Immunity against post-translationally modified proteins in autoimmune diseases

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The cover presents confocal microscopy images showing THP-1 macrophages (nuclei, blue) phagocytosing complement-opsonized post-translationally modified proteins coupled to fluorescent beads (red). Cells were exposed to the six modifications used in this thesis (nitration, citrullination, carbamylation, acetylation, MAA-modified and AGE-modified) as well as non-modified protein. Representative images of all conditions are also shown at the different chapters. This is non-modified next to chapter 1, nitration next to chapter 2, citrullination next to chapter 3, carbamylation next to chapter 4, acetylation next to chapter 5, MAA-modified next to chapter 6 and AGE-modified next to chapter 7.

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Immunity against post-translationally modified proteins in autoimmune diseases

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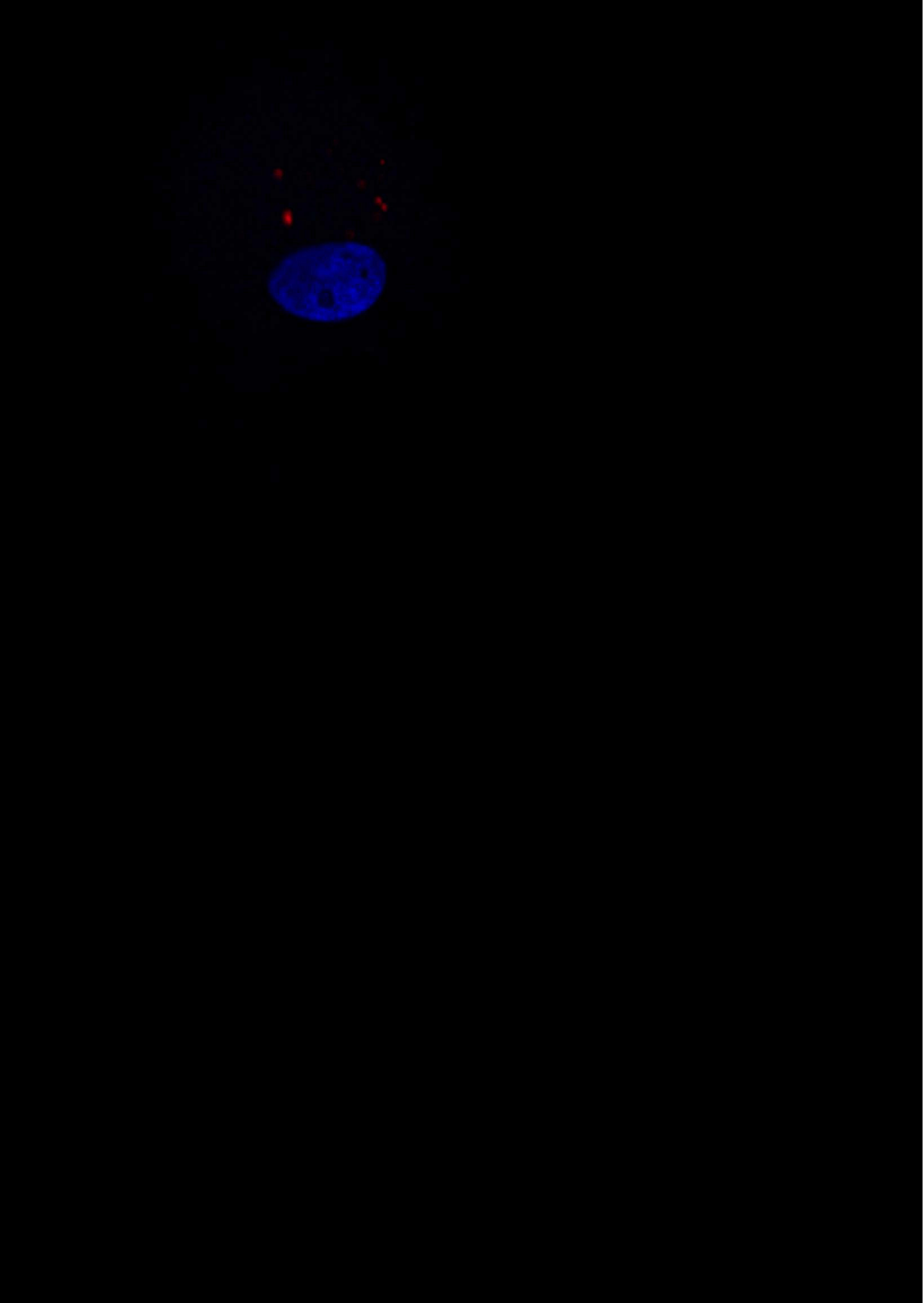
Table of contents

Chapter 1	General introduction	7
Chapter 2	Antibodies against advanced glycation end-products and malondialdehyde-acetaldehyde adducts identify a new specific subgroup of hitherto patients with seronegative arthritis with a distinct clinical phenotype and an HLA class II association	31
Chapter 3	Autoantibodies against specific post-translationally modified proteins are present in patients with lupus and associate with major neuropsychiatric manifestations	57
Chapter 4	Antibodies against multiple post-translationally modified proteins aid in diagnosis of autoimmune hepatitis and associate with complete biochemical response to treatment. *Original publication + commentary*	87
Chapter 5	Post-translationally modified proteins bind and activate complement with implications for cellular uptake and autoantibody formation	119
Chapter 6	General discussion and future perspectives	157
Chapter 7	Appendices	
	English summary	172
	Nederlandse samenvatting	177
	List of publications	183
	Curriculum vitae	185
	Dankwoord	186



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General introduction

Autoimmune diseases

Our immune system defends the body against disease and infection. However, in cases where the immune system falters, it may mistakenly direct an attack on healthy cells, tissues, or organs. Such malfunction can lead to serious disease, resulting in chronic inflammation and autoimmunity that affect various parts of the body. Conditions such as rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), and autoimmune hepatitis (AIH) exemplify the diversity and complexity of autoimmune disorders, each with unique symptoms and challenges.

Rheumatoid arthritis

RA is a chronic autoimmune disorder that primarily affects the joints (Figure 1). Worldwide, in many populations, the prevalence of RA is between 0.5 and 1% and occurs more often in females than in males (1). RA is characterized by an inappropriate immune response that leads to inflammation in the synovium, the lining of the membranes surrounding the joints. This inflammation can cause significant pain, swelling, and stiffness, particularly in the morning or after periods of inactivity. This often leads to a decreased range of motion and functional impairment (2). Currently, RA is classified based on the 2010 rheumatoid arthritis classification criteria (3). A hallmark feature of RA is the presence of specific autoantibodies, including rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPAs) (4). Additionally, an antibody that targets another post-translational modification (PTM), carbamylation, is associated with the development of joint damage (5). Despite the high diagnostic value of ACPA, a group of RA patients is seronegative for RF and ACPA (6). Therefore, novel biomarkers are needed to improve diagnosis of RA. The etiology of rheumatoid arthritis is multifactorial, involving a complex interplay of genetic predisposition, environmental triggers and immune dysregulation. The genetic predisposition to this immune driven disease is exemplified best with the strongest genetic association with genes associated with antigen presentation, e.g. human leukocyte antigen (HLA-)DRB1*01 and HLA-DRB1*04 alleles (7). The environmental factors, impacting on the development of this disease is best exemplified by smoking, an environmental factor that, although not only associated with RA, will chronically stimulate the immune system, triggering the disease in genetically susceptible individuals (8). Despite current sophisticated treatments, the majority of RA patients do not reach complete drug free remission, or cure, for their disease. (9). Drugs used in these patients are called disease-modifying antirheumatic drugs (DMARDs) and include methotrexate or sulfasalazine and leflunomide. A substantial proportion of the patients, however, does not responds sufficiently. If treatment is not effective, methotrexate can be combined with other DMARDs such as TNF inhibitors or the CD20 rituximab, based on Fc-fusion proteins or antibody formats. Additionally, corticosteroids can be provided for short-term management during flare-ups or until DMARDs take effect (9). These

drugs have working mechanisms that are not specific for RA, but aim to achieve systemic immunosuppression.

Systemic lupus erythematosus

SLE is a multifaceted autoimmune disease characterized by the production of autoantibodies and widespread inflammation that can affect multiple organ systems, including the skin, joints, kidneys, heart, and nervous system (Figure 1) (10). The estimated prevalence of SLE ranges from 20 to 70 cases per 10,000 individuals (11). The disease predominantly affects woman, with an average female-to-male ratio of 9:1, which varies with age (12). Currently, the 2019 EULAR/ACR classification criteria for SLE is used for diagnosing SLE (13). One of the hallmark features of SLE is the presence of a diverse range of autoantibodies, including anti-nuclear antibodies (ANA), anti-double-stranded DNA (anti-dsDNA), and anti-Smith (anti-Sm) antibodies (14). These autoantibodies can serve as important diagnostic markers and are often used to monitor disease activity (15). The pathophysiology of SLE involves immune complex formation and deposition in various tissues, leading to inflammation and damage, which can result in serious complications such as nephritis, cardiovascular disease, and lead to increased morbidity and mortality (16, 17). Genetic predisposition, environmental factors, and hormonal influences contribute to the development of SLE, with certain genetic markers, such as those in the complement system and HLA, being associated with increased susceptibility (18). Given the variable manifestations leading to a great spectrum of disease presentations and severity in SLE, also a variety of therapeutic options are available, all suppressing the immune system with the goal to achieve sustained remission (19). Treatment options to achieve this include amongst others corticosteroids, hydroxychloroquine and immunosuppressants/biological agents (12). Recently spectacular results were obtained using anti-CD19 CAR-T cells in small number of patients, reaching B cell depletion and drug-free remission of systemic disease for 12 months even after reconstitution of B cells (20). However, this treatment is not yet available for all patients and substantially more data is needed to understand the full potential of this treatment in autoimmunity. Additionally, feasibility to use CAR-T cell treatment in large patient cohorts needs to be assessed.

Autoimmune liver disease and autoimmune hepatitis

Autoimmune liver disease (AILD) encompasses a group of chronic liver disorders. This category includes AIH, primary biliary cholangitis and primary sclerosing cholangitis. The prevalence of AILD varies widely with AIH being the most frequently diagnosed form. AIH is a chronic autoimmune disease that primarily affects the liver (Figure 1). The prevalence of AIH ranges from 0.1% to 2% in populations in different regions worldwide and is more commonly diagnosed in females compared to males (21). AIH is characterized by an inappropriate immune response that leads to inflammation of the liver, resulting in hepatocellular injury and potentially progressing to cirrhosis, hepatocellular carcinoma,

or death (22). The classification of autoimmune hepatitis is based on clinical, serological, and histological criteria, with the most common types being type 1 and type 2 AIH (23). The etiology of AIH is multifactorial, involving a complex interplay of genetic predisposition, environmental factors, and immune dysregulation. Specific autoantibodies, such as ANA and smooth muscle antibodies (SMA), are often present in affected individuals and can aid in diagnosis (21). Current treatment options for AIH focus on immunosuppression to reduce liver inflammation and prevent disease progression. Corticosteroids, such as prednisone, are commonly used as first-line therapy, often in combination with azathioprine, a disease-modifying immunosuppressive agent (22). While many patients respond well to treatment, some may require additional therapies or adjustments to their treatment regimen to achieve optimal control of the disease (22). Overall, while AIH cannot be cured, effective management strategies can lead to remission and improve long-term outcomes for patients. Regular monitoring and follow-up care are essential to assess liver function and adjust treatment as necessary (23).

Noteworthy is that, in many autoimmune diseases, including RA, SLE and AILD, inflammation occurs in specific anatomical locations, such as the joints, the kidney or the liver, with systemic inflammation contributing to (co)morbidities (2, 24, 25). While several immunopathological processes, may not be disease- or organ-specific, they may contribute to the disease severity in the affected organ and may also contribute to the (chronic) inflammation that is damaging other organ systems. One example is the occurrence of cardiovascular disease in patients with autoimmune diseases like RA and SLE (26).

In several autoimmune diseases autoantibodies are present in the circulation and in the target organs. The success of B-cell targeted therapies in many autoantibody-positive diseases, indicate that B cell-mediated autoimmunity is playing a direct (pathogenic) role (27). Autoantibodies that target PTMs are being included as diagnostic and prognostic biomarkers in RA (5, 28). What triggers the induction of (chronic) inflammation and formation of autoantibody responses is unknown, but if unraveled this will provide the opportunity to design (site-)specific interventions as opposed to the non-specific immune suppression or non-antigen specific depletion of B cells that is currently used.

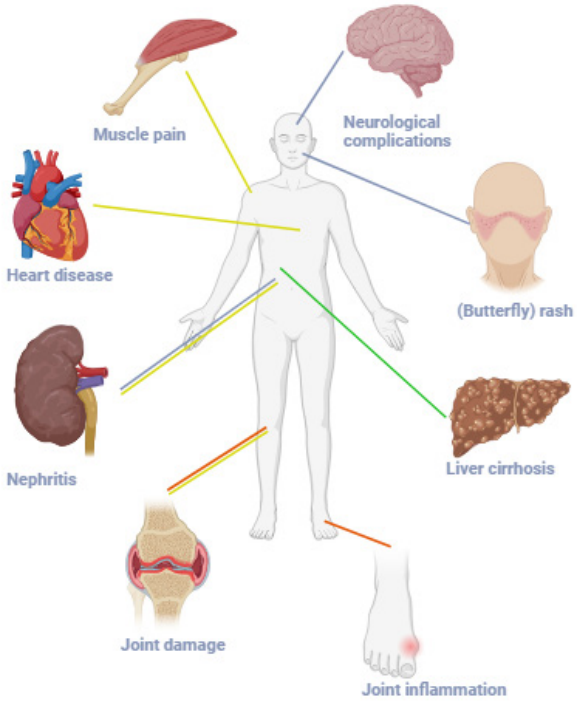

Figure 1: affected sites in RA, AILD and SLE. In some autoimmune diseases specific sites are primarily affected e.g. joints in RA and liver in AILD whereas other diseases display multiple organ involvement such as SLE. However, autoimmune diseases often express similar symptoms, mainly as a consequence of (chronic) inflammation such as coronary artery disease. RA is for instance characterized by inflammation of the joint leading to joint damage (red). Butterfly rash, lupus nephritis and neurological complications are typical manifestations in SLE (blue), as result of systemic inflammation and deposition of immune complexes. In different subtypes of AILD immune-mediated liver injury is playing a central role (green). Additionally, patients with AILD may present with extrahepatic disorders such as arthritis and nephritis (yellow). Higher risk of coronary artery disease is associated with severe systemic inflammation and is observed in RA and SLE patients (yellow). *Image was created using Biorender.com.*

Post-translationally modified proteins

Over the years it has become apparent that the presence of PTMs and/or anti-PTM antibodies is associated with a variety of inflammatory and autoimmune diseases. The role of PTMs and anti-PTMs is however unclear. In this thesis we anticipated that disease associated PTMs, and especially the more readily detectable anti-PTM antibodies, may provide important insight into the disease processes of autoimmune diseases and chronic inflammation. Therefore, we set out to study PTM and anti-PTM reactivities in autoimmunity more closely.

PTM of proteins refers to the changes that occur after the protein has been produced and have a central, mostly physiological, function (29). PTMs occur both inside and outside of the cell and modify many different amino acids. Additionally, both reversible and irreversible PTMs are observed in the body and are commonly employed to regulate protein function. A well-known example is phosphorylation, which is typically a reversible process. Other PTMs are largely irreversible and therefore may persist in the body. It is likely that long-lived proteins, such as laminin and elastin, with half-lives up to 70 years, are more prone to accumulation of PTMs as they are exposed for a longer time period especially during sustained inflammation as compared to for example plasma proteins with a much shorter half-life (29).

Notably, some PTMs are associated with disease (30). Some individuals develop an antibody response against PTM-modified proteins. Interestingly, not every individual that harbors disease-associated PTMs will develop anti-PTM antibodies and only a subset of individuals with PTM and anti-PTM antibodies will develop disease conceivably depending on several factors such as genetic and environmental factors. Noteworthy is that, some individuals develop an anti-PTM antibody response against certain specific PTMs, or combinations of anti-PTM antibody responses, while others develop an anti-PTM antibody response against other PTMs, or develop no anti-PTM antibody response at all (31, 32). In several major human diseases the presence of PTMs or anti-PTM antibodies is associated with disease progression (30-32). Interestingly, in some diseases more emphasis was put on the presence of the PTM proteins e.g. AGEs in diabetes, while in others more emphasis was put on the anti-PTM antibodies, e.g. ACPA in RA. We therefore set out to

study six PTMs; nitration (Nt), citrullination (Cit), carbamylation (Ca), acetylation (Ac), malondialdehyde-acetaldehyde adducts (MAA) and advanced glycation end-products (AGE). We focus on these PTMs as these are observed in a variety of disease including cardiovascular disease, autoimmune disease such as RA, and diabetes type 2. Additionally, some of these PTMs are structurally very different, whereas others are very similar (Figure 2). Notably, the chosen PTMs occur on specific amino acids such as lysine, arginine or tyrosine.

Nitration (Nt)

Protein nitration is an irreversible modification of tyrosine (Figure 2) and may alter the structure and/or diminish the function of proteins (33). Mechanisms of protein nitration include reactions with peroxynitrous acid (ONOOH) or nitrosoperoxycarbonate ($ONOOCO_2$-) (33). peroxynitrous acid (ONOOH) is the protonated form of peroxynitrite (ONOO-) and can directly nitrate free or protein bound tyrosine. Peroxynitrite can also react with CO_2 to form a reactive CO_2 adduct of peroxynitrite, nitrosoperoxycarbonate

($ONOOCO_2$-). Nitrosoperoxycarbonate in turn decomposes to carbonate radical and nitrogen dioxide which can react with tyrosine to form 3-nitrotyrosine.

ONOO- is a result of a reaction of nitric oxide (NO) and superoxide (O_2-). Under physiological conditions, NO is an important small molecule involved in various biochemical processes such as neurotransmission and modulation of blood flow (34-36). Production of excess NO, however, can have detrimental effects and lead to the formation of reactive nitrogen species (RNS) such as ONOO-. During inflammatory processes, cells produce high levels of NO but also superoxide (O_2-) which is a reactive oxygen species (ROS) (37). In case NO and O_2- react, peroxynitrite (ONOO-) formation is inevitably leading to 3-nitrotyrosine formation in protein. Enhanced peroxynitrite formation is associated with a variety of conditions as a consequence of oxidative stress (38). On top of that, 3-nitrotyrosine is strongly associated with cardiovascular disease and diabetes (39, 40). More specifically, immunohistochemistry of atherosclerotic plaques showed 3-nitrotyrosine staining in various studies (41). In some studies, evidence for co-localization with specific extracellular matrix species has been found, including long-lived proteins such as laminin (42) and elastin (43). In cardiovascular disease accumulation of PTMs is suggested to alter for instance viscoelasticity, increasing the risk of coronary artery disease (44).

Citrullination (Cit)

Citrullination is an enzymatic conversion of arginine residues into citrulline residues (Figure 2). This process is mediated by the peptidyl arginine deiminase enzyme (PAD) family, of which PAD1-4 have citrullinating activity (45). PAD activity is regulated by calcium. PADs are activated when calcium homeostasis is lost. PAD activity and the resulting citrullination has been implicated in several physiological processes including apoptosis and terminal differentiation of the epidermis. For example, citrullination may alter the structure of cytoskeletal proteins such as filaggrin and trichohyalin (46, 47).

Under physiological conditions, nuclear substrates such as histones are citrullinated, thereby affecting nucleosome stability. It has been suggested that this process causes the nucleosome to open up, and render DNA more accessible to nucleases (48, 49). Interestingly, granulocytes and more specifically neutrophils, use this histone citrullination, among other things, to form neutrophil extracellular traps (NETs). By histone citrullination, PAD4 will decondense chromatin essential for NET formation. These fibrous chromatin-based NETs therefore contain (citrullinated) histones, DNA and granular proteins (50). During NETosis, NETs are released into the extracellular space to capture and eliminate pathogens or emerge infections. In case of sustained inflammation, PAD enzyme is continuously released in the inflamed site which may lead to localized protein citrullination (49). For example, such sustained inflammation and citrullination of proteins is seen in the joints of rheumatoid arthritis patients. In addition,

it has been shown that expression of PAD2 and 4 is increased in rheumatoid synovial tissue and fluid (51). Importantly, in RA it is well known that autoantibodies are present that bind such citrullinated proteins, called ACPAs.

Carbamylation (Ca)

During carbamylation, the amino acid lysine is converted into the amino acid homocitrulline (Figure 2). The adduct NH_2CO- that is formed is a carbamoyl group, a process nowadays referred to as carbamylation (52). Noteworthy is that citrulline and homocitrulline only differ by one methylene group and are structurally very similar (53) yet occur on a different amino acid residue: citrulline on arginine and homocitrulline on lysine. Protein carbamylation is driven by a chemical reaction with cyanate. Urea is in equilibrium (ratio 1 to 500,000) with ammonium cyanate and thus is a source of cyanate in the body (54). High levels of urea as a result of uremia are observed in case of renal insufficiency (55). Increased levels of cyanate can also be due to the conversion of thiocyanate into cyanate by myeloperoxidase (56). Thiocyanate is therefore another source of cyanate. Both thiocyanate and cyanate itself are observed in smokers (53, 57). Carbamylation dose dependently changes the structure and function of a broad range of proteins and small molecules (32). This may affect cellular functions and trigger systemic disorders. Carbamylation of proteins is shown to be related to chronic kidney disease and (sustained) inflammation, but has also been described in other conditions such as cardiovascular disease (32). Additionally, carbamylated proteins have been detected in synovial tissue and synovial fluid of RA patients (58). In RA patients, next to ACPA also antibodies that bind to carbamylated proteins (anti-CarP) are detected (5). The presence of anti-CarP is associated with joint damage in these patients, especially in the ACPA negative subgroup (5).

Acetylation (Ac)

Acetylation is a reversible enzymatic process during which acetyl groups are added to free amines of lysine residues in the presence of acetyl-CoA (59). Notably, two mechanisms for acetylation of amino groups of proteins have been described: lysine acetylation (Nε-acetylation) and N-terminal protein acetylation Nα-acetylation). Acetylation of histones (Nε-acetylation) is extensively studied in the context of transcription regulation (60-62). Next to histone acetylation also non-histone protein acetylation is described and reviewed by Narita *et al.* (63). Non-histone acetylation is involved in key cellular processes such as gene transcription, signal transduction and protein folding. During lysine acetylation the size of the side chain is increased and the charge neutralized (+1 to 0). Consequently, this may alter protein stability, protein-protein interaction and protein-DNA interactions. Acetylated lysine resembles homocitrulline except for one side-chain terminal amine, which is replaced by a methyl group (Figure 2). Therefore Juarez *et al.* studied whether antibodies recognizing these acetylated proteins are present in sera of RA patients as they harbor antibodies against the structurally very similar citrulline and

homocitrulline (64). Indeed, they noticed that in approximately 40% of the RA patients harbored antibodies against acetylated vimentin. Interestingly, Juarez *et al.* observed that the presence of anti-acetylated vimentin was largely confined to the ACPA-positive subgroup of RA patients. Next to RA, altered protein acetylation is reported in several neurodegenerative diseases, cardiovascular disease and cancer (65, 66).

Malondialdehyde acetaldehyde-adducts (MAA)

Malondialdehyde acetaldehyde-adducts are a highly stable product of oxidative stress and form a ring structure (Figure 2). During inflammation, oxidative stress is induced and reactive oxygen species (ROS) may be formed. Upon exposure of cells to ROS, lipid peroxidation occurs followed by rupture of the cell membrane and membrane lipid oxidation, forming malondialdehyde (MDA) (67). MDA can spontaneously break down and form acetaldehyde (AA) (67). Both MDA and AA are highly reactive aldehydes and together they have shown to modify proteins to produce an MDA-AA protein adduct, termed the malondialdehyde-acetaldehyde adduct (MAA) (68). MAA have been proven to be immunogenic and have pro-inflammatory capacities (69). MDA, AA and MAA, as well as antibodies against these adducts, have been observed in several diseases such as atherosclerosis, alcohol induced liver injury and RA (48, 70-73).

Advanced glycation end-product (AGE)

Advanced glycation end-products are a result of reactive sugar groups that bind to amino acid residues with a free amino group. During this process a reaction occurs between the free amino groups and carbonyl group of reducing sugars such as reducing aldose and reducing ketose. These processes include the Maillard or Polyol pathway (74). Condensation of carbonyl groups of reducing sugars, form reversible reactions yielding the so-called Schiff base. Next, these undergo rearrangement resulting in so-called stable Amadori or Heyns products. These products are considered ''early glycation products''. To become AGEs, the end-compounds (e.g. Schiff bases), must undergo further rearrangements and involve the formation of reactive carbonyls. Reactive carbonyls can undergo further condensation with available amine groups from e.g. lysines. In the end this reaction yield a great variety of final AGEs. Excessive glycation is a response to oxidative stress and inflammation leading to AGEs. AGEs in turn bind to AGE receptors on e.g. macrophages and endothelial cells leading to the perpetuation of inflammation (75). Additionally, dietary intake of AGEs is associated with higher levels of AGEs in plasma (76). AGEs are typically found in patients with diabetes and is associated with cardiovascular disease in these patients (77, 78). Carboxymethyllysine (CML) is a frequently measured AGE and a marker for oxidative stress. CML increases slowly with age and is significantly increased in coronary artery disease and age-related macular degeneration, but is also observed in synovial tissue of RA patients (79-81).

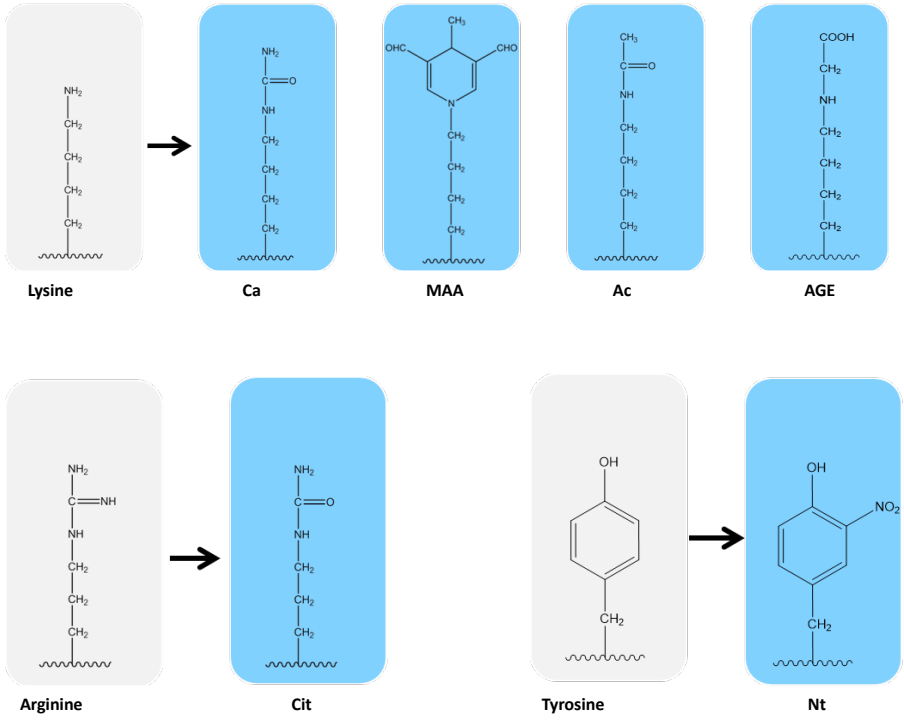

Figure 2: structures of PTMs nitration, citrullination, carbamylation, acetylation, malondialdehyde-acetaldehyde adducts and advanced glycation end-products on different amino acids. *Abbreviations: Ac, Acetylation; AGE, advanced glycation end-products (exemplified by carboxymethyl lysine); Ca, carbamylation; Cit, citrullination; MAA, malondialdehyde-acetaldehyde adducts; Nt, nitration.*

Complement

Since many PTMs are associated with disease but simultaneously are also found in healthy individuals we hypothesized that complement could be a mechanism that could be involved in clearance in health but in breach in tolerance in autoimmunity. If complement is activated directly on PTM-modified proteins, these PTM-modified proteins become opsonized. Complement opsonized PTM-modified proteins, can in turn be recognized by cells expressing complement receptors eliciting further response e.g. clearance by phagocytic cells or triggering autoreactive B-cells. In the following paragraphs basics on the complement system, complement receptors and how this would lead to a breach in tolerance will be introduced.

Research into the origin of the complement system revealed that a primitive form of the complement system dates back more than 500 million years ago (82). The ''modern''

complement system was established upon appearance of the jawed vertebrates. The complement system is activated within seconds after a pathogen has entered (83). Complement is a major component of the innate immune system but is more recently also recognized to play a role in modulating adaptive immunity (84). The complement system consists of over 30 proteins which are involved in opsonization, chemotaxis, activation of leukocytes and cytolysis of target cells through the C5b9-membrane attack complex (85, 86). Additionally, complement is well-known for its role in clearance of apoptotic cells and immune complexes.

Complement can be activated through three pathways: classical (CP), lectin (LP) and alternative pathway (AP) (Figure 3). Each complement pathway has its own initiators and/or activation mechanisms but all form C3 convertases resulting in the formation of the membrane attack complex (MAC, C5b9) at the end.

The CP is initiated by C1q (87). C1q is composed six globular target recognition domains (gC1q) attached to a collagen-like region (CLR) forming a ''bouquet of tulips'' like structure. C1q is able to bind to immune complexes but also serves as a pattern recognition molecule with its ability to sense i.e. eat-me signals on apoptotic. C1q is bound to C1r and C1s in a calcium dependent manner and forms a C1 complex. C1r and C1s from the C1 complex are the first enzymes in this pathway to trigger a series of enzymatic events. After C1q has bound to the target its conformation will change and C1r will be activated. C1r cleaves C1s, forming activated C1s which in turn binds to C4 and enzymatically liberates C4a and C4b. C1s will additionally cleave C2, into C2a and C2b. Together with C2b, C4b will form the C3 convertase C4bC2b formerly referred to as C4b2a (88).

The LP can be activated through binding of collectins (mannan binding lectin (MBL), collectin-10, collectin-11) or ficolins (ficolin-1, ficolin-2, ficolin-3) to sugar groups on the surface of a pathogen in a calcium dependent manner (85). Just like C1q, collectins and ficolins have a N-terminal collagenous region serving as pattern-recognition molecule and form a complex with MBL-associated serine proteases (MASPs). Despite the similarities between the architecture of the C1 an MBL/MASP complexes, the mechanism of activation of the LP is different from the CP. In the CP each C1 complex carries both C1r and C1s, and each complex can therefore activate complement. However, the majority of the MBL molecules are associated with only one homodimer of either MASP-1 or MASP-2 (89). In order to activate the LP both MASP-1 and MASP-2 are required. MASP-1 is able to cleave C2 and is required to activate MASP-2. MASP-2 in turn can cleave both C2 and C4. During activation, MBL or ficolin, associated with either MASP-1 or MASP-2, will bind to its target bringing MASP-1 and MASP-2 into close proximity resulting in cleavage of C2 and C4 (90). After C2 and C4 are cleaved, C2b and C4b will form the C3 convertase C4bC2b.

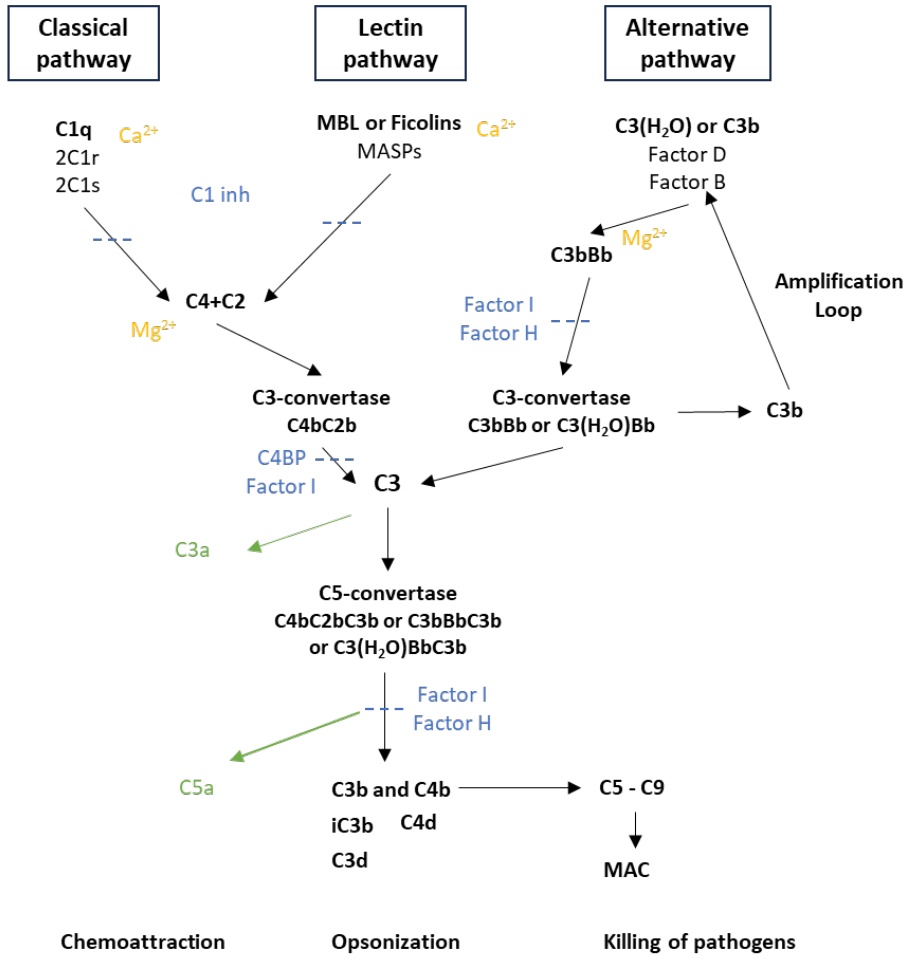

Figure 3: Schematic overview of the activation of the three complement pathways. The complement system consists of three different pathway namely the classical, lectin and alternative pathway. The classical and lectin pathway are calcium-dependent (depicted in orange) whereas the alternative pathway has a magnesium-dependent step (depicted in orange). All pathways come together at the level of C3. Subsequently, C3 is cleaved by C3 convertases formed in the different pathways into C3a (anaphylatoxin depicted in green) and C3b. Prolonged complement activation can result in the addition of C3b to C3 convertases forming C5 convertases. C5 in turn will be cleaved forming C5a (anaphylatoxin depicted in green) and C5b. C5b will subsequently trigger the formation of the membrane attack complex (MAC; C5b-9). Complement is regulated by regulators (C1 inhibitor, Factor I, Factor H, C4BP) at different levels (depicted in blue). C3b can for instance be cleaved by factor I with the help of e.g. factor H, serving as cofactor resulting in iC3b and finally C3d. C3b derivatives can in turn serve as opsonin to elicit further response.

The AP can be activated spontaneously, maintaining a constitutive activation by a process called tick-over (91). During this process a labile thioester bond is hydrolyzed and converts C3 into C3(H_2O). Upon hydrolysis, the thioester domain (TED) of C3 undergoes a structural change exposing a binding site for factor B. Factor B bound to C3(H_2O) is cleaved by factor D allowing formation of the fluid phase C3 convertase C3(H_2O)Bb. This minor form of C3 convertase is responsible for a constant low level of C3a and C3b (tick-over) (92). The formed C3b can bind covalently to the cell surface sugar carbohydrates or immune aggregates (opsonization). In case of a pathogen, factor B associates with C3b in magnesium dependent manner and is cleaved by factor D forming the major form of C3 convertase, namely C3bBb. Properdin, in the AP, serves as a positive regulator recruiting C3b to the surface but also stabilizes C3 convertase. On host cells, bound C3b is rapidly inactivated by surface or fluid phase regulatory complement components maintaining homeostasis.

In summary, for the CP and LP clear recognition molecules have been identified which trigger each pathway only at sites where activation is necessary. These recognition molecules undergo structural changes in order to elicit activation of enzymes which in turn are able to cleave subsequent molecules of the complement cascade. All pathways will form the central enzymatic complexes called C3 convertases (C4bC2b, C3bBb or C3(H_2O)Bb). The AP, lacks a traditional initiator. However, several molecules such as properdin and P-selectin have been described to recruit C3(H_2O) and C3b to the cell surface and serve as a local initiator of the AP.

All three pathways converge at the level of C3 as all pathway specific C3 convertases use C3 as a substrate. C3 convertases cleave C3 into the anaphylatoxin C3a and opsonin C3b. Prolonged complement activation results in addition of one or more molecules of C3b to C3 convertases forming C5 convertases: C4bC2bC3b, C3bBbC3b and C3(H_2O)BbC3b (93, 94). C5 convertases cleaves C5 into the anaphylatoxin C5a, which diffuses away, and C5b which triggers the assembly of the membrane attack complex (MAC; C5b-9). C5b in turn binds C6 which acquires the ability to interact with the lipid bilayer stabilizing the complex (95). Subsequently, C7 and C8 bind to C5b and form the C5b-7 and C5b-8 complexes. Lastly, one molecule of C9 binds to the membrane inserted C8, followed by multiple C9 molecules to complete the MAC complex.

Next to formation of further C3 convertases, C3b undergoes successive proteolytic cleavages leading to inactive C3 products which is mediated by factor I. Factor I requires a cofactor such as factor H, C4BP, CR1 or MCP, in order to bind and inactivate C3b, generating iC3b (inactive form of C3b) and C3f. These iC3b molecules are not able to form new C3 convertases protecting host cells from complement mediated attack. After iC3b is cleaved, factor I will together with CR1 as cofactor cleaves iC3b into C3dg,

and C3c, eventually leading to C3d formation induced by plasma proteases (96). C3b derivatives can in turn bind to complement receptors expressed on a variety of cells and elicit further response.

Complement receptors

Complement receptors (CRs) are critical components of the immune system, facilitating the interaction between immune cells and the complement system, which is a key element of innate immunity and adaptive immunity. These receptors are expressed on various immune cell types, including macrophages, neutrophils, dendritic cells, and B cells, and they play pivotal roles in mediating immune responses such as opsonization, phagocytosis, and the regulation of inflammation. Complement receptors that recognize complement opsonized target include CR1, -2, -3, -4 and CRIg.

CR1 (CD35) is primarily expressed on erythrocytes, macrophages, and dendritic cells, but is also found on B cells and some T cells (97, 98). It binds to C3b and C4b, facilitating the clearance of immune complexes and pathogens. CR1 plays a significant role in regulating complement activation by promoting the decay of C3 convertase and acting as a cofactor for factor I, which inactivates C3b and C4b (99). On B cells, CR1 may function both as a processing molecule, converting C3b to iC3b (100), and as a contrasting signal to CR2 down-modulating B cell responses to C3b-coated antigens (101).

CR2 (CD21) is predominantly found on B cells and follicular dendritic cells. It binds especially to C3d and also to iC3b and C3dg, enhancing B cell activation and signaling through the B cell receptor (97, 102). On follicular dendritic cells within lymphoid tissues the interaction of CR2 and C3 fragment-opsonized antigens is important in trapping and retaining immune complexes (97).

CR3 (CD11b/CD18) is expressed on myeloid cells, including neutrophils and macrophages. But are also found on NK cells and activated B and T cells (103). It binds to iC3b, and is essential for the phagocytosis of opsonized pathogens. Additionally, engagement of CR3 is involved in leukocyte adhesion and migration, and induction of both inflammatory and tolerogenic responses fulfilling an immune modulatory function (104).

Similar to CR3, CR4 (CD11c/CD18) is expressed on myeloid cells and binds to iC3b. It plays a role in phagocytosis and is involved in the activation of antigen-presenting cells, thereby contributing to the initiation of adaptive immune responses. CR4 has also been implicated in the regulation of inflammation (103).

The binding of complement fragments to their respective receptors has several important consequences for immune cell function. It enhances phagocytosis, whereby immune cells engulf and digest opsonized pathogens, which is crucial for effective pathogen clearance. Additionally, the interaction between complement receptors and their ligands triggers intracellular signaling pathways that promote cell activation, leading to the release of pro-inflammatory cytokines and chemokines, and regulation of inflammation. This results in the recruitment of additional immune cells to the site of infection or injury. Next to CR1, -2, -3 and -4, the complement receptor repertoire also encompasses: CRIg and C3aR, C5aR1 and C5aR2. CRIg is a complement receptor of the immunoglobulin family binding C3b and iC3b (105). In contrast to the other C3 fragment receptors (CR1 to CR4), CRIg is found on a constitutive recycling pool of membrane vesicles where it participates in the internalization of C3-opsonized particles from the bloodstream by Kupffer cells and tissue resident macrophages. Notably, functional effect of C3b binding to CRIg is confined to the C3b of the alternative pathway, as it fails to inhibit C3 and C5 convertases of the other pathways. C3aR binds to the anaphylatoxin C3a and C5aR1/2 binds C5a. All anaphylatoxin receptors are G protein-coupled receptors and regulate innate and adaptive immune responses upon ligand binding.

Taken together, cells expressing complement receptors are vital for orchestrating immune responses against pathogens and maintaining homeostasis. Disbalance in immune activation may result in (sustained) inflammation and breach in tolerance.

Breach in tolerance

The breach of immune tolerance is a fundamental mechanism in the development of autoimmune diseases, where the immune system mistakenly targets the body's own tissues. PTMs could trigger the immune system resulting in inflammation or autoimmunity through complement opsonization. Anti-PTM antibodies can form immune complexes on which the complement system will be activated preventing precipitation. In autoimmunity, continued production of antibodies leads to extended formation of immune complexes that can overwhelm the complement system. Consequently, immune complexes will be poorly cleared and may deposit in various tissues, further driving inflammation and tissue damage (106).

One of the primary mechanisms underlying the breach of immune tolerance involves genetic predisposition. Certain genetic factors, such as specific alleles of the HLA complex, have been linked to an increased risk of autoimmune diseases. For instance, the HLA-DRB1 gene is associated with RA, influencing the presentation of autoantigens (107). These genetic variations can alter the immune response, predisposing individuals to a loss of tolerance.

Environmental factors also play a significant role in breaking immune tolerance. Viral and bacterial infections, for instance, can trigger autoimmune responses through mechanisms like molecular mimicry, where pathogens share structural similarities with host tissues. This can lead to cross-reactivity, where the immune system targets both the pathogen and the host tissues (108). Additionally, environmental triggers such as smoking and certain dietary components may result in irreversible post-translational modification on self-proteins (32, 109). The immune system will target the modified proteins and depending on genetic predisposition tolerance will break, further complicating the interplay between genetics and the environment.

In conclusion, the breach of immune tolerance in autoimmunity results from a complex interplay of genetic, environmental, and immunological factors. Understanding these mechanisms is crucial for developing targeted therapies aimed at restoring immune tolerance and mitigating the effects of autoimmune diseases.

Implications for therapeutic interventions

Treatments for autoimmune diseases such as RA, SLE and AILD often include (cortico) steroids and/or biologics. The ultimate goal using these interventions is sustained (drug free) remission, as cure is not often reached for these diseases. Designing an ''one cure fits all'' therapeutic is not possible as patients with these types of autoimmune disease are highly heterogenous. Nonetheless, for many patients B-cell targeted therapy, using for instance rituximab, has proven to be effective (27). B-cell targeted therapy depletes all CD20 positive B cells using monoclonal antibodies (mAb). However, only a subset of B cells produce autoantibodies targeting ''self''. Other ''healthy'' B cells will also be targeted using this type of therapeutics. Over the years, mAb therapeutics have evolved and nowadays bispecific antibodies (bsAbs) are generated and proven to be effective (110). Such bsAbs are able to bind two different antigens as opposed to regular mAb binding only one antigen or epitope. This format would allow to bridge two cell types or to engage two molecules and therefore making treatment more (site-)specifically enhancing therapeutic efficacy (111).

A strategy would be to prevent B cells from becoming autoreactive by targeting the triggers of these B cells. In this thesis we describe that the complement system could be triggered and opsonize PTM-modified proteins. These opsonized PTM-modified proteins could in turn induce (chronic) inflammation and induce autoimmunity under certain circumstances. In patients, blocking the whole complement system systemically, although successful, is unwanted, as the complement is needed to fight infection, heal injury and kill bacteria and viruses (112). BsAbs could in this context also provide

opportunities for interventions. In this case, bsAbs targeting the PTM of interest with one arm and complement inhibitors with the other will provide local complement inhibition (113). Such targeted therapy could inhibit immune reactivity locally, preventing PTM specific B cells to become autoreactive. In RA for example citrullinated and carbamylated proteins could be targeted in the joints, potentially regulating sustained inflammation and dampening of antibody responses. In case of SLE and AIH several PTMs could be targeted and immune regulators brought into close proximity to potentially dampen or prevent anti-PTM antibody responses.

Outline of this thesis

This thesis is mainly focused on PTMs and antibodies targeting these PTMs in the context of autoimmunity. PTMs and anti-PTM responses are described in many (autoimmune) diseases and, in RA patients, antibodies targeting the modifications citrullination and carbamylation are nowadays used as diagnostic and prognostic tools. We focused on six different PTMs namely, Nt, Cit, Ca, Ac, MAA and AGE. With these PTMs we have an broad set of PTMs associating with different diseases, have different structures and/or occur on different amino acids. In the **Chapters 2, 3 and 4** we first measured anti-PTM responses and explored clinical associations in three autoimmune diseases, namely RA, SLE and AILD (including AIH), respectively. In **Chapter 2** anti-MAA and anti-AGE are observed to be significantly increased in RA. These anti-PTM antibodies identify a group of RA patients that are hitherto seronegative (anti-CarP negative and ACPA-negative). These data contribute to closing the serological gap that currently exists in the classification of RA patients. In **Chapter 3** we focused on SLE and SLE patients with neuropsychiatric manifestations (NPSLE). Here we identified three anti-PTM antibodies namely anti-Ca, anti-MAA and anti-AGE that are present more frequently in patients with SLE as compared to healthy controls. Several anti-PTM antibodies (anti-Ca and anti-MAA) were more prevalent in patients with major NPSLE, a disease manifestation currently lacking a suitable biomarker. In addition, all three anti-PTM antibodies also correlated with brain volumes. Next to RA and SLE, we investigated anti-PTM responses in AILD in **Chapter 4**. In these patients the presence of anti-Ca, anti-MAA and anti-AGE correlated with the presence of AIH. In AIH, harboring at least three anti-PTM antibody responses is positively associated with complete biochemical response.

Now that we observed anti-PTM antibody responses in several autoimmune diseases we moved to identifying triggers resulting in these antibody responses. In **Chapter 5** we identified complement components as proteins that increasingly bound to PTM-modified proteins compared to non-modified proteins. We verified complement activation was independent of antibodies, and observed increased binding and/or uptake

of complement opsonized PTM-coupled beads by leukocytes and phagocytic cells (THP-1 macrophages). Furthermore, correlation analysis on complement SNPs from 587 RA patients were performed highlighting a role for complement in driving production of anti-PTM antibodies. Finally, in **Chapter 6** the major findings in this thesis are summarized and the implications of the presented results for future research are discussed.

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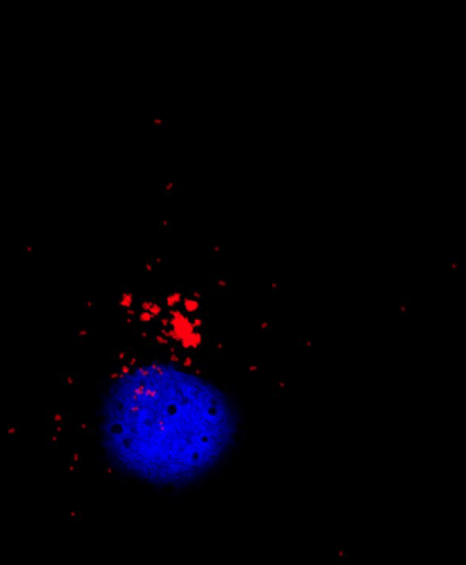

2

Antibodies against advanced glycation end-products and malondialdehyde-acetaldehyde adducts identify a new specific subgroup of hitherto patients with seronegative arthritis with a distinct clinical phenotype and an HLA class II association

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Key messages

What is already known?

Rheumatoid arthritis (RA) patients can be divided into seropositive and seronegative subgroups. The presence of antibodies against post-translationally modified (PTM) proteins such as citrullinated proteins is nowadays used as a diagnostic and prognostic marker in RA. Antibodies directed against carbamylated proteins have more recently been shown to be present in a subset of the seronegative patients and are associated with bone erosions in that group.

