number of autoantibodies can be found, with the presence of several autoantibodies indicating a break of tolerance to more autoantigens. Yet the specific clinical implications of a broader humoral autoimmune response remain unclear, although this could be very important since broadening of the autoantibody response could be amenable to therapeutic intervention. Therefore this study investigates the association between the number of autoantibodies, as proxy of a broader humoral autoimmune response, and initial clinical presentation in RA patients.

Patients and Methods

Patients

Two independent early RA cohorts from the Netherlands and Sweden were analysed: the Leiden Early Arthritis Clinic (EAC) and the Better Anti-Rheumatic Farmaco Therapy project (BARFOT) (details described elsewhere) (7, 8). All patients had a short symptom duration (<24 months EAC, <12 months BARFOT), were DMARD-naive and had known status for all three autoantibodies (RF, ACPA, anti-CarP). All patients fulfilled the 1987 ACR criteria for RA (in EAC within 1 year of follow-up and in BARFOT at inclusion).

Informed consent was obtained and the studies were approved by the local medical ethics committees. At baseline, information about demographics, smoking, family history and disease characteristics was recorded. The location of initial symptoms was documented only in EAC.

Autoantibody measurements

All antibody measurements were performed in baseline sera. IgM-RF was measured by commercial enzyme-linked immunosorbent assay (ELISA) in EAC and agglutination test (SERODIA-RA) in BARFOT. In both cohorts, ACPA were determined by anti-CCP2 ELISA (Euro-Diagnostica) with manufacturer's cut-offs. Antibodies against carbamylated fetal calf serum (anti-CarP-FCS) were measured using in-house ELISAs in Leiden for both cohorts as described previously (2). The cut-off was set at the mean plus two times the standard deviation of the specific anti-CarP antibody reactivity of healthy controls matched for country of origin (Netherlands and Sweden). The presence of ACPA isotypes, ACPA fine specificities and SE alleles was determined in EAC for a subset of patients as described previously (9, 10).

Statistical analysis

Multiple imputations were used in both cohorts to deal with random missing data. Markov Chain Monte Carlo imputations (m=20) using linear and logistic regression for continuous and categorical variables respectively were done in SPSS version 23. Ordinal regression was performed to compare baseline characteristics between patients harbouring 0-3 autoantibodies and results of each imputed dataset were pooled to yield odds ratios (ORs) and 95% confidence intervals (95% CI), which signify the increase in association with every additional autoantibody. The Holm-Bonferroni method was applied to correct for multiple testing (11). Variables with a univariable p-value <0.10 were included in a multivariable model, excluding highly correlated variables. Associations between SE positivity and baseline characteristics were analysed using Mann-Whitney U tests or Chi-square tests.

Results

In both cohorts, the distribution of the number of autoantibodies was similar. The majority of patients was either seronegative (31% EAC, 33% BARFOT) or triple-positive for RF, anti-CCP2 and anti-CarP (35% EAC, 29% BARFOT). Baseline characteristics differed between the two cohorts at several points (see online supplementary table S1), reflecting differences in inclusion criteria and referral systems.

Several phenotypic characteristics were significantly associated with the number of autoantibodies in both EAC and BARFOT. In univariable and multivariable analysis, the following independent associations were found in both cohorts (Table 1, 2): patients with additional RA-associated antibodies were younger, more often smokers, had longer symptom duration and higher ESR at presentation (Figure 1). Furthermore some associations were found in one cohort but could not be replicated, like BMI, SJC28 and TJC28.

To investigate whether underlying genetic risk factors could partly explain our findings, we analysed the association between shared epitope alleles (presence versus absence) and initial clinical presentation in EAC. SE-positive patients indeed had a significant longer symptom duration and a trend towards being younger, more often smokers and having a higher ESR, but associations were less strong than with the number of autoantibodies.

Thereafter it was investigated whether the increasing prevalence of one autoantibody in particular among patients with an increasing number of autoantibodies, might be responsible for the observed observations. Stratifications for all three autoantibodies were performed, comparing single-positive patients with patients harbouring 1 or 2 additional autoantibodies (data for anti-CCP2 shown in online supplementary tables S2, S3). This resulted in small groups with limited power, but for most variables there was still a trend visible for increasing numbers of autoantibodies. Overall, the effect of the higher number of autoantibodies seemed stronger than the effect of individual autoantibodies.

Besides the number of autoantibodies, other features of a broad autoantibody response are increased levels, ACPA fine specificity and isotype usage. In EAC, there was an association between clinical phenotype and these features (see online supplementary table S4-11), although not as significant and consistent as with the number of autoantibodies. This could be due to inclusion of only ACPA-positive individuals in these sub-analyses.

Table 1: EAC univariable and multivariable ordinal regression analysis for increasing number of autoantibodies present.

n=828	0 (n=261)	1 (n=138)	2 (n=139)	3 (n=290)	Ordinal OR (95% CI)	p-value	Multivariable Ordinal OR (95% CI)	p-value
Age, years, M ± SD	60 ± 16	59 ± 17	53± 16	55± 15	Per 10 years: 0.85 (0.79-0.92)	**<0.001***	0.87 (0.79-0.95)	**0.001**
Female, n (%)	171 (66)	99 (72)	95 (68)	190 (66)	0.98 (0.75-1.27)	0.868		
BMI, kg/m^2, M ± SD	26.5 ± 4.0	26.0 ± 3.8	25.1 ± 3.1	25.3 ± 3.8	0.93 (0.90-0.97)	**<0.001***	0.95 (0.92-0.99)	**0.022**
Smoking (ever), n (%)	115 (45)	70 (52)	71 (53)	167 (59)	1.51 (1.17-1.94)	**0.001***	1.52 (1.17-1.97)	**0.001**
Family history of RA, n (%)	50 (20)	35 (26)	37 (28)	85 (30)	1.47 (1.11-1.96)	**0.008**	1.45 (1.08-1.95)	**0.015**
(Sub-)Acute onset symptoms, n (%)	157 (66)	82 (59)	66 (48)	145 (51)	0.70 (0.54-0.90)	**0.005***	0.80 (0.61-1.05)	0.104
Symptom duration, months, M ± SD	4.6 ± 4.9	4.9 ± 4.9	6.0 ± 5.3	6.1 ± 5.3	Per 3 months: 1.14 (1.05-1.23)	**0.001***	1.09 (1.00-1.18)	**0.044**
Location start of symptoms, n (%)								
· Small joints	150 (60)	75 (54)	78 (56)	166 (58)	1 (ref)			
· Large joints	36 (14)	22 (16)	20 (14)	53 (18)	1.22 (0.85-1.73)	0.279		
· Both	64 (26)	41 (30)	41 (30)	68 (24)	0.96 (0.72-1.29)	0.806		
Symmetric start of symptoms, n (%)	179 (77)	101 (79)	93 (72)	177 (68)	0.70 (0.52-0.94)	**0.017**		
Start symptoms in, n (%)								
· Upper extremity	120 (54)	71 (60)	46 (37)	92 (36)	1 (ref)			
· Lower extremity	24 (11)	14 (12)	17 (14)	46 (18)	2.18 (1.46-3.24)‡	**<0.001**		
· Both	78 (35)	34 (29)	61 (49)	115 (46)	1.71 (1.29-2.27)‡	**<0.001***		

Table 1: Continued

n=828	0 (n=261)	1 (n=138)	2 (n=139)	3 (n=290)	Ordinal OR (95% CI)	p-value	Multivariable Ordinal OR (95% CI)	p-value
ESR, mm/hour, M ± SD	36 ± 25	37 ± 26	38 ± 27	42 ± 29	Per 10 mm/hr: 1.06 (1.02-1.11)	**0.009**	1.14 (1.08-1.21)	**<0.001**
CRP, mg/liter, M ± SD	32 ± 36	27 ± 31	30 ± 42	30 ± 33	1.00 (1.00-1.00)	0.743		
SJC in 28 joints, M ± SD	9 ± 6	8 ± 6	7 ± 5	7 ± 5	0.95 (0.93-0.97)	**<0.001***	0.95 (0.93-0.97)	**<0.001**
TJC in 28 joints, M ± SD	11 ± 8	9 ± 6	8 ± 7	9 ± 6	0.97 (0.95-0.99)	**0.002***		
HAQ score, M ± SD	1.2 ± 0.7	1.2 ± 0.7	1.0 ± 0.7	1.0 ± 0.7	0.81 (0.67-0.97)	**0.023**	0.85 (0.69-1.05)	0.126
SHS score, M ± SD	8 ± 9	9 ± 10	9 ± 14	9 ± 11	1.01 (0.99-1.02)	0.435		
VAS general health, M ± SD	41 ± 26	44 ± 24	41 ± 25	41 ± 25	1.00 (0.99-1.01)	0.760		
DAS28-ESR, M ± SD	5.2 ± 1.4	5.1 ± 1.1	4.8 ± 1.4	5.1 ± 1.1	0.93 (0.84-1.03)	0.143		

ORs of imputed datasets are pooled. *Significant after correction for multiple testing (only performed in univariable analysis). ǂTest of parallel lines significant. Joint symptoms refer to any signs or symptoms of synovitis (e.g. pain, swelling, tenderness). Onset of symptoms was (sub-)acute when symptoms started within one week. Patient data were partly missing for a number of baseline variables, in particular for BMI, TJC28, HAQ, VAS general health and DAS28-ESR. Multivariable analysis was performed after exclusion of variables not available in BARFOT and of TJC28 due to high correlation.

Table 2: BARFOT univariable and multivariable ordinal regression analysis for increasing number of autoantibodies present.

n=802	0 (n=265)	1 (n=111)	2 (n=195)	3 (n=231)	Ordinal OR (95% CI)	p-value	Multivariable Ordinal OR (95% CI)ǂ	p-value
Age, years, M ± SD	61 ± 17	56 ± 17	55 ± 15	58 ± 14	Per 10 years: 0.92 (0.85-0.99)ǂ	**0.034**	0.85 (0.78-0.93)	**<0.001**
Female, n (%)	169 (64)	78 (70)	136 (70)	143 (62)	0.95 (0.73-1.24)	0.707		
BMI, kg/m^2, M ± SD	25.7 ± 4.2	25.1 ± 4.2	25.0 ± 4.4	25.5 ± 4.6	0.99 (0.96-1.03)	0.629		
Smoking (ever), n (%)	137 (52)	58 (53)	121 (63)	153 (67)	1.59 (1.23-2.06)	**<0.001***	1.62 (1.25-2.10)	**<0.001**
Family history of RA, n (%)	87 (34)	34 (31)	71 (37)	78 (35)	1.07 (0.82-1.40)	0.628		
(Sub-)Acute onset symptoms, n (%)	141 (55)	51 (47)	93 (49)	119 (52)	0.91 (0.71-1.17)	0.452		
Symptom duration, months, M ± SD	5.6 ± 3.1	5.9 ± 3.1	6.4 ± 3.0	6.3 ± 3.0	Per 3 months: 1.19 (1.05-1.34)	**0.006**	1.22 (1.07-1.39)	**0.002**
ESR, mm/hour, M ± SD	31 ± 25	31 ± 23	37 ± 24	46 ± 27	Per 10 mm/hr: 1.19 (1.13-1.25)	**<0.001***	1.24 (1.17-1.30)	**<0.001**
CRP, mg/liter, M ± SD	29 ± 34	26 ± 31	31 ± 30	44 ± 44	1.01 (1.01-1.01)ǂ	**<0.001***		
SJC in 28 joints, M ± SD	11 ± 6	10 ± 6	10 ± 5	11 ± 5	1.00 (0.98-1.03)	0.786		
TJC in 28 joints, M ± SD	9 ± 7	8 ± 7	6 ± 6	8 ± 6	0.98 (0.96-1.00)ǂ	**0.024**	0.98 (0.95-1.00)	**0.018**
HAQ score, M ± SD	1.0 ± 0.7	0.9 ± 0.6	1.0 ± 0.6	1.1 ± 0.6	1.05 (0.86-1.28)ǂ	0.638		
SHS score, M ± SD	3 ± 8	3 ± 5	5 ± 8	4 ± 7	1.01 (0.99-1.03)	0.351		
VAS general health, M ± SD	45 ± 25	44 ± 25	45 ± 26	46 ± 25	1.00 (1.00-1.01)	0.758		
DAS28-ESR, M ± SD	5.1 ± 1.3	5.0 ± 1.4	5.1 ± 1.2	5.5 ± 1.1	1.19 (1.07-1.32)ǂ	**0.001***		

ORs of imputed datasets are pooled. *Significant after correction for multiple testing (only performed in univariable analysis) ǂTest of parallel lines significant. Joint symptoms refer to any signs or symptoms of synovitis (e.g. pain, swelling, tenderness). Onset of symptoms was (sub-)acute when symptoms started within one week. Patient data were partly missing for a number of baseline variables, in particular for BMI. Multivariable analysis was performed after exclusion of DAS28-ESR and CRP due to high correlation.

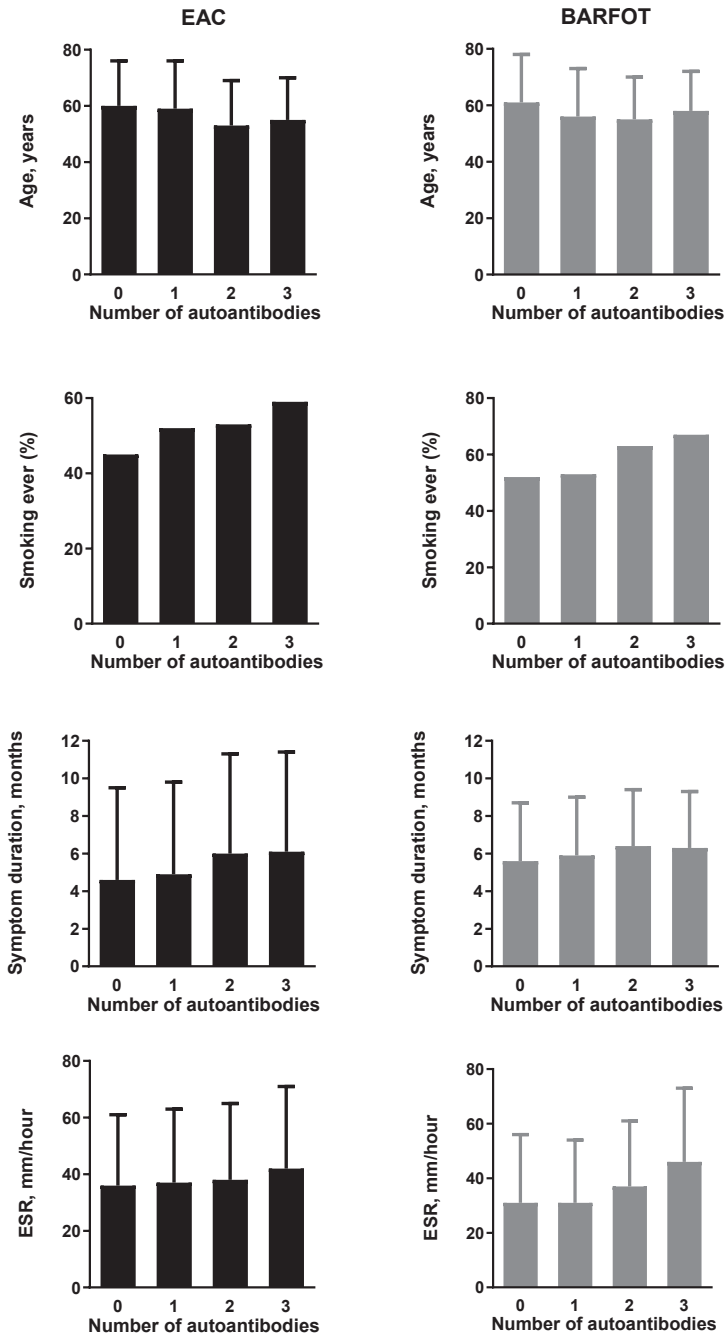

Figure 1: Number of autoantibodies and clinical characteristics. Means and standard deviations or percentages of clinical characteristics, which were significant in multivariable analysis in both cohorts, for increasing number of autoantibodies.

Discussion

This study describes for the first time the association between initial clinical presentation and the number of autoantibodies. We found that younger age, smoking, longer symptom duration and higher ESR were independently associated with having additional autoantibodies. We replicated these results in a second independent cohort.

Patients with a higher number of autoantibodies also have higher autoantibody levels, more fine specificities and broadened isotype usage. These features also appeared to be associated with clinical phenotype. Since all reflect the breadth of underlying autoimmune pathophysiology, this suggests an association between initial clinical presentation and the extent of humoral autoimmunity.

The underlying genetic predisposition associated with autoantibody positivity could possibly explain part of the observations, e.g. why patients with multiple autoantibodies develop RA at a younger age (12). This idea is supported by the association between clinical presentation and HLA SE alleles, representing part of the genetic predisposition. The association between the presence of additional autoantibodies and longer symptom duration is in line with a previous publication (13). It was recently reported that seronegative individuals have higher joint counts at presentation (5). We found in addition that increasing numbers of autoantibodies are associated with lower tender joint counts (and swollen joint counts in EAC). This may possibly reflect the higher number of joints required for seronegative individuals to fulfil classification criteria as well as intrinsic pathophysiologic differences. Overall, the findings suggest that the genetic predisposition underlying autoantibody-positive RA may result in an early, smouldering disease onset which is difficult to recognize promptly by patients and their general practitioners.

Our study has several limitations. Some variables had a large amount of missing data. However, since these data were generally missing at random and multiple imputations yielded very similar results, it seems unlikely that missing data biased our conclusions. Although this study focuses on the simultaneous presence of autoantibodies, stratifications for the different autoantibodies were performed to determine whether the observed effects are due to the presence of a single autoantibody. This analysis lacks power to draw solid conclusions, but there were still trends visible. Although we cannot exclude certain effects of specific autoantibodies, overall the effect of a higher number of autoantibodies seemed stronger than the effect of individual autoantibodies. Another limitation is the dissimilarity in baseline characteristics between both cohorts, which might be due to difference in inclusion criteria or referral systems between the Netherlands and Sweden (7, 8).

Nevertheless, the use of two large, independent cohorts enabled us to distinguish between patients with 1 versus 2 versus 3 autoantibodies and thereby discover new dose-dependent associations consistent across different populations. To further enhance our understanding of the link between humoral autoimmunity and clinical phenotype, more studies are warranted investigating disease evolution, possibly by studying immunological processes in arthralgia patients progressing towards RA. Our study also has implications for the early recognition and treatment of RA. The fact that patients with additional autoantibodies had a longer symptom duration, while they are particularly predisposed to a more severe disease course, might indicate the need for better targeted early recognition strategies to reach these patients.

In conclusion, the number of autoantibodies present is associated with clinical phenotype at presentation, indicating that the breadth of the humoral autoimmune response affects the initial clinical presentation of RA.

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Supplementary information

Supplementary table S1: Baseline characteristics of EAC and BARFOT.

	EAC (n=828)	BARFOT (n=802)	p-value
Age, years M ± SD	57 ± 16	58 ± 16	0.384
Female, no. (%)	555 (67)	526 (66)	0.561
BMI, kg/m², M ± SD	25.8 ± 3.9	25.3 ± 4.4	0.080
Smoking (ever), no. (%)	423 (52)	469 (59)	**0.006**
Family history of RA, no. (%)	207 (26)	270 (34)	**<0.001**
(Sub-)Acute onset symptoms, no. (%)	450 (54)	403 (52)	0.096
Symptom duration, months, M ± SD	5.4 ± 5.2	6.0 ± 3.0	**<0.001***
ESR, mm/hour, M ± SD	39 ± 27	37 ± 26	0.125*
CRP, mg/liter, M ± SD	30 ± 35	34 ± 37	**0.002***
SJC in 28 joints, M ± SD	7 ± 6	10 ± 6	**<0.001***
TJC in 28 joints, M ± SD	9 ± 7	8 ± 6	**<0.001***
HAQ score, M ± SD	1.1 ± 0.7	1.0 ± 0.7	0.241
SHS score, M ± SD	9 ± 11	4 ± 7	**<0.001***
VAS general health, M ± SD	42 ± 25	45 ± 25	**0.038***
DAS28-ESR, M ± SD	5.1 ± 1.3	5.2 ± 1.3	0.144

The cohorts are compared using T-tests or Mann-Whitney U tests (the latter indicated by *) for continuous data and Chi-square tests for categorical data. Symptoms refer to any signs of synovitis.

Supplementary table S2: In EAC stratification for anti-CCP2.

n=433	Anti-CCP2 only (n=20)	Anti-CCP2 +1 (n=123)	Anti-CCP2 +2 (n=290)	Ordinal OR (95% CI)	p-value
Age, years M ± SD	53 ± 15	53 ± 16	55 ± 15	1.09 (0.96-1.25)[#]	0.188
Female, no. (%)	17 (85)	87 (71)	190 (66)	0.69 (0.45-1.07)	0.098
BMI, kg/m², M ± SD	25.5 ± 3.3	25.1 ± 3.2	25.3 ± 3.8	1.00 (0.94-1.06)	0.996
Smoking (ever), no. (%)	8 (42)	62 (52)	167 (59)	1.44 (0.96-2.17)	0.077
Family history of RA, no. (%)	5 (25)	34 (28)	85 (30)	1.12 (0.72-1.75)	0.608
(Sub-)Acute onset symptoms, no. (%)	13 (65)	55 (46)	145 (51)	1.08 (0.73-1.62)	0.691
Symptom duration, months, M ± SD	4.6 ± 3.8	6.2 ± 5.4	6.1 ± 5.3	1.01 (0.90-1.13)[#]	0.919
Location start of symptoms, no. (%)					
Small joints	9 (45)	73 (59)	166 (58)	reference	
Large joints	5 (25)	17 (14)	53 (19)	1.14 (0.66-1.99)	0.641
Both	6 (30)	33 (27)	68 (24)	0.84 (0.53-1.34)	0.469
Symmetric start of symptoms, no. (%)	14 (74)	82 (72)	177 (68)	0.84 (0.53-1.33)	0.450
Start of symptoms in, no. (%)					
Upper extremity	9 (47)	42 (38)	92 (36)	reference	
Lower extremity	4 (21)	16 (15)	46 (18)	1.25 (0.66-2.35)	0.496
Both	6 (32)	52 (47)	115 (46)	1.13 (0.71-1.78)	0.610
ESR, mm/hour, M ± SD	30 ± 27	39 ± 28	42 ± 29	1.07 (0.99-1.15)[#]	0.085
CRP, mg/liter, M ± SD	20 ± 27	29 ± 41	30 ± 33	1.00 (1.00-1.01)	0.616
SJC in 28 joints, M ± SD	5 ± 3	7 ± 6	7 ± 5	1.01 (0.97-1.05)	0.537
TJC in 28 joints, M ± SD	5 ± 4	8 ± 6	9 ± 6	1.01 (0.98-1.05)	0.538
HAQ score, M ± SD	0.9 ± 0.6	1.0 ± 0.7	1.0 ± 0.7	1.06 (0.77-1.45)	0.738
SHS score, M ± SD	7 ± 6	9 ± 14	9 ± 11	1.00 (0.98-1.02)	0.699
VAS general health, M ± SD	54 ± 14	40 ± 25	41 ± 25	1.00 (0.99-1.01)	0.524
DAS28-ESR, M ± SD	4.4 ± 1.4	4.7 ± 1.4	4.7 ± 1.4	1.13 (0.95-1.34)	0.160

Single positive patients are compared to patients harbouring additional autoantibodies using ordinal regression analysis. ORs of imputed datasets are pooled. [#]OR for age is per 10 years, OR for symptom duration is per 3 months, OR for ESR is per 10 mm/hr.

