Clinical and etiological meaning of anti-carbamylated protein antibodies in rheumatoid arthritis

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ABSTRACT
Several autoantibodies against proteins with post-translational modifications have been detected in patients with rheumatoid arthritis (RA) and are called anti-modified protein antibodies (AMPAs). Anti-carbamylated protein antibodies (Anti-CarP Ab) are the second most vigorously researched AMPAs following anti-citrullinated protein/peptide antibodies (ACPA). Anti-CarP Ab and ACPA show cross-reactivity to some extent and frequently co-exist with each other in RA, but are two distinct antibodies. Although the diagnostic efficacy of anti-CarP Ab is inferior to that of ACPA, the diagnostic specificity of RA may improve when used in combination with ACPA and rheumatoid factor. Anti-CarP Ab and ACPA are also useful for identifying patients at high risk of more severe joint destruction and cardiovascular diseases. The high prevalence of the co-existence of both antibodies suggests a common factor in their production, and this is important for the development of RA because both antibodies emerge before the onset of clinical symptoms. Neutrophils may also be crucially involved. It is important to distinguish citrullinated antigens from carbamylated antigens because the methods commonly used to detect the former are now known to be cross-reactive with the latter. Research on anti-CarP Ab will provide novel insights into the pathology and etiology of RA.

ARTICLE HISTORY
Received 24 August 2018
Accepted 4 September 2018

KEYWORDS
Anti-modified protein antibodies; anti-carbamylated protein antibodies; autoantibodies; rheumatoid arthritis; neutrophil; post-translational modification

1. Introduction
Autoantibodies are important biomarkers of rheumatoid arthritis (RA). Anti-citrullinated protein/peptide antibodies (ACPA) are most widely used in daily clinical practice, along with rheumatoid factor (RF). They are the antibodies of proteins with citrullination, which is one of the post-translational modifications of proteins in which arginine is converted to citrulline by the catalysis of peptidyl arginine deiminase (PAD).

Several autoantibodies against proteins with other post-translational modifications have recently been reported, and are called anti-modified protein antibodies (AMPAs) [1–4]. The second most researched AMPAs following ACPA are anti-carbamylated protein antibodies (anti-CarP Ab), which were reported by Shi et al. [1]. They are the antibodies of proteins with carbamylation. This post-translational modification involves the conversion of lysine to homocitrulline, which is irreversibly induced by cyanate. Cyanate is in equilibrium with urea in blood, and, thus, the carbamylation of proteins commonly occurs in end-stage renal disease patients [5]. On the other hand, thiocyanate, which is taken into the body by cigarette smoking or eating brassica vegetables [6,7], is catalyzed into cyanate by myeloperoxidase (MPO) and hydrogen peroxide, and this induces carbamylation in an inflammatory milieu [8].

Citrullination and carbamylation both cause the loss of charges on lysine and arginine, which may, in turn, affect the 3D structures and solubilities of proteins. Homocitrulline and citrulline themselves are very similar to each other (Figure 1). Therefore, the possibility of a cross-reaction is a cause of great concern. Previous studies reported that anti-CarP Ab and ACPA are cross-reactive to some extent [9–11]. However, since many patients who have only anti-CarP Ab or ACPA [1], or both without showing cross-reactivity [9], they are regarded as similar but different antibodies.

The characteristics, clinical efficacy and etiological meaning of anti-CarP Ab in RA, sometimes with comparisons with ACPA, have been discussed herein.
2. Characteristics of anti-CarP Ab

2.1. Target antigens of anti-CarP Ab

Anti-CarP Ab is generally detected by ELISA using carbamylated fetal calf serum (FCS) as the immobilized antigen on plates following Shi et al.’s method [1]. Shi et al. selected FCS because it contains many types of proteins and, thus, was considered to be suitable for screening [1]. After this study, fibrinogen, vimentin, alpha-enolase and 78-kDa glucose-regulated protein (GRP78/BIP) were identified as target antigens of anti-CarP Ab [1,3,10,12–14]. However, they are also target antigens of ACPA. Since cross-reactivity between anti-CarP Ab and ACPA is a matter of great concern, they may have been detected as a result of the cross-reaction. Accordingly, the detection of carbamylation-specific antigens was anticipated. In 2017, albumin and alpha 1 anti-trypsin were identified as anti-CarP-specific antigens [15,16]. However, it currently remains unclear whether these carbamylation-specific antigens are the most important antigens of anti-CarP. This is similar that the principal antigen of ACPA also has not yet been identified. Although there have been no studies on the epitope spreading of anti-CarP Ab, which is observed in ACPA [17], anti-CarP Ab react with many antigens, as demonstrated by the many bands detected by Western blotting [15]. On the other hand, common antigens of ACPA and anti-CarP Ab may have been detected not as a result of a cross-reaction. Given that the mechanisms of antigen production involved common factors, which will be described below, they may be true ‘double-modified’ antigens.