What does this study add?

In this study, two different anti-PTM antibodies are investigated: anti-Advanced Glycation End-product modified protein antibodies (anti-AGE) and anti-Malondialdehyde-Acetaldehyde Adduct modified protein antibodies (anti-MAA). These antibodies can be detected in several forms of inflammatory arthritis. Within seronegative RA (negative for rheumatoid factor (RF), anti-citrullinated protein antibodies (ACPA) and anti-carbamylated protein antibodies (anti-CarP)), 16.9% percent of patients are positive for anti-MAA and/or anti-AGE antibodies. This subgroup is characterized by an association with HLA-DRB1*03, increased radiographic joint damage, and (for anti-MAA) inflammation.

How might this impact on clinical practice or future developments?

The presence of anti-PTM antibodies like anti-AGE and anti-MAA in RA patients and other inflammatory arthritis patients previously considered to be seronegative, may not only serve as a prognostic marker, but importantly may contribute to understanding the pathogenesis of these conditions, including a subset of RA.

Abstract

Objective

In rheumatoid arthritis (RA) around two-thirds of patients are autoantibody-positive for rheumatoid factor (RF), anti-citrullinated protein antibodies (ACPA) and/or anti-carbamylated protein antibodies (anti-CarP). The remaining seronegative subgroup of patients is clinically heterogeneous and thus far, biomarkers predicting the disease course are lacking. Therefore, we analyzed the value of other autoantibodies in RA directed against malondialdehyde-acetaldehyde adducts (MAA) and advanced glycation end-products (AGE).

Methods

In sera of 648 RA patients and 538 non-RA arthritis patients from the Leiden Early Arthritis Clinic, anti-MAA and anti-AGE IgG antibody levels were measured using ELISA. Associations between genetic risk factors, acute phase reactants, radiological joint damage, remission and anti-PTM positivity were investigated using regression, correlation and survival analyses.

Results

Anti-AGE and anti-MAA were most prevalent in RA (44.6% and 46.1% respectively) but were also present in non-RA arthritis patients (32.9% and 30.3% respectively). Anti-AGE and anti-MAA antibodies were associated with HLA-DRB1*03 within seronegative RA (OR=1.98, p=0.003, and OR=2.37, p<0.001, respectively) and, for anti-AGE also in non-RA arthritis patients (OR=2.34, p<0.001). Presence of anti-MAA antibodies was associated significantly with markers of inflammation, ESR and CRP, in all groups independent of anti-AGE. Interestingly, the presence of anti-AGE and anti-MAA antibodies was associated with radiologic progression in seronegative RA patients, but not evidently with sustained drug-free remission.

Conclusions

Anti-AGE and anti-MAA were present in around 45% of RA patients and 30% of non-RA patients, and although not specific for RA, their presence associated with HLA, inflammation and, for RA, with clinical outcomes especially in seronegative RA patients.

Introduction

In rheumatoid arthritis (RA) around two-thirds of patients are autoantibody-positive for rheumatoid factor (RF), anti-citrullinated protein antibodies (ACPA) and/or anti-carbamylated protein (anti-CarP) antibodies (1). The remaining seronegative subgroup of RA is clinically heterogeneous and thus far no reliable biomarkers are available to identify these patients or predict their disease course (1).

ACPA and anti-CarP are antibodies that recognize proteins that have undergone post-translational modification (PTM), citrullination of arginine and carbamylation of lysine respectively (2, 3). However, many other types of PTMs exist (4). Two examples of PTMs that are found to associate with disease are Advanced Glycation End-products (AGE) and Malondialdehyde-Acetaldehyde Adducts (MAA). AGEs are a result of oxidative stress and tissue damage (5) and are for example present in patients with diabetes mellitus type 2 (6). Interestingly, in these patients also antibodies directed against this PTM were observed (6). MAA modifications are a result of reactive oxygen species (ROS) that are formed during inflammation and oxidative stress (7). MAA modified proteins as well as anti-MAA antibodies are found in patients with RA, as well as in other diseases (7). AGE and MAA are both highly immunogenic PTMs (8, 9). Therefore it is plausible that antibodies against AGE and MAA are also present in patients with arthritis.

Seronegative RA is associated with HLA-DRB1*03, suggesting a role for immunopathology driven by e.g. B cell immunity (10). Indeed, within the ACPA-negative patients the presence of anti-CarP was associated with HLA-DRB1*03 (11, 12). However, it did not yet explain the full HLA-DRB1*03 association, raising the possibility that other anti-PTM responses may be present in 'seronegative' RA that are present in the remainder of the HLA-DRB1*03 positive individuals (13, 14). On top of this haplotype association, within these ACPA-negative RA patients anti-CarP was found to associate with a more severe radiological progression (3). Seronegative RA patients are a diverse group of patients that in many ways resemble undifferentiated arthritis. Presence of antibodies, like anti-PTM antibodies, might help to better understand and characterize subgroups that possibly belong to this so called seronegative RA.

We therefore investigated whether anti-AGE and anti-MAA antibodies are present in patients with RA and other forms of arthritis, and whether they could potentially close the so-called serological gap (1) in seronegative RA.

Methods

Patients

1186 patients with arthritis of at least one joint and a symptom duration of less than 2 years were included in the Leiden Early Arthritis Clinic (EAC) cohort (15). Data was collected at baseline and follow-up (4, 12 months and yearly thereafter). Patients were being followed as long as the patient remained being seen clinically by the rheumatologist. RA was classified based on the 1987 ACR criteria (n=648) (16). Definitive diagnoses other than RA (n=538), were made by the treating physician after 1 year of follow-up and were predominantly psoriatic arthritis (PsA) (n=100), inflammatory osteoarthritis (OA) (n=95) and gout (n=93) besides other more rare forms of arthritis. For this manuscript, the following diagnoses were termed autoimmune (AI): RA, psoriatic arthritis, spondyloarthritis, sarcoidosis, systemic lupus erythematosus (SLE) and paraneoplastic arthritis. The diagnoses termed as non-autoimmune (non-AI) were: gout, pseudogout and septic arthritis. The protocols were approved by the Leiden University Medical Center ethics committee and written informed consent was obtained. Clinical and demographic patient characteristics were collected as described previously (17).

Genotyping, radiological progression and remission

From all patients, HLA genotypes were established as described previously (18). The alleles that were marked as shared epitope-encoding HLA (HLA-SE) positive were: HLA-DRB1*01:01, 01:02, 04:01, 04:04, 04:05, 04:08, 10:01, 14:02. For the radiological progression analyses, 2853 X-ray sets of the hands and feet of 635 RA patients were scored as described previously using the Sharp-van der Heijde score (SHS) (19, 20). Sustained drug-free remission (SDFR) was defined as absence of clinical synovitis after discontinuation of disease-modifying antirheumatic drug (DMARD) treatment, that persisted for the entire follow-up, being at least 1 year (21).

Anti-AGE and anti-MAA measurements

Anti-AGE and anti-MAA antibodies were detected using an in-house ELISA based on modified fetal calf serum (FCS) as described previously (22). Briefly, modified and non-modified FCS were coated to a Nunc Maxisorp ELISA plate (430341, Thermofisher). In between each sequential step plates were washed 3 times using Phosphate Buffered Saline (PBS)/0.05%Tween (Sigma, P1379). After blocking (PBS/1%Bovine Serum Albumin (BSA)) for 6 hours at 4°C plates were incubated overnight at 4°C with 1/100 or 1/1000 diluted serum for anti-AGE and anti-MAA respectively. Each plate contained a standard of anti-PTM positive serum to calculate arbitrary units. After incubation, IgG levels were detected using Rabbit-anti-Human IgG-HRP (Dako, P0214). Plates were developed by incubating with 2,2'-azino-bis[3-ethylbenzothiazoline-6-sulfonic acid] (ABTS)/0.015% H2O2 (A1888 and 7722-84-1, both from Merck) and absorbance at 415nm was measured

using a microplate reader (Bio-Rad iMark). The cut-off for positivity was set as the mean arbitrary units plus two times the standard deviation of 80 healthy controls, excluding values higher than 10x the mean.

Statistical analysis

Independent samples T-test and Mann-Whitney U tests were used to analyze the baseline characteristics. The association of HLA-DRB1*03 with autoantibodies was assessed with logistic regression, and stratified for anti-cyclic citrullinated peptide 2 (anti-CCP2) and anti-CarP if relevant. Correlations between anti-PTM antibodies and inflammatory markers were calculated using Spearman's rank correlation. For the radiologic progression analyses, a multivariate normal regression model for longitudinal data was used with SHS as response variable. The model controlled for the age, sex, and inclusion-year of the patients (19). SDFR-development until follow-up was calculated using Kaplan Meier survival analysis and Cox's regression. All statistical analysis were performed using SPSS statistics version 25 (IBM).

Results

Anti-AGE and anti-MAA in arthritis patients

Baseline characteristics are described in *Table 1*. Anti-PTM antibody levels were measured in RA and non-RA arthritis patients and compared to healthy controls (*Figure 1A and B, and Supplementary Table 1)*. The non-RA arthritis group was divided into subgroups and separately depicted based as Auto-Immune (AI) arthritis (without RA) including psoriatic arthritis, paraneoplastic arthritis, SLE, sarcoidosis and spondyloarthritis and as non-Auto-Immune (non-AI) arthritis including septic arthritis, gout and pseudogout.

Compared to healthy controls anti-AGE and anti-MAA were most prevalent in RA (anti-AGE: 7.5% in HC versus 44.6% in RA and anti-MAA: 3.8% in HC versus 46.1% in RA but were also present in other types of early arthritis. Within non-RA arthritis patients anti-AGE and anti-MAA were present in 32.9% and 30.3%, respectively and in non-RA autoimmune arthritis anti-AGE and anti-MAA were found in 38.5% and 41.5% respectively. These data indicate that the presence of anti-PTM antibodies is not specific for RA. When analyzing combinations of autoantibodies, the largest subgroup of RA patients (n=99) had all four anti-PTM antibodies (anti-AGE, anti-MAA, anti-CarP, anti-CCP2) as well as RF, after which the second largest group (n=63) was characterized by the combination of RF, anti-CCP2 and anti-CarP (*Figure 1C*).

Interestingly, 67 (34.0%) and 57 (28.9%) of seronegative (RF-, ACPA- and anti-CarP negative) RA patients were positive for anti-AGE and anti-MAA respectively. Moreover, 40 (20.3%) of

these seronegative RA patients were positive for both anti-AGE and anti-MAA. These anti-PTM responses may identify a new subgroup in the otherwise seronegative RA patients.

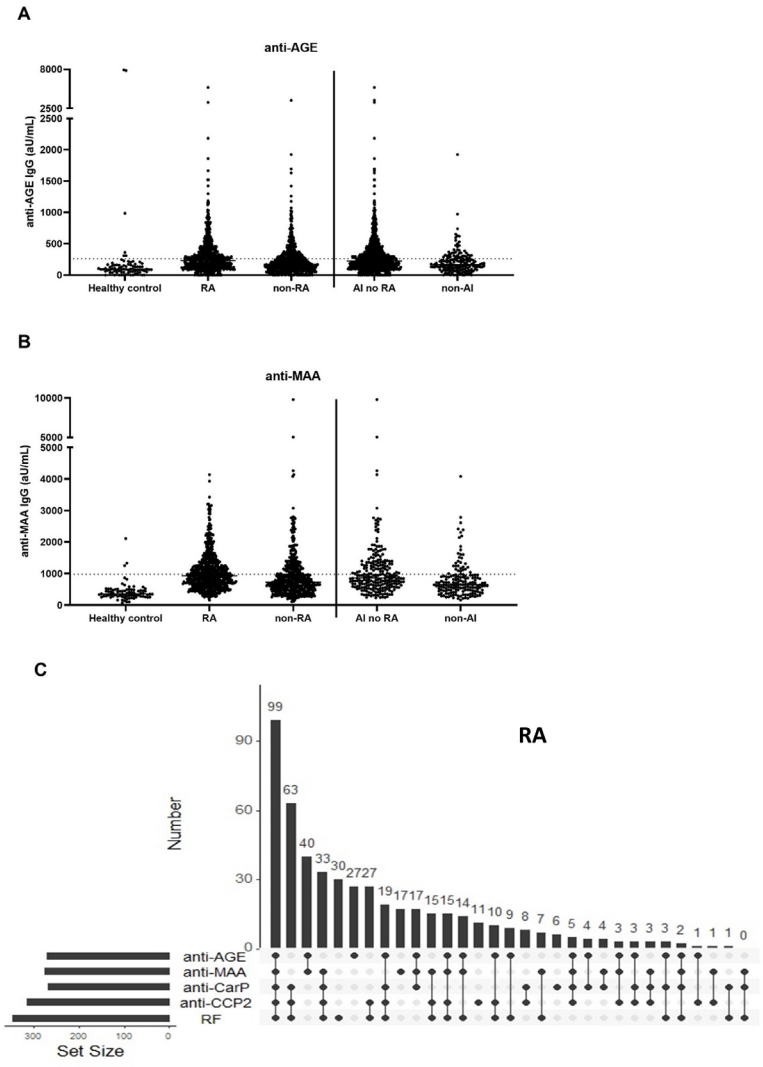


Figure 1: Anti-AGE and anti-MAA show higher levels in RA and occur in a subgroup of anti-CarP anti-CCP2 negative RA patients. IgG antibody levels of anti-AGE (A) and anti-MAA (B) in patients with (n=648) and without (n=538) RA. Early arthritis patients were separately depicted as groups: AI without RA (including psoriatic arthritis, paraneoplastic arthritis, SLE, sarcoidosis and spondyloarthritis) and non-AI (including septic arthritis, gout, pseudogout). (C) Upset plots of groups of RA patients (n=499*) positive for anti-PTM combinations; anti-AGE, anti-MAA, anti-CarP, anti-CCP2 and RF. *data for anti-CarP was missing for 149 RA patients. *Abbreviations: AGE, Advanced Glycation End-product; aU/mL, Arbitrary Units per mL; AI, autoimmune; CarP, Carbamylated Protein; CCP2, citrullinated cyclic peptide 2; MAA, Malondialdehyde Acetaldehyde Adduct; RA, Rheumatoid Arthritis; RF, Rheumatoid Factor.*

Table 1: Baseline characteristics of the RA, non-RA arthritis, autoimmune no RA and non-autoimmune group.

	RA (n=648)*	non-RA (n=538)*	AI no RA (n=233)*	non-AI (n=226)*
Female (n,%)	432 (66.7%)	269 (50.0%)	165 (49.8%)	112 (49.6%)
Age (mean, SD)	57.3 (17.4)	50.9 (15.8)	43.9 (15.7)	61.2 (13.5)
BMI (mean, SD)	25.9 (3.9)	26.5 (4.5)	25.7 (4.3)	27.5 (4.4)
Sympt. Dur. Weeks (median, IQR)	18 (9-36)	9 (2-27)	11 (4 – 28)	10 (2 – 31)
SJC (in 28joints) (median, IQR)	6 (3 - 11)	1 (1 - 4)	2 (0 - 4)	1 (1 – 4)
TJC (in 28joints) (median, IQR)	8 (4 - 14)	4 (1 - 9)	5 (2 – 9)	4 (1 – 8)
VAS (0-100) (median, IQR)	42 (20 – 58)	40 (19-60)	40 (20 – 60)	35 (19 – 52)
ESR (median, IQR)	34 (19 - 54)	27 (11 - 50)	33 (13 – 56)	19 (9 – 37)
CRP (median, IQR)	18 (8 - 41)	13 (4 - 34)	18 (6 – 41)	9 (3 – 23)
HAQ (median, IQR)	1 (0.62-1.62)	0.75 (0.25-1.13)	0.63 (0.25 – 1.13)	0.75 (0.25 – 1.13)
Smoking+ (n,%)	159 (24.5%)	102 (19.0%)	63 (20.5%)	41 (20.8%)
HLA-SE+ (n,%)	410 (63.3%)	128 (23.8%)	411 (48.5%)	N/A**
ACPA	317 (51.3%)	22 (5.1%)	17 (7.0%)	5 (2.7%)
RF	365 (56.3%)	52 (9.8%)	28 (10.4%)	24 (10.9%)

* = numbers differ slightly per analyses due to missing variables
** = data not shown (47.8% missing)
Diagnoses were termed non-RA: all diagnoses other than RA within the EAC cohort.
Diagnoses were termed autoimmune (AI) no RA: psoriatic arthritis, spondyloarthritis, sarcoidosis, SLE and paraneoplastic arthritis.
Diagnoses termed as non-autoimmune (non-AI) were: gout, pseudogout and septic arthritis.
Abbreviations: BMI, Body Mass Index; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; HAQ, Health Assessment Questionnaire; HLA-SE, HLA Shared Epitope; IQR, interquartile range; SD, standard deviation; SJC, swollen joint count; Sympt. Dur. Weeks, Symptom duration in weeks; TJC, tender joint count; VAS, visual analog scale.

HLA-DRB1*03 associates with anti-AGE and anti-MAA independently of anti-CarP in anti-CCP2-negative RA patients

Since HLA class II alleles are known to associate with autoantibody positivity in RA, we sought to investigate the presence of HLA-SE and its association with anti-AGE and anti-MAA antibodies. Of all RA patients 63.3% were HLA-SE+ (*Table 1*). Based on the well-known association between HLA-SE and RA, the HLA-SE alleles were assessed and were significantly more prevalent in all RA subgroups compared to healthy controls. In the anti-AGE-positive group, as compared to anti-AGE-negative RA patients however, the prevalence of HLA-SE alleles was similar (*Table 2*). The same was true for anti-MAA, therefore both anti-AGE and anti-MAA antibodies were not associated with HLA-SE.

Table 2: Association between anti-AGE and anti-MAA antibodies and HLA-SE and HLA-DRB1*03 presence in RA and HLA-DRB1*03 presence in non-RA patients from the Leiden EAC cohort.

Part I	**RA n=648**								
	HLA-SE-	**HLA-SE+**	**Total**	**OR**	**95%CI**	**p-value**	**OR**	**95%CI**	**p-value**
Healthy controls	674 (55.7)	537 (44.3)	1211	1 (ref)					
Anti-AGE-	139 (38.7%)	220 (61.3%)	359	**1.99**	**(1.56, 2.53)**	**<0.001**	1 (ref)		
Anti-AGE+	99 (34.3%)	190 (65.7%)	289	**2.41**	**(1.84, 3.15)**	**<0.001**	1.21	(0.88, 1.67)	0.24
Anti-MAA-	130 (37.2%)	219 (62.8%)	349	**2.11**	**(1.66, 2.70)**	**<0.001**	1 (ref)		
Anti-MAA+	108 (36.1%)	191 (63.9%)	299	**2.22**	**(1.71, 2.88)**	**<0.001**	1.04	(0.76, 1.45)	0.77
	HLA-DRB1*03-	**HLA-DRB1*03+**	**Total**	**OR**	**95%CI**	**p-value**	**OR**	**95%CI**	**p-value**
Healthy controls	941 (77.7%)	270 (22.3%)	1211	1 (ref)	-	-	-	-	-
Anti-AGE-	271 (75.5%)	88 (24.5%)	359	1.13	(0.86, 1.49)	0.38	1 (Ref)	-	-
Anti-AGE+	211 (73.0%)	78 (27.0%)	289	1.29	(0.96, 1.73)	0.09	1.14	(0.80, 1.62)	0.47
Anti-MAA-	266 (76.2%)	83 (23.8%)	349	1.09	(0.82, 1.44)	0.56	1 (ref)	-	-
Anti-MAA+	216 (72.2%)	83 (27.8%)	299	**1.34**	**(1.01, 1.78)**	**0.05**	1.23	(0.87, 1.75)	0.25
	non-RA n=246#								
	HLA-DRB1*03-	**HLA-DRB1*03+**	**Total**	**OR**	**95%CI**	**p-value**	**OR**	**95%CI**	**p-value**
Healthy controls	941 (77.7%)	270 (22.3%)	1211	1 (ref)	-	-	-	-	-
Anti-AGE-	99 (76.7%)	30 (23.3%)	129	1.06	(0.69, 1.62)	0.80	1 (Ref)	-	-
Anti-AGE+	70 (59.8%)	47 (40.2%)	117	**2.34**	**(1.58, 3.47)**	**<0.001**	**2.22**	**(1.28, 3.84)**	**0.01**
Anti-MAA-	97 (72.4%)	37 (27.6%)	134	1.33	(0.89, 1.99)	0.17	1 (ref)	-	-
Anti-MAA+	72 (64.3%)	40 (35.7%)	112	**1.94**	**(1.29, 2.92)**	**0.002**	1.46	(0.85, 2.50)	0.17

Table 2: Continued.

Part II	anti-CCP2-negative RA n=301##								
	HLA-DRB1*03-	**HLA-DRB1*03+**	**Total**	**OR**	**95%CI**	**p-value**	**OR**	**95%CI**	**p-value**
Healthy controls	941 (77.7%)	270 (22.3%)	1211	1 (ref)	-				
Anti-AGE- Anti-CarP-	125 (72.3%)	48 (27.7%)	173	1.34	(0.93, 1.92)	0.11	1 (ref)	-	-
Anti-AGE+ Anti-CarP-	60 (63.8%)	34 (36.2%)	94	**1.98**	**(1.27, 3.07)**	**0.003**	1.48	(0.86, 2.52)	0.16
Anti-AGE- Anti-CarP+	6 (50.0%)	6 (50.0%)	12	**3.49**	**(1.11, 10.89)**	**0.03**	2.60	(0.80, 8.47)	0.11
Anti-AGE+ Anti-CarP+	14 (50.0%)	14 (50.0%)	28	**3.49**	**(1.64, 7.40)**	**0.001**	**2.60**	**(1.16, 5.87)**	**0.02**
	anti-CCP2-negative RA n=307###								
	HLA-DRB1*03-	**HLA-DRB1*03+**	**Total**	**OR**	**95%CI**	**p-value**	**OR**	**95%CI**	**p-value**
Healthy controls	941 (77.7%)	270 (22.3%)	1211	1 (ref)	-				
Anti-MAA- Anti-CarP-	135 (73.8%)	48 (26.2%)	183	1.24	(0.87, 1.77)	0.24	1 (ref)	-	-
Anti-MAA+ Anti-CarP-	50 (59.5%)	34 (40.5%)	84	**2.37**	**(1.50, 3.74)**	**<0.001**	**1.91**	**(1.11, 3.30)**	**0.02**
Anti-MAA- Anti-CarP+	6 (42.9%)	8 (57.1%)	14	**4.65**	**(1.60, 13.51)**	**0.01**	**3.75**	**(1.24, 11.36)**	**0.02**
Anti-MAA+ Anti-CarP+	14 (53.8%)	12 (46.2%)	26	**2.99**	**(1.37, 6.54)**	**0.01**	**2.41**	**(1.04, 5.58)**	**0.04**

Table 2: Continued.

Part II	SE-negative RA n=271[##+]								
	HLA-DRB1*03-	**HLA-DRB1*03+**	**Total**	**OR**	**95%CI**	**p-value**	**OR**	**95%CI**	**p-value**
Healthy controls	941 (77.7%)	270 (22.3%)	1211	1 (ref)	-	-			
Anti-AGE- Anti-CarP-	110 (82.7)	23 (17.3)	133	0.73	0.46 – 1.17)	0.29	1 (ref)	-	-
Anti-AGE+ Anti-CarP-	36 (58.1)	26 (41.9)	62	**2.52**	**(1.49 – 4.24)**	**<0.001**	**3.45**	**(1.76 – 6.79)**	**<0.001**
Anti-AGE- Anti-CarP+	23 (59.0)	16 (41.0)	39	**2.42**	**(1.26 - 4.66)**	**0.008**	**3.33**	**(1.52 – 7.26)**	**0.003**
Anti-AGE+ Anti-CarP+	23 (62.2)	14 (37.8)	37	**2.12**	**(1.08 – 4.18)**	**0.03**	**2.91**	**(1.31 – 6.49)**	**0.009**
	SE-negative RA n=269[++]								
	HLA-DRB1*03-	**HLA-DRB1*03+**	**Total**	**OR**	**95%CI**	**p-value**	**OR**	**95%CI**	**p-value**
Healthy controls	941 (77.7%)	270 (22.3%)	1211	1 (ref)	-	-			
Anti-MAA- Anti-CarP-	114 (85.7%)	19 (14.3)	133	**0.58**	**(0.35 – 0.96)**	**0.04**	1 (ref)	-	-
Anti-MAA+ Anti-CarP-	35 (58.3%)	25 (41.7)	60	**2.49**	**(1.46 - 4.23)**	**<0.001**	**4.29**	**(2.11 – 8.69)**	**<0.001**
Anti-MAA- Anti-CarP+	15 (51.7)	14 (48.3)	29	**3.25**	**(1.56 – 6.82)**	**0.002**	**5.60**	**(2.33 – 13.44)**	**<0.001**
Anti-MAA+ Anti-CarP+	31 (66.0)	16 (34.0)	47	1.80	(0.97 – 3.34)	0.06	**3.10**	**(1.43 – 6.72)**	**0.004**

[#]HLA-DRB1*03 data was present from 246 of 538 non-RA patients.
[##]HLA-DRB1*03 as well as anti-CarP and anti-AGE data was present from 301 of 648 RA patients.
[###]HLA-DRB1*03 as well as anti-CarP and anti-MAA data was present from 307 of 648 RA patients.
[+]HLA-SE as well as anti-CarP and anti-AGE data was present from 271 of 648 RA patients.
[++]HLA-SE as well as anti-CarP and anti-MAA data was present from 269 of 648 RA patients.
Statistically significant difference between patient group and healthy controls (p≤0.05)
Abbreviations: AGE, Advanced Glycation End-products; CarP, Carbamylated Protein; CCP2, citrullinated cyclic peptide 2; HLA-SE, human leukocyte antigen shared epitope; MAA, Malondialdehyde Acetaldehyde Adducts; RA, rheumatoid arthritis; 95%CI, 95% confidence interval.

Since HLA-DRB1*03 is associated with seronegative RA and anti-CarP antibodies in this disease subset, we sought to investigate the association of HLA-DRB1*03 with anti-AGE and anti-MAA. In RA patients HLA-DRB1*03 was more prevalent in anti-AGE-positive and anti-MAA-positive patients as compared to healthy controls with OR values of 1.34 (95% CI 1.01 to 1.78, p=0.05) and 1.29 (95% CI 0.96 to 1.73, p=0.09), although this did not achieve statistical significance compared to anti-AGE-negative or anti-MAA-negative patients respectively (*Table 2, part I*). To investigate whether HLA-DRB1*03 is associated with anti-MAA and anti-AGE in anti-CCP2-negative RA, we focused on this subset and stratified the analysis for anti-CarP. Within the anti-CCP2 negative RA patients, anti-AGE and anti-MAA antibodies were associated with HLA-DRB1*03 compared to healthy controls (OR: 1.98, 95% CI 1.27 to 3.07, p=0.003, and OR: 2.37, 95% CI 1.50 to 3.74, p<0.001, respectively). Anti-MAA was associated with HLA-DRB1*03 in the anti-CCP2 negative stratum independent of anti-CarP (OR: 1.91, 95% CI 1.11 to 3.30, p=0.02) (*Table 2, part II*). In this stratified analysis, anti-AGE showed the same trend for association but did not reach significance (OR: 1.48, 95% CI 0.86 to 2.52, p=0.16). Since anti-AGE and anti-MAA often co-occur, we next stratified the association analysis for these autoantibodies, to dissect whether the observed association to HLA-DRB1*03 could be attributed to one of them in particular. After stratification for anti-AGE or anti-MAA, only double positive RA patients showed a significant association with HLA-DRB1*03 compared to healthy controls (*Supplementary Table 2, part I*). Since some controversy exists on the association of HLA-DRB1*03 in anti-CCP2 negative RA patients, we investigated the association between anti-AGE and anti-MAA with HLA-DRB1*03 within HLA-SE negative RA patients. In both HLA-SE negative and anti-CCP2 negative stratum we find similar associations with anti-AGE/-MAA and HLA-DR1*03 (*Table 2, part III*).

In non-RA arthritis patients, both anti-AGE and anti-MAA showed a similar association with HLA-DRB1*03 with OR values of 2.34 (95% CI 1.58 to 3.47, p<0.001) and 1.94 (95% CI 1.29 to 2.92, p=0.002) compared to healthy controls (*Table 2, part I*). In a comparison within the non-RA arthritis patients, HLA DRB1*03 remained significantly associated with anti-AGE-positive compared to anti-AGE-negative patients (OR: 2.22, 95% CI 1.28 to 3.84, p=0.01), while the association with anti-MAA did not remain significant. To disentangle the effects of anti-AGE and anti-MAA, analyses were again stratified, after which only the presence of anti-AGE in anti-MAA-negative non-RA arthritis patients remained significantly associated with HLA-DRB1*03 (*Supplementary Table 2, part II*).

Taken together, these data indicate that anti-AGE and anti-MAA associate with HLA-DRB1*03 in RA and non-RA arthritis patients, and that this association (which cannot be ascribed to anti-AGE or anti-MAA in particular) is mainly present in anti-CCP2 negative RA patients. Similar associations were observed in HLA-SE negative RA patients.

Inflammation markers associate with anti-MAA positivity in RA and non-RA arthritis

Next we sought to investigate whether anti-PTM antibodies correlate with inflammation markers erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) (*Table 3*). Higher inflammation parameters in anti-AGE- and anti-MAA-positive individuals were observed in RA and non-RA arthritis patients, and in both the autoimmune and non-autoimmune subgroups of arthritis patients. To investigate whether both anti-MAA and anti-AGE were associated with acute phase reactants in RA independently, anti-AGE and anti-MAA were stratified for each other. After this stratification, anti-AGE was no longer associated with either CRP or ESR whereas the association of anti-MAA with these inflammation markers remained significant (*Supplementary Table 3)*. These data indicate that anti-PTM responses, especially anti-MAA, is associated with markers of inflammation in early arthritis in both RA and non-RA arthritis patients.

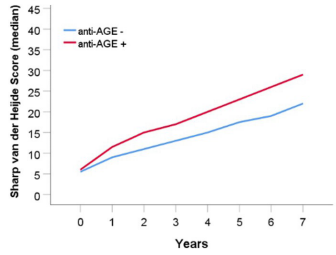
Anti-AGE and anti-MAA associate with radiological progression in anti-CCP2-negative RA patients

We next analyzed if the presence of anti-AGE and anti-MAA is associated with radiological progression in RA. Anti-AGE-positive patients displayed more radiographic damage per year than anti-AGE-negative patients (p<0.001) (*Figure 2A*). Data were then stratified for anti-CCP2, which revealed that this association was mainly present in the anti-CCP2-negative subgroup (*Figure 2B)*. When anti-CCP2 negative patients were further stratified for anti-CarP, the association between anti-AGE and radiographic progression remained significant (*Figure 2C)*. This indicates that in anti-CCP2 negative RA patients, anti-AGE is associated with radiological progression independent of anti-CarP, suggesting that this anti-PTM antibody could discriminate a different subgroup. Anti-MAA positivity was also associated with radiological progression (p=0.002) (*Figure 2D*). This effect was also observed in the anti-CCP2-negative stratum (Figure 2E), although no longer significant after stratifying for anti-CCP2. The latter could be a consequence of power as the effect size (beta) which decreased only slightly to 1.03/year, p=0.16 (*Figure 2E)*.

Presence of anti-MAA or anti-AGE is not associated with SDFR in RA

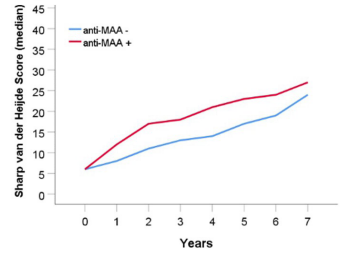
Next we sought to investigate whether anti-AGE and anti-MAA were associated with SDFR over time (*Supplementary Figure 1*). Anti-AGE was not associated with SDFR, hazard ratio (HR): 0.93 (95% CI 0.66 to 1.30; p=0.66) which did not differ after adjusting for CCP2 status (HR: 1.14, 95% CI 0.81 to 1.61, p=0.46). Anti-MAA-positive patients were less likely to achieve SDFR, compared to anti-MAA-negative patients, HR: 0.72 (95% CI 0.51 to 1.00, p=0.053). After adjusting for CCP2 status, there was no longer an association between anti-MAA and SDFR, HR: 1.05 (95% CI 0.74 to 1.50, p=0.80).

A.

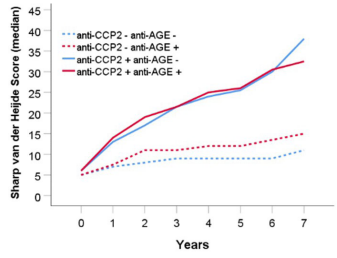


Anti-AGE+	**1.05 (1.03, 1.07), p<0.001**	Anti-MAA+	**1.04 (1.01, 1.06), p=0.002**

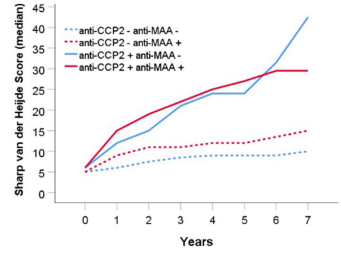
D.



B.

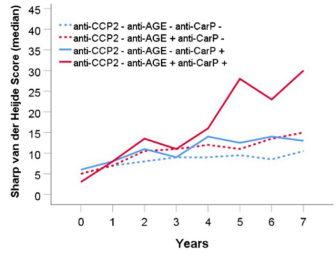


E.



CCP2- Anti-AGE-	Reference		CCP2- Anti-MAA-	Reference	
CCP2+ Anti-AGE-	**1.12 (1.09, 1.16), p<0.001**	Reference	CCP2+ Anti-MAA-	**1.12 (1.09, 1.16), p<0.001**	Reference
CCP2- Anti-AGE+	**1.04 (1.003, 1.08), p=0.03**		CCP2- Anti-MAA+	1.03 (0.99, 1.06), p=0.16	
CCP2+ Anti-AGE+	**1.14 (1.11, 1.18), p<0.001**	1.02 (0.99, 1.05), p=0.26	CCP2+ Anti-MAA+	**1.13 (1.10, 1.16), p<0.001**	1.01 (0.97, 1.04), p=0.68

C.



CCP2- Anti-AGE-Anti-CarP-	Reference	
CCP2- Anti-AGE+Anti-CarP-	**1.04 (1.003, 1.95), p-0.027**	
CCP2- Anti-AGE-Anti-CarP+	1.04 (0.97, 1.12), p=0.26	Reference
CCP2- Anti-AGE+Anti-CarP+	**1.12 (1.06, 1.18), p<0.001**	**1.04 (10004, 1.92), p=0.03**

Figure 2: Anti-AGE and anti-MAA associate with radiological progression in RA patients (n=600). (A) radiological progression in anti-AGE positive and negative RA. (B) data stratified for CCP2. (C) data stratified for anti-CarP in anti-CCP2-negative stratum. (D) radiological progression in anti-MAA positive and negative RA. (E) data stratified for CCP2. Data presented as estimate (95%CI), p-value. *Abbreviations: AGE, Advanced Glycation End-product; CarP, Carbamylated Protein; CCP2, citrullinated cyclic peptide 2; MAA, Malondialdehyde Acetaldehyde Adduct.*

Table 3: Association between anti-AGE and anti-MAA antibodies and ESR and CRP levels.

	RA n=648*		non-RA n=538*		AI no RA n=233*		non-AI n=131*	
	ESR (median, IQR)	CRP (median, IQR)	ESR (median, IQR)	CRP (median, IQR)	ESR (median, IQR)	CRP (median, IQR)	ESR (median, IQR)	CRP (median, IQR)
n, % anti-AGE+	289 (44.6%)		177 (32.9%)		90 (38.6%)		31 (23.7%)	
Anti-AGE-	32.0 (19.0-52.8)	17.0 (7.0-36.0)	21.5 (9.0-41.0)	11.0 (3.8-29.0)	24.0 (10.0-47.0)	14.0 (4.0-32.0)	22.0 (9.0-93.0)	14.0 (4.0-34.0)
Anti-AGE+	38.0 (19.0-57.0)	19.0 (9.0-48.0)	39.0 (21.5-59.5)	19.0 (7.0-55.0)	43.5 (28.5-64.8)	21.0 (9.0-56.0)	42.0 (26.0-55.0)	25.0 (6.5-93.0)
p-value	**0.02**	**0.03**	**<0.001**	**<0.001**	**<0.001**	**0.004**	**0.001**	0.095
n, % anti-MAA+	299 (46.1%)		163 (30.3%)		96 (41.2%)		19 (14.5%)	
Anti-MAA-	30.0 (16.8-48.3)	15.0 (6.0-31.0)	20.5 (9.0-39.0)	10.0 (3.0-27.0)	25.0 (10.0-45.0)	13.0 (4.0-32.0)	22.0 (11.0-41.0)	13.8 (4.0-34.0)
Anti-MAA+	41.0 (22.0-61.5)	22.5 (10.0-48.3)	42.0 (27.0-61.0)	21.0 (9.0-48.8)	44.5 (27.0-62.5)	21.5 (8.8-56.0)	45.0 (31.0-58.0)	25.0 (9.0-79.2)
p-value	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**0.003**	**0.005**	0.06

*ESR and CRP levels were not determined for all patients and numbers might therefore slightly differ per variable

Statistically significant difference between groups (p≤0.05)

Abbreviations: AGE, Advanced Glycation End-products; AI, Autoimmune (RA, psoriatic arthritis, paramalignant arthritis, SLE, sarcoidosis, spondyloarthropathy); CRP, C-reactive protein;

ESR, erythrocyte sedimentation rate; IQR, interquartile range; MAA, Malondialdehyde Acetaldehyde Adducts; non-AI, non-autoimmune (septic arthritis, gout and pseudogout);

RA, rheumatoid arthritis.

Discussion

In this study we demonstrated that anti-AGE and anti-MAA are present in RA patients, and interestingly also in a substantial part of otherwise seronegative RA patients. This is not specific for RA, as anti-AGE and anti-MAA antibodies were also present in other forms of early arthritis. Both anti-AGE and anti-MAA are associated with HLA-DRB1*03 in RA, and anti-AGE is also associated with HLA-DR1*03 in non-RA arthritis patients. Anti-AGE and anti-MAA are associated with a distinct clinical phenotype: anti-AGE associates with radiological progression in RA whereas anti-MAA only showed a trend with radiological progression but associated with increased inflammatory parameters in both RA and non-RA arthritis.

Associations with particular HLA class II alleles have been described to occur in many seropositive auto-immune diseases (13, 14). More specifically, HLA-DRB1*03, initially reported to be associated with anti-CCP2 negative RA, was later associated with presence of anti-CarP, although not all HLA DRB1*03-positive patients were anti-CarP-

positive (10, 12). In this study we observed that HLA-DRB1*03 was associated with anti-AGE and anti-MAA in anti-CCP2 negative RA patients which was independent of anti-CarP, thereby identifying another subgroup of anti-CCP2 negative RA that is associated with HLA-DRB1*03. In addition, anti-AGE associated with HLA-DRB1*03 in non-RA arthritis confirming the robustness of this finding. Together, these observations provide additional insight into the association of HLA DBR1*03 with (rheumatoid) arthritis; although these alleles are not associated with the presence of ACPA, they do appear to predispose to the formation of other autoantibodies (anti-CarP, anti-AGE and anti-MAA) in a process in which HLA class II-associated T-cell-dependent immune responses are likely to be involved.

Interestingly, in RA, anti-AGE associated with radiological progression independent of anti-CCP2 and anti-CarP suggesting an additive value of anti-AGE in determining disease evolution as it could define a new subgroup of RA patients. Strikingly, anti-AGE was not associated with SDFR. In RA and non-RA arthritis, a subgroup of patients is characterized by more extensive inflammation and the presence of anti-MAA antibodies, while a subgroup of CCP2 negative RA patients is characterized by radiological progression and presence of anti-AGE antibodies. Based on these results distinct subgroups within RA and non-RA arthritis can be delineated based on their specific clinical phenotype.

The presence of AGE modified proteins and anti-AGE antibodies has been observed in diabetes and hypertension (6, 23). Also in synovial tissue and sera of RA patients, AGE-modified proteins have been detected (24-26). In addition, MAA-modified proteins have been observed before in RA tissue (7) and it is clear that both modifications can be induced by inflammation and oxidative stress in the inflamed joint (5, 7). Our study now adds that in a subset of the RA patients antibodies against these PTMs are present. Additionally, anti-AGE and anti-MAA have been found to be associated with ESR in previous studies in RA and SLE (22). PTMs and anti-PTMs such as anti-AGE and anti-MAA add to the understanding that the combined presence of the antigen and the antibody could trigger effector mechanisms and contribute to the overall process of arthritis and joint damage, in RA and also in non-RA arthritis. It would therefore be interesting to investigate whether next to carbamylated proteins (27) also the modifications AGE and MAA are present in cartilage and synovium. Additionally, experimental pathogenicity studies on anti-AGE and anti-MAA specifically should be performed to elucidate on the contribution of these anti-PTM antibodies to pathogenesis.

There are some limitations to our study. Data on anti-CarP antibody levels was missing for 149 RA patients, therefore analysis using stratification including anti-CarP could only be performed in a subgroup of all RA patients. However, this group still consists of 499 RA patients and therefore still appears a good representation of the RA population.

Radiological progression was assessed in 635 RA patients included before 2006. Thereafter, radiographs have not been scored since radiographic damage has become rare/nearly non-existent with current treatment strategies. This effectively enabled us to detect differences in the, earlier, informative part of the cohort. When stratifying radiological progression data, groups became small and therefore could suffer from insufficient power implicating that significance could not always be reached. It is therefore important to verify associations using different and/or bigger cohorts to be able to generalize findings to the whole RA population. Additionally, in order to verify the results obtained in this study, a replication cohort is needed. In such a study IgA and IgM responses could be included to elaborate on the full anti-PTM antibody responses in (rheumatoid) arthritis patients (28, 29). One of the strengths of this study is that the EAC is a well-defined cohort containing RA and non-RA early arthritis patients with extensive information on the HLA haplotype and radiological progression for RA patients (15). Secondly, antibody responses have been investigated on the PTM-modified proteins and their control proteins. All PTMs were created on the same antigen backbone and reactivity against FCS itself was subtracted from the results. This results in reliable measurements that capture truly PTM-specific signals and decreases the chance of false observations (30). Additionally, correlation analyses were performed (data not shown) and data was stratified for the other investigated anti-PTM and to verify that anti-AGE and anti-MAA are solely responsible for the observed result and not cross-reactive.

In conclusion, anti-AGE and anti-MAA antibodies are both prevalent in RA patients, and other inflammatory rheumatic conditions, and although not specific for RA they each correlate with specific parameters. Anti-MAA associates with HLA-DRB1*03 in CCP2 negative (RA) patients independent of anti-CarP and associates with inflammation. Anti-AGE associates with HLA-DRB1*03 in CCP2- negative RA patients and is associated with a worse radiological progression especially in anti-CCP2- and anti-CarP-negative RA patients. With this study we have now characterized a seropositive subgroup within the heterogeneous group of RA patients that have been thus far been considered seronegative.

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Supplementary files

Supplementary Table 1: Prevalence of antibodies against AGE and MAA in healthy controls (n= 80) versus RA (n=648), non-RA (n=538), AI no RA (n=234) and non-AI (n=166) patient groups.

	Anti-AGE		**Anti-MAA**	
	aU/mL	***n, % positive***	**aU/mL**	***n, % positive***
HC (n=80)	94,2 [52,7 – 160,6]	6 (7,5)	358,4 [282,4 – 480,8]	3 (3,8)
RA (n=648)	233,1 [130,4 – 367,4]	289 (44,6)*	931,8 [663,2 – 1277,4]*	299 (46,1)*
non-RA (n=538)	177,8 [93,8 – 309,4]	177 (32,9)*	728,3 [485,1 – 1111,0]*	163 (30,3)*
AI no RA (n=234)	192,6 [102,3 – 328,6]	90 (38,5)*	853,7 [592,1 – 1246,5]*	97 (41,5)*
non-AI (n=166)	171,0 [107,2 – 286,4]	46 (27,7)*	666,1 [485,5 – 922,4]*	33 (19,9)*

Results are presented as median [IQR] and n (%)

* Statistically significant difference between patient group and healthy controls (p≤0.001)

Abbreviations: AGE, advanced glycation end-product; AI, Autoimmune (including psoriatic arthritis, paraneoplastic arthritis, SLE, sarcoidosis and spondyloarthritis); HC, Healthy controls; IQR, interquartile range; *MAA, malondialdehyde-acetaldehyde adduct; non-AI, non-autoimmune (including septic arthritis, gout, pseudogout); RA rheumatoid arthritis.*

Supplementary Table 2: Association anti-AGE and anti-MAA responses with HLA-DRB1*03 cross stratified for anti-AGE and anti-MAA for RA in part I and non-RA arthritis in part II.

Part I		RA n=648								
		HLA-DRB1*03-	**HLA-DRB1*03+**	**Total**	**OR**	**95%CI**	**p-value**	**OR**	**95%CI**	**p-value**
Anti-AGE-	**Healthy controls**	941 (77.7%)	270 (22.3%)	1211	1 (ref)	-	-	-	-	-
	Anti-MAA-	203 (75.5%)	66 (24.5%)	269	1.13	(0.83, 1.54)	0.43	-	-	-
	Anti-MAA+	68 (75.6%)	22 (24.4%)	90	1.13	(0.68, 1.86)	0.64	-	-	-
		HLA-DRB1.03-	**HLA-DRB1.03+**	**Total**	**OR**	**95%CI**	**p-value**	**OR**	**95%CI**	**p-value**
Anti-AGE+	**Healthy controls**	941 (77.7%)	270 (22.3%)	1211	1 (ref)	-	-	-	-	-
	Anti-MAA-	63 (78.8%)	17 (21.3%)	80	0.94	(0.54, 1.63)	0.83	1 (ref)	-	-
	Anti-MAA+	148 (70.8%)	61 (29.2%)	209	**1.44**	**(1.04, 1.99)**	**0.03**	1.53	(0.83, 2.82)	0.18
		HLA-DRB1*03-	**HLA-DRB1*03+**	**Total**	**OR**	**95%CI**	**p-value**	**OR**	**95%CI**	**p-value**
Anti-MAA-	**Healthy controls**	941 (77.7%)	270 (22.3%)	1211	1 (ref)	-	-	-	-	-
	Anti-AGE-	203 (75.5%)	66 (24.5%)	269	1.13	(0.83, 1.54)	0.43	-	-	-
	Anti-AGE+	63 (78.8%)	17 (21.3%)	80	0.94	(0.54, 1.63)	0.83	**-**	**-**	**-**
		HLA-DRB1.03-	**HLA-DRB1.03+**	**Total**	**OR**	**95%CI**	**p-value**	**OR**	**95%CI**	**p-value**
Anti-MAA+	**Healthy controls**	941 (77.7%)	270 (22.3%)	1211	1 (ref)	-	-	-	-	-
	Anti-AGE-	68 (75.6%)	22 (24.4%)	90	1.13	(0.68, 1.86)	0.64	1 (ref)	-	-
	Anti-AGE+	148 (70.8%)	61 (29.2%)	209	**1.44**	**(1.04, 1.99)**	**0.03**	1.27	(0.72, 2.24)	0.40

Supplementary Table 2: Continued.

