Unravelling the anti-carbamylated protein antibody response in rheumatoid arthritis

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Chapter 1

Introduction

During a lifetime, the human body encounters a large number of immunological challenges, such as infections with viruses and bacteria. If the immune system is sufficiently competent, such a challenge will be met by the activation of one or more immune pathways that will attack and clear the infection. However, there are situations in which complications may arise. In the first situation, a weakened immune system might not be able to control the pathogen, resulting in prolonged infection with accompanying consequences. Often, this situation can be prevented upfront by vaccination or countered during the infection by treatment with antibiotics or passive immunization therapy[1]. In the second situation, the immune system may not attack a foreign pathogen but direct it's action towards (part of) the human body. Diseases resulting from such an unwanted self-reactive immune response have been named autoimmune diseases. Examples of common diseases often categorized as autoimmune diseases are Graves' disease, type I diabetes and rheumatoid arthritis (RA)[2].

Clinical aspects of rheumatoid arthritis

Although RA has been described as an autoimmune disease, it has also been called an inflammatory disease with autoimmune aspects[3]. The fact that multiple of such descriptions are available already indicates that many aspects of the disease, especially with regards to pathogenic processes, are relatively unclear. In the Netherlands, more than 1.3% of the population suffers of a chronic inflammatory disease, belonging to the rheumatology disease category[4]. A large part of this category consists of RA patients. It has been estimated the general prevalence of RA is between 0.5% and 1%[5,6]. The diagnosis of an RA patient is often based on both clinical presentation and serology. The clinical presentation of RA involves inflammation of one or multiple joints. Involvement of larger joints, inflammation in multiple joints and the duration of the complaints each contribute to increased susceptibility of RA. Additionally, two autoantibodies, called rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPA) are often measured in serum, possibly combined with other markers of inflammation such as CRP and ESR. Positivity or high levels of each of these biomarkers further adds to increased susceptibility for RA. Most of these factors are also part of the most recent classification criteria for RA[7]. An overview of the most recent classification criteria, compared to the 1987 criteria can be found in table 1[8]. The main difference between these two criteria is the increased attention to the presence of autoantibodies. When studying the presence of autoantibodies in clinical cohorts, results may therefore vary depending on the selection criteria.

The 2010 classification criteria are able to capture a large proportion of RA patients[9,10]. These criteria are not diagnostic criteria and may still miss out on the group of (early) RA patients in which few joints are affected and serology is negative[11]. At the moment it is considered important to identify RA patients as early as possible, as early treatment allows the most favourable long-term outcome.

Likewise, during the early phase of disease, there may exist a so-called "window of opportunity" during which treatment is more effective and which may even result in the prevention of chronic disease development[12].

At the moment, there is no cure for RA, although the development of chronic disease may be prevented by effective and early treatment. Therefore, medication prescribed aims to modify the disease or reduce the symptoms. Methotrexate is one of the disease-modifying anti-rheumatic drugs (DMARDs) and is often part of the standard treatment strategy after diagnosis of RA. An important mode of action of methotrexate is to suppress the immune system. This drug is effective in the majority of patients but a substantial proportion of the patients does not respond sufficiently. If the treatment is not effective, methotrexate can be combined with other DMARDs such as TNF inhibitors or rituximab, which both target separate aspects of the immune system. In order to further alleviate symptoms such as pain and inflammation, non-steroidal anti-inflammatory drugs (NSAIDs) can be prescribed as well[13]

Criteria	1987 Criteria	2010 Criteria
Morning Stiffness	Yes	No
Number of joints involved	Yes	Yes
Arthritis of hand joints	Yes	Yes (a subset of joints)
Symmetric arthritis	Yes	No
Rheumatoid nodules	Yes	No
Rheumatoid factor	Yes	Yes
Radiographic changes	Yes	No
Anti-Citrulline antibodies	No	Yes
Duration of symptoms	Yes	Yes
CRP or ESR	No	Yes

Table 1 – Comparison between the 1987 and 2010 diagnostics criteria for rheumatoid arthritis

Risk factors for rheumatoid arthritis

Known risk factors for diseases can often be divided into inheritable and environmental. In RA, it has been estimated using twin studies that the genetic contribution of the disease is approximately 60%[14]. By now, many studies investigating genetics have been carried out for RA. By far the largest genetic components in RA is the presence of certain HLA-DR alleles which can either predispose for the development of RA, in the case of the "shared epitope" alleles, but can also be protective in the case of HLA-DRB1*13[15,16]. Also, PTPN22 is a commonly discussed gene in which SNPs have been associated with the development of RA, although many more genes, which each seem to contribute minimally to RA development have been identified[17].