Supplementary table S3: In BARFOT stratification for anti-CCP2.

n=461	Anti-CCP2 only (n=46)	Anti-CCP2 +1 (n=184)	Anti-CCP2 +2 (n=231)	Ordinal OR (95% CI)	p-value
Age, years, M ± SD	51 ± 15	55 ± 15	58 ± 14	1.23 (1.08-1.39)#	**0.001***
Female, n (%)	38 (83)	130 (71)	143 (62)	0.57 (0.39-0.84)	**0.004***
BMI, kg/m², M ± SD	24.2 ± 3.8	25.0 ± 4.4	25.5 ± 4.6	1.03 (0.98-1.08)	0.270
Smoking (ever), n (%)	21 (47)	113 (62)	153 (67)	1.48 (1.02-2.13)	**0.037**
Family history of RA, n (%)	15 (33)	67 (37)	78 (35)	0.97 (0.67-1.41)	0.887
(Sub-)Acute onset symptoms, n (%)	24 (52)	86 (48)	118 (52)	1.10 (0.77-1.56)	0.606
Symptom duration, months, M ± SD	6.7 ± 3.0	6.5 ± 2.9	6.3 ± 3.0	0.92 (0.77-1.10)#	0.368
ESR, mm/hour, M ± SD	32 ± 25	36 ± 24	46 ± 27	1.18 (1.10-1.27)#	**<0.001***
CRP, mg/liter, M ± SD	26 ± 35	30 ± 30	44 ± 44	1.01 (1.01-1.02)	**<0.001***
SJC in 28 joints, M ± SD	8 ± 5	10 ± 5	11 ± 5	1.05 (1.01-1.09)	**0.018**
TJC in 28 joints, M ± SD	7 ± 6	7 ± 6	8 ± 6	1.02 (0.98-1.05)	0.371
HAQ score, M ± SD	1.0 ± 0.6	1.0 ± 0.6	1.1± 0.6	1.29 (0.96-1.73)	0.094
SHS score, M ± SD	4 ± 6	4 ± 8	4 ± 7	0.99 (0.96-1.02)	0.647
VAS general health, M ± SD	45 ± 23	46 ± 25	46 ± 25	1.00 (0.99-1.01)	0.866
DAS28-ESR, M ± SD	4.8 ± 1.2	5.1 ± 1.1	5.5 ± 1.1	1.34 (1.14-1.58)	**<0.001***

Single positive patients are compared to patients harbouring additional autoantibodies using ordinal regression analysis. ORs of imputed datasets are pooled. #OR for age is per 10 years, OR for symptom duration is per 3 months, OR for ESR is per 10 mm/hr. *Significant after correction for multiple testing by the Holm-Bonferroni method.

Supplementary table S4: Amount of ACPA isotypes according to number of autoantibodies present.

	Number of autoantibodies present (RF, anti-CCP2 and anti-CarP)		
n=231	**1 (n=14) (only ACPA positive), n (%)**	**2 (n=60) n (%)**	**3 (n=157) n (%)**
1 ACPA isotype	7 (50)	24 (40)	33 (21)
2 ACPA isotypes	6 (43)	15 (25)	21 (13)
3 ACPA isotypes	1 (7)	21 (35)	103 (66)

The number of ACPA isotypes (IgG, IgA and IgM) present in patients grouped according to the number of autoantibodies present (RF, anti-CCP2 and anti-CarP) in EAC. ACPA isotype measurements were restricted to anti-CCP2 positive patients.

Supplementary table S5: The number of ACPA isotypes present and clinical characteristics at presentation in EAC.

	Number of ACPA isotypes present (ACPA IgG, IgA and IgM)				
n=231	**1 (n=64)**	**2 (n=42)**	**3 (n=125)**	**Ordinal OR (95% CI)**	**p-value**
Age, years, M ± SD	54 ± 17	53 ± 17	58 ± 13	1.18 (1.00-1.40)[#]	**0.048**
Smoking (ever), n (%)	23 (38)	20 (48)	76 (62)	2. 16 (1.30-3.60)	**0.003**
Symptom duration, months, M ± SD	6.0 ± 5.3	5.2 ± 3.7	6.7 ± 5.3	1.10 (0.94-1.28)[#]	0.259
ESR, mm/hour, M ± SD	42 ± 28	41 ± 23	49 ± 30	1.09 (1.00-1.20)[#]	0.052

Clinical characteristics are compared between patients with an increasing number of ACPA isotypes (IgG, IgA and IgM) using ordinal regression analysis. ACPA isotype measurements were restricted to anti-CCP2 positive patients. ORs of imputed datasets are pooled. [#]OR for age is per 10 years, OR for symptom duration is per 3 months, OR for ESR is per 10 mm/hr.

Supplementary table S6: Amount of ACPA fine specificities according to number of autoantibodies present.

	Number of autoantibodies present		
n=273	**1 (n=15) (only ACPA positive), n (%)**	**2 (n=77) n (%)**	**3 (n=181) n (%)**
0 fine specificities	4 (27)	10 (13)	14 (8)
1 fine specificity	3 (20)	22 (29)	27 (15)
2 fine specificities	4 (27)	18 (23)	33 (18)
3 fine specificities	2 (13)	15 (19)	40 (22)
4 fine specificities	2 (13)	7 (9)	41 (23)
5 fine specificities	0	4 (5)	21 (12)
6 fine specificities	0	1 (1)	5 (3)

The number of ACPA fine specificities present in patients grouped according to the number of autoantibodies present (RF, anti-CCP2 and anti-CarP) in EAC. ACPA fine specificities: vimentin 1-16: STCitSVSSSSYCitCitMFGG, vimentin 59-74: VYATCitSSAVCitLCitSSVP, fibrinogen α 27-43: FLAEGGGVCitGPRVVERH, fibrinogen β 36-52: NEEGFFSACitGHRPLDKK, α-enolase 5-20: KIHACitEIFDSCitGNPTV and myelin basic protein (MBP). ACPA fine specificity measurements were restricted to anti-CCP2 positive patients.

Supplementary table S7: The number of ACPA fine specificities present and clinical characteristics at presentation in EAC.

	Number of ACPA fine specificities present				
n=273	**0 or 1 (n=80)**	**2 or 3 (n=112)**	**4, 5 or 6 (n=81)**	**Ordinal OR (95% CI)**	**p-value**
Age, years, M ± SD	52 ± 16	57 ± 15	54 ± 15	1.06 (0.92-1.22)[#]	0.443
Smoking (ever), n (%)	38 (51)	59 (54)	46 (58)	1.25 (0.80-1.95)	0.329
Symptom duration, months M ± SD	6.5 ± 5.0	5.7 ± 5.1	6.5 ± 5.5	0.99 (0.87-1.13)[#]	0.914
ESR, mm/hour, M ± SD	37 ± 24	42 ± 28	45 ± 28	1.08 (1.00-1.18)[#]	0.056

ACPA fine specificity measurements were restricted to anti-CCP2 positive patients. Clinical characteristics are compared between patients with 0 or 1, 2 or 3 and 4, 5 or 6 ACPA fine specificities using ordinal regression analysis. ORs of imputed datasets are pooled. [#]OR for age is per 10 years, OR for symptom duration is per 3 months, OR for ESR is per 10 mm/hr.

Supplementary table S8: The autoantibody levels for patients grouped according to the number of autoantibodies present (RF, anti-CCP2, anti-CarP) in EAC.

	Number of autoantibodies present		
	1	**2**	**3**
Levels RF, M ± SD	(n=77) 22 ± 28	(n=102) 53 ± 51	(n=275) 62 ± 59
Levels anti-CCP2, M ± SD	(n=17) 490 ± 678	(n=99) 702 ± 867	(n=262) 1329 ± 2223
Levels anti-CarP, M ± SD	(n=39) 345 ± 99	(n=46) 607 ± 402	(n=290) 699 ± 455

All levels are in aU/ml. Only the levels of patients positive for that specific autoantibody are taken into account.

Supplementary table S9: RF levels and clinical characteristics at presentation in EAC.

	RF negative (n=318)	**Low RF positive (n=230)**	**High RF positive (n=224)**	**Ordinal OR (95% CI)**	**p-value**
Age, years M ± SD	59 ± 16	56 ± 16	56 ± 15	0.91 (0.83-0.98)[#]	**0.018**
Smoking (ever), n (%)	138 (44)	118 (52)	134 (63)	1.72 (1.31-2.25)	**<0.001**
Symptom duration, months, M ± SD	4.8 ± 4.9	5.7 ± 4.9	5.9 ± 5.4	1.11 (1.02-1.20)[#]	**0.014**
ESR, mm/hour, M ± SD	36 ± 24	40 ± 27	42 ± 27	1.06 (1.01-1.12)[#]	**0.019**

Clinical characteristics are compared between RF negative patients, patients that are low positive for RF (levels below median) and patients that are high positive for RF (levels above median) using ordinal regression analysis. ORs of imputed datasets are pooled. [#]OR for age is per 10 years, OR for symptom duration is per 3 months, OR for ESR is per 10 mm/hr.

Supplementary table S10: Anti-CCP2 levels and clinical characteristics at presentation in EAC.

	Anti-CCP2 negative (n=348)	Low anti-CCP2 positive (n=189)	High anti-CCP2 positive (n=189)	Ordinal OR (95% CI)	p-value
Age, years M ± SD	60 ± 16	54 ± 16	56 ± 14	0.86 (0.79-0.94)‡#	**0.001**
Smoking (ever), n (%)	161 (47)	94 (52)	107 (59)	1.40 (1.06-1.84)	**0.018**
Symptom duration, months, M ± SD	4.6 ± 4.9	5.5 ± 4.9	6.6 ± 5.4	1.19 (1.10-1.30)#	**<0.001**
ESR, mm/hour, M ± SD	37 ± 25	37 ± 26	44 ± 28	1.07 (1.02-1.13)#	**0.010**

Clinical characteristics are compared between anti-CCP2 negative patients, patients that are low positive for anti-CCP2 (levels below median) and patients that are high positive for anti-CCP2 (levels above median) using ordinal regression analysis. ORs of imputed datasets are pooled. #OR for age is per 10 years, OR for symptom duration is per 3 months, OR for ESR is per 10 mm/hr. ‡Test of parallel lines significant.

Supplementary table S11: Anti-CarP levels and clinical characteristics at presentation in EAC.

	Anti-CarP negative (n=453)	Low anti-CarP positive (n=188)	High anti-CarP positive (n=187)	Ordinal OR (95% CI)	p-value
Age, years M ± SD	58 ± 16	55 ± 16	55 ± 15	0.89 (0.82-0.97)#	**0.006**
Smoking (ever), n (%)	216 (49)	102 (55)	105 (58)	1.34 (1.03-1.75)	**0.029**
Symptom duration, months, M ± SD	4.9 ± 5.0	5.7 ± 5.5	6.2 ± 5.0	1.11 (1.03-1.20)#	**0.010**
ESR, mm/hour, M ± SD	37 ± 26	39 ± 27	43 ± 29	1.06 (1.01-1.11)#	**0.020**

Clinical characteristics are compared between anti-CarP negative patients, patients that are low positive for anti-CarP (levels below median) and patients that are high positive for anti-CarP (levels above median) using ordinal regression analysis. ORs of imputed datasets are pooled. #OR for age is per 10 years, OR for symptom duration is per 3 months, OR for ESR is per 10 mm/hr.

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

Emma C. de Moel[1#], Veerle F.A.M. Derksen[1#], Gerrie Stoeken[1], Leendert A. Trouw[1], Holger Bang[2], Robbert J. Goekoop[3], Irene Speyer[4], Tom W.J. Huizinga[1], Cornelia F. Allaart[1], René E.M. Toes[1], Diane van der Woude[1]

[1] Department of Rheumatology, Leiden University Medical Center, Leiden, the Netherlands
[2] Orgentec Diagnostika GmbH, Mainz, Germany
[3] Department of Internal medicine and Rheumatology, Haga Hospital, the Hague, the Netherlands
[4] Department of Rheumatology, Haaglanden Medical Center, the Hague, the Netherlands

[#] These authors contributed equally

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Abstract

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

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

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

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

Background

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

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

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

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

Methods

Study design

The IMPROVED study is a multicentre, randomized controlled trial that enrolled 610 patients with early (<2 years) untreated RA or undifferentiated arthritis. It was steered at change in disease activity score -remission (DAS44<1,6), and for those achieving remission, at drug-free remission, with treatment adjusted every 4 months according to whether treatment targets had been reached. Initial treatment comprised methotrexate (MTX) and high-dose prednisone, followed by either tapering of medication or randomization to one of two treatment arms: MTX, prednisone, hydroxychloroquine, and sulphasalazine combination (multi-DMARD arm), or MTX and adalimumab combination as described previously (2). According to protocol, patients tapered and discontinued methotrexate at 8 months if they achieved early remission, allowing them to become drug-free and remain so until the DAS increased to >1.6.

Patient selection and outcomes

All 479 patients fulfilling the 2010 ACR/EULAR RA criteria were selected. Of these patients, those seropositive for routine clinical testing for anti-cyclic citrullinated peptide-2 (anti-CCP2 IgG), rheumatoid factor (RF IgM), or our in-house assay for anti-carbamylated protein antibodies (anti-CarP IgG)(10) at baseline were selected (n=395; see Figure 1 for detailed selection algorithm). If patients were fully seronegative at baseline, we measured anti-CCP2 IgG, RF IgM and anti-CarP IgG in serum collected after 1 year of follow-up to include any patients that seroconverted to positive, yielding 399 seropositive patients, of which 356 had baseline, untreated sera available and 209 had 8-month treated sera available for further serological measurements as described below (2).

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

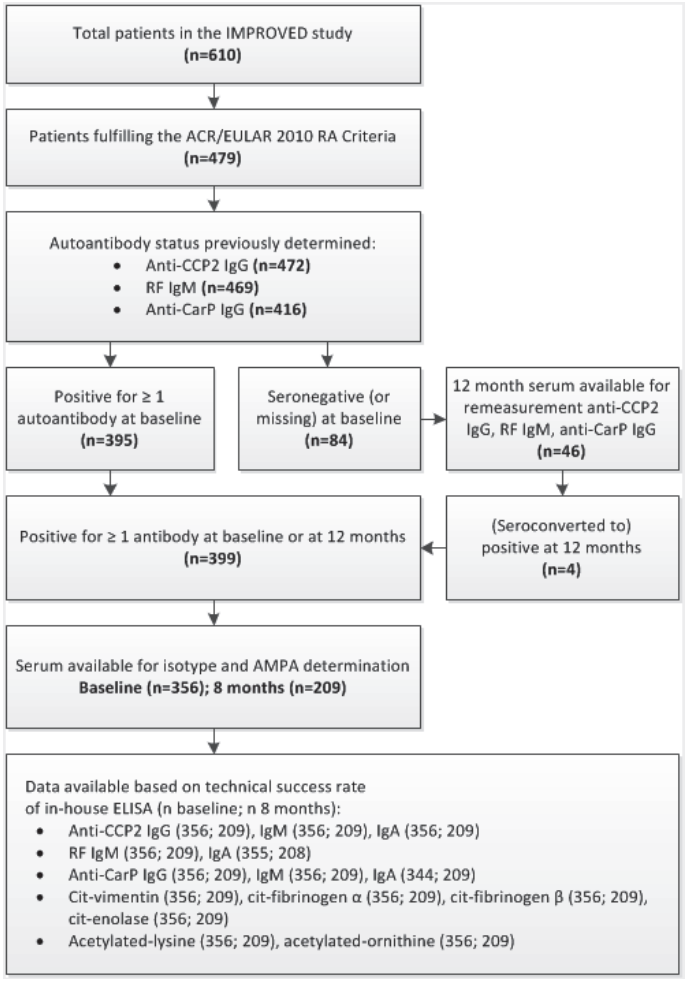


Figure 1: Extended patient selection algorithm.

Serological measurements

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

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

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

Statistical analysis

We constructed categories reflecting the breadth of the antibody response that consisted of the sum of positive antibody tests. First, the number of isotypes present; both in total (anti-CCP2 IgG, IgM, IgA; RF IgM, IgA; anti-CarP IgG, IgM, IgA; range: 1-8) and per family (range: anti-CCP2 and anti-CarP: 1-3; RF: 1-2). Second, the number of IgG anti-modified peptide responses present, both in total (anti-CCP2 IgG, anti-CarP IgG, anti-citrullinated-vimentin 59-74 IgG, anti-citrullinated-fibrinogen β 36-52 IgG and α 27-43 IgG, anti-citrullinated-enolase 5-20 IgG, anti-acetylated-lysine IgG, anti-acetylated-ornithine IgG; range 1-8) and per modification (range: citrullinated peptides: 1-4; acetylated peptides: 1-2). Differences between categories were calculated using analysis of variance (ANOVA) for continuous outcomes (DAS and ΔDAS 0-4 months), adjusted for age, gender, and smoking (ever/never), as well as baseline body mass index and Health Assessment Questionnaire (HAQ) score, which were independent predictors of early remission in the IMPROVED study (5). Binary logistic regression was used for categorical outcomes, adjusted as above, with the analyses of initial DFR additionally adjusted for baseline DAS and the analyses of long-term sustained DFR additionally adjusted for baseline DAS and treatment arm. Holmes-Bonferroni methods were used to correct the alpha level for multiple testing, assuming the same number of hypotheses as pairwise comparisons made. All reported p-values are derived from the analysis models following correction; only p-values that remained significant after correction for multiple testing are reported in the figures.

Results

Antibody positivity at baseline and 8 months

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

Initial change in DAS

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

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

Table 1: Baseline characteristics and antibody positivity.

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

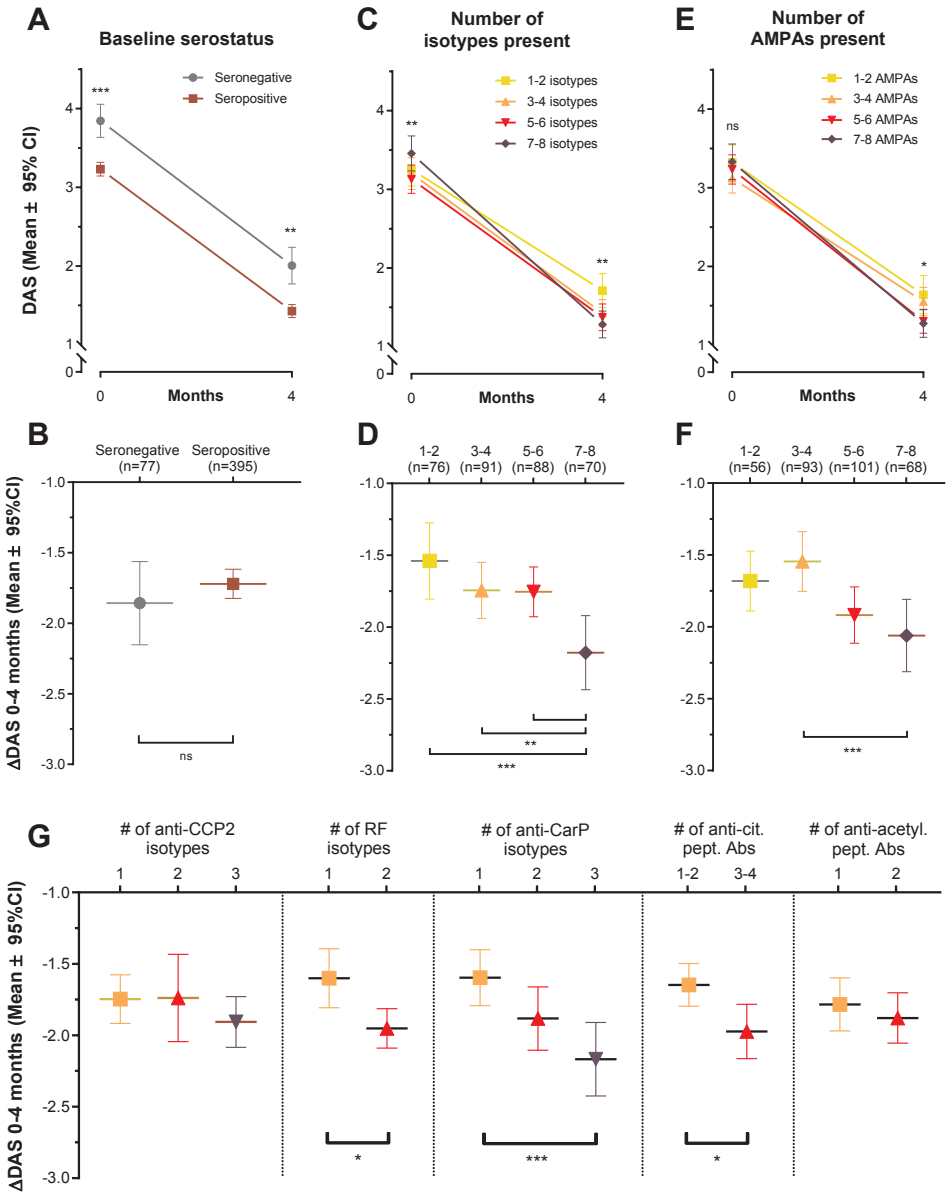
¥ Some missing values. For number of data available of individual antibody measurements, see Figure 1. Available data for symptom duration & smoking: N=355; for anti-CarP IgA: N baseline=344; for Number of isotypes: N baseline=343; N 8 months=208. Vim: vimentin; Fib: fibrinogen; Eno: enolase; Lys: lysine; Orn: ornithine; IQR: interquartile range; SD: standard deviation.

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

Initial successful drug discontinuation

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

Interestingly, while a broad baseline autoantibody response was favourable for initial DAS response, it was unfavourable for the chance of achieving initial DFR (Figure 3). Within patients seropositive for anti-CCP2 IgG, RF IgM, or anti-CarP IgG at baseline, there was a non-significant trend indicating that patients with more isotypes achieve less initial DFR (1-2, 3-4, and 5-6 isotypes, vs 7-8 isotypes respectively: 21% (p=0.07), 20% (p=0.13), and 20% (p=0.10) vs 3%) (Figure 3A). Patients with more AMPAs present also achieved significantly less initial DFR (1-2 AMPAs vs 3-4, 5-6, and 7-8 AMPAs, respectively: 37% vs 13% (p=0.004), 14% (p=0.007), and 11% (p=0.005) (Figure 3B).


