Tolerance and immune regulation in rheumatoid arthritis

Dekkers, J.S.

Chapter 1

Introduction and outline

Rheumatoid arthritis

Rheumatoid arthritis (RA) is a common autoimmune disease characterized by synovial inflammation, autoantibody production and destruction of cartilage and bone (1). Classically, the disease affects the small joints of the hands and feet in a symmetrical pattern. As RA is a systemic disease, extra-articular manifestations can be present in almost any system of the body e.g. the skin, heart, lungs and blood vessels (1). The diagnosis of RA is predominantly a clinical one, as a history of progressive joint swelling, stiffness and increased pain after a period of inactivity is indicative of an inflammatory joint disease. Early diagnosis and timely initiation of disease-modifying antirheumatic drugs, has been shown to have a favourable effect on the course of disease (2-4). However, timely diagnosis remains a challenge as classical signs of structural changes may be missing in early disease and subtle synovitis may escape notice during clinical examination.

Over the past few years, research in the field of RA has focused on the earliest stages of disease, leading to the discovery that circulating autoantibodies, such as rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPA) which precede the clinical onset of disease (5-7). ACPA are highly specific for RA (8) and the presence of the self-reactive antibodies reflects the complexity of disease. As RA seems to be a highly heterogenous disease, classification criteria have been developed with the aim to identify more homogenous patient groups to facilitate comparison with international studies. Using a quantitative scoring system patients can be classified based on scores obtained from; joint involvement, serologic markers, inflammatory markers and duration of symptoms. Although RA is a clinical diagnosis and diagnostic criteria for RA do not exist, classification criteria have been developed to arrive at homogeneous inclusion in clinical trials. The 1987 American College of Rheumatology (ACR) classification criteria for RA were not very well suited for the inclusion of recent onset RA as they rely on the expression of clinical symptoms, a feature of established RA (9). These findings led to the inclusion of an additional early serological marker, ACPA, to the current 2010 American College of Rheumatology/European League Against Rheumatism (2010 ACR/EULAR criteria) classification criteria for rheumatoid arthritis (10), (Table 1).

Although biomarkers provide valuable information to identify patients at an early disease phase, interpretation within the clinical context remains vital. For example, if the prior probability of RA is relatively low, such as in patients in primary care who have only knee pain or those who meet no other ACR criteria, measuring antibodies reactive to cyclic citrullinated peptide (CCP) alone seems to be a reasonable strategy that avoids too many false-positive results. If however, the prior probability of RA is relatively high, such as in patients seen in rheumatology clinics, measuring both autoantibodies seems to be a reasonable strategy that avoids missing potentially treatable patients.

Table 1. ACR/EULAR 2010 classification criteria for rheumatoid arthritis.

Joint involvement	**Score**
1 large joint	0
2-10 large joints	1
1-3 small joints (large joints not counted)	2
4-10 small joints	3
>10 joints (at least one small joint)	5
Serology	
Negative RF and negative ACPA status	0
Low-positive RF or low-positive ACPA	2
High-positive or high-positive ACPA	3
Acute phase-reactants	
Normal CRP and normal ESR	0
Abnormal CRP or abnormal ESR	1
Duration of symptoms	
<6 weeks	0
≥ 6 weeks	1

A score ≥ 6 is the cut point for rheumatoid arthritis. Patients can also be classified as having rheumatoid arthritis in the presence of 1) erosive disease, 2) long-standing disease according to the previous classification criteria. Target population: patients who have at least one joint with definite clinical synovitis, not better explained by another disease. Joint involvement: any swollen or tender joint on examination. Large joint: shoulders, elbows, hips, knees and ankles. Small joint: joints in the hands, writs and feet. Negative serology: below or equal to upper limited of normal (ULN). Low-positive serology: higher than ULN, less then 3 times ULN. High-positive serology: more than 3 times ULN. Acute phase reactants: according to local standards. Duration of symptoms: reported by the patient or symptoms of synovitis at the time of assessment.
RF= rheumatoid factor, ACPA=anti-citrullinated protein antibodies, CRP= C-reactive protein, ESR= erythrocyte sedimentation rate (10).