The main environmental risk factor thought to induce RA is smoking, which is interestingly enough a risk factor for many other (autoimmune) diseases as well[18,19]. Other risk factors described include infections, for example during periodontitis or changes in the microbiome, but these are less evident, or were investigated less when compared with smoking[20].

Pathogenesis of Rheumatoid arthritis

At the moment, little formal evidence is present for the pathogenesis of RA. Also, a large proportion of data at this point is derived from mouse models which may not resemble the human disease process. However, most agree that the interaction between large numbers of different genetic and environmental factors result in a complex interplay of the immune system, possibly inducing a positive feedback loop and eventually chronic inflammation. Although many hypotheses and theories have been developed regarding the pathogenesis of RA, there still remains much to be elucidated.

One of the more general theories for the development of autoimmune diseases is based on molecular mimicry[21]. Initially, an immune response will develop towards a foreign (pathogen-derived) epitope. However, (part of) this epitope may be similar or equal to epitopes already present in the human body, resulting in a break of tolerance against these particular epitopes. A specific example of possible molecular mimicry in RA focuses on the DERAA-sequence which are sequences that fit well in the HLA-DR shared epitope molecules. This sequence is present in multiple pathogens, including influenza A, but can also be found in human self-proteins, such as vinculin. Eventually reactivity towards these pathogens may result in autoimmunity towards (citrullinated) vinculin[22]. This particular theory can be well combined with the genetic risk factors for RA.

A second model focusses on mucosal sites, mainly the lung, as an initiation site for the break of tolerance in RA patients[23]. An initial indication for the involvement of the lung in RA comes from studies that show that early, untreated RA patients seem to have increased inflammation in the lungs[24]. This theory also incorporates the increased risk on RA when smoking. It has been described that smoking may increase inflammation (and citrullination) in the lung, resulting in a break of tolerance and eventually autoantibody production. Why the joint would be targeted in such a situation is currently unclear.

Besides these models of initiation, there are also several cells that are known to play a role in the pathogenesis of RA, although their exact role and whether their presence is cause or consequence are not known. Cells that are very common in the synovial fluid of RA patients, namely neutrophils may be involved in disease in several manners. One of the aspects may be through their general immune functions such as degranulation or the formation of neutrophil extracellular traps (NETs)[25,26], which may contribute to the general immune reaction within the synovium.

Also, neutrophils may provide several antigens in the form of post-translational modifications such as carbamylation, citrullination or malondialdehyde-acetaldehyde modifications, by providing components that promote these modifications [27,28].

Furthermore, T cells are often thought to contribute or even cause autoimmune diseases. Also for RA, the genetic associations seem to indicate that a role for T cells seems likely. However, directly targeting the T cells by depleting them in RA, does generally not result in resolution of the disease, and it has been suggested that a the correct balance of T cell subsets, such as the right amount Tregs, Th1 and Th2 cells is important[29,30]. Another role for T cells may come in the form of T-B cell interactions during which B cells undergo class switching and somatic hypermutation. This may also occur within the joint of RA patients, since tertiary lymphoid structures have been detected in some patients[31].

A final cell type that is thought to be involved in RA is the B cell. Targeting this cell type, for example with rituximab, can be an effective treatment strategy in RA[32], indicating that there is indeed a role for B cells as well. What this role is, is at the moment unclear, but at this point, most research with regards to B cells in RA seems to focus on antibody production and their respective antigens in RA patients.

Autoantibodies in rheumatoid arthritis

The production of autoantibodies in RA has become an important disease characteristic, also indicated by their incorporation into the diagnostic criteria. One of the first autoantibodies discovered in RA is RF, which targets the Fc tail of other antibodies and therefore has the potential to form large immune complexes[33]. However, the specificity of RF for RA is relatively low when compared to ACPA, antibodies that recognize citrullinated proteins or peptides[34,35]. Citrullination is a process of post-translational modification that is carried out by PAD enzymes, which are able to convert arginine residues into citrulline residues.