A
Baseline serostatus
Seronegative
Seropositive
DAS (Mean ± 95% CI)
Months
C
Number of isotypes present
1-2 isotypes
3-4 isotypes
5-6 isotypes
7-8 isotypes
E
Number of AMPAs present
1-2 AMPAs
3-4 AMPAs
5-6 AMPAs
7-8 AMPAs
B
Seronegative (n=77)
Seropositive (n=395)
ΔDAS 0-4 months (Mean ± 95%CI)
D
1-2 (n=76)
3-4 (n=91)
5-6 (n=88)
7-8 (n=70)
F
1-2 (n=56)
3-4 (n=93)
5-6 (n=101)
7-8 (n=68)
G
# of anti-CCP2 isotypes
# of RF isotypes
# of anti-CarP isotypes
# of anti-cit. pept. Abs
# of anti-acetyl. pept. Abs

◂ **Figure 2: Breadth of the autoantibody response and initial treatment response.** DAS (mean +/- 95% confidence intervals) over 4 months of treatment and mean initial change in DAS from baseline to 4 months (ΔDAS 0-4 months), separated by baseline serological status and breadth of autoantibody response. A & B DAS over time and ΔDAS 0-4 months separated for baseline autoantibody seropositivity based on anti-CCP2 IgG, RF IgM, or anti-CarP IgG positivity. Based on availability of antibody data, the total number of patients included in 2A&D is 472. C & D Within baseline seropositive patients, DAS over time and ΔDAS 0-4 months separated for the total number of isotypes present (anti-CCP2 IgG, IgM, IgA; RF IgM, IgA; anti-CarP IgG, IgM, IgA). Due to the technical success rate of isotype measurements, and some seropositive patients testing negative upon re-measurement (see Supplementary Figure S1), the total number of patients included in 2B&E is 325. E & F Within baseline seropositive patients, DAS over time and ΔDAS 0-4 months separated for the total number of anti-modified peptide antibodies present (anti-CCP2 IgG, anti-CarP IgG, anti-citrullinated-vimentin 59-74 IgG, anti-citrullinated-fibrinogen β 36-52 IgG and α 27-43 IgG, anti-citrullinated-enolase 5-20 IgG, anti-acetylated-lysine IgG, anti-acetylated-ornithine IgG). Thirty-eight patients were RF IgM positive but had no AMPAs (not shown). Due to the technical success rate of isotype measurements and some anti-CCP2 IgG and anti-CarP IgG positive patients testing negative upon re-measurement (see Supplementary Figure S1), the total number of patients included in 2C&F is 318. G Within baseline seropositive patients, ΔDAS 0-4 months separated for the number of isotypes present per antibody family and for the number of antibodies present to citrullinated or acetylated peptides. Reported P-values are adjusted for multiple testing using Holmes-Bonferroni methods. ns: not significant ($p \geq 0.05$); *: $p < 0.05$; **: $p < 0.01$, ***: $p < 0.001$

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

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

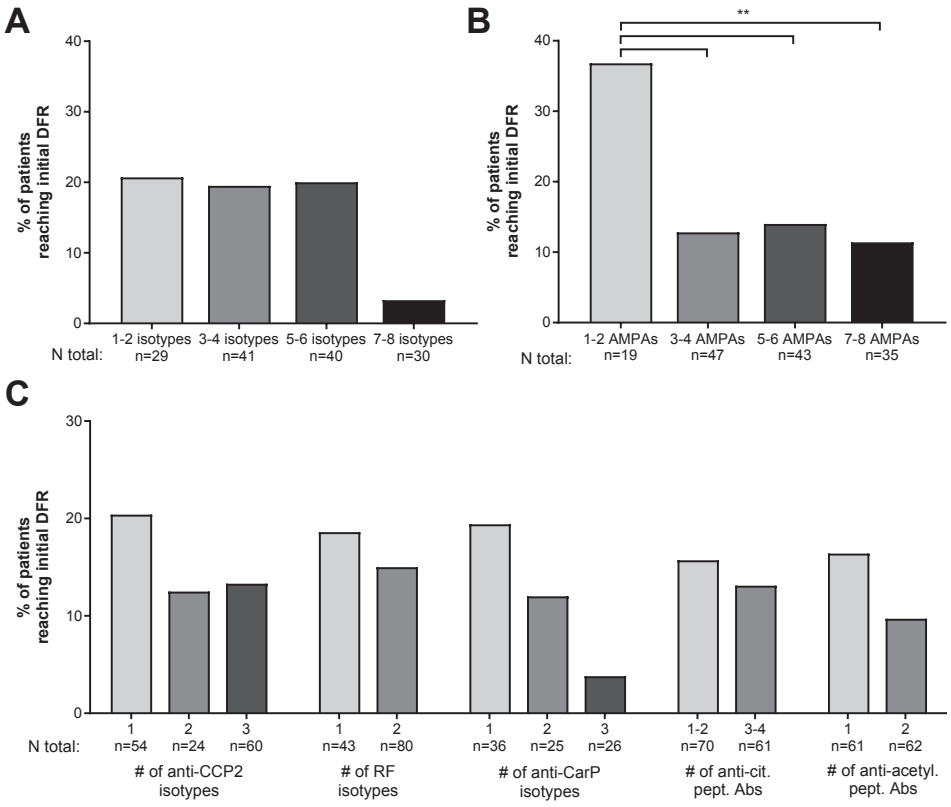


Figure 3: Baseline autoantibody profile and initial drug-free remission (DFR). Association of baseline autoantibody profile with initial drug-free remission in patients seropositive for anti-CCP2 IgG, RF IgM, or anti-CarP IgG at baseline that had serum available for re-measurement (n=155). Pairwise comparisons between each group were not significant after multiple testing. A Percentage of patients with the specified number of isotypes present reaching initial DFR. The composite number of isotypes consists of the positivity count for: anti-CCP2 IgG, IgM, IgA; RF IgM, IgA; anti-CarP IgG, IgM, IgA. Due to the technical success rate of isotype measurements, and some seropositive patients testing negative upon re-measurement (see Supplementary Figure S1), the total number of patients included in 3A is 140. B Percentage of patients with the specified number of AMPAs present reaching initial DFR. The composite number of AMPAs consists of the positivity count for: anti-CCP2 IgG, anti-CarP IgG, anti-citrullinated-vimentin 59-74 IgG, anti-citrullinated-fibrinogen β 36-52 IgG and α 27-43 IgG, anti-citrullinated-enolase 5-20 IgG, anti-acetylated-lysine IgG, anti-acetylated-ornithine IgG. Eleven patients were RF IgM positive but had no AMPA antibodies (not shown). C Percentage of patients with the specified number of antibodies present reaching initial DFR. Reported P-values are adjusted for multiple testing Holmes-Bonferroni methods. ns: not significant ($p \geq 0.05$); *: $p < 0.05$; **: $p < 0.01$, ***: $p < 0.001$.

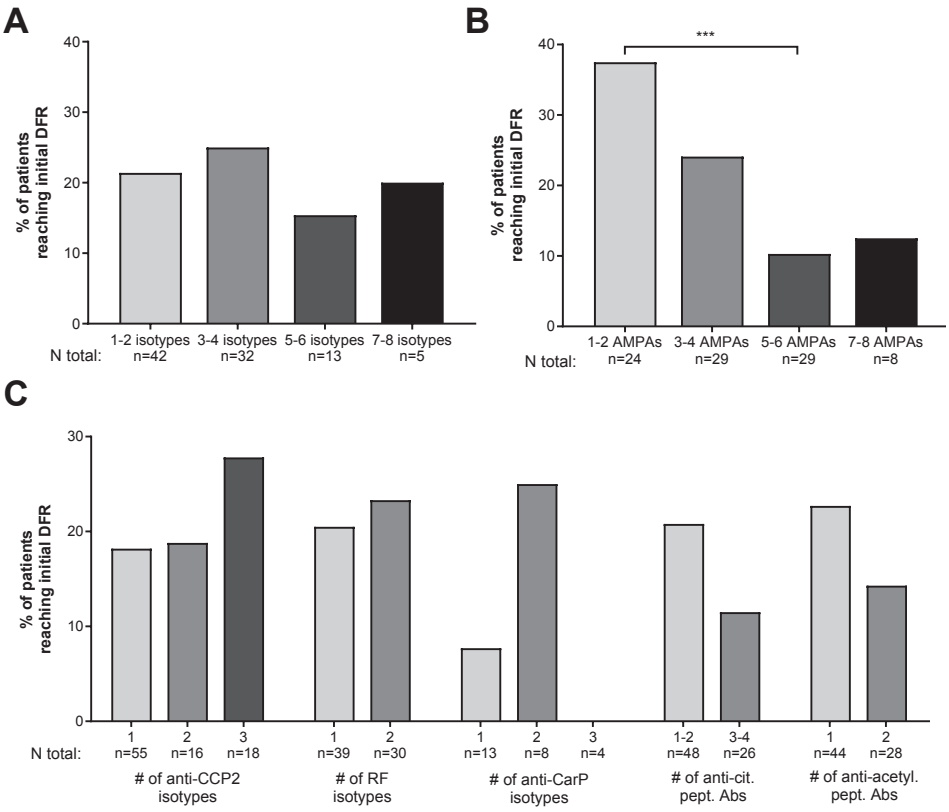


Figure 4: Autoantibody profile at 8 months and initial drug-free remission (DFR). Association of 8-month autoantibody profile with initial DFR in patients seropositive for anti-CCP2 IgG, RF IgM, or anti-CarP IgG at baseline that had serum available for re-measurement at 8 months (n=103). Pairwise comparisons between each group were not significant after multiple testing (see in text). A Percentage of patients with the specified number of isotypes present reaching initial DFR. The composite number of isotypes consists of the positivity count for: anti-CCP2 IgG, IgM, IgA; RF IgM, IgA; anti-CarP IgG, IgM, IgA. Due to some seropositive patients testing negative upon re-measurement or seroconverting to negative by 8 months, the total number of patients with any isotypes present was 92. B Percentage of patients with the specified number of AMPAs present reaching initial DFR. The composite number of AMPAs consists of the positivity count for: anti-CCP2 IgG, anti-CarP IgG, anti-citrullinated-vimentin 59-74 IgG, anti-citrullinated-fibrinogen β 36-52 IgG and α 27-43 IgG, anti-citrullinated-enolase 5-20 IgG, anti-acetylated-lysine IgG, anti-acetylated-ornithine IgG. Thirteen patients were RF IgM positive but had no AMPA antibodies or had seroconverted to negative by 8 months (not shown). C Percentage of patients with the specified number of antibodies present reaching initial DFR. Reported P-values are adjusted for multiple testing Holmes-Bonferroni methods. ns: not significant ($p \geq 0.05$); *: $p < 0.05$; **: $p < 0.01$, ***: $p < 0.001$.

Long-term sustained drug-free remission

Finally, we wished to determine whether the baseline autoantibody profile was associated with the most favourable long-term outcome: long-term sustained drug-free remission. Fifty-seven percent of patients that had initial DFR also achieved long-term sustained DFR, defined as at least 1 year of DFR lasting until last follow-up, an outcome approximating cure of disease. For patients that were not in early remission at 4 months and therefore could not achieve initial DFR, it was still possible to taper medication at a later stage and reach long-term sustained DFR. In the full RA cohort, baseline anti-CCP2 IgG positive patients reached this outcome significantly less often than their negative counterparts (10% vs 26% ($p<0.001$)); RF IgM and anti-CarP IgG positive patients showed a similar trend (14% vs 19% ($p=0.05$) and 12% vs 18% ($p=0.13$), respectively).

In contrast to the previous results regarding initial DFR (broad baseline autoantibody response: decreased chance of initial DFR), there was no difference in rates of long-term sustained DFR between seropositive patients with many isotypes versus few isotypes present, or between patients with many AMPAs versus few AMPAs present (Figure 5A-B). Furthermore, when separately assessing the number of isotypes in a family and the number of antibodies present against citrullinated/acetylated peptides, there were also no differences in long-term sustained DFR rates (Figure 5C). Only anti-CarP isotypes showed a trend that was no longer present after correction for multiple testing: 1 isotype vs 2 isotypes and 3 isotypes respectively: 20% vs 12% ($p=0.49$) and 5% ($p=0.02$). The presence of an anti-CCP2 IgM and/or IgA isotype within patients positive for anti-CCP2 IgG did not decrease the chance of long-term sustained DFR, nor did the presence of a RF IgA in patients positive for conventional RF IgM (data not shown). Lastly, positivity to a specific isotype or antibody did not confer increased or decreased chances of reaching long-term sustained DFR (Supplementary Figure S4C).

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

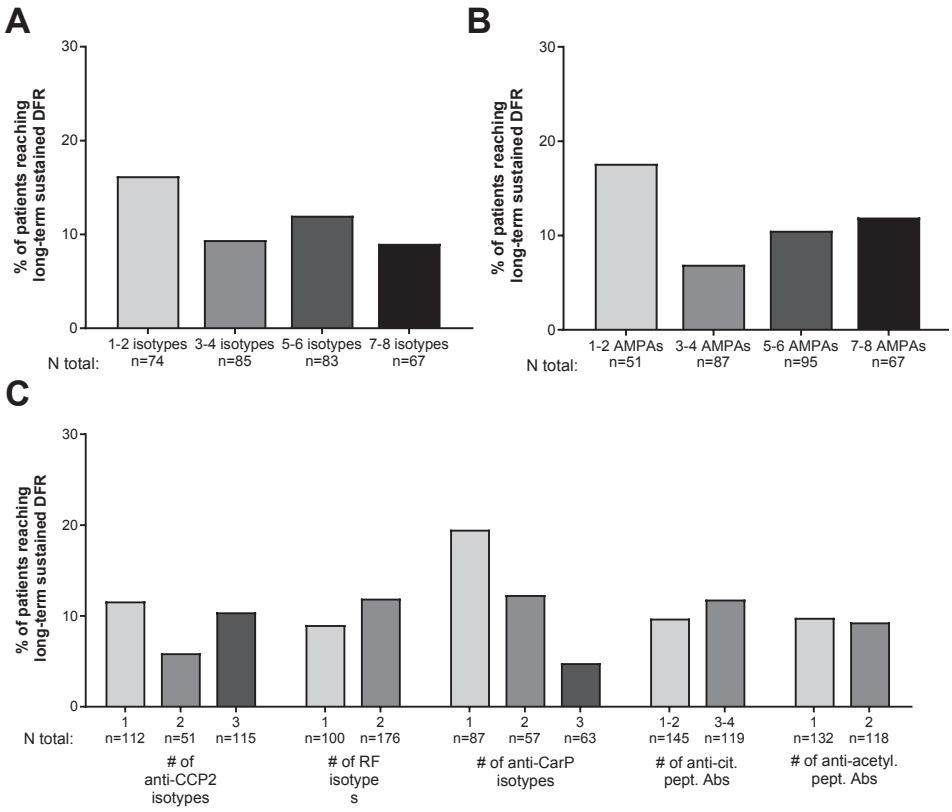


Figure 5: Baseline autoantibody profile and long-term sustained drug-free remission (DFR). Association of baseline autoantibody profile with long-term sustained DFR in patients seropositive for anti-CCP2 IgG, RF IgM, or anti-CarP IgG at baseline (n=336). Pairwise comparisons between each group were not significant after multiple testing (see in text). A Percentage of patients with the specified number of isotypes present reaching long-term sustained DFR. The composite number of isotypes consists of the positivity count for: anti-CCP2 IgG, IgM, IgA; RF IgM, IgA; anti-CarP IgG, IgM, IgA. Due to the technical success rate of isotype measurements, and some seropositive patients testing negative upon re-measurement (see Supplementary Figure S1), the total number of patients included in 4B is 309. B Percentage of patients with the specified number of AMPAs present reaching long-term sustained DFR. The composite number of AMPAs consists of the positivity count for: anti-CCP2 IgG, anti-CarP IgG, anti-citrullinated-vimentin 59-74 IgG, anti-citrullinated-fibrinogen β 36-52 IgG and α 27-43 IgG, anti-citrullinated-enolase 5-20 IgG, anti-acetylated-lysine IgG, anti-acetylated-ornithine IgG. Thirty-six patients were RF IgM positive but had no AMPA antibodies (not shown). D Percentage of patients with the specified number of antibodies present reaching long-term sustained DFR.

Discussion

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

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

As for the second outcome, initial DFR, Figueiredo et al. recently showed that patients with a broad pattern of AMPA response are at high risk of disease relapse in the first year after DMARD tapering (27). Although the trend we found for initial DFR was significant for the number of AMPAs, our findings do not fully support Figueiredo's observation that a broad autoantibody profile is unfavourable for DFR because we do not find a dose-dependent effect. The most likely reason is that we used a different, more stringent outcome (i.e.: maintaining DFR for a full year) and that we only measured seropositive patients. As such, we had no patients with zero antibodies at baseline as Figueiredo et al. did, and the contrast with patients with more antibodies was less striking. We also investigated whether the autoantibody profile at the moment of drug-tapering (8 months in the IMPROVED study) instead of at baseline determines the chance of successfully discontinuing medication without disease flare. We found that a broad profile at this moment was not associated with initial DFR. These findings are relevant as they indicate that characterising the autoantibody profile at the moment of tapering does not yield additional information over baseline.

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

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

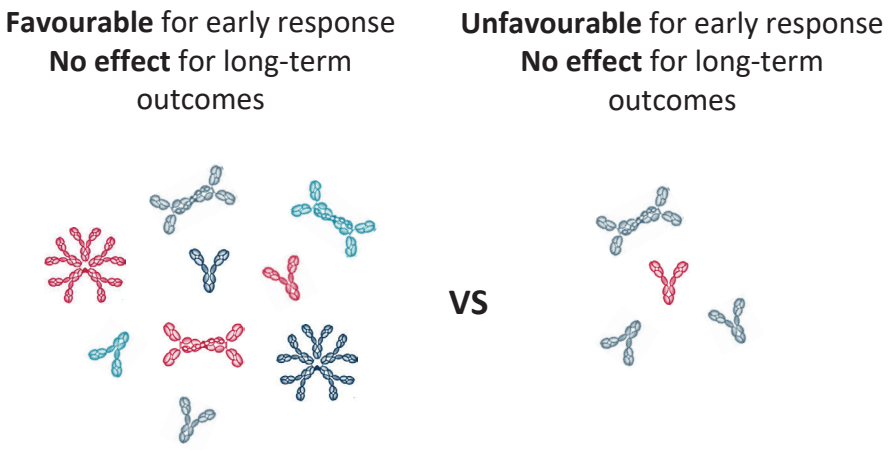


Figure 6: Summary of results. Colours of the antibodies indicate diversity in antigenic targets, and structures indicate diversity in the isotype usage. A broad baseline profile (left) is favourable for early response, but has no association with long-term outcomes like DFR, as compared to a less broad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

The association between the breadth of the autoantibody profile and disease outcomes diminished in magnitude as the outcome investigated became further removed from baseline. This implies that breadth of baseline autoantibody profile is mostly relevant for short-term outcomes and has implications for the mechanisms underlying disease chronicity. It has recently been shown that memory B-cells expressing anti-citrullinated

peptide antibodies (ACPA) persist in the circulation (28, 29), despite conventional DMARD use and remission of synovial disease (30). These data indicate the persistent presence of a population of auto-reactive B cells that is not affected by therapies. Perhaps the best indicator of this long-lived autoimmunity that accounts for the inability to become drug-free in the long run is presence of this persistent B cell population (whose presence can be best measured by the anti-CCP IgG test), rather than the recognition of multiple modified antigens or the presence of multiple autoantibody isotypes at baseline. This would explain why anti-CCP2 IgG positivity (firmly established in literature) but not more antibodies (this study) is a poor prognostic factor for DFR.

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

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

Strengths of the current study include that, to the best of our knowledge, it is the broadest autoantibody profile investigation in RA to date (eight isotypes and six fine-specificities within four autoantibody systems), in a cohort with an exceptionally long follow-up. Furthermore, it is the first study that investigates the relationship between the number of autoantibodies and early response to conventional DMARD therapy. The associations we found cannot be explained by differences in treatment or in demographic characteristics, as we adjusted all analyses for these. Lastly, we characterized the antibody profile both at baseline and at the moment of attempted drug-tapering, something that, to our knowledge, has not been investigated thus far.

Conclusions

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

List of abbreviations

RA: rheumatoid arthritis; anti-CCP2; anti-citrullinated protein 2; RF: rheumatoid factor; DFR: drug-free remission; anti-CarP: anti-carbamylated; DMARD: disease-modifying anti-rheumatic drug; IMPROVED: Induction therapy with Methotrexate and Prednisone in Rheumatoid Or Very Early arthritic Disease; DAS: disease activity score; MTX: methotrexate; ELISA: enzyme-linked immunosorbent assay; AMPA: anti-modified protein antibodies; ANOVA: analysis of variance; HAQ: Health Assessment Questionnaire