Part II		**non-RA n=246**								
		HLA-DRB1*03-	**HLA-DRB1*03+**	**Total**	**OR**	**95%CI**	**p-value**	**OR**	**95%CI**	**p-value**
Anti-AGE-	**Healthy controls**	941 (77.7%)	270 (22.3%)	1211	1 (ref)	-	-	-	-	-
	Anti-MAA-	77 (78.6%)	21 (21.4%)	98	0.95	(0.58, 1.57)	0.84	1 (ref)	-	-
	Anti-MAA+	22 (71.0%)	9 (29.0%)	31	1.43	(0.65, 3.13)	0.38	1.50	(0.60, 3.74)	0.38
		HLA-DRB1.03-	**HLA-DRB1.03+**	**Total**	**OR**	**95%CI**	**p-value**	**OR**	**95%CI**	**p-value**
Anti-AGE+	**Healthy controls**	941 (77.7%)	270 (22.3%)	1211	1 (ref)	-	-	-	-	-
	Anti-MAA-	20 (55.6%)	16 (44.4%)	36	**2.79**	**(1.43, 5.46)**	**0.003**	1 (ref)	-	-
	Anti-MAA+	50 (61.7%)	31 (38.5%)	81	**2.16**	**(1.35, 3.45)**	**0.001**	0.78	(0.35, 1.72)	0.53
		HLA-DRB1*03-	**HLA-DRB1*03+**	**Total**	**OR**	**95%CI**	**p-value**	**OR**	**95%CI**	**p-value**
Anti-MAA-	**Healthy controls**	941 (77.7%)	270 (22.3%)	1211	1 (ref)	-	-	-	-	-
	Anti-AGE-	77 (78.6%)	21 (21.4%)	98	0.95	(0.58, 1.57)	0.84	1 (ref)	-	-
	Anti-AGE+	20 (55.6%)	16 (44.4%)	36	**2.79**	**(1.43, 5.46)**	**0.003**	**2.93**	**(1.30, 6.63)**	**0.01**
		HLA-DRB1.03-	**HLA-DRB1.03+**	**Total**	**OR**	**95%CI**	**p-value**	**OR**	**95%CI**	**p-value**
Anti-MAA+	**Healthy controls**	941 (77.7%)	270 (22.3%)	1211	1 (ref)	-	-	-	-	-
	Anti-AGE-	22 (71.0%)	9 (29.0%)	31	1.43	(0.65, 3.13)	0.38	1 (ref)	-	-
	Anti-AGE+	50 (61.7%)	31 (38.5%)	81	**2.16**	**(1.35, 3.45)**	**0.001**	1.52	(0.62, 3.71)	0.36

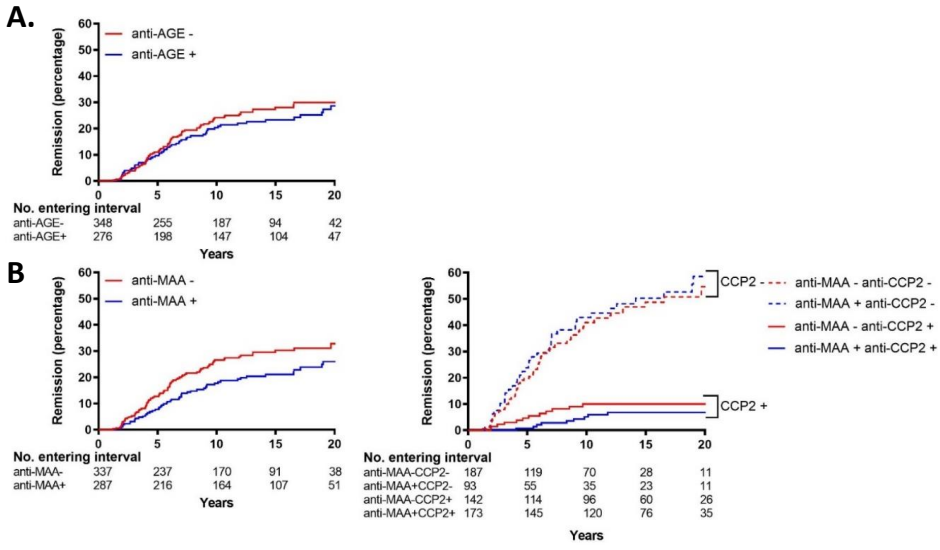
Results are presented as n (%) and OR (95% confidence interval)
Statistically significant difference between patient group and healthy controls (p≤0.05)
Abbreviations: AGE, advanced glycation end-product; MAA, malondialdehyde-acetaldehyde adduct; HC, Healthy controls; RA rheumatoid arthritis.

Supplementary Table 3: Association anti-PTM responses with ESR and CRP cross stratified for anti-AGE and anti-MAA for RA and non-RA arthritis.

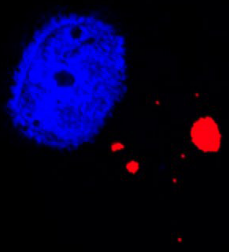
RA	Anti-AGE-			Anti-AGE+			Anti-MAA-			Anti-MAA+		
	Anti-MAA- n=267	Anti-MAA+ n=89	p-value	Anti-MAA- n=79	Anti-MAA+ n=208	p-value	Anti-AGE- n=267	Anti-AGE+ n=79	p-value	Anti-AGE- n=89	Anti-AGE+ n=208	p-value
ESR (median, IQR)	30.0 (16.0-49.0)	41.0 (22.5-57.0)	**0.009**	30.0 (18.0-47.0)	41.0 (20.0-64.8)	**0.010**	30.0 (16.0-49.0)	30.0 (18.0-47.0)	0.83	41.0 (22.5-57.0)	41.0 (20.0-64.8)	0.72
CRP (median, IQR)	15.0 (6.0-33.0)	24.0 (10.0-41.8)	**0.008**	12.0 (8.0-29.0)	22.0 (9.8-52.3)	**0.005**	15.0 (6.0-33.0)	12.0 (8.0-29.0)	0.81	24.0 (10.0-41.8)	22.0 (9.8-52.3)	0.77

non-RA	Anti-AGE-			Anti-AGE+			Anti-MAA-			Anti-MAA+		
	Anti-MAA- n= 302*	Anti-MAA+ n=54*	p-value	Anti-MAA- n=68*	Anti-MAA+ n=109*	p-value	Anti-AGE- n=302*	Anti-AGE+ n=68*	p-value	Anti-AGE- n=54*	Anti-AGE+ n=109*	p-value
ESR (median, IQR)	17.5 (8.0-36.0)	38.5 (23.0-63.5)	**<0.001**	31.0 (14.0 – 55.8)	42.00 (27.0-60.5)	**0.05**	17.5 (8.0-36.0)	31.0 (14.0 – 55.8)	**<0.001**	38.5 (23.0-63.5)	42.0 (27.0-60.5)	0.59
CRP (median, IQR)	9.0 (3.0-25.8)	21.0 (9.0-44.0)	**<0.001**	15.9 (5.0- 48.3)	21.0 (8.7-55.5)	0.09	9.0 (3.0-25.8)	15.9 (5.0- 48.3)	**0.05**	21.0 (9.0-44.0)	21.0 (8.7-55.5)	0.66

Results are presented as median (IQR)
*ESR and CRP levels were not determined for all patients and numbers might therefore slightly differ per variable
Statistically significant difference between groups (p≤0.05)
Abbreviations: AGE, Advanced Glycation End-products; *CRP,* C-reactive protein; *ESR,* erythrocyte sedimentation rate; *IQR,* interquartile range; *MAA,* Malondialdehyde Acetaldehyde Adducts*; RA,* rheumatoid arthritis.



Supplementary Figure 1: Presence of anti-MAA or anti-AGE is not associated with SDFR in RA patients (n=624). (A) Kaplan Meier curves presenting percentage remission in anti-AGE positive and -negative RA. (B) left panel: percentage remission in anti-MAA positive and -negative RA. Right panel: data stratified for CCP2 status. The number of patients entering the time interval is shown under each graph. *Abbreviations: AGE, Advanced Glycation End-product; CarP, Carbamylated Protein; CCP2, citrullinated cyclic peptide 2; MAA, Malondialdehyde-Acetaldehyde Adduct.*



3

Autoantibodies against specific post-translationally modified proteins are present in patients with lupus and associate with major neuropsychiatric manifestations

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Key messages

What is already known?

- Post-translationally modified (PTM) proteins and anti-PTM antibodies are described in many diseases, such as rheumatoid arthritis, in which anti-PTM antibodies are associated with disease progression;

What does this study add?

- We demonstrate the presence of several anti-PTM antibodies (anti-MAA, anti-AGE and anti-CarP) in patients with systemic lupus erythematosus (SLE) and their association with different aspects of disease activity in SLE and neuropsychiatric SLE (NPSLE);

How might this impact on clinical practice or future developments?

- As virtually no biomarker exists for NPSLE, anti-PTM Abs are a potential candidate. Future studies should further establish the potential role of anti-PTM antibodies in NPSLE.

Abstract

Background

Although autoantibodies are an important hallmark of systemic lupus erythematosus (SLE), most are not specific for SLE or any of its clinical manifestations. Autoantibodies against post-translationally modified (PTM) proteins have been studied extensively in rheumatoid arthritis and associate with disease progression. While PTMs have also been detected in SLE patients, studies on anti-PTM antibodies remain scarce. We studied the presence of anti-PTM antibodies in SLE and neuropsychiatric SLE (NPSLE), a manifestation that lacks serological markers.

Methods

IgG antibody responses against six PTMs (malondialdehyde-acetaldehyde adducts (MAA), advanced glycation end-products (AGE), carbamylation (CarP), citrullination (Cit), acetylation (AL) and nitration (NT)) were tested using ELISA in sera of 349 SLE patients (mean age 44 ± 13 years; 87% female) and compared to 108 healthy controls. Levels and positivity were correlated with clinical features and SLE manifestations.

Results

Anti-MAA, -AGE and -CarP antibodies were more prevalent in SLE compared to controls (MAA: 29 vs 3%, AGE: 18 vs 4%, CarP: 14 vs 5%, all $p \leq 0.0001$). Anti-MAA and anti-AGE antibodies correlated with clinical manifestations and serological inflammatory markers. Patients with major NPSLE showed higher positivity of anti-MAA (39 vs 24%, $p=0.01$) and anti-CarP antibodies (20 vs 11%, $p=0.04$) than patients without major NPSLE. In addition, anti-PTM Abs levels correlated with brain volumes, an objective measure of nervous system involvement.

Conclusions

In our NPSLE cohort, a subset of SLE patients have anti-PTM antibodies against MAA, AGE and CarP modified proteins. Interestingly, anti-MAA and anti-CarP were more prevalent in NPSLE, a manifestation for which no biomarkers exist.

Introduction

Systemic lupus erythematosus (SLE) is a heterogenous autoimmune disease characterized by a global loss of self-tolerance. Although autoantibodies are an important hallmark of SLE, many autoantibodies are not specific for SLE or specific SLE manifestations, such as neuropsychiatric involvement (NPSLE) (1).

Many different types of biomarkers exist and may be used in different contexts for diagnostic, prognostic and predictive purposes. In rheumatoid arthritis (RA), the identification of anti-citrullinated protein antibodies (ACPA), antibodies directed against a post-translational modification (PTM), has facilitated the diagnostic process and created new insights in its pathophysiology (2,3). Noteworthy, the presence of specific anti-PTM antibodies (Abs) also facilitate discrimination between phenotypes within RA, as they associate with more severe RA (4-7). It is possible that antibodies against PTMs may also contribute to the identification of specific phenotypes in patients with SLE.

PTMs can occur naturally, as part of physiological functions, or may be the result of enzymatic or chemical processes (8,9). SLE has been associated with a dysregulated metabolic state and elevated levels of reactive oxygen species (10), which enhances the formation of PTMs. In some situations, immune responses against PTMs can develop, leading to anti-PTM Abs (11). To date, anti-PTM Ab studies in SLE have mainly focused on ACPA and anti-carbamylated protein Abs (anti-CarP), which associated with increased joint damage (12-15). In addition, several anti-PTM Abs have been associated with general disease activity (SLE disease activity index (SLEDAI)) in lupus (16-18). Phospholipid B2-glycoproteine-1 is reported to be modified by PTMs making it more antigenic (19). Around 35% of all SLE patients are positive for antibodies against these phospholipids (aPL), which are associated with antiphospholipid antibody syndrome (APS) (20). Overall, studies on anti-PTM Abs in SLE patients remain limited and techniques to measure anti-PTM Abs vary greatly (17,18)

Based on previous studies, we hypothesized that SLE activity can lead to the generation of PTMs on relevant antigens and that there is specificity in breaking tolerance towards these neoantigens. In this study, we focused on IgG antibodies against six different PTMs, selected based on their association with activity in other diseases and variation in location in the protein, configuration and reversibility. We aimed to firstly study the presence of these six anti-PTM Abs in patients with SLE using a standardized method to assess specific anti-PTM Ab reactivities. Secondly, we aimed to assess the association between anti-PTM Abs and clinical phenotypes of SLE, in particular NPSLE, for which virtually no biomarkers exist. Additionally, both the subjective clinical diagnosis of NPSLE and objective evidence of nervous system involvement, namely radiological measurements, are assessed.

Methods

Study design and population

Patients visiting the NPSLE clinic of the LUMC between 2007-2019 with the clinical diagnosis of SLE and signed informed consent were included in this study. The NPSLE clinic is a tertiary referral center in which patients with neuropsychiatric (NP) symptoms, potentially caused by SLE, are assessed multidisciplinary. This evaluation process has been described in detail previously (21,22). In short, NP symptoms attributed to SLE by multidisciplinary consensus requiring immunosuppressive or anticoagulant treatment are classified as 'major NPSLE'. NP symptoms not attributed to SLE, mild NP symptoms that do not require additional treatment other than symptomatic treatment or NP symptoms due to other causes, are classified as minor/non-NPSLE. Patients with major NPSLE are further classified as having an ischemic, inflammatory or combined (both ischemic and inflammatory) phenotype, based on the suspected pathogenetic mechanism (21). Major NPSLE diagnoses are classified according to the 1999 ACR case definitions for NPSLE syndromes (23). Approval for this study was obtained (Leiden-the Hague-Delft ethical committee, *B18.040). Supplementary Figure 1* depicts the inclusion procedure.

Patient and Public Involvement statement

No patients were involved in the concept or design of this study.

Patient characteristics

Demographic and clinical patient characteristics were collected from electronical medical files of the visit to the NPSLE clinic: age, sex, smoking status, BMI, 1997 SLE classification criteria (24), SLE duration, SLEDAI-2K (25), SLICC/ACR Damage Index (SDI) (24), presence and phenotype of NPSLE and medication use. Current renal involvement was defined as hematuria, proteinuria, urinary casts or pyuria as according to the SLEDAI-2K (25). The presence of active arthritis was established during physical assessment. Serum samples from each participant were collected at time of visit to the NPSLE clinic. In some patients with inflammatory NPSLE, immunosuppressive treatment was already initiated at this timepoint (median treatment duration: 1 month). Details of routine laboratory assessment are provided in *Supplementary files Part II.*

Assessment of anti-PTMs

Generation of antigens

Modified proteins and their corresponding control non-modified protein were produced by either enzymatic or chemical reactions as previously described with some adaptations (6, 26-29). For more details, see *Supplementary files Part II.*

Detection of anti-PTM IgG antibodies by ELISA

Modified Fetal Calf Serum (FCS) and non-modified FCS were coated at 10µg/mL in 0.1M carbonate-bicarbonate buffer pH 9.6 (CB) on Nunc Maxisorp plates (430341, Thermofisher) overnight at 4°C. All sequential incubation steps were done at 4°C, blocking for 6 hours, serum incubation overnight and detection antibody for 3.5 hours. In between each step plates were washed with PBS/0.05% Tween (P1379, Sigma). After washing, plates were blocked using PBS/1%BSA for 6 hours at 4°C. Following washing, wells were incubated with serum at a 1/50 dilution in PBS/0.05%Tween/1%BSA (PTB) for CarP, Cit, AL and NT and at a 1/100 or 1/1000 dilution in PTB for AGE and MAA respectively. For each PTM, a standard of a pool of anti-PTM positive sera was taken along in serial dilutions on each plate. Human IgG was detected using rabbit anti-human IgG-HRP (P0214, Dako) diluted in PTB and incubated at 4°C for 3.5 hours. After the final wash, HRP enzyme activity was visualized using ABTS (A1888, Merck) with 0.05% H202 (107209, Merck) and absorbance at 415 nm was read using a microplate reader (Bio-Rad). Serum samples of 108 healthy controls from the Leiden area were also tested.[30 31] Absorbance was transformed to arbitrary units per milliliter (aU/mL) using a corresponding standard line for each PTM. Background aU/mL of FCS was subtracted from the aU/mL signal on FCS-PTM to analyze specific anti-PTM reactivity. Negative outcomes were changed to 0. Positivity for specific anti-PTM Abs was defined as a value larger than the mean plus two times the standard deviation (SD) in the healthy controls (HC). HCs with a value ≥10x the mean were excluded in calculating the cut-off. Additionally, serum of 54 NPSLE patients at their second visit (<2 years after 1st visit) were analyzed for anti-PTM antibody reactivity.

Brain volume

Brain volume measurements were available for 182 patients visiting the NPSLE clinic between 2007-2015. An extensive description thereof has been published previously (32,33). In short, white matter volume, grey matter volume, white matter hyperintensity volume and total brain volume were assessed using the CAT12 toolbox from the statistical parametric mapping software and the Lesion Segmentation Toolbox version 2.0.15 (34).

Statistical analysis

Differences in levels of anti-PTM Abs between HC, SLE patients and specific SLE manifestations were assessed using the Mann-Whitney test and Chi-Square test. Median, median difference and 95% confidence intervals (CI) were calculated using quantile regression. Further analyses were only performed if anti-PTM Ab positivity between HC and SLE differed by at least a factor of two. Spearman rank analyses were used to assess correlation between anti-PTM Ab levels and all continuous clinical variables (including brain volumes). Point-biserial correlations were used to assess correlation between the level of anti-PTM Abs (continuous) and other antibodies (dichotomous). As anti-PTM Ab level was non-normally distributed, the levels were natural log transformed for the point-biserial

correlations. Kruskal-Wallis test was used to compare level of anti-PTM Abs in patients with different NPSLE phenotypes. Wilcoxon signed-rank test was used to compare anti-PTM Ab level at baseline vs follow-up. P-values ≤0.05 were considered significant.
All statistical analyses were performed using STATA statistical software version 16.

Results

Study cohort

349 SLE patients were included in this study: 87% female and mean age 43.7 ± 13.4 years. At time of enrollment, median disease duration was four years [interquartile range (IQR): 1 – 13] and median disease activity (SLEDAI-2K) was four [IQR: 2 – 8] (*Table 1A)*. The most common ACR 1997 criteria were ANA positivity ever (97%), immunologic disorder (76%) and non-erosive arthritis (59%). 104 patients were diagnosed with major NPSLE (30%), of which 51 patients had an inflammatory, 28 patients an ischemic and 25 patients a combined phenotype. NPSLE syndromes (1999 ACR case definitions) are presented in *Supplementary Table 1*. Active nephritis and arthritis were present in 85 and 17 patients respectively. Most patients were ANA positive at inclusion (89%) and complement consumption was present in 34% of patients (*Table 1B)*.

Anti-MAA, anti-AGE and anti-CarP levels and positivity differ between HC and lupus patients

IgG antibody levels against six PTMs (MAA, AGE, CarP, Cit, AL and NT) from serum of all SLE patients were compared to serum of 108 HCs (*Table 2 and Supplementary Figure 2A-F*). Median difference (95% CI) between antibody levels in SLE patients vs HCs were 12 (95% CI: 7; 18) for anti-MAA, 32 (3; 60) for anti-AGE, 91 (60; 123) for anti-CarP, 0 (-1; 1) for anti-Cit, 4 (-2; 9) for anti-AL and 33 (-1; 67) for anti-NT.

Cut-off for anti-PTM positivity was defined as values larger than 2 times standard deviation above the mean of HCs. Anti-MAA, -AGE and -CarP showed significant higher positivity in SLE patients compared to HCs *(Table 2*).

Anti-MAA and anti-AGE correlate with measures of systemic inflammation

Next, we sought to investigate whether these increased anti-PTM Abs correlated with clinical and serological markers (*Figure 1A-C* and *Supplementary Table 2*). Anti-MAA and anti-AGE both negatively correlated with complement factors C3 and C4 ($p \leq 0.002$) and correlated positively with ESR ($p < 0.001$), ANA ($p = 0.02/0.03$), anti-dsDNA ($p \leq 0.005$) and anti-SM ($p = 0.02$). Anti-MAA negatively correlated with disease duration ($p = 0.03$) and showed positive correlations with disease activity ($p = 0.03$) and anticardiolipin ($p = 0.04$). Compared to anti-MAA, anti-AGE correlated slightly stronger with disease activity ($p = 0.001$). All correla-

tions found were modest (correlation coefficients ≤0.30). Anti-CarP only correlated significantly with age (p = 0.01). There was no significant difference in anti-PTM antibody levels between patients with and without immunosuppressive treatment.

Table 1A: Characteristics of study population with systemic lupus erythematosus at time of inclusion

Patient characteristics	**SLE (n = 349)**
Female	303 (87)
Age (years)	43.7 ± 13.4
Duration of SLE (years)	4 [1 – 13]
SLEDAI-2K	4 [2 – 8]
SDI	1 [0 – 2]
BMI	24.9 ± 5.1
Current Smoking	99 (28)
Comorbidities	
Hypertension	120 (35)
Diabetes	17 (4.9)
ACR 1997 criteria for SLE	
Malar rash	135 (39)
Discoid rash	65 (19)
Photosensitivity	179 (51)
Oral ulcers	149 (43)
Non-erosive arthritis	206 (59)
Pleuritis or pericarditis	90 (26)
Renal disorder (ever)	94 (27)
Neurologic disorder (psychosis/epilepsy)	43 (12)
Hematologic disorder	175 (50)
Immunologic disorder	265 (76)
Positive ANA	340 (97)
Current immunosuppressive medication	
Hydroxychloroquine	226 (65)
Prednisolone	187 (54)
Azathioprine	55 (16)
Methotrexate	22 (6)
Belimumab	2 (1)
Other*	11 (3)
Current organ involvement	
Major NPSLE	
Inflammatory	51 (15)
Ischemic	28 (8)
Combined	25 (7)
Renal involvement	67 (19)
Arthritis	17 (5)

Results are presented as n (%), mean ± sd or median [interquartile range; IQR]
*Other: includes cyclophosphamide (n = 9), tacrolimus (n = 2)
ANA, anti-nuclear antibodies; SDI, SLICC/ACR damage index; SLE, systemic lupus erythematosus; SLEDAI, systemic lupus erythematosus disease activity index.

Table 1B: Routine laboratory assessment of study population with systemic lupus erythematosus at time of inclusion*

	SLE (n = 349)
Nuclear antibodies	
ANA	309 (89)
Anti-dsDNA	91 (26)
Anti-ENA	166 (48)
Anti-SSA	131 (38)
Anti-SSB	39 (11)
Anti-SM	24 (7)
Anti-RNP	46 (13)
Antiphospholipid antibodies	
Lupus anticoagulant	101 (29)
Anti-cardiolipin IgG	46 (13)
β2-glycoprotein IgG	40 (15)
Complement factors	
Low C1q	42 (12)
Low C3	119 (34)
Low C4	86 (25)
Inflammation	
CRP	0.8 [0.8 - 7]
ESR	17 [9 - 39]

Results are presented as n (%) or median [interquartile range; IQR]
* Missing data nuclear antibodies: ANA + anti-dsDNA: n = 1, other: n = 2; antiphospholipid antibodies: β2-glycoprotein n = 79, LAC n = 5, aCl = 1. Complement factors: n = 2, Inflammation: n = 2. Percentages are given for the number of positive patients divided by the number of patients tested
ANA, anti-nuclear antibodies; anti-dsDNA, anti-double stranded DNA; anti-RNP, anti-ribonucleoprotein; anti-SM, anti-Smith; anti-SSA/B, anti–Sjögren's-syndrome-related antigen A/B autoantibodies; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; ENA, extractable nuclear antigen antibodies; SLE, systemic lupus erythematosus.

Table 2: Prevalence of antibodies against specific post-translational modifications in patients with systemic lupus erythematosus (n = 349) and healthy controls (n = 108)

	SLE (n = 349)		Healthy controls (n = 108)		
	aU/mL	***n, % positive***	**aU/mL**	***n, % positive***	**Median difference (95% CI)**
Anti-MAA	35 [23 – 52]*	101 (29)	23 [18-29]	3 (3)	12 (7;18)
Anti-AGE	112 [51 – 200]*	63 (18)	80 [41 – 122]	4 (4)	32 (3; 60)
Anti-CarP	126 [50– 206]*	49 (14)	35 [0 – 11]	5 (5)	91 (60; 123)
Anti-Cit	3 [2 – 6]	22 (6)	3 [2 – 6]	3 (3)	0 (-1; 1)
Anti-AL	8 [0 – 23]	29 (8)	4 [0 – 19]	8 (7)	4 (-2; 9)
Anti-NT	44 [0 - 177]	17 (5)	11 [0 – 132]	8 (7)	33 (-1; 67)

Results are presented as n (%) or median [IQR].
* Statistically significant difference between SLE patients and heathy controls ($p \leq 0.0001$)
AGE, advanced glycation end-product; AL, acetylated protein; CarP, carbamylated protein; Cit, citrullinated protein; MAA, malondialdehyde-acetaldehyde adduct; NT, nitrated protein.

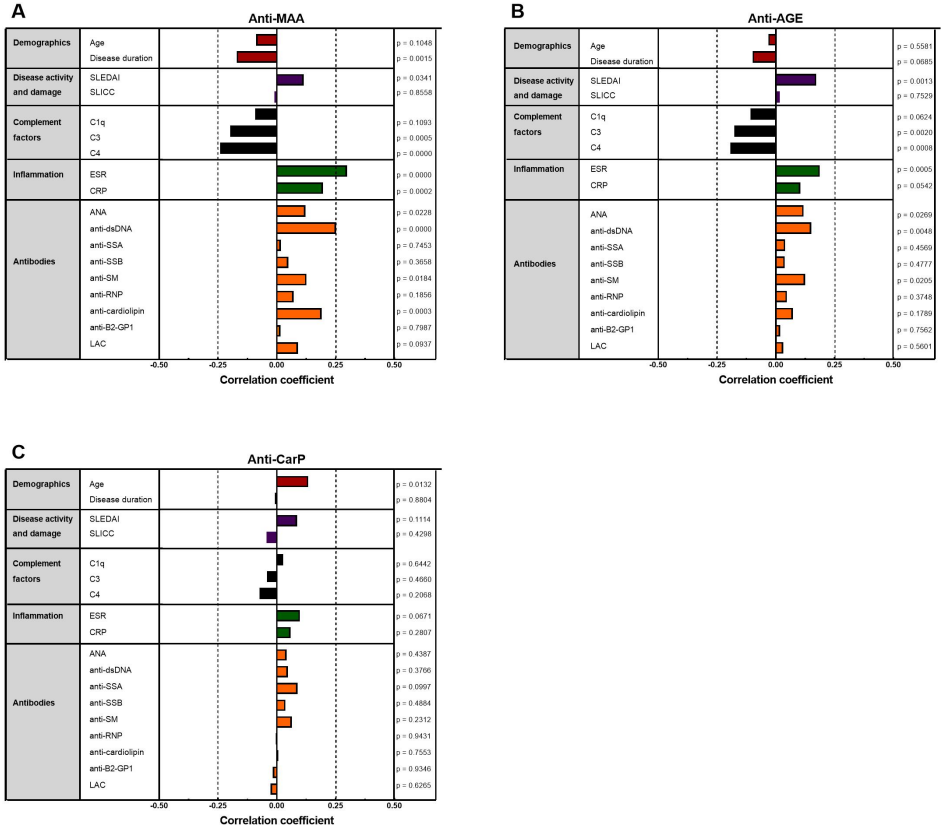

Figure 1: Correlation between (A) anti-MAA IgG (B) anti-AGE IgG and (C) anti-CarP IgG and clinical and laboratory markers in patients with systemic lupus erythematosus (n = 349). Measured by Spearman correlation analyses (demographics – inflammation) and point biserial correlation analyses after transformation (antibodies). AGE, advanced glycation end-product; ANA, anti-nuclear antibodies; anti-B2-GP1, anti-beta-2-glycoprotein; anti-dsDNA, anti-double stranded DNA; anti-RNP, anti-ribonucleoprotein; anti-SM, anti-Smith; anti-SSA/B, anti–Sjögren's-syndrome-related antigen A/B autoantibodies; CarP, carbamylated protein; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; LAC = lupus anticoagulant; MAA, malondialdehyde-acetaldehyde adduct; SLEDAI, systemic lupus erythematosus disease activity index; SLICC, systemic lupus International Collaborating Clinics damage index.

Anti-MAA and anti-CarP are more common in major NPSLE than in other SLE organ manifestations

Levels and positivity for different anti-PTM Abs were compared between patients with and without specific SLE manifestations (*Table 3* and *Figure 2A-C*). As our cohort comprises patients visiting an expertise center for NPSLE, we primarily focused on this manifestation. Patients with major NPSLE more frequently harbored anti-MAA Abs (39 vs 24%, p = 0.01) and anti-CarP Abs (20 vs 11%, p = 0.04) compared to patients without major NPSLE, whereas the prevalence of anti-AGE Abs did not differ as clearly (23 vs 16%, p =

0.13). In our cohort, for patients with active nephritis or active arthritis, differences in anti-PTM Ab positivity were less pronounced. Associations between the different anti-PTM Abs and ever having major organ manifestations is presented in *Supplementary Table 3*.

Table 3: The association between anti-PTMs (IgG) antibodies and specific organ manifestations of systemic lupus erythematosus (n = 349)

	NPSLE		**Active nephritis**		**Arthritis**	
	Yes *n* = 104	No *n* = 245	Yes n = 85	No *n* = 264	Yes n = *17*	No *n* =332
Anti-MAA						
aU/mL	41 [24 – 61]	34 [23 – 48]	36 [22 – 52]	35 [23 – 52]	30 [24 - 71]	35 [22 - 51]
Positive	**41 (39)***	60 (24)	24 (28)	77 (29)	6 (35)	95 (29)
Anti-AGE						
aU/mL	134 [48 - 217]	103 [52 –188]	124 [70 - 220]	107 [50 - 187]	115 [70 - 325]	112 [50 – 198]
Positive	24 (23)	39 (16)	20 (23)	43(16)	6 (35)	57 (17)
Anti-CarP						
aU/mL	133 [52– 245]	123 [50– 203]	139 [55 - 261]	121 [50 - 204]	157 [65 - 258]	126 [50 - 206]
Positive	**21 (20)****	28 (11)	16 (19)	33 (13)	3 (18)	46 (14)

Results are presented as n (%) or median [IQR].
*Chi-Square tests were used to assess the difference between the presence and absence of the specific manifestations. NPSLE yes vs no: *p = 0.01 ** p = 0.04, other values were not significant.
AGE, advanced glycation end-product; AL, acetylated protein; CarP, carbamylated protein; Cit, citrullinated protein; MAA, malondialdehyde-acetaldehyde adduct; NT, nitrated protein.

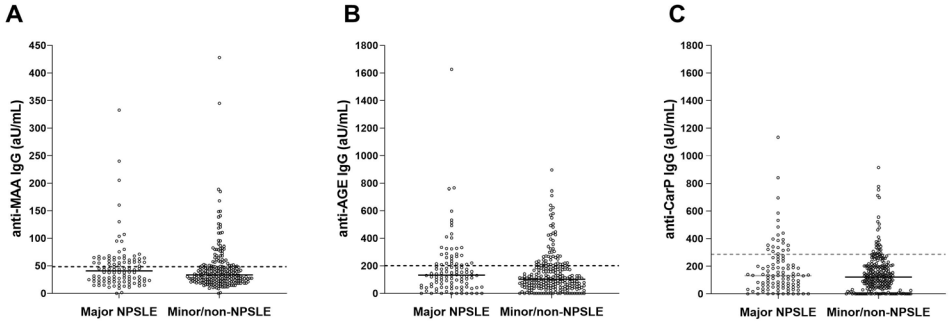

Figure 2: Levels of (A) anti-MAA, (B) anti-AGE and (C) anti-CarP IgG in patients with (n = 104) and without (n = 245) major NSPLE. Reactivity was determined using ELISA and cut-off was calculated using mean + plus two times the SD of the healthy controls (dashed line), as described in the Material and Methods. Reactivity is depicted as arbitrary units per milliliter (aU/mL). AGE, advanced glycation end-product; CarP, carbamylated protein; NSPLE, neuropsychiatric systemic lupus erythematosus; MAA, malondialdehyde-acetaldehyde adduct.

Anti-PTM Abs are similarly present in different major NPSLE phenotypes

As NPSLE has different pathophysiological origins, levels and positivity for different anti-PTM Abs were compared within specific phenotypes of major NPSLE (*see Supplementary Table 4*). Patients with a combined NPSLE phenotype showed the most anti-PTM Ab positivity (anti-MAA = 40%, anti-AGE = 28%, anti-CarP = 32%), followed by patients with an inflammatory phenotype (41, 27 and 14% respectively) and an ischemic phenotype (36, 11 and 21% respectively). These differences were not statistically significant. In addition, no difference was observed in the presence of anti-PTM Abs between inflammatory NPSLE patients that did and did not initiate immunosuppressive treatment prior to the clinic visit.

Brain volume and anti-PTM Abs

Previous analyses were based on the distinction between major and minor NP involvement, of which the diagnosis was based on multidisciplinary assessment. As this has the risk for phenotypic misclassification, we sought to study the correlation between anti-PTM Abs and an objective marker of central nervous system involvement in SLE. For 182 patients (52%), assessment of brain volumes was available (*see Supplementary Table 5*). The strongest correlations were between anti-MAA and white matter volume (WMV) and total brain volume (TBV) (Spearman ρ: –0.20 and –0.18; both $p < 0.02$) and anti-AGE and WMV and TBV (Spearman ρ: –0.16 and –0.15; both $p = 0.03$). Anti-CarP showed a significant association with white matter hyperintensity volume (Spearman ρ: 0.19, $p = 0.03$)

Longitudinal study comparing anti-PTM Ab responses overtime

In order to study anti-PTM Abs over time, serum samples from 54 patients, that were taken within 2 years after the first visit, were analyzed. Levels of anti-MAA, -AGE and -CarP Abs generally showed a decrease (*Figure 3A-C*). This decrease was significant for anti-MAA and anti-AGE ($p \leq 0.0001$), but not for anti-CarP ($p = 0.20$). Change in anti-MAA and anti-AGE Ab levels associated with change in SLEDAI-2K (SR: 0.29 and 0.28 ($p = 0.04$) respectively, but change in anti-CarP Ab level did not (SR: 0.11, ($p = 0.41$)).

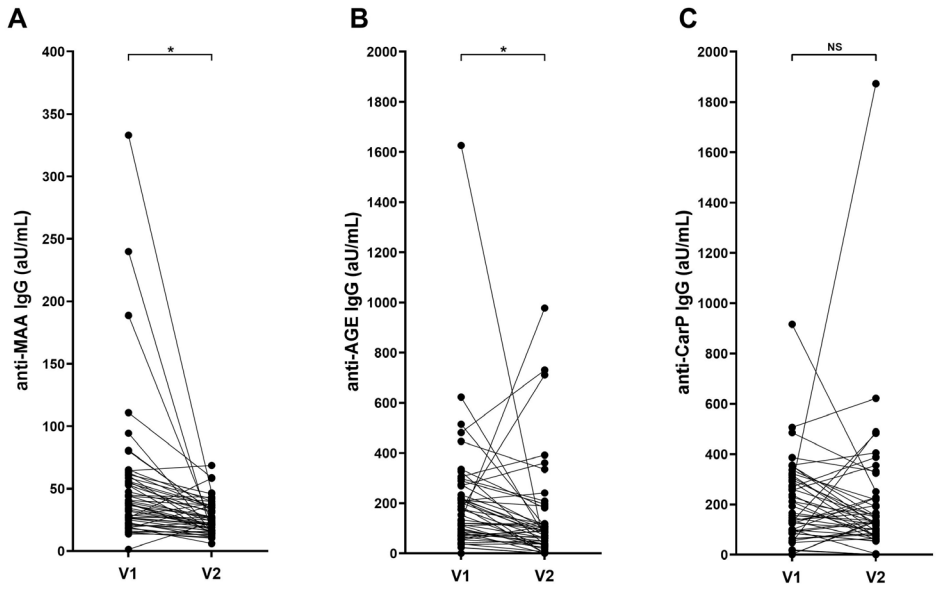

Figure 3: Levels of (A) anti-MAA, (B) anti-AGE and (C) anti-CarP in NPSLE patients (n = 54) overtime within 2 years after first visit. Reactivity was determined using ELISA and cut-off was calculated using mean + plus two times the SD of the healthy controls, as described in the Material and Methods. Reactivity is depicted as arbitrary units per milliliter (aU/mL). *p <0.01, NS, not significant. AGE, advanced glycation end-product; CarP, carbamylated protein; NPSLE, neuropsychiatric systemic lupus erythematosus; MAA, malondialdehyde-acetaldehyde adduct; V1, first visit; V2; second visit.

Discussion

We hypothesized that in SLE there is generation of PTMs on relevant antigens and that the presence of anti-PTM autoantibodies may be associated with clinical presentation and/or disease activity. Therefore, we investigated the presence of antibodies against six different PTMs on the same antigen backbone (FCS). We indeed observed that breaking of tolerance in SLE results in production of antibodies against the PTMs, predominantly MAA, AGE and CarP, and less pronounced against Cit, AL and NT. Furthermore, anti-MAA Abs and anti-AGE Abs associated with markers of inflammation. Finally, we searched for antibodies specific for NPSLE and observed that anti-MAA and anti-CarP Abs associated with major NPSLE.

Many different PTMs occur in both health and disease. Carbamylation and citrullination have been identified in RA patients and antibody responses against these PTMs are nowadays used as a clinical measure (2,3). While it is currently unknown why a subset of the patients produce anti-PTM antibodies, there is substantial insight into the processes

that drive the PTM modification of proteins. Inflammation and oxidative stress can lead to formation of reactive oxygen species (ROS) or induction of enzymes, which may lead to the formation of PTMs. ROS can lead to malondialdehyde (MDA) following peroxidation of lipids from for instance cell walls leading to MAA modification (35). Additionally, the ROS peroxynitrite is the reactive compound for nitration (36). Citrullination is the Peptidyl Arginine Deiminase (PAD) enzyme mediated conversion of arginine (37). The conversion of lysine into homocitrulline is driven by a chemical reaction with cyanate, a compound in equilibrium with urea and induced by smoking and inflammation (38). Excessive glycation is also a response to oxidative stress and inflammation leading to AGEs. AGEs in turn bind to AGE receptor (RAGE) leading to the perpetuation of inflammation (39). Excessive acetylation is a result of dysregulation of acetylation and deacetylation pathways (40). Taken together specific PTMs are a consequence of inflammation and oxidative stress. It is therefore well possible that these modifications occur in SLE patients in which widespread inflammation is going on. We previously observed that the PTM carbamylation is present in the joint of RA patients, but also in the joint of healthy controls, while the anti-CarP Abs are only found in a subset of RA patients (41). Why only a subset of RA and SLE patients produce such anti-PTM antibodies is still unknown. In our study, we demonstrated that three anti-PTM Abs are more prevalent in patients with SLE than in HCs, in increasing prevalence: anti-CarP, anti-AGE and anti-MAA. As only three out of six tested anti-PTMs showed increased reactivity, a specific induction process is implied. MAA, AGE and CarP are all modifications that occur on the lysine residue. However, acetylation (AL) is also a modification of lysine residue, but no difference in reactivity between SLE patients and HCs was observed there. Whether a specific underlying pathological mechanism for reactivity against these three PTMs exists, needs to be further investigated. As SLE patients are known for their global loss of self-tolerance, it is plausible that PTMs, that are persistently or abundantly present, are targeted. However, other factors, such as genetics and environmental triggers might play a role in breaking tolerance towards PTMs (42,43). In order to understand specific anti-PTM reactivities, the location of specific PTMs in different organ tissues in SLE patients needs to be evaluated. Furthermore, studies on monoclonal antibodies obtained from SLE patients are required to further pinpoint specific reactivity.

The three notable anti-PTM antibody responses have been studied to some degree in SLE before. Anti-CarP Abs, prevalent in 8 – 53% of SLE patients in other studies, have been associated with articular involvement, joint damage and disease activity (SLEDAI) (12,15,16). Our study was unable to confirm these findings, possibly because of the low number of patients with arthritis in our cohort. We can however exclude that the observed association between the presence of certain anti-PTM Abs and major NPSLE is driven by the association between anti-PTM Abs and arthritis and the concurrent presence of arthritis and major NPSLE. An increase of anti-CarP Abs was observed in patients

with major NPSLE, but the clinical meaning of this remains to be elucidated as limited correlation with other clinical markers was observed. Anti-AGE Abs have not been studied previously in SLE. However, increased amounts of AGEs have been identified in skin tissue of SLE patients and increased AGEs in blood plasma are described, which correlated with disease activity (44). We demonstrated an association between anti-AGE Abs and different markers of systemic inflammation. In our study, anti-MAA associated most clearly with different markers of inflammation. Few previous studies have investigated the role of malondialdehyde (MDA), the unstable predecessor of MAA in SLE. One study demonstrated that anti-MDA IgG positively correlated with disease activity, ESR and CRP and negatively correlated with complement factors (18). This is in line with the results of our study. In this same study, an association with renal involvement was found, using a different definition for active nephritis (proteinuria > 5 g per day or greater than 3+ by dipstick, and/or cellular casts), whereas our study demonstrated an association with major NPSLE, a disease for which virtually no biomarker exists.

Several antibodies have been suggested as biomarker for NPSLE (45). In particular, anti-ribosomal P and anti-N-methyl-D-aspartate receptor antibodies which have been implicated in the pathogenesis of NPSLE, although their exact role remains inconclusive and their clinical value limited (46-48). It is thought that breaches of the neuroimmune interface (amongst others, the blood-brain barrier) might enable neuropathic antibodies in the serum of patients to enter the central nervous system (49). Interestingly, we demonstrated that several anti-PTM Abs are more prevalent in patients with major NPSLE. Discrimination between the presence and absence of NPSLE is important, as it influences treatment. The diagnosis is based on multiple clinical factors, and the presence or absence of anti-PTM Abs could give further direction. Additional studies need to clarify whether specific anti-PTM responses are specific enough for NPSLE or should be interpreted as part of a series of markers to point towards specific subgroups. The observed association between anti-PTM Abs and NPSLE is supported by the correlation between specific anti-PTM Abs and white matter and white matter hyperintensity brain volume, objective measures of CNS involvement linked to NPSLE. MAA has previously been linked to brain injury and neurodegenerative diseases (50,51). Furthermore, in aging individuals, increased levels of MDA are seen in the temporal lobes, occipital lobes and hippocampus, underlying the potential relevance of anti-MAA Abs (52). We demonstrated that all three anti-PTM Abs (anti-MAA, -AGE and -CarP) showed a decrease in reactivity over time, of which anti-MAA and anti-AGE correlated with disease activity. To further uncover the role of anti-PTM Abs in NPSLE, future studies should assess the presence of anti-PTMs in cerebrospinal fluid.

Our study has several strengths: we used a standardized controlled ELISA set-up with one antigen backbone for each of the PTMs and a well-defined cohort of patients with

SLE and specifically NPSLE.

There are also several limitations to our study. A relatively large number of patients were negative or weakly positive for ANA at inclusion, which was tested at a dilution of 1:40. Therefore, we repeated the analyses in the patients positive for ANA (see *Supplementary files part III*), which led to similar results as the main analyses. Furthermore, as this study cohort is part of a tertiary referral for NPSLE, other clinical subsets (such as arthritis) are less prevalent. In this exploratory study within a well-defined cohort we found anti-PTM antibodies as potential biomarker for NPSLE and now additional studies need to be performed to determine the discriminative value of anti-PTM in different clinical settings, such as the outpatient clinic of a non-academic hospital. In addition, the clinical correlations identified in this study were modest and need further investigation. Lastly, the diagnosis of major NPSLE is made on clinical grounds. Although this is a clinically relevant phenotype, there still might be different underlying biological processes. Therefore, we used an objective marker (brain volumes) for central nervous system involvement and found an association with anti-PTM antibodies.

In conclusion, we identified three anti-PTM Abs (anti-MAA, -AGE and -CarP) that are present more frequently in patients with SLE, of which anti-MAA and anti-AGE correlate with measurements of systemic inflammation. Furthermore, several anti-PTMs Abs (anti-MAA and -CarP) were more prevalent in patients with major NPSLE, a disease manifestation currently lacking a suitable biomarker. In addition, all three anti-PTM Abs also correlated with brain volumes. Further research should confirm the role of anti-PTM Abs as well as its discriminative value for (NP)SLE.

Acknowledgements

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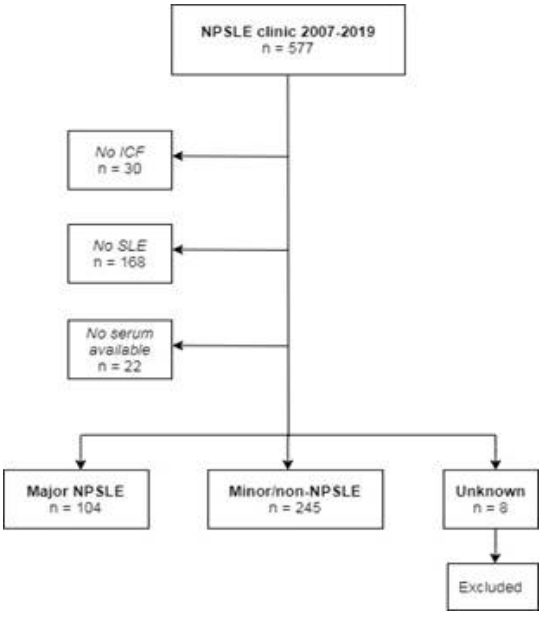
Supplementary files

Part I: Additional cohort information

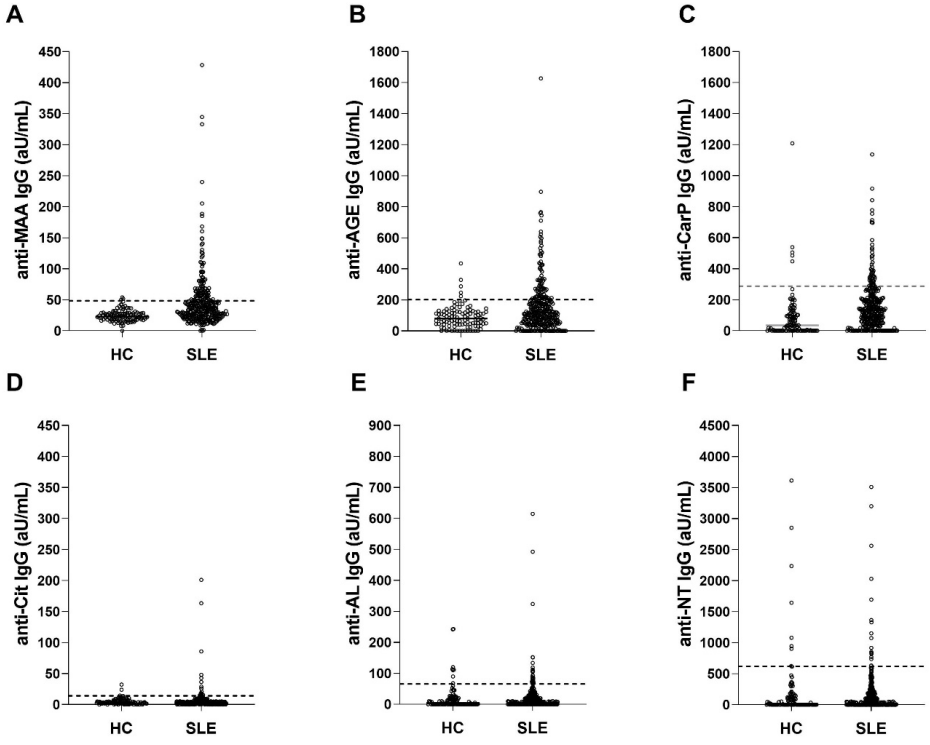


Supplementary Figure 1: Patient inclusion. The diagram illustrates the number of patients that were included and excluded from this study. The resulting number of patients include patients from whom SLE has been confirmed and required data was available. Namely, 104 patients with major NPSLE and 245 minor/non-NPSLE, making a total of 349 patients.
ICF = informed consent form; *NPSLE* = neuropsychiatric systemic lupus erythematosus

Supplementary Table 1: NPSLE syndromes (n = 176) according to 1999 ACR criteria of patients with major NPSLE (n = 104)

NPSLE syndrome (n, % of patients with major NPSLE)	
Aseptic meningitis	1 (1)
Cerebrovascular disease	45 (43)
Demyelinating syndrome	0 (0)
Headache	8 (8)
Movement disorder (chorea)	3 (1)
Myelopathy	9 (9)
Seizure disorders	9 (9
Acute confusional state	8 (8)
Anxiety disorder	1 (1)
Cognitive dysfunction	39 (38)
Mood disorder	13 (13)
Psychosis	7 (7)
AIDP[b]	0 (0)
Autonomic disorder	0 (0)
Mononeuropathy	0 (0)
Myasthenia gravis	0 (0)
Neuropathy, cranial	6 (2)
Plexopathy	0 (0)
Polyneuropathy	4 (4)
Other[c]	31 (30)

[a] Patients with neuropsychiatric symptoms attributed to SLE
[b] Acute inflammatory demyelinating polyneuropathy.
[c] Other NPSLE symptoms: cerebral vasculitis (n = 15), organic brain syndrome (n = 3), lethargia (n = 1) visual disturbance other than optic neuritis (n =2), apraxia (n =2), apathy (n = 2) walking disorder (n =2), motor disorder left arm (n =1), paresis left arm and dysarthria (n = 1), increased intracranial pressure (n =1), mononeuritis multiplex (n =1).