Autoantibodies in RA

The first RA associated autoantibody was RF which was identified in 1940 (11). RFs are antibodies that bind immunoglobulin (Ig)G and recognizes epitopes in the fragment crystallizable (Fc) region, which is responsible for effector functions like complement binding (12). Although usually measured as IgM-RF, RF activity can also be found in other subclasses of Ig (13). RF can be detected in 50-90% of RA patients, but are also found in the sera of patients with other connective tissue diseases, patients with infectious diseases and elderly healthy individuals (14). RF was the most valuable diagnostic marker for RA before

the discovery of ACPA and is still part of the 2010 ACR/EURLAR classification criteria. The presence of RF and ACPA represents an important early biomarker as these autoantibodies can be detected early in the disease course and can precede the clinical onset of disease (5-7). RF an ACPA can be found in sera of patients a median of 5 years before onset of clinical symptoms (6). Subjects with arthralgia which are seropositive for these autoantibodies have an approximately 30% chance of developing RA within one year (15). These findings highlight an important role for autoantibodies in the preclinical phase of RA which precedes the onset clinical signs and symptoms. Most research on the role of autoantibodies in RA has focused on ACPA, which are directed against citrullinated proteins. In the past several years it has become clear that the autoantibody response in RA extends to several other modified proteins, such as proteins modified by carbamylation and acetylation. As all these auto-antibodies recognize post-translationally modified proteins, these antibodies are collectively called anti-modified protein antibodies (AMPA). A variety of AMPAs against different protein modifications (anti-citrullinated (16), -carbamylated (17) and -acetylated protein antibodies (18)) have now been described in RA suggesting a shared common 'developmental' basis.

Post-translational protein modifications

Post-translational protein modification refers to the covalent and generally enzymatic modification of proteins following biosynthesis. Protein modifications can exist in different forms e.g. addition of small chemical groups, fatty acids or sugar chains, and can occur on the amino acids side chains or at the protein's C- or N- termini (19). Most post-translational modifications can modulate the protein conformation, function, activity, stability and/or location of a protein. For example, phosphorylation is a very general post-translational protein modification involved in the regulation of enzyme activity.

AMPA responses directed against post-translational modified proteins are highly specific for RA as they are, by and large, not found in other auto-immune or inflammatory diseases (20). As many proteins in the joint are long-lived especially extra-cellular matrix proteins expressed in the cartilage, it is not surprising that modified proteins can be found at higher levels in the joint compartment (21). Interestingly, the increased expression of modified proteins in the joints and cartilage might contribute to the production of AMPAs and possibly explain why AMPAs are associated with rheumatic diseases especially RA. As these post-translational modified proteins are prolongedly expressed and possibly more exposed by extracellular expression in the synovial compartment and cartilage, AMPA responses can contribute to increased susceptibility and risk of chronic inflammatory responses of the joints (20). For example, plasma proteins which carry post-translational modifications are readily recognised by AMPAs which can result in immune complex formation, complement

activation and subsequent clearance of the circulation. By contrast, modified matrix molecules recognised by AMPAs also induce immune complex formation and complement formation, however as these proteins are expressed in the synovial compartment they are immobile and these immune responses do not result in clearance of the modified proteins (22). This enables a prolonged process of complement activation, attraction of immune cells and release of mediators like myeloperoxidase (MPO) which stimulates local inflammation and protein modification contributing to chronic inflammatory responses.

Anti-citrullinated protein antibodies

Citrullination is a post-translational modification of proteins, in which a positively charged peptidylarginine residue is converted into a neutral peptidylcitrulline (Figure 1) (8). The conversion is catalysed by the calcium dependent peptidylarginine deiminases (PAD) enzyme. This modification takes place during a variety of biological processes including inflammation. ACPA are a hallmark of RA as these autoantibodies can be present many years prior to disease onset, and their presence is associated with an increased risk of developing RA (6, 15, 23). In the pre-RA phase the ACPA response matures which is characterised by an increase in isotype diversity, the range of epitopes recognized and the levels of antibodies (24). Both the presence of ACPA as well as the extent of the ACPA response are associated with clinical outcomes (25). ACPA positive patients respond different to therapy compared to ACPA negative patients (23, 24), have an more destructive disease course (26) and have an increased risk of relapse after tapering of disease-modifying antirheumatic drug (DMARD) therapy (27). The presence of ACPA can be detected by several commercially available laboratory tests. The first assay was based om the artificial CCP. Antibodies reactive to this assay are referred to as anti-CCP antibodies. While ACPA and RF are highly prevalent in RA they can also be identified in a small percentage of healthy individuals. The anti-CCP assay has a moderate sensitivity for RA (67%), comparable to IgM-RF (69%), and a very high specificity (95%) at the optimal cut-off value, whereas the sensitivity of IgM-RF is reported to be around 85% (16, 28).