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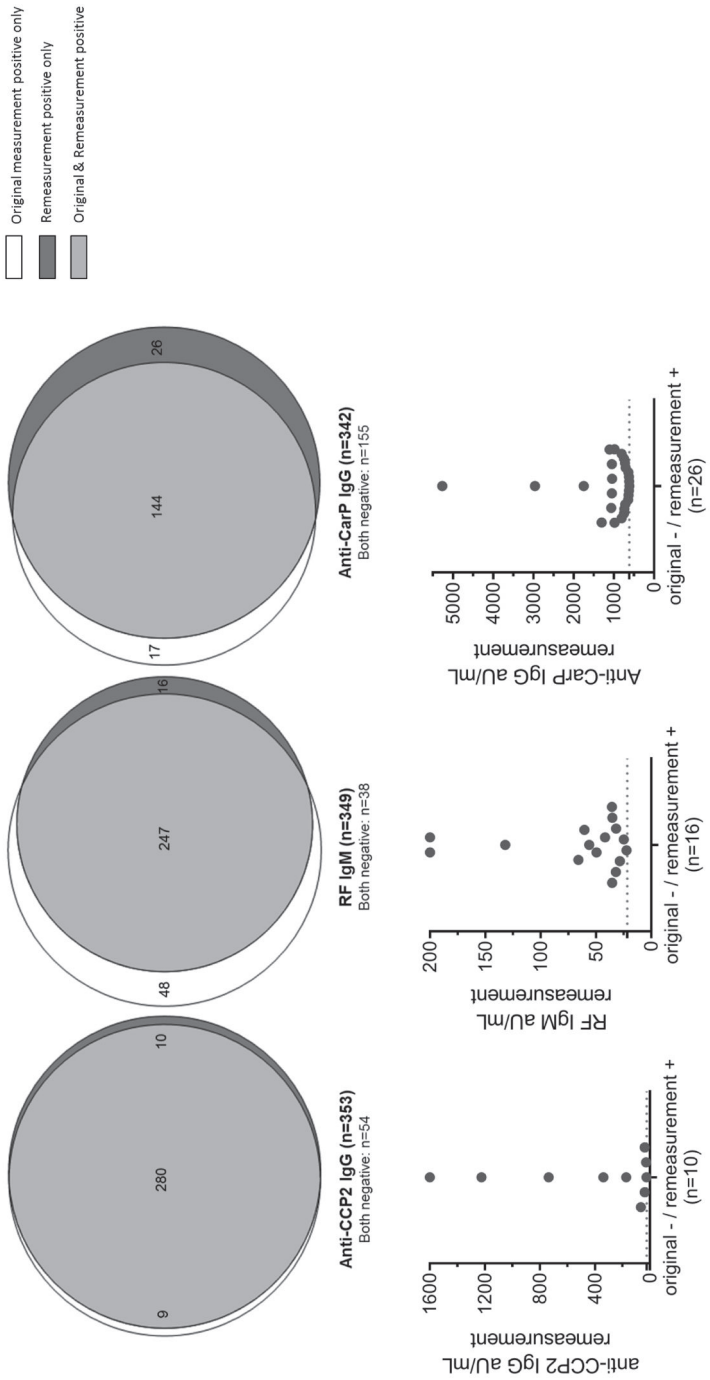
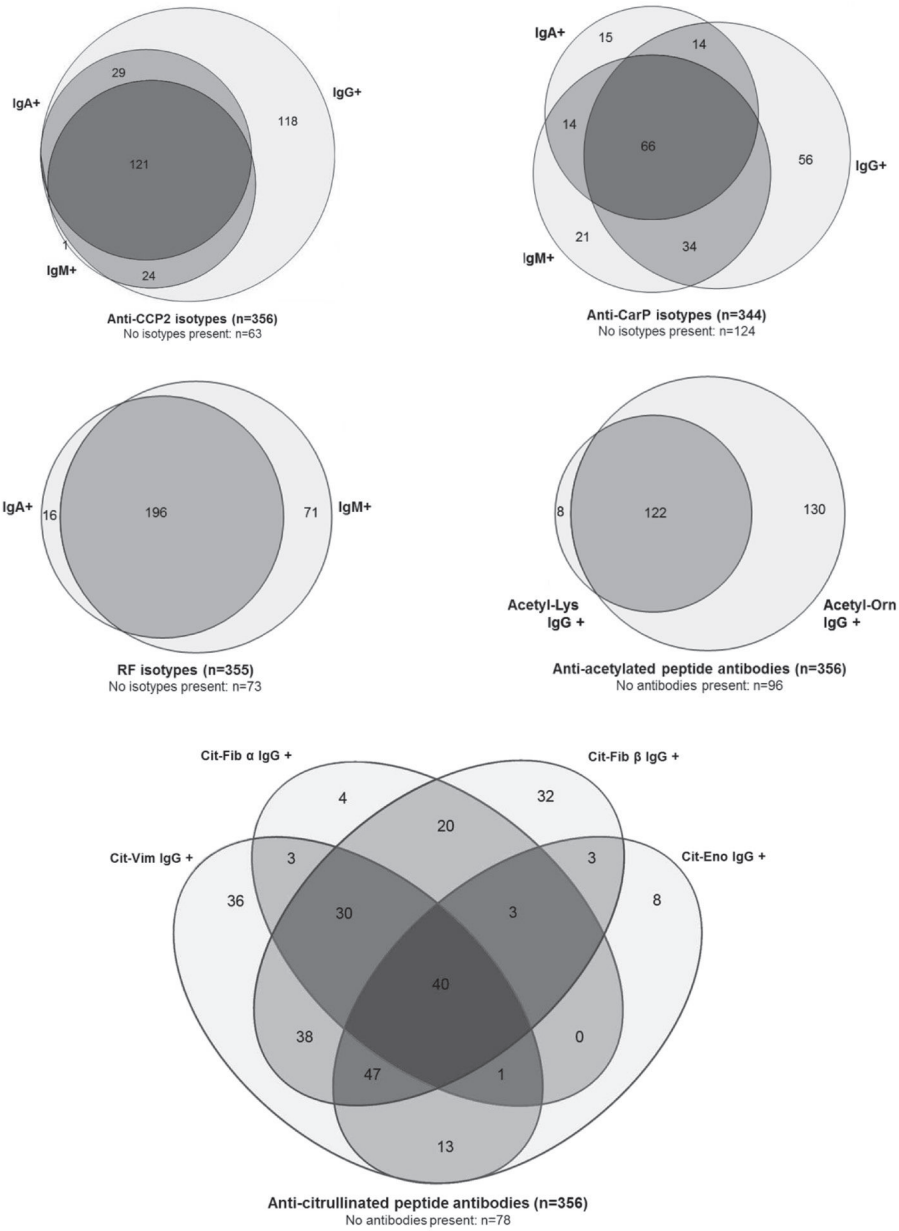
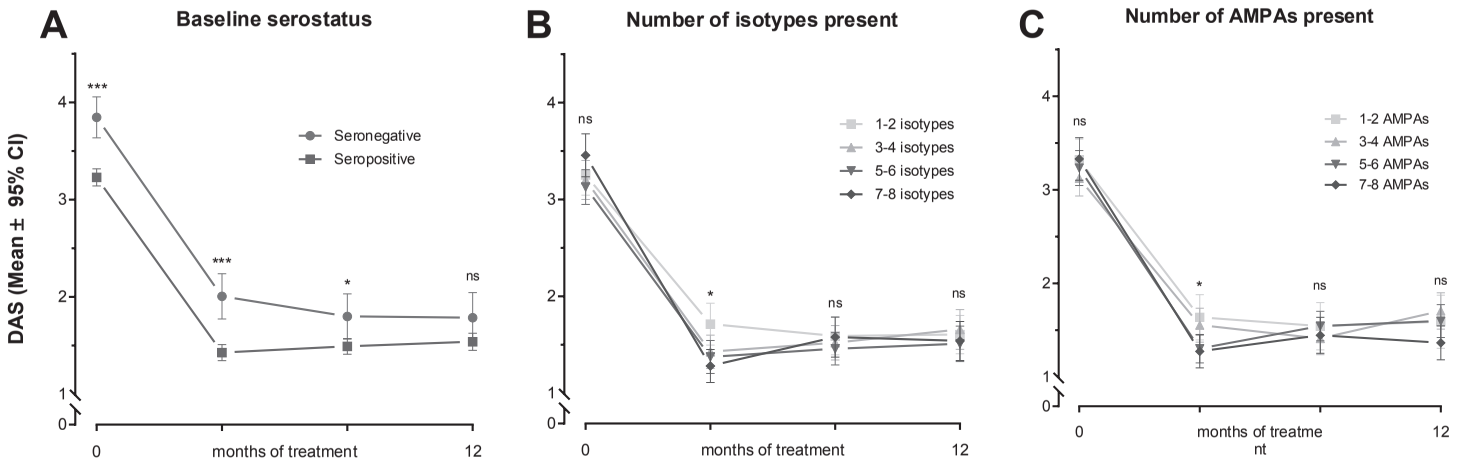
Supplementary information



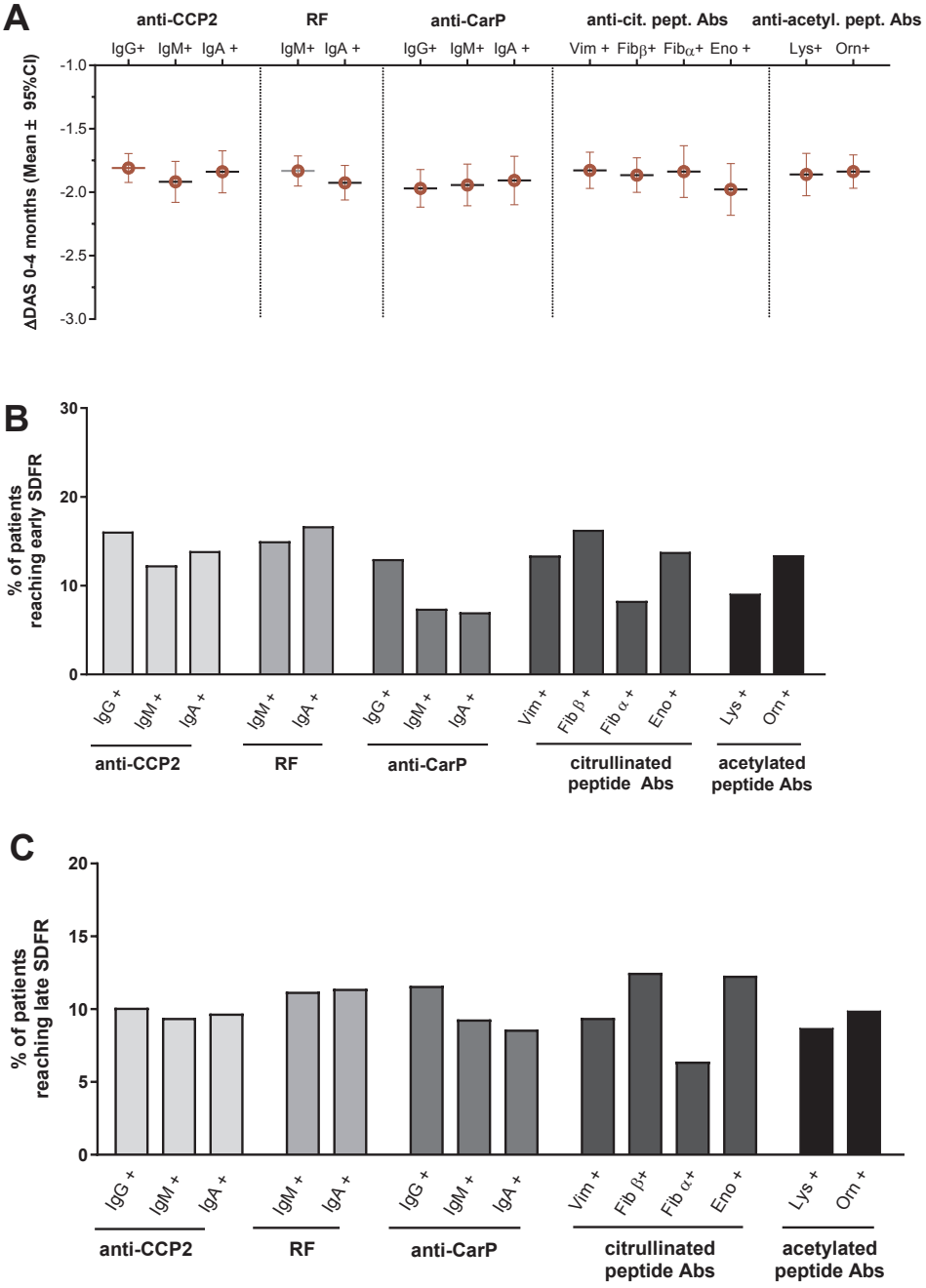
◂ **Supplementary figure S1: Agreement between previously determined antibody status and remeasurement by ELISA (n=356).** A Proportional Venn diagrams displaying agreement between positivity in the previously determined antibody measurement (for anti-CCP2 IgG and RF IgM, by commercial testing; for anti-CarP, in-house ELISA) and the remeasurement (all by in-house ELISA). All samples positive for any antibody were remeasured for the presence of all antibodies. According to Cohen's κ, there was moderate to good agreement between measurements: anti-CCP2 IgG κ=0.82 ($p<0.001$); RF IgM κ=0.44 ($p<0.001$); anti-CarP IgG κ=0.75 ($p<0.001$). The number of patients included in each Venn diagram may be less than 356 due to missing values in the previously determined measurement. B Dotplots representing the arbitrary units (aU/mL) of each antibody for the remeasurement by in-house ELISA within the subset of patients that was previously determined to be negative. Levels upon remeasurement are generally low, suggesting that the discrepancy in positivity between previously determined and remeasurement may be due to cut-off or inter-test variation. The three patients with high levels upon remeasurement in all three ELISAs are not the same patients.



Supplementary figure S2: Overlap of isotypes and antibodies at baseline upon remeasurement by ELISA. Two- and three-way Venn diagrams are proportional to the number of patients indicated in overlap. There was strong overlap of anti-CCP2 IgG positivity and reactivity to citrullinated peptides. Of 292 anti-CCP2 IgG positive patients, only 8,2% had no reactivity to any citrullinated peptide. Conversely, of 64 anti-CCP2 IgG negative patients, 15,6% harboured reactivity to at least one citrullinated peptide. Cit = citrullinated. Vim = vimentin; Fib = fibrinogen; Eno = enolase; Lys = lysine; Orn = ornithine. Acetyl = acetylated. Lys = lysine; Orn=ornithine.



Supplementary figure S3: DAS (mean +/- 95% confidence intervals) over first year of treatment (4 month intervals), separated by baseline serological status and breadth of autoantibody response. Adjusted for age, gender, smoking, body mass index, baseline Health Assessment Questionnaire and baseline DAS. A DAS over time separated for baseline autoantibody seropositivity based on anti-CCP2 IgG, RF IgM, or anti-CarP IgG positivity. B Within baseline seropositive patients, DAS over time separated for the total number of isotypes present (anti-CCP2 IgG, IgM, IgA; RF IgM, IgA; anti-CarP IgG, IgM, IgA). C Within baseline seropositive patients, DAS over time separated for the total number of anti-modified peptide antibodies present (anti-CCP2 IgG, anti-CarP IgG, citrullinated-vimentin 59-74, citrullinated-fibrinogen β 36-52 and α 27-43, citrullinated-enolase 5-20, acetylated-lysine, acetylated-ornithine). Thirty-eight patients were RF IgM positive but had no AMPAs (not shown). Reported p-values are adjusted for multiple testing using Holmes-Bonferroni methods. ns: not significant (p≥0.05); *: p<0.05; **: p<0.01, ***: p<0.001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Lys+ Orn+
ΔDAS 0-4 months (Mean ± 95%CI)
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citrullinated peptide Abs
acetylated peptide Abs
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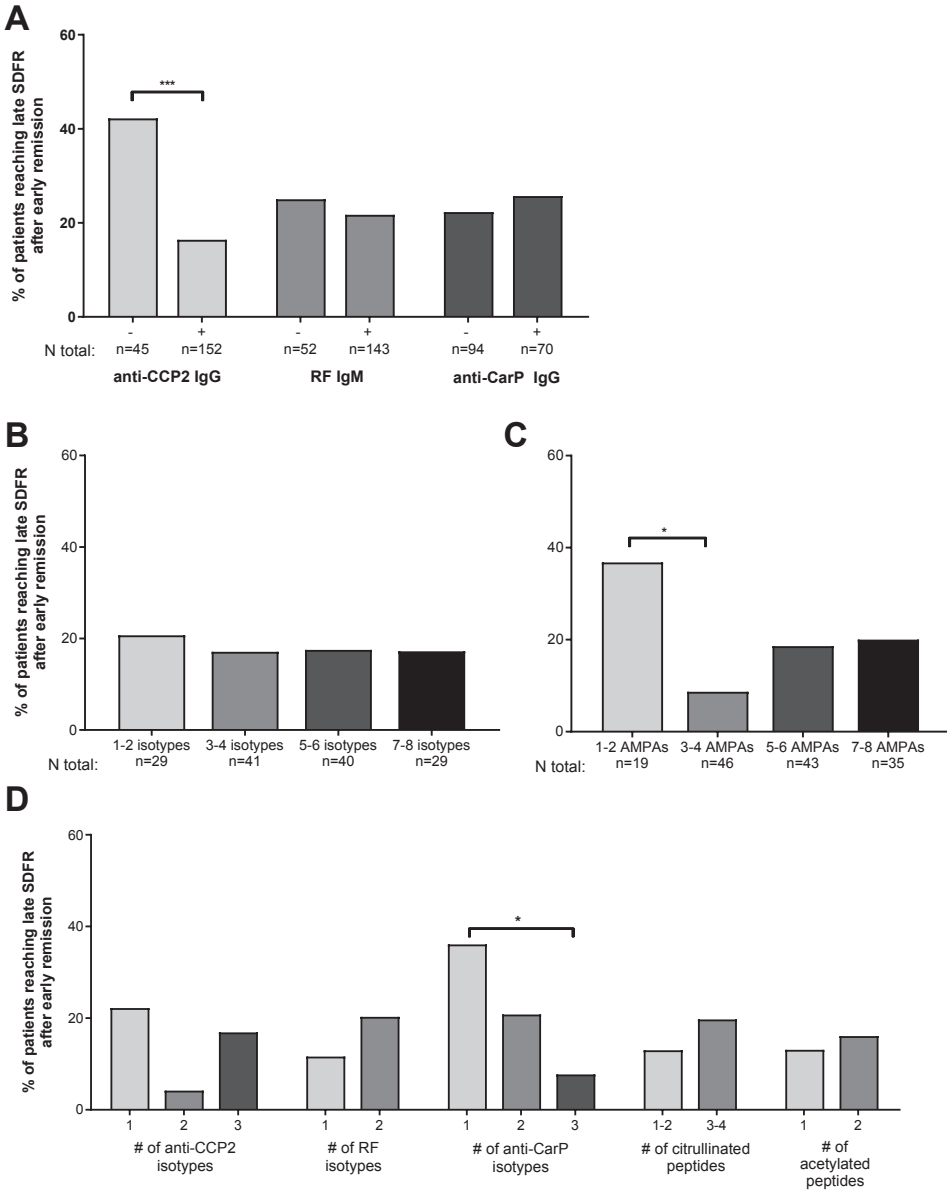
citrullinated peptide Abs
acetylated peptide Abs

◂ **Supplementary figure S4: Initial change in DAS and DFR outcomes within patients positive for the indicated individual antibody.** No statistical testing was possible as patient groups overlap. A Initial change in DAS (mean +/- 95% confidence intervals) from baseline to 4 months within patients positive for anti-CCP2 IgG, RF IgM, or anti-CarP IgG. B Percentage of patients positive for the specified antibody that reached initial DFR. C Percentage of patients positive for the specified antibody that reached long-term sustained DFR. Anti-cit. pept. Abs = anti-citrullinated peptide antibodies; Anti-acetyl. pept. Abs = anti-acetylated peptide antibodies; Vim = vimentin; Fib = fibrinogen; Eno = enolase; Lys = lysine; Orn = ornithine.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RF IgM
anti-CarP IgG
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% of patients reaching late SDFR after early remission
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5-6 isotypes n=40
7-8 isotypes n=29
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3-4 AMPAs n=46
5-6 AMPAs n=43
7-8 AMPAs n=35
N total:
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% of patients reaching late SDFR after early remission
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1
2
3
# of anti-CCP2 isotypes
# of RF isotypes
# of anti-CarP isotypes
1-2
3-4
# of citrullinated peptides
# of acetylated peptides

◂ **Supplementary figure S5: Association of baseline autoantibody profile with long-term sustained drug-free remission within patients that reached early remission and had outcome data available (A; n=199) and in only patients seropositive for anti-CCP2 IgG, RF IgM, *or* anti-CarP IgG (B-D; n=154).** Adjusted for age, gender, smoking, body mass index, baseline Health Assessment Questionnaire and baseline DAS. A Percentage of anti-CCP2 IgG, RF IgM, or anti-CarP IgG positive and negative patients reaching long-term sustained DFR after early remission. B Within baseline seropositive patients, percentage of patients with the specified number of isotypes present reaching long-term sustained DFR after early remission. The composite number of isotypes consists of the positivity count for: anti-CCP2 IgG, IgM, IgA; RF IgM, IgA; anti-CarP IgG, IgM, IgA. Due to the technical success rate of isotype measurements and some seropositive patients testing negative upon re-measurement (see Figure S2), the total number of patients included in S6B is 139. C Within baseline seropositive patients, percentage of patients with the specified number of AMPAs present reaching long-term sustained DFR after early remission. The composite number of AMPAs consists of the positivity count for: anti-CCP2 IgG, anti-CarP IgG, anti-citrullinated-vimentin 59-74 IgG, anti-citrullinated-fibrinogen β 36-52 IgG and α 27-43 IgG, anti-citrullinated-enolase 5-20 IgG, anti-acetylated-lysine IgG, anti-acetylated-ornithine IgG. Eleven patients were RF IgM positive but had no AMPA antibodies (not shown). D Within baseline seropositive patients, percentage of patients with the specified number of antibodies present reaching long-term sustained DFR after early remission. Reported P-values are adjusted for multiple testing using Holmes-Bonferroni methods. ns: not significant ($p \geq 0.05$); *: $p < 0.05$; **: $p < 0.01$, ***: $p < 0.001$

Anti-cit. pept. Abs = anti-citrullinated peptide antibodies; Anti-acetyl. pept. Abs = anti-acetylated peptide antibodies

Inflammation rather than Anti-Citrullinated Protein Antibodies is associated with CardioVascular Mortality in RA: Insights from Rheumatoid Arthritis and Coronary Artery Disease Cohorts

Veerle F.A.M. Derksen[1], Jane Lindschou[2], Per Winkel[2], Graciela E Delgado[3], Emma C. de Moel[1], Tom W.J. Huizinga[1], Winfried März[3], Janus C. Jakobsen[2,4], Annette H.M. van der Helm - Van Mil[1,5], René E.M. Toes[1], Sofia Ajeganova[6], Christian Gluud[2,4], Jan Leipe[3,7#], Diane van der Woude[1#], on behalf of the CLARICOR Trial Group* and the LURIC Study Group**.

[1] Department of Rheumatology, Leiden University Medical Center, Leiden, The Netherlands
[2] Copenhagen Trial Unit, Centre for Clinical Intervention Research, The Capital Region, Copenhagen University Hospital – Rigshospitalet, Copenhagen, Denmark
[3] Vth Department of Medicine (Nephrology, Hypertensiology, Rheumatology, Endocrinology, Diabetology), Medical Faculty Mannheim, University of Heidelberg, Mannheim, Germany
[4] Department of Regional Health Research, The Faculty of Health Sciences, University of Southern Denmark, Odense, Denmark
[5] Department of Rheumatology, Erasmus Medical Centre, Rotterdam, the Netherlands
[6] Department of Medicine Huddinge, Karolinska Institutet, Stockholm, Sweden
[7] Division of Rheumatology, Department of Internal Medicine I, University Medical Centre Schleswig-Holstein, Kiel, Germany

[#] These authors contributed equally

* ***CLARICOR Trial Group*** **members:** Hans-Jørn Kolmos, Gorm Boje Jensen, Erik Kjøller, Jens Kastrup, Ahmad Sajadieh, Per Winkel, Janus C Jakobsen, Jane Lindschou and Christian Gluud
** ***LURIC Study Group*** **members**: Graciela E Delgado, Marcus Kleber, Jan Leipe and Winfried März

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Abstract

Objectives Anti-citrullinated protein antibodies (ACPA) are associated with increased mortality in rheumatoid arthritis (RA) patients. Previous data suggest ACPA might be associated with worse disease outcomes in non-RA patients with coronary artery disease (CAD). Therefore, we investigated ACPA prevalence and its association with mortality in non-RA CAD patients. Furthermore, the role of systemic inflammation in the relation between ACPA and mortality was investigated in RA.

Methods The prevalence of ACPA in non-RA CAD was investigated in two large CAD cohorts (LURIC n=2189 patients and 656 controls; CLARICOR n=959 patients) using commercial ELISA. Multivariable Cox proportional hazards models were performed to investigate the association between ACPA and all-cause mortality. In two cohorts of RA patients (EAC n=764; BARFOT n=794), joint models were applied to investigate the role of CRP on the association between ACPA and (cardiovascular) mortality.

Results Average follow-up time in the cohorts was 8.2-11.8 years. In both CAD cohorts, ACPA prevalence was low (0.9% and 4.6%), and no association was found between seropositivity and all-cause mortality. In RA patients, the association between ACPA positivity and all-cause mortality was no longer significant after adjustment for CRP. In contrast, CRP was significantly associated with all-cause and cardiovascular mortality in RA (indirect effect hazard ratio (95% confidence interval): EAC 1.24 (1.14-1.34), BARFOT 1.33 (1.24-1.42)).

Conclusions ACPA prevalence is not increased in CAD non-RA patients. In RA, the association between ACPA positivity and increased (cardiovascular) mortality was primarily explained by CRP. This highlights the impact of chronic inflammation in cardiovascular outcomes in RA.

Introduction

Rheumatoid arthritis (RA) is a prevalent autoimmune disease characterized not only by chronic synovial inflammation, but also by an increased risk of cardiovascular disease (CVD) (1, 2). Epidemiological evidence indicates that patients with RA have 43% increased risk of myocardial infarction and a 55% increased risk of experiencing any major adverse cardiac event compared to the general population (3, 4). Approximately half of this excess risk appears to be attributable to traditional cardiovascular risk factors (5), some of which are also risk factors for the development of RA, such as smoking (6) and obesity (7). About one-third of the excess cardiovascular risk in RA is thought to result directly from the disease itself (5).