Supplementary Figure 2: Levels of different IgG antibodies against specific post-translational modifications (A) MAA, (B) AGE, (C) CarP, (D) Cit, (E) AL and (F) NT in healthy controls (HC) and patients with systemic lupus erythematosus (SLE). Reactivity was determined using ELISA and cut-off was calculated using mean + plus two times the SD of the healthy controls (dashed line), as described in the Material and Methods. Reactivity is depicted as arbitrary units per milliliter (aU/mL). AGE, advanced glycation end-product; AL, acetylated protein; CarP, carbamylated protein; Cit, citrullinated protein; MAA, malondialdehyde-acetaldehyde adduct; NT, nitrated protein.

Supplementary Table 2: Details regarding correlation coefficients (including 95% confidence intervals) provided in Figure 2

	Anti-MAA		Anti-AGE		Anti-CarP	
		95% CI*	corr	95% CI	corr	95% CI
Demographics						
Age	-0.09	-0.19; 0.02	-0.02	-0.13; 0.09	0.13	0.03; 0.23
Disease duration	-0.17	-0.27; -0.07	-0.10	-0.20; 0.01	-0.02	-0.12; 0.09
Disease activity and damage						
SLEDAI	0.12	0.01; 0.22	0.18	0.08; 0.28	0.09	-0.02; 0.19
SDI	-0.02	-0.12; 0.09	0.02	-0.09; 0.12	0.05	-0.15; 0.06
Complement factors						
C1q	-0.08	-0.19; 0.02	-0.09	-0.19; 0.02	0.05	-0.06; 0.15
C3	-0.20	-0.30; -0.09	-0.17	-0.28; -0.06	-0.04	-0.15; 0.07
C4	-0.24	-0.35; -0.13	-0.19	-0.30; -0.08	-0.07	-0.18; 0.04
Inflammation						
ESR	0.30	0.20; 0.39	0.19	0.08; 0.28	0.10	0.00; 0.20
CRP	0.19	0.09; 0.30	0.10	0.00; 0.20	0.07	-0.04; 0.17
Antibodies						
ANA	0.12	0.02; 0.22	0.12	-0.01; 0.22	0.04	-0.06; 0.15
Anti-dsDNA	0.25	0.15; 0.35	0.15	0.05; 0.25	0.05	-0.06; 0.15
Anti-SSA	0.02	-0.09; 0.12	0.04	-0.07; 0.15	0.09	-0.02; 0.19
Anti-SSB	0.05	-0.09; 0.12	0.04	-0.07; 0.14	0.04	-0.07; 0.14
Anti-SM	0.13	-0.02; 0.23	0.12	0.02; 0.23	0.06	-0.04; 0.17
Anti-RNP	0.08	-0.03; 0.18	0.05	-0.06; 0.15	0.00	-0.11; 0.10
Anti-cardiolipin	0.19	0.09; 0.29	0.07	-0.03; 0.18	-0.02	-0.12; 0.09
Anti-B2-GP1	0.02	-0.10; 0.14	0.02	-0.10; 0.14	0.01	-0.11; 0.12
LAC	0.09	-0.02; 0.19	0.03	-0.07; 0.14	-0.03	-0.13; 0.08

All confidence intervals were calculated using Fisher's transformation

*Correlation between anti-MAA IgG, anti-AGE IgG and anti-CarP IgG and clinical and laboratory markers in patients with systemic lupus erythematosus. Measured by Spearman correlation analyses (demographics – inflammation) and point biserial correlation analyses after transformation (antibodies).

AGE, advanced glycation end-product; ANA, anti-nuclear antibodies; anti-B2-GP1, anti-beta-2-glycoprotein; anti-dsDNA, anti-double stranded DNA; anti-RNP, anti-ribonucleoprotein; anti-SM, anti-Smith; anti-SSA/B, anti–Sjögren's-syndrome-related antigen A/B autoantibodies; CarP, carbamylated protein; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; LAC = lupus anticoagulant; MAA, malondialdehyde-acetaldehyde adduct; SLEDAI, systemic lupus erythematosus disease activity index; SLICC, systemic lupus International Collaborating Clinics damage index.

Supplementary Table 3: The association between anti-PTMs (IgG) and major organ manifestations according to the 1997 ACR criteria for SLE[53]

	Renal disease		Arthritis		Serositis	
	Yes *n = 94*	No *n = 255*	Yes *n = 206*	No *n = 143*	Yes *n = 90*	No *n = 259*
Anti-MAA						
aU/mL	31 [23-45]	37 [23-56]	38 [24-56]	31 [21-50]	38 [24-56]	32 [22-51]
Positive	18 (19)	83 (33)	63 (31)	38 (27)	27 (30)	74 (29)
Anti-AGE						
aU/mL	104 [47-190]	115 [52-102]	112 [46-209]	114 [55-192]	128 [59-215]	110 [47-187]
Positive	14 (15)	49 (19)	22 (15)	41 (20)	20 (22)	43 (17)
Anti-CarP						
aU/mL	133 [70-186]	125 [44-212]	123 [50-204]	130 [50-212]	138 [68-209]	122 [50-205]
Positive	11 (12)	38 (15)	18 (13)	31 (15)	16 (18)	33 (13)

Results are presented as n (%) or median [IQR]. Levels are AU/mL
AGE, advanced glycation end-product; CarP, carbamylated protein; MAA, malondialdehyde-acetaldehyde adduct.

Supplementary Table 4: The association between anti-PTMs (IgG) and specific NPSLE phenotypes

	Major NPSLE		
	Ischemic *n = 28*	Inflammatory *n = 51*	Combined phenotype *n = 25*
Anti-MAA			
aU/mL	29 [20 – 64]	44 [27 – 57]	39 [24 – 65]
Positive	10 (36)	21 (41)	10 (40)
Anti-AGE			
aU/mL	111 [37 – 84]	154 [70 – 253]	125 [44 – 260]
Positive	3 (11)	14 (27)	7 (28)
Anti-CarP			
aU/mL	90 [41 – 245]	137 [55 – 209]	182 [54 – 323]
Positive	6 (21)	7 (14)	8 (32)

Results are presented as n (%) or median [IQR].
AGE, advanced glycation end-product; CarP, carbamylated protein; NPSLE, neuropsychiatric systemic lupus erythematosus; MAA, malondialdehyde-acetaldehyde adduct.

Supplementary Table 5: The association between anti-PTMs (IgG) and brain volumes (n = 182)

	Grey matter	White matter	Total	White matter hyperintensity
	r_s	r_s	r_s	r_s
Anti-MAA	-0.13 (-0.27; 0.01)	-0.20 (-0.34; -0.06)	-0.18 (-0.31; -0.03)	-0.06 (-0.20; 0.09)
Anti-AGE	-0.13 (-0.27; 0.02)	-0.16 (-0.30; -0.02)	-0.16 (-0.29; -0.01)	0.06 (-0.09; 0.20)
Anti-CarP	-0.13 (-0.27; 0.01)	-0.14 (-0.28; 0.01)	-0.14 (-0.28; 0.01)	0.19 (0.05; 0.33)

Results are presented as Spearman R correlation coefficients (95% CI), as calculated by Spearman rank-order correlation.
AGE, advanced glycation end-product; CarP, carbamylated protein; MAA, malondialdehyde-acetaldehyde adduct; $r_{s,}$ spearman rank coefficient.

Supplementary files

Part II: Methods

Routine laboratory assessment

IgG anti-dsDNA antibodies were detected using the *Crithidia luciliae* indirect immune fluorescence technique (Immuno Concepts, Sacramento, CA, USA). IgG antibodies against SS-A/Ro-52, SS-B/La, Sm, RNP and IgG and IgM anticardiolipin (aCL) and anti-β2 glycoprotein 1 (anti-β2GP1) antibodies were determined using a Phadia® 250 EliA fluorescence enzyme immunoassay (FEIA) (Thermo Scientific, Freiburg, Germany). Anti-β2GP1 (IgG/IgM) and IgG antibodies against SS-A, SS-B, SM and RNP were considered positive if levels were >10 U/ml. aCL (IgG/IgM) was considered positive if levels were >30 GPL/U/ml. Lupus anticoagulant (LAC) was determined using STA-Rack and STA Evolution coagulation analyzers (Stago, Parsippany, NJ, USA). ANA analysis was performed with an immunofluorescence assay test on Hep-2 cells using a dilution of 1:40. ANA was defined as positive according to the normal limits of the LUMC laboratory (positive, 74% or weakly positive, 15%).

C1q, C3 and C4 were measured in serum using laser nephelometry. Based on the normal limits for our laboratory, C1q, C3 and C4 were defined as low or normal/high.

Erythrocyte sedimentation rate (ESR) was measured using the Westergren-method (StaRRsed Compact). C-reactive protein (CRP) was measured with turbidimetric assays using Roche COBAS 8000 Modular - c702. CRP <3 was under the detection limit until 2017, thereafter high sensitivity CRP was reported (hsCRP). The values of 2017 with CRP < 3 (no exact measurement) were imputed with the median of CRP < 3 after 2017 (exact measurement, median 0.8, range 0.3 – 2.5).

Generation of antigens

For all modifications backbone Fetal Calf Serum (FCS, Bodinco, Alkmaar, the Netherlands) was used. For malondialdehyde acetaldehyde adducts (MAA), first an MDA solution was prepared using 0.5M 1,1,3,3,-Tetramethoxypropane (108383, Sigma) and 0.3% hydrochloric acid (1003171000, EMSURE) and incubated for 12 minutes at 37C in a water bath. [29] Meanwhile 20mg/mL protein was prepared and diluted 1:5 with 20% MilliQ, 20% freshly prepared MDA solution, and 4% acetaldehyde (402788, Sigma), pH was adjusted to pH=4.8 and MilliQ was added till 5 times volume of protein was reached. The solution was then incubated for 2hr at 37C in a water bath. Advanced glycation end-products (AGE) were created by incubating 2mg/mL protein with 1:100 diluted 3.3M glycolaldehyde (23147-58-

2, Sigma). [30] The solution was filtered using a 0.2um whatmann filter (10462200, GE Healthcare) and incubated for 10 days at 37C while shaking. For carbamylation (CarP) of proteins, first a 2M potassium cyanate (KOCN, 215074, Sigma-Aldrich) solution in PBS was prepared. [6] 4mg/mL protein backbone was incubated to a 1:1 volume-to-volume ratio and incubated for 12hr at 37C. Citrullinated (Cit) proteins were created by incubating 1mg protein in a volume of 1mL containing 0.1M TRIS-HCl pH 7.6, 5mM DL-DTT (D0632, Sigma), 1mg/mL protein, 1U Peptidyl Arginine Deiminase 4 enzyme/100uL (PAD enzyme, 1584, Sigma) and CaCl2 10mM (21097, Sigma), volume was supplemented with MilliQ. [27] The mixture was incubated for 6hr at 53C. After incubation citrullinated protein was spun down and stored at -80C. Acetylation (AL) was performed using protein at a concentration of 1mg/mL in 0.1M Na2CO3. 50uL of acetic anhydride (100042, Merck) and 200uL of pyridine (109728, Merck) was added per 10mL of protein solution. [28] Proteins were incubated for 5hr at 30C. After incubation, the reaction was stopped using 200uL (per 10mL solution) of 1M TRIS. Samples were concentrated using Amicon Ultra – 15 centrifugal filter unit (10kDa, UFC901024, Sigma) and buffer was exchanged to PBS using Zeba Spin Desalting columns (89890, Thermofisher). For nitration (NT) 6 equal shots of peroxynitrite (14042-01-4, Cayman Chem) were directly used from stock solution to create an end concentration of 6mM. [26] After every shot the mix was directly vortexed for 30 seconds and left on ice for one minute before adding the next shot. After the last shot the samples were incubated for 1hr at 37C. Following incubation, all modifications (except for Cit, which was spun down) and their non-modified counterpart were extensively dialyzed against PBS using 10kDa cut-off SnakeSkin dialyzing tube (88243, Thermofisher). All modifications were stored at -20C until used. Protein concentrations were measured using Bradford solution (500-0006, Biorad) or Nanodrop. Modifications were verified using an ELISA based assay using anti-PTM specific antibodies: goat anti-MAA-HRP, ab20703, abcam; mouse anti-AGE 4B5, kindly provided by prof. H de Boer; rabbit anti-CBL, STA-078, biolabs; mouse anti-citrulline F95, MABN328, MilliPore; rabbit anti-acetyllysine, ADI-KAP-TF120-E, Enzo Lifesciences; mouse anti-nitrotyrosine, ab61392, abcam.

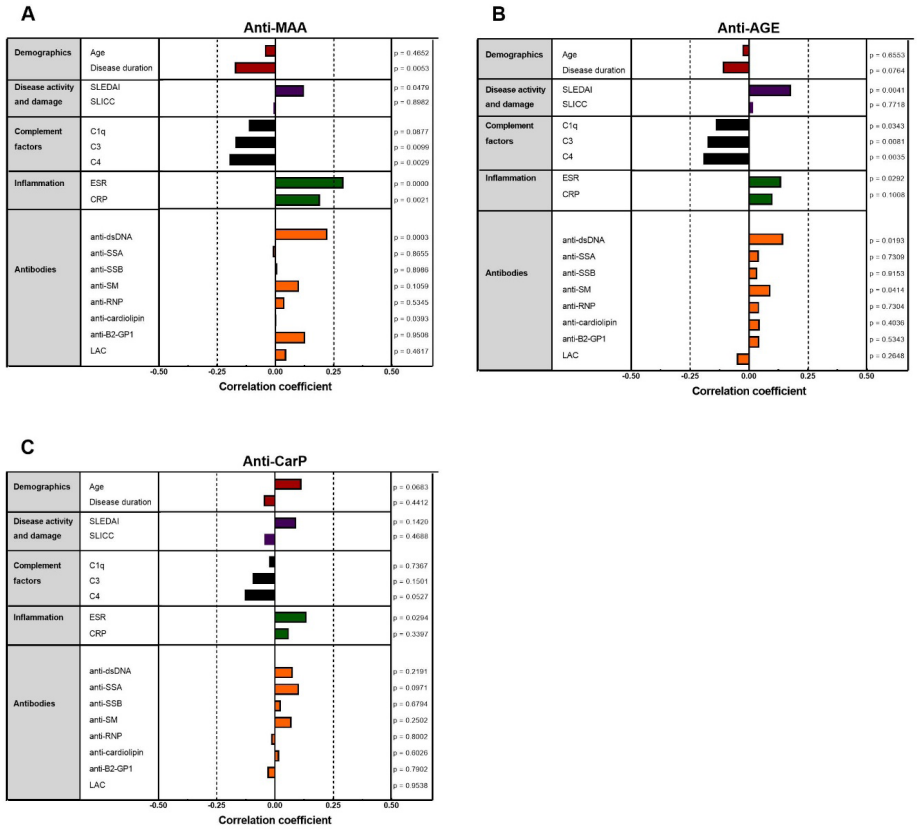
Supplementary files

Part III: Results repeated for patients with positive anti-nuclear antibodies (ANA) at time of visit

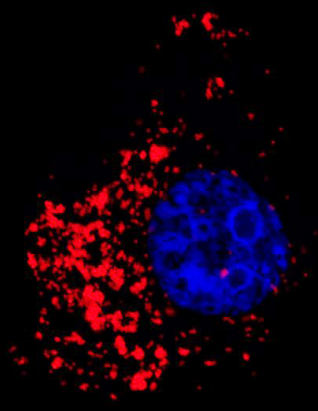
Supplementary Table 6: Prevalence of antibodies against specific post-translational modifications in healthy control and patients with systemic lupus erythematosus with positive ANA

	SLE patients (n = 258)		Healthy controls (n = 108)		Median difference (95% CI)
	aU/mL	*n,% positive*		*n,% positive*	
Anti-MAA	39 [1 - 57]*	91 (35)	23 [18 - 29]	3 (3)	16 (10; 22)
Anti-AGE	125 [56 – 215]*	56 (22)	80 [41 – 122]	4 (4)	45 (11; 79)
Anti-CarP	126 [50 – 220]*	41 (16)	35 [0 – 11]	5 (5)	91 (55; 128)
Anti-Cit	3 [2 – 6]	14 (5)	3 [2 – 6]	3 (3)	0 (-1; 1)
Anti-AL	8 [0 – 26]	23 (9)	4 [0 – 19]	8 (7)	4 (-3; 10)
Anti-NT	46 [0 – 180]	10 (4)	10 [0 – 132]	8 (7)	36 (0; 72)

Results are presented as n (%) or median [IQR]. * $p \leq 0.0001$
AGE, advanced glycation end-product; AL, acetylated protein; CarP, carbamylated protein; Cit, citrullinated protein; MAA, malondialdehyde-acetaldehyde adduct; NT, nitrated protein.



Supplementary Figure 3: Correlation between (A) anti-MAA IgG (B) anti-AGE IgG and (C) anti-CarP IgG and clinical and laboratory markers in ANA positive patients. Measured by Spearman correlation analyses (demographics – inflammation) and point biserial correlation analyses after transformation (antibodies). AGE, advanced glycation end-product; ANA, anti-nuclear antibodies; anti-B2-GP1, anti-beta-2-glycoprotein; anti-dsDNA, anti-double stranded DNA; anti-RNP, anti-ribonucleoprotein; anti-SM, anti-Smith; anti-SSA/B, anti–Sjögren's-syndrome-related antigen A/B autoantibodies; CarP, carbamylated protein; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; LAC = lupus anticoagulant; MAA, malondialdehyde-acetaldehyde adduct; SLEDAI, systemic lupus erythematosus disease activity index; SLICC, systemic lupus International Collaborating Clinics damage index.



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Antibodies against multiple post-translationally modified proteins aid in diagnosis of autoimmune hepatitis and associate with complete biochemical response to treatment.

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Abstract

Background

(Auto)immune mediated and cholestatic liver disease (AILD) includes autoimmune hepatitis (AIH), primary biliary cholangitis (PBC) and primary sclerosing cholangitis (PSC). Especially AIH is characterized by the presence of autoantibodies and elevated serum immunoglobulins. In rheumatoid arthritis, autoantibodies against post-translational modifications (PTMs) such as citrullination (Cit) and carbamylation (CarP) are used as diagnostic and prognostic markers, respectively. We studied the presence of six anti-PTM antibodies in patients with the three AILDs and non-AILD.

Methods

Antibodies against six PTMs (malondialdehyde–acetaldehyde adducts (MAA), advanced glycation end-products (AGE), CarP, acetylation (AL), Cit, and nitration (NT)) were tested in sera of patients with AILD (n=106), non-AILD (n=101) and compared with healthy controls (HC) (n=100). Levels and positivity were correlated with clinical and biochemical features in a well-defined cohort of untreated AIH patients.

Results

Anti-PTM antibodies were more often detectable in sera from AILD patients compared with HCs (anti-MAA: 67.9% vs 2.0%, anti-AGE: 36.8% vs 4.0%, anti-CarP: 47.2% vs 5.0% and anti-AL: 18.9% vs 5.0%). In untreated AIH, time to complete biochemical response (CBR) was associated with anti-MAA, anti-AGE, anti-CarP and anti-AL antibodies. Significantly more patients with at least three anti-PTM antibodies attained CBR at 12 months of treatment (13 vs 3 p=0.01).

Conclusions

Anti-PTM antibodies are frequently present in AILD. The presence of anti-MAA, anti-AGE and anti-CarP antibodies correlates with the presence of AIH within this cohort. In AIH, harboring at least three anti-PTM antibody responses is positively associated with CBR. Determination of anti-PTM antibodies in liver disease may have diagnostic and prognostic value.

Introduction

(Auto)immune mediated and cholestatic liver disease (AILD) is a heterogeneous group of both cholestatic and hepatocellular diseases, consisting of primary biliary cholangitis (PBC), primary sclerosing cholangitis (PSC), autoimmune hepatitis (AIH) and overlap variants. AIH and PBC are characterized by the presence of autoantibodies and elevated total immunoglobulin (Ig) G and IgM, respectively (1). The presence of autoantibodies against for example smooth muscle (SMA) and mitochondria (AMA) play an important role in the diagnostic scoring of AIH and PBC, respectively (2, 30). Although testing for different autoantibodies is implemented in the standard diagnostic work-up for liver disease with an unknown origin, they are not disease specific (4).

In another autoimmune disease, namely rheumatoid arthritis (RA), autoantibodies are also present, but in this disease antibodies frequently target proteins that have undergone post-translational modifications (PTM) (5). In particular, antibodies that target citrullination (anti-citrullinated antibodies: ACPA) and anti-carbamylated protein (anti-CarP) antibodies are used as diagnostic and prognostic markers in RA, respectively (6, 7). During inflammation, peptidyl arginine deiminases and cyanate are formed resulting in extracellular citrullination of arginine and carbamylation of lysine amino acids, respectively (8, 9). More recently, we have discovered antibody responses against the modifications malondialdehyde-acetaldehyde adducts (MAA) and advanced glycation end-products (AGE) in patients with systemic lupus erythematosus (SLE), defining a group of patients with neuropsychiatric manifestations (10). Both MAA and AGE are a result of oxidative stress and modify lysine amino acids (11, 12). Additionally, under oxidative stress nitration (NT) of the tyrosine amino acids and acetylation of lysines occur as a result of a reaction with peroxynitrite species and dysregulation of acetylation and deacetylation pathways, respectively (13, 14).

Inflammation occurs in both AIH and cholestatic liver disease, albeit at different sites (hepatocytes versus biliary tract). Oxidative stress occurs more frequently in patients with AIH compared to patients with cholestatic liver disease(15, 16). PTMs that are the result of oxidative stress have been reported to be highly immunogenic which could therefore result in anti-PTM antibody production, also in the context of AILD (17-19). However, studies assessing anti-PTM antibody responses in AILD are limited. Antibodies against cyclic citrullinated peptide (CCP) have been studied and were found in 9-11% of patients with type 1 AIH (20, 21), commonly in the absence of RA (21). Additionally, MAA modifications have shown to induce liver damage and to cause an autoimmune like pathophysiology in mice (22).

Since AIH, PBC and PSC are often considered (auto)immune mediated diseases that, like RA and SLE, display a variety of autoantibodies, we hypothesized that anti-PTM antibodies may be present in AILD and could have diagnostic or prognostic associations. Here we report that anti-PTM antibodies are present in AILD, allow discrimination between subgroups of AILD and are related to treatment response in AIH.

Materials and methods

Study design and population

Patients visiting the Department of Gastroenterology and Hepatology of the Leiden University Medical Centre (LUMC) between 1996 and 2020 who signed informed consent for the Biobanking facility were eligible for inclusion. Patients visiting the Department of Gastroenterology and Hepatology of Erasmus Medical Centre, Rotterdam, with no objection against the use of residual material, were also included. The biobank protocol (B21.032) was prospectively approved by the Medical Ethical Committee of the LUMC. For the purpose of this study, patients were divided into three groups: AILD, (i.e. AIH, PBC or PSC), miscellaneous chronic liver diseases (non-AILD) and healthy controls (HC). HC were preselected from a biobank containing serum from healthy individuals. They were matched based on sex and age to the AILD cohort. No data on medical history of medication use was available, mimicking the general population. Although clinical, biochemical and histological overlap can occur, patients with overlap variants (AIH-PBC or AIH-PSC) were not included in the AILD cohort. AIH was diagnosed using the *revised original* or *simplified* criteria for the diagnosis for AIH (2, 23, 24). Patients with AIH were included at diagnosis. Of all AILD patients, 66 were diagnosed with AIH. Of these patients 8 patients already started treatment before inclusion. PBC and PSC were diagnosed according to the diagnostic criteria in the European guidelines and were included during follow-up (25). As the AIH cohort was the largest cohort with complete data, this was the cohort in which the final analyses were done.

Patient characteristics

Demographics and patient characteristics were collected from electronic patient files at the time of visit to the outpatient clinic. This included: age, sex, comorbidities, disease duration, presence of liver cirrhosis, simplified criteria for the diagnosis of AIH (24), revised original criteria for AIH (23), presence of self-reported arthralgia (i.e., extrahepatic manifestation of AIH) and medication use. Follow-up data (i.e., time to complete biochemical response (CBR), treatment response, mortality and liver transplantation) was also collected. CBR was defined as normalization of aminotransferases and IgG below the upper limit of normal (26). Time to CBR was defined as the time from treatment initiation until the first time CBR was reached.

In addition to routine laboratory assessments (aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT), IgG, gamma-glutamyl transferase (GGT), alkaline phosphatase (AP) and presence of autoantibodies), serum samples from each patient were collected (*Supplementary Table 1*). For patients with cholestatic liver disease, data regarding cholangiographic findings and laboratory assessments (GGT, AP and autoantibodies) were also collected (*Supplementary Table 1*).

Generation of PTMs

Modified proteins and their corresponding control non-modified protein were produced by either enzymatic or chemical reactions as previously described [10).

Assessment of anti-PTM antibodies

Anti-PTM antibodies were detected using an in-house enzyme-linked immunosorbent assay (ELISA), based on modified fetal calf serum (FCS) as described previously (10). Briefly, modified and non-modified FCS were coated to a Nunc Maxisorp ELISA plate (430341, Thermofisher). In between each sequential step plates were washed three times using Phosphate Buffered Saline (PBS)/0.05%Tween (Sigma, P1379). After blocking (PBS/1% Bovine Serum Albumin (BSA)) for 6 hours at 4°C, plates were incubated overnight at 4°C with 1/50, 1/100 or 1/1000 diluted serum. Each plate contained a standard of anti-PTM antibody positive serum to calculate arbitrary units. After incubation, IgG levels were detected using horseradish peroxidase (HRP) labelled Rabbit-anti-Human IgG (Dako, P0214). Plates were developed by incubating with 2,2'-azino-bis[3-ethylbenzothiazoline-6-sulfonic acid] (ABTS)/0.015% H_2O_2 (A1888 and 7722-84-1, both from Merck) and absorbance at 415nm was measured using a microplate reader (Bio-Rad iMark). The cut-off for positivity was set as the mean arbitrary units plus two times the standard deviation of 100 HCs, excluding values higher than 10x the mean.

Statistical analysis

Statistical analyses were performed using IBM SPSS 25.0 (IBM, Armonk, NY). Baseline characteristics were evaluated using descriptive statistics. Differences in levels of anti-PTM antibodies between HCs, AILD and non-AILD were assessed using Kruskall-Wallis Test and Chi-2 test. Analyses of correlation between anti-PTM antibody levels and clinical variables were done using Spearman rank analyses for continuous clinical variables and point biserial correlation (i.e. mathematical equivalent of Pearson correlation) for dichotomous clinical variables. The anti-PTM antibody levels were transformed to natural logarithms to perform point-biserial correlations. Wilcoxon signed-rank test was used to compare anti-PTM antibody levels at baseline versus levels at the second visit.

Correlations between the difference in anti-PTM antibody levels at baseline versus the second visit and the change in levels of ALAT, ASAT and IgG were done using Spearman's

rho (r_s). Landmark analysis was used for the evaluation of CBR, with pre-determined timepoints at 3, 6 and 12 months, to prevent immortal time bias. P-values <0.05 were considered statistically significant.

Results

Study cohort

We studied 207 patients with liver disease comprising an AILD cohort (n=106) and a non-AILD cohort (n=101). The AILD cohort consisted of patients with AIH (n=66), PBC (n=10) and PSC (n=30) and was subsequently divided into two separate cohorts: AIH and cholestatic liver disease (CLD) (i.e. PBC and PSC). The non-AILD cohort consisted of patients with alcoholic liver disease (ALD) (n=29), chronic hepatis B (HBV) (n=4), chronic hepatitis C (HCV) (n=22), non-alcoholic fatty liver disease (NAFLD) (n=30), non-alcoholic steatohepatitis (NASH) (n=1), or a combination of these (n=15) (*Table 1*). In the AILD and non-AILD cohort 63.2% and 33.7% of the patients were female (p <0.001) with a mean age of 48.2±16.6 years and 54.0±11.0 years, respectively (p = 0.003) (*Table 1*). Cirrhosis was present in 39.6% of the AILD cohort and in 56.4% of non-AILD patients (p= 0.035). In the AILD cohort, 96.7% of patients with PSC had large duct PSC on cholangiographic imaging. Eighty percent of PBC patients was AMA positive. The mean age of the HCs was 50.2±10.5 years and 49% were female.

Anti-MAA, anti-AGE, anti-CarP and anti-AL antibodies are more prevalent in AILD compared to HC and non-AILD and are more likely to be positive for more than one anti-PTM antibody

Anti-PTM IgG antibody levels directed against 6 PTMs were measured in 207 patients with liver disease and 100 HCs (*Figure 1 and Supplementary Table 2*). Anti-MAA, anti-AGE, anti-CarP and anti-AL antibody levels differed significantly between AILD and HCs (1036.0, 234.5, 352.5 and 13.3 aU/mL vs 266.9, 88.9, 74.0 and 0.0 aU/mL respectively, all p<0.01). Only anti-MAA and anti-CarP antibodies were significantly increased comparing non-AILD compared to HCs (495.8 and 241.0 aU/mL vs 266.9 and 74.0 aU/mL, respectively, both p<0.01). Additionally, AILD showed significantly higher median levels of anti-MAA, anti-AGE, anti-CarP and anti-AL antibodies compared to non-AILD (anti-MAA, anti-AGE and anti-AL 1036.0, 234.5, 13.3 aU/mL vs 495.8, 130.0, 6.4 aU/mL, respectively, p<0.01 and anti-CarP 352.5aU/mL vs 241.0 aU/mL, p<0.05). Median levels of anti-NT and anti-Cit differed significantly between AILD and HCs (269 and 3.1 aU/mL vs 108 and 1.3 aU/mL, p<0.01 and p<0.05, respectively) but did not differ significantly between non-AILD and HCs.

Table 1: Characteristics of study population with autoimmune mediated and cholestatic liver disease (AILD) and non-AILD at time of inclusion. Results are presented as n (%), mean ±SD or median (IQR). P<0.05 is considered statistically significant (*). *Abbreviations: AIH, autoimmune hepatitis; AILD, autoimmune liver disease; ALD, alcoholic liver disease; HBV, chronic hepatis B; HCV, chronic hepatitis C; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; PBC, primary biliary cholangitis; PSC, primary sclerosing cholangitis.*

Patient characteristics	Auto-immune liver disease (AILD) (n=106)	Non-autoimmune liver disease (non-AILD) (n=101)	p-value
Primary diagnosis			
AIH	66 (62.3)	-	
PBC	10 (9.4)	-	
PSC	30 (28.3)	-	
NAFLD	-	30 (29.7)	
ALD	-	29 (28.7)	
HCV	-	22 (21.8)	
HBV	-	4 (4.0)	
NASH	-	1 (1.0)	
Hemochromatosis	-	0 (0.0)	
Combination†	-	15 (14.9)	
Female sex	67 (63.2)	34 (33.7)	<0.001*
Age sample (years)	48.2±16.6	54.0±11.00	0.003*
Cirrhosis	42 (39.6)	57 (56.4)	0.035*
Yes, compensated	28 (26.4)	27 (26.7)	-
Yes, decompensated	14 (13.2)	30 (29.7)	-
No cirrhosis	59 (55.7)	44 (43.6)	-
Unknown	5 (4.7)	0 (0.0)	-

† Combinations: ALD + HBV (N=1), ALD + HCV + HBV (N=1), ALD + HCV (N=6), ALD and hemochromatosis (N=2), ALD + NASH (N=2), ALD + PSC (N=1), HBV + HDV (N=1), HCV + HIV (N=1)

Comparing the frequency of positivity, AILD patients showed significantly increased positivity of anti-MAA, anti-AGE, anti-CarP and anti-AL antibodies compared to HCs (67.9, 36.8, 47.2 and 18.9% vs 2.0, 4.0, 5.0 and 5.0%, all p<0.01). Increased positivity for anti-MAA, anti-AGE and anti-CarP antibodies (28.7, 17.8 and 27.7% vs 2.0, 4.0 and 5.0%, respectively, all p<0.01) was observed when comparing non-AILD and HCs. Additionally, increased positivity between non-AILD and AILD was observed for anti-MAA, anti-AGE and anti-CarP antibodies (67.9, 36.8 and 47.2% vs 26.7, 17.8 and 27.7%, respectively, all p<0.01). Also when the non-AILD control group is limited to a more stringent set of conditions, excluding HBV, NASH and hemochromatosis, all statistical associations remain intact (data not shown). Anti-PTM antibody positivity for different anti-PTM antibodies were combined to calculate positivity for multiple anti-PTM antibodies (*Figure 2*). Patients with AILD more frequently harbored at least one type of anti-PTM antibody compared to non-AILD and HCs (AILD: 81.2%, non-AILD: 58.4% and HCs: 20%). The data in Figure 2 also indicate that AILD patients are more likely to be positive for multiple

anti-PTM antibodies. Overall, these data indicate that anti-PTM antibodies are especially present in patients with AILD.

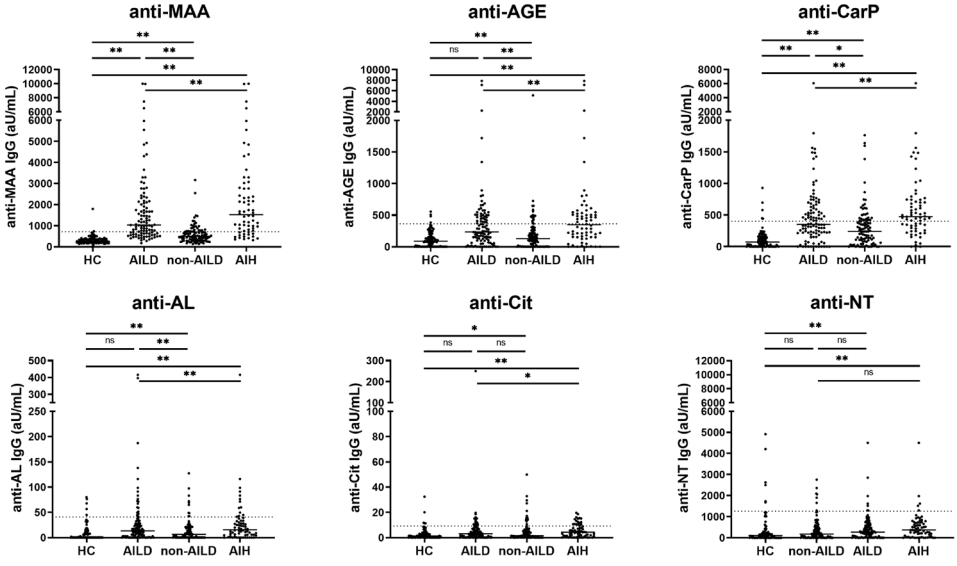

Figure 1: Anti-MAA, anti-AGE, anti-CarP, and anti-AL antibodies are increased in patients with AILD, and especially in patients with AIH. IgG antibody levels are presented as arbitrary units per milliliter (aU/mL) and cut-off for each PTM is indicated by the dashed line. *p<0.05, **p<0.01. *Abbreviations: Autoimmune Liver Disease: AIH, PBC and PSC; non-Autoimmune Liver Disease: NAFLD, HCV, HBV, ALD, Combination, NASH. AGE, advanced glycation end-product; AIH, autoimmune hepatitis; AL, acetylated protein; CarP, carbamylated protein; Cit, citrullinated protein; MAA, malondialdehyde–acetaldehyde adduct; ns, not significant; NT, nitrated protein.*

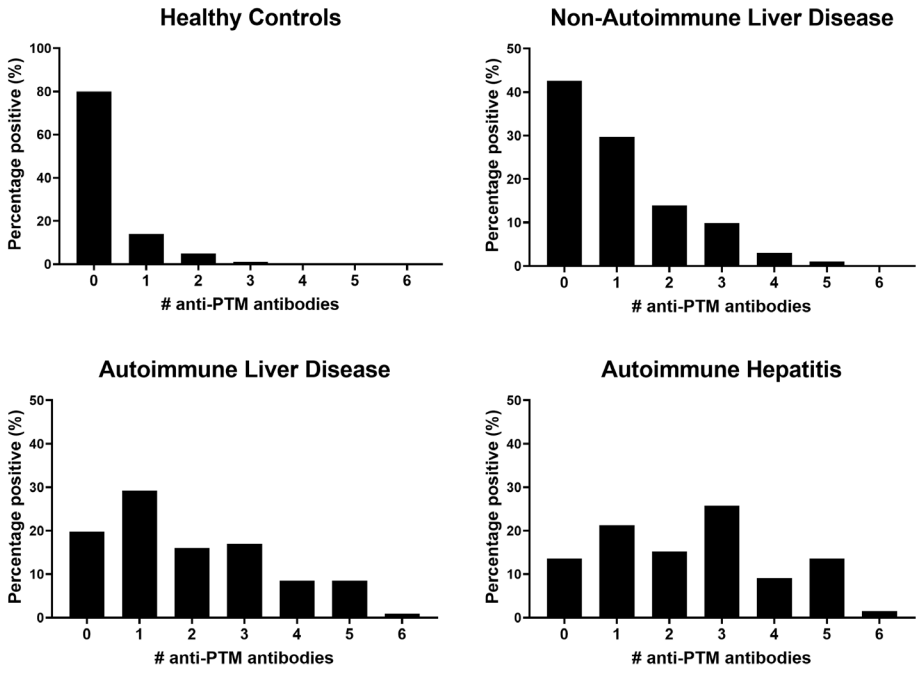

Figure 2: Patients with AILD, and especially AIH, are more likely to be positive for more than one anti-PTM antibody. Data is presented as percentage positive (%) patients for a number of anti-PTM antibodies in (from left to right) healthy controls, non-autoimmune liver disease autoimmune liver disease and autoimmune hepatitis.

Within AILD, patients with AIH harbor anti-PTM antibodies more often and present with specific combinations of anti-PTM antibodies

Next, AILD was dissected into the three major immune liver disease subgroups, namely AIH, PBC and PSC. Presence of anti-MAA, anti-AGE, anti-CarP and anti-AL antibodies were assessed in these subgroups, or AIH alone, and compared to non-AILD *(Figure 1 and 2, and Supplementary Table 3*). Interestingly, patients with AIH harbored significantly more of these antibodies compared to non-AILD patients (anti-MAA: 77.3% vs 26.7%, anti-AGE: 48.5% vs 17.5% and anti-CarP: 63.6% vs 27.7%, all $p<0.001$, respectively). Within the AILD cohort, predominantly patients with AIH harbored anti-PTM antibodies. We did however also see some patients with CLD who were positive for some. Subsequently, the AILD was divided into two cohorts: AIH and CLD. Patients with AIH were significantly more often positive for anti-MAA, anti-AGE, anti-CarP and anti-Cit (77.3, 48.5, 63.6 and 25.8% vs 52.2, 17.5, 20.0 and 2.5% respectively, all $p<0.01$) compared to patients with CLD (*Table 2*).

Table 2: The association between the presence of anti-PTM antibodies in HC, non-AILD, AIH and cholestatic liver disease.

	Healthy Controls *n=100*				**Non-Autoimmune Liver Disease** *n=101*				**AIH** *n=66*				**Cholestatic liver disease** *n=40*			
	aU/mL [IQR]		**n (% positive)**		**aU/mL [IQR]**		**n (% positive)**		**aU/mL [IQR]**		**n (% positive)**		**aU/mL [IQR]**		**n (% positive)**	
Anti-MAA	266.9	[200.4 – 370.2]	2	(2.0)	495.8	[315.2– 726.8]	27	(26.7)	1519.5	(760.0 – 2775.3)	51	(77.3)	771.5	538.3 – 1247.7)**,#	21	(52.5)**,#, +
Anti-AGE	88.9	[0.0 – 182.5]	4	(4.0)	130.0	[4.2 – 261.2]	18	(17.8)	349.0	(156.0 – 537.0)	32	(48.5)	143.5	(27.0 – 304.0)+	7	(17.5)*,+
Anti-CarP	74.0	[1.5 – 157.9]	5	(5.0)	241.0	[83.5 – 422.0]	28	(27.7)	475.5	(293.2 – 741.8)	42	(63.6)	226.5	(9.8 – 328.5)**,++	8	(20.0)*,++
Anti-AL	0.0	[0.0 – 9.8]	5	(5.0)	6.4	[0.0 – 10.0]	10	(9.9)	15.4	(4.7 – 33.4)	13	(19.7)	10.8	(0.5 – 25.4)*	7	(17.5)*
Anti-Cit	1.3	[0.0 – 3.2]	6	(6.0)	1.6	[0.0 – 5.9]	15	(14.9)	4.3	(1.2 – 9.7)	17	(25.8)	1.8	(0.0 – 4.2)	1	(2.5)#,+
Anti-NT	108.0	[0.0 – 250]	6	(6.0)	179	[0.0 – 501.5]	7	(6.9)	369.0	(65.8 – 732.5)	5	(7.6)	212.0	(0.0 – 400.8)	2	(5.0)

Results are presented as median (IQR) of n (%). Chi-2-tests were used to assess the difference between the presence of the specific manifestations and non-AILD patients. HC versus cholestatic liver disease *p<0.005, **p<0.001; non-AILD versus cholestatic liver disease #p<0.005, ##p<0.001; and AIH versus Cholestatic liver disease +p<0.005, ++p<0.001. *AGE, advanced glycation end-product; AIH, autoimmune hepatitis; AL, acetylated protein; aU/mL, arbitrary units per milliliter; CarP, carbamylated protein; Cit, citrullinated protein; IQR, interquartile range; MAA, malondialdehyde–acetaldehyde adduct; NT, nitrated protein; non-AILD, non-autoimmune liver disease; PBC, Primary Biliary Cirrhosis; PSC, Primary Sclerosing Cholangitis*

Analysis of different anti-PTM antibody combinations showed that AILD patients mostly harbored a combination of anti-MAA, anti-AGE and anti-CarP antibodies (15/85 = 17.6%) or anti-MAA and anti-CarP antibodies (7/85 = 8.2%) compared to non-AILD (anti-MAA/-AGE /-CarP: 5/58 = 8.6% and anti-MAA/-CarP: 3/58 = 5.2%) (*Supplementary Figure 1A and B*). Strikingly, comparing AIH patients with total AILD, all double (anti-MAA/-CarP), almost all (except 1) triple (anti-MAA/-AGE /-CarP) and all quintuple (anti-MAA/-AGE /-CarP /-AL/-Cit) positive patients from the AILD group belonged to the AIH group (*Supplementary Figure 1C*). Taken together, patients with AIH harbored anti-PTM antibodies more often compared to other subgroups of AILD.

There are no significant associations between anti-PTM antibody positivity, presence of ANA and SMA, cirrhosis and sex in AIH patients

In AIH several other antibodies have been described such as ANA and SMA. We have analyzed to what extent these antibodies occur together with the anti-PTM antibody responses or to what degree detectable anti-PTM antibody responses differ depending on the positivity status for ANA or SMA. We did not observe a significant difference in the presence of anti-PTM antibodies in patients positive or negative for ANA or SMA, with the exception of anti-MAA positivity and ANA positivity in patients with AIH (Chi-2 (1)> = 4.687, p=0.030). We further analyzed the positivity for anti-PTM antibodies in patients with AIH who were negative for both ANA and SMA. Despite it being a small cohort (n=11), we observed that the absolute percentages for positivity of anti-MAA, anti-AGE, anti-CarP, anti-AL, anti-Cit and anti-NT was in general higher in patients who were both ANA and SMA negative compared to patients who were either ANA negative of SMA negative (*Table 3*). This further supports the idea that anti-PTM antibodies provide different information compared to the already known antibodies ANA and SMA. Additionally, in the AIH cohort positivity for any of the anti-PTM antibodies did not show significant differences between patients when stratifying for cirrhosis. Furthermore, in the AILD cohort, we observed a significant association between anti-CarP and female sex (Chi-2 (1)> = 4.740, p=0.029). In the AIH cohort however, none of the anti-PTM antibodies showed significant associations with sex.

Anti-MAA and anti-CarP antibodies significantly correlate with measures of biochemical treatment response

We investigated if increased anti-PTM antibodies correlated with commonly used serological and clinical markers in patients with AIH (*Figure 3A-D and Supplementary Table 4*). As treatment for AIH consists of immunomodulatory treatment, and might therefore influence biochemical markers, only patients with treatment naïve AIH were included in these analyses (*Table 4*). Both anti-MAA and anti-CarP correlated positively with serum IgG (p<0.000/p=0.001) and antinuclear antibodies (ANA) (p=0.001). Anti-CarP correlated positively with ASAT (p=0.009). We demonstrated correlations between anti-MAA,

self-reported arthralgia and antibodies against soluble liver antigen (SLA) approaching statistical significance (p=0.082 and 0.059 respectively). No significant correlations were found for anti-AGE and anti-AL.

Anti-MAA, anti-AGE and anti-CarP antibodies positively correlate with CBR

Time to CBR negatively correlated with the presence of anti-PTM antibodies in patients with AIH, reaching significance for anti-AGE (p=0.042) (*Figure 3A-D*). In line with these findings, anti-MAA and anti-AGE correlated positively with CBR at 3 months (p=0.015 and 0.036, respectively). In addition, anti-MAA, anti-AGE, and anti-CarP positively correlated with CBR at 12 months (p=0.014, 0.005, and 0.012, respectively) (*Figure 3A-D*). A trend towards significance was found for anti-AL and CBR at 12 months. No association between the presence of anti-PTM antibodies and long-term follow-up (i.e. liver transplantation or mortality) was found. A logistic regression was performed to analyze the effects of positivity for all six individual anti-PTM antibodies on the likelihood of reaching CBR at 3, 6 and 12 months. Positivity for any individual anti-PTM antibody was not independently associated with an increased or decreased likelihood of reaching CBR at 3, 6 or 12 months (data not shown).

Table 3: Percentage of positivity for anti-PTM antibodies in ANA positive, ANA negative, SMA positive, SMA negative and double negative (ANA and SMA) patients with AIH.