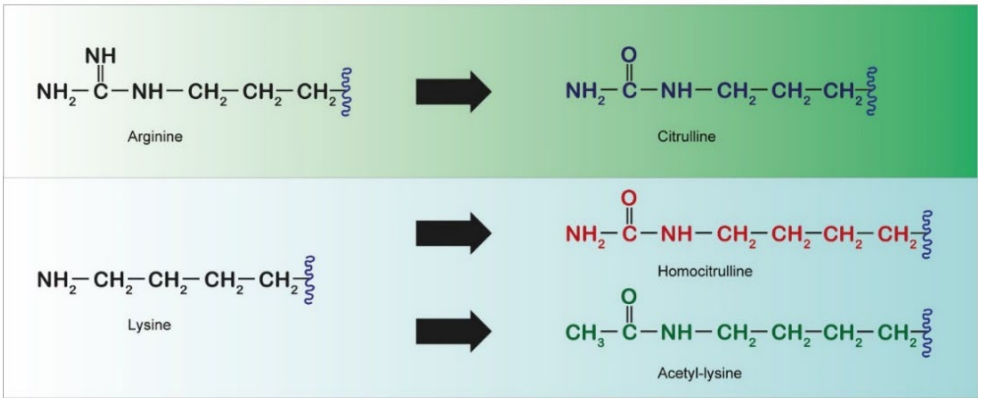

Figure 1. Chemical structures of post-translational protein modifications. Conversion of arginine into citrulline by PAD enzymes. Chemical modification of lysine into homocitrulline through cyanate. Enzymatic modification of lysine to acetyl-lysine by Acetyl-CoA.

Anti-carbamylated protein antibodies

When compared to ACPA, anti-carbamylated protein antibodies (anti-CarP antibodies) were discovered more recently (17). Anti-CarP antibodies target proteins that contain homocitrullines, also referred to as carbamylated proteins. Carbamylation is a chemical modification which occurs in the presence of cyanate. The formation of cyanate may result in a reaction with any accessible primary amine, including that of the lysine side chain and the amine at the N-terminus of polypeptides (29). The product formed by carbamylation, peptidyl-homocitrulline, highly resembles peptidyl-citrulline but contains one additional CH_2 group (Figure 1). Conversion of lysine into homocitrulline, similar to the conversion of arginine into citrulline, results in the loss of a positive charge which influences protein structure and function. Cyanate can be increased upon chronic inflammation (30) due to the conversion of thiocyanate into cyanate by myeloperoxidase, atherosclerosis (31) and smoking (32). Cyanate, a dissociation product of urea, is also increased in patients with renal failure as urea accumulates with decline of renal function (33).

The observation that the chemical structure of homocitrulline strongly resembles citrulline inspired experiments to analyse whether autoantibodies exist that target carbamylated proteins as well. In the first experiments to identify anti-CarP antibodies, carbamylated fetal calf serum (FCS) was used as a model antigen (17). Subsequent studies revealed that anti-CarP antibodies recognize carbamylated self-antigens like human fibrinogen(34), demonstrating that anti-CarP antibodies are autoantibodies, able to recognize modified self-protein. Using molecularly-defined peptides (containing carbamylated epitopes) anti-CarP antibodies were found to be reactive to peptides from α-enolase (35), collagen (36),

fillagrin (37) and vimentin (18). These studies revealed that protein modification is crucial for antibody recognition. Although several human proteins are currently used in the laboratory for the detection of anti-CarP antibody response, there is little information available on the specific nature and location of carbamylated proteins present in the (inflamed) joint.

Recent clinical studies revealed that anti-CarP antibodies are specific for RA and can be found in 45% of patients with early RA (17, 34, 36, 38, 39). Similar to ACPA, anti-CarP antibodies and can be identified in sera of patients years before onset of clinical symptoms (39-41). In addition, anti-CarP antibodies are associated with bone erosions, disability and mortality, independent of anti-CCP antibodies (17, 38, 39, 41). As anti-CarP antibodies occur in 10-20% of RA patients seronegative for ACPA (17, 38, 40), anti-CarP antibodies are thought to represent an additional serological marker distinct from ACPA. The sensitivity of the anti-CarP assay is reported to be moderate (44%) and with a high sensitivity (89%) (42). With the arrival of anti-CarP antibodies as a serological marker, it is intriguing whether these autoantibodies could contribute to the early identification and classification of patients with RA. Although anti-CarP antibodies are distinct from ACPAs, it has been described that some ACPA can bind peptides or proteins containing homocitrulline (18, 34, 35). However, the degree of cross-reactivity of ACPA for homocitrullinated proteins varies between studies. These discrepancies may be a result of proteins that contain different numbers and locations of homocitrulline and citrulline. Current studies using the same peptide backbone are inconclusive as the affinity of antibodies to citrulline and homocitrullinated peptides was not investigated (18, 35, 36, 43). However, the relative cross-reactivity between ACPA and anti-CarP antibodies suggests that these autoantibodies may originate from the same B-cell population.