Systemic inflammation is known to contribute to accelerated atherosclerosis and increased cardiovascular events (8-10). However, there are also indications that RA-specific autoantibodies, especially ACPA, might play a direct role in the increased incidence of cardiovascular disease in RA patients. Several disease-specific autoantibodies can be detectable in serum of RA patients, including rheumatoid factor (RF), anti-citrullinated protein antibodies (ACPA) and antibodies against carbamylated proteins (anti-CarP) (11). ACPA are routinely determined in clinical practice and are specific for RA. These autoantibodies are detected in approximately two-thirds of RA patients and can be present in serum years before the onset of arthritis (12). Although their role in RA pathophysiology remains incompletely understood, ACPA are believed to contribute to chronic inflammation, as ACPA have been shown to be able to activate the complement system (13) and trigger Fc-receptors leading to pro-inflammatory cytokine production (14). Several studies have indicated that ACPA may be independently associated with increased all-cause mortality in RA patients, particularly from cardiovascular causes (15-18), although findings remain inconsistent (1, 19-23). Histological studies have identified citrullinated proteins within atherosclerotic plaques (24) and experimental data demonstrated that ACPA can activate platelets via FcγRIIa-dependent pathways (25). Taken together, these findings might indicate that ACPA can contribute directly to the increased cardiovascular risk observed in RA.

There is also evidence that ACPA may augment cardiovascular risk in individuals who do not have RA. Two clinical studies reported increased ACPA seropositivity in non-RA cohorts based on commercial ACPA assays: 10.4% (15/144) of patients with coronary artery disease (CAD) compared to 3.8% (11/288) of healthy controls was ACPA-positive in one cohort (26), and 11% (29/275) of patients with ST-elevation myocardial infarction (STEMI) was seropositive compared to 2% (3/160) in healthy

controls in another cohort (27). In the latter study, ACPA were independently associated with worse long-term mortality in STEMI patients during a median follow of 9.4 years (27). Notably, none of the 29 ACPA-positive patients developed RA during follow-up. These findings point to a potential direct role for ACPA in cardiovascular risk in individuals without RA, but require confirmation in larger well-characterized cardiovascular cohorts. Furthermore, it remains unclear whether other RA-associated autoantibodies such as RF or anti-CarP are also present in patients with CAD, and how the presence of autoantibodies is related to cardiovascular mortality in both non-RA and RA populations.

To address these questions, we examined the prevalence of ACPA and other RA-associated antibodies in two large, independent non-RA CAD cohorts and evaluated the association between seropositivity and all-cause mortality. In parallel, two early RA cohorts were analysed to assess whether systemic inflammation plays a role in the relationship between ACPA and all-cause or cardiovascular mortality. We hypothesized that the observed association between ACPA and adverse cardiovascular outcomes in RA might (partly) reflect underlying inflammatory burden instead of a consequence of autoantibody presence by itself.

Methods

Study design

For the analyses reported here, we utilized baseline laboratory data, patient demographics and characteristics, and mortality data collected from two large cardiovascular and two RA cohorts (see cohort descriptions below). For the cardiovascular cohorts, participants with a reported comorbidity of RA were excluded based on the data available to allow more accurate comparisons between the cardiovascular and RA cohorts. For all four cohorts, written informed consent was obtained from all participants and the studies were approved by local ethics committees (28-31).

CAD non-RA cohorts

To investigate the prevalence of ACPA and other autoantibodies and their association with mortality in non-RA populations in coronary artery disease, baseline samples from two large independent cohorts of patients with CAD were used: the CLARIthromycin for patients with stable CORonary heart disease (CLARICOR) trial (959 patients analysed) (31) and the LUdwigshafen Risk and Cardiovascular Health (LURIC) study (2189 patients and 656 controls analysed) (28).

CLARICOR is a Danish, randomised multicentre trial that enrolled patients with a previous diagnosis of acute myocardial infarction or angina pectoris between 1993-1999 (detailed information is described elsewhere (31)). Enrolled patients were randomized to receive clarithromycin or placebo for a period of two weeks between October 1999 and April 2000 (31). 959 randomly selected patients from both the intervention and control arm were analysed. 10 years follow-up data on cardiovascular outcomes, comorbidities, and mortality were obtained from the Danish National Patient Register, which records diagnoses using International Statistical Classification of Diseases (ICD) with a coverage of almost 100%.

The LURIC study is a German prospective cohort study that enrolled patients with CAD defined as luminal narrowing on angiography between 1997 and 2000 (detailed information is described elsewhere (28)). Clinical indications for angiography were suspected CAD based on symptoms such as acute chest pain or non-invasive tests consistent with myocardial ischaemia. Follow-up mortality data for this cohort were available until 2010. Although severe chronic disease was an exclusion criterion in LURIC (28), additional screening using baseline comorbidity- and medication data as well as self-reported (incomplete) follow-up comorbidity surveys was performed to identify and exclude RA cases from the analyses reported here, leading to the exclusion of 29 CAD patients and 4 controls. Of the included participants, 2,189 CAD patients and 656 controls (family members and patients who underwent angiography, but did not have CAD) were tested for ACPA and RF.

RA cohorts

To evaluate the role of systemic inflammation in the relation between ACPA and mortality in RA, data from two prospective early RA cohorts were examined: the Leiden Early Arthritis Clinic (EAC) (29) and the Better Anti-rheumatic Farmaco-therapy (BARFOT) cohort (30).

In the EAC, 764 patients with early RA, enrolled between 1993 and 2010, were studied (detailed information is described elsewhere (29)). Seventy-nine patients were excluded due to missing data on smoking status. CRP levels were measured annually for up to a maximum of 10 years. All-cause mortality data were available through April 2012, while cause-specific mortality could only be assessed through 2008 due to changes in privacy legislation, limiting the scope of the cardiovascular mortality sub-analyses to 575 patients (15).

The Swedish BARFOT cohort included 803 patients with newly diagnosed early RA recruited between 1993 and 1999. Nine patients were excluded due to missing smoking status, six patients due to missing causes of death. CRP was measured at baseline and at follow-up visits at 6, 12, 24, 60, and 96 months. Mortality data were available until December 2010 (detailed information is described elsewhere (30).

Autoantibody measurements

In CLARICOR, ACPA were measured in baseline serum using the QUANTA Lite CCP3 IgG enzyme-linked immunosorbent assay (ELISA) (Inova Diagnostics). Positivity was calculated according to the manufacturer instructions using the standard-curve method. RF IgM and anti-CarP IgG were determined using validated in-house ELISA (Supplementary Methods S1). 78 healthy individuals from the Leiden area aged 20 and 70 years were taken along as control. In LURIC, ACPA and RF positivity were assessed in samples (either heparin plasma, citrated plasma, or acidified citrated plasma (Stabilyte)) using the Alinity i anti-CCP IgG and Alinity c total RF assays (Abbott), according to manufacturer protocols. In both EAC and BARFOT, ACPA were measured at baseline using the anti-CCP2 ELISA (Euro-Diagnostica) with a cut-off >25 units.

Statistical analysis

In CLARICOR and LURIC, baseline characteristics and risk factors were compared between autoantibody-positive and autoantibody-negative patients using the appropriate statistical tests (chi-square, Fisher's exact, Student's t test, or Mann-Whitney U test). Associations between autoantibodies and all-cause mortality were evaluated using Kaplan-Meier curves and log-rank tests. Furthermore, Cox proportional hazards models with adjustment for age and sex were performed, with the autoantibody-negative patient group as reference. In CLARICOR, all-cause mortality analyses were performed separately for the clarithromycin and placebo groups, as previous research indicated higher mortality in the clarithromycin group (31). The small number of antibody-positive patients prohibited meaningful analyses concerning cause-specific mortality outcomes.

In EAC and BARFOT, previous analyses showing an association between ACPA positivity and both higher all-cause and cardiovascular mortality (15) were extended to explore the role of systemic inflammation on this relation. CRP repeatedly measured over time was used as a proxy for inflammation. As depicted in Supplementary Figure S1, inflammation might be a potential mediating factor in the association between ACPA and mortality in RA. The relationship between ACPA and CRP trajectories was analysed using linear mixed-effects models (LMM) with random intercept and random slope using restricted maximum likelihood estimation. In all analyses CRP is log-transformed. Joint models, which incorporate both a LMM and a Cox regression model, were employed to simultaneously use the repeatedly measured CRP values for each patient and the time-to-event (mortality) data, with the Cox model adjusted for age, sex, smoking status and year of inclusion as proxy of changed treatment strategy over time, in line with previous analyses

(15). Mediation effects were assessed by calculating the indirect effect of CRP on mortality in these joint models (32-34). As a sensitivity analysis, a joint model with a non-weighted cumulative association structure was calculated, using the CRP area under the curve. Hazard ratios (HRs) with 95% confidence intervals (95% CIs) are reported. Further details are provided in Supplementary Methods S2.

Results

Baseline demographics and mortality outcomes across patient populations in all CAD and RA cohorts are shown in Table 1. The mean follow-up time was approximately 9 years. Mortality rates during follow-up ranged from 16% in the LURIC control group to nearly 39% in CLARICOR.

Table 1: Demographic data at baseline and information on deaths during follow-up in coronary artery disease (CAD) and rheumatoid arthritis (RA) cohorts.

	CAD cohorts			RA cohorts	
	CLARICOR	LURIC		BARFOT	EAC
	CAD (n=959)	CAD (n=2189)	Controls (n=656)	RA (n=794)	RA (n=764)
Baseline					
Age in years, mean ± SD	65.3 ± 10.4	63.6 ± 10.0	58.3 ± 12.1	57.5 ± 15.6	56.8 ± 15.7
Female, n (%)	293 (30.6)	532 (24.3)	313 (47.7)	524 (66.0)	514 (67.3)
Smoking ever, n (%)	814 (84.9)	1503 (68.7)	319 (48.6)	469 (59.1)	415 (54.3)
BMI in kg/m², mean ± SD	-	27.5 ± 3.9	27.5 ± 4.4	25.4 ± 5.0	25.8 ± 3.9
Myocardial infarction, n (%)	630 (65.7)	1157 (52.9)	-	-	-
Follow-up					
Follow-up in years, mean ± SD	8.2 ± 2.9	8.7 ± 3.1	9.5 ± 2.4	11.8 ± 3.7	9.0 ± 4.8
Death, n (%)	372 (38.8)	694 (31.7)	105 (16.0)	228 (28.7)	137 (17.9)
Death from cardiac cause, n (%)	170 (17.7)	394 (18.0)	49 (7.5)	141 (17.8)	40 (5.2)

SD: standard deviation

Autoantibody prevalence in CAD patients

In CLARICOR, 4.6% (44/959) of non-RA CAD patients tested positive for ACPA (Figure 1A), which is lower than the 10% to 11% ACPA seropositivity described previously in other CAD patient populations (26, 27). RF IgM and anti-CarP were detected in 8.6%

(82/959) and 3.0% (26/959) of patients, respectively (Figure 1B, C). In contrast to what is commonly observed in RA patients (35), dual seropositivity for ACPA and either RF or anti-CarP was rare (3% and 1%) and no triple-positive CAD patients were identified.

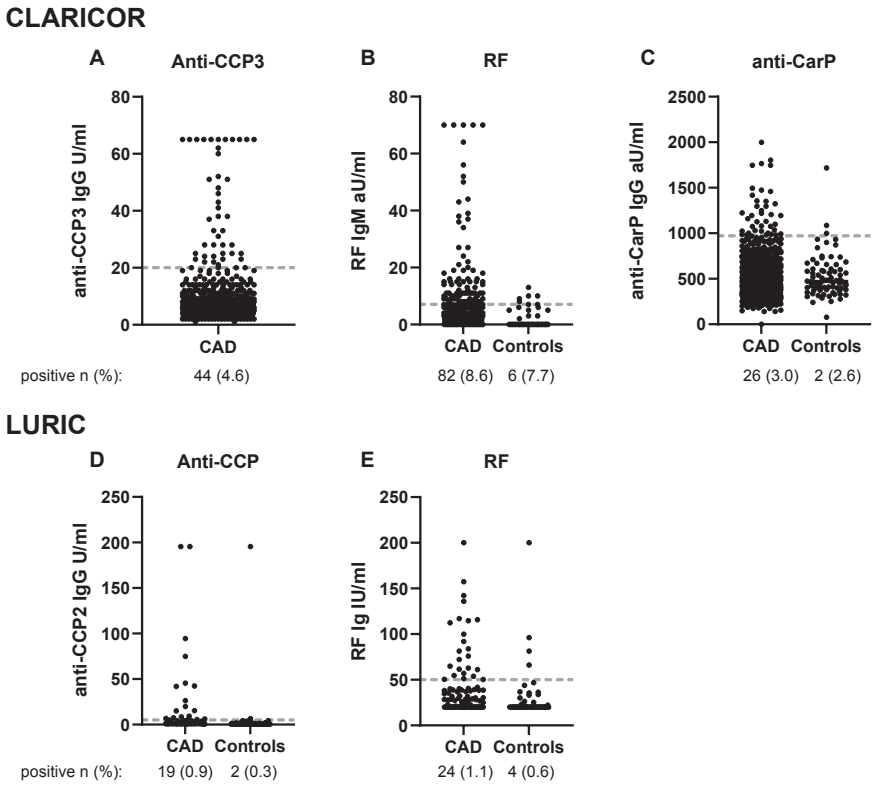

Figure 1: Autoantibody measurements in CAD cohorts. CLARICOR: A anti-CCP3 IgG in CAD patients only, B RF IgM in CAD patients and Leiden healthy controls, C anti-CarP IgG in CAD patients and Leiden healthy controls. LURIC: D anti-CCP IgG and E total RF in CAD patients and LURIC controls. Dashed lines represent assay cut-offs.

In the LURIC cohort, 0.9% (19/2189) of patients with CAD and 0.3% (2/656) of controls were positive for ACPA (Figure 1D) and 1.1% (24/2189) of CAD patients and 0.6% (4/656) of controls were positive for RF (Figure 1E). There was no significant difference in the frequency of autoantibody positivity between patients with CAD and controls (anti-CCP: p=0.19; RF: p=0.38). Dual autoantibody positivity was low with only two CAD patients being both RF- and ACPA-double positive.

To investigate whether ACPA are found more frequently in CAD patients with high cardiovascular risk, cardiovascular risk factors at baseline were compared between ACPA-positive and ACPA-negative individuals in both cohorts. Age, sex, smoking status, diagnosis of hypertension, low-density lipoprotein (LDL) levels and percentage of patients with previous myocardial infarction did not significantly differ between ACPA-positive and ACPA-negative patients in either cohort (Tables 2, 3). Similar findings were observed for RF (CLARICOR and LURIC, Supplementary Table S1-2 respectively) and anti-CarP (CLARICOR only, Supplementary Table S3) with the exception of RF-positive individuals in LURIC being significantly older than RF-negative individuals. Overall, autoantibody-positive and autoantibody-negative patients were comparable concerning cardiovascular risk factors.

Table 2: Cardiovascular risk factors at baseline in CLARICOR by anti-CCP3 IgG status.

	anti-CCP3 – (n=915)	Anti-CCP3 + (n=44)	p-value
Age in years, mean ± SD	65.2 ± 10.4	67.1 ± 9.6	0.20
Female, n (%)	281 (30.7)	12 (27.3)	0.75
Smoking ever, n (%)	775 (84.7)	39 (88.6)	0.62
Clarithromycin group, n (%)	452 (49.4)	28 (63.6)	0.09
Hypertension, n (%)	371 (40.5)	19 (43.2)	0.85
LDL cholesterol in mmol/L, median (IQR)	2.5 (2.1-3.0)	2.5 (2.0-3.1)	0.90
Previous myocardial infarction, n (%)	597 (65.2)	33 (75.0)	0.24

SD: standard deviation, IQR: inter-quartile range

Table 3: Cardiovascular risk factors at baseline in LURIC by anti-CCP IgG status.

	Anti-CCP – (n=2170)	anti-CCP + (n=19)	p-value
Age in years, mean ± SD	63.6 ± 10.0	62.7 ± 11.1	0.72
Female, n (%)	527 (24.3)	5 (26.3)	0.79
Smoking ever, n (%)	1489 (68.6)	14 (73.7)	0.82
BMI in kg/m², mean ± SD	27.5 ± 3.9	25.9 ±3.7	0.07
Hypertension, n (%)	1623 (74.8)	15 (78.9)	0.80
LDL-cholesterol in mmol/L, median (IQR)	2.9 (2.4-3.5)	2.8 (2.3-3.2)	0.36
Previous myocardial infarction, n (%)	1144 (52.7)	13 (68.4)	0.26

SD: standard deviation, IQR: inter-quartile range

Autoantibodies and mortality in patients with CAD

Kaplan-Meier curves for all-cause mortality showed no differences between ACPA-positive and ACPA-negative patients in the clarithromycin and placebo arms of CLARICOR (Figures 2A-B) and in LURIC (Figure 2C) (log-rank tests not significant). Age- and sex-adjusted Cox regression analyses confirmed these findings (CLARICOR clarithromycin group HR 0.94 (95% CI 0.52-1.68); CLARICOR placebo group HR 0.63 (95% CI 0.26-1.53); LURIC HR 0.87 (95% CI 0.39-1.94); Supplementary Table S4). Similar results were obtained for RF and anti-CarP in CLARICOR (Supplementary Figure S2A-D), which were further confirmed with adjusted Cox regression analyses (Supplementary Table S4). In the LURIC study, RF-positive patients displayed worse survival compared to their RF-negative counterparts (log-rank test p=0.011; Supplementary Figure S2E), but after correction for age and sex this difference was no longer significant (HR 1.40 (95% CI 0.81-2.43); Supplementary Table S4). Based on these results, it seems that the presence of ACPA does not associate with worse all-cause mortality in patients with coronary artery disease.

ACPA, CRP and mortality in RA patients

In contrast to the results in the coronary artery disease cohorts, ACPA positivity has been associated with increased all-cause mortality in multiple cohorts of RA patients (15, 18, 36). This raises the question whether the increase in mortality rates in ACPA-positive RA patients can be solely attributed to the presence of autoantibodies or whether other factors such are systemic inflammation are involved. If so, CRP over time, as a proxy for systemic inflammation, could be a mediator in the association between ACPA and mortality in RA patients (as depicted in Supplementary Figure S1). To investigate this hypothesis, we reanalysed data from two early RA cohorts, the EAC and the BARFOT cohort. During follow-up 137/764 patients (17.9%) died in EAC and 228/794 (28.7%) in BARFOT. Multivariable Cox models on the association between ACPA and all-cause mortality including age, sex, smoking status, and year of inclusion as covariates were repeated and provided results consistent with previous findings (EAC HR 1.66 (95% CI 1.17–2.37); BARFOT HR 1.50 (95% CI 1.13–1.99); Table 4) (15). Furthermore, CRP over time was significantly associated with ACPA positivity (EAC β=0.13 (95% CI 0.08–0.18); BARFOT β=0.16 (95% CI 0.12–0.20)), a prerequisite for CRP to be a potential mediator. When CRP was added using a joint model, the association between ACPA and all-cause mortality was attenuated and became non-significant (EAC HR 1.22 (95% CI 0.70–2.17); BARFOT HR 1.14 (95% CI 0.73–1.75); Table 4, for complete models see Supplementary Tables S5-S6). In contrast, there was a significant indirect effect of logCRP on mortality (EAC HR 1.24 (95% CI 1.14–1.34); BARFOT HR 1.33 (95% CI 1.24–1.42)). Thus, the association between ACPA and all-cause mortality found earlier in BARFOT and EAC is most likely largely explained by CRP.

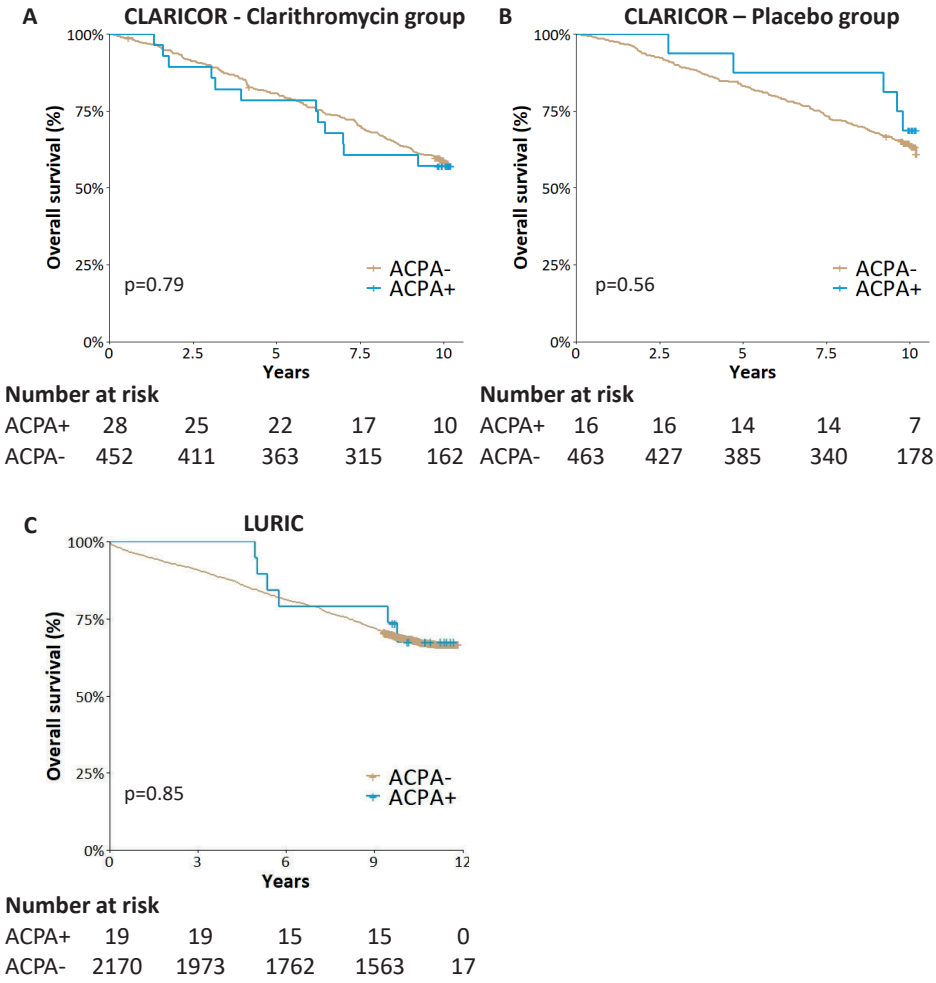

Figure 2: Association between ACPA positivity and all-cause mortality depicted using Kaplan-Meier curves. A CLARICOR clarithromycin treatment group, B CLARICOR placebo group, C LURIC. p-values calculated with log-rank tests. Number of patients at risk in both strata over time is listed below each graph.

Analyses were repeated using cardiovascular mortality instead of all-cause mortality as outcome measure. 40/575 patients (7.0%) in EAC and 141/788 (17.9%) in BARFOT died from cardiovascular causes. A significant association between ACPA positivity and cardiovascular mortality was observed in BARFOT (HR 2.10 (95% CI 1.46–3.01)), but not in EAC (HR 1.57 (95% CI 0.82–2.99)) in multivariable Cox regression. Adjustment for CRP in the joint model reduced the HRs, now being non-significant in both cohorts (EAC HR 0.92 (95% CI 0.31-2.53); BARFOT HR 1.65 (95% CI 0.95–2.92); Table 4, for complete

models see Supplementary Tables S7–S8). The calculated indirect effect for logCRP over time on cardiovascular mortality was significant in both cohorts (EAC HR 1.45 (95% CI 1.27–1.66); BARFOT HR 1.42 (95% CI 1.31–1.54)). Sensitivity analysis using the cumulative CRP model yielded similar results (Supplementary Table S9).

Table 4: Association between ACPA and mortality in two RA cohorts.