2.2. Isotype and avidity of anti-CarP Ab

In the first study in 2011, Shi et al. [1] reported that IgG- and IgA-anti-CarP Ab were detected in RA patients. A recent study demonstrated that IgM-anti-CarP Ab and all subtypes of IgG-anti-CarP Ab were detectable [18]. These findings clearly showed that class-switching occurs and suggested that continuous antigen presentation exists in RA.

On the other hand, the avidity of anti-CarP Ab is lower than that of the anti-tetanus toxoid antibody [19]. While the avidity of ACPA is also low [20], that of anti-CarP Ab is lower than that of ACPA [19]. These findings raise questions about the maturation of these antibodies. Affinity (avidity) maturation and class switching are generally coupled; however, this does not appear to be applicable to the development of ACPA and anti-CarP Ab. The mechanisms responsible for the induction of auto-antibodies in RA may differ from those for the induction of antibodies against extrinsic pathogens.

3. Clinical aspect

3.1. Diagnostic efficacy of anti-CarP, particularly in ACPA-negative RA

Anti-CarP Ab have been attracting increasing attention as a new diagnostic biomarker of RA because
they are detected in 8–16% of ACPA-negative RA patients [1,21]. According to a recent meta-analysis, sensitivity and specificity were 42 and 96%, respectively [22]. However, high specificity was achieved using a healthy population as the control. Since the target population in clinical settings is patients with arthritis or at least arthralgia, efficacy needs to be assessed in these patients and the prevalence of anti-CarP Ab in other rheumatic diseases needs to be clarified.

Shi et al. assessed the diagnostic efficacy of the combined measurement of anti-CarP Ab, ACPA and RF in patients with arthritis in at least one joint with a duration of less than 2 years [23]. In this cohort, 47% of patients were classified as having RA while the others were classified as having, for example, undifferentiated arthritis, psoriatic arthritis and osteoarthritis. The sensitivity and specificity of anti-CarP Ab in this cohort were 44 and 89%, respectively. However, most patients with anti-CarP Ab also have ACPA or RF, and its sensitivity and specificity were less than those of ACPA and RF. Furthermore, anti-CarP Ab was detected in approximately 10% of the control group, and the AUC of anti-CarP Ab in the ACPA-negative group was 0.52 in a receiver operator curve (ROC) analysis.

On the other hand, anti-CarP Ab may be detected in 9.1–22.0% of patients with systemic lupus erythematosus (SLE) [24,25], and in 26.9–35.6% of those with primary Sjögren’s syndrome (SS) [25,26]. These percentages are similar to the prevalence of anti-CarP Ab in ACPA-negative RA. In addition, anti-CarP levels are lower in ACPA-negative RA than in ACPA-positive RA and similar to those of other rheumatic diseases [25]. We also performed an ROC analysis, and the AUC of anti-Car Ab in the ACPA-negative group in our rheumatic disease cohort was 0.46 [25].

Therefore, anti-CarP Ab cannot be used to diagnose ACPA-negative RA.

3.2. Other possibilities for anti-CarP Ab in clinical settings

Although anti-CarP Ab cannot be applied to the diagnosis of ACPA-negative RA as expected, they may be useful for other purposes.

One of its applications is pre-clinical diagnoses in population screening. Anti-CarP Ab becomes detectable before the onset of RA, similar to ACPA [27–31]. Even if a treatment option is available that effectively stops the development of RA before its onset, careful elimination of people who will not develop RA is important because they cannot accept the potential risk of side effects and the cost. Accordingly, the specificity of the diagnosis rather than the sensitivity is important in this situation. The combined assessment of anti-CarP Ab, ACPA and RF will fit this need because it may increase the specificity of the diagnosis of RA, but with reductions in sensitivity [23]. Since ACPA may be detected in 5% of the healthy population [32], the addition of anti-CarP Ab may be useful for discriminating future RA from an ACPA-positive healthy population.