	AIH (n=66)				
	ANA positive (n=42)	**ANA negative (n=24)**	**SMA positive (n=35)**	**SMA negative (n=31)**	**ANA negative / SMA negative (n=11)**
Anti-MAA positive	85.7%	62.5%	71.4%	83.9%	81.8%
Anti-AGE positive	54.8%	37.5%	40.0%	58.1%	63.6%
Anti-CarP positive	69.0%	54.2%	62.9%	64.5%	72.7%
Anti-AL positive	23.8%	12.5%	20.0%	19.4%	27.3%
Anti-Cit positive	31.0%	16.7%	22.9%	29.0%	27.3%
Anti-NT positive	4.8%	12.5%	8.6%	6.5%	18.2%

AGE, advanced glycation end-product; AL, acetylated protein; ANA, anti-nuclear antibodies; CarP, carbamylated protein;Cit, citrullinated protein; MAA, malondialdehyde–acetaldehyde adduct; NT, nitrated protein; SMA, smooth muscle antibody

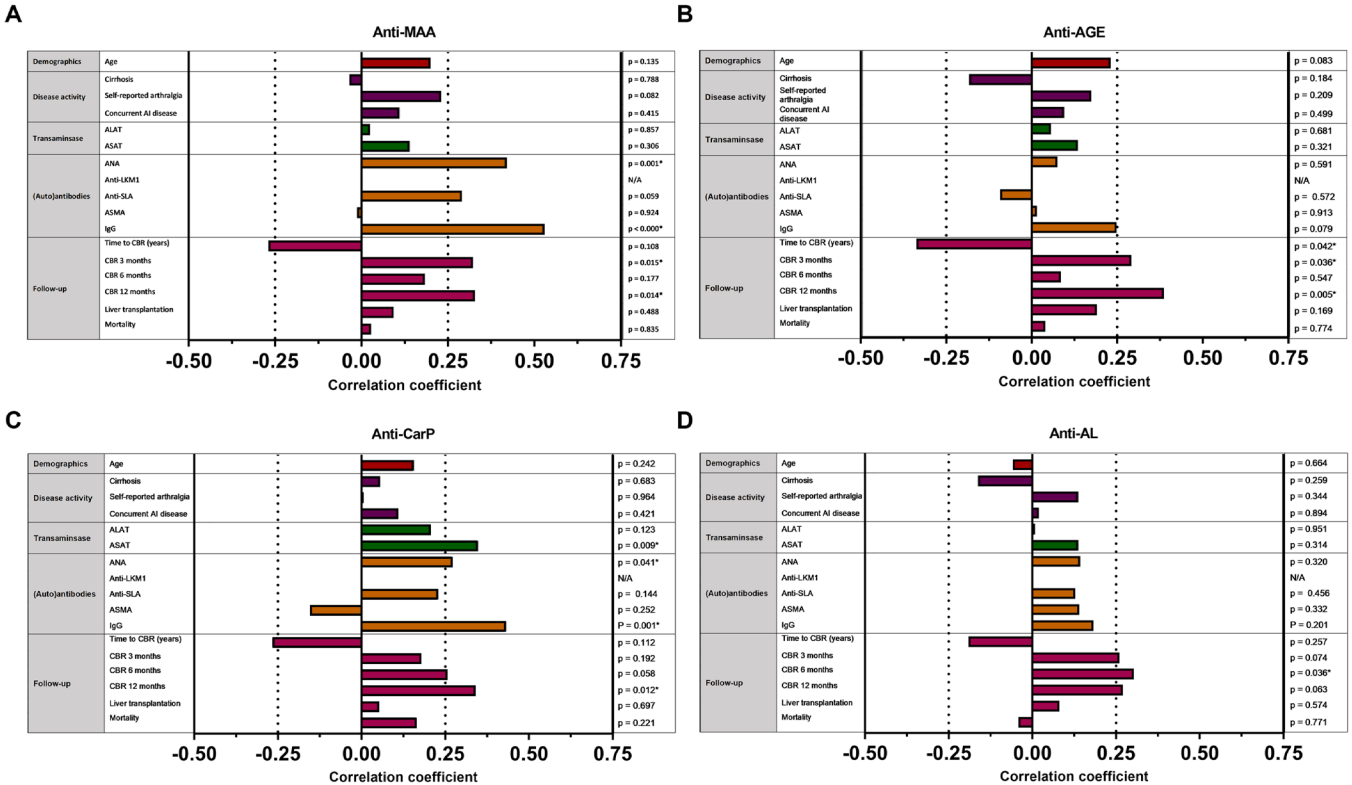

Figure 3: Correlation between (A) anti-MAA IgG, (B) anti-AGE IgG, (C) anti-CarP IgG and (D) anti-AL IgG antibodies and clinical and serological markers in patients with untreated auto-immune hepatitis (n=58). Correlation analyses are done using Spearman's rho correlation analysis and point-biserial correlation analysis. P<0.05 is considered statistically significant (*). *AGE, advanced glycation end-product; ALAT, alanine aminotransferase; ANA, anti-nuclear antibodies; ASAT, aspartate aminotransferase; SMA, smooth muscle antibody; CBR, complete biochemical response; IgG, immunoglobulin gamma; LKM, Liver Kidney microsomal antibody; SLA, soluble liver antigen;*

Table 4: Characteristics of untreated AIH patients in the AILD cohort (n=58).

Patient characteristics	Autoimmune hepatitis (n=58)
Female sex	43 (74.1)
Age diagnosis (years)	46.4±19.4
Simplified criteria for the diagnosis of AIH	8 (6 – 8)
Original revised criteria for AIH	16.7±3.3
Positive antibodies	
ANA (n=58)	38 (65.5)
SMA (n=58)	29 (50.0)
Anti-LKM (n=43)	0 (0.0)
Anti-SLA	2 (3.4)
*Others**	8 (13.8)
IgG (n=57)	24.80 (19.85-32.65)
Histology	
Typical	45 (77.6)
Compatible	9 (15.5)
Atypical/biopsy not done	3 (5.2)
Negative viral hepatitis serology	57 (98.3)
Cirrhosis	23 (39.7)
Yes, compensated	14 (24.1)
Yes, decompensated	9 (15.5)
No cirrhosis	35 (60.3)
Unknown	0 (0.0)
Self-reported arthralgia	12 (20.7)
(More than one) concomitant auto immune disease**	17 (29.3)

Results are presented as n(%), mean ±SD or median (IQR). *AIH, autoimmune hepatitis; ANA, anti-nuclear antibodies; SMA, smooth muscle antibody; IgG, immunoglobulin gamma; IQR, interquartile range; LKM, Liver Kidney microsomal antibody; SD, standard deviation; SLA, soluble liver antigen. *Others: pANCA (n=8) **Other: auto-immune hemolysis (n=1), celiac disease (n=1), diabetes mellitus type 1 (n=2), granulomatosis with polyangiitis (n=1), Henloch-Schönlein purpura (n=1), Hyperthyroidism (n=3), hypothyroidism (n=6), myastenia gravis (n=1), sclerodermia (n=2) ulcerative colitis (n=1)*

Patients with AIH and positive for at least three anti-PTM antibodies reach CBR quicker after initiating treatment

Based on the discovery of multiple anti-PTM antibody positivity in patients with AIH, we attempted to discover the clinical relevance of harboring these multiple anti-PTM antibodies. The median follow-up was 8.7 years (4.6 - 15.3) (*Supplementary Table 5)*. Patients with at least three anti-PTM antibodies scored significantly higher on the revised original score for AIH and had significantly higher levels of IgG at time of diagnosis (*Supplementary Table 5)*. Aminotransferase levels were higher in the group with at least three positive anti-PTM antibodies, albeit not significant. Anti-MAA and anti-CarP correlated positively with ASAT at baseline in the group with less than three anti-PTM antibodies present (r_s=0.37 and r_s=0.45, p=0.037 and p=0.009 respectively), but

not in AIH patients with at least three anti-PTM antibodies. After 3 months treatment, significantly more AIH patients with at least three anti-PTM antibodies had reached CBR (p=0.03). After 12 months of treatment, the difference was still significant (p=0.01). Overall, a trend towards significance for time to CBR (in years) was found in favor of multiple anti-PTM antibody positivity.

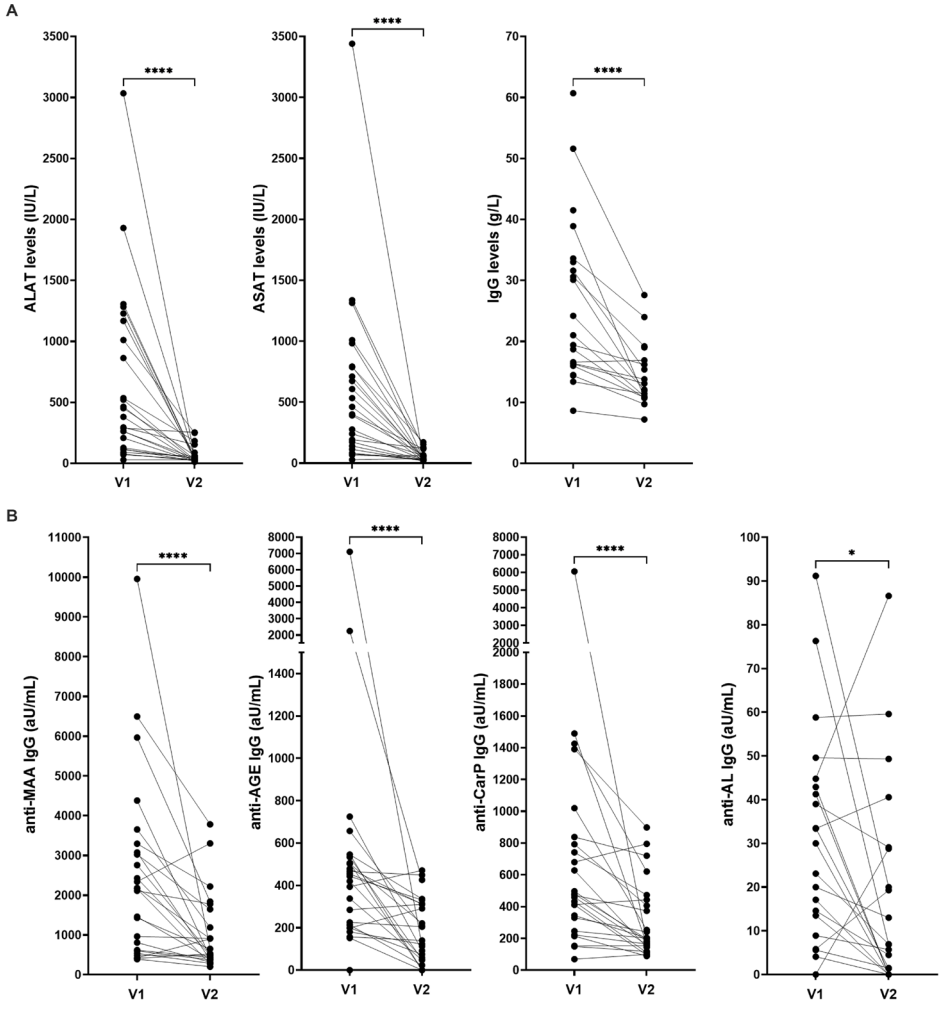

Figure 4: Levels of (A) ALAT, ASAT, IgG and (B) anti-PTM antibodies over time in patients with AIH (n=25). ALAT, ASAT and IgG levels were determined as standard procedure after inclusion. V1: before commencing treatment, V2: during treatment. Median time interval: 65 months (6 – 138). Reactivity towards anti-PTM antibodies was determined using ELISA and is depicted as arbitrary units per milliliter (aU/mL). *p=0.0244 and ****p≤0.0001. *AGE, advanced glycation end-product; AL, acetylated protein; ALAT, Alanine aminotransferase; ASAT, Aspartate aminotransferase; CarP, carbamylated protein; IgG, immunoglobulin gamma; MAA, malondialdehyde–acetaldehyde adduct; V1, first visit; V2, second visit.*

Anti-PTM antibody responses decrease over time and show distinct associations with ALAT, ASAT or total IgG levels.

Clinical data of two different timepoints were available of 25 AIH patients and antibody responses over time was investigated *(Figure 4)*. The first sample was taken before commencing treatment, the second sample during treatment. The median time interval between visit one and two was 65 months (6 – 138). Median ΔALAT, ΔASAT and ΔIgG were 352IU/L (951 – 79) , 324IU/L (722 – 83) and 8g/L (14 – 2) respectively. Levels of all four anti-PTM antibody responses decreased significantly over time (anti-MAA, anti-CarP, and anti-AL (p≤0.0001) and anti-AGE (p=0.024)). Change in anti-AL antibody titers associated significantly with change in ASAT and ALAT (r_s: 0.46 and 0.40 p=0.02 and 0.05 respectively) but did not associate with change in total IgG (r_s: 0.17 p=0.53) (*Supplementary Table 6*). Change in anti-AGE antibody titers significantly associated with change in IgG (r_s: 0.63 p=0.007). Change in anti-MAA and anti-CarP antibody levels was not associated with decrease in ALAT, ASAT and IgG. However, change in anti-CarP antibody levels did show a positive trend towards significant association with decrease of IgG (r_s: 0.48 p=0.052) (*Supplementary Table 6*).

Discussion

To the best of our knowledge, this is the first report on the presence of anti-PTM antibodies in AILD. The presence of anti-PTM antibodies has been described in several other autoimmune diseases where they can serve as diagnostic or prognostic markers (6, 7, 27). Based on these results, we hypothesized that anti-PTM antibodies are also generated in patients with AILD. Additionally, we speculated that patterns in the presence of anti-PTM antibodies might serve diagnostic or prognostic purposes in AILD. In this study there were five significant findings: First, four anti-PTM antibodies were more prevalent in patients with AILD compared to HCs and to non-AILD: anti-MAA, anti-AGE, anti-CarP, and anti-AL. Second, patients with AILD and particularly patients with AIH often harbored multiple types of anti-PTM antibodies. Third, anti-MAA and anti-CarP antibody positivity significantly correlated with markers for biochemical response in AIH. Fourth, AIH patients with at least three types of anti-PTM antibodies reached CBR at 12 months after initiating treatment more frequently. Lastly, after initiating immunosuppressive treatment next to aminotransferases and IgG also anti-AGE and anti-AL antibody titers decreased. These findings confirmed that anti-PTM antibodies are present in AILD and moreover multiple anti-PTM antibodies identify a group of AIH patients in which these anti-PTM antibodies associate with CBR. Interestingly, we observed that several anti-PTM antibodies are present and even more prevalent in AIH patients who were 'sero-negative' for the classical autoantibodies at diagnosis, compared to patients who were positive for either ANA or SMA. This is particularly captivating since conventional antibodies

are not disease specific and may be expressed at a later stage of the disease in 'sero-negative' AIH patients. We suggest that anti-PTM antibodies may be present in patients with AIH before conventional antibodies can be detected. Therefore anti-PTM antibody assessment could especially be interesting in the diagnostic work-up for 'sero-negative' AIH patients. Future studies should further determine the possible implementation of anti-MAA, anti-AGE or anti-CarP assessment, all associated with CBR at 12 months, in the diagnostic algorithm for AIH.

The clinical presentation of AIH is very heterogeneous and can vary from asymptomatic disease to acute (on chronic) liver failure. Occasionally, polyarthralgia without arthritis is present in patients with AIH (1, 28), and is considered an extra hepatic manifestation of AIH. However, this is often not recognized and is underreported. In clinical practice, reoccurrence of arthralgia is often seen during corticosteroid withdrawal (28). Next to arthralgia, RA is sometimes seen in AIH. We have previously reported that, in the context of RA, anti-CarP (29) and anti-Cit (30) antibodies in arthralgia predict development of RA. Additionally, anti-PTM antibodies have been described in the context of rheumatic disease (10, 31, 32). In this study only a trend was found for the correlation between self-reported arthralgia and anti-MAA antibodies in AIH. This could be a result of the small cohort size and would require further investigation.

Previous research showed that IgG levels are not associated with long-term outcomes in AIH, whereas normalization of aminotransferases is the main treatment goal in AIH, as this positively associates with survival in the first 12 months after diagnosis (33). Additionally, Hartl *et al*. found that patients with normal IgG levels showed a comparable treatment response to patients with elevated IgG (34). On the contrary, CBR is defined as normalization of ALAT, ASAT and IgG (26). The role of IgG remains a pivoting point in disease progression in AIH. The results of this study suggest that specific subsets of anti-PTM antibodies are associated with treatment response.

In this study, patients with AIH positive for at least three types of anti-PTM antibodies had significantly higher IgG levels at diagnosis and tended to reach CBR more often at 12 months of treatment than patients with AIH with less than three anti-PTM antibodies. By choosing more than three anti-PTM reactivities as a cut-off in this analysis we achieved an equal number of AIH patients in each group (26 with less and 32 with at least three anti-PTM antibodies). Larger studies could determine whether combinations of anti-PTM antibodies, also combined with serum levels of IgG, ALAT, and ASAT at baseline could be better predictors for the likelihood of treatment response. The anti-PTM antibody response is an IgG mediated response and is part of the significantly elevated IgG in this specific group of patients. Positivity for multiple autoantibodies has been reported to provide more reliable information than single biomarkers in for example diabetes (35) and pre-RA (36).

Our study has some limitations: the cohort is heterogeneous and has a limited size. We found that forty percent of AIH patients identify with specific combinations of anti-PTM antibodies (anti-MAA/anti-CarP; anti-MAA/-AGE /-CarP; anti-MAA /-AGE /-CarP /-AL/-Cit) within the AILD group. These specific combinations might aid in the diagnostic work-up for AIH. Noteworthy is that anti-PTM antibodies are not solely found in AIH, but are also found in other liver diseases possibly as a result of breach in tolerance against PTMs that are formed during inflammation. For several autoimmune diseases it is well known that certain autoantibodies are already present many years before the patients develop clinically overt disease for example anti-Cit and anti-CarP Ab in the context of RA (6,7). Whether this is also the case in these autoimmune liver diseases is currently unknown. PTMs formed as a consequence of inflammation together with impaired liver function may well accumulate and mediate a breach in tolerance, and in this setting anti-PTM antibodies can be formed as a consequence of liver disease. The same set of six anti-PTMs were studied in SLE, and anti-MAA, anti-AGE and anti-CarP antibodies were also most frequently found in patients with SLE compared to healthy controls (10). Interestingly, anti-MAA and anti-CarP associated with neuropsychiatric manifestations of SLE, a manifestation that lacked a biomarker. These findings are in the same range as anti-PTM antibody responses found in AILD. Anti-CarP and anti-Cit are well studied anti-PTM antibodies in RA and are found in approximately 50% of RA patients (6,7). Discovery of new anti-PTM antibodies in RA helped in diagnosis and in following disease progression, and can potentially help to distinguish groups within so-called seronegative RA (5). In order to further validate these findings and prove the sensitivity and specificity of these anti-PTM antibody combinations in the diagnostic work-up of AIH, anti-PTM antibodies need to be assessed extensively in a larger cohort. This could provide the opportunity to set a cut-off titer level and perhaps even distinguish AIH from other liver diseases. In this limited cohort it was not possible to evaluate the prognostic value of anti-PTM antibodies for disease progression as 40% of patients already had cirrhosis at diagnosis. A larger cohort study should be conducted in patients with AILD and no cirrhosis at diagnosis with set follow-up timepoints.

Different clinical parameters are measured to monitor disease activity for AIH, PBC and PSC. As a result, the three groups within the AILD cohort are incomparable. However, in the AILD cohort we have included PBC and PSC, which are not pure auto-immune diseases (where immune injury results in cholestasis) (25, 37-43). We hoped to evaluate possible differences in anti-PTM antibodies patterns in AIH, PBC and PSC. When analyzing AIH patients, only untreated patients were included to prevent impact of treatment. One of the strengths of this study is that the group of interest, AILD, is well-defined according to simplified or original revised score for AIH. Therefore, we can state that the results found for these subgroups are representative.

Combining the prevalence data of all 6 anti-PTM antibodies tested we observe that approximately 20% of healthy controls harbored at least one anti-PTM antibody (*Figure 2*). PTM of proteins occurs in all individuals, these PTMs may represent neo-epitopes towards which antibodies can be formed. Interestingly, this is apparently often not associated with disease, but is known to predispose to disease.

The standard therapy for patients with AIH consists of a combination of glucocorticoids and azathioprine (2). Most patients with AIH in this cohort were initially treated with this preferred treatment. Pape *et al.* demonstrated that a higher or lower initial predniso(lo)ne dose does not have impact on reaching CBR. In our study, stratification of the results by the initial steroid dose was not possible, as 42 patients (85.7%) received an initial predniso(lo)ne dose above 30mg/day (44). When patients do not reach CBR, the treating physician may decide to intensify or adjust treatment regimens. The nature of the disease, characterized by intermittent loss of remission and flares, may give reason to frequently adjust therapy. The size of the studied cohort limited us to correct for change in therapy over time. Since it was not possible to obtain the necessary data we were not able to correct the correlation analyses regarding CBR for duration of steroid treatment, duration of tapering schemes, dose modification or drug withdrawal during follow-up. The median ASAT and ALAT did not differ between the patients who were prescribed budesonide compared to predniso(lo)ne, although this has been previously reported (45).

However, according to the guidelines and Delphi consensus on treatment response, treatment effect is first evaluated 6 months after commencing treatment (2, 26). We additionally did see more patients reaching CBR at 12 months of treatment if they had at least three positive anti-PTM antibodies. This may imply that having anti-PTM antibodies for at least three PTMs may be prognostically favorable regarding treatment response. Despite higher ALAT and ASAT levels at baseline in the AIH patients with at least three anti-PTM antibodies present, no association between transaminase levels and multiple positivity could be found. Only in patients with less than three anti-PTM antibodies present, a positive correlation between anti-MAA, anti-CarP and ASAT was found. This strengthens the implication that multiple positivity for at least three anti-PTM antibodies may be beneficial for treatment response and may guide treating physicians to earlier treatment intensification.

In conclusion: anti-PTM antibodies are present in patients with AILD. Some patients are positive for multiple anti-PTM antibodies. Having three or more anti-PTM antibody responses is associated with a favorable response to treatment in AIH.

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Supplementary files

Supplementary Table 1: Characteristics of AIH, PBC and PSC patients in the AILD cohort. Results are presented as n (%), or median (IQR).

	AIH (n=66)	PBC (n=10)	PSC (n=30)
Laboratory assessments			
ALAT	338.0 (125.0 - 1057.5)	37.5 (28.0 – 76.8)	54.0 (37.0 – 114.0)
ASAT	420.0 (142.8 - 935.3)	50.5 (34.5 – 53.8)	48.0 (36.0 – 99.0)
IgG	24.8 (19.4-33.2)	-	-
ALP	178.5 (123.0 – 279.0)	277.0 (158.0 – 433.0)	229.0 (132.0 – 405.0)
GGT	212.0 (116.0 – 371.5)	147.0 (58.0 – 571.0)	166.0 (96.5 – 278.5)
Positive antibodies			Insufficient data
ANA	42 (63.6)	3 (33.3)	-
SMA	35 (53.0)	-	-
Anti-LKM	1 (1.5)	-	-
Anti-SLA	2 (3.0)	-	-
AMA	-	8 (80.0)	-
*Other**	9 (13.6)	-	-
Large duct anomalies **	N/A	N/A	29 (96.70)

AIH, autoimmune hepatitis; ALAT, alanine aminotransferase; ALP, alkaline phosphatase; AMA, anti-mitochondrial antibodies; ANA, anti-nuclear antibodies; ASAT, aspartate aminotransferase; SMA, smooth muscle antibody; CBR, complete biochemical response; GGT, gamma-glutamyl transferase; IgG, immunoglobulin gamma; LKM, Liver Kidney microsomal antibody; PBC, primary biliary cholangitis; PSC, primary sclerosing cholangitis; SLA, soluble liver antigen. * pANCA (n=8), anti-parietal cell (n=1) ** based on cholangiographic findings with magnetic resonance/endoscopic retrograde cholangiopancreatography.

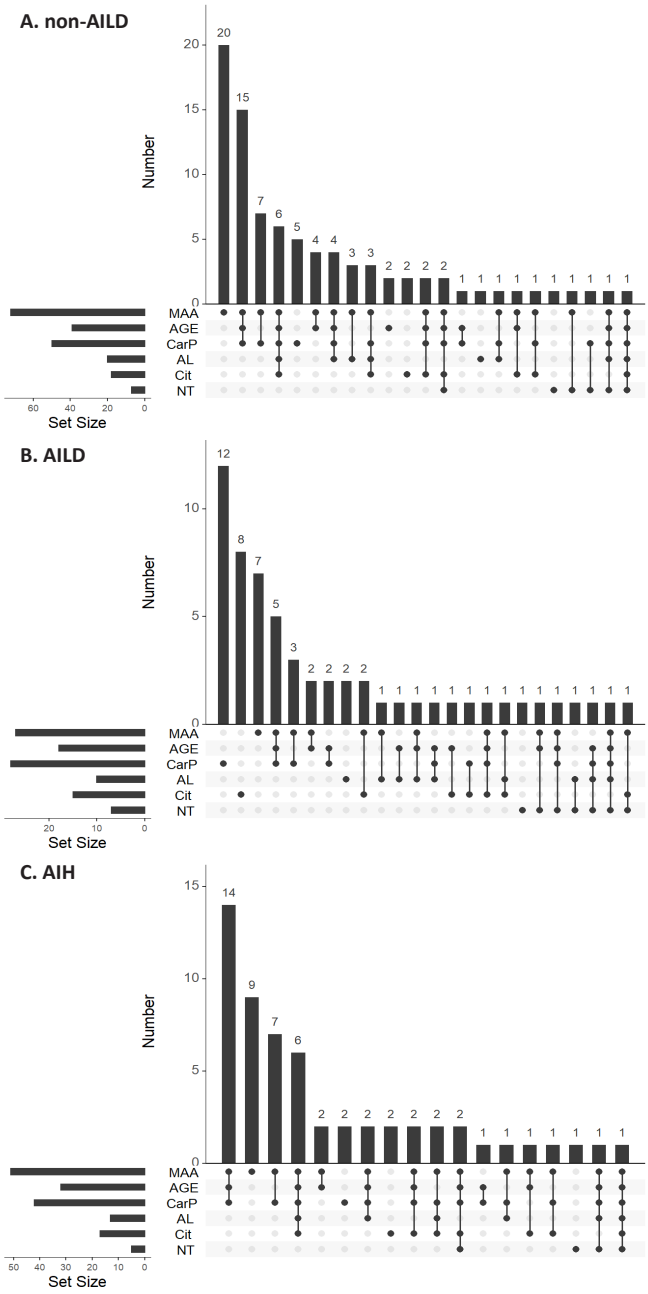
Supplementary Table 2: Prevalence of antibodies against post-translationally modified proteins in patients with autoimmune (n=106) or non-autoimmune liver disease (n=101) and healthy controls (n=100).

	Healthy Controls n=100			Autoimmune Liver Disease n=106				Non-Autoimmune Liver Disease n=101			
	aU/mL [IQR]		n (% positive)	aU/mL [IQR]		n (% positive)		aU/mL [IQR]		n (% positive)	
Anti-MAA	266,9	[200,4 – 370,2]	2 (2,0)	1036,0	[625,5 – 2119,6]**	72	(67,9)**	495,8	[315,2– 726,8]$^{\#\#,++}$	27	(26,7)$^{\#\#,++}$
Anti-AGE	88,9	[0,0 – 182,5]	4 (4,0)	234,5	[95,5 – 480,5]**	39	(36,8)**	130,0	[4,2 – 261,2]$^{++}$	18	(17,8)$^{\#\#,++}$
Anti-CarP	74,0	[1,5 – 157,9]	5 (5,0)	352,5	[213,5 – 633,0]**	50	(47,2)**	241,0	[83,5 – 422,0]$^{\#\#,+}$	28	(27,7)$^{\#\#,++}$
Anti-AL	0,0	[0,0 – 9,8]	5 (5,0)	13,3	[2,8 – 30,7]**	20	(18,9)**	6,4	[0,0 – 10,0]$^{++}$	10	(9,9)
Anti-Cit	1,3	[0,0 – 3,2]	6 (6,0)	3,1	[0,5 – 7,2]*	18	(17,0)*	1,6	[0,0 – 5,9]	15	(14,9)$^{\#}$
Anti-NT	108,0	[0,0 – 250]	6 (6,0)	269,0	[33,8 – 602,5]**	7	(6,6)	179	[0,0 – 501,5]	7	(6,9)

Results are presented as median [IQR] or n (%). *p<0.05, **<0.01 between HC and AILD #p<0.05, ##<0.01 between HC and non-AILD +p<0.05, ++<0.01 between AILD and non-AILD Autoimmune Liver Disease: AIH, PBC and PSC; non-Autoimmune Liver Disease: NAFLD, HCV, HBV, ALD, Combination, NASH. *AGE, advanced glycation end-product; AL, acetylated protein; aU/mL, arbitrary units per milliliter; CarP, carbamylated protein; Cit, citrullinated protein; IQR, interquartile range; MAA, malondialdehyde–acetaldehyde adduct; NT, nitrated protein;*

Supplementary Table 3: The association between the presence of anti-PTM antibodies and the three major autoimmune liver diseases. Results are presented as median (IQR) of n (%). Chi-2-tests were used to assess the difference between the presence of the specific manifestations and non-AILD patients. *p=0.006, **p<0.001. *AGE, advanced glycation end-product; AIH, autoimmune hepatitis; AL, acetylated protein; aU/mL, arbitrary units per milliliter; CarP, carbamylated protein; IQR, interquartile range; MAA, malondialdehyde–acetaldehyde adduct; non-AILD, non-autoimmune liver disease; PBC, Primary Biliary Cirrhosis; PSC, Primary Sclerosing Cholangitis*

	AIH		**PBC**		**PSC**		**Non-AILD (reference)**	
	Yes, n=66		**Yes, n=10**		**Yes, n=30**		**No, n=101**	
Anti-MAA								
aU/mL	1519.5	(760.0 – 2775.3)	787.6	(436.3 – 1669.7)	771.5	(545.9 – 1223.0)	495.8	(315.2– 726.8)
Positive	**51**	**(77.3)** **	5	(50.0)	**16**	**(53.3)** *	27	(26.7)
Anti-AGE								
aU/mL	349.0	(156.0 – 537.0)	76.5	(0.0 – 177.9)	172.5	(59.3 – 359.3)	130.0	(4.2 – 261.2)
Positive	**32**	**(48.5)** **	0	(0.0)	7	(23.3)	18	(17.8)
Anti-CarP								
aU/mL	475.5	(293.2 – 741.8)	148.7	(0.0 – 376.0)	230.5	(41.3 – 336.0)	241.0	(83.5 – 422.0)
Positive	**42**	**(63.6)** **	2	(20.0)	6	(20.0)	28	(27.7)
Anti-AL								
aU/mL	15.4	(4.7 – 33.4)	15.9	(0.8 – 24.8)	9.9	(0.2 – 28.0)	6.4	(0.0 – 10.0)
Positive	13	(19.7)	1	(10.0)	6	(20.0)	10	(9.9)



Supplementary Figure 1: Patients with AILD present with double or triple positivity for different anti-PTM antibodies compared to non-AILD and define a group of AIH patients harboring three anti-PTM antibodies. Upset plot indicating the frequency of combinations of anti-PTM antibodies in (A) non-AILD, (B) AILD, and (C) AIH. Horizontal bars represent the number of individuals in each of the anti-PTM antibodies of interest with a table on the right indicating single or combinations of anti-PTM antibodies. Vertical bars represent the number of patients positive for that anti-PTM antibody or combinations thereof.

Supplementary Table 4: Detailed overview of correlation coefficients (including 95% confidence intervals) as provided in Figure 5.

	Anti-MAA		Anti-AGE		Anti-CarP		Anti-AL	
	corr	95% CI*	corr	95% CI*	corr	95% CI*	corr	95% CI*
Demographics								
Age	0.199	-0.06 – 0.43	0.230	-0.03 – 0.46	0.156	-0.11 – 0.40	-0.058	-0.31 – 0.20
Disease activity								
Cirrhosis	-0.36	-0.29 – 0.22	-0.183	-0.43 – 0.09	0.055	-0.21 – 0.31	-0.163	-0.42 – 0.12
Self-reported arthralgia	0.230	-0.03 -0.46	0.174	-0.10 – 0.42	0.006	-0.25 – 0.26	0.137	-0.15 – 0.40
Concurrent AI disease	0.110	-0.15 – 0.36	0.095	-0.18 – 0.35	0.110	-0.16 – 0.36	0.019	-0.26 – 0.30
Transaminases								
ALAT	0.024	-0.24 – 0.28	0.056	-0.21 – 0.31	0.207	-0.06 – 0.44	0.008	-0.25 – 0.27
ASAT	0.139	-0.13 – 0.38	0.135	-0.13 – 0.38	0.348	0.09 – 0.56	0.137	-0.13 – 0.38
(Auto) antibodies								
ANA	0.420	0.18 – 0.61	0.075	-0.19 – 0.33	0.272	0.01 – 0.49	0.143	-0.14 – 0.40
Anti-LKM1	-	-	-	-	-		-	
Anti-SLA	0.290	-0.03 – 0.51	-0.093	-0.39 – 0.23	0.229	-0.08 – 0.49	0.128	-0.21 – 0.43
SMA	-0.013	-0.31 – 0.29	0.015	-0.25 – 0.28	-0.154	-0.40 – 0.11	0.140	-0.14 – 0.40
IgG	0.529	0.29 – 0.70	0.248	-0.03 – 0.49	0.435	0.18 – 0.63	0.182	-0.10 – 0.43
Follow-up								
Time to CBR (years)	-0.269	-0.54 – 0.06	-0.337	-0.59 – 0.01	-0.266	-0.54 – 0.06	-0.191	-0.48 – 0.14
CBR 3 months	0.322	0.06 – 0.54	0.291	0.02 – 0.52	0.179	-0.09 – 0.42	0.260	-0.03 – 0.50
CBR 6 months	0.183	-0.08 – 0.42	0.085	-0.19 – 0.35	0.257	-0.01 – 0.49	0.303	0.02 – 0.54
CBR 12 months	0.328	0.07 – 0.54	0.386	0.12 – 0.59	0.341	0.08 – 0.55	0.270	-0.02 – 0.51
Liver transplantation	0.093	-0.17 – 0.34	0.190	-0.08 – 0.43	0.053	-0.21 – 0.31	0.081	-0.20 – 0.35
Mortality	0.028	-0.23 – 0.28	0.040	-0.23 – 0.30	0.165	-0.10 – 0.41	-0.042	-0.31– 0.24

All confidence intervals were calculated using Fisher's transformation. *AGE, advanced glycation end-product; AI, autoimmune*; *AL, acetylated protein; ALAT, alanine aminotransferase; ALP, alkaline phosphatase; AMA, anti-mitochondrial antibodies; ANA, anti-nuclear antibodies; ASAT, aspartate aminotransferase; SMA, anti-smooth muscle antibody; CarP, carbamylated protein; CBR, complete biochemical response; CI, confidence interval; corr, correlation coefficient; IgG, immunoglobulin gamma; MAA, malondialdehyde–acetaldehyde adduct; LKM, Liver Kidney microsomal antibody; SLA, soluble liver antigen.*

Supplementary Table 5: Baseline characteristics of patients with AIH and at least 3 positive anti-PTM antibodies versus less than 3.

	≥3 anti-PTM positive	<3 anti-PTM positive	p-value
Demographics			
Patients	32 (55.2)	26 (44.8)	-
Female sex	24 (75.0)	19 (73.1)	0.87
Age diagnosis (years)	47.7 (±20.0)	44.8 (±18.9)	0.571
Simplified criteria for the diagnosis of AIH	8.0 (6.0 – 8.0)	7.0 (5.8 – 8.0)	0.19
Revised original criteria for AIH	17.9 (±2.6)	15.3 (±3.6)	<0.05*
Cirrhosis	12 (37.5)	11 (42.3)	0.71
Yes, compensated	8 (25.0)	6 (23.1)	-
Yes, decompensated	4 (12.5)	5 (19.2)	-
No cirrhosis	20 (62.5)	15 (57.7)	-
Self-reported arthralgia	7 (21.9)	5 (19.2)	0.81
Auto immune comorbidities	11 (34.4)	6 (23.1)	0.31
Laboratory			
ALAT	535.0 (186.0 – 1028.0)	333.0 (118.3 – 1136.0)	0.65
ASAT	580.0 (190.8 – 989.3)	304.0 (111.3 – 827.8)	0.29
IgG	28.8 (22.2 – 39.6)	21.0 (14.0 – 31.1)	0.02*
Treatment started			0.47
Azathioprine	1 (3.1)	0 (0.0)	-
Budesonide	0 (0.0)	2 (7.7)	-
Budesonide + azathioprine	2 (6.3)	1 (3.8)	-
Prednisolone	2 (6.3)	1 (3.8)	-
Prednisolone + azathioprine	25 (78.1)	20 (76.9)	-
Prednisolone + thioguanine	1 (3.1)	0 (0.0)	-
Thioguanine	0 (0.0)	1 (3.8)	-
Unknown	1 (3.1)	0 (0.0)	-
No treatment started	0 (0.0)	1 (3.8)	-
Follow-up			
Duration of follow-up	9.8 (4.9 – 18.2)	7.01 (4.0 – 12.26)	0.15
Time to CBR (years)	0.8 (0.3 – 2.3)	2.0 (0.8 – 4.1)	0.06
CBR 3 months	8 (25.0)	1 (3.8)	0.03*
CBR 6 months	9 (28.1)	3 (11.5)	0.12
CBR 12 months	13 (40.6)	3 (11.5)	0.01*
Liver transplantation	2 (6.3)	1 (3.8)	0.68
Mortality during follow-up	8 (25.8)	5 (19.2)	0.60
Switch in medication	15 (46.9)	10 (38.5)	0.36

Results are presented as n (%), mean ±SD or median (IQR). *AIH, autoimmune hepatitis; ALAT, alanine aminotransferase; ASAT, aspartate aminotransferase; CBR, complete biochemical response; IgG, immunoglobulin gamma.*

Supplementary Table 6: Correlations between change in anti-PTM antibody levels and changes in aminotransferases and immunoglobulin gamma (IgG). All confidence intervals were calculated using Fisher's transformation. *AGE, advanced glycation end-product; AL, acetylated protein; ALAT, alanine aminotransferase; ASAT, aspartate aminotransferase; CarP, carbamylated protein; CI, confidence interval; corr, correlation coefficient; IgG, immunoglobulin gamma; MAA, malondialdehyde–acetaldehyde adduct.*

	Δ Anti-MAA			**Δ Anti-AGE**			**Δ Anti-CarP**			**Δ Anti-AL**		
	corr	p-value	95% CI*	corr	p-value	95% CI*	corr	p-value	95% CI*	corr	p-value	95% CI*
Δ ALAT	0.1	0.6	-0.3 – 0.5	0.28	0.2	-0.1 – 0.6	0.2	0.4	-0.2 -0.5	0.4	0.0*	0.0 – 0.78
Δ ASAT	0.2	0.4	-0.2 – 0.5	0.29	0.2	-0.1 – 0.6	0.3	0.1	-0.1 – 0.6	0.5	0.0*	0.18 – 0.7
Δ IgG	0.3	0.3	-0.2 – 0.7	0.6	0.007*	0.2 – 0.8	0.5	0.1	0.0 – 0.8	0.2	0.5	-0.3 – 0.6

Response to commentary on

Antibodies against multiple post-translationally modified proteins aid in diagnosis of autoimmune hepatitis and associate with complete biochemical response to treatment

We read with great interest the commentary of Taubert and colleagues (1) on our article "Antibodies against multiple post-translationally modified proteins aid in diagnosis of autoimmune hepatitis and associate with complete biochemical response to treatment (2)". In their kind commentary the authors bring up the very important and relevant subject of polyreactive IgG (pIgG) as they have described to occur in autoimmune hepatitis (AIH) (3). The team of Taubert have identified such pIgG using an experimental set up roughly similar to the enzyme-linked immunosorbent assay (ELISA) set up as we have used for the detection of the antibodies against post-translationally modified proteins (anti-PTM). In their commentary they raise the concern that part of the antibodies identified in our assays as anti-PTM antibodies may in fact be pIgG. We can reassure the authors and readers that we are specifically detecting anti-PTM antibodies in our assay. Importantly, this is because of the setup of our ELISA system. Ever since the identification of antibodies binding to carbamylated antigens (anti-CarP) (4) we have used both carbamylated fetal calf serum (Ca-FCS) and unmodified FCS as control antigens for the coating of the ELISA plates. In practice one half of the ELISA plate is coated with Ca-FCS and the other with unmodified, control FCS. The entire plate is blocked with bovine serum albumin (BSA). Each serum sample is tested on both the Ca-FCS and the control FCS. The levels of antibody binding are calculated from absorbance values into arbitrary units per milliliter based on a standard line on the same plate. Next, the level of carbamylation specific antibodies is defined as the level of antibody binding to the Ca-FCS minus the level of antibodies binding to the control FCS. Hence, we report the PTM-specific response. In many of the analyses that we have run for rheumatoid arthritis (RA) and for systemic lupus erythematosus (SLE) (5) the reactivity of the control protein is very low. Indeed, we have observed that in AIH this was somewhat higher, but importantly we have subtracted this from the anti-PTM response, allowing us to conclude on the PTM-specific antibodies and avoiding undesired interference from pIgG. We realize that we may not have stressed this to the greatest extend in our manuscript and thank the authors for bringing up this point and for the opportunity to clarify this.

In our manuscript we have used six different PTMs. To make the best comparisons, we have not used the same control FCS for all the PTMs but have actually generated a separate control FCS for each of the conditions. For example, the control for carbamylation is an aliquot of the same FCS, incubated at the same time point, for the same duration at

the same temperature and dialysis steps as the carbamylated FCS, but only without the addition of the KOCN, the carbamylating chemical. For the modification with Advanced Glycation End-products, we have performed the incubations of the control FCS also for 10 days at 37° C, all to ensure that we make the best possible comparisons.

In the original paper we already reported that each of the anti-PTM reactivities has clearly different sensitivities, while all of the assays are based on FCS coating and bovine serum albumin (BSA) blocking, indicating that the assays do not detect pIgG. We have tested if there was any correlation between the signals observed on PTM-FCS versus control FCS. For the four anti-PTMs with the highest percentage of positive samples we did not find any correlation, again indicating that the anti-PTM antibodies are specifically binding to the PTM. The authors raise interesting questions regarding the nature of the antibody response to the PTM proteins. As can be seen in Supplementary Figure 1 of the manuscript (2), we studied how often the different anti-PTM antibodies can be found together in the same patients, as this may be an indication of either co-induction or cross-reactivity. We clearly observe different patterns with some individuals positive for one anti-PTM and other positive for several others (2), again indicating that the different assays are clearly identifying different antibodies. Additionally, while between some anti-PTM responses we do observe a correlation (as observed before), for other anti-PTM responses we do not detect any correlations. Importantly, some patients can be highly positive for one anti-PTM reactivity and simply negative for the other.

We did find that overall levels of some anti-PTM antibodies (weakly) associate with levels of IgG, but this may simply reflect that a polyclonal B cell stimulation (6) will stimulate the anti-PTM reactive B cells as well as other B cells, but it will only result in positivity in individuals that actually have anti-PTM reactivity. In the context of RA, we have observed that many of the anti-PTM antibodies are isotype switched but are of low-avidity (7, 8) indicating that there has been T-cell help, but lack of avidity maturation. The authors finally raise the point of serum storage time. This is an important issue and difficult to address experimentally. We have previously studied this in detail for our cohort in the context of our previous paper on AIH, focused on other biomarkers (9), where we concluded that the quality of the samples was good, as there was no difference in the sensitivity of the markers in the samples that were stored for a long time (i.e., ≥10 years) versus the samples that were stored more recently (i.e., <10 years) , suggesting that the storage was not a major factor in these analyses. Also for the current study on anti-PTM antibodies we have now carefully plotted the levels of all the 6 anti-PTM reactivities versus the time of storage of the sample and observed that positivity for the anti-PTM antibodies is not influenced by storage time (data not shown).

Importantly, for the anti-PTM responses in AIH we do observe associations with response to treatment while in the work of Taubert *et al.* (3) no such association is observed for pIgG, again indicating that the anti-PTM detection does measure different antibodies. For a subset of patients we have analyzed changes in anti-PTM antibody levels over time, and we observed that upon treatment the levels decrease. The data obtained from these two time points does not reveal if the anti-PTM positivity will completely seroconvert.

In conclusion, we agree with the authors of the commentary that unintentional detection of pIgG is an important factor to consider when running ELISA experiments on sera of patients with AIH. However, we are convinced that the careful set up of our experiments excluded the detection of pIgG and specifically measures anti-PTM antibodies.

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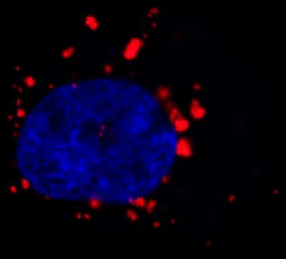

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Post-translationally modified proteins bind and activate complement with implications for cellular uptake and autoantibody formation

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Abstract

Introduction

Autoimmune diseases, such as rheumatoid arthritis (RA), are characterized by the presence of autoantibodies including those targeting self-proteins modified by post-translational modifications (PTMs). The complement system is known for its role in innate immune defense, but also in clearing debris and induction of antibody responses. We therefore hypothesized that complement could directly bind to PTMs and target PTM-modified proteins for clearance, or stimulate (chronic) inflammation and development of anti-PTM autoimmunity.

Methods

Six PTMs were investigated: nitration (Nt), citrullination (Cit), carbamylation (Ca), acetylation (Ac), malondialdehyde-acetaldehyde adducts (MAA) and advanced glycation end-products (AGE). We used mass spectrometry and plate-bound assays to analyze binding of serum proteins to PTM-modified proteins. The impact of complement activation on cellular uptake was studied in phagocytosis assays. The relationship between complement SNPs, and presence of anti-PTM autoantibodies was analyzed in 587 RA patients.

Results

Mass spectrometry analysis revealed a strong binding of complement to proteins modified with Ca, Ac, MAA and AGE but not to Nt and Cit. These observations were confirmed by plate-bound assays revealing that Ca-, MAA- and AGE-modified proteins activated the classical pathway, without involving antibodies. Ac activated the lectin pathway through ficolin-3. Complement activation on Ca-, Ac-, MAA- and AGE-coupled beads enhanced phagocytosis. SNPs in complement genes, associated with higher complement activity, were strongly associated with the presence of anti-PTM antibodies in RA patients.

Conclusion

Proteins containing the PTMs Ca, Ac, MAA or AGE activate complement. These complement opsonized PTMs increase phagocytosis and may lead to the development of anti-PTM antibodies.

Introduction

In patients with rheumatoid arthritis (RA) autoantibodies against self-proteins that have undergone a post-translational modification (PTM) are used as diagnostic and prognostic markers (1). These include anti-citrullinated protein antibodies (ACPA) and anti-carbamylated protein (anti-CarP) antibodies. Remarkably, these anti-PTM antibodies are present before onset of clinical symptoms and correlate with increased joint destruction over time (2, 3). RA patients benefit from B-cell targeted therapy, suggesting that B-cell mediated (anti-PTM) autoimmunity is playing a pathogenic role (4). However, what triggers the induction of autoantibody responses is currently unknown. Importantly, how the PTMs themselves contribute to inflammation is currently unknown. Unraveling the impact of PTM-driven immune activation on formation of anti-PTM antibody responses and on (chronic) inflammation would allow the design of specific early interventions.

In healthy individuals, proteins are frequently subjected to PTMs. There are more than 650 PTMs described affecting many aspects of protein functions (5). Some PTMs are essential for cellular processes and some take place outside the cell, maintaining homeostasis. A well-known example is phosphorylation, which is typically a reversible process. Other PTMs are largely irreversible such as glycosylation and glycation. Some irreversible PTMs alter the primary structure and lead loss of protein function forming neoantigens. In case long lived proteins are subjected to such irreversible PTMs, they may persist in the body, exposed to the immune system to be detected. While PTMs will occur in all individuals, only a subset of the individuals will produce autoantibodies against certain PTM-modified proteins (6, 7). To date, there is no understanding on how the human body processes these PTM-modified proteins and how these PTM-modified proteins impact (chronic) inflammation and autoimmunity. However, it is likely that PTMs that disrupt protein function are targeted for clearance. In addition to Ca and Cit, implicated in RA, several other PTMs have been reported to be immunogenic (8-10). One immunological system that is particularly known for its clearance function is the complement system.

Binding of complement recognition molecules such as C1q (11) (e.g. to cellular debris, dead cells and immune complexes), results in complement activation, opsonization of the target and cell-mediated clearance. Individuals that lack the early components of the classical pathway (e.g. C1q), frequently develop the autoimmune disease systemic lupus erythematosus (SLE), in part as a consequence of defective clearance (11, 12). In case of aberrant recognition or opsonization, a strong immune response against components of the accumulating debris may occur, including the generation of anti-PTM antibodies. Such a breach in tolerance has been described for several PTMs in the context of RA (13), SLE (14), cardiovascular diseases (15) and autoimmune hepatitis (16). We set out to

identify triggers that induce anti-PTM autoantibodies. Since the complement system is known for its role in clearance of debris and for stimulating adaptive immune responses, we hypothesize that complement can bind directly to PTM-modified proteins and impact on clearance and immune activation. Opsonized PTM-modified proteins can then be recognized by cells expressing complement receptors (CRs). CR3- and CR4-expressing phagocytic cells, such as macrophages and neutrophils, could then clear these opsonized PTM-modified proteins. B-cells specifically express CR2 (CD21) which is known to bind to complement opsonization fragment C3d, enabling co-ligation of the BCR and CR2, thus lowering the threshold for B-cell activation (17, 18).

Whether an individual will clear a PTM-modified protein or mount an autoreactive B-cell response is dependent on both genetic and environmental factors (19, 20). Certain combinations of single nucleotide polymorphisms (SNPs) in complement genes (the so called complotype), may result in high or low complement-activating profiles (19), that may predispose to the breaking of tolerance in only some individuals. In addition, several SNPs in complement activating protein C1q were described to be associated with development of RA (21) and SLE (22-24).

In this study we focus on six PTMs: nitration (Nt), citrullination (Cit), carbamylation (Ca), acetylation (Ac), malondialdehyde acetaldehyde adducts (MAA) and advanced glycation end-products (AGE). Each of these PTMs and/or antibodies against these PTMs are associated with a variety of disease such as RA, diabetes and coronary artery disease (1, 25-28). Additionally, some of these PTMs are structurally very different. Nt for example is a modification of tyrosine, Cit of arginine and Ca, Ac, MAA and AGE are all modifications of the amino acid lysine. We studied the capacity of PTM-modified proteins to bind and activate complement and enhance cellular binding and phagocytosis. Additionally, the relationship between SNPs in complement genes and positivity for anti-PTM antibodies was analyzed to obtain insight into the relevance of these findings in patients.