	HR (95% CI) in EAC	HR (95% CI) in BARFOT
All-cause mortality		
Adjusted Cox regression	**1.66 (1.17-2.37)**	**1.50 (1.13-1.99)**
Joint model including CRP	1.22 (0.70- 2.17)	1.14 (0.73- 1.75)
Cardiovascular mortality		
Adjusted Cox regression	1.57 (0.82-2.99)	**2.10 (1.46-3.01)**
Joint model including CRP	0.92 (0.31-2.53)	1.65 (0.95-2.92)

Hazard ratios (HR) with 95% confidence interval (95% CI) for ACPA in both adjusted Cox regression (without CRP) and joint models (including CRP) in EAC and BARFOT are given. Outcome was either all-cause mortality or cardiovascular death. Survival analysis was adjusted for age, sex, inclusion period and smoking status.

Discussion

In this study, the relationship between ACPA positivity and mortality was investigated in two large cohorts of CAD patients without RA as well as in two cohorts of RA patients. In contrast to previous findings (26, 27), the prevalence of ACPA in individuals with CAD without RA was not significantly higher than in controls without CAD. In both CLARICOR and LURIC, the presence of ACPA was not associated with worse all-cause mortality in CAD patients. Additional analyses investigating other RA-associated autoantibodies similarly showed no significant association between RF and anti-CarP and mortality in CAD patients without RA. Regarding RA on the other hand, multiple studies have reported a significant association between ACPA positivity and increased all-cause mortality (15, 17, 18, 36). However, using joint modelling analyses we found that the HR for the effect of ACPA on all-cause and cardiovascular mortality became non-significant after addition of CRP over time, while increased CRP levels were significantly associated with mortality. Thus, high inflammation over time seems to be a more important factor for mortality and cardiovascular disease in patients with RA than the presence of ACPA in itself.

The prevalences of ACPA in CAD patients in LURIC and in CLARICOR were lower than the 10% to 11% ACPA positivity previously described in two other CAD non-RA cohorts (26, 27). There might be several explanations for this disparity. Test characteristics might vary between different commercial assays, but this seems unlikely to fully explain the different findings. Differences in size and inclusion criteria between cohorts may further explain differences in ACPA frequencies and outcomes. For example, in CLARICOR 959 patients with previous diagnosis of myocardial infarction or angina pectoris were included, a diagnosis which could have been established several years before, while one of the previous studies included 275 patients at the time of STEMI diagnosis (27). In the other study by Cambridge et al (26). 3052 healthy middle-aged men without clinical cardiovascular disease were included with a follow-up of 5 years, while in LURIC 2189 patient with coronary artery disease were included. Moreover, using the Danish National Patient Register, we could reliably exclude patients with a concomitant diagnosis of RA from the CAD patients in CLARICOR, whereas in the other studies there might have been unidentified RA patients left in CAD groups, skewing the results. The low amount of CAD non-RA patients positive for multiple RA-related autoantibodies further supports the successful exclusion of RA patients from our investigation.

In both RA cohorts, chronic inflammation seemed to play a more important role in the increased all-cause and cardiovascular mortality risk in ACPA-positive RA patients than the presence of ACPA in itself. It is known that systemic inflammation can lead to atherosclerosis and CAD via increased oxidative stress and endothelial dysfunction (1, 2, 8, 9). ACPA positivity might reflect a disease state in which there is more ongoing inflammation, explaining why multiple studies have reported a significant association between ACPA positivity and increased all-cause mortality before (15, 17, 18, 36). Our findings are in line with previous reports that found that the risk of cardiovascular events was higher in RA patients with higher disease activity (37, 38) and increased CRP levels (39, 40).

This insight that inflammation seems a strong driver of increased cardiovascular risk in ACPA-positive RA is relevant for developing new treatment strategies. Early intense treatment in RA was shown to normalise excess mortality rates in ACPA-negative RA patients, but in ACPA-positive patients mortality rates are still higher than in the general population (36). In seropositive RA, ACPA-expressing B cells maintain an active and proliferating phenotype despite treatment, even in patient with clinical disease remission (41). Thus, clinical remission does not appear to equate immunological remission in these patients. Based on our current results, unceasing subclinical inflammation might contribute to the persistent excess mortality in ACPA-positive patients. Hence, therapies specifically targeting this continuous underlying inflammation might be the key to improving long-term outcomes in ACPA-positive RA.

Our study has several limitations. The number of identified ACPA-positive CAD patients was limited despite using large CAD non-RA cohorts, which restricted analyses, for example on cardiovascular mortality. Furthermore, there was no uniformity in ACPA tests among cohorts and differences in specificity and sensitivity might have led to differences in positivity rates. Except for LURIC, no population specific control groups were available. While in CLARICOR RA patients were excluded from the CAD non-RA group with high certainty, comorbidity registration over time was less thorough in LURIC. Using chart review of medication at inclusion and survey data on comorbidity and medication use over time, we tried to exclude RA patients to the best of our possibilities. The low number of LURIC patients that are positive for more than one RA-specific autoantibody indicates this approach was successful, but the possibility that a few RA patients might have remained in the analyses cannot be excluded. Moreover, in both RA cohorts CRP was measured on a yearly basis, providing only a rough estimate of cumulative inflammation over time. In the CAD cohorts, there was no information about inflammation over time available. In the EAC cohort cause-specific mortality data were only available for a subset of patients due to changes in privacy regulations, thereby decreasing the power of the analyses on cardiovascular mortality in this cohort.

Despite these drawbacks, our study also has considerable strengths. Two large CAD cohorts including follow-up data were used, making this, to the best of our knowledge, the most extensive study into the prevalence of ACPA in the non-RA CAD population to date. In addition to ACPA, we also evaluated RF and anti-CarP, which also have been indicated to potentially impact cardiovascular disease and mortality (15, 42). Furthermore, findings in CAD cohorts were complemented with analyses of two large early RA cohorts with well-documented CRP and mortality data. Advanced joint modelling techniques, which are only rarely used in the field of rheumatology despite its appealing name, were applied to adjust mortality analyses in these RA patients for CRP, a time-varying covariate, to optimize the modelling of inflammation over time. In this manner, we feel this study provides the most comprehensive answer to date on the question whether ACPA are associated with cardiovascular disease in individuals with and without RA.

Taken together, our research shows that ACPA positivity is not associated with cardiovascular disease in non-RA patients. The association between ACPA positivity and cardiovascular disease in RA, described before by others and in our cohorts, is likely explained by the higher inflammation over time in seropositive RA patients. Therefore, ACPA positive RA represents a disease subset in which the ongoing chronic inflammatory process seems to be especially critical in the development of cardiovascular and is therefore an important therapeutic target to improve long-term outcomes for RA patients.

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Supplementary information

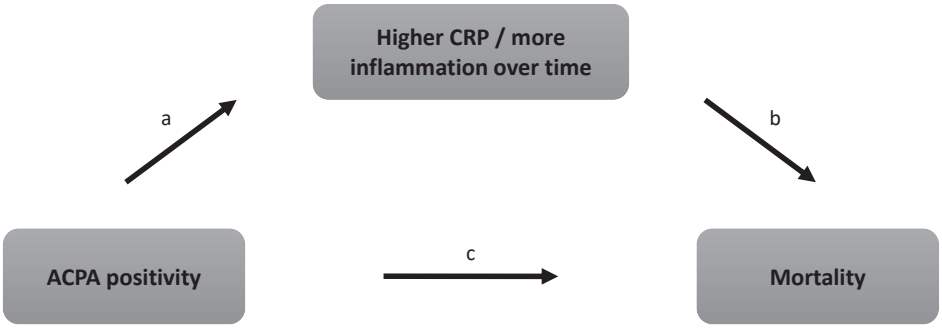
Supplementary Methods S1: Rheumatoid factor (RF) and anti-carbamylated protein antibodies (anti-CarP) in-house ELISA in CLARICOR

Rheumatoid factor

For the RF ELISA, Nunc maxisorp plates (VWR, 430341) were incubated overnight at room temperature (RT) with 10 µg/ml human IgG (Jackson Immunoresearch; 009-000-003). After blocking with PBS/1%BSA for 1 hour at RT, serum samples were diluted 1:100 in PBT and incubated for 1 hour at 37°C. A commercial standard (N/T Rheumatology control SL/2, Siemens) was taken along to calculate arbitrary units. Plates were incubated with goat anti-human-IgM-HRP (Millipore; AP114P) 0,3 µg/ml in PBS/ 1% BSA/ 0.05% Tween (PBT) for 1 hour at 37°C. RF, as well as anti-CARP ELISA's were visualized with ABTS/H_2O_2. Between each step plates were washed with PBS/0.005% Tween 20. Samples were considered RF positive when their value was above the cut-off based on the mean plus 2 times the standard deviation of the optical density (OD) of healthy controls.

Anti-CarP

Anti-CarP IgG was analysed using in-house ELISA with carbamylated fetal calf serum as antigen as described earlier (Shi J, et al. Proc Natl Acad Sci U S A. 2011), with the exception that rabbit anti-human-IgG-HRP was used as single detection antibody. Samples were considered anti-CarP positive when the OD value on the modified protein was at least 2 times the OD on the unmodified protein and samples values were above the cut-off based on the mean plus 2 times the standard deviation of the OD of healthy controls.



Supplementary Figure S1: Graphical depiction of the hypothesis that higher inflammation over time may function as a mediator in the association between ACPA positivity and mortality in RA patients. a·b is the indirect effect of CRP over time and c is the direct effect of ACPA positivity on mortality.

Supplementary Methods S2: Additional information on statistical analyses in RA cohorts
In all analyses CRP was log-transformed and the ACPA-negative group was used as reference. In RA patients, initiation of treatment in treatment-naïve patients usually leads to a steep decline in CRP in the first months, which flattens out during follow-up, leading to a non-linear curve over time. To accommodate for this flexibility, natural cubic splines with 2 degrees of freedom (df) in both the fixed- and random effects were included in the linear mixed model (LMM).

To compute the joint model, a Cox proportional hazard model needed to be specified. The same covariates as in the original study, i.e. age, sex, smoking status and year of inclusion as proxy of changed treatment strategy over time, were included (Ajeganova S, et al. Ann Rheum Dis. 2016). Martingale residuals for age showed a strong deflection of the curve around the age of 55-60. As it is biologically plausible that the risk of cardiovascular morbidity and mortality increases significantly above that age, the age variable was modelled using a natural cubic spline with 2 df. In the analysis of all-cause mortality in EAC, Schoenfeld residuals for smoking status were significant and stratification for smoking status was applied. A similar procedure was followed for sex in the all-cause mortality analysis in BARFOT, both with very little impact on model outcomes. The joint model gives the direct effect (item c in Supplementary Figure S1) of ACPA on mortality. The mediating effect of CRP over time can be obtained by multiplying the effect of ACPA on CRP over time and the effect of CRP on mortality (a·b in Supplementary Figure S1) under the assumption of sequential ignorability. The joint models were computed under a Bayesian approach using package JMbayes2 in R version 4.4.1. Confidence intervals of the indirect effect were calculated using Monte Carlo simulation using the RMediation package.

Supplementary Table S1: Cardiovascular risk factors at baseline in CLARICOR by RF IgM status.

	RF – (n=877)	RF + (n=82)	p-value
Age in years, mean ± SD	65.2 ± 10.4	65.7 ± 10.6	0.66
Female, n (%)	270 (30.8)	23 (28.0)	0.70
Smoking ever, n (%)	741 (80.5)	73 (89.0)	0.35
Clarithromycin group, n (%)	434 (49.5)	46 (56.1)	0.30
Hypertension, n (%)	353 (40.3)	37 (45.1)	0.46
LDL cholesterol in mmol/L, median (IQR)	2.5 (2.1-3.0)	2.3 (2.1-3.1)	0.80
Previous myocardial infarction, n (%)	568 (64.8)	62 (75.6)	0.06

SD: standard deviation, IQR: inter-quartile range

Supplementary Table S2: Cardiovascular risk factors at baseline in LURIC by RF Ig status.

	RF – (n=2164)	RF + (n=24)	p-value
Age in years, mean ± SD	63.5 ± 10.0	69.0 ± 8.3	**0.004**
Female, n (%)	528 (24.4)	4 (16.7)	0.52
Smoking ever, n (%)	1486 (68.7)	16 (66.7)	1
BMI in kg/m², mean ± SD	27.5 ± 3.9	27.6 ± 4.5	0.87
Hypertension, n (%)	1617 (74.7)	20 (83.3)	0.47
LDL-cholesterol in mmol/L, median (IQR)	3.0 (2.4-3.5)	3.1 (2.2-3.9)	0.70
Previous myocardial infarction, n (%)	1144 (52.9)	16 (66.7)	0.25

SD: standard deviation, IQR: inter-quartile range

Supplementary Table S3: Cardiovascular risk factors at baseline in CLARICOR by anti-CarP IgG status.

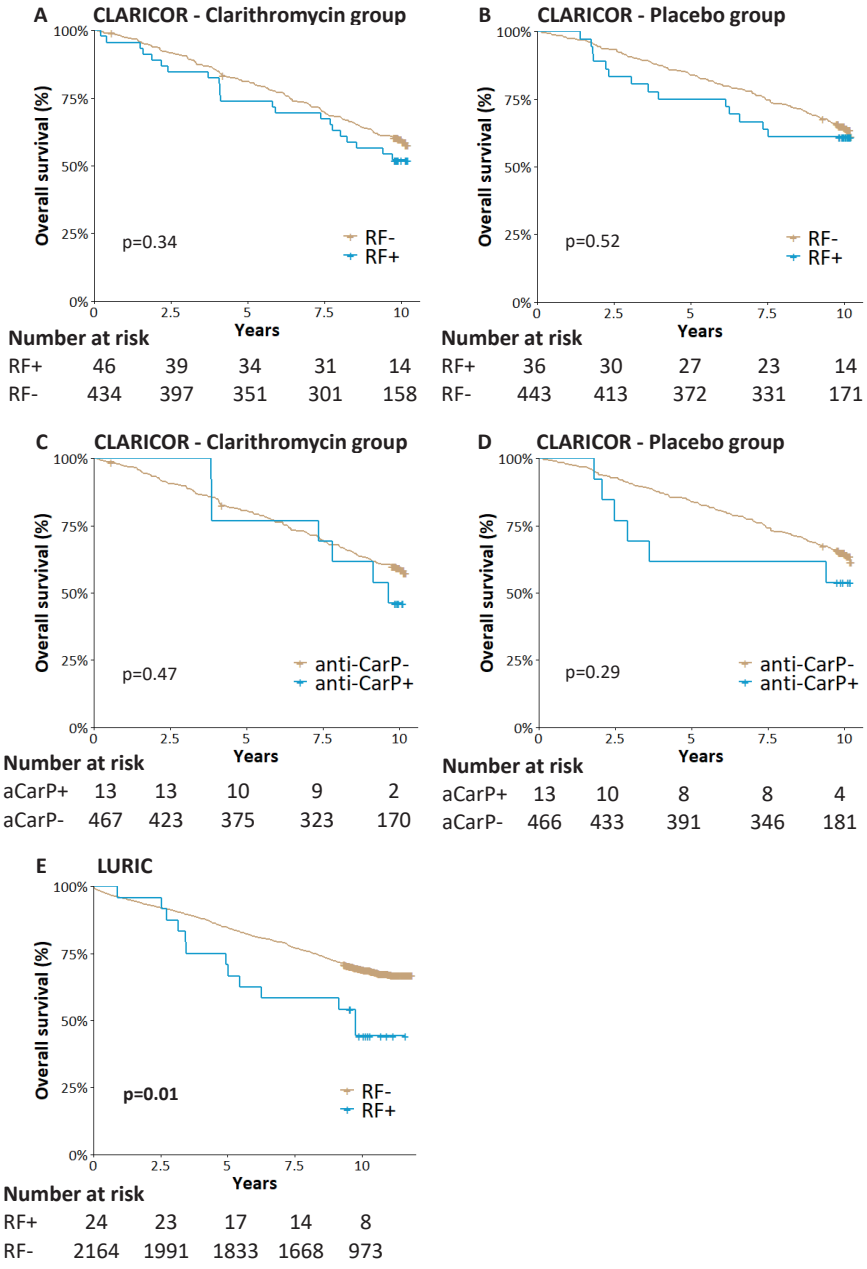
	Anti-CarP – (n=933)	Anti-CarP + (n=26)	p-value
Age in years, mean ± SD	65.3 ± 10.4	64.5 ± 8.8	0.65
Female, n (%)	284 (30.4)	9 (34.6)	0.81
Smoking ever, n (%)	793 (85.0)	21 (80.8)	0.58
Clarithromycin group, n (%)	467 (50.1)	13 (0.5)	1
Hypertension, n (%)	383 (41.1)	7 (26.9)	0.21
LDL cholesterol in mmol/L, median (IQR)	2.5 (2.1-3.0)	2.4 (1.9-2.8)	0.25
Previous myocardial infarction, n (%)	610 (65.4)	20 (76.9)	0.31

SD: standard deviation, IQR: inter-quartile range

Supplementary Table S4: Cox regression for autoantibody positivity and all-cause mortality in CAD patients, adjusted for age and sex.

	CLARICOR Clarithromycin group	CLARICOR Placebo group	LURIC
ACPA (HR, 95% CI)	0.94 (0.52-1.68)	0.63 (0.26-1.53)	0.87 (0.39-1.94)
RF (HR, 95% CI)	1.00 (0.64-1.56)	1.55 (0.90-2.69)	1.40 (0.81-2.43)
Anti-CarP (HR, 95% CI)	1.51 (0.71-3.22)	1.85 (0.81-4.19)	-

Analysis was performed for each autoantibody separately. Hazard ratio (HR) with 95% confidence interval (95% CI) is stated.



Supplementary Figures S2: Kaplan-Meier curves for all-cause mortality stratified for either RF or anti-CarP positivity in CLARICOR (per treatment group) and LURIC. A RF in CLARICOR clarithromycin treatment group, B RF in CLARICOR placebo group, C anti-CarP in CLARICOR clarithromycin treatment group, D Anti-CarP in CLARICOR placebo group, E RF in LURIC. p-values are calculated with log-rank test. Number of patients at risk over time in both strata is stated below each graph.

Supplementary Table S5: Regression coefficients of the survival outcome of the joint model in EAC using all-cause mortality.

	Mean	95% CI	p-value
ACPA positivity	0.197	-0.351 – 0.776	0.51
Male sex	0.224	-0.173 – 0.641	0.27
ns(Age,1)	9.780	5.828 – 14.301	<0.001
Ns(Age,2)	6.275	5.202 – 7.357	<0.001
Year of inclusion 1996-1998	0.185	-0.271 – 0.675	0.44
Year of inclusion ≥1999	-0.366	-0.875 – 0.167	0.15
Log(CRP)	1.623	0.733 – 2.515	<0.001

Coefficients (listed under mean) can be exponentiated to obtain hazard ratios. 95% confidence intervals (95% CI) of the coefficients are given. 764 patients included in the analysis, number of deaths: 137 (17.9%), number of CRP values over time: 3389. Reference for year of inclusion is inclusion <1995.

Supplementary Table S6: Regression coefficients of the survival outcome of the joint model in BARFOT using all-cause mortality.

	Mean	95% CI	p-value
ACPA positivity	0.129	-0.317 – 0.560	0.56
ns(Age,1)	11.293	7.022 – 17.653	<0.001
Ns(Age,2)	7.276	6.160 – 8.914	<0.001
Inclusion after 1-7-1996	-0.351	-0.645 – -0.049	0.02
Smoking ever	0.678	0.390 – 0.983	<0.001
Log(CRP)	1.776	0.996 – 2.582	<0.001

Coefficients (listed under mean) can be exponentiated to obtain hazard ratios. 95% confidence intervals (95% CI) of the coefficients are given. 794 patients included in the analysis, number of deaths: 228 (28.7%), number of CRP values over time: 4203. Reference for year of inclusion is inclusion before 1-7-1996.

Supplementary Table S7: Regression coefficients of the survival outcome of the joint model in EAC using cardiovascular mortality.

	Mean	95% CI	p-value
ACPA positivity	-0.086	-1.161 – 0.927	0.90
Male sex	-0.301	-1.069 – 0.383	0.41
ns(Age,1)	18.980	6.991 – 30.931	<0.001
Ns(Age,2)	7.670	4.972 – 10.418	<0.001
Year of inclusion 1996-1998	0.177	-0.587 – 0.989	0.67
Year of inclusion ≥1999	-1.346	-2.750 – 0.151	0.03
Smoking ever	1.031	0.336 – 1.749	0.003
Log(CRP)	2.349	0.479 – 4.445	0.014

Coefficients (listed under mean) can be exponentiated to obtain hazard ratios. 95% confidence intervals (95% CI) of the coefficients are given. 575 patients included in the analysis, number of deaths from cardiovascular causes: 40 (7%), number of CRP values over time: 2672. Reference for year of inclusion is inclusion <1995.

Supplementary Table S8: Regression coefficients of the survival outcome of the joint model in BARFOT using cardiovascular mortality.

	Mean	95% CI	p-value
ACPA positivity	0.503	-0.047 – 1.072	0.08
Male sex	0.135	-0.286 – 0.504	0.50
ns(Age,1)	28.722	15.275 – 47.967	<0.001
Ns(Age,2)	12.617	9.470 – 17.385	<0.001
Inclusion after 1-7-1996	-0.544	-0.930 – -0.153	0.005
Smoking ever	0.784	0.388 – 1.172	<0.001
Log(CRP)	2.165	1.112 – 3.355	<0.001

Coefficients (listed under mean) can be exponentiated to obtain hazard ratios. 95% confidence intervals (95% CI) of the coefficients are given. 788 patients included in the analysis, number of deaths from cardiovascular causes: 141 (17.9%) number of CRP values over time: 4195. Reference for year of inclusion is inclusion before 1-7-1996.

Supplementary Table S9: Sensitivity analysis using cumulative CRP regarding the association between ACPA and mortality in two RA cohorts.

	EAC	BARFOT
All-cause mortality		
ACPA	1.21 (0.69- 2.13)	1.11 (0.70- 1.73)
Cardiovascular mortality		
ACPA	0.91 (0.34-2.41)	1.54 (0.91-2.61)

Hazard ratios (HR) with 95% confidence interval (95% CI) for ACPA are given, calculated based on a joint model including CRP area under the curve in EAC and BARFOT. Outcome was either all-cause mortality or cardiovascular death. Survival analysis was adjusted for age, sex, inclusion period and smoking status.

General discussion

General discussion

The immune system provides continuous protection from invasive microorganisms, but sometimes an interplay of genetic and environmental factors may lead to reactivity against self-proteins. Autoimmunity can, but does not have to, result in the development of autoimmune disease such as rheumatoid arthritis (RA), a condition causing joint inflammation and eventually joint destruction. Over half of RA patients are positive for antibodies directed against post-translationally modified proteins (AMPA), which are considered to be a reflection of underlying disease pathology. The most clinically important AMPA are anti-citrullinated protein antibodies (ACPA), while anti-carbamylated protein antibodies (anti-CarP) and anti-acetylated protein antibodies (AAPA) can also be found in RA patients (1-3). The processes leading to the break of tolerance against proteins carrying post-translational modifications (PTMs) and the progression from autoimmunity to clinical disease are only partly understood. The current hypothesis is that 'multiple hits' over the course of years in combination with genetic susceptibility can eventually lead to the development of seropositive RA. Better understanding of the mechanisms involved might provide new opportunities for the development of targeted therapies. In this thesis several aspects of the AMPA response are investigated. In **part 1,** the effect of Fc glycosylation on ACPA B-cell receptor function is studied. **Part 2** is focussed on the role of the mucosal immune system in the origin of the AMPA response in RA and in **part 3** the association between the AMPA profile, clinical phenotype and disease outcomes is examined.