ACPAs were shown to associate with joint damage and can often be identified before disease onset[36,37]. Furthermore, epitope spreading before disease onset has been observed for these autoantibodies[38,39]. Importantly, most of the genetic association discussed are mainly found for the ACPA+ RA subset. Besides the presence of RF and ACPA, many other autoantibodies have been described in RA. Just as ACPA, some of these antibodies also target a posttranslational modification. Previously described modifications that can be recognized by autoantibodies in RA include acetylation, malondialdehyde modifications and carbamylation[40-42]. Of these, autoantibodies targeting carbamylation proteins have been described most extensively[43].

Carbamylation

Carbamylation is a post-translational modification mainly affecting lysine residues.

The carbamylation of a lysine residue is a chemical process carried out in the presence of cyanate and resulting in a homocitrulline (figure 1)[44]. Besides lysine modification, some other amino acids and the n-terminus can also be modified by cyanate, although these are thought to be less common than lysine modification. The homocitrulline, as the name implies, is rather similar to a citrulline, the last being only a Ch2 group shorter.

At the moment, there are two pathways known that can result in cyanate in the human body. It is not clear how much each of the pathways contributes to carbamylation, although one pathway is constantly present while the other pathway seems to require inflammatory conditions. The more constant production of cyanate is derived from urea, which is in equilibrium with cyanate[45]. This is a pathway that is increasingly involved in renal disease, since an increase in urea can be observed in patients with renal failure. The second method to generate cyanate involves the conversion of thiocyanate in a reaction with myeloperoxidase (MPO) and H2O2[46]. This pathway may be driven by inflammation, which increases the presence of MPO. Smoking may also contribute by increasing thiocyanate levels[47,48].

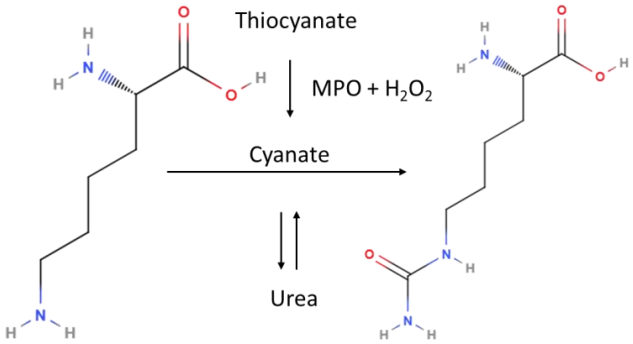

Figure 1 – A lysine is converted into a homocitrulline in the presence of cyanate. Cyanate is generally in equilibrium with urea but can also be formed from thiocyanate by myeloperoxidase (MPO). Structures were made with MolView.

The knowledge on carbamylation in health and disease at this moment is limited. Most studies investigating carbamylation focus on renal disease. Here the amount of carbamylation in serum may help to predict mortality[49], and to distinguish between acute or chronic disease[50]. Upon hemodialysis, levels of carbamylation in the blood of renal disease patients decrease again[51]. Furthermore, the levels of carbamylation may also associate with heart failure in renal disease patients[52], or with the severity of coronary artery disease in the general population[53]. Interesingly, in mice the total amount of carbamylation seems to increase with age[54].

With regards to RA, only one study investigating carbamylation in the foot of one RA patient has been carried out[55]. Homocitrulline residues were indeed detectable in several joint biopsies, but no obvious differences were observed between inflamed and non-affected biopsies.

The direct effect of the carbamylation process on the function of a protein is difficult to study, since the modified lysines of the protein in vivo might be different from the modied lysines when in vitro carbamylation is carried out. However, studies have shown that carbamylation of IgG may prevent complement activation[56] and carbamylation of low-density lipoproteins may increase cell death of endothelial cells[57]. However, the question remains whether the carbamylation in vivo is enough to actually establish such effects.

Anti-CarP antibodies

The existence of anti-CarP antibodies was described by Shi et al, showing that anti-CarP antibodies are increased in RA [43]. The presence of anti-CarP antibodies has also been described for other conditions, including juvenile arthritis, psoriatic arthritis, sjögren syndrome and other non-RA arthritic diseases[58-61]. However, levels and percentage of people positive for anti-CarP antibodies are much higher in RA patients than in the other conditions in which these antibodies have been described.