Interestingly, anti-CarP antibodies can be detected in sera of mice with collagen-induced arthritis, even in the absence of ACPAs (44). Studies revealed that anti-CarP antibodies can be generated by immunization of rodents and rabbits with carbamylated proteins (37, 45). In rabbits, the induced response was partially cross-reactive. Comparison of different mouse and rat models of arthritis revealed that anti-CarP antibodies are only present in models that require active immunisation (46). Time course experiments in collagen-induced arthritis models indicate that the presence of anti-CarP antibodies precedes the onset of clinical symptoms (44), an observation similar to findings in patients with RA. These data suggest that anti-CarP antibodies or B-cell may play a pathogenic role in the development of arthritis.

Anti-acetylated protein antibodies

The most recent identified AMPA responses in RA are the anti-acetylated protein antibodies, directed against the post-translationally modification lysine by acetylation. Anti-acetylated protein antibodies have been described in approximately 40% of RA patients, mainly in the ACPA positive group (18). The identification of another AMPA response in RA provides interesting new insights in the pathophysiology of RA.

Lysine acetylation is a common post-translational modification and is involved in various biological processes such as chromatin remodelling, activation of transcription factors and regulation of metabolic enzymes. Protein lysine acetylation refers to the post-translational addition of an acetyl group to the ε-amino group of the side chain of a lysine residue (47). Acetylated lysine resembles homocitrulline except for the side-chain terminal amine, which is replaced by a methyl group (Figure 1). Multiple acetyltransferases are responsible for lysine acetylation and the first lysine acetylation was discovered on histones (48). Imbalance in histone acetylation has been found to change the chromatin structure and is associated with dysregulation of genes involved in cell-proliferation, differentiation and apoptosis (49-51). Protein acetylation has historically been considered a predominant eukaryotic phenomenon. Recent evidence, however, shows that also many bacterial species are able to acetylate proteins (52), including bacteria postulated as link between periodontal infection and RA (53, 54). These bacteria include *Porphyromonas Gingivalis* as well as *Aggregatibacter Actinomycetemcomitans*. It has been hypothesised that bacterial acetylation could play a role in the development of AMPA responses. Epidemiological data suggest that periodontal disease is more common in RA (35%) compared to controls (37%) (55). Interestingly, citrullinated, carbamylated and malondialdehyde-acetyladehyde (MAA) adduct modified proteins have been found in inflamed periodontal tissues (56). It has been shown that MAA adduct formation, as a result of oxidative stress, are increased in RA patients (57). Current studies reveal that the AMPA response is diverse and heterogenous between patients, but also point at the possibility of a shared developmental basis and role in pathophysiology.

Outline of this thesis

The general aim of this thesis was to elucidate the immune regulation and breach of tolerance towards modified proteins in RA. AMPA responses are a hallmark of disease and are implicated in the pathogenesis of RA. Recent studies have shown that these autoantibodies can serve as diagnostic and prognostic biomarkers.

The studies described in **chapter 2** describe the current evidence supporting the notion that autoimmunity against citrullinated proteins is already present at a preclinical phase of RA and how the ACPA response matures over time. Recent clinical studies have demonstrated that effective treatment of arthritis can lead to reduced levels of ACPA or a change in composition of ACPA. Many autoimmune diseases including RA, are characterized by the production of antibodies that can bind self-antigens. Animal studies have shown that the immunogenicity of proteins is enhanced upon protein modification (58-60). However, how these self-reactive B cells escape negative selection in the thymus is not known. To understand how autoreactive B cell responses are generated to post-translational modified proteins we hypothesised that exposure to carbamylated self-antigens is sufficient for a breach of immunological tolerance. The studies described in **chapter 3** show that exposure of mice to carbamylated self-antigens can lead to the formation of anti-CarP antibodies. Our observations reveal that not only carbamylation of self- but also foreign proteins is sufficient to induce self-reactive AMPA responses. In line with these findings, it was recently shown that homocitrulline containing peptides are also immunogenic in SE-expressing DR4tg mice and lead to T and B cell responses directed to carbamylated antigens (61).

Activation of naïve B cells by T cell dependent signals is characterised by the formation of germinal centers. Activated T-cells provide a stimulatory signal to B cells via CD40L-CD40 interaction (62) which subsequently leads to B cell proliferation and differentiation. In **chapter 4** we analysed whether carbamylation of an antigen can result in the generation of neo-epitopes and the subsequent induction of a breach tolerance at T cell level towards self-antigens. Our results indicate that carbamylated self-proteins are sufficient for a breach of immunological tolerance and are able to trigger primary immune responses, including autoantibody formation, T cell activation and cytokine production. In our studies we describe the concept that post-translational modification of self-proteins can create “new” antigens for which immune tolerance does not yet exist. To better understand the origin of AMPA responses and development of AMPA-producing B cells we immunized mice with different post-translational modified proteins and studied the induced humoral responses. These studies are described in **chapter 5** and indicate that exposure to a particular class of modified proteins (carbamylated or acetylated) can induce an immune response against another class of modified proteins as well. Likewise, AMPA from RA-patients purified against one PTM can recognize different classes of PTMs. These findings are important as

they indicate that the different AMPA-responses observed in RA-patients can be derived from the same inciting antigen(s) carrying only one particular modification.