Material and Methods

Protein modification

Modified proteins were produced by either enzymatic or chemical reactions as previously described with minor adaptations for some of the modifications. For all modifications, fetal calf serum (FCS, Bodinco, Alkmaar, the Netherlands), IgG- and IgA-depleted fibrinogen (Fib, F4883, Sigma) and human serum albumin (HSA, A1653, Sigma) were used as backbones. Fibrinogen was depleted of IgG and IgA using protein G Plus/Protein A agarose suspension (IP05, Invitrogen).

For nitration 6 equal amounts of peroxynitrite (14042-01-4, Cayman Chem) were used directly from the stock solution to create an end concentration of 6 mM (29) for 1mg/mL protein. After addition of every aliquot the mix was directly vortexed for 30 seconds and left on ice for one minute before adding the next aliquot. After addition of the last aliquot the samples were incubated for 1 hr at 37°C. Citrullinated proteins were created by incubating 1 mg protein in a volume of 1 mL MilliQ containing 0.1 M TRIS-HCl pH 7.6, 5 mM DL-DTT (D0632, Sigma), 1U peptidyl arginine deiminase 4 enzyme/100µL (PAD enzyme, 1584, Sigma) and 10 mM $CaCl_2$ (21097, Sigma) (30). The mixture was incubated for 6 hr at 53°C. After incubation, citrullinated protein was spun down and stored at -80°C. For carbamylation of proteins, first a 2 M potassium cyanate (KOCN, 215074, Sigma) solution in PBS was prepared (3). 4 mg/mL protein was added to 2 M KOCN in a 1:1 volume-to-volume ratio and incubated for 12 hr at 37°C. Acetylation was performed using protein at a concentration of 1 mg/mL in 0.1 M Na_2CO_3. 50 µL of acetic anhydride (100042, Merck) and 200 µL of pyridine (109728, Merck) were added per 10 mL of protein solution (31). Proteins were incubated for 5hr at 30°C. After incubation, the reaction was stopped using 200 µL (per 10 mL solution) of 1 M TRIS. Samples were concentrated using Amicon Ultra – 15 centrifugal filter unit (10 kDa, UFC901024, Sigma) and buffer was exchanged to PBS using Zeba Spin desalting columns (89890, Thermofisher). For malondialdehyde-acetaldehyde adducts, first an malondialdehyde solution was prepared using 0.5 M 1,1,3,3,-Tetramethoxypropane (108383, Sigma) and 0.3% hydrochloric acid (1003171000, EMSURE) and incubated for 12 min at 37°C in a water bath (32). Meanwhile, 20 mg/mL protein was prepared and diluted 1:5 with 20% MilliQ, 20% freshly prepared MDA solution, and 4% acetaldehyde (402788, Sigma); pH was adjusted to 4.8 and MilliQ was added till 5 times volume of protein was reached. The solution was then incubated for 2 hr at 37°C in a water bath. Advanced glycation end-products were created by incubating 2 mg/mL protein with 1:100 diluted 3.3 M glycolaldehyde (23147-58-2, Sigma) (33). The solution was filtered using a 0.2 µm Whatman filter (10462200, GE Healthcare) and incubated for 10 days at 37°C while shaking. Following incubation, all (modified) proteins (except for Cit, which was spun down) were extensively dialyzed against PBS using 10 kDa cut-off SnakeSkin dialyzing tubes (88243, Thermofisher). All modifications were stored at -20°C until used. Protein concentrations were measured using Bradford protein assay (500-0006, Biorad) or Nanodrop.

The presence of PTM on the proteins was verified in an ELISA-based assay using anti-PTM specific antibodies. To detect Nt, mouse anti-nitrotyrosine (ab61392, abcam) was used. Mouse anti-citrulline (F95, MABN328, MilliPore) was used to detect Cit, rabbit anti-CBL (STA-078, biolabs) to detect Ca, rabbit anti-acetyllysine (ADI-KAP-TF120-E, Enzo Lifesciences) to detect Ac, goat anti-MAA-HRP (ab20703, abcam) to detect MAA and mouse anti-AGE (4B5, kindly provided by Dr. H de Boer, LUMC, Leiden, The Netherlands) (34) to detect AGE. Additionally, to verify that the PTM of interest is solely on the

modified proteins, mass spectrometry analysis was performed as described before and peptide-spectrum matches (PSMs) were determined to assess the concurrent presence of all six PTMs in each protein preparation (35, 36).

Anti-PTM IgG detection

Anti-PTM IgG antibodies were detected in sera from patients out of three cohorts consisting of patients with RA, SLE or AILD. RA samples were obtained from the Leiden Early Arthritis Cohort (EAC) (37). Details on SLE and AILD cohorts used in this manuscript are previously published in detail. Briefly, SLE samples were obtained from patients visiting the NPSLE clinic of LUMC between 2007 and 2019 with the clinical diagnosis of SLE (14). AILD samples were obtained from patients visiting the Department of Gastroenterology and Hepatology of the LUMC or Department of Gastroenterology and Hepatology between 1996 and 2020 (16). Modified FCS and nonmodified FCS were coated at 10 µg/mL in 0.1 M carbonate-bicarbonate buffer pH 9.6 (CB) on Nunc MaxiSorp plates (430341, Thermofisher) overnight at 4°C. In between each step plates were washed using washing buffer containing PBS and 0.05% Tween (P1379, Sigma). After washing, plates were blocked using PBS/1%BSA (blocking buffer) for 6 hr at 4°C. Following washing, wells were incubated with serum at a 1/50 dilution in PBS/0.05% Tween/1% BSA (PTB) for Nt, Cit, CarP and Ac and at a 1/100 or 1/1000 in PTB for AGE and MAA respectively. For each PTM, a standard of a pool of anti-PTM antibody-positive sera was taken along in serial dilutions on each plate. Human IgG was detected using rabbit anti-human IgG-HRP (P0214, Dako) diluted in PTB and incubated at 4°C for 3.5 hr. After the final wash, HRP enzyme activity was visualized using ABTS (A1888, Merck) with 0.05% H_20_2 (107209, Merck), and absorbance at 415 nm was read using a microplate reader (Bio-Rad). Absorbance was transformed to arbitrary units per milliliter (aU/mL) using the corresponding standard line. Background aU/mL of FCS was subtracted from the aU/mL signal on FCS-PTM to analyze specific anti-PTM antibody reactivity. The cut-off was calculated based on the mean plus two times the SD of the specific anti-PTM antibody reactivity of the healthy controls (HC). Specific signals of HC ten times higher than the mean were excluded in calculating the cut-off.

Binding of serum components to PTM-modified proteins

Binding of serum components to PTM-modified proteins was assessed in an unbiased way using mass spectrometry analysis. For this purpose we biotinylated FCS using EZ-link Sulfo-NHS-SS-biotin kit (21331, Pierce) according to manufacturer's instructions and modified biotinylated FCS as previously described. Biotinylated PTM-modified proteins and biotinylated non-modified proteins were incubated with streptavidin beads (CP01000-CP01N, Bangs Laboratories) at a concentration of 500 µg/mL and 3 µg proteins/µL beads, for 1 hr at 20°C in a shaking heat block at 400 rpm. After incubation, beads were washed twice using PBS/0.05%Tween and once using PBS and centrifuged

for 5 minutes at 10,000 rpm. Next, coupled beads were incubated in 10% normal human serum (NHS) in RPMI in a total volume of 150 µL for 1 hr at room temperature (RT). After incubation, beads were washed and resuspended in 15 µL PBS. Samples were incubated with 1:1 sample buffer with the addition of β-mercaptoethanol (1610737, Bio-Rad and M650, Sigma) for 10 minutes at 95°C. After incubation, samples were centrifuged for 2 min at 13,000 rpm. Samples were run a 4-15% gradient gel (456-1083, Biorad) according to manufacturer's instructions and proteins were visualized using instant stain (ISB1L, Expedeon). Bands of interest were subsequently excised for mass spectrometry analysis.

Analysis of gel bands

Gel slices were washed, subjected to reduction with dithiothreitol, alkylation with iodoacetamide and in-gel trypsin digestion using a Proteineer DP digestion robot (Bruker). Tryptic peptides were extracted from the gel slices and lyophilized.

Peptides were dissolved in 95/3/0.1 v/v/v water/acetonitril/formic acid (FA) and analyzed by online C18 nanoHPLC MS/MS with a system consisting of an Easy nLC 1000 gradient HPLC system (Thermo, Bremen, Germany), and a LUMOS mass spectrometer (Thermo). Samples were injected onto an in-house made precolumn (100 µm × 15 mm; Reprosil-Pur C18-AQ 3 µm, Dr. Maisch, Ammerbuch, Germany) and eluted via an in-house made analytical nano-HPLC column (30 cm × 50 µm; Reprosil-Pur C18-AQ 3 µm). The gradient was run from 10% to 40% solvent B (20/80/0.1 water/acetonitrile/ FA v/v) in 30 min. The nano-HPLC column was drawn to a tip of ~10 µm and acted as the electrospray needle of the MS source. The LUMOS mass spectrometer was operated in data-dependent MS/MS mode for a cycle time of 3 seconds, with an HCD collision energy at 32% and recording of the MS2 spectrum in the orbitrap. In the master scan (MS1) the resolution was 120,000, the scan range 400-1500, at an AGC target of 'standard'. A lock mass correction on the background ion m/z=445.12003 was used. Dynamic exclusion was performed after n=1 with exclusion duration of 10 s. Charge states 2-5 were included. For MS2 precursors were isolated with the quadrupole with an isolation width of 1.2 Da. The MS scan range was set to 'auto'. The MS2 scan resolution was 30,000 with an AGC target of 'standard' with a maximum fill time of 60 ms. In a post-analysis process, raw data were first converted to peak lists using Proteome Discoverer version 2.5 (Thermo Scientific), and then submitted to the Uniprot Human minimal database (20596 entries), using Mascot v. 2.2.07 (www.matrixscience.com) for protein identification. Mascot searches were done with 10 ppm and 0.02 Da deviation for precursor and fragment mass, respectively, and trypsin was specified as the enzyme. Methionine oxidation was set as a variable modification. Carbamidomethyl on cysteine was set as a fixed modification. The false discovery rate was set < 1%.

Subsequently, the abundance as well as abundance ratio (Abundance (Grouped) PTM / Abundance (Grouped) Control) were determined using Proteome Discoverer. Ratios

above 2 were considered enriched and proteins were sorted accordingly. Top 20s of proteins being enriched on the PTM-modified proteins were combined and duplicates removed, resulting in a list of 60 proteins. Proteins were sorted for being enriched on the number of PTMs for which they show a ratio above 2. Next, for each PTM-modified protein with a ratio above 2 data was entered in STRING pathway analysis program (https://string-db.org/) using the ''Proteins with Values/Ranks – Functional Enrichment Analysis'' option. Using the STRING database, a list of biological processes were generated.

Complement activation assay

Complement activation on PTM-modified proteins was assessed by coating PTM-modified proteins or unmodified control on Nunc MaxiSorp plates (430341, Thermofisher) at a concentration of 10 µg/mL in bicarbonate coating buffer (0.1 M Na_2CO_3/$NaHCO_3$ pH=9.6) overnight at RT. After each step, plates were washed 3 times using PBS containing 0.05% Tween 20 (P1379, Sigma). After overnight incubation plates were blocked using blocking buffer (PBS/1% BSA) for 1 hr at 37°C. Next, plates were incubated with serial dilutions of NHS in RPMI for 1 hr at 37°C. Then, bound complement components were detected using 1:1000 rabbit anti-human C1q (A0136, Dako), 1:1000 rabbit anti-human C3d (A0063, Dako), 1:333 mouse anti-HuC5b9 (M0777, Dako) or 1:1000 goat anti-human Factor H (A312, Quidel) diluted in PTB buffer. Plates were incubated for 1hr at 37°C whereafter primary antibodies were detected using matched HRP-labeled antibodies; goat anti-rabbit IgG-HRP (P0448, Dako), goat anti-mouse IgG-HRP (P0447, Dako) or rabbit anti-goat IgG-HRP (P0449, Dako) diluted in PTB buffer. Secondary antibodies were incubated for 1hr at 37°C after which plates were washed and developed using ABTS (A1888, Merck) with 0.05% H_20_2 (107209, Merck). Absorbance at 415 nm was read using a microplate reader (Bio-Rad).

Binding assay of purified components

To assess binding of purified serum components, Nunc MaxiSorp plates (430341, Thermofisher) were coated with PTM-modified proteins or non-modified counterpart at a concentration of 10 µg/mL in bicarbonate coating buffer (0.1 M Na_2CO_3/$NaHCO_3$ pH=9.6) overnight at RT. As a standard curve, purified serum components (C1q, A099, Complement Technology; or Factor H, A137, Complement Technology) were directly coated on the plate in bicarbonate coating buffer. After each step, plates were washed 3 times using PBS containing 0.05% Tween 20 (P1379, Sigma). After overnight incubation plates were blocked using blocking buffer (PBS/1% BSA) for 1 hr at 37°C. Next, plates were incubated with serial dilutions of purified C1q or purified Factor H and their binding was detected using rabbit anti-human C1q or goat anti-human Factor H (A0136, Dako and A312, Quidel). Primary antibodies were detected using goat anti-rabbit IgG-HRP (P0448, Dako) or rabbit anti-goat IgG-HRP (P0449, Dako) diluted in PTB buffer. Secondary antibodies were incubated for 1 hr at 37°C after which plates were washed

and developed using ABTS (A1888, Merck) with 0.05% H_20_2 (107209, Merck). Absorbance at 415 nm was read using a microplate reader (Bio-Rad).

Complement activation routes

To study which of the three complement pathways (classical (CP), lectin (LP) or alternative (AP)) were activated by the PTM-modified proteins we performed plate-bound complement activation assays. First we established that we could detect the specific activation of each of the pathways. For this purpose we analyzed the three routes using IgM, LPS and mannan as ligands for the CP, LP and AP, respectively. Nunc MaxiSorp plates (430341, Thermofisher) were coated with human IgM at 2 µg/mL (1838-8, Protos Immunoresearch), LPS at 10 µg/mL (L6386, Sigma) or mannan at 100 µg/mL (M7504, Sigma) in coating buffer (0.1M Na_2CO_3/$NaHCO_3$ pH=9.6) overnight at RT. After we verified that we could allocate each pathway correctly, we bound PTM-modified proteins and non-modified proteins at a concentration of 10 µg/mL. After each step, plates were washed 3 times using PBS containing 0.05% Tween 20 (P1379, Sigma). After overnight incubation with the ligands, plates were blocked using blocking buffer (PBS/1%BSA) for 1 hr at 37°C. To analyze activity of only one complement pathway, different complement-deficient/depleted sera and buffers were used. After washing, plates were incubated with serial dilutions (10%, 1%, 0.1%) of NHS or C1q-depleted human serum (A300, Complement Technology) in RPMI to analyze CP, MBL-deficient human serum (collected from a MBL deficient individual) in RPMI to analyze LP, or Factor B-depleted human serum (A335, Complement Technology) in RPMI-5 mM/10 mM MgEGTA ($MgCl_2$*$6H_2O$: 7791-18-6, Sigma and EGTA: E4378, Sigma) to analyze the AP and incubated for 1 hr at 37°C. Sera diluted in RPMI-10mM EDTA were taken along as negative control. After washing, bound C3 (AP) or C4 (CP and LP) was detected using 1:1000 rabbit anti-human C3d (A0063, Dako) or 1:1000 goat anti-human C4 (A305, Quidel) respectively, diluted in PTB Buffer. Additionally, Ficolin-1, -2 and -3 were detected in NHS diluted in RPMI using mouse anti-human Ficolin-1 (clone 7G1,HM2196, Hycult), DIG-labeled mouse anti-human Ficolin-2 (L-ficolin clone GN5, HM2091, Hycult) and mouse anti-human Ficolin-3 (H-ficolin clone 4H5, HM2089, Hycult) to confirm LP activation on acetylated protein as described before (38). Plates were incubated for 1 hr at 37°C whereafter primary antibodies were detected using matched HRP-labeled antibodies: goat anti-rabbit IgG-HRP (P0448, Dako), rabbit anti-goat IgG-HRP (P0449, Dako), goat anti-mouse IgG-HRP (P0447, Dako) or sheep anti-DIG-HRP (1120773910, Roche) diluted in PTB buffer. Secondary antibodies were incubated for 1 hr at 37°C after which plates were washed and developed using ABTS (A1888, Merck) with 0.05% H_20_2 (107209, Merck). Absorbance at 415 nm was read using a microplate reader (Bio-Rad).

Preparing beads for binding and phagocytosis assays

To assess binding and phagocytosis of complement-opsonized PTM-modified proteins we employed phagocytosis assays using whole blood and differentiated THP-1

macrophages. For this purpose we biotinylated FCS using EZ-link Sulfo-NHS-SS-biotin kit (21331, Pierce) according to manufacturer's instructions and modified biotinylated FCS as previously described. Biotinylated PTM-modified proteins and biotinylated non-modified proteins were incubated with 0.4 µm or 1 µm fluorescent streptavidin beads (CFFR002 or CFFR004, Bangs Laboratories) at a concentration of 300 µg/mL and 5 µg protein per µL beads, for 1 hr at 20°C in a shaking heat block at 400 rpm. Beads were washed 3 times using 10x volume PBS at 10,000 rpm for 5 min. Supernatant was carefully removed to prevent pellets from being disturbed. For phagocytosis assays using differentiated THP-1 macrophages, PTM-modified protein-coupled beads (PTM-coupled beads) were incubated in the presence of RPMI, 10% NHS in RPMI, or 10% NHS in RPMI/10 mM EDTA for 1 hr at 37°C in a shaking heat block (400 rpm). Beads were washed 3 times using 10x volume PBS at 10,000 rpm for 5 min, resuspended at 10 times starting volume. Next, beads were analyzed for presence of C1q, C3c and added to whole blood (1 µm beads, 150 beads/cell) or added to THP-1 macrophages (0.4 µm beads 3000 beads/cell in FCS-free THP-1 medium) to investigate phagocytosis.

Binding assay using whole blood

Fresh heparin blood was collected by staff of the LUMC voluntary donor service. One mL blood was washed using PBS, to wash away the heparin, at 500 g for 5 min. Plasma was removed and blood resuspended in 1 mL RPMI. Five µL washed blood was added to a 96 wells plate and subsequently 1 µL serum/PBS, 1 µL PTM-coupled beads (+/-150 beads/cell; prepared as described before, without serum incubation) and 3 µL RPMI were added resulting in a total volume of 10 µL. The joined volumes were incubated for 1 hr at 37°C to allow binding and uptake of the PTM-coupled beads. 3 µL sample was stained using CD45-FITC (clone 2D1, 345808, BD), CD61-PE (clone VIPL2, 12-0619-42, eBioscience) and CD235a-PE-Cy7 (clone HI264, 349112, Biolegend) to identify leukocytes, platelets and erythrocytes respectively. Samples were acquired using a BD LSR Fortessa equipped with FACSDiva software. Geometric mean fluorescence intensity (GMFI) was determined and normalized to the control condition in the presence of 10% NHS, which was included in each experiment. As a control intravenous immunoglobulin (IVIg) was biotinylated and coupled to the streptavidin beads similar to the biotinylated PTM-modified proteins. Subsequently, data was corrected for the signal on control FCS.

Phagocytosis assay THP-1 macrophages

THP-1 acute monocytic leukemia cells (DSMZ ACC 16) were plated in 6, 24 or 96 wells culture plate at 0.2*10^6 cells/mL. Cells were incubated for 72 hr at 37°C/5% CO_2 in THP-1 medium (Dutch modified RPMI-1640 + 10% FCS + 2% penicillin/streptomycin + 2% Glutamax) in the presence of 400 nM PMA (Sigma, P8139). After 72 hr the medium was removed and fresh THP-1 medium without PMA was added. THP-1 macrophages were subsequently rested for 5 days at 37°C/5% CO_2 before use.

Medium was removed from differentiated THP-1 cells. Serum-free THP-1 medium (RPMI, Pen/Strep/Glutamax) was added to differentiated THP-1 cells. As a negative control, cells were incubated with 10 µM cytochalasin D (Cyto D, Sigma, C8273). Beads were resuspended in serum-free THP-1 medium (with or without 10 µM Cytochalasin D) at 3000 beads/cell and added to the plate. Plates were spun down at 200 g for 1 min in order for the beads to be close to the cells. Phagocytosis took place for 1 hr in the incubator at 37°C/5% CO_2. After incubation, residual beads were washed away and cells were detached using trypsin (25300-054, Gibco). Simultaneously, THP-1 cells were stained using 1/13 CD15-BV10 and 1/40 CD11b-APC antibodies (563141 and 333143, respectively, both BD Biosciences) for 30 min at 4°C in the dark. Samples were acquired using a BD Canto I. Percentage of differentiated THP-1 cells was determined based on expression of CD15 and CD11b. Phagocytosis was determined using GMFI of the APC channel. Data was corrected for Cytochalasin D which was taken along for each condition.

Genetics

587 RA samples from the Leiden Early Arthritis Cohort (EAC) study were sequenced for complement SNPs containing the major alleles rs800292 (*FH*), rs1061179 (*FH*), rs2230199 (*C3*) and rs641153 (*CFB*) and rs665691 (*C1qA*), rs292001 (*C1qA*), rs294179 (*C1qC*) (21, 37). The SNPs in *CFH*, *C3* and *CFB* are part of the complotype (19) and the SNPs in *C1q* have been described as a risk factor for developing RA and SLE (21, 22, 39). We first ran a global test for each anti-PTM antibody with a logistic regression model using the glm function with family binomial (40). These regression models contained all seven SNPs as independent variable, and one of the anti-PTM antibodies (anti-Nt, -Cit, -Ca, -Ac, -MAA, -AGE) as dependent variable. We compared the log-likelihood of these models with a null-model. We used the McFadden pseudo-R-squared (R^2) to estimate which proportion of the variance in anti-PTM antibody presence was explained by the total SNP set.
For each anti-PTM antibody where the genetics significantly improved the model's fit, we ran a logistic regression for each individual SNP against the anti-PTM antibody.

Statistical analysis

Where applicable, statistical analysis was performed using paired t-tests or repeated-measures (RM) one-way ANOVA with Dunnett's multiple comparisons test. Significance was defined as p-value ≤ 0.05. For statistical analysis and graphical representations, GraphPad Prism v10.2.3 was used (San Diego, USA). For analysis on genetics first a logistic regression model was run using the glm function with family binomial and Bonferroni correction using R v4.3.1. For the global genetic test we considered p< 0.008 as significant (correcting the p-value of 0.05 for six tests (one for each anti-PTM antibody) following Bonferroni's correction). When the global trend was positive, we ran the individual SNP tests where a p-value of 0.05 was considered significant (40). Next, the McFadden pseudo-R-squared (R^2) was used to estimate which proportion of the

variance in anti-PTM antibody presence was explained by the total SNP set.

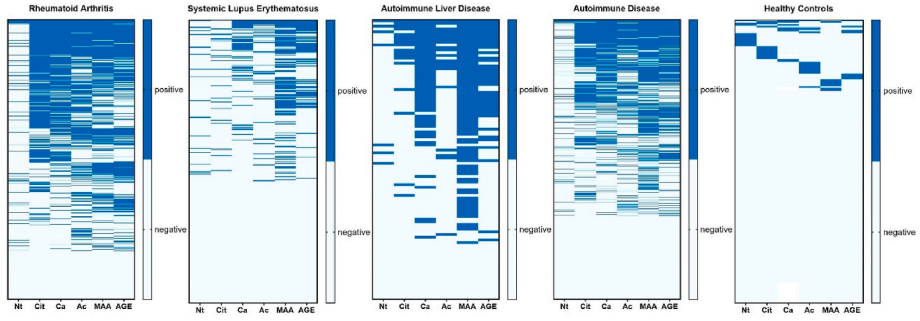
Results

Anti-PTM antibodies occur in many patients suffering from autoimmune diseases. In this study we focused on six PTMs: Nt, Cit, Ca, Ac, MAA and AGE. Fetal calf serum (FCS) was used as a model protein mix for post-translational modification. We confirmed that the desired PTM was present on the modified but not on the non-modified FCS using monoclonal antibodies against each PTM (Supplementary Figure 1A). Next, we confirmed, using mass spectrometry, that the procedures to achieve PTMs indeed resulted only in the desired PTM and did not trigger formation of other PTMs (Supplementary Figure 1B). Since ACPA and anti-CarP are associated with RA we analyzed the presence of anti-PTM autoantibodies against six PTMs in large cohorts of patients suffering from an autoimmune disease.

We screened the sera of 614 RA patients, 360 SLE patients and 106 patients suffering from auto-immune liver disease (AILD) relative to 197 healthy controls for the presence of anti-PTM antibodies (Supplementary Table 1 and Figure 1A). We observed that in all three autoimmune diseases there are substantial numbers of patients positive for anti-PTM antibodies. The clinical associations of positivity for anti-PTM antibodies have been published separately for each cohort (13, 14, 16). Here we focus on the presence and co-occurrence of these antibodies in autoimmune diseases

In RA patients 81,9% were positive for at least one anti-PTM antibody (Supplementary Table 1). In SLE and AILD 47,5% and 80,2% of all patients, respectively, were positive for at least one anti-PTM antibody. Interestingly, the highest percentage positivity was observed for anti-MAA in both SLE and AILD. In RA, anti-MAA was found in 46,7% of all patients and was therefore the second most common anti-PTM antibody in RA next to anti-Cit (49,3%). When all data of all autoimmune diseases were pooled, we observed that antibodies targeting Cit, Ca, Ac, MAA and AGE mostly co-occurred in autoimmune disease (Figure 1B). From these data we conclude that anti-PTM antibodies co-occur in many patients suffering from autoimmune diseases and that different combinations of anti-PTM antibodies exist. These data emphasize on the fact that anti-PTM antibodies are present and therefore relevant in autoimmunity.

A.



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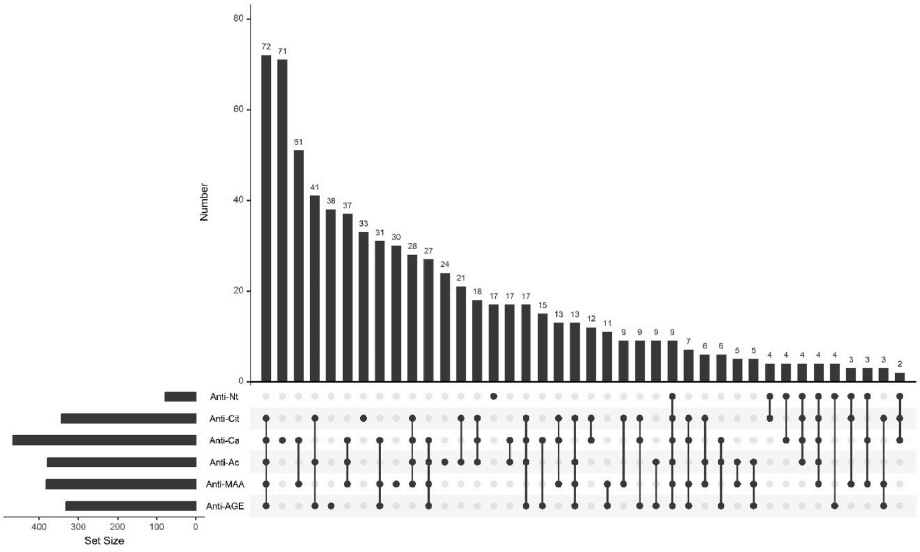

Figure 1: Anti-PTM antibodies against all six PTM-modified proteins are present in autoimmune disease. (A) Anti-PTM IgG positivity for each modification is presented in heatmaps for (from left to right) RA, SLE, AILD and all three auto immune diseases (RA, SLE and AILD) combined and HCs. Positivity was determined by calculating the mean reactivity of the HCs plus two times the standard deviation. (B) Upset plots of prevalence of anti-PTM antibody combinations in autoimmune disease. *Abbreviations: AGE, advanced glycation end-product; Ac, acetylated protein; Ca, carbamylated protein; Cit, citrullinated protein; HC, healthy control; MAA, malondialdehyde-acetaldehyde adduct; Nt, nitrated protein.*

Complement proteins bind more strongly to PTM-modified proteins compared to their non-modified controls.

To study how PTM-modified proteins could be involved in these chronic autoimmune diseases we set out to screen for plasma proteins that bind to PTM-modified proteins. For this purpose, we incubated PTM-modified-FCS-coupled beads (PTM-coupled beads)

with complement-active normal human serum (NHS). Of note, this NHS was prepared from sera of individuals with no detectable anti-PTM antibodies towards either of the six PTMs. The beads were washed and bound proteins were analyzed by mass spectrometry. We compared the relative abundance of specific proteins, based on the same unique peptides, on the PTM-coupled beads versus the non-modified-FCS-coupled beads and obtained insight into the enrichment of binding to the PTM-coupled beads by evaluating the ratios (Figure 2 and Supplementary Table 2).

Analysis of the data revealed strong enrichment of complement proteins binding to four of the six PTM-modified proteins compared to non-modified FCS. Seven of the top 10 hits were complement components including C1q, C4, C3 and Factor H (Figure 2). There were pronounced differences in the specific complement proteins binding to each of the PTM-modified proteins. We observed that proteins modified with the PTMs Ca, MAA and AGE bound proteins of the classical pathway in particular. The Ac-modified protein bound a similar set of complement proteins but did not show enhanced binding for C1q subunit A and C, suggesting a different activation pathway. Noteworthy is that Ac has been described to bind ficolin-3 (38). Indeed, we confirmed in our mass spectrometry analysis that ficolin-3 is enriched on Ac (Supplementary Table 2). The protein containing the PTMs Nt and Cit bound fewer proteins and did not reveal a similar enrichment of complement proteins (Figure 2).

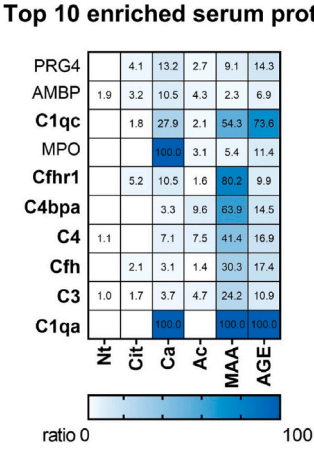

Figure 2: Seven out of top 10 enriched serum components binding to PTM-modified proteins are complement components. Heatmap of top ten enriched proteins binding to PTM-modified proteins compared to its control non-modified protein indicated by ratio abundance PTM / abundance Control. Ratio above two (filled blue boxed) were considered enriched. *Abbreviations: AGE, advanced glycation end-product; Ac, acetylation; AMBP, alpha-1-microglobulin; C1qA, complement C1q subcomponent subunit A; C1qc, complement C1q subcomponent subunit C; C3, complement C3; C4b, complement C4b; C4bpa, C4b-binding protein alpha chain; Ca, carbamylation; Cit, citrullination; Cfh, complement factor H; Cfhr1, complement factor H-related protein 1; MAA, malondialdehyde-acetaldehyde adduct; MPO, myeloperoxidase; Nt, nitration; PRG4, proteoglycan 4.*

STRING pathway analyses were performed on proteins with a ratio above two, revealing that most proteins were involved in protein activation cascades and complement activation (data not shown).

PTM-modified proteins activate the complement system

To investigate whether PTM-modified proteins were able to activate the complement system we performed an ELISA-based assay. Complement binding and activation on PTM-modified proteins was assessed by detecting plate-bound C1q and Factor H as well as deposition of C3 and C5b9, respectively. We determined optimal assay conditions using carbamylated proteins first. Complement was increasingly activated on carbamylated proteins at the level of C1q upon increasing complement active serum concentrations until 1% NHS (Figure 3A). C1q binding was not observed on non-modified protein, indicating that C1q binding is PTM-specific. For all other modifications one concentration (1% or 3% NHS, depending on the read out) was chosen and depicted in heatmaps. Next to Ca, also MAA- and AGE-modified proteins were able to bind C1q.

When analyzing downstream complement activation markers C3d and C5b9, we observed that Ca, MAA, AGE and also Ac were able to activate complement at the level of C3 and C5b9. Likewise, Factor H binding was observed for Ca, Ac, MAA and AGE. Since the FCS used in our experiments is a rich mix of different proteins we next confirmed that the same observations could be made using defined purified proteins. Indeed, using PTM-modified versions of purified human fibrinogen and human serum albumin in the complement activation assays, we observed a very similar complement activation profile indicating that complement activation was PTM-specific and not dependent on the protein backbone where the modifications are present (Figure 3A).

PTM-modified proteins bind complement independently of antibodies

After establishing that complement was activated by PTM-modified proteins, we assessed binding of purified complement components to investigate whether complement components bind directly to the PTM-modified proteins or indirectly through the presence of antibodies in the serum (Figure 3B). Ca, MAA and AGE modified proteins were able to bind purified C1q in a dose-dependent fashion. In addition, we verified whether Factor H was binding directly to the PTM-modified proteins or bound the deposited C3b. Binding of purified Factor H was readily observed for MAA-modified proteins, while Ca-, Ac- and AGE-modified proteins did not bind factor H. These data indicate that for Ca, Ac and AGE the Factor H binding observed in the complement activation assays is mainly indirect and a consequence of C3b binding. Additionally, similar assays were performed using the non-complement plasma proteins human serum albumin (HSA), alpha-2-macroglobulin (A2M) and alpha-1-antitrypsin (A1AT) in order to exclude that not just any plasma protein was binding to the PTM-modified protein (Supplementary Figure 2).

Indeed, we observed that these abundantly present plasma proteins were not binding to the PTM-modified proteins.

Ca, Ac, MAA and AGE activate complement through different complement pathways

Next, we set out to investigate which of the three complement pathways, classical (CP), lectin (LP) or alternative (AP), was activated by the PTM-modified proteins. For this purpose, we employed complement activation assays using serum depleted or deficient for specific complement components and made use of pathway-specific buffers. On top of that, we detected complement activation at the level of C4 and C3 to further discriminate between the pathways. We verified the experimental set up by making use of IgM (CP), mannan (LP) or LPS (AP) as a coating and C1q-depleted serum in RPMI, MBL-deficient serum in RPMI and Factor B-depleted serum in RPMI Mg-EGTA, respectively, as a source of complement. We compared these sera and buffer combination to NHS in RPMI to verify that we could correctly allocate the activation pathway of these positive controls. (Supplementary Figure 3). Next, the PTM-modified proteins were used as the coating and the same set of reagents was used (Figure 3C). With increasing concentrations of NHS, the complement system was activated on FCS-Ca. No complement activation was observed when C1q-depleted serum was used, indicating that the CP was activated on FCS-Ca. For the other PTM-modified proteins a dilution of 1% serum was chosen to assess the CP and LP and 10% to assess the AP. The complement system was not activated on Nt and Cit, as was observed before, and no pathway could be allocated. Similarly to FCS-Ca, also FCS-MAA and FCS-AGE activated complement in the presence of NHS and did not activate complement when using C1q-depleted serum, indicating that the CP was activated and essential on these PTM-modified proteins. For FCS-Ac, complement activation was still observed even when using C1q-depleted serum and MBL-deficient serum. However, earlier reports indicate that acetylated proteins may trigger the LP through binding of ficolins and not MBL (38). We therefore tested ficolin binding to FCS-Ac. Indeed, we observed that ficolin-3 was specifically binding to FCS-Ac, leading to complement activation (Figure 3D). Altogether, these data indicate that the CP was activated on FCS-Ca, -MAA and -AGE and the LP on FCS-Ac.

C1q binding to Ca-, MAA- and AGE-modified proteins was significantly decreased when C1q was preincubated with F(ab')2 fragments of an anti-C1q-head but not anti-C1q-tail antibody (data not shown). Additionally, C1q binding was abrogated only in the presence of 2M NaCl and not in the presence of heparin or low pH (data not shown). These data indicate that C1q binds to Ca, MAA and AGE through its globular heads and that the interaction is an electrostatic interaction.

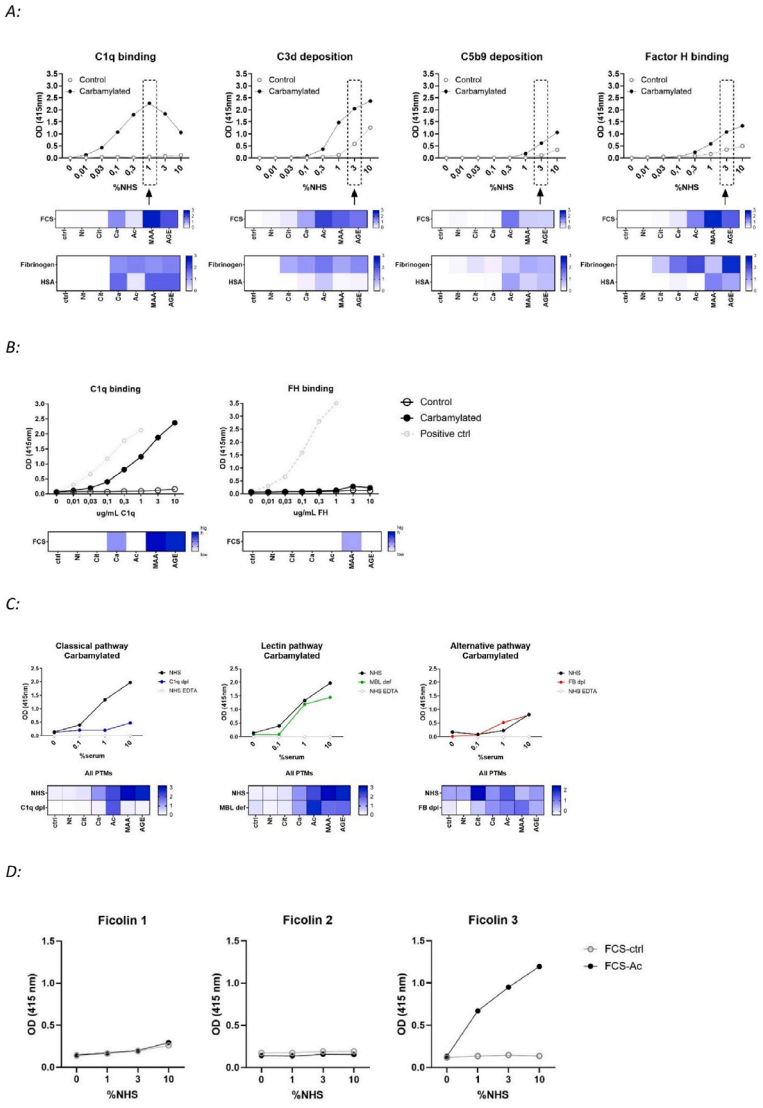

Figure 3: Complement binds directly and is activated on specific PTM-modified proteins. (A) Both complement binding (C1q and Factor H) and complement activation (C3d and C5b9) were measured using ELISA using a pool of complement active serum. (B) Direct binding of C1q and Factor H was assessed using purified C1q and Factor H, respectively. Directly coated C1q and Factor H were taken along as positive controls. (A,B) In the upper figures, plates were coated with carbamylated or control protein and serially diluted serum or purified complement was added and assessed. Below, one concentration was chosen and heatmaps were created to visualize reactivity for all six PTM-modified proteins and non-modified protein. Complement activation pathways (CP, LP and AP) were activated on (C) the different PTM-modified proteins and (D) ficolin detected on acetylated FCS. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$ by one-way ANOVA and Dunnet's multiple comparison. Data are an representative of three independent experiments. *Abbreviations: AGE, advanced glycation end-product; Ac, acetylated protein; Ca, carbamylated protein; Cit, citrullinated protein; FB, factor B; FCS, fetal calf serum; HSA, human serum albumin; MAA, malondialdehyde-acetaldehyde adduct; MBL, mannan binding lectin; NHS, normal human serum; Nt, nitrated protein; OD, optical density; PTM, post-translational modification.*

As the mass spectrometry data indicated presence of some of the FH related proteins, we further investigated if next to Factor H also members of the FHR family were binding to PTM-modified proteins (Supplementary Figure 4). We observed that Cit, Ca, MAA and AGE modified proteins bind FHR-1, -3 and -5.

In whole blood PTM-modified proteins bind to leukocytes and not to erythrocytes or platelets

To study functional consequences of the PTM-complement interaction we set out to investigate PTM-modified protein binding to cells expressing complement receptors, namely leukocytes, platelets and erythrocytes from whole blood. Therefore, a whole blood assay was performed with PTM-modified FCS coupled to fluorescent beads, with and without serum (Figure 4A). We observed that those PTMs that activated complement (Ca, Ac, MAA, AGE) and Cit were able to bind to leukocytes. For Nt we did not observe binding to leukocytes, erythrocytes or platelets. We observed an increased binding of PTM-modified protein loaded beads in the presence of complement active serum compared to complement inactivated serum. Interestingly, we did not observe a noticeable binding to erythrocytes or platelets. These data indicate that PTM-modified proteins mainly bind to leukocytes, a process that is increased in the presence of active complement.

PTM-modified proteins that activate complement show increased uptake by differentiated THP-1 cells.

Since macrophages are the main phagocytic cells in tissues that express complement receptors, we set out to investigate uptake of PTM-modified proteins by THP-1 cells that were differentiated into macrophages using phorbol 12-myristate 13-acetate (PMA). For these experiments, beads containing PTM-modified FCS or non-modified FCS were incubated without serum, with complement-active serum or with complement-inactive serum (EDTA). We first confirmed that also for PTM-modified proteins coupled to beads, the same complement activation profiles were present as observed in the plate-bound assays (data not shown). These sets of beads were added to THP-1 macrophages and uptake was assessed (Figure 4B). Non-modified FCS, Nt and Cit coupled beads were not taken up by differentiated THP-1 macrophages. Ca-, Ac-, MAA- and AGE-coupled beads were taken up by THP-1 macrophages, and uptake was significantly increased in the presence of complement-active serum. When using complement-inactive serum the signal does not decrease to the level of no serum, indicating that the uptake is partially depending on complement activation and partially dependent on serum factors that bind but do not require complement activation. In addition, we have used complement receptor blocking antibodies to study the relative importance of complement activation on the uptake of PTM-coupled beads. We observed that by blocking CR1, -3 and -4 the uptake of complement opsonized PTM-coupled beads is partially inhibited (Supplementary Figure 5). These results indicate that the PTM-modified proteins capable

of activating complement demonstrate enhanced uptake by THP-1 macrophages, which is partially dependent on complement activation.

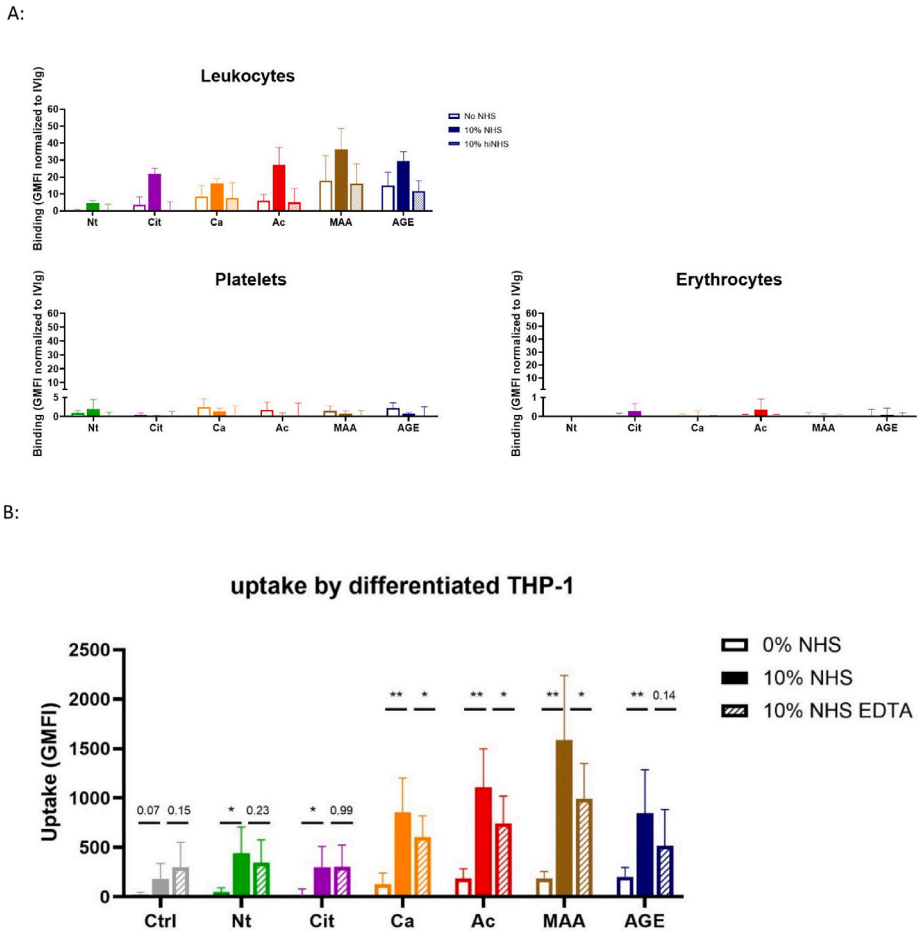

Figure 4: Leukocytes and THP-1 macrophages bind PTM-coupled beads in a complement dependent manner. (A) Binding of PTM-coupled beads to different cell populations from whole blood that express complement receptors (i.e. leukocytes, platelets and erythrocytes) in the presence of RPMI (open bar), RPMI/10% NHS (solid bar) or RPMI/10% hiNHS (striped bar). (B) PTM-coupled beads that were incubated with 0% NHS (open bar), 10%NHS (solid bar) and 10%NHS in the presence of 10mM EDTA (striped bar) and incubated with THP-1 macrophages and phagocytosis was assessed. $^{*}P < 0.05$, $^{**}P < 0.01$ by multiple paired t-tests. Data shown as mean +/- standard deviation and are representative of three independent experiments. *Abbreviations: AGE, advanced glycation end-product; Ac, acetylated protein; Ca, carbamylated protein; Cit, citrullinated protein; GMFI, geometric mean fluorescence intensity; hiNHS: heat inactivated normal human serum; IVIg,* intravenous immunoglobulin; *MAA, malondialdehyde-acetaldehyde adduct; NHS, normal human serum; Nt, nitrated protein.*

Combinations of complement SNPs associate with anti-PTM antibody positivity.
Environmental and genetic risk factors play an important role in the induction of autoantibody responses in RA patients. We therefore hypothesized that individuals that harbor genetic variants of complement genes, associated with a more active complement system, opsonization and activation of immune cells leading to phagocytosis and/or induction of antibody responses may be different from individuals with genetic variants that result in a less active complement system. Therefore, we assessed the association of complement SNPs with positivity for anti-PTM autoantibodies in RA patients. For that purpose, we used samples from RA patients enrolled in the Leiden Early Arthritis Clinic cohort (n = 587). We used genotype data for the complotype SNPs including *CFH*, *CFB* and *C3* (19) as well as SNPs in *C1q* known to associate with risk for developing RA and SLE (21, 22, 39).

The global test, where we ran a model including all SNPs to predict anti-PTM antibody positivity, showed a significant association of the complement SNPs with each of the anti-PTM autoantibodies (Supplementary Table 3). The SNPs together explained 17 to 20 percent of the variance of the individual anti-PTM antibodies.

Since the global tests were statistically significantly different, we further explored which of the individual SNPs were significantly associated with the anti-PTM antibodies (Table 1). Here, we found an association of rs292001 (*C1qA*) with anti-Cit (OR 1.27, 95%CI 1.0-1.6, *P-value 0.05*), rs2230199 (*C3*) with anti-Ca (OR 1.4, 95%CI 1.0-2.0, *P-value 0.04*), rs1061170 (*CFH*) with anti-Ac (OR 1.35, 95%CI 1.0-1.8, *P-value 0.04*), rs1061170 (*CFH*) with anti-MAA (OR 1.38, 95%CI 1.1-1.8, *P-value 0.01*), rs1061170 (*CFH*) with anti-AGE (OR 1.36, 95%CI 1.1-1.7, *P-value 0.02)*. These significantly associated SNPs captured often only part of the proportion of the variance of anti-PTM antibody presence explained by the total SNP set. Possibly the combination of SNPs, also known as the complotype, is relevant for developing antibody responses against PTM-modified proteins. Overall, these data indicate that having genetic variants that are known to result in a more active complement system, is associated with positivity for anti-PTM antibodies.