Besides an increase of autoantibodies in RA, many other clinical aspects have been investigated. For example, the presence of anti-CarP antibodies associates with joint damage over time, also when patients are ACPA-negative[43]. This indicates that there may be a role for the immune response against carbamylated proteins in the pathogenesis of RA. Supporting this hypothesis are the data that show that anti-CarP antibodies are present years before disease onset[62], which also indicates that anti-CarP antibodies alone are not sufficient to induce RA. In arthralgia patients, the presence of anti-CarP antibodies does however associate with the development of RA, indicating that anti-CarP antibodies may also be an interesting biomarker in the early phase of RA development[63]. Another aspect that has been investigated with regards to anti-CarP antibodies are genetics. Interestingly, at the moment, no such associations are known. One study found no independent association with well-known RA genes such as the HLA shared epitope alleles and *PTPN22*[64]]. This study also showed no correlation between anti-CarP antibodies and smoking.

Outline of this thesis

Although quite some data is available on anti-CarP antibodies, several questions remain unanswered, including the reproducibility of the clinical data on anti-CarP antibodies, such as their presence before disease onset and association with joint damage. It is also unknown whether these findings can be expanded to non-caucasian populations. Furthermore, it is unclear how anti-CarP antibodies are induced, what proteins they recognize, whether they are able to recognize multiple carbamylated proteins and what the characteristics of these autoantibodies are. Here, some of these questions will be answered.
In this thesis, I will first discuss the clinical implications of the presence of anti-CarP antibodies compared to RA-specific autoantibodies in both RA patients other relevant conditions (chapters 2 till 7). This is followed by more detailed investigations into the characteristics of anti-CarP antibodies and their antigens (chapters 8 till 12).

References

1 Sparrow, E., Friede, M., Sheikh, M. & Torvaldsen, S. Therapeutic antibodies for infectious diseases. *Bull World Health Organ* **95**, 235-237, doi:10.2471/BLT.16.178061 (2017).

2 Wang, L., Wang, F. S. & Gershwin, M. E. Human autoimmune diseases: a comprehensive update. *J Intern Med* **278**, 369-395, doi:10.1111/joim.12395 (2015).

3 Chemin, K., Klareskog, L. & Malmstrom, V. Is rheumatoid arthritis an autoimmune disease? *Current opinion in rheumatology* **28**, 181-188, doi:10.1097/bor.0000000000000253 (2016).

4 Sloot R, F. L., Heins M, Lafeber M, Boeije H, Poos MJJC Reumatische aandoeningen in Nederland: Ervaringen en kengetallen. *NIVEL, Utrecht* (2016).

5 Silman, A. J. & Pearson, J. E. Epidemiology and genetics of rheumatoid arthritis. *Arthritis Res* **4 Suppl 3**, S265-272, doi:10.1186/ar578 (2002).

6 Smolen, J. S., Aletaha, D. & McInnes, I. B. Rheumatoid arthritis. *Lancet* **388**, 2023-2038, doi:10.1016/S0140-6736(16)30173-8 (2016).

7 Aletaha, D. *et al.* 2010 Rheumatoid arthritis classification criteria: an American College of Rheumatology/European League Against Rheumatism collaborative initiative. *Arthritis and rheumatism* **62**, 2569-2581, doi:10.1002/art.27584 (2010).

8 Arnett, F. C. *et al.* The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis and rheumatism* **31**, 315-324 (1988).

9 van der Linden, M. P., Knevel, R., Huizinga, T. W. & van der Helm-van Mil, A. H. Classification of rheumatoid arthritis: comparison of the 1987 American College of Rheumatology criteria and the 2010 American College of Rheumatology/European League Against Rheumatism criteria. *Arthritis and rheumatism* **63**, 37-42, doi:10.1002/art.30100 (2011).

10 Humphreys, J. H. *et al.* The incidence of rheumatoid arthritis in the UK: comparisons using the 2010 ACR/EULAR classification criteria and the 1987 ACR classification criteria. Results from the Norfolk Arthritis Register. *Annals of the rheumatic diseases* **72**, 1315-1320, doi:10.1136/annrheumdis-2012-201960 (2013).

11 Mjaavatten, M. D. & Bykerk, V. P. Early rheumatoid arthritis: the performance of the 2010 ACR/EULAR criteria for diagnosing RA. *Best practice & research. Clinical rheumatology* **27**, 451-466, doi:10.1016/j.berh.2013.09.001 (2013).

12 van Nies, J. A. *et al.* What is the evidence for the presence of a therapeutic window of opportunity in rheumatoid arthritis? A systematic literature review. *Annals of the rheumatic diseases* **73**, 861-870, doi:10.1136/annrheumdis-2012-203130 (2014).