Since the discovery of autoantibodies in RA it has been hypothesized that patients may benefit from treatment tailored to "autoantibody status". As methotrexate is the most widely used anti-rheumatic drug in clinical practice, it would be important to know whether the presence of autoantibodies is associated with better treatment response. In **chapter 6** we show that autoantibody status is not associated with early remission in newly diagnosed RA-patients receiving methotrexate. The results from our study in 1826 RA-patients from the METEOR database (an international rheumatoid arthritis registry) indicate that methotrexate is effective as initial treatment strategy regardless of autoantibody status.

The studies described in **chapter 7** focus on the preclinical disease phase of RA as it has been thought that treatment initiation in pre-arthritis stages might result in an improved treatment efficacy and possibly the prevention of a full-blown disease. In animal studies with the collagen-induced arthritis and adjuvant-induced arthritis model we aimed to study whether treatment initiation in a prophylactic or pre-arthritis setting could reveal differences in treatment efficacy between antirheumatic drugs. In this systematic literature study we describe the evidence that both prophylactic and pre-arthritis treatment strategies can lead to a significant reduction of arthritis severity scores, which hints at a possibility for preventive treatment strategies in RA.

Several studies have shown a clinical and epidemiological association between periodontitis and RA (63). Periodontal disease is characterized by gingivitis and a chronic inflammatory process of the bone. *Aggregatibacter actinomycetemcomitans* (Aa), a specific periodontitis-associated bacterium and its lytic toxin (Leukotoxin A or LtxA) were hypothesized to be involved in the initiation of ACPA responses in genetically predisposed individuals (54). In **chapter 8** we studied whether RA patients of Leiden Early Arthritis Clinic had an increased prevalence of Aa, measured by the presence of anti-LtxA antibodies. Furthermore, we aimed to replicate the finding that the association between human leukocyte antigens shared epitope (HLA SE) alleles and ACPA-positive RA is limited to the anti-LtxA-positive subset of patients. Using sera of arthritis patients we found that the presence of anti-LtxA antibodies was not specifically associated with RA. In addition, no association with the presence of ACPA or HLA SE alleles among RA patients was found. Finally, **chapter 9** discusses and summarizes the results of the studies described in this manuscript and describes possible directions for future research.

References

1. McInnes IB, Schett G. The pathogenesis of rheumatoid arthritis. N Engl J Med. 2011;365(23):2205-19.
2. Lard LR, Visser H, Speyer I, vander Horst-Bruinsma IE, Zwinderman AH, Breedveld FC, et al. Early versus delayed treatment in patients with recent-onset rheumatoid arthritis: comparison of two cohorts who received different treatment strategies. Am J Med. 2001;111(6):446-51.
3. Nell VP, Machold KP, Eberl G, Stamm TA, Uffmann M, Smolen JS. Benefit of very early referral and very early therapy with disease-modifying anti-rheumatic drugs in patients with early rheumatoid arthritis. Rheumatology (Oxford). 2004;43(7):906-14.
4. van der Linden MP, le Cessie S, Raza K, van der Woude D, Knevel R, Huizinga TW, et al. Long-term impact of delay in assessment of patients with early arthritis. Arthritis Rheum. 2010;62(12):3537-46.
5. Berglin E, Padyukov L, Sundin U, Hallmans G, Stenlund H, Van Venrooij WJ, et al. A combination of autoantibodies to cyclic citrullinated peptide (CCP) and HLA-DRB1 locus antigens is strongly associated with future onset of rheumatoid arthritis. Arthritis Res Ther. 2004;6(4):R303-8.
6. Nielen MM, van Schaardenburg D, Reesink HW, van de Stadt RJ, van der Horst-Bruinsma IE, de Koning MH, et al. Specific autoantibodies precede the symptoms of rheumatoid arthritis: a study of serial measurements in blood donors. Arthritis Rheum. 2004;50(2):380-6.
7. Rantapaa-Dahlqvist S, de Jong BA, Berglin E, Hallmans G, Wadell G, Stenlund H, et al. Antibodies against cyclic citrullinated peptide and IgA rheumatoid factor predict the development of rheumatoid arthritis. Arthritis Rheum. 2003;48(10):2741-9.
8. van Venrooij WJ, van Beers JJ, Pruijn GJ. Anti-CCP Antibody, a Marker for the Early Detection of Rheumatoid Arthritis. Ann N Y Acad Sci. 2008;1143:268-85.
9. Arnett FC, Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS, et al. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum. 1988;31(3):315-24.
10. Aletaha D, Neogi T, Silman AJ, Funovits J, Felson DT, Bingham CO, 3rd, et al. 2010 Rheumatoid arthritis classification criteria: an American College of Rheumatology/European League Against Rheumatism collaborative initiative. Arthritis Rheum. 2010;62(9):2569-81.
11. Waaler E. On the occurrence of a factor in human serum activating the specific agglutintion of sheep blood corpuscles. 1939. Apmis. 2007;115(5):422-38; discussion 39.
12. Moore TL, Dorner RW. Rheumatoid factors. Clin Biochem. 1993;26(2):75-84.
13. Tarkowski A, Nilsson LA. Isotype-specific measurement of rheumatoid factor with reference to clinical features of rheumatoid arthritis. J Clin Lab Immunol. 1983;12(3):129-35.
14. Shmerling RH, Delbanco TL. The rheumatoid factor: an analysis of clinical utility. Am J Med. 1991;91(5):528-34.
15. van de Stadt LA, van der Horst AR, de Koning MH, Bos WH, Wolbink GJ, van de Stadt RJ, et al. The extent of the anti-citrullinated protein antibody repertoire is associated with arthritis development in patients with seropositive arthralgia. Ann Rheum Dis. 2011;70(1):128-33.