Another promising usage is the discrimination of patients with a poor prognosis. Anti-CarP Ab is a risk factor for future joint destruction independent of ACPA [1,33–37]. A previous study reported that the anti-carbamylated albumin antibody was associated with a history of cardiovascular disease (CVD) [15] and anti-CarP Ab was associated with multiple indices of arteriosclerosis [38]. The intensity of treatment and screening plans for CVD may be modulated in accordance with the antibody profile.

3.3. Possibility of better methods for the detection of anti-CarP Ab

Antibody detection assays themselves may be improved. The most commonly used assay is currently direct ELISA in which carbamylated FCS is immobilized onto plates; however, this may not be the optimal method for the detection of anti-CarP Ab. A previous study reported that ELISA using a synthetic cyclic peptide with homocitrulline (HomoCitJED) improved diagnostic efficacy [11]. Diagnostic efficacy may be heightened by selecting promising synthetic carbamylated peptides, such as the second generation cyclic citrullinated peptide (CCP2) for ACPA.

4. Factors potentially involved in anti-CarP Ab production

4.1. Involvement of neutrophils in the production of antigens

Although the co-existence of anti-CarP Ab and ACPA abrogates the diagnostic efficacy of anti-CarP Ab, it provides interesting insights into the etiology of RA. Previous studies showed not only that anti-CarP Ab and ACPA frequently co-exist [21,23,39,40], but also that anti-CarP Ab precedes the onset of RA, similar to ACPA [27–31], and the co-existence of these antibodies was limited to RA [25]. Furthermore, citrullinated and carbamylated proteins co-exist in rheumatoid nodules and synovial tissue [41]. These findings suggest the presence of a common factor in the development of these antibodies and its relationship with the etiology of RA.
Therefore, neutrophil extracellular traps (NETs) are attracting increasing attention. This is one of the reactions of neutrophils against infection, in which they release web-like chromatin fibers with an abundance of bactericidal proteins, such as MPO, lactoferrin and LL37 [42,43]. Peptidylarginine deiminase 4 (PAD4) is considered to play an important role in NETs [44], and previous studies reported that NET fibers are a source of citrullinated antigens [45-50].

On the other hand, MPO is the key enzyme for in vivo carbamylation [8], and serum MPO levels are known to be associated with the presence of the anti-carbamylated albumin antibody [15]. Neutrophils activated by phorbol-12-myristate-13-acetate (PMA) in vitro may carbamylate albumin in culture medium [51].

These findings suggest that neutrophils are a key player in the induction of these antibodies and also in the subsequent development of RA.

4.2. HLA

ACPA is strongly associated with several HLA-DR4 haplotypes with a common amino acid sequence, which is called the shared epitope (SE) [52-55]. Although few genomic association studies have been conducted between HLA-DR4 SE and anti-CarP Ab, anti-CarP Ab was shown to be associated with the HLA-DR4 SE allele, similar to ACPA.

Although Jiang et al. [21] denied the relationship between anti-CarP Ab and HLA-DR4 SE using 2 RA cohorts, caution is needed because they attempted to confirm this relationship in an ACPA-negative group. In simple calculations using the data provided in their study, anti-CarP Ab was associated with HLA-SE.

On the other hand, Scinocca et al. [12] suggested using the findings of in silico experiments showing that carbamylated fibrinogen binds to SE. A recent study reported that the positivity of HLA-SE was greater in anti-CarP IgG patients with more isotypes and subtypes of anti-CarP Ig [18].

Although further studies are needed, it is reasonable to assume that anti-CarP Ab is also associated with HLA-DR4 SE.

5. Technical issues for discriminating citrullinated antigens from carbamylated antigens

As described above, citrullination and carbamylation are closely related with each other. Therefore, careful discrimination is required when evaluating the roles of these post-translational modifications in RA. However, a difficulty is associated with distinguishing citrullinated proteins from carbamylated proteins: not only the cross-reaction of antibodies in patient serum, but also that of commercially available antibodies for experiments. Although the anti-modified citrulline (AMC)-Senshu antibody [56] and antibody against the deca-citrulline peptide (F95) [57] have been used in many studies on citrullinated antigens, both were shown to bind to carbamylated antigens [9,58,59]. These findings indicate the potential misinterpretation of carbamylated antigens as citrullinated antigens in many previous studies because citrullinated proteins and carbamylated proteins may both be produced by activated neutrophils, including NETs-forming neutrophils, as discussed above.