13 Burmester, G. R. & Pope, J. E. Novel treatment strategies in rheumatoid arthritis. *Lancet* **389**, 2338-2348, doi:10.1016/s0140-6736(17)31491-5 (2017).

14 MacGregor, A. J. *et al.* Characterizing the quantitative genetic contribution to rheumatoid arthritis using data from twins. *Arthritis and rheumatism* **43**, 30-37, doi:10.1002/1529-0131(200001)43:1<30::aid-anr5>3.0.co;2-b (2000).

15 Nepom, G. T. Major histocompatibility complex-directed susceptibility to rheumatoid arthritis. *Advances in immunology* **68**, 315-332 (1998).

16 Lundstrom, E. *et al.* Opposing effects of HLA-DRB1*13 alleles on the risk of developing anti-citrullinated protein antibody-positive and anti-citrullinated protein antibody-negative rheumatoid arthritis. *Arthritis and rheumatism* **60**, 924-930, doi:10.1002/art.24410 (2009).

17 Messemaker, T. C., Huizinga, T. W. & Kurreeman, F. Immunogenetics of rheumatoid arthritis: Understanding functional implications. *Journal of autoimmunity* **64**, 74-81, doi:10.1016/j.jaut.2015.07.007 (2015).

18 Sugiyama, D. *et al.* Impact of smoking as a risk factor for developing rheumatoid arthritis: a meta-analysis of observational studies. *Annals of the rheumatic diseases* **69**, 70-81, doi:10.1136/ard.2008.096487 (2010).

19 Costenbader, K. H. & Karlson, E. W. Cigarette smoking and autoimmune disease: what can we learn from epidemiology? *Lupus* **15**, 737-745, doi:10.1177/0961203306069344 (2006).

20 Hoovestol, R. A. & Mikuls, T. R. Environmental exposures and rheumatoid arthritis risk. *Current rheumatology reports* **13**, 431-439, doi:10.1007/s11926-011-0203-9 (2011).

21 Kohm, A. P., Fuller, K. G. & Miller, S. D. Mimicking the way to autoimmunity: an evolving theory of sequence and structural homology. *Trends in microbiology* **11**, 101-105 (2003).

22 van Heemst, J. *et al.* Crossreactivity to vinculin and microbes provides a molecular basis for HLA-based protection against rheumatoid arthritis. *Nature communications* **6**, 6681, doi:10.1038/ncomms7681 (2015).

23 Catrina, A. I., Ytterberg, A. J., Reynisdottir, G., Malmstrom, V. & Klareskog, L. Lungs, joints and immunity against citrullinated proteins in rheumatoid arthritis. *Nature reviews. Rheumatology* **10**, 645-653, doi:10.1038/nrrheum.2014.115 (2014).

24 Reynisdottir, G. *et al.* Signs of immune activation and local inflammation are present in the bronchial tissue of patients with untreated early rheumatoid arthritis. *Annals of the rheumatic diseases* **75**, 1722-1727, doi:10.1136/annrheumdis-2015-208216 (2016).

25 Darrah, E. & Andrade, F. NETs: the missing link between cell death and systemic autoimmune diseases? *Frontiers in immunology* **3**, 428, doi:10.3389/fimmu.2012.00428 (2012).

26 Thieblemont, N., Wright, H. L., Edwards, S. W. & Witko-Sarsat, V. Human neutrophils in auto-immunity. *Seminars in immunology* **28**, 159-173, doi:10.1016/j.smim.2016.03.004 (2016).

27 Darrah, E. & Andrade, F. Editorial: citrullination, and carbamylation, and malondialdehyde-acetaldehyde! Oh my! Entering the forest of autoantigen modifications in rheumatoid arthritis. *Arthritis & rheumatology (Hoboken, N.J.)* **67**, 604-608, doi:10.1002/art.38970 (2015).

28 Corsiero, E., Pratesi, F., Prediletto, E., Bombardieri, M. & Migliorini, P. NETosis as Source of Autoantigens in Rheumatoid Arthritis. *Frontiers in immunology* **7**, 485, doi:10.3389/fimmu.2016.00485 (2016).

29 Cope, A. P. T cells in rheumatoid arthritis. *Arthritis research & therapy* **10 Suppl 1**, S1, doi:10.1186/ar2412 (2008).

30 Cope, A. P., Schulze-Koops, H. & Aringer, M. The central role of T cells in rheumatoid arthritis. *Clinical and experimental rheumatology* **25**, S4-11 (2007).