16. Schellekens GA, Visser H, de Jong BA, van den Hoogen FH, Hazes JM, Breedveld FC, et al. The diagnostic properties of rheumatoid arthritis antibodies recognizing a cyclic citrullinated peptide. Arthritis Rheum. 2000;43(1):155-63.
17. Shi J, Knevel R, Suwannalai P, van der Linden MP, Janssen GM, van Veelen PA, et al. Autoantibodies recognizing carbamylated proteins are present in sera of patients with rheumatoid arthritis and predict joint damage. Proc Natl Acad Sci U S A. 2011;108(42):17372-7.
18. Juarez M, Bang H, Hammar F, Reimer U, Dyke B, Sahbudin I, et al. Identification of novel antiacetylated vimentin antibodies in patients with early inflammatory arthritis. Ann Rheum Dis. 2016;75(6):1099-107.
19. Walsh CT, Garneau-Tsodikova S, Gatto GJ, Jr. Protein posttranslational modifications: the chemistry of proteome diversifications. Angew Chem Int Ed Engl. 2005;44(45):7342-72.
20. Darrah E, Andrade F**.** Editorial: citrullination, and carbamylation, and malondialdehyde-acetaldehyde! Oh my! Entering the forest of autoantigen modifications in rheumatoid arthritis. Arthritis Rheumatol. 2015;67(3):604-8.
21. Courties A, Gualillo O, Berenbaum F, Sellam J**.** Metabolic stress-induced joint inflammation and osteoarthritis. Osteoarthritis Cartilage. 2015;23(11):1955-65.
22. Trouw LA, Rispens T, Toes REM**.** Beyond citrullination: other post-translational protein modifications in rheumatoid arthritis. Nat Rev Rheumatol. 2017;13(6):331-9.
23. Klareskog L, Widhe M, Hermansson M, Ronnelid J**.** Antibodies to citrullinated proteins in arthritis: pathology and promise. Curr Opin Rheumatol. 2008;20(3):300-5.
24. Willemze A, Trouw LA, Toes RE, Huizinga TW**.** The influence of ACPA status and characteristics on the course of RA. Nat Rev Rheumatol. 2012;8(3):144-52.
25. van der Woude D, Syversen SW, van der Voort EI, Verpoort KN, Goll GL, van der Linden MP, et al. The ACPA isotype profile reflects long-term radiographic progression in rheumatoid arthritis. Ann Rheum Dis. 2010;69(6):1110-6.
26. Syversen SW, Gaarder PI, Goll GL, Odegard S, Haavardsholm EA, Mowinckel P, et al. High anti-cyclic citrullinated peptide levels and an algorithm of four variables predict radiographic progression in patients with rheumatoid arthritis: results from a 10-year longitudinal study. Ann Rheum Dis. 2008;67(2):212-7.
27. van der Woude D, Young A, Jayakumar K, Mertens BJ, Toes RE, van der Heijde D, et al. Prevalence of and predictive factors for sustained disease-modifying antirheumatic drug-free remission in rheumatoid arthritis: results from two large early arthritis cohorts. Arthritis Rheum. 2009;60(8):2262-71.
28. Nishimura K, Sugiyama D, Kogata Y, Tsuji G, Nakazawa T, Kawano S, et al. Meta-analysis: diagnostic accuracy of anti-cyclic citrullinated peptide antibody and rheumatoid factor for rheumatoid arthritis. Ann Intern Med. 2007;146(11):797-808.
29. Shi J, van Veelen PA, Mahler M, Janssen GM, Drijfhout JW, Huizinga TW, et al. Carbamylation and antibodies against carbamylated proteins in autoimmunity and other pathologies. Autoimmun Rev. 2014;13(3):225-30.
30. Wang Z, Nicholls SJ, Rodriguez ER, Kummu O, Horkko S, Barnard J, et al. Protein carbamylation links inflammation, smoking, uremia and atherogenesis. Nat Med. 2007;13(10):1176-84.