Table 1: Association between individual complement SNPs with each anti-PTM antibody

	SNP	rs800292	rs1061170	rs665691	rs292001	rs294179	rs2230199	rs641153
Anti-PTM	**Gene**	*CFH*	*CFH*	*C1qA*	*C1qA*	*C1qC*	*C3*	*CFB*
Anti-Nt	OR	3.54	0.77	1.66	1.79	2.36	1.51	0.30
	P	*0.16*	*0.79*	*0.56*	*0.52*	*0.33*	*0.73*	*0.60*
	R2	0.01	0.01	0.01	0.02	0.02	0.15	0.01
Anti-Cit	*OR*	*1.05*	*1.19*	*1.22*	*1.27*	*1.22*	*1.03*	*1.15*
	P	*0.73*	*0.18*	*0.09*	*0.05**	*0.09*	*0.83*	*0.56*
	R2	0.05	0.05	0.05	0.06	0.05	0.19	0.05
Anti-Ca	OR	1.13	0.98	1.13	1.13	1.12	1.4	1.19
	P	*0.35*	*0.85*	*0.32*	*0.34*	*0.35*	*0.04**	*0.48*
	R2	0.05	0.05	0.05	0.06	0.05	0.19	0.05
Anti-Ac	OR	1.02	1.35	0.98	1.09	0.94	1.06	0.96
	P	*0.89*	*0.04**	*0.9*	*0.52*	*0.66*	*0.76*	*0.88*
	R2	0.05	0.05	0.05	0.05	0.05	0.16	0.05
Anti-MAA	OR	0.73	1.38	1.11	1.04	1.03	0.88	0.76
	P	*0.16*	*0.01**	*0.42*	*0.75*	*0.83*	*0.43*	*0.27*
	R2	0.06	0.06	0.05	0.05	0.05	0.18	0.05
Anti-AGE	OR	0.96	1.36	1.12	1.13	1.13	0.88	1.15
	P	*0.78*	*0.02**	*0.36*	*0.31*	*0.3*	*0.42*	*0.55*
	R2	0.05	0.06	0.05	0.06	0.05	0.19	0.05

* significant association

Abbreviations: AGE, advanced glycation end-product; Ac, acetylated protein; Ca, carbamylated protein; CFB, co-factor B; Cit, citrullinated protein; FH, Factor H; MAA, malondialdehyde-acetaldehyde adduct; Nt, nitrated protein; OR, odds ratio; PTM, post-translational modification; R2, McFadden Pseudo R squared; SNP, single nucleotide polymorphism.

Discussion

Autoimmune diseases, such as RA and SLE, are characterized by chronic inflammation and the presence of autoantibodies, including autoantibodies that target PTM-modified proteins. What triggers B-cells to generate these autoantibodies is largely unknown. Unravelling B-cell triggers by PTM-modified proteins will provide more specific treatment options as compared to the broad B-cell depletion used nowadays (4). Therefore, we investigated six irreversible PTMs (Nt, Cit, Ca, Ac, MAA, and AGE). We generated these six PTMs on a mix of proteins (FCS) and also generated mock-modified FCS by performing exactly the same procedure but leaving out the key-reagent for the modification. We verified the presence of the PTMs using both mass spectrometry and ELISA (36, 41). Importantly, we not only confirmed that the modified FCS preparations contained the desired PTM, but also that they did not contain any of the other PTMs. AGEs consist of a number of different structures and for the mass spectrometry analysis we therefore focused on carboxymethyl lysine, the key and most common AGE structure (42). Therefore, the number of peptide-spectrum matches (PSMs) are lower as compared to other modifications. However, compared to its non-modified counterpart there is clearly increased modification (Supplementary Figure 1B). Additionally, we verified that equal levels of PTM-modified protein and control protein was coated on the plate (data not shown).

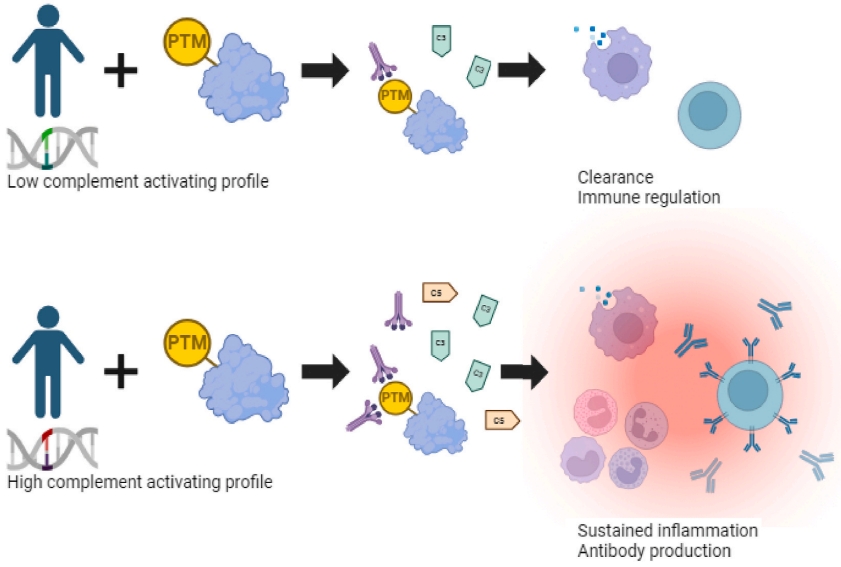

Figure 5: Model.
The so-called complotype of individuals results in a low (upper part) or high (lower part) complement activating profile. Depending on the complotype, complement opsonization of PTM-modified proteins could lead to clearance and immune regulation or (sustained) inflammation and autoantibody production, respectively.

We investigated autoantibody responses against the six PTM-modified proteins in three cohorts of patients suffering from the autoimmune diseases RA, SLE or AILD. In previous publications we established that in these three clinical conditions the presence of anti-PTM antibodies is associated with disease severity and/or response to therapy (13, 14, 16). These studies, and others, indicate that in many autoimmune diseases both PTM-modified proteins and/or anti-PTM antibodies, are present and could contribute to the disease process (36, 43-46). In this work, we hypothesized that complement proteins could directly bind to PTM-modified proteins to enhance clearance but that depending on the complotype this process could lead to (sustained) inflammation and autoantibody production (Figure 5).

Using mass-spectrometry we identified all human serum proteins that were bound to our PTM-modified proteins, therefore providing an unbiased analysis. The data indicated that complement proteins were specifically binding to 4 of the 6 PTM-modified proteins tested, namely Ca-, Ac-, MAA- and AGE-modified proteins. As we, and others, previously established the presence of antibodies against PTM-modified proteins, we needed to make sure that the observed complement activation was not simply the result of anti-PTM antibodies in the human serum (47). Our further experiments, using purified C1q, revealed that indeed there is a direct binding of C1q to the PTM-modified proteins as was previously also indicated for AGE (48). For Ac, we confirmed that, ficolins were

directly binding to the PTM-modified protein (38). Binding of complement recognition molecules, C1q and ficolin, resulted in complement activation for Ca, Ac, MAA and AGE at the level of C3d and C5b9. Complement activation is regulated by Factor H and FHR proteins acting on the AP and amplification loop of all pathways (49). Similar to other studies, we observed direct binding of Factor H (50) and FHR-1, -3 and -5 (51-54) to MAA when using purified proteins. No competition was observed between C1q and factor H for binding to MAA modified proteins (data not shown). Next to MAA, we observed minor direct binding of Factor H to Ca only at high concentrations. Additionally, when employing complement activation assays using C1q depleted serum, no residual complement activation is observed. These data indicate that the binding of FHR-3 and -5 to Ca, MAA and AGE did not induce AP activation.

Interestingly, Ca, Ac, MAA and AGE are all modifications of the amino acid lysine. However, we observed that not all lysine modifications induce similar complement activation pathways and activate complement to the same extent, thereby indicating that not all modifications of lysine behave similarly. In patients, direct complement activation on certain PTMs may contribute to sustained inflammation, in the patients anti-PTM antibodies could trigger additional complement activation (47). Complement activation assays including anti-CarP IgG (WT and LALAPG) showed that with the addition of increasing concentrations LALAPG mutated anti-CarP antibodies (that lack the capacity to activate complement), complement activation by Ca modified FCS itself was inhibited (Supplementary Fig. 6). Addition of the WT anti-CarP antibody further enhanced complement activation, as evidenced by increased levels of C5b-9. These data indicate that complement activation occurs directly on PTM-modified proteins and is further amplified in the presence of anti-PTM antibodies.

We coupled PTM-modified proteins to beads and then examined their binding and phagocytosis by leukocytes, platelets and erythrocytes from whole blood. Additionally, THP-1 macrophages were included to mimic a tissue-resident phagocytic cell population. We observed an increased uptake and binding for complement opsonized Ca-, Ac-, MAA- and AGE-coupled beads for THP-1 macrophages and leukocytes.

While we expected to see predominant binding to erythrocytes because of their high CR1 expression, we observed a clear binding particularly to leukocytes. This interaction is not antigen-specific as all leukocyte types were involved. This process of complement-dependent antigen transport has been described before (55) and would allow accumulation of the PTM-modified proteins e.g. in liver and spleen where they could trigger immune responses. The B-cells and follicular dendritic cells could serve a specific function as they express CR2 (17). Several studies based on mouse experiments indicate a clear enhancement of B-cell activation if next to the BCR also CR2 is engaged (17, 56)

resulting in enhanced antibody responses to complement-opsonized targets. However, in a human setting contradictory results are observed (18, 57).

Genetic and environmental factors are important factors that contribute to disease onset (19, 20). In case of polymorphisms in complement genes, certain combinations, so called complotype, may result in high or low complement activating profiles (19). We observed that some genetic variants that are part of the complotype are associated with the presence of anti-PTM antibodies. Namely, the minor allele of *CFH* rs1061170, being associated with higher complement activity (58), was associated with the presence of anti-Ac, -MAA and -AGE. Moreover, this minor allele was previously shown to alter FH binding abilities to malondialdehyde (50). Others showed that the minor allele of *C3* rs2230199, associated with anti-Ca, bound FH less strongly and was less efficiently inactivated, thereby increasing AP activity (59). Noteworthy is that, studies showed that minor allele of *C1q* SNPs associate with change in C1q serum levels but do not show increase in complement activity (21, 60, 61). Interestingly, the minor allele of *C1qA* rs292001 associated with anti-Cit, contributing to the findings that *C1q* SNPs are described in the context of RA (21). These data suggest a link between complotype and development of anti-PTM antibody reactivity. Inhibiting the complement system systemically, may not be a realistic option to limit complement activation mediated by PTM, because of costs and because of infectious risk associated with systemic complement inhibition. For future purposes we therefore propose to explore bispecific antibodies targeting both the PTM-modified protein and bringing complement inhibitors into close proximity (62).

Future *in vivo* studies should unravel the importance of complement in the induction of anti-PTM antibodies. In such studies complement deficient mice could be used and compared to mice with a fully functioning complement system. We previously established that in mice several anti-PTM antibody reactivities can be observed and induced (63, 64). However, the role of complement has not been studied. Additionally, specified cell populations as well as cells from (at risk) patients should be further investigated to elucidate how complement leads to (chronic) inflammation and autoimmunity.

In conclusion, PTM-modified proteins can bind and activate complement, which increases inflammation and phagocytosis, and may lead to development of anti-PTM antibodies.

Data availability

All data are available upon request. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [1] partner repository with the dataset identifier PXD054220".

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Supplementary files

Additional materials and methods on supplementary figures

Complement activation through anti-PTM antibodies versus PTM-modified proteins

To assess relative complement activation through anti-PTM antibodies compared to PTM-modified proteins complement activation assays were employed using monoclonal IgG antibodies with and without LALAPG mutations. First, carbamylated FCS was coated on Nunc MaxiSorp plates (430341, Thermofisher) at a concentration of 10 µg/mL in bicarbonate coating buffer (0.1 M Na_2CO_3/$NaHCO_3$ pH=9.6) overnight at RT. After each step, plates were washed 3 times using PBS containing 0.05% Tween 20 (P1379, Sigma). After overnight incubation plates were blocked using blocking buffer (PBS/1% BSA) for 1 hr at 37°C. Next, plated were incubated with or without IgG antibodies (with and without LALAPG mutations) against Ca. The LALAPG mutation prevents Fc mediated complement activation. Then, plates were incubated with 3% NHS in RPMI for 1 hr at 37°C. Bound IgG and C5b-9 were detected using 1:2000 goat anti-rabbit IgG-HRP (P0448, Dako) and 1:333 mouse anti-HuC5b9 (M0777, Dako), respectively, diluted in PTB buffer. Plates were incubated for 1hr at 37°C whereafter mouse-anti-HuC5b9 was detected using goat anti-mouse IgG-HRP (P0447, Dako). Secondary antibodies were incubated for 1hr at 37°C after which plates were washed and developed using ABTS (A1888, Merck) with 0.05% H_20_2 (107209, Merck). Absorbance at 415 nm was read using a microplate reader (Bio-Rad).

Binding of Factor H related proteins

To assess binding of Factor H-related (FHR) proteins, modified FCS and non-modified FCS were coated on Nunc MaxiSorp plates (430341, Thermofisher) at 10 µg/mL in coating buffer (0.1 M Na_2CO_3/$NaHCO_3$ pH 9.6) overnight at RT. The next day, and after each incubation step, plates were washed 5 times using PBS containing 0.05% Tween 20 (P1379, Sigma). After washing, plates were blocked using blocking buffer (PBS/1% BSA) for 30 min at RT while shaking. After blocking, His-tagged FHR proteins 1, 2, 3, 4A and 5 (all produced at Sanquin Research) were diluted to equal molar concentrations, starting the concentration curve at 80 nM for all FHR proteins. As positive and negative controls His-tagged recombinant Factor H (produced in house at Sanquin Research) and His-tagged irrelevant protein FcRn (neonatal Fc Receptor; produced in house at Sanquin Research) were taken along. Since all FHR proteins and recombinant Factor H contained a His tag, FHR proteins could be detected using the same mouse anti-His tag at 1 µg/mL followed by a rat anti-mouse kappa HRP conjugated secondary antibody (Clone RM-19, produced in house at Sanquin Research) at 0.25 µg/mL. Both were incubated for 1 hr at RT while shaking. Plates were developed using TMB and absorbance was read at 415 nm using a microplate reader (Bio-Rad).

Complement receptor blocking experiments

To assess the importance of complement receptors in phagocytosis of complement-opsonized PTM-coupled beads by THP-1 macrophages we analyzed uptake in the presence or absence of complement receptor blocking antibodies. Blocking antibodies against CR1, CR3 and CR4 (CR1, clone J3D3 sc-59022 Santa Cruz; CR3, anti-CD11b, clone ICRF44, 301302, Biolegend; CR4, anti-CD11c, clone 3.9, 301602, Biolegend; CR3/4, anti-CD18, clone 1B4, 373402, Biolegend) were used. Additionally, blocking antibodies against CR2 (not expressed by THP-1, anti-CD21, 552727, BD Pharmingen) and isotype controls (IgG1 21335011 and IgG2 21335021, both Immunotools) were included as controls. THP-1 macrophages were first incubated with Fc block (564220, BD) for 10 min. Without washing, cells were incubated with a combination of all three blocking antibodies against CR1, 3 and 4 or their controls at a concentration of 10 µg/mL for 30 min at 4°C. Additionally, THP-1 macrophages were incubated without blocking antibodies or with Cyto D (C8273, Sigma) as reference conditions. Without washing, beads were added at 3000 beads/cell. Phagocytosis took place for 1 hr in the incubator at 37°C/5% CO_2. After incubation, beads were washed away and cells were detached using trypsin (25300-054, Gibco). Samples were acquired using BD Canto I. Phagocytosis was determined using GMFI of the APC channel.

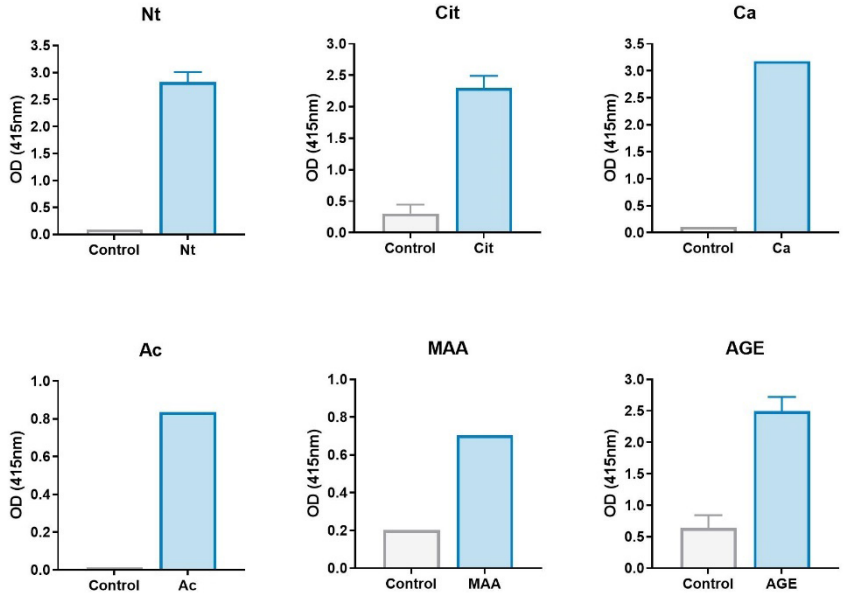
Supplementary Tables and Figures

Supplementary Table 1: Prevalence of anti-PTM antibodies in three autoimmune diseases (RA, SLE and AILD, respectively), and the combination of these autoimmune diseases (AID), compared to anti-PTM antibodies in HCs.

	RA n=614	SLE n=360	AILD n=106	AID n=1080	HC n=197
Anti-Nt n, (%)	54 (8.8)	18 (5.0)	7 (6.6)	79 (7.3)	12 (6.1)
Anti-Cit n, (%)	303 (49.3)	22 (6.1)	18 (17.0)	343 (31.8)	12 (6.1)
Anti-Ca n, (%)	276 (45.0)	53 (14.7)	50 (47.2)	379 (35.1)	8* (4.5)
Anti-Ac n, (%)	278 (45.3)	33 (9.2)	20 (18.9)	331 (30.6)	13 (6.6)
Anti-MAA n, (%)	287 (46.7)	108 (30.0)	72 (67.9)	467 (43.2)	8 (4.1)
Anti-AGE n, (%)	277 (45.1)	67 (18.6)	39 (36.8)	383 (35.5)	7 (3.6)
At least one anti-PTM	503 (81.9)	171 (47.5)	85 (80.2)	759 (70.3)	48 (24.4)
At least two anti-PTMs	407 (66.3)	85 (23.6)	54 (50.9)	546 (50.6)	9 (4.6)

*for 20 HCs data on anti-CarP was missing. *Abbreviations: AGE, advanced glycation end-product; AID, autoimmune disease; AILD, autoimmune liver disease; Ac, acetylated protein; Ca, carbamylated protein; Cit, citrullinated protein; HC, healthy control; MAA, malondialdehyde-acetaldehyde adduct; Nt, nitrated protein; PTM, post-translational modification; RA, rheumatoid arthritis; SLE, systemic erythematosus.*

A:



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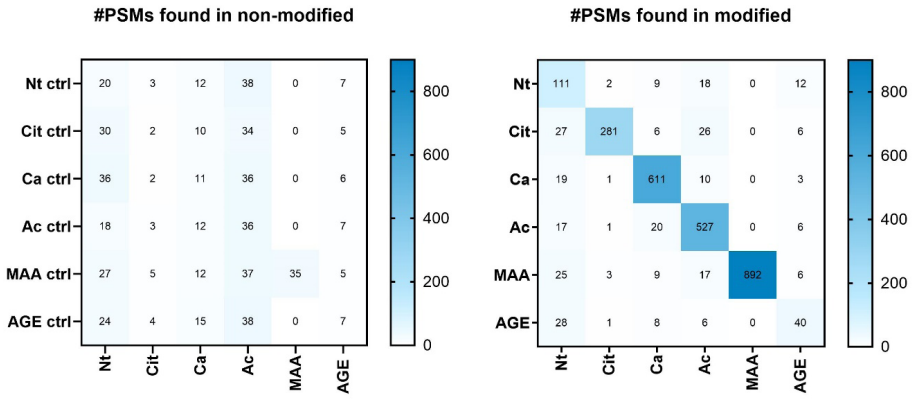


Supplementary Figure 1: Confirmation of PTM on FCS. (A) PTMs were detected using specific antibodies against the PTM or (B) the amount of modification on each modified protein represented by peptide-spectrum matches (PSMs), analyzed using mass spectrometry. Data are representative of three (A) or one (B) independent experiment(s). *Abbreviations: AGE, advanced glycation end-product; Ac, acetylated protein; Ca, carbamylated protein; Cit, citrullinated protein; MAA, malondialdehyde-acetaldehyde adduct; Nt, nitrated protein; OD, optical density; PSMs,* peptide-spectrum matches; *PTM, post-translational modification.*

Supplementary Table 2: Combined data of enriched serum components binding to PTM-modified proteins. Top 20 enriched proteins binding to each PTM-modified proteins compared to its control non-modified protein were combined. List is based on ratio abundance PTM / abundance Control determined by Proteome Discoverer. Duplicates were removed and ratio above 2 (bold and underlined) were considered enriched. Complement proteins are depicted in yellow and immunoglobulin fragments in pink. Data are representative of two independent experiments.

	Accession #	Description	Nt	Cit	Ca	Ac	MAA	AGE	# ratio >2
1	Q92954	Proteoglycan 4 OS=Homo sapiens	-	**4,1**	**13,2**	**2,7**	**9,1**	**14,3**	5
2	P02760	Protein AMBP OS=Homo sapiens	1,9	**3,2**	**10,5**	**4,3**	**2,3**	**6,9**	5
3	P02747	Complement C1q subcomponent subunit C	-	1,8	**27,9**	**2,1**	**54,3**	**73,6**	4
4	P05164	Myeloperoxidase	-	-	**100**	**3,1**	**5,4**	**11,4**	4
5	Q03591	Complement factor H-related protein 1	-	**5,2**	**10,5**	1,6	**80,2**	**9,9**	4
6	P04003	C4b-binding protein alpha chain	-	-	**3,3**	**9,6**	**63,9**	**14,5**	4
7	P0C0L5	Complement C4-B	1,1	-	**7,1**	**7,5**	**41,4**	**16,9**	4
8	P08603	Complement factor H	-	**2,1**	**3,1**	1,4	**30,3**	**17,4**	4
9	P01024	Complement C3	1,0	1,7	**3,7**	**4,7**	**24,2**	**10,9**	4
10	P02745	Complement C1q subcomponent subunit A	-	-	**100**	-	**100**	**100**	3
11	P02746	Complement C1q subcomponent subunit B	-	-	**25,4**	**2,1**	-	**100**	3
12	P05155	Plasma protease C1 inhibitor	-	-	-	**2,4**	**52,5**	**11,7**	3
13	P01008	Antithrombin-III	1,0	1,7	**6,8**	1,2	**45,6**	**3,9**	3
14	P00734	Prothrombin	1,6	1,8	**6,8**	0,9	**37,0**	**6,0**	3
15	P05546	Heparin cofactor 2	-	1,9	10,0	**2,4**	**16,8**	-	3
16	P04004	Vitronectin	1,4	1,5	**5,1**	0,8	**17,9**	**4,0**	3
17	P05154	Plasma serine protease inhibitor	-	1,5	**4,9**	1,7	**9,1**	**6,7**	3
18	O43866	CD5 antigen-like L	1,0	**9,8**	1,5	**5,5**	**3,5**	1,1	3
19	Q14624	Inter-alpha-trypsin inhibitor heavy chain H4	-	-	**6,3**	1,8	**6,6**	**3,0**	3
20	P02649	Apolipoprotein E	1,3	**4,8**	**2,2**	1,0	**3,8**	1,2	3
21	P01137	Transforming growth factor beta-1	-	2,0	**5,8**	**2,6**	**3,3**	-	3
22	P55056	Apolipoprotein C-IV	**2,2**	1,1	1,8	**2,1**	0,01	**2,0**	3
23	O75636	Ficolin-3	-	-	**100**	**100**	-	-	2
24	Q9BXR6	Complement factor H-related protein 5	-	-	**22,3**	-	**75,5**	-	2
25	B4DPQ0	Complement C1r subcomponent	-	-	-	-	**62,5**	**20,0**	2
26	P09871	Complement C1s subcomponent	-	-	-	-	**23,7**	**5,5**	2
27	P02788	Lactotransferrin	-	1,0	1,4	1,3	**7,2**	**7,7**	2
28	P27918	Properdin	-	-	**6,7**	-	**6,8**	-	2
29	A0A0A0MRJ7	Coagulation factor V	0,8	**2,4**	0,6	0,7	**7,9**	1,0	2
30	P01591	Immunoglobulin J chain	-	-	1,9	**2,5**	**8,2**	-	2
31	P02647	Apolipoprotein A-I	1,8	**2,6**	1,3	0,8	**2,7**	1,6	2
32	Q13201	Multimerin-1	1,9	1,1	**2,7**	**2,3**	0,2	1,6	2
33	P02675	Fibrinogen beta chain	-	1,4	**2,9**	**4,9**	0,1	-	2
34	H0YAC1	Plasma kallikrein (Fragment)	-	**2,3**	0,7	1,0	**3,6**	-	2
35	Q9Y2J8	Protein-arginine deiminase type-2	-	**100**	-	-	-	-	1
36	O00187	Mannan-binding lectin serine protease 2	-	-	-	**100**	-	-	1
37	P20851	C4b-binding protein beta chain	-	-	-	-	**100**	-	1
38	A0A075B6I9	Immunoglobulin lambda variable 7-46	-	-	-	**92,9**	-	-	1
39	Q15485	Ficolin-2	-	-	-	**51,7**	-	-	1
40	P48740	Mannan-binding lectin serine protease 1	-	-	-	**26,1**	-	-	1
41	B1AHL2	Fibulin-1 GN=FBLN1	-	-	-	-	**20,0**	-	1
42	P0C0L4	Complement C4-A	-	-	-	-	**19,6**	-	1
43	P01767	Immunoglobulin heavy variable 3-53	-	-	-	-	**19,1**	-	1
44	P02743	Serum amyloid P-component	-	-	-	-	**16,2**	-	1
45	P01876	Immunoglobulin heavy constant alpha 1	1,5	1,4	1,2	1,1	**4,7**	1,1	1
46	Q14766	Latent-transforming GF beta-binding protein 1	-	1,5	**6,9**	1,4	1,0	-	1
47	P02776	Platelet factor 4	**4,8**	0,9	1,4	1,2	0,1	1,0	1
48	P06312	Immunoglobulin kappa variable 4-1	1,4	1,0	1,3	1,2	1,8	**2,7**	1
49	P04196	Histidine-rich glycoprotein	-	**8,3**	-	1,2	-	-	1
50	P02654	Apolipoprotein C-I	1,5	**2,1**	0,6	1,5	0,8	1,5	1
51	P01042	Kininogen-1	0,8	**2,5**	0,8	1,1	0,6	1,1	1
52	P02751	Fibronectin	0,6	1,3	1,1	**2,7**	0,3	0,7	1
53	P02671	Fibrinogen alpha chain	0,5	1,1	1,1	**2,9**	0,4	-	1
54	P06727	Apolipoprotein A-IV	-	**3,7**	0,8	-	1,1	-	1
55	P62805	Histone H4	**5,4**	-	-	0,2	-	-	1
56	P0DOY3	Immunoglobulin lambda constant 3	-	1,1	1,4	**3,0**	-	-	1
57	P01031	Complement C5	-	-	-	**2,8**	1,9	-	1
58	P02749	Beta-2-glycoprotein 1	-	**4,4**	-	-	-	-	1
59	D6RF35	Vitamin D-binding protein	-	**2,3**	0,3	-	-	-	1
60	P18428	Lipopolysaccharide-binding protein	-	**2,5**	-	-	-	-	1

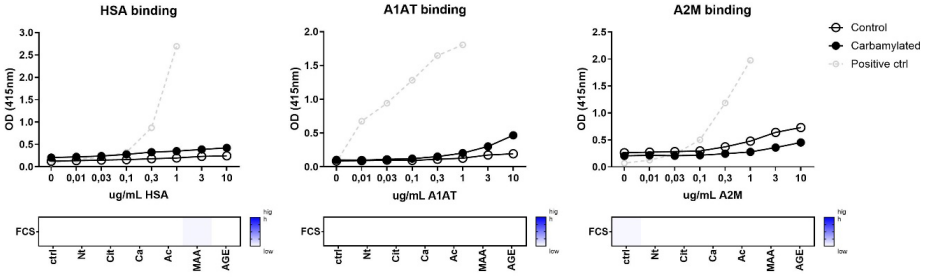
Ratio: Abundance ratio: (Grouped Abundances PTM)/(Grouped Abundances ctrl)

Complement factor

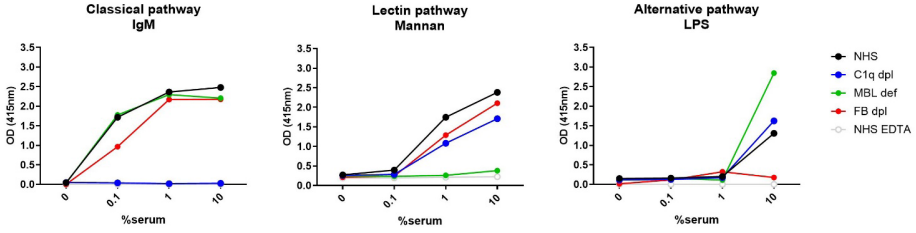
Immunoglobulin fragment

- : no data

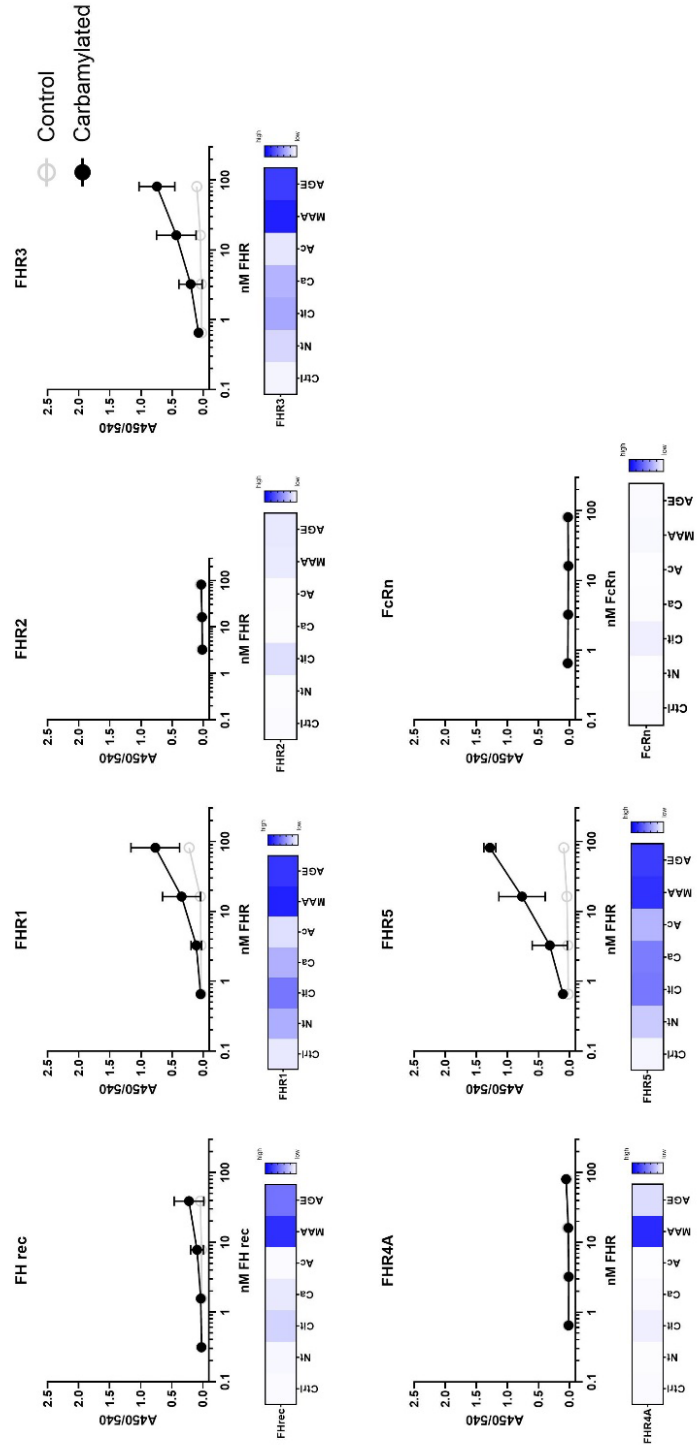
Abbreviations: AGE, advanced glycation end-product; Ac, acetylated protein; Ca, carbamylated protein; Cit, citrullinated protein; MAA, malondialdehyde-acetaldehyde adduct; Nt, nitrated protein; PTM, post-translational modification.



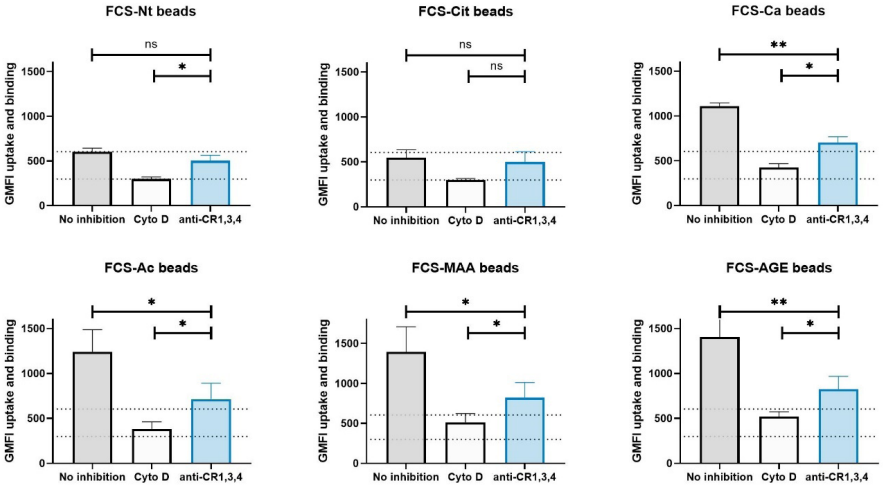
Supplementary Figure 2: Abundantly present serum proteins (HSA, A1AT and A2M) do not bind PTM-modified proteins. Direct binding of HSA, A1AT and A2M to all six PTM-modified proteins was assessed using plate-bound assays. Plates coated with either non-modified FCS (black open symbols) or PTM-FCS (black closed symbols) were incubated with serial dilutions of the purified proteins HSA, A1AT and A2M (left to right). Additionally, plates were coated with each protein (HSA, A1AT or A2M) as a positive control (light grey). Data are representative of three independent experiments. *Abbreviations: A1AT, alpha-1 antitrypsin; A2M, alpha-2-macroglobulin; AGE, advanced glycation end-product; Ac, acetylated protein; Ca, carbamylated protein; Cit, citrullinated protein; HSA, human serum albumin; MAA, malondialdehyde-acetaldehyde adduct; Nt, nitrated protein; PTM, post-translational modification.*



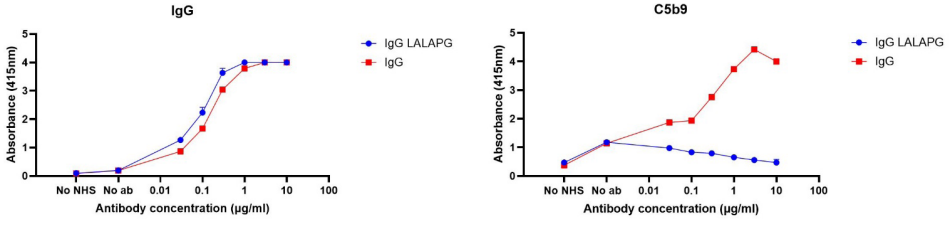
Supplementary Figure 3: Controls for complement activation pathways. Complement activation pathways (from left to right: CP, LP and AP) were activated on IgM, mannan or LPS, respectively, coated ELISA plates. Coated wells were incubated with NHS, C1q-depleted serum, MBL-deficient or Factor B-depleted serum in the presence of RPMI, RPMI/10mM EGTA/5mM MgCl2 or RPMI/10mM EDTA. C4 (CP and LP) and C3 (AP) were analyzed. Data are representative of three independent experiments. *Abbreviations: FB dpl, Factor B-depleted; MBL def, mannan-binding lectin-deficient; NHS, normal human serum.*



Supplementary Figure 4: Factor H and FHR proteins bind to specific PTM-modified proteins. Binding of purified Factor H and FHR-family (FHR-1, -2, -3, -4A, -5) to the PTM-modified proteins was assessed. Graphs are representative of two independent experiments. *Abbreviations: AGE, advanced glycation end-product; Ac, acetylated protein; Ca, carbamylated protein; Cit, citrullinated protein; MAA, malondialdehyde-acetaldehyde adduct; Nt, nitrated protein; PTM, post-translational modification.*



Supplementary Figure 5: Complement receptor blocking experiments. PTM-coupled beads were subjected to differentiated THP-1 cells in the presence of CR blocking antibodies. After incubation, THP-1 cells were detached and analyzed by flowcytometry. Data are representative of three independent experiments. *Abbreviations: AGE, advanced glycation end-product; Ac, acetylated protein; Ca, carbamylated protein; Cit, citrullinated protein; CR, complement receptor; Cyto D, cytochalasin D; FCS, fetal calf serum. MAA, malondialdehyde-acetaldehyde adduct; Nt, nitrated protein;*



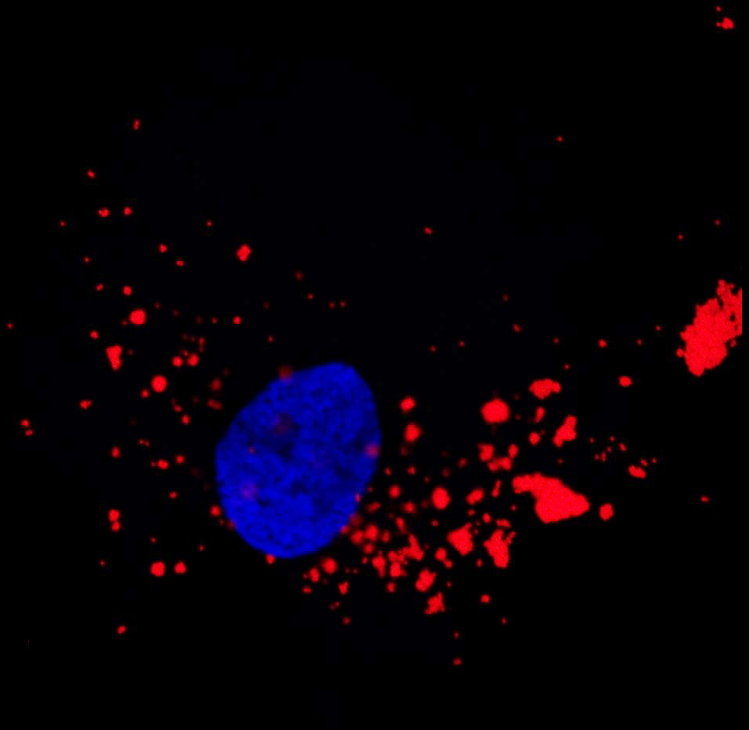
Supplementary Figure 6: Complement activation in the presence or absence of anti-CarP IgG antibodies with or without LALAPG mutation. Binding of IgG (WT or LALAPG) and subsequently complement activation was assessed on carbamylated protein using 3% NHS.

Supplementary Table 3: P-values for the global test of all seven complement SNPs against the individual anti-PTM antibodies.

Anti-PTM	***P-value*** **models improvement**	***McFadden*** **Pseudo-R-squared**
Anti-Nt	*0.002*	0.17
Anti-Cit	*2.5e-34**	0.20
Anti-Ca	*5.9e-32**	0.19
Anti-Ac	*1.4e-23**	0.17
Anti-MAA	*5.7e-35**	0.20
Anti-AGE	*4.0E-34**	0.20

* significant association

Abbreviations: AGE, advanced glycation end-product; Ac, acetylated protein; Ca, carbamylated protein; Cit, citrullinated protein; MAA, malondialdehyde-acetaldehyde adduct; Nt, nitrated protein; PTM, post-translational modification; SNP, single nucleotide polymorphism.



6

General discussion

Autoimmune diseases are a diverse group of conditions in which the immune system mistakenly damages healthy cells, tissues, or organs. Many autoimmune diseases are therefore characterized by the presence of autoantibodies. Some of these antibodies are disease specific and some are observed in several conditions. One particular example are antibodies that target post-translational modifications (PTMs), which are the main focus of this thesis. Antibodies targeting PTMs are observed in several major autoimmune diseases such as rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE). Antibodies targeting the modification citrullination (ACPA) in RA are disease specific and are therefore included in the 2010 rheumatoid arthritis classification criteria (1). Other anti-PTM antibodies, such as antibodies targeting carbamylated protein (anti-CarP) are associated with in joint damage in RA (2), but also in arthralgia and SLE joint damage occurs (3, 4). In these patients, anti-CarP antibodies may serve as a promising marker to predict joint damage. In this thesis we focused on anti-PTM antibodies directed against six different PTMs namely, nitration (Nt), citrullination (Cit), carbamylation (Ca), acetylation (Ac), malondialdehyde acetaldehyde adduct (MAA) and advanced glycation end-product (AGE). We selected these 6 PTMs as they are relatively easy to produce, were either structurally different, occurred on the same or on different amino acid residues and because these 6 PTMs are differentially associated with several types of disease. The relevance of these six PTMs was investigated by analyzing anti-PTM antibody reactivities and performing association studies in patients with RA (**Chapter 2**), SLE (**Chapter 3**) and autoimmune liver disease (AILD) (**Chapter 4**). In an attempt to understand how tolerance is broken towards PTM proteins we studied the interaction between complement and PTM-modified proteins (**Chapter 5**). With these studies we provide insight into the presence of anti-PTM antibodies and mechanisms by which PTM and anti-PTM antibodies are contributing to (chronic) inflammation of autoimmune diseases.

The clinical relevance of anti-PTM antibodies in RA

Autoantibodies targeting PTMs are frequently present in patients with RA. More specifically, anti-PTM antibodies that target citrullinated (ACPA) and carbamylated (anti-CarP) proteins are widely used as diagnostic and prognostic markers (2, 5). However, despite the high diagnostic value of ACPA and rheumatoid factor (RF) a substantial group of RA patients is reported to be seronegative (6). Therefore, there is a need for novel serological markers to further improve early diagnosis of RA also in such seronegative patients. Many research groups are focusing on new targets of autoantibody reactivity. It is becoming apparent that next to ACPA and anti-CarP also other PTMs are targeted in these patients (7-10). In this thesis we investigated the six different anti-PTM antibody reactivities (anti-Nt, -Cit, -Ca, -Ac, -MAA and -AGE).. These anti-PTM antibody reactivities were assessed in a well-established cohort of RA patients, namely the Leiden Early

Arthritic Cohort. In **Chapter 2** we focus on anti-PTM antibodies targeting MAA- and AGE-modified proteins and their clinical relevance in RA (11). RA patients were significantly more frequently positive for the presence of anti-MAA and anti-AGE antibodies. On top of that, presence of anti-MAA and anti-AGE identified a subgroup of RA patients that was seronegative for ACPA, anti-CarP and rheumatoid factor (RF). These findings underpin that patients with RA display a heterogeneous array of autoantibodies, each associated with more severe disease and therefore possibly contributing to disease pathogenesis. Noteworthy is that a subgroup of RA patients remains seronegative. Serological markers for these seronegative RA patients may be identified in the future, further identifying subgroups. However, it is possible that a subgroup of RA patients remains seronegative, as the association with HLA is present especially in the currently seropositive patients and not with the seronegative patients. Recently, the immune response to PTM-modified proteins in RA was reviewed in detail (12). Here they focused on cross-reactive capabilities of anti-PTM antibodies and suggest that citrullinated proteins are probably the dominant antigen in the anti-PTM response in patients with RA (12). Intriguingly, PTM-directed B cell responses in RA does not seem to transition into a resting state, but remain consistently activated (13). When aiming for sustained clinical remission, this may imply that disease-associated immune response most likely remain active despite treatment-induced clinical remission. This provides an immunological rationale for the observed disease flares on drug tapering withdrawal.

The clinical relevance of anti-PTM antibodies in other autoimmune diseases

Next to RA, many other autoimmune diseases display a variety of autoantibodies targeting (modified) self-proteins. Additionally, PTMs and antibodies targeting PTMs have been identified in several non-autoimmune diseases such as age-related macular degeneration, cardiovascular disease and diabetes type 2 (14-16). It is therefore likely that anti-PTM antibodies, or combinations of several anti-PTM antibodies, are present in many autoimmune diseases. Next to RA, we therefore investigated the set of six different anti-PTM antibody reactivities in cohorts with SLE patients and patients with AILD. In **Chapter 3**, we had the unique opportunity to investigate SLE patients with neuropsychiatric symptoms since the Leiden University Medical Center is a tertiary referral center for these patients (17). No serological markers are currently available to identify patients with neuropsychiatric SLE (NPSLE), therefore such research was warranted. We observed that patients with SLE more frequently had anti-PTM antibodies targeting CarP-, AGE- and MAA-modified proteins. On top of that, anti-CarP and anti-MAA were more frequently present in patients with NPSLE. Additionally, anti-PTM antibodies negatively correlated with brain volumes, an objective marker of central nervous system

involvement. In **Chapter 4**, patients with AILD were assessed for the presence of the six anti-PTM antibodies (18). Anti-PTM antibodies (anti-CarP, anti-Ac, anti-MAA, anti-AGE) were more often detected in sera from AILD patients compared to healthy controls. The presence of anti-MAA, anti-AGE and anti-CarP antibodies correlated with the presence of autoimmune hepatitis (AIH) within this cohort. On top of that, in AIH, harboring at least three anti-PTM antibody responses is positively associated with complete biochemical response. In a reply on a commentary written on **Chapter 4** (included in Chapter 4) we further explained the methods used to measure anti-PTM antibodies. The experimental set-up is based on the anti-CarP assay (2, 19). This method includes, and corrects for, antibody responses towards the backbone of non-modified FCS we are therefore certain to measure specifically anti-PTM antibodies in serum samples.

Overall, we have shown that measuring anti-PTM antibodies in patients with autoimmune diseases may have diagnostic and prognostic value. In addition, the presence of anti-PTM antibodies may provide information on the response to therapy. Further research is needed to establish sensitivity and specificity. We observed that similar PTMs were targeted by anti-PTM antibodies throughout different autoimmune diseases. Noteworthy is that, many biomarkers have already been suggested in several autoimmune diseases, but for many of these suggested biomarkers the role is yet to be determined and clinical value limited (20, 21). It is therefore unlikely that within the whole population one of these anti-PTM antibodies on its own distinguish (at risk) patients from healthy individuals. We, however, have observed that within a specific cohort anti-PTM antibodies, or combinations of anti-PTM antibodies, may determine a relevant subgroup of patients. Many autoimmune diseases namely consist of a heterogenous group of patients. Determination of subgroups may provide better insight in disease progression or treatment response.

During our studies we observed that anti-AGE and anti-MAA correlate with inflammatory markers. Previous studies have shown that the PTM AGE binds to the receptor RAGE amplifying immune and inflammatory responses (22). Both AGE- and MAA-modified proteins have previously shown to be immunogenic (23, 24). The role of MAA-modified proteins as mediators of inflammation in cardiovascular diseases have been reviewed in detail elsewhere (25). These studies, together with our observations, suggest that PTMs themselves may be a result of inflammation and oxidative stress but also play a role in perpetuation of inflammation. This vicious circle, may under the ''right'' circumstances, lead to chronic inflammation and autoimmunity. In addition, **Chapter 5** we show that the PTMs Ca, Ac, MAA and AGE have the capacity to trigger complement activation. Collectively indicating that both the PTMs and the anti-PTM antibodies have the capacity to stimulate (chronic) inflammation.