31 Takemura, S. *et al.* Lymphoid neogenesis in rheumatoid synovitis. *Journal of immunology (Baltimore, Md. : 1950)* **167**, 1072-1080 (2001).

32 Edwards, J. C. *et al.* Efficacy of B-cell-targeted therapy with rituximab in patients with rheumatoid arthritis. *The New England journal of medicine* **350**, 2572-2581, doi:10.1056/NEJMoa032534 (2004).

33 Waaler, E. On the occurrence of a factor in human serum activating the specific agglutintion of sheep blood corpuscles. 1939. *APMIS : acta pathologica, microbiologica, et immunologica Scandinavica* **115**, 422-438; discussion 439, doi:10.1111/j.1600-0463.2007.apm_682a.x (2007).

34 Nishimura, K. *et al.* Meta-analysis: diagnostic accuracy of anti-cyclic citrullinated peptide antibody and rheumatoid factor for rheumatoid arthritis. *Annals of internal medicine* **146**, 797-808 (2007).

35 Vander Cruyssen, B. *et al.* Anti-citrullinated protein/peptide antibodies (ACPA) in rheumatoid arthritis: specificity and relation with rheumatoid factor. *Autoimmunity reviews* **4**, 468-474, doi:10.1016/j.autrev.2005.04.018 (2005).

36 Kroot, E. J. *et al.* The prognostic value of anti-cyclic citrullinated peptide antibody in patients with recent-onset rheumatoid arthritis. *Arthritis and rheumatism* **43**, 1831-1835, doi:10.1002/1529-0131(200008)43:8<1831::aid-anr19>3.0.co;2-6 (2000).
37 van der Helm-van Mil, A. H., Verpoort, K. N., Breedveld, F. C., Toes, R. E. & Huizinga, T. W. Antibodies to citrullinated proteins and differences in clinical progression of rheumatoid arthritis. *Arthritis research & therapy* **7**, R949-958, doi:10.1186/ar1767 (2005).
38 Willemze, A. *et al.* The ACPA recognition profile and subgrouping of ACPA-positive RA patients. *Annals of the rheumatic diseases* **71**, 268-274, doi:10.1136/annrheumdis-2011-200421 (2012).
39 van Beers, J. J. *et al.* ACPA fine-specificity profiles in early rheumatoid arthritis patients do not correlate with clinical features at baseline or with disease progression. *Arthritis research & therapy* **15**, R140, doi:10.1186/ar4322 (2013).
40 Gronwall, C. *et al.* Autoreactivity to malondialdehyde-modifications in rheumatoid arthritis is linked to disease activity and synovial pathogenesis. *Journal of autoimmunity* **84**, 29-45, doi:10.1016/j.jaut.2017.06.004 (2017).
41 Figueiredo, C. P. *et al.* Antimodified protein antibody response pattern influences the risk for disease relapse in patients with rheumatoid arthritis tapering disease modifying antirheumatic drugs. *Annals of the rheumatic diseases* **76**, 399-407, doi:10.1136/annrheumdis-2016-209297 (2017).
42 Juarez, M. *et al.* Identification of novel antiacetylated vimentin antibodies in patients with early inflammatory arthritis. *Annals of the rheumatic diseases* **75**, 1099-1107, doi:10.1136/annrheumdis-2014-206785 (2016).
43 Shi, J. *et al.* Autoantibodies recognizing carbamylated proteins are present in sera of patients with rheumatoid arthritis and predict joint damage. *Proceedings of the National Academy of Sciences of the United States of America* **108**, 17372-17377, doi:10.1073/pnas.1114465108 (2011).
44 Ryan, W. L. & Wells, I. C. HOMOCITRULLINE AND HOMOARGININE SYNTHESIS FROM LYSINE. *Science (New York, N.Y.)* **144**, 1122-1127 (1964).
45 Fluckiger, R., Harmon, W., Meier, W., Loo, S. & Gabbay, K. H. Hemoglobin carbamylation in uremia. *The New England journal of medicine* **304**, 823-827, doi:10.1056/nejm198104023041406 (1981).
46 Wang, Z. *et al.* Protein carbamylation links inflammation, smoking, uremia and atherogenesis. *Nature medicine* **13**, 1176-1184, doi:10.1038/nm1637 (2007).
47 Kalim, S., Karumanchi, S. A., Thadhani, R. I. & Berg, A. H. Protein carbamylation in kidney disease: pathogenesis and clinical implications. *American journal of kidney diseases : the official journal of the National Kidney Foundation* **64**, 793-803, doi:10.1053/j.ajkd.2014.04.034 (2014).
48 Shi, J. *et al.* Carbamylation and antibodies against carbamylated proteins in autoimmunity and other pathologies. *Autoimmunity reviews* **13**, 225-230, doi:10.1016/j.autrev.2013.10.008 (2014).
49 Koeth, R. A. *et al.* Protein carbamylation predicts mortality in ESRD. *Journal of the American Society of Nephrology : JASN* **24**, 853-861, doi:10.1681/asn.2012030254 (2013).
50 Desmons, A. *et al.* Homocitrulline: a new marker for differentiating acute from chronic renal failure. *Clinical chemistry and laboratory medicine* **54**, 73-79, doi:10.1515/cclm-2015-0398 (2016).
51 Jaisson, S. *et al.* Homocitrulline as marker of protein carbamylation in hemodialyzed patients. *Clinica chimica acta; international journal of clinical chemistry* **460**, 5-10, doi:10.1016/j.cca.2016.06.009 (2016).