31. Holzer M, Gauster M, Pfeifer T, Wadsack C, Fauler G, Stiegler P, et al. Protein carbamylation renders high-density lipoprotein dysfunctional. Antioxid Redox Signal. 2011;14(12):2337-46.
32. Roberts JM, Veres PR, Cochran AK, Warneke C, Burling IR, Yokelson RJ, et al. Isocyanic acid in the atmosphere and its possible link to smoke-related health effects. Proc Natl Acad Sci U S A. 2011;108(22):8966-71.
33. Kalim S, Karumanchi SA, Thadhani RI, Berg AH**.** Protein carbamylation in kidney disease: pathogenesis and clinical implications. Am J Kidney Dis. 2014;64(5):793-803.
34. Scinocca M, Bell DA, Racape M, Joseph R, Shaw G, McCormick JK, et al. Antihomocitrullinated fibrinogen antibodies are specific to rheumatoid arthritis and frequently bind citrullinated proteins/peptides. J Rheumatol. 2014;41(2):270-9.
35. Reed E, Jiang X, Kharlamova N, Ytterberg AJ, Catrina AI, Israelsson L, et al. Antibodies to carbamylated alpha-enolase epitopes in rheumatoid arthritis also bind citrullinated epitopes and are largely indistinct from anti-citrullinated protein antibodies. Arthritis Res Ther. 2016;18(1):96.
36. Turunen S, Hannonen P, Koivula MK, Risteli L, Risteli J**.** Separate and overlapping specificities in rheumatoid arthritis antibodies binding to citrulline- and homocitrulline-containing peptides related to type I and II collagen telopeptides. Arthritis Res Ther. 2015;17:2.
37. Mydel P, Wang Z, Brisslert M, Hellvard A, Dahlberg LE, Hazen SL, et al. Carbamylation-dependent activation of T cells: a novel mechanism in the pathogenesis of autoimmune arthritis. J Immunol. 2010;184(12):6882-90.
38. Jiang X, Trouw LA, van Wesemael TJ, Shi J, Bengtsson C, Kallberg H, et al. Anti-CarP antibodies in two large cohorts of patients with rheumatoid arthritis and their relationship to genetic risk factors, cigarette smoking and other autoantibodies. Ann Rheum Dis. 2014;73(10):1761-8.
39. Brink M, Verheul MK, Ronnelid J, Berglin E, Holmdahl R, Toes RE, et al. Anti-carbamylated protein antibodies in the pre-symptomatic phase of rheumatoid arthritis, their relationship with multiple anti-citrulline peptide antibodies and association with radiological damage. Arthritis Res Ther. 2015;17:25.
40. Shi J, van de Stadt LA, Levarht EW, Huizinga TW, Hamann D, van Schaardenburg D, et al. Anti-carbamylated protein (anti-CarP) antibodies precede the onset of rheumatoid arthritis. Ann Rheum Dis. 2014;73(4):780-3.
41. Gan RW, Trouw LA, Shi J, Toes RE, Huizinga TW, Demoruelle MK, et al. Anti-carbamylated protein antibodies are present prior to rheumatoid arthritis and are associated with its future diagnosis. J Rheumatol. 2015;42(4):572-9.
42. Shi J, van Steenbergen HW, van Nies JA, Levarht EW, Huizinga TW, van der Helm-van Mil AH, et al. The specificity of anti-carbamylated protein antibodies for rheumatoid arthritis in a setting of early arthritis. Arthritis Res Ther. 2015;17:339.
43. Lac P, Racape M, Barra L, Bell DA, Cairns E**.** Relatedness of Antibodies to Peptides Containing Homocitrulline or Citrulline in Patients with Rheumatoid Arthritis. J Rheumatol. 2018;45(3):302-9.
44. Stoop JN, Liu BS, Shi J, Jansen DT, Hegen M, Huizinga TW, et al. Antibodies specific for carbamylated proteins precede the onset of clinical symptoms in mice with collagen induced arthritis. PLoS One. 2014;9(7):e102163.