Fc glycosylation and ACPA IgG B cell receptor function

Glycans are attached to B-cell receptors (BCRs) and antibodies at specific locations. For example, all IgG contain a conserved N-glycosylation site at position 297 in the CH2 domain of the Fc region (4). Fc glycans have a substantial impact on the structure of IgG antibodies and are essential for the recruitment of antibody effector functions such as complement activation (4, 5). The effect of Fc glycans on ACPA IgG BCR function was investigated in **chapter 2**, using a human germinal center-derived (GC) B-cell line derived from Burkitt lymphoma cells in which the endogenous BCR was knocked out. These cells were transfected with BCR sequences obtained from ACPA IgG B cells in sera of RA patients or with similar sequences containing a mutation at position 297 in the CH2 domain precluding glycan attachment at this site. The absence of the BCR heavy chain glycan did not affect the expression of functional IgG BCRs on the surface of the B cells, despite the previously described involvement of glycans in shaping the 3D structure of BCRs (6). Furthermore, no differences in antigen binding, BCR signalling, and internalization of BCR-antigen complexes were seen in absence of Fc glycans.

Based on the proximity between N(297)-linked glycans and the ITAM-bearing Igα/Igβ signalling complex of the BCR, a functional effect of the Fc glycan on BCR signalling could have been expected. However, the long cytoplasmic tail of the BCR, which amplifies signalling independent of the Igα/Igβ signalling complex (7), might have limited the effect of absent Fc glycans on B-cell activation. It is possible that the impact of constant domain glycans might vary between isotypes and perhaps also between B-cell stages, whereas this study was limited to an IgG GC-derived cell line. Furthermore, in vivo, Fc glycans might interact with lectins expressed on the cell surface or on surrounding immune cells not present in the B cell culture system used in our experiments. Our findings regarding Fc glycans are remarkably different compared to the effects of ACPA IgG BCR variable domain glycans (VDG) in a similar experimental set-up. VDG glycans seem to affect BCR functioning though delayed BCR internalisation, possibly due to differences in spatial BCR organisation (8). Taken together, the results of this study seem to suggest that IgG constant domain glycosylation mainly evolved as a way to modify IgG antibody effector functions and does not play an essential role in IgG BCR functioning. Modulation of Fc glycans is thus essential for the production of antibody-based therapeutics, while unravelling the factors regulating BCR variable domain glycosylation might provide opportunities for development of B cell targeted therapies in RA.

The role of mucosal inflammation in AMPA development

The events leading to the break of tolerance to citrullinated and other post-translational modified proteins and the development of the humoral AMPA response in RA patients remain unclear. One of the current hypotheses, the mucosal origin hypothesis, suggests that AMPA reactivity might originate at mucosal sites. In **chapter 3**, **chapter 4,** and **chapter 5** the potential involvement of mucosal immunity in the AMPA response in RA was investigated using several different methods.

ACPA IgA subclasses

The main antibody isotype present at mucosal surfaces is IgA, of which two subclasses can be produced, IgA1 and IgA2. IgA2 has a shorter hinge region compared to IgA1, making it less susceptible to bacterial proteases (9). This might be one of the reasons why IgA subclass ratios differ throughout the body, with IgA2 being mainly produced in the intestines and to a lesser extent at other mucosal surfaces (10). Furthermore, IgA2 has been reported to have a stronger pro-inflammatory effect on neutrophils and macrophages than IgA1, possibly due to glycosylation differences influencing Fc-receptor binding (11). In **chapter 3**, we found that both total IgA1- and IgA2-levels were increased in seropositive RA patients, compared to seronegative patients and healthy donors. In ACPA IgA seropositive patients, both

circulating ACPA IgA1 and ACPA IgA2 could be detected. Comparable results were found for rheumatoid factor (RF) IgA subclasses. Unfortunately, quantification of ACPA IgA subclass ratios proved challenging, since RF interfered with the ACPA IgA2 ELISA.

The mechanisms behind the elevated IgA (subclass) levels in RA patients are not clear, but there could be a relation with mucosal inflammation. In the bronchial mucosa around 25% of all IgA is of the IgA2 subclass, compared to less than 10% is serum (10). Pulmonary inflammation could thus lead to local production of large quantities of IgA(2), providing a possible explanation for the significantly increased serum levels of total IgA2 and higher RF IgA1 levels in RA patients who smoke. However, research in celiac disease showed that mucosal and serum IgA are clonally related, but do not have the same antibody characteristics and are likely to be produced by different plasma cells (12). Thus, mucosal responses might lead to elevated levels of (antigen-specific) IgA in serum without a typical 'mucosal antibody phenotype' in seropositive RA patients, making it difficult to determine the origin of the elevated serum total IgA(2) levels. Alternatively, elevated IgA subclass levels could be part of a general immunoglobulin hyperproduction in RA patients, for example due to aspecific B-cell hyperreactivity in a proinflammatory context, as IgG and IgM levels can also be increased in RA patients (13-15).

Chapter 3 also explored potential pro-inflammatory effects of IgA2 by examining the association between IgA2 levels and markers of inflammation in RA patients. No association between total IgA2- levels in serum and either CRP or disease activity score were found. Therefore, a substantial contribution of serum IgA2 to systemic inflammation in RA patients does not appear very likely, despite the pro-inflammatory effects described in vitro before. This does not preclude possible effects of IgA2 on local mucosal inflammation in RA patients.

<u>AMPA in mucosal excretions</u>

Several studies have provided evidence of the local production of ACPA at mucosal sites in RA patients. ACPA can be present in both sputum, bronchoalveolar fluid and saliva of RA patients (16-18) and could even be detected in sputum of first-degree relatives of RA patients who did not have detectable ACPA in serum (19). Autoantibody production at the largest mucosal surface, the intestines, was not investigated thus far. There are several indications that the gut is involved in RA pathophysiology. For example, RA patients can be distinguished from healthy controls based on dysbiosis of the gut microbiome (20). Furthermore, circulating plasmablasts in individuals at risk of RA can bind both citrullinated autoantigens and bacteria in faeces (21). The MUCOSA study,

described in **chapter 4,** was designed to investigate whether evidence for local AMPA responses could be found at several different mucosal sites. We assessed not only ACPA, but also anti-CarP, AAPA and RF. To this end, paired serum, saliva and faeces samples of RA patients were collected and tested for autoantibody positivity. The fact that not only citrullinated, but also carbamylated and acetylated proteins can be present at mucosal surfaces (22), and that immunisation with acetylated bacterial proteins could induce a cross-reactive AMPA response in a previous study in mice (23), illustrates the importance of investigating the full AMPA profile.

ACPA, anti-CarP, AAPA IgA antibodies could all be detected in saliva of established seropositive RA patients, although these salivary antibodies were only present in a limited subset of patients. Positivity for a specific AMPA in saliva always coincided with the presence of that specific AMPA in serum, although the detected isotype could differ. This indicates that AMPA can be produced locally at mucosal sites. RF IgA was more abundant in saliva of seropositive patients compared to AMPA and not all saliva RF IgA-positive patients tested positive for RF IgM in serum. No association between saliva ACPA positivity and local inflammatory markers such as saliva total protein content, matrix metalloproteinase 8 (MMP-8) levels and total IgA were observed, although numbers were small. All patients in our study received treatment at the time of sample collection, potentially affecting both local inflammation as well as salivary autoantibody production. Another possible explanation for the low number of saliva AMPA-positive patients is that prior salivary autoantibody responses might have declined over time and titers decreased below the detection limit, similar to the dynamics of anti-pathogen IgA antibodies induced by (upper) airway infections (24).

Although AMPA can be produced and secreted locally in the oral mucosa, no AMPA could be found in faeces samples. Replication in faeces samples of a different Dutch RA cohort and in ileal wash samples of RA patients obtained by colonoscopy in a Swedish study led to similar results. The faecal samples did contain intact antibodies, since total IgA and anti-E. coli IgA antibodies could be readily detected. These data suggest that production of AMPA is limited to certain mucosal sites, with local production of all AMPA taking place in the oral cavity, but not to a substantial degree in the lower intestinal tract (figure 1). However, several other factors might explain the lack of AMPA positivity in faeces, for example strong binding of antibodies to local PTM-containing antigens, degradation of antibodies by digestive enzymes, or the lack of sensitivity of our assays. Unfortunately, the COVID-19 pandemic prohibited the collection of paired sputum samples of RA patients in the MUCOSA study as originally planned.

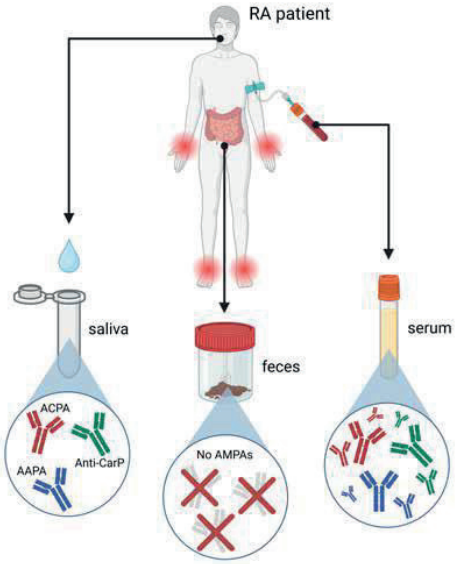


Figure 1: Summary of finding of the MUCOSA study. All AMPA can be found in saliva, but not in faeces of RA patients.

Activated B cells circulate and re-enter to the same tissue where they were activated based on their homing marker expression, although there might be some crossover to other anatomical sites (10). This suggests that plasma cells producing AMPA in the oral mucosa and in the lungs are likely to be derived from B cells activated locally in mucosal associated lymphoid tissue (MALT) or local ectopic lymphoid follicles. Characteristics of the local micro-environment, such as antigen availability and inflammation, might lead to spatial variation in mucosal AMPA production. Neutrophil extracellular traps (NETs), which are increased in saliva and synovial fluid of RA patients (25, 26), might be a potent inducer of ACPA responses, presenting citrullinated proteins in a pro-inflammatory context. In combination with the epidemiological association between RA and both toxic inhalants and periodontitis (27, 28), these data suggest that there is both increased inflammation and a high amount of post-translationally modified proteins present in the oral and pulmonary mucosa. These conditions might be less pronounced in the gut of RA patients, providing a possible explanation for the detection of AMPA in saliva and sputum, but not in faeces of RA patients. Nonetheless, determination of the specific proteins or post-translational modifications driving the AMPA response in patients is difficult, as mice models have shown that initial exposure to a PTM-antigen induces cross-reactive towards other PTMs, a response that can be boosted and skewed upon repeated exposure to different PTM-containing antigens (29).

Onset of RA after COVID-19

Not only bacterial infections, but also inflammation caused by viruses might lead to autoimmunity. The research outlined in this thesis was largely performed in times of the COVID-19 pandemic caused by the Severe Acute Respiratory Syndrome CoronaVirus 2 (SARS-CoV-2), a virus that can lead to severe mucosal inflammation in the lungs. Multiple studies have reported the presence of autoantibodies in patients with severe COVID-19, such as anti-cardiolipin and anti-nuclear antibodies (30). In **chapter 5**, the seroprevalence of ACPA after COVID-19 was investigated among patients visiting the post-COVID outpatient clinic of the Leiden University Medical Center 5 weeks after hospitalization. None of these patients tested positive for ACPA, except two patients previously diagnosed with ACPA-positive RA. Thus, in this limited sample size we could not observe an increase in ACPA-positivity after COVID-19. In addition, five patients who presented in other clinics with polyarthritis compatible with RA after SARS-CoV-2 infection were investigated. Autoantibody measurements in these patients revealed patterns similar to early RA patients presenting before 2019, with two patients being completely seronegative and three patients testing positive for a range of AMPA at presentation. In all post-COVID ACPA-positive RA patients the percentage of ACPA V-domain glycosylation was increased compared to total IgG, similar to ACPA glycosylation in RA patients without preceding SARS-CoV-2 infection. A detailed examination of the specific ACPA IgG V-domain glycan traits in the post-COVID RA patients was performed, as previous research showed inflammatory conditions can induce changes in antibody glycan composition (31). These analyses revealed a significant decrease in bisecting N-Acetylglucosamine-containing moieties, similar to changes observed in total IgG Fc-glycosylation post-COVID. No longitudinal samples were available to investigate whether the glycan profile changed after COVID-infection. The biological consequences of the differences in glycan composition remain unclear, but there is evidence indicating variable domain glycans are able to modulate antibody function (32). Therefore, it is tempting to speculate that changes in antibody glycosylation patterns induced by infections might modulate their pathogenicity.

ACPA responses mature towards RA onset, with rising ACPA IgG levels, epitope spreading and increased variable domain glycosylation, a process which most likely requires multiple events or 'hits'. Given the short time window between COVID-19 and onset of seropositive RA, the similarity in ACPA profile compared to other early RA patients and the lack of ACPA positivity in our post-COVID hospitalisation cohort, it seems more likely that the patients who developed RA shortly after COVID-19 already had quite a mature ACPA response, instead of de novo development of ACPA positivity after infection. In this case, COVID-19 might have been one of the last 'hits' needed to develop clinical disease in these ACPA-positive individuals prone to develop RA, and other (inflammatory) triggers would probably also have provoked disease onset.

The mucosal origin hypothesis

Based on the work presented in this thesis and the research done by others, evidence for a role of mucosal immunity in the development of the AMPA response in seropositive RA accumulates. This model is summarized in figure 2. AAPA IgM can be present in healthy individuals and might thus be part of the physiological immune repertoire, for instance targeting anti-acetylated bacterial proteins (33). Interestingly, germline AMPA IgM can already cross-reactive (34). Environmental risk factors such as smoking and exposure to silica dust might create pro-inflammatory conditions in the oral and airway mucosa and could also increase the amount of post-translationally modified proteins, for example via NETosis (25). Likewise, bacterial dysbiosis and the associated changes in the microbiome metabolome could provide a source of PTMs in combination with inflammatory triggers (20, 35). It is likely that AAPA IgM B cells directed against bacterial components can receive T-cell help, for example from mucosal Th17 T cells. Under the proinflammatory conditions described above, these physiological AAPA response might give rise to a class-switched AMPA clone, reactive to multiple post-translationally modified antigens. This cross-reactive AMPA response could be shaped and matured further over time by repeated expose to different PTM-containing triggers (29), which may vary between individuals. Based on the data described in this thesis, the oral and respiratory mucosa seem the preferred sites of mucosal AMPA production.

It remains unclear how local mucosal B cell responses, whether they target foreign antigens or self-antigens like PTM containing proteins, are related to circulating (auto) antibodies (36, 37). One of the hypotheses is that transient episodes of subclinical bacteraemia might occur, caused by damage to mucosal barriers during infections or by mechanical stress for example during toothbrushing (38). This systemic exposure to either pathogenic bacteria, bystander microorganisms or other pro-inflammatory proteins carrying PTMs, could give rise to a systemic (IgG) AMPA response. It has even been suggested that bacteraemia may lead to translocation of bacteria to the synovial compartment, where they might trigger a local inflammatory reaction. The detection of DNA from Prevotella copri and other bacteria in synovial fluid of RA patients (39, 40) supports this idea. However, mucosal antibody responses might also directly lead to IgG production, as IgG is abundant in the lower respiratory tract (41, 42), where it might be transported to the lumen by binding to the epithelial neonatal Fc receptor (FcRn) (43). ACPA IgG has indeed been detected in sputum of RA patients and individuals at risk for RA (19). Over time, the AMPA response might enter the next stage of development under influence of HLA-shared epitope-restricted T cells (44), although the circumstances provoking this process are currently unclear. These events may lead to broadening of the AMPA profile, including epitope spreading and increased VDG-glycosylation (45, 46), features associated with the transition from autoimmunity to autoimmune disease.

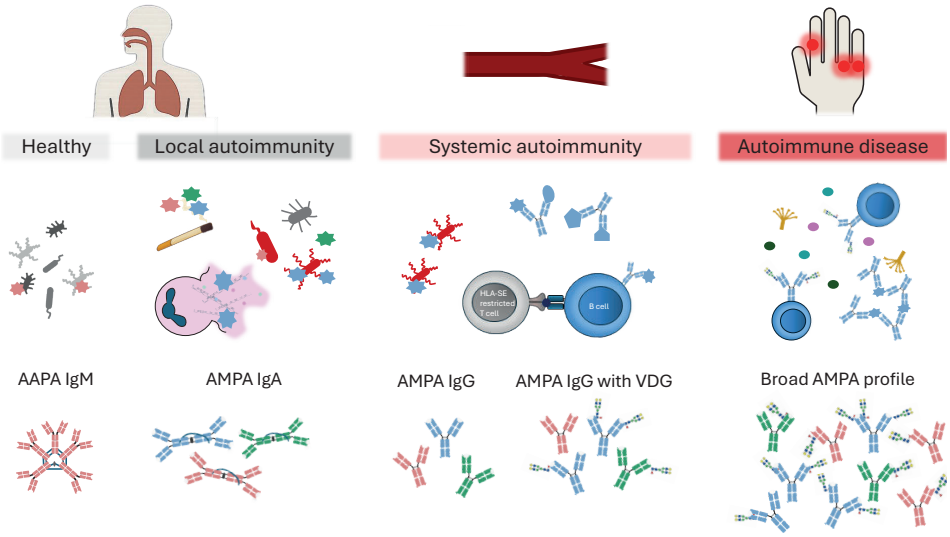


Figure 2: The mucosal origin hypothesis in RA. AAPA IgM, for example targeting acetylated bacterial components seems part of the physiological immune repertoire. Smoke inhalation, microbiome dysbiosis and NETs may lead towards a broader local humoral immune response in the oral and respiratory mucosa. Further 'hits' lead to the development of a systemic AMPA response, for example due to bacteraemia. Over time more epitopes are recognised and VDG glycans are introduced. Eventually arthritis occurs.

Although this model is named the 'mucosal origin hypothesis', the described mucosal immunological processes might be involved in different stages during disease development, such as the introduction of AMPA reactivity, diversification of AMPA response, but a potential role in disease flares should also be considered.

AMPA positivity and clinical disease presentation

The **third part** of this thesis is focused on the AMPA profile in relation to clinical phenotype and disease outcomes in RA. It is known that ACPA-positive patients have worse disease outcomes with more radiological damage over time (47). Whether the AMPA profile is also associated with disease presentation is investigated in **chapter 6**, by analysing the relationship between several clinical features at baseline and the number of autoantibodies detectable in serum. The autoantibodies included in this study were RF IgM, ACPA IgG and anti-CarP IgG, as data on the presence of AAPA in RA patients were first published at the time the manuscript was already drafted (3). In two early RA cohorts, positivity for multiple autoantibodies was associated with younger age of disease onset, smoking, longer symptom duration and higher inflammatory markers at presentation. This effect was not driven by the presence

of a specific individual autoantibody, but rather by the combined positivity for multiple autoantibodies. The observation that patients with multiple autoantibodies develop RA at a younger age might indicate a stronger genetic predisposition in these individuals, accelerating the development of autoimmunity and/or the transition from autoimmunity to autoimmune disease (48). Smoking can potentially increase the amount of protein citrullination, carbamylation and acetylation in the lung (49-51) in pro-inflammatory conditions, providing a possible explanation for the association between smoking and the break of tolerance against multiple PTMs. The AMPA response develops and expands over a long period of time. Less potent last 'hits' might be required to trigger RA onset in individuals with a broad AMPA profile, which could result in a more insidious disease onset in these patients. Moreover, patients who were positive for various autoantibodies also showed higher levels of these autoantibodies, a broader isotype usage and reactivity against more fine specificities. Thus, the number of autoantibodies present can be viewed as a marker for the breath/activity of the humoral autoimmune response which might therefore explain the association between number of autoantibodies present and increased systemic inflammation at disease onset. In conclusion, the extent of the autoantibody response is related to disease presentation in RA patients, which implies a role for the AMPA response in disease development. However, it should be taken into consideration that variations in the AMPA profile could also represent a by-effect of underlying inflammatory processes, given that no direct pathogenic effect of AMPA has been established.

The AMPA profile and treatment response in RA

Whether the autoantibody profile is not only related to clinical presentation, but also to treatment response, was investigated in **chapter 7**. To this end, positivity for RF, ACPA, anti-CarP and AAPA isotypes was measured in serum samples collected over time of patients in the IMPROVED study. Patients who tested positive for a range of autoantibodies at disease presentation showed a larger decrease in disease activity score (DAS) within the first four months after treatment initiation with methotrexate and high-dose prednisone, compared to patients with a more limited autoantibody profile. According to the IMPROVED protocol, medication was tapered in patients with low DAS after initial treatment. The presence of multiple AMPA at baseline was associated with unfavourable outcomes after drug tapering, with a high chance of disease flares within one year after medication was withdrawn. AMPA measurements at the moment of drug tapering did not provide additional prognostic value compared to the baseline autoantibody profile regarding the risk of disease flares after treatment discontinuation. In the subset of patients that did reach drug-free remission for over a year after drug tapering, AMPA-positivity was a risk factor for the relapse of arthritis, but no association between disease flares and the breath of the autoantibody profile was

observed. These data suggest that patients with a broad humoral auto-immune profile respond well to initial treatment with methotrexate and steroids with rapid lowering of disease activity, but that this therapy seems to suppress rather than definitively resolve the inflammation. In most AMPA-positive patients, disease flares at some point after drug tapering, except for a small subset of AMPA-positive patients that reaches sustained drug-free remission. Currently, it is unclear which immunological features are associated with this lasting medication-free remission, the closest approximation of disease cure to date. AMPA levels remain detectable in these patients after all. It is tempting to speculate that the underlying immune response in the patients with sustained drug-free remission has been reverted to a state similar to pre-disease, where there is autoimmunity, but no autoimmune disease. Identification of the autoantibody and B-cell characteristics related to sustained drug-free remission in AMPA-positive patients could provide new insights in the pathogenicity of AMPA in RA.

Long-term disease outcomes in ACPA-positive individuals

Long-term disease outcomes in RA patients are currently not determined by joint destruction, but by the development of potentially life-threatening extra-articular manifestations like interstitial lung disease and premature atherosclerotic disease. The introduction of early intensive treatment in RA has normalised mortality rates in ACPA-negative RA patients, but in ACPA-positive RA excess mortality remains, mainly from cardiac causes (52). An association between ACPA and increased cardiovascular mortality was reported in RA patients (53, 54), although these finding could not be replicated in other studies (55, 56). Therefore, in **chapter 8** the role of ACPA in coronary artery disease (CAD) was investigated in more detail. These analyses were not only performed in RA patients, but also in non-RA patients, as two studies reported the presence of ACPA in approximately 10% of patients with CAD without concomitant RA (57, 58). In these non-RA patients, ACPA was associated with unfavourable CAD outcome (57). However, in the study presented in this thesis, we found no increased prevalence of ACPA in two large CAD non-RA cohorts. Furthermore, presence of ACPA did not lead to worse all-cause survival in these patients.

Given the conflicting results regarding the relationship between ACPA and cardiovascular mortality, we hypothesised that in seropositive RA the inflammatory burden rather than the sole presence of the autoantibodies themself might be associated with increased cardiovascular disease development, as systemic inflammation is a known risk factor for accelerated atherosclerosis (59). Therefore, data from two RA cohorts, in which an association between ACPA and all-cause mortality was found before, were reanalysed using a joint modelling approach to include longitudinal CRP measurements, a marker for inflammation. The hazard ratios for the effect of ACPA on all-cause and cardiovascular

mortality became non-significant after addition of CRP over time to the analyses, while higher CRP levels were significantly associated with mortality. Thus, high inflammation over time seems to be a more decisive factor for cardiovascular disease development and mortality in patients with RA than the presence of ACPA by itself. A direct role of ACPA in cardiovascular disease, for example via binding to citrullinated proteins in the atherosclerotic plague or via FcγRIIa-dependent activation of platelets, may be considered less likely based on these data. Despite the fact that CRP was only measured at intervals of a year or longer and thus only gives a rough estimation of total inflammation, these findings infer that current intensive treatment is insufficient to subdue the continuous inflammatory processes in seropositive RA patients.