The observation that next to RA also other diseases are characterized by anti-PTM antibodies, provides important insight into the pathophysiological processes that take place in these diseases. However, it does not necessarily indicate that the detection of anti-PTM antibodies in RA for diagnostic or prognostic purposes would be less valuable. The assays set up to determine ACPA, often the CCP2, CCP3 or CCP4 based assays are still very relevant. Also the assay to detect anti-CarP antibodies, can still be employed for their diagnostic and prognostic purposes in RA. These assays have been established employing sets of relevant disease controls and cut-offs for positivity have been determined for optimal use in the clinical setting. However, the data do importantly indicate that in several autoimmune conditions, known to involve autoantibodies, anti-PTM antibodies can readily be detected in a large part of the patients. Importantly, the anti-PTM antibodies are not present in all the patients as some patients with RA, SLE or AIH harbor none of the anti-PTM antibodies investigated. Combining the data from the three cohorts, as well as studying the cohorts separately, we observed that there are several patterns of positivity. Patients can be positive for all six, or for none, but all other combinations were observed as well. The highest percentage positivity was observed for anti-MAA in both SLE and AILD. In RA, anti-MAA was found in 46,7% of all patients and was therefore the second most common anti-PTM antibody in RA, next to anti-Cit (49,3%). When all data of all autoimmune diseases were pooled, we observed that antibodies targeting Cit, CarP, Ac, MAA and AGE mostly co-occurred in autoimmune disease followed by the combination of anti-CarP and anti-MAA antibodies. Taken together, our observations indicate that despite the fact that all these patients are characterized by major inflammatory events, that only a part of the patients will actually break tolerance and produce such autoantibodies.

Several reports have looked into the cross-reactive nature of anti-PTM antibodies and while there is certainly a degree of cross-reactivity present for some of the anti-PTM antibodies it is also obvious that PTM specific, non-cross reactive antibodies are present. For example evidenced by patients that are single positive for one anti-PTM. The degree of cross-reactivity may appear different when analyzing anti-PTM antibodies on PTM containing CCP peptides as compared to PTM containing proteins. Work that is currently in progress at the Department of Rheumatology is highlighting that apparent cross-reactivity is generally higher in CCP based assays as compared to protein based assays. Conceivably, this is because of the structure of the CCP peptide backbone that was optimized to capture as many RA patients as possible compared to controls. Future work, based on human monoclonal anti-PTM antibodies will have to disclose the cross-reactive nature also including the full set of six PTM reactivities.

The possible role of complement in the induction of anti-PTM antibodies

PTMs and antibodies targeting these PTMs are the main subjects of this thesis. It has become apparent that PTMs and anti-PTMs are frequently present, in both health and disease (26). In **Chapter 5** we aimed to shed light on how the human body deals with PTM-modified proteins and identified triggers that may lead to autoimmunity (Figure 1) (27). Specifically we asked the question if serum proteins could bind to PTMs to modify their clearance and immune stimulating capacity. We observed that, from human serum, complement components bind directly to certain PTM-modified proteins (Ca, Ac, MAA and AGE). Notably, seven out of the top ten hits observed using mass spectrometry were complement components, suggesting an important role of complement binding to PTM-modified proteins. The complement system is perceived as a central constituent of innate immunity, defending the host against pathogens, coordinating various events during inflammation, and bridging innate and adaptive immune responses.

We have established that complement not only binds directly to PTMs, but also that complement binding to PTM-modified proteins stimulates the uptake of PTM-modified proteins by macrophages. Next to macrophages, leukocytes bind complement opsonized PTM-modified proteins more compared to non-opsonized PTM-modified proteins.

Interestingly, most people have PTM-modified proteins at several locations in their body and have a functional complement system, but not all people develop an anti-PTM antibody response. We therefore set out to analyze genetic variants of complement single nucleotide polymorphisms (SNPs), associated with a more active complement profile, so called complotype, and correlated these with anti-PTM reactivities. We observed that patients with a more complement activating profile, were associated with anti-PTM antibodies. We observed that minor allele factor H SNPs, previously shown to increase complement activity, associated with presence of anti-Ac, -MAA and -AGE. The minor allele of rs2230199 (C3) associated with anti-Ca. Interestingly, the minor allele of C1qA rs292001 associated with anti-Cit, contributing to the findings that C1q SNPs are described in the context of RA. However, the PTM Cit itself did not show complement activating properties, suggesting a different mechanism of antibody induction for this particular PTM.

We hypothesized that the complement system will be activated on PTM-modified proteins regardless of the complotype and that C1q or ficolins will bind to specific PTM-modified proteins. Following binding of C1q or ficolins the complement cascade will be further activated resulting in amongst others deposition of C3 fragments (C3b, C3d, iC3b) serving as opsonins for cells to be detected through complement receptors. The complotype will

determine how strong this reaction will be. Next to opsonization, complement activation will also trigger the release of the anaphylatoxins C3a and C5a stimulating immune cell influx and immune cell activation. Ultimately, complement activation can result in the formation of the membrane attack complex, leading to cellular activation or even cellular death by apoptosis or direct lysis. Therefore we hypothesized that, depending on the complotype, people will clear the PTM-modified proteins, or alternatively upon highly active complement sustain inflammation and might mount an antibody response. Nonetheless, we think that it is an interplay of genetic and environmental factors that will eventually lead to disease in certain people.

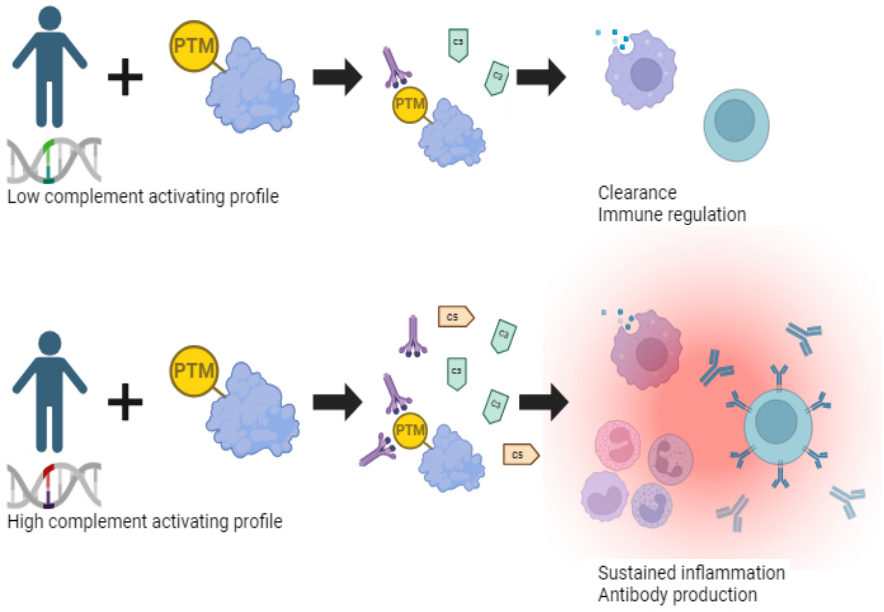


Figure 1: Key hypothesis. The so-called complotype of individuals results in a low (upper part) or high (lower part) complement activating profile. Depending on the complotype, complement opsonization of PTM-modified proteins could lead to clearance and immune regulation or (sustained) inflammation and autoantibody production, respectively.

To study the impact of complement opsonization of PTM-modified proteins on the triggering and activation of anti-PTM specific B-cells it would have been great to stimulate patient derived anti-PTM reactive B-cells with PTM-modified protein or with complement opsonized PTM-modified protein. However, only a few B-cells in these patients will be PTM specific. Since the amount of PTM-specific B-cells is limited, no experiments could be performed to investigate the induction of antibodies by our six PTM-modified proteins in the presence or absence of complement. However, several groups have studied the impact of complement

opsonization of proteins in their capacity to stimulate B-cell responses. The concept is that complement receptor 2, expressed on B-cells, binds to C3d lowering the B-cells activation threshold. Kovacs *et al.* has shown that, in mice, tolerance of B-cells is decreased when the B-cell receptor was activated non-specifically the presence of complement factor C3d, using anti-IgG/A/M F(ab')2 fragments coupled to C3d (28). In the near future this should be investigated in human in order to strengthen the hypothesis that a breach in tolerance could be induced against complement opsonized PTM-modified proteins by PTM specific B-cells.

Others have investigated anti-PTM reactivities in a different approach by assessing cross-reactivity (29, 30). They hypothesized that only one PTM may lead to the induction of several anti-PTM reactivities. In the context of RA, they have created B-cells with citrullinated protein-reactive IgG B-cell receptors. Indeed, they observed that these B-cells showed activation on stimulation with various types of PTM-antigens (Cit, Ca and Ac) (29). The B-cell model used in this study were Ramos cells. Unfortunately, the cell line Ramos lacks expression complement receptor 2. Additionally, Cit was one of the PTMs that did not show complement activation in our studies. Therefore, this model could not be used to investigate whether complement lowers the B-cell activating threshold in PTM-specific B-cells.

The same group recently published findings on acetylated gut-resident bacteria induce cross-reactive anti-modified protein antibodies in mice (31). It is postulated that gut microbiome is involved in the breach in tolerance to modified self-proteins. Additionally, the genetic-environmental interaction has been found for HLA-SE alleles and smoking (32, 33). Smoking namely leads to increased citrullination in pulmonary tissue which could be subsequently presented in HLA-SE alleles. This hypothesis was backed up by data including presence of citrullinated antigens, ACPA-positive B-cells and ACPA in bronchoalveolar lavage samples before and after onset of RA (34-36).

Combining data from these studies with our observations it is theoretically possible that, depending on environmental and genetic factors, strong immune reactions are induced against specific PTM-modified proteins. It is however important, based on the phases of disease evolution, to distinguish risk factors that contribute to the initial development of autoantibodies, from those that affect later stages such as symptom onset (12). Our findings showed that certain PTMs could directly activate complement which led to increased binding and uptake by leukocytes and macrophages. Under the ''right'' circumstances this could lead to the breach in tolerance of PTM-specific B-cells that will produce anti-PTM antibodies with cross-reactive potential targeting several different PTM-modified proteins. These anti-PTM reactivities will be detectable in patients. We would however, like to note that not all patients display similar cross-reactivity. We

namely observed that patients can be positive for multiple anti-PTM reactivities but clearly not all for the same combinations.

The findings that PTM-modified proteins activate complement directly may have important implications for the understanding of smoldering, chronic inflammation. PTM-modified proteins present in joints, kidneys or vessels may be a chronic stimulus for low level complement activation leading to a vicious circle of enhanced PTM formation, complement activation, triggering of inflammation, which in turn again leads to PTM formation.

Notably, not all PTM-modified protein induce anti-PTM antibody reactivities in all patients. Anti-Nt antibodies, namely, were only observed in a few individuals. These data suggests that anti-Nt antibodies do not play a substantial role in the autoimmune diseases investigated in this thesis. On top of that, both Nt and Cit did not activate complement suggesting a different type of mechanism that lead to the induction of anti-PTM antibodies. Besides, in this thesis all lysine modifications induced complement activation. However, we would like to point out that not every lysine modification does this similarly and to the same extend. Ca-, MAA- and AGE-modified proteins activate the classical pathway and Ac-modified proteins activate the lectin pathway. Additionally, MAA-modified proteins bind C1q already at very low concentrations exerting great complement activating potential. For future studies it is important to keep in mind that not all PTM-modified proteins react similarly and different mechanism leading to disease onset, but also at a later stage considering disease symptoms, might be involved for each PTM-antigen.

Conclusion and future perspective

At this point, we have established that anti-PTM antibodies are present in a variety of autoimmune diseases including RA (**Chapter 2**), SLE (**Chapter 3**) and AILD (**Chapter 4**). In the cohorts tested we observed that anti-PTM antibodies and combinations of anti-PTM antibodies distinguish subgroups, correlate with specific manifestations or correlate with a treatment outcome. Since many autoimmune diseases are characterized by autoantibodies, including antibodies targeting PTMs, it is challenging to pin-point specific PTMs associating with specific disease. It is more likely that certain anti-PTM antibodies or combinations of anti-PTM antibodies are present in specific subgroups and associate with disease outcome. Prospective studies comprising different autoimmune diseases could shed light on diagnostic and prognostic value of these anti-PTM antibodies.

We have established that the complement system is activated by specific PTMs (**Chapter**

5). Additionally, complement-opsonized PTM-modified proteins show an increase in cellular uptake. This data is based on plate bound assays using complement active human serum and human cells or cell lines. To further elucidate on the relevance of the complement system in the induction of anti-PTM antibodies, mouse studies should be performed. In such studies mice deficient for specific complement components should be used to identify the role of complement in the breach in tolerance. For example, mice lacking complement component C1q, important for the classical pathway, should be exposed to Ca-, AGE- or MAA-modified protein and subsequently anti-PTM reactivities measured. When antibody reactivity is compared to antibody reactivity in mice with a normal complement system, this would give insight in the contribution of complement activation to the induction of anti-PTM antibodies.

Results observed in **Chapter 5**, however, are convincing and could be a great basis for further immunotherapeutic purposes. In RA, but also in other autoimmune diseases, broad immunosuppression is current treatment and sustained clinical remission the golden standard (37-39). The possible role of complement in the induction of anti-PTM reactivity provides opportunities to modify complement activation and therefore lower the chance of autoimmunity. However, therapeutic targeting of the complement system is easier said than done (40). Suppressing complement systemically for instance is undesired as the complement system is needed to fight infection, heal injury and kill bacteria and viruses (41). Data provided in this manuscript provides ground work for new therapeutic interventions. Proteins modified with Ca, Ac, MAA or AGE activate the complement system directly and are shown to occur in several autoimmune diseases. Direct complement activation implies that complement inhibition may serve as therapeutic intervention in these patients. Using prolonged and systemic complement inhibition may be disproportional to the chronic complement activation by the PTMs, especially in light of the risk of infections during such therapy. Suppressing complement activation locally, only on PTM-modified proteins, for example using bispecific antibodies could serve this purpose very well (42). This strategy would fit in the proposed interventions aiming for sustained immunological remission leading to sustained (drug-free) remission (12). In the mentioned strategy, bispecific antibodies, are able to bind two different antigens as opposed to normal antibodies only targeting one antigen. In this way, a bispecific antibody was designed that with one arm can bind to the PTM and the other arm to endogenous complement regulator Factor H, bringing complement inhibitors into close proximity to the PTMs regulating complement activation.

Altogether, throughout this thesis, I described a role for immunity against PTMs in a variety of autoimmune diseases. Further exploration of triggers towards PTMs and anti-PTM antibody induction is warranted to establish treatment options for patients aiming for sustained immunological remission.

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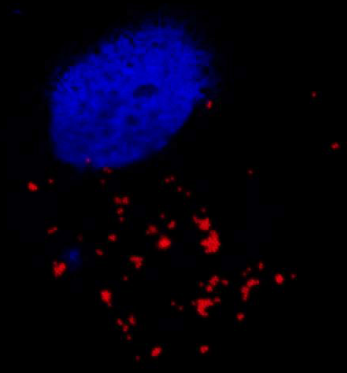

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Appendices

English summary
Nederlandse samenvatting
List of publications
Curriculum vitae
Dankwoord

English summary

Autoimmune diseases are conditions in which the immune system mistakenly attacks its own healthy cells, tissues, or organs. Such diseases are often characterized by chronic inflammation. Conditions such as rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), and autoimmune liver disease (AILD) exemplify the diversity and complexity of autoimmune disorders. These heterogenous diseases are characterized by the presence of a wide array of autoantibodies. Patients with RA harbor antibodies against proteins that have undergone a post-translational modification (PTM). Antibodies targeting the modification citrullination (anti-citrullinated protein antibodies; ACPA) are included in the 2010 rheumatoid arthritis classification criteria. Antibodies targeting the modification carbamylation (anti-carbamylated protein; anti-CarP) are reported to be associated with radiological progression, especially in ACPA negative individuals. Other autoimmune diseases such as SLE and AILD are hallmarked by the presence of amongst others anti-nuclear antibodies. These antibodies have shown to be pathogenic and associate with severe clinical complication in SLE and aid in the diagnosis of AILD.

For all three autoimmune diseases (RA, SLE and AILD) it is important to note that within each disease, many subgroups of patients exist. These subgroups may be characterized by specific autoantibodies and vary in speed of progression and/or display specific disease manifestations. In addition, patients in such subgroups can also respond differently to specific therapies. It is therefore warranted to investigate serological markers that could identify relevant subgroups within these autoimmune diseases to provide better treatment for patients. Throughout this thesis we focused on six different PTMs namely, nitration (Nt), citrullination (Cit), carbamylation (Ca), acetylation (Ac), malondialdehyde acetaldehyde adduct (MAA) and advanced glycation end-product (AGE) in the context of autoimmunity. In **Chapter 2**, we investigated anti-PTM antibodies targeting MAA- and AGE-modified proteins in a well-established RA cohort namely the Leiden Early Arthritic Cohort which contained information on ACPA as measured using the anti-CCP2 assay and anti-CarP. We observed that the presence of anti-MAA and anti-AGE antibodies identified a subgroup of RA patients that was seronegative for anti-CCP2, anti-CarP and Rheumatoid Factor (RF). Since HLA-DRB1*03 is associated with seronegative RA and anti-CarP antibodies in this disease subset, we sought to investigate the association of HLA-DRB1*03 with anti-MAA and anti-AGE in this study. We observed that anti-MAA was associated with HLA-DRB1*03 in the anti-CCP2 negative stratum, independent of anti-CarP. Anti-AGE followed the same trend but did not reach significance. We also observed that anti-MAA is associated with markers of inflammation in early arthritis in both RA and non-RA arthritis patients. Previous studies showed that anti-CarP was found to be associated with increased bone erosion in anti-CCP2 negative RA patients. We therefore investigated associations with radiographic joint damage in these patients. We observed

that in patients with ACPA-negative RA, anti-AGE is associated with radiological progression independent of anti-CarP, suggesting that this anti-PTM antibody could discriminate a different subgroup. Finally, correlation analyses were performed to investigate associations between anti-PTM antibody positivity and sustained drug free remission. We did not find any association of anti-MAA nor anti-AGE with sustained drug free remission, also not after stratifying for ACPA status. In conclusion, anti-MAA and anti-AGE antibodies are both prevalent in patients with RA, and other inflammatory rheumatic conditions, and although not specific for RA they each correlate with specific parameters. Anti-MAA associates with HLA-DRB1*03 in ACPA-negative (RA) patients independent of anti-CarP and associates with inflammation. Anti-AGE associates with HLA-DRB1*03 in patients with ACPA-negative RA and is associated with a worse radiological progression especially in patients with ACPA-negative and anti-CarP-negative RA. With this study, we have characterized a seropositive subgroup (anti-MAA and anti-AGE positive) within the heterogeneous group of patients with RA that have thus far been considered seronegative (ACPA-, anti-CarP and RF-negative).

Now that we have established that in RA, next to antibody responses against proteins modified by citrulline or homocitrulline by citrullination and carbamylation, respectively, also proteins modified by other PTMs could be targeted by autoantibodies, we further explored different autoimmune diseases. Autoimmune diseases such as SLE and AILD display a variety of autoantibodies targeting self-proteins. Therefore it is plausible that these patients harbor antibodies that bind specifically to PTM-modified proteins. In **Chapter 3** we investigated anti-PTM antibody reactivities against all six different PTMs (Nt, Cit, Ca, Ac, MAA and AGE), in SLE patients. SLE patients are known for their global loss of self-tolerance. It is therefore plausible anti-PTM antibodies will be present. Since the Leiden University Medical Centre is a tertiary referral center for SLE patients with neuropsychiatric manifestations (NPSLE), we had the unique opportunity to investigate this specific manifestation. Identifying patients with NPSLE in the complex disease course of SLE is challenging and it would be highly desirable to have serological biomarkers to support the diagnosis. In the whole SLE cohort we observed that anti-MAA, anti-AGE and anti-CarP levels and positivity were significantly increased compared to healthy controls. On top of that, levels of anti-MAA and anti-AGE negatively correlated with complement C3 and C4 levels and positively with erythrocyte sedimentation rate (ESR) pointing towards systemic inflammation. Interestingly, we observed that anti-MAA and anti-CarP were more common in patients with major NPSLE than in patients with other SLE organ manifestations. On top of that, anti-MAA and anti-AGE correlated with white matter volume (WMV) and total brain volume (TBV), and anti-CarP with white matter hyperintensity volume. Since WMV, TBV and white matter hyperintensity volume are objective markers for the involvement of the central nervous system in SLE, they provide objective evidence next to the multidisciplinary assessment to diagnose these patients.

All other NPSLE activity markers are more subjective, such as mood for example. Taken together, anti-PTM antibodies could aid in the diagnosis and/or subgrouping of (NP)SLE. Future studies should further establish the potential role of anti-PTM antibodies in (NP) SLE.

In **Chapter 4** we investigated anti-PTM responses against our set of six PTM-modified proteins in patients with AILD. AILD is a heterogenous group of both cholestatic and hepatocellular diseases, consisting of primary biliary cholangitis (PBC), primary sclerosing cholangitis (PSC), autoimmune hepatitis (AIH) and overlap variants. PBC, PSC and AIH are often considered (auto)immune mediated diseases and AIH and PBC are characterized by the presence of autoantibodies and elevated total immunoglobulins. Additionally, autoantibodies are nowadays implemented in the diagnostic work-up but are not disease specific. We observed increased anti-PTM antibodies targeting Ca-, Ac-, MAA- and AGE-modified proteins in patients with AILD compared to healthy controls. Next to single positivity also positivity for more than one anti-PTM antibodies was analyzed. We observed that patients with AILD more frequently harbored at least one type of anti-PTM antibody compared to non-AILD and HCs. Since AILD consists of three subgroups, anti-PTM responses were assessed in these groups. Patients with AIH were significantly more often positive for anti-MAA, anti-AGE, anti-CarP and anti-Cit compared to patients with cholestatic liver disease (PBC and PSC). For patients with AIH, presence of anti-MAA and anti-AGE correlated positively with complete biochemical remission (CBR) at 3 months. In addition, anti-MAA, anti-AGE, and anti-CarP positively associated with achieving CBR at 12 months. Based on the discovery of multiple anti-PTM antibody positivity in patients with AIH, we attempted to discover the clinical relevance of harboring these multiple anti-PTM antibodies. After 3 months treatment, significantly more AIH patients with at least three anti-PTM antibodies had reached CBR. After 12 months of treatment, the difference was still significant. Overall, a trend toward significance for time to CBR (in years) was found in favor of multiple anti-PTM antibody positivity. In conclusion, anti-PTM antibodies are present in patients with AILD. Some patients are positive for multiple anti-PTM antibodies. Having three or more anti-PTM antibody responses is associated with a favorable response to treatment in AIH. A commentary was published based on **Chapter 4** (included in **Chapter 4**) questioning whether we were measuring anti-PTM antibodies or (also) measuring polyreactive IgG. In a reply on the commentary (included in **Chapter 4**) we clarified our methods explaining that in our experimental set-up we namely include mock-modified FCS to correct for reactivities towards FCS, reassuring that we are measuring IgG antibodies that specifically target PTM modified proteins.

Interestingly, many patients with autoimmune disease, including RA, SLE and AIH, benefit for B-cell targeted therapy suggesting a (pathogenic) role for B-cells and/or antibodies. However, what triggers these B-cells to become autoreactive is currently under debate.

In **Chapter 5** we shed light on PTM-modified proteins that could trigger the complement system which in turn may lead to clearance, chronic inflammation and/or autoimmunity.

In this context we investigated our set of six PTMs (Nt, Cit, Ca, Ac, MAA and AGE). Proteins (fetal calf serum) modified with these PTMs were previously used to establish IgG antibody reactivities in the RA, SLE and AILD cohorts in **Chapters 2, 3 and 4**. We have now combined the antibody profiles of RA, SLE and AILD against all six PTMs in **Chapter 5** in order to investigate combinations of anti-PTM antibodies occurring in autoimmunity. Interestingly, we observed that antibodies targeting Cit-, Ca-, Ac-, MAA- and AGE-modified proteins mostly co-occurred. To study how PTM-modified proteins could be involved in these chronic autoimmune diseases we set out to screen for plasma proteins that bind to PTM-modified proteins. Using mass spectrometry we could identify that from human serum it were mostly complement components that were binding specifically to PTM-modified proteins with the PTMs Ca, Ac, MAA and AGE compared to non-modified proteins. In order to verify direct binding and activation of complement to Ca-, Ac-, MAA- and AGE-modified proteins binding assays with purified complement components and complement activation assays were employed. We verified that complement indeed binds directly to the PTM-modified proteins, without the involvement of antibodies. Since the complement system entails three activation pathways (classical, lectin and alternative) we sought to allocate a complement pathway to each PTM-modified protein. We observed that Ca-, MAA- and AGE-modified proteins activate the classical pathway and verified that Ac-modified proteins activate the lectin pathway. The complement system is known for its clearance function but is also known to link innate and adaptive immunity through complement receptors. We therefore set out to investigate cells from whole blood and THP-1 macrophages as a model for more tissue resident cells. For this purpose we coupled PTM-modified proteins to beads using strep-biotin interaction to mimic long lived proteins that are frequently exposed to PTMs. Next, we incubated those beads with complement active serum to opsonize the beads with complement. We observed that leukocytes and THP-1 macrophages showed increased binding and uptake of complement opsonized PTM-modified protein coupled beads compared to non-opsonized PTM-modified protein coupled beads or non-modified protein coupled beads. At last, we had the opportunity to use data on single-nucleotide polymorphisms (SNPs) of complement genes in a set of RA patients in which we also measured anti-PTM reactivities. For this purpose we performed association studies between presence of anti-PTM antibodies and SNPs in complement genes known to be involved the activity of the complement system. We observed that minor allele factor H SNPs, previously shown to increase complement activity, associated with presence of anti-Ac, -MAA and -AGE. The minor allele of rs2230199 (C3) associated with presence of anti-CarP. These data indeed suggest a link between complotype and development of anti-PTM antibody reactivity. With this study we emphasize that complement activation is triggered on several specific

PTMs, but clearly not all PTMs react similarly. In conclusion, PTM-modified proteins can bind and activate complement, which increases inflammation and phagocytosis, and may lead to development of anti-PTM antibodies.

As summarized and discussed in **Chapter 6**, the research described in this thesis shows that PTMs and anti-PTM antibodies associate with disease progression and treatment response. Additionally, detection of anti-PTM antibodies could be utilized to discriminate subgroups in several autoimmune diseases. Further studies should be employed to pinpoint potential diagnostic and prognostic value within each group. It has become apparent that some PTMs and anti-PTMs are involved in (chronic) inflammation, as we have observed associations between anti-PTMs and inflammatory markers throughout different cohorts. Whether these anti-PTMs are causing inflammation or are a result of (chronic) inflammation is to be explored in the future. We however established that complement proteins could directly bind to PTM-modified proteins to enhance clearance but that depending on the complotype this process could lead to (sustained) inflammation and autoantibody production. The research presented therefore urges further investigation of complement in the induction of anti-PTM antibodies *in vivo* by making use of complement deficient mice. Next to this fundamental research also studies into therapeutic opportunities are warranted. Strategies need to be developed to specifically deplete anti-PTM reactive B cells and also strategies to specifically inhibit complement activation driven by PTM. Our team has recently developed bispecific antibodies that with one antibody arm bind to PTM protein and with the other recruit an endogenous complement inhibitor to dampen complement activity on PTMs. This strategy would fit in the proposed interventions aiming for sustained immunological remission leading to sustained (drug-free) remission.

Altogether, throughout this thesis, evidence is provided that immunity against PTMs play a role in a variety of autoimmune diseases. Further exploration of triggers towards PTMs and anti-PTM antibody induction is warranted to establish specific targeted treatment options for patients aiming for sustained immunological remission.

Nederlandse samenvatting

Auto-immuunziekten zijn aandoeningen waarbij het immuunsysteem ten onrechte zijn eigen gezonde cellen, weefsels of organen aanvalt. Dergelijke ziekten worden vaak gekenmerkt door chronische ontstekingen. Aandoeningen zoals reumatoïde artritis (RA), systemische lupus erythematodes (SLE) en auto-immuun leverziekte (AILD) illustreren de diversiteit en complexiteit van auto-immuunziekten. Deze heterogene ziektebeelden worden gekenmerkt door de aanwezigheid van een breed scala aan autoantilichamen. Patiënten met RA hebben antilichamen tegen eiwitten die een post-translationele modificatie (PTM) hebben ondergaan. Antilichamen gericht tegen de modificatie citrullinatie (anti-citrullinated protein antibodies; ACPA) zijn opgenomen in de 2010 reumatoïde artritis classificatie criteria. Antilichamen gericht tegen de modificatie carbamylatie (anti-carbamylated protein; anti-CarP) zijn naar verluidt geassocieerd met radiologische progressie, vooral bij ACPA-negatieve personen. Andere auto-immuunziekten zoals SLE en AILD worden gekenmerkt door de aanwezigheid van onder andere anti-nucleaire antilichamen. Deze antilichamen zijn pathogeen gebleken en geassocieerd met ernstige klinische complicaties bij SLE en helpen bij de diagnose van AILD.

Het is belangrijk om op te merken dat binnen alle drie de auto-immuunziekten (RA, SLE en AILD) veel subgroepen aan patiënten bestaan. Deze subgroepen worden gekarakteriseerd door aanwezigheid van specifieke autoantilichamen, verschillen in de snelheid van progressie en/of door de aanwezigheid van specifieke ziekte manifestaties. Daarnaast kunnen patiënten in dergelijke subgroepen verschillend reageren op specifieke behandelingen. Het is daarom belangrijk om onderzoek te doen naar serologische markers die relevante subgroepen binnen auto-immuunziekten kunnen identificeren om patiënten een betere behandeling te kunnen geven. In dit proefschrift richt ik mij op zes verschillende PTMs namelijk, nitratie (Nt), citrullinatie (Cit), carbamylatie (Ca), acetylatie (Ac), malondialdehyde acetaldehyde adducten (MAA) en advanced glycation end-producten (AGE) in de context van auto-immuniteit. In **Hoofdstuk 2**, hebben we anti-PTM antilichamen gericht tegen MAA- en AGE-gemodificeerde eiwitten onderzocht in een gerenommeerd RA cohort, namelijk het Leiden Early Arthritis Cohort, welke informatie bevatten over ACPA en anti-CarP. We zagen dat de aanwezigheid van anti-MAA en anti-AGE antilichamen een subgroep van RA patiënten identificeerde dat seronegatief was voor anti-CCP2, anti-CarP en reumafactor (RF). Aangezien HLA-DRB1*03 geassocieerd is met seronegatieve RA en met anti-CarP antilichamen in deze subgroep, hebben we de associatie tussen HLA-DRB1*03 en anti-MAA en anti-AGE onderzocht in deze studie. We zagen dat anti-MAA associeerde met HLA-DRB1*03 in het anti-CCP2 negatieve stratum, onafhankelijk van anti-CarP. Anti-AGE volgde dezelfde trend maar bereikte geen significantie. We zagen ook dat anti-MAA associeerde met inflammatie markers in vroege

artritis in zowel RA en non-RA artritis patiënten. Eerdere studies toonden aan dat anti-CarP geassocieerd was met verhoogde bot erosie bij anti-CCP2-negatieve RA patiënten. Om deze reden onderzochten we de associaties met radiologische gewrichtsschade bij deze patiënten. We zagen dat in patiënten met ACPA-negatieve RA, anti-AGE associeerde met radiologische progressie onafhankelijk van anti-CarP, wat kan suggereren dat deze anti-PTM een andere subgroep kan onderscheiden. Tot slot zijn er correlatie analyses gedaan om associaties tussen anti-PTM antilichaam positiviteit en aanhoudende medicatie vrije remissie te onderzoeken. Hier vonden we geen associatie tussen anti-MAA of anti-AGE met aanhoudende medicijnvrije remissie, ook niet na stratificeren voor ACPA status. Concluderend zijn anti-MAA en anti-AGE antilichamen beide relevant voor patiënten met RA, en andere inflammatoire reumatische aandoeningen. Hoewel niet specifiek voor RA, correleren ze beide met specifieke parameters. Anti-MAA associeert met HLA-DRB1*03 in ACPA-negatieve (RA) patiënten, onafhankelijk van anti-CarP, en associeert met inflammatie. Anti-AGE associeert met HLA-DRB1*03 in patiënten met ACPA-negatieve RA en associeert met een slechtere radiologische progressie, vooral bij patiënten met ACPA-negatieve en anti-CarP negatieve RA. In deze studie, hebben we een seropositieve subgroep (anti-MAA en anti-AGE positief) gekarakteriseerd binnen een heterogene groep van patiënten met RA dat tot op heden werd beschouwd als seronegatief (ACPA-, anti-CarP en RF-negatief).

Nu we hebben vastgesteld dat in RA, naast antilichaamresponsen tegen eiwitten gemodificeerd met citrulline of homocitrulline door citrullinatie en carbamylatie, respectievelijk, ook eiwitten gemodificeerd met andere PTMs kunnen worden herkend door autoantilichamen, hebben we dit daarom ook in andere auto-immuunziekten onderzocht. Auto-immuunziekten zoals SLE en AILD worden gekenmerkt door een breed scala aan autoantilichamen gericht tegen lichaamseigen eiwitten. Het is daarom aannemelijk dat deze patiënten antilichamen hebben die binden aan PTM-gemodificeerde eiwitten. In **Hoofdstuk 3**, hebben we anti-PTM antilichaam reactiviteiten onderzocht tegen alle zes de verschillende modificaties (Nt, Cit, Ca, Ac, MAA en AGE), in SLE patiënten. SLE patiënten staan bekend om hun brede verlies van zelftolerantie. Het is daarom aannemelijk dat anti-PTM antilichamen aanwezig zullen zijn. Aangezien het Leids Universitair Medisch Centrum een tertiair verwijscentrum is voor SLE-patiënten met neuropsychiatrische manifestaties (NPSLE), hadden wij de unieke gelegenheid om deze specifieke manifestatie nader te onderzoeken. Het identificeren van patiënten met NPSLE in het complexe ziektebeloop van SLE is een uitdaging en het zou zeer wenselijk zijn om serologische biomarkers te hebben om de diagnose te ondersteunen. In het gehele SLE cohort zagen we dat anti-MAA, anti-AGE en anti-CarP levels en positiviteit significant verhoogd waren ten opzichte van gezonde controles. Bovendien, anti-MAA en anti-AGE antilichaam levels correleerde negatief met complement C3 en C4 levels en correleerde positief met erytrocytbezinkingssnelheid (erythrocyte sedimentation rate; ESR), welke

systemische inflammatie impliceren. Interessant genoeg zagen we dat anti-MAA en anti-CarP meer voorkwamen in patiënten met major NPSLE dan in patiënten met andere SLE orgaan manifestaties. Daarbovenop, correleerde anti-MAA en anti-AGE met witte stof volume (white matter volume; WMV) en totaal brein volume (total brain volume; TBV), en anti-CarP met witte stof hyperintensiteit volume. Omdat WMV, TBV en witte stof hyperintensiteit volume objectieve markers zijn voor de betrokkenheid van het centrale zenuwstelsel bij SLE, leveren zij objectief bewijs, naast de multidisciplinaire beoordeling, om deze patiënten te diagnosticeren. Alle andere NPSLE activiteitsmarkers zijn meer subjectief, zoals bijvoorbeeld stemming. Samengenomen, anti-PTM antilichamen zouden kunnen helpen bij de diagnose en/of subgroepering van (NP)SLE. Toekomstige studies moeten de potentiële rol van anti-PTM antilichamen in (NP)SLE vaststellen.

In **Hoofdstuk 4** onderzochten we anti-PTM antilichaam responsen tegen onze set van zes PTM-gemodificeerde eiwitten in patiënten met autoimmuun lever ziektes (AILD). AILD is een heterogene groep van cholestatische en hepatocellulaire ziekten, bestaande uit primaire biliaire cholangitis (PBC), primaire scleroserende cholangitis (PSC), autoimmuun hepatitis (AIH) en overlappende varianten. PBC, PSC en AIH worden vaak gezien als (auto-)immuun gemedieerde ziekten en AIH en PBC worden gekarakteriseerd door de aanwezigheid van autoantilichamen en verhoogd totaal immunoglobuline. Daarnaast worden autoantilichamen tegenwoordig gebruikt in de diagnostische work-up, maar zijn ze niet ziekte specifiek. We observeerden dat anti-PTM antilichamen gericht tegen Ca-, Ac-, MAA- en AGE-gemodificeerde eiwitten waren verhoogd in patiënten met AILD ten opzicht van gezonde controles. Naast enkel-positiviteit werd ook positiviteit voor meer dan één anti-PTM antilichamen geanalyseerd. We zagen dat patiënten met AILD vaker minstens één type anti-PTM antilichaam hadden ten opzichte van niet-AILD en gezonde controles. Omdat AILD uit drie subgroepen bestaat, zijn anti-PTM antilichaam responsen vastgesteld in deze subgroepen. Patiënten met AIH waren significant vaker positief voor anti-MAA, anti-AGE, anti-CarP en anti-Cit vergeleken met patiënten met cholestatische lever ziekten (PBC en PSC). Binnen patiënten met AIH correleerde de aanwezigheid van anti-MAA en anti-AGE positief met complete biochemische remissie (CBR) na 3 maanden. Daarnaast associeerde anti-MAA, anti-AGE, en anti-CarP positief met het bereiken van CBR na 12 maanden. Gebaseerd op de bevinding dat positiviteit voor meerdere anti-PTM antilichamen meer voorkwam in patiënten met AIH, hebben we gepoogd de klinische relevantie van het hebben van deze meerdere anti-PTM reactiviteiten te onderzoeken. Na 3 maanden behandeling hadden significant meer AIH patiënten met minstens drie anti-PTM antilichamen CBR bereikt. Na 12 maanden behandeling was het verschil nog steeds significant. Over het geheel genomen werd een trend naar significantie voor de tijd tot CBR (in jaren) gevonden wanneer patiënten positief waren voor meerdere anti-PTM antilichamen. Samenvattend zijn anti-PTM antilichamen aanwezig in patiënten met AILD. Sommige patiënten zijn positief voor meerdere anti-PTM antilichamen. Het hebben

van drie of meer anti-PTM antilichaam responsen is geassocieerd met een gunstigere reactie op de behandeling in AIH. Direct na publicatie van onze bevindingen in Hoofdstuk 4 werd er een reactie gepubliceerd (toegevoegd aan **Hoofdstuk 4**) waarin de auteurs zich afvroegen of we enkel anti-PTM antilichamen detecteerde of (ook) polyreactive IgG. In een antwoord op dit commentaar (toegevoegd aan **Hoofdstuk 4**) verduidelijkte wij onze methoden en konden we toelichten dat we in onze experimentele set-up mock-gemodificeerde FCS includeerde om te kunnen corrigeren voor reactiviteit tegen FCS. Dit bevestigd dat we IgG antilichamen detecteerde die specifiek gericht zijn tegen PTM gemodificeerde eiwitten.

Interessant is dat veel patiënten met auto-immuunziekten, waaronder RA, SLE en AIH, baat hebben bij B-cel gerichte therapie, wat wijst op een (pathogene) rol voor B-cellen en/of antilichamen. Wat deze B-cellen aanzet tot autoreactiviteit staat momenteel ter discussie. In **Hoofdstuk 5** wordt beschreven dat PTM-gemodificeerde eiwitten het complement systeem kunnen activeren welke op haar beurt kan leiden tot klaring, chronische inflammatie en/of auto-immuniteit.

In deze context hebben we onze set van zes PTMs (Nt, Cit, Ca, Ac, MAA and AGE) onderzocht op hun vermogen om complement eiwitten te binden en te activeren. Eiwitten (fetal calf serum) gemodificeerd met deze PTMs werden eerder gebruikt om IgG antilichaam reactiviteit te bepalen in de RA, SLE en AILD cohorten beschreven in de **Hoofdstukken 2, 3 en 4**. We hebben nu de antilichaam profielen zoals gevonden in RA, SLE en AILD tegen alle zes de PTMs gecombineerd in **Hoofdstuk 5** om zo de aanwezigheid van combinaties van anti-PTM antilichamen in auto-immuniteit te onderzoeken. Interessant genoeg, zagen we dat antilichamen tegen Cit-, Ca-, Ac-, MAA- en AGE-gemodificeerde eiwitten het vaakst samen voorkwamen. Om te bestuderen hoe PTM-gemodificeerde eiwitten betrokken zouden kunnen zijn bij deze chronische auto-immuunziekten zijn we op zoek gegaan naar plasma eiwitten die binden aan PTM-gemodificeerde eiwitten. Met behulp van massa spectrometrie hebben we vanuit humaan serum vooral complement factoren geïdentificeerd die specifiek binden aan PTM-gemodificeerde eiwitten met de PTMs Ca, Ac, MAA en AGE in vergelijking met niet-gemodificeerde eiwitten. Om directe binding en activatie van complement op Ca-, Ac-, MAA- en AGE-gemodificeerde eiwitten aan te tonen hebben we bindingsassays met gezuiverd complement eiwitten en complement activatie assays uitgevoerd. We bevestigde dat complement inderdaad direct bindt aan PTM-gemodificeerde eiwitten, zonder de tussenkomst van antilichamen. Aangezien het complement systeem bestaat uit drie activatie routes (klassieke, lectine en alternatieve) hebben we ernaar gestreefd om te kunnen vaststellen welke complement route betrokken was bij complement activatie door de verschillende types PTM-gemodificeerd eiwitten. We zagen dat Ca-, MAA- en AGE-gemodificeerde eiwitten de klassieke route activeerde en verifieerde dat Ac-gemodificeerde eiwitten de lectine route activeren.

Het complement systeem staat bekend om haar opruimfunctie maar is ook bekend als link tussen aangeboren en verworven immuniteit via complement receptoren. We zijn daarom begonnen met het onderzoeken van cellen uit volbloed en THP-1 macrofagen, als model voor meer weefsel macrofagen. Voor dit doel hebben we PTM-gemodificeerde eiwitten gekoppeld aan beads om langlevende eiwitten na te bootsen die PTMs kunnen accumuleren. Vervolgens hebben deze beads geïncubeerd met complement actief serum om de beads te opsoniseren met complement. We zagen dat leukocyten en THP-1 macrofagen een verhoogde binding en opname van complement geopsoniseerde PTM-gemodificeerde eiwit gekoppelde beads lieten zien ten opzichte van niet-geopsoniseerde PTM-gemodificeerde eiwit gekoppelde beads of niet-gemodificeerde eiwit gekoppelde beads. Als laatste hadden we de mogelijkheid om data betreffend single nucleotide polymorfismen (SNPs) van complement genen in een set van RA patiënten, waarin we ook anti-PTM reactiviteiten hebben gemeten, te gebruiken. Hiervoor hebben we associatiestudies uitgevoerd tussen de aanwezigheid van anti-PTM antilichamen en SNPs in complement genen waarvan bekend is dat ze belangrijk zijn bij de activiteit van het complement systeem. We zagen dat minor allel factor H SNPs, waarvan eerder was aangetoond dat ze de complementactiviteit verhogen, geassocieerd waren met de aanwezigheid van anti-Ac, -MAA en -AGE. Aanwezigheid van het minor allel van rs2230199 (C3) associeerde met de aanwezigheid van anti-CarP. Deze data suggereert inderdaad een link tussen het zogenaamde ''complotype'' en het ontstaan van anti-PTM antilichamen. Met deze studie benadrukken we dat complement geactiveerd wordt door specifieke PTMs en dat verschillende complement activatie routes geactiveerd worden door verschillende PTMs. Concluderend kunnen we stellen dat PTM-gemodificeerde eiwitten complement binden en activeren, wat inflammatie en fagocytose verhoogd, en wat kan bijdragen aan de vorming van anti-PTM antilichamen.

Zoals samengevat en bediscussieerd in **Hoofdstuk 6**, laat het onderzoek beschreven in dit proefschrift zien dat PTMs en anti-PTM antilichamen associëren met ziekte progressie en respons op behandeling. Daarnaast kan detectie van anti-PTM antilichamen worden gebruikt om subgroepen te onderscheiden in een aantal auto-immuunziekten. Verdere studies moeten worden uitgevoerd om de potentiële diagnostische en prognostische waarde binnen elke groep vast te stellen. Het is duidelijk geworden dat sommige PTMs en anti-PTM antilichamen betrokken zijn bij (chronische) inflammatie, omdat we in de verschillende cohorten terugkerende associaties zagen tussen anti-PTM antilichamen en markers van inflammatie. Of deze antilichamen ontstekingen veroorzaken of een gevolg zijn van (chronische) ontstekingen moet in de toekomst worden onderzocht. Wij hebben echter vastgesteld dat complement eiwitten direct binden aan PTM-gemodificeerde eiwitten en dat zij daarmee klaring verhogen. Afhankelijk van het complotype kan dit proces leiden tot (aanhoudende) inflammatie en autoantilichaam productie. Het gepresenteerde onderzoek dringt daarom aan op verder onderzoek naar complement

in de inductie van anti-PTM antilichamen *in vivo* en daarbij gebruik te maken van complement deficiënte muizen. Naast dit fundamentele onderzoek moeten ook studies naar therapeutische mogelijkheden worden opgezet. Strategieën moeten worden ontwikkeld om specifiek anti-PTM reactive B cellen te depleteren en ook mogelijkheden om specifiek complement activatie gedreven door PTMs te inhiberen. Ons team heeft recent bispecifieke antilichamen ontwikkeld welke met één arm binden aan het PTM-gemodificeerde eiwit en met de andere arm een endogene complement inhibitor aantrekt om zo complement activatie te remmen op de PTMs. Deze strategie zou passen in de voorgestelde interventies die gericht zijn op aanhoudende immunologische remissie die leidt tot aanhoudende (geneesmiddelvrije) remissie.

Alles bij elkaar genomen, wordt in dit proefschrift bewijs geleverd dat immuniteit tegen PTMs een rol speelt in een variëteit aan auto-immuunziekten. Verder onderzoek naar PTMs en anti-PTM antilichaam inductie is nodig om specifiek gerichte behandelingsopties vast te stellen voor patiënten die streven naar aanhoudende immunologische remissie.

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Curriculum Vitae

Michelle Dominique van den Beukel werd geboren op 27 juni 1991 in Raamsdonksveer. Na het behalen van haar vwo-diploma in 2011 aan het Dongemond College in Raamsdonksveer, begon Michelle met de bachelor Biomedische Wetenschappen aan de Radboud Universiteit in Nijmegen. Tijdens haar bachelor heeft ze stage gelopen op de afdeling Matrix Biochemie aan het Radboud Instituut voor Moleculaire Levenswetenschappen (RIMLS) onder begeleiding van Els van de Westerlo en prof. dr. Toin van Kuppevelt. Hier focuste ze zich op het proces van phage display bij het vinden van biomarkers tegen glycosaminoglycanen. Na haar bachelor, volgde Michelle in 2014 een master Pathobiologie. Tijdens haar master heeft ze, ter verdieping, een extra keuzevak ''Advanced Immunology'' gevolgd aan de Universiteit van Amsterdam. In 2015 liep Michelle stage op de afdeling Tumorimmunologie in het RIMLS onder begeleiding van dr. Annette Sköld. Hier deed ze onderzoek naar de interactie tussen myeloïde en plasmacytoïde dendritische cellen. De data gegenereerd tijdens deze stage vormde het eerste wetenschappelijke artikel van Michelle. Michelle liep in 2017 haar tweede stage bij Genmab B.V. in Utrecht, onder begeleiding van dr. Frank Beurskens. Hier deed ze onderzoek naar de rol van complement en Fc:Fc versterking op verschillende antistof effector functies. Na afloop van deze tweede stage, werd de master in september 2017 met bene meritum afgerond.

In januari 2018 begon Michelle aan haar promotietraject op de afdeling Immunologie (voorheen Immuno-Haematologie en Bloedtransfusie) aan het Leids Universitair Medisch Centrum. Hierbij werd ze begeleid door prof. dr. Leendert Trouw en prof. dr. Paul Parren. Haar onderzoek richtte zich op immuniteit tegen post-translationeel gemodificeerde (PTM) eiwitten in auto-immuunziekten. Hierbij werden anti-PTM antistoffen gemeten in verschillende cohorten van patiënten met auto-immuunziekten en analyses uitgevoerd met betrekking tot klinische data. Overkoepelend deed Michelle onderzoek naar de mechanismen, gefocust op complement, waarmee deze PTMs mogelijk auto-immuniteit kunnen induceren. De resultaten van het onderzoek staan beschreven in dit proefschrift. Na haar PhD-traject werkte Michelle als postdoc op de afdeling Tumorimmunologie aan het RIMLS in de groep van prof. dr. Annemiek van Spriel. Hier deed ze onderzoek naar de mechanismen van antistof therapieën in de behandeling van B-cel lymfomen.

Dankwoord

Hora est! Het is tijd. Dat was hem dan. Mijn promotietraject komt hier ten einde en mijn proefschrift is geschreven. Het product van een turbulent PhD traject waarin velen mij hebben bijgestaan in experimenten, analyses, het schrijven van artikelen, maar ook mentaal. In dit dankwoord wil ik graag een aantal mensen in het bijzonder bedanken.

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