52 Tang, W. H. *et al.* Protein carbamylation in chronic systolic heart failure: relationship with renal impairment and adverse long-term outcomes. *Journal of cardiac failure* **19**, 219-224, doi:10.1016/j.cardfail.2013.02.001 (2013).

53 Jaisson, S. *et al.* Increased serum homocitrulline concentrations are associated with the severity of coronary artery disease. *Clinical chemistry and laboratory medicine* **53**, 103-110, doi:10.1515/cclm-2014-0642 (2015).

54 Gorisse, L. *et al.* Protein carbamylation is a hallmark of aging. *Proceedings of the National Academy of Sciences of the United States of America* **113**, 1191-1196, doi:10.1073/pnas.1517096113 (2016).

55 Turunen, S. *et al.* Different amounts of protein-bound citrulline and homocitrulline in foot joint tissues of a patient with anti-citrullinated protein antibody positive erosive rheumatoid arthritis. *Journal of translational medicine* **11**, 224, doi:10.1186/1479-5876-11-224 (2013).

56 Koro, C. *et al.* Carbamylation of immunoglobulin abrogates activation of the classical complement pathway. *European journal of immunology* **44**, 3403-3412, doi:10.1002/eji.201444869 (2014).

57 Ok, E., Basnakian, A. G., Apostolov, E. O., Barri, Y. M. & Shah, S. V. Carbamylated low-density lipoprotein induces death of endothelial cells: a link to atherosclerosis in patients with kidney disease. *Kidney international* **68**, 173-178, doi:10.1111/j.1523-1755.2005.00391.x (2005).

58 Muller, P. C. *et al.* Anticarbamylated protein (anti-CarP) antibodies are present in sera of juvenile idiopathic arthritis (JIA) patients. *Annals of the rheumatic diseases* **72**, 2053-2055, doi:10.1136/annrheumdis-2013-203650 (2013).

59 Chimenti, M. S. *et al.* Auto-reactions, autoimmunity and psoriatic arthritis. *Autoimmunity reviews* **14**, 1142-1146, doi:10.1016/j.autrev.2015.08.003 (2015).

60 Bergum, B. *et al.* Antibodies against carbamylated proteins are present in primary Sjogren's syndrome and are associated with disease severity. *Annals of the rheumatic diseases* **75**, 1494-1500, doi:10.1136/annrheumdis-2015-207751 (2016).

61 Shi, J. *et al.* The specificity of anti-carbamylated protein antibodies for rheumatoid arthritis in a setting of early arthritis. *Arthritis research & therapy* **17**, 339, doi:10.1186/s13075-015-0860-6 (2015).

62 Shi, J. *et al.* Anti-carbamylated protein (anti-CarP) antibodies precede the onset of rheumatoid arthritis. *Annals of the rheumatic diseases* **73**, 780-783, doi:10.1136/annrheumdis-2013-204154 (2014).

63 Shi, J. *et al.* Anti-carbamylated protein antibodies are present in arthralgia patients and predict the development of rheumatoid arthritis. *Arthritis and rheumatism* **65**, 911-915, doi:10.1002/art.37830 (2013).

64 Jiang, X. *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. *Annals of the rheumatic diseases* **73**, 1761-1768, doi:10.1136/annrheumdis-2013-205109 (2014).