In RA patients, the presence of ACPA seems to reflect a disease state in which there is more ongoing inflammation. It is known that ACPA-expressing B cells retain an active and proliferating phenotype during treatment, even in patient in clinical disease remission (60), indicating that clinical remission is not equal to immunological remission. It cannot be excluded that the continuous activation of AMPA B cells could contributes to the chronic systemic inflammation in seropositive RA patients and thus to premature CAD development. Better insights in the processes underlying the sustained inflammatory response in AMPA-positive RA patients might provide new opportunities to improve long-term disease outcomes, as cardiovascular mortality is still a major problem in seropositive RA.

Conclusion and further perspectives

In RA patients, AMPA are related to phenotype at disease presentation and treatment response. Furthermore, ACPA-positive patients seem to have more chronic inflammation, which can affect long-term prognosis. The pathophysiological processes underlying AMPA development and their role in disease onset are unclear. Data presented in this thesis suggest that local mucosal immune responses in the airway and oral cavity may be involved in AMPA production. The higher levels of total IgA1 and IgA2 observed in seropositive RA patients also suggest that mucosal antibody production is increased in these patients. No evidence of de novo AMPA development after COVID-19, a potentially severe mucosal infection, was found, but this viral infection could be one of the final 'hits' leading to RA development in seropositive asymptomatic individuals, as several cases of new-onset RA after COVID-19 have been reported.

To gain further insights in the factors associated with the development of local and eventually systemic autoimmunity in RA, it is key to investigate AMPA responses in mucosal excretions and tissue in more detail. Exploring the relationship between bacterial disbalance, the microbial metabolome and local barrier function in mucosal

tissue, including the respiratory and the female reproductive tract, could provide useful new insights. Moreover, better understanding of the interactions between mucosal AMPA B cells and T cells and the crosstalk between mucosal and systemic immunity in different stages before and after onset of disease, might provide new pieces of the puzzle that is RA pathophysiology. Investigating AMPA B cell homing to mucosal tissue, bone marrow and the synovial compartment, could be one of the first steps in this process.

The influence of current RA treatments, including non-steroidal anti-inflammatory drugs (NSAIDS) known to cause intestinal damage, on mucosal (auto)immune responses in RA also remains an open question. Yet studies into these mechanisms are complex, as collection and usage of mucosal samples is more demanding compared to serum. Furthermore, the mucosal humoral responses involved in the development of AMPA can occur years before onset of symptoms. Fortunately, increasing attention for mucosal immunity in many diseases in combination with novel experimental techniques present new research possibilities. Integration of insights found in RA and other (autoimmune) diseases has the potential to broaden our understanding of general and disease-specific mucosal humoral immune responses.

Meanwhile, we should not hesitate to the translate the insights gained so far on RA pathophysiology into actions to prevent disease and improve quality of life for RA patients. For years, it has been clear that smoking is a major risk factor for RA development and that RA patients who smoke respond worse to treatment while having a higher risk of cardiovascular complications (61). Therefore, both healthcare workers and policy makers should give smoking cessation priority. The power of lifestyle adjustments in RA care was recently further illustrated by the 'Plants for Joints' study, which showed that a 16-week lifestyle program including a whole-food plant-based diet, physical activity and stress management decreased disease activity in RA patients (62). High fibre intake is associated with beneficial alterations in the gut microbiome (63) and might even alter systemic immunity in RA patients (64). These data illustrate the need for larger studies on dietary and microbiome interventions in RA, although funding of these studies could prove more challenging compared to studies on pharmacotherapy. However, the increase in lifestyle-related diseases worldwide justifies prompt implementation of lifestyle education for the whole population, including seropositive RA patients and their relatives.

To conclude, it is an exciting time in translational RA research with new opportunities to study the immunological depths of the mucosal and systemic AMPA response and momentum for novel ideas regarding therapeutic interventions to improve the clinical course for RA patients.

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Addendum

Nederlandse samenvatting
Curriculum vitae
List of publications
Acknowledgements

Nederlandse samenvatting

Seropositieve reumatoïde artritis

Het immuunsysteem beschermt het lichaam continu tegen ziekmakende micro-organismen. Door een combinatie van verschillende factoren, waaronder genetische en omgevingsfactoren, valt het immuunsysteem soms lichaamseigen structuren aan, bijvoorbeeld door antilichamen tegen lichaamseigen eiwitten te maken. Dit wordt auto-immuniteit genoemd. Auto-immuniteit kan, maar hoeft niet, te leiden tot een auto-immuunziekte, zoals reumatoïde artritis (RA). RA is een ziekte waarbij ontsteking en uiteindelijk schade van de gewrichten optreedt, met name in de kleine gewrichten van de handen en voeten. RA kan ook ontstekingen veroorzaken buiten de gewrichten en kan bijvoorbeeld leiden tot interstitiële longziekten en het vroegtijdig ontwikkelen van hartvaatziekten. Bij meer dan de helft van de RA-patiënten zijn autoantilichamen tegen post-translationeel gemodificeerde eiwitten (AMPA) te meten in het bloed. Post-translationele modificaties (PTMs) zijn veranderingen in de biochemische structuur van aminozuren, de bouwstenen van eiwitten, nadat het eiwit al is gevormd. Er zijn verschillende post-translantionele modificaties waartegen patiënten met RA kunnen reageren. De meeste bekende AMPA zijn autoantilichamen gericht tegen gecitrullineerde eiwitten (ACPA). ACPA worden door reumatologen gebruikt als hulpmiddel om RA vast te stellen. ACPA kunnen aan verschillende gecitrullineerde eiwitten binden, zowel aan lichaamseigen als aan lichaamsvreemde gecitrullineerde eiwitten. Daarnaast worden ook autoantilichamen tegen gecarbamyleerde eiwitten (anti-CarP) en autoantilichamen tegen geacetyleerde eiwitten (AAPA) gevonden bij RA-patiënten. Er is een bepaalde mate van kruisreactiviteit tussen deze AMPA, waarbij bijvoorbeeld sommige antilichamen tegen gecitrullineerde eiwitten ook kunnen binden aan gecarbamyleerde eiwitten. AMPA kunnen al jaren voor het ontstaan van gewrichtsontstekingen in het bloed aanwezig zijn. Verder kan ook reumafactor (RF), een ander soort autoantilichaam, aanwezig zijn in RA-patiënten. Bij een deel van de patiënten met RA is geen van bovengenoemde autoantilichamen aantoonbaar in het bloed. RA-patiënten zonder autoantilichamen worden seronegatief genoemd, terwijl patiënten met autoantilichamen seropositief zijn. Seronegatieve en seropositieve RA-patiënten hebben een verschillend ziektebeloop en ook de onderliggende risicofactoren voor het ontwikkelen van RA verschillen tussen beide groepen.

AMPA zijn heel specifiek voor RA, maar het is niet duidelijk hoe auto-immuniteit tegen gemodificeerde eiwitten ontstaat. Daarnaast is ook hun rol in het ziekteproces niet zeker. Er is bijvoorbeeld nooit aangetoond dat AMPA reumatoïde artritis veroorzaken. Een van de hypotheses over het ontstaan van AMPA is dat een combinatie tussen genetische aanleg en meerdere gebeurtenissen in de loop van tijd zorgen voor de ontwikkeling van deze

autoantilichamen en dat de onderliggende immuunprocessen uiteindelijk leiden tot RA. Mogelijk spelen de slijmvliezen hier een rol in. In dit proefschrift worden verschillende aspecten van de AMPA-response onderzocht, zowel factoren die een rol kunnen spelen bij het ontstaan van AMPA als het effect van AMPA op het ziektebeloop van patiënten.

ACPA B-cellen en glycanen

In **deel 1, hoofdstuk 2** is gekeken naar B-cellen gericht tegen gecitrullineerde eiwitten. B-cellen zijn witte bloedcellen die uiteindelijk leiden tot de productie van antilichamen. De B-cel receptor (BCR) heeft dezelfde structuur als een antilichaam, maar zit op het celmembraan van de B-cel gebonden. Aan zowel de B-cel receptor als aan een antilichaam zijn op specifieke plaatsen suikerstructuren (glycanen) gebonden. ACPA-antilichamen en ACPA B-cel receptoren van de IgG klasse hebben de bijzondere eigenschap dat ze niet alleen suikerstructuren dragen in het constante domein (Fc), maar ook in de antigeen bindende regio, oftewel het variabele domein. Deze variabele domein glycanen (VDG) verhogen de activeringsstatus van de B-cel wanneer de B-cel receptor aan gecitrullineerde eiwitten bindt. In **hoofdstuk 2** is de invloed van Fc suikerstructuren, die op alle IgG B-cel receptoren voorkomen, op de ACPA B-cel onderzocht. De afwezigheid van de Fc-glycaan heeft geen invloed op de binding van de B-cel receptor aan gecitrullineerde eiwitten en ook geen invloed op de activering van de B-cellen. In tegenstelling tot variabele domein glycanen, lijken de Fc-glycanen dus minder belangrijk voor de B-cel receptor functie. Voor de functie van antilichamen zijn de Fc-glycanen wel heel belangrijk, omdat ze een rol spelen in de binding aan Fc-receptoren op verschillende witte bloedcellen en aan eiwitten van het complementsysteem, die een essentiële rol spelen in de immuunrespons.

De rol van de slijmvliezen bij het ontstaan van AMPA

Een van de hypotheses is dat de slijmvliezen betrokken zijn bij het ontstaan van AMPA. In **deel 2** van dit proefschrift wordt deze hypothese op verschillende manieren onderzocht. In **hoofdstuk 3** is gekeken naar antilichamen van de IgA klasse. IgA wordt veel meer geproduceerd ter hoogte van de slijmvliezen dan in het bloed. Er zijn twee subklassen van IgA, namelijk IgA1 en IgA2. De hoeveelheid IgA1 en IgA2 is niet gelijk verdeeld. In het bloed is bijna al het IgA van de IgA1 subklasse, terwijl bij de longslijmvliezen een kwart van het totale IgA uit IgA2 bestaat. In de dikke darm is het meeste IgA2 aanwezig, daar is ongeveel de helft van al het IgA van de IgA2 subklasse. Uit ons onderzoek bleek dat zowel de totale IgA1 waardes als de totale IgA2 waardes verhoogd zijn in het bloed van seropositieve RA-patiënten, vergeleken met seronegatieve RA-patiënten en gezonde vrijwilligers. ACPA-IgA antilichamen kunnen zowel in de IgA1 als de IgA2 vorm voorkomen in het bloed van seropositieve RA-patiënten, net als reumafactor IgA1 en IgA2. De precieze verhouding tussen ACPA IgA1 en IgA2 kon niet worden vastgesteld,

omdat reumafactor de testen verstoorde. De reden van de verhoogde IgA (subklassen) waardes in RA is niet geheel duidelijk, maar een van de mogelijke verklaringen is dat RA-patiënten meer ontsteking bij de slijmvliezen hebben. Dit wordt ondersteund door de bevinding dat RA-patiënten die roken een hoger totaal IgA2 en RF IgA1 hebben dan in RA-patiënten die nooit hebben gerookt. Een andere denkbare verklaring is dat de B-cellen in RA-patiënten veel te actief zijn, aangezien andere antilichaamklassen zoals IgG en IgM ook verhoogd kunnen zijn in RA. Eerder is beschreven dat IgA2 ontsteking sterk kan bevorderen, meer dan IgA1 dat doet. In onze studie vonden we geen relatie tussen de hoogte van de IgA2-waardes en een ontstekingswaarde (CRP) in het bloed en er was ook geen relatie tussen IgA2 en ziekteactiviteit in RA-patiënten.

Eerdere onderzoeken hebben aangetoond dat ACPA aanwezig kunnen zijn in slijm uit de longen en in speeksel van seropositieve RA-patiënten. De aanwezigheid van ACPA in de darm, het grootste slijmvliesoppervlakte in het lichaam, was nog niet onderzocht. Daarnaast was ook niet bekend of andere AMPA dan ACPA aanwezig zijn bij de slijmvliezen. Om deze vragen te onderzoeken, hebben we de MUCOSA-studie opgezet, waarin we bloed, speeksel en ontlasting van patiënten met RA en gezonde individuen hebben verzameld. De resultaten, beschreven in **hoofdstuk 4**, laten zien dat zowel ACPA, anti-CarP en AAPA IgA antilichamen aanwezig kunnen zijn in speeksel van seropositieve RA-patiënten, hoewel ze slechts bij een klein deel van de patiënten werden gevonden. In het bloed van deze patiënten waren dezelfde AMPA aanwezig, maar niet altijd van de IgA klasse. Dit betekent dat de IgA AMPA in speeksel lokaal geproduceerd zijn. RF IgA kwam vaker voor in speeksel van seropositieve RA-patiënten. De aanwezigheid van autoantilichamen was niet geassocieerd met hogere ontstekingswaarden in het speeksel, maar alle patiënten in de studie kregen behandeling met medicijnen voor RA op het moment dat de lichaamsmaterialen afgenomen werden.

In tegenstelling tot speeksel waren er geen AMPA aantoonbaar in ontlasting. Om deze resultaten te bevestigen zijn ontlastingsmonster van RA-patiënten die deelnamen aan een andere Nederlandse studie onderzocht, net als monsters die zijn afgenomen tijdens een kijkonderzoek van de dikke darm in Zweedse RA-patiënten. In al deze monsters was geen AMPA meetbaar, terwijl er wel IgA antilichamen en antilichamen tegen de darmbacterie E. coli aantoonbaar waren. Onze onderzoeksresultaten suggereren dat er lokale productie is van AMPA in de mondholte, maar niet in de darm.

In de periode dat de onderzoeken beschreven in dit proefschrift zijn uitgevoerd, werd de wereld geconfronteerd met de COVID-19 pandemie, veroorzaakt door het nieuwe coronavirus SARS-CoV-2. Dit virus veroorzaakt ernstige ontsteking met name in de luchtwegen en kan daarnaast leiden tot de vorming van autoantilichamen. Gezien het

mogelijke verband tussen de slijmvliezen van de luchtwegen en ACPA, hebben we in **hoofdstuk 5** onderzocht of ACPA vaker voorkomen na een ernstige COVID-19 infectie. Geen van de patiënten die de speciale COVID polikliniek in het LUMC bezochten na ziekenhuisopname wegens COVID-19 testten positief op ACPA in het bloed, behalve twee patiënten die al voor COVID-19 gediagnostiseerd waren met seropositieve RA. We vonden dus geen toename van ACPA na COVID-19, hoewel het aantal mensen wat aan de studie meedeed beperkt was. Daarnaast hebben samen met verschillende ziekenhuizen en klinieken in Nederland vijf patiënten geïdentificeerd die RA ontwikkelden na hun COVID-19 infectie. Drie van de vijf patiënten waren seropositief. Het AMPA-profiel en de hoeveelheid suikerstructuren in het variabele domain van ACPA waren vergelijkbaar met patiënten die RA hadden gekregen voor de coronapandemie. Het is bekend dat de uitgebreide AMPA-reacties zich vaak meerdere jaren voor de eerste gewrichtsklachten ontwikkelen. Daarom is het aannemelijk dat de patiënten die RA kregen net na COVID-19 al een uitgebreid autoantilichaam profiel hadden en dat dit niet door de virusinfectie is ontstaan. Het is wel mogelijk dat de ontstekingsreactie in COVID-19 het 'laatste zetje' heeft gegeven voor het ontwikkelen van RA, maar waarschijnlijk had een infectie met een ander virus of bacterie ook tot het ontwikkelen van RA geleid bij deze patiënten.

De relatie tussen AMPA en ziekte kenmerken

In **deel 3** van dit proefschrift is gekeken naar de samenhang tussen het AMPA-profiel, ziektepresentatie en ziektebeloop in RA. In **hoofdstuk 6** is de associatie tussen AMPA en symptomen/kenmerken bij eerste presentatie van de RA-patiënt bekeken. Het blijkt dat patiënten met meer autoantilichamen jonger zijn als de ziekte zich openbaart in vergelijking met patiënten met minder AMPA-autoantilichamen. Mogelijk hebben patiënten met meer autoantilichamen een sterkere genetische aanleg, wat zou kunnen verklaren dat ze de ziekte op jongere leeftijd ontwikkelen. Daarnaast roken patiënten met meerdere autoantilichamen vaker en hebben ze hogere ontstekingswaarden in het bloed. Ook hebben ze langer klachten voordat RA daadwerkelijk wordt vastgesteld dan RA-patiënten met weinig autoantilichamen. Deze effecten worden niet veroorzaakt door de aanwezigheid van één specifiek autoantilichaam, maar door de aanwezigheid van meerdere autoantilichamen tegelijkertijd. Dit impliceert dat AMPA een rol spelen in de ontwikkeling van RA, maar omdat dit nooit is aangetoond, kunnen de AMPA-autoantilichamen ook een bijproduct zijn van een onderliggend ontstekingsproces wat uiteindelijk zorgt voor de ontwikkeling van RA.

In **hoofdstuk 7** is onderzocht of AMPA gerelateerd zijn aan de mate waarop RA-patiënten reageren op de eerste behandeling met medicatie. In de eerste maanden van de behandeling met methotrexaat en hoge dosis prednison daalt de ziekteactiviteit van patiënten met meerdere autoantilichamen harder dan in patiënten met weinig

autoantilichamen. Na het afbouwen van de medicijnen was een uitgebreide AMPA-reactie juist geassocieerd met meer ziekte opvlammingen. De hoogte van de autoantilichamen op het moment van afbouwen leverde geen extra informatie op over het risico op ziekte opvlammingen vergeleken met de hoogte van AMPA gemeten aan het begin van de ziekte. Deze data suggereren dat RA-patiënten met een breed AMPA-profiel goed reageren op medicijnen en dat deze medicijnen de ziekte goed kunnen onderdrukken, maar dat de gewrichtsontstekingen in de meeste seropositieve patiënten terugkomen na het staken van de medicijnen. Een uitzondering is een kleine groep AMPA-positieve patiënten die na het staken van de medicijnen lange tijd geen gewrichtsontstekingen meer hadden. In al deze patiënten blijven AMPA meetbaar. Het immuunsysteem van deze patiënten lijkt op een of andere manier terug te zijn gegaan naar de situatie voordat de ziekte zich openbaarde, waarbij er wel autoantilichamen aanwezig zijn, maar geen ziekte. Waarom dit bij sommige mensen wel gebeurt, maar bij de meeste niet, moet verder worden onderzocht.

De langetermijnprognose wordt in RA-patiënten niet bepaald door gewrichtsontstekingen, maar door het ontwikkelen van potentieel dodelijke RA-gerelateerde aandoeningen buiten de gewrichten, zoals longproblemen en hartvaatziekten. Door betere behandeling is de levensverwachting van RA-patiënten zonder ACPA genormaliseerd, maar ACPA-positieve RA-patiënten hebben nog steeds een kortere levensverwachting ten opzichte van de algehele bevolking, met name door hartvaatziekten. In sommige eerdere onderzoeken in RA is een associatie tussen ACPA en hartvaatziekten gevonden, maar in andere studies werd dit verband niet gezien. Daarom hebben we de relatie tussen ACPA en sterfte (door hartvaatziekten) in **hoofdstuk 8** nader onderzocht. Hiervoor hebben we niet alleen RA-patiënten bestudeerd, maar ook patiënten zonder RA met hartvaatziekten. Uit eerdere onderzoeken bleek dat 10% van deze hartvaatziekten patiënten zonder RA ook meetbaar ACPA in het bloed hadden en dat ACPA geassocieerd waren met slechtere ziekte uitkomsten. Dat was verrassend, omdat ACPA specifiek gevonden worden bij mensen met RA. We hebben dit onderzoek herhaald in twee grotere groepen patiënten met hartvaatziekten, na het zorgvuldig uitsluiten van RA-patiënten. Uit ons onderzoek blijkt dat ACPA niet vaker voorkomen in hartvaatziekten patiënten zonder RA dan in de algemene bevolking. Daarnaast was de aanwezigheid van ACPA niet geassocieerd met de overleving in deze individuen.

Op basis van deze resultaten en eerdere onderzoeken vroegen we ons af of in ACPA-positieve RA niet de aanwezigheid van autoantistoffen, maar chronisch verhoogde ontstekingswaardes belangrijk zijn voor het ontwikkelen van hartvaatziekten. Om dit te onderzoeken is een eerdere studie in RA-patiënten, waarin een verband was gevonden

tussen ACPA en sterfte (met name door hartvaatziekten), opnieuw geanalyseerd. Door middel van nieuwere statistische methoden hebben we CRP, een maat voor ontsteking in het bloed, toegevoegd aan de analyse. CRP was over de jaren heen op meerdere momenten gemeten in deze studie. Na het toevoegen van CRP aan de analyse was het verband tussen ACPA en sterfte niet meer aantoonbaar. In tegenstelling waren de langdurige verhoogde ontstekingswaardes wel geassocieerd met sterfte in RA. Het lijkt dus niet zozeer de aanwezigheid van ACPA, maar verhoogde ontstekingsparameters in ACPA-positieve RA-patiënten die kunnen leiden tot sterfte aan hartvaatziekten.

Conclusie

In dit proefschrift zijn verschillende aspecten van de AMPA-response in reumatoïde artritis patiënten onderzocht: van nieuwe aanwijzingen dat de slijmvliezen betrokken zijn bij de productie van AMPA tot de relatie van AMPA met ziektepresentatie en ziekte uitkomsten. In **hoofdstuk 9** worden de bevindingen beschreven in dit proefschrift besproken in de context van de bestaande literatuur. De resultaten geven aanleiding tot verder onderzoek, bijvoorbeeld naar de details van de relatie tussen de slijmvliezen, het microbioom en RA.

Curriculum vitae

Veerle Derksen was born on the 17th of May 1995 in Veldhoven, the Netherlands. In 2012 she graduated from the Gymnasium Camphusianum summa cum laude, after which she started medical school at Leiden University. During her second year of medical training, she was accepted in the MD/PhD honours program, allowing her to take her first steps in research. Given her strong interest in immunology, she started to study rheumatoid arthritis under supervision of professor Tom Huizinga and (later promoted to) professor Diane van der Woude, which would form the foundation of this thesis. In 2015 she obtained her bachelor diploma in medicine cum laude. During her master, she did an extracurricular semester at Edinburgh University taking several courses on immunology. After a final clinical internship at the internal medicine & rheumatology department of the Haga Hospital and a research internship in the rheumatology lab under supervision of professor Diane van der Woude and professor René Toes, she obtained her medical degree at Leiden University in 2019. In the same year she received a competitive grant from the Leiden University Medical Center (LUMC) board of directors, allowing her to continue her research in the rheumatology lab as a fulltime PhD student for three years, which led to the publication of this thesis.

In 2022 she started her medical specialty training in rheumatology. During the first three years, she worked in the internal medicine department of the Alrijne hospital in Leiderdorp, as well as in the Curaçao Medical Center in Willemstad and in the LUMC. In 2026 she continued her residency in the rheumatology department of the Haga hospital in the Hague.

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* Authors contributed equally

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