While several other methods for detecting citrullination have been reported [60], the most reliable is mass spectrometry (MS); however, this involves a detailed procedure to identify citrullinated proteins from many other proteins (reviewed by Verheul et al. [59]). High-performance liquid chromatography (HPLC) is also useful for focusing on one specific protein and discriminating citrullination from carbamylation [61].

On the other hand, carbamylated proteins are specifically detected by the anti-carbamyl-lysine (CBL) antibody, which was reported to not show cross-reactivity against citrullinated proteins [59]. Therefore, methods need to be carefully selected, particularly for the detection of citrullinated proteins. At least, the possibility of the presence of carbamylated antigens needs to be excluded using the anti-CBL antibody, when antibody-based methods are used.

6. Anti-CarP Ab in animal models

While ACPA is generally not detected in RA model mice, a previous study reported that anti-CarP Ab was present in collagen-induced arthritis (CIA) before the appearance of clinical symptoms [29]. CIA is one of the most commonly used models of RA, and is induced by an injection of type II collagen with complete Freund’s adjuvant (CFA). Anti-CarP Ab were detected in mice that did not develop arthritis, or even in those injected with CFA only; however, the levels of anti-CarP Ab in these mice were significantly lower than those in mice that developed arthritis. This finding may be equivalent to human data showing that anti-CarP Ab are detected in other connective tissue diseases, but at lower levels than that of RA [25].

On the other hand, Mydel et al. [62] reported that immunization with the citrullinated peptide did not induce the production of antibodies against it, whereas immunization with the carbamylated peptide did. Furthermore, mice immunized with the
carbamylated peptide developed severe arthritis after an intra-articular injection of the citrullinated peptide, while those immunized with the citrullinated peptide did not develop arthritis after the injection of the citrullinated or carbamylated peptide. They subsequently reported that the transfer of lymphocytes from carbamylated peptide-immunized mice to naïve mice induced arthritis, whereas those from citrullinated peptide-immunized mice did not. They also showed that sera containing the antibody against the carbamylated peptide did not induce arthritis when it was transferred to naïve mice.

These findings suggest that anti-CarP Ab is more easily induced than ACPA, at least in mice, and CarP antigens trigger immune responses more strongly than citrullinated antigens, whereas anti-CarP Ab itself is not arthrogenic.

7. Conclusions

Anti-CarP Ab is a distinct antibody from, but frequently co-exists with ACPA. The diagnostic efficacy of anti-CarP Ab in daily clinical practice is currently limited; however, it may be used for preclinical diagnoses and the selection of patients with a poor prognosis, and its diagnostic efficacy may be increased by improving assays for anti-CarP Ab.

While its co-existence with ACPA abrogates diagnostic efficacy, this provides us with a new viewpoint on the etiology and pathology of RA. Since anti-CarP Ab and ACPA may be detected before the onset of clinical symptoms, the common factor in their production must play an important role in the development of RA. Neutrophils may also be a key player.

Previous findings on citrullinated antigens may need to be reevaluated because several techniques cannot discriminate citrullinated arginine from carbamylated lysine. Further research is warranted.

Disclosure statement

No potential conflict of interest was reported by the author.

References

[1] Shi J, Knevel R, Suwannalai P, et al. Autoantibodies recognizing carbamylated proteins are present in sera of patients with rheumatoid arthritis and predict joint damage [Research Support, Non-U.S. Gov't]. Proc Natl Acad Sci USA. 2011;108:17372–17377.

[2] Thiele GM, Durpee MJ, Anderson DR, et al. Malondialdehyde-acetaldehyde adducts and antimalondialdehyde-acetaldehyde antibodies in rheumatoid arthritis. Arthritis Rheumatol. 2015;67:645–655.

[3] Juarez M, Bang H, Hammar F, et al. Identification of novel antiacylated vimentin antibodies in patients with early inflammatory arthritis. Ann Rheum Dis. 2016;75:1099–1107.

[4] Trouw LA, Rispens T, Toes REM. Beyond citrullination: other post-translational protein modifications in rheumatoid arthritis. Nat Rev Rheumatol. 2017;13:331–339.

[5] Oimomi M, Ishikawa K, Kawasaki T, et al. Plasma carbamylated protein in renal failure. N Engl J Med. 1983;308:655–656.

[6] Butts WC, Kueheman M, Widdowson GM. Automated method for determining serum thiocyanate, to distinguish smokers from nonsmokers. Clin Chem. 1974;20:1344–1348.

[7] Felker P, Bunch R, Leung AM. Concentrations of thiocyanate and goitrin in human plasma, their precursor concentrations in brassica vegetables, and associated potential risk for hypothyroidism. Nutr Rev. 2016;74:248–258.

[8] Wang Z, Nicholls SJ, Rodriguez ER, et al. Protein carbamylation links inflammation, smoking, uremia and atherogenesis [Comparative Study Research Support, N.I.H., Extramural]. Nat Med. 2007;13:1176–1184.

[9] Shi J, Willemze A, Jansen GM, et al. Recognition of citrullinated and carbamylated proteins by human antibodies: specificity, cross-reactivity and the ‘AMC-Senshu’ method. Ann Rheum Dis. 2013;72:148–150.

[10] Reed E, Jiang X, Kharlamova N, 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;18:96.

[11] Lac P, Racape M, Barra L, et al. Relatedness of antibodies to peptides containing homocitrulline or citrulline in patients with rheumatoid arthritis. J Rheumatol. 2018;45:302–309.

[12] Scinocca M, Bell DA, Racape M, et al. Anti-homocitrullinated fibrinogen antibodies are specific to rheumatoid arthritis and frequently bind citrullinated proteins/peptides. J Rheumatol. 2014;41:270–279.

[13] Martinez G, Gomez JA, Bang H, et al. Carbamylated vimentin represents a relevant autoantigen in Latin American (Cuban) rheumatoid arthritis patients. Rheumatol Int. 2016;36:781–791.

[14] Yu HC, Lai PH, Lai NS, et al. Increased serum levels of anti-carbamylated 78-kDa glucose-regulated protein antibody in patients with rheumatoid arthritis. Int J Mol Sci. 2016;17. DOI:10.3390/ijms17091510

[15] Nakabo S, Hashimoto M, Ito S, et al. Carbamylated albumin is one of the target antigens of anti-carbamylated protein antibodies. Rheumatology (Oxford). 2017;56:1217–1226.

[16] Verheul MK, Yee A, Seaman A, et al. Identification of carbamylated alpha 1 anti-trypsin (A1AT) as an antigenic target of anti-CarP antibodies in patients with rheumatoid arthritis. J Autoimmun. 2017;80:77–84.

[17] Sokolove J, Bromberg R, Deane KD, et al. Autoantibody epitope spreading in the pre-clinical phase predicts progression to rheumatoid arthritis.
van Delft MAM, Verheul MK, Burgers LE, et al. The isotype and IgG subclass distribution of anti-carbamylated protein antibodies in rheumatoid arthritis patients. Arthritis Res Ther. 2017;19:190.

Suwannalai P, Scherer HU, van der Woude D, Jiang X, Trouw LA, van Wesemael TJ, et al. Anti-carbamylated protein antibody response is of overall low avidity despite extensive isotype switching. Rheumatology (Oxford). 2018;57:1583–1591.

van Delft MAM, Verheul MK, Burgers LE, et al. The anti-carbamylated protein antibody response is of overall low avidity despite extensive isotype switching. Rheumatology (Oxford). 2018;57:1583–1591.

Ziegelasch M, van Delft MA, Wallin P, et al. Antibodies against carbamylated proteins and cyclic citrullinated peptides in systemic lupus erythematosus: results from two well-defined European cohorts. Arthritis Res Ther. 2016;18:289.

Shi J, van Steenbergen HW, van Nies JA, et al. The specificity of anti-carbamylated protein antibodies for rheumatoid arthritis in a setting of early arthritis. Arthritis Res Ther. 2015;17:339.

Li L, Deng C, Chen S, et al. Meta-analysis: diagnostic accuracy of anti-carbamylated protein antibody for rheumatoid arthritis. PLoS One. 2016;11:e0159000.

Jiang X, Trouw LA, van Wesemael TJ, 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;73:1761–1768.

Suwannalai P, Scherer HU, van der Woude D, et al. Anti-citrullinated protein antibodies have a low avidity compared with antibodies against recall antigens. Ann Rheum Dis. 2011;70:373–379.

Shi J, van Steenbergen HW, van Nies JA, et al. The specificity of anti-carbamylated protein antibodies for rheumatoid arthritis in a setting of early arthritis. Arthritis Res Ther. 2015;17:339.

Ziegelasch M, van Delft MA, Wallin P, et al. Antibodies against carbamylated proteins and cyclic citrullinated peptides in systemic lupus erythematosus: results from two well-defined European cohorts. Arthritis Res Ther. 2016;18:289.

Nakabo S, Yoshifuji H, Hashimoto M, et al. Antibodies against carbamylated protein antibodies are detectable in various connective tissue diseases. J Rheumatol. 2017;44:1384–1388.

Bergum B, Koro C, Delaleu N, et al. Antibodies against carbamylated proteins are present in primary Sjögren’s syndrome and are associated with disease severity. Ann Rheum Dis. 2016;75:1494–1500.

Shi J, van de Stadt LA, Levarht EW, et al. Anti-carbamylated protein antibodies are present in arthralgia patients and predict the development of rheumatoid arthritis. Arthritis Rheum. 2013;65:911–915.

Shi J, van de Stadt LA, Levarht EW, et al. Anti-carbamylated protein (anti-CarP) antibodies precede the onset of rheumatoid arthritis. Ann Rheum Dis. 2014;73:780–783.

Stoop JN, Liu BS, Shi J, et al. Antibodies specific for carbamylated proteins precede the onset of clinical symptoms in mice with collagen induced arthritis. PLoS One. 2014;9:e102163.

Brink M, Verheul MK, Ronnelid J, 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.

Gan RW, Trouw LA, Shi J, et al. Anti-carbamylated protein antibodies are present prior to rheumatoid arthritis and are associated with its future diagnosis. J Rheumatol. 2015;42:572–579.
autoantigens and stimulate inflammatory responses in rheumatoid arthritis. Sci Transl Med. 2013;5:178ra40.

[46] Dwivedi N, Neeli I, Schall N, et al. Deimination of linker histones links neutrophil extracellular trap release with autoantibodies in systemic autoimmunity. FASEB J. 2014;28:2840–2851.

[47] Dwivedi N, Radic M. Citrullination of autoantigens implicates NEToxis in the induction of autoimmunity [Review]. Ann Rheum Dis. 2014;73:483–491.

[48] Wright HL, Moots RJ, Edwards SW. The multifactorial role of neutrophils in rheumatoid arthritis. Nat Rev Rheumatol. 2014;10:593–601.

[49] Spengler J, Lugonja B, Ytterberg AJ, et al. Release of active peptidyl arginine deiminases by neutrophils can explain production of extracellular citrullinated autoantigens in rheumatoid arthritis synovial fluid. Arthritis. Rheumatol. 2015;67:3135–3145.

[50] Carmona-Rivera C, Carlucci PM, Moore E, et al. Synovial fibroblast-neutrophil interactions promote pathogenic adaptive immunity in rheumatoid arthritis. Sci Immunol. 2017;2. doi:10.1126/sciimmunol.aag3358.

[51] Nakabo S, Ohmura K, Akizuki S, et al. Protein carbamylation is induced by activated neutrophil: ex vivo analysis [abstract]. Arthritis Rheumatol. 2017;69(Supplement S10):479.

[52] Hill JA, Southwood S, Sette A, et al. Cutting edge: the conversion of arginine to citrulline allows for a high-affinity peptide interaction with the rheumatoid arthritis-associated HLA-DRB1*0401 MHC class II molecule. J Immunol. 2003;171:538–541.

[53] Huizinga TW, Amos CI, van der Helm-van Mil AH, et al. Refining the complex rheumatoid arthritis phenotype based on specificity of the HLA-DRB1 shared epitope for antibodies to citrullinated proteins. Arthritis Rheum. 2005;52:3433–3438.

[54] Irigoyen P, Lee AT, Wener MH, et al. Regulation of anti-cyclic citrullinated peptide antibodies in rheumatoid arthritis: contrasting effects of HLA-DRB3 and the shared epitope alleles. Arthritis Rheum. 2005;52:3813–3818.

[55] Taneda V, Behrens M, Basal E, et al. Delineating the role of the HLA-DR4 "shared epitope" in susceptibility versus resistance to develop arthritis. J Immunol. 2008;181:2869–2877.

[56] Senshu T, Sato T, Inoue T, et al. Detection of citrulline residues in deiminated proteins on polyvinylidene difluoride membrane. Anal Biochem. 1992;203:94–100.

[57] Nicholas AP, Whitaker JN. Preparation of a monoclonal antibody to citrullinated epitopes: its characterization and some applications to immunohistochemistry in human brain. Glia. 2002;