45. Steinbrecher UP, Fisher M, Witztum JL, Curtiss LK. Immunogenicity of homologous low density lipoprotein after methylation, ethylation, acetylation, or carbamylation: generation of antibodies specific for derivatized lysine. J Lipid Res. 1984;25(10):1109-16.
46. Stoop JN, Fischer A, Hayer S, Hegen M, Huizinga TW, Steiner G, et al. Anticarbamylated protein antibodies can be detected in animal models of arthritis that require active involvement of the adaptive immune system. Ann Rheum Dis. 2015;74(5):949-50.
47. Guan KL, Yu W, Lin Y, Xiong Y, Zhao S. Generation of acetyllysine antibodies and affinity enrichment of acetylated peptides. Nat Protoc. 2010;5(9):1583-95.
48. Phillips DM. The presence of acetyl groups of histones. Biochem J. 1963;87:258-63.
49. Grunstein M. Histone acetylation in chromatin structure and transcription. Nature. 1997;389(6649):349-52.
50. Paolinelli R, Mendoza-Maldonado R, Cereseto A, Giacca M. Acetylation by GCN5 regulates CDC6 phosphorylation in the S phase of the cell cycle. Nat Struct Mol Biol. 2009;16(4):412-20.
51. Gu W, Roeder RG. Activation of p53 sequence-specific DNA binding by acetylation of the p53 C-terminal domain. Cell. 1997;90(4):595-606.
52. Hu LI, Lima BP, Wolfe AJ. Bacterial protein acetylation: the dawning of a new age. Mol Microbiol. 2010;77(1):15-21.
53. Butler CA, Veith PD, Nieto MF, Dashper SG, Reynolds EC. Lysine acetylation is a common post-translational modification of key metabolic pathway enzymes of the anaerobe Porphyromonas gingivalis. J Proteomics. 2015;128:352-64.
54. Konig MF, Abusleme L, Reinholdt J, Palmer RJ, Teles RP, Sampson K, et al. Aggregatibacter actinomycetemcomitans-induced hypercitrullination links periodontal infection to autoimmunity in rheumatoid arthritis. Sci Transl Med. 2016;8(369):369ra176.
55. Mikuls TR, Payne JB, Yu F, Thiele GM, Reynolds RJ, Cannon GW, et al. Periodontitis and Porphyromonas gingivalis in patients with rheumatoid arthritis. Arthritis Rheumatol. 2014;66(5):1090-100.
56. Bright R, Thiele GM, Manavis J, Mikuls TR, Payne JB, Bartold PM. Gingival tissue, an extrasynovial source of malondialdehyde-acetaldehyde adducts, citrullinated and carbamylated proteins. J Periodontal Res. 2018;53(1):139-43.
57. Thiele GM, Duryee MJ, Anderson DR, Klassen LW, Mohring SM, Young KA, et al. Malondialdehyde-acetaldehyde adducts and anti-malondialdehyde-acetaldehyde antibodies in rheumatoid arthritis. Arthritis Rheumatol. 2015;67(3):645-55.
58. Lundberg K, Nijenhuis S, Vossenaar ER, Palmblad K, van Venrooij WJ, Klareskog L, et al. Citrullinated proteins have increased immunogenicity and arthritogenicity and their presence in arthritic joints correlates with disease severity. Arthritis Res Ther. 2005;7(3):R458-67.
59. Hill JA, Bell DA, Brintnell W, Yue D, Wehrli B, Jevnikar AM, et al. Arthritis induced by posttranslationally modified (citrullinated) fibrinogen in DR4-IE transgenic mice. J Exp Med. 2008;205(4):967-79.
60. Thiele GM, Tuma DJ, Willis MS, Miller JA, McDonald TL, Sorrell MF, et al. Soluble proteins modified with acetaldehyde and malondialdehyde are immunogenic in the absence of adjuvant. Alcohol Clin Exp Res. 1998;22(8):1731-9.

61. Lac P, Saunders S, Tutunea-Fatan E, Barra L, Bell DA, Cairns E**.** Immune responses to peptides containing homocitrulline or citrulline in the DR4-transgenic mouse model of rheumatoid arthritis. J Autoimmun. 2018;89:75-81.
62. Foy TM, Laman JD, Ledbetter JA, Aruffo A, Claassen E, Noelle RJ**.** gp39-CD40 interactions are essential for germinal center formation and the development of B cell memory. J Exp Med. 1994;180(1):157-63.
63. Bingham CO, 3rd, Moni M**.** Periodontal disease and rheumatoid arthritis: the evidence accumulates for complex pathobiologic interactions. Curr Opin Rheumatol. 2013;25(3):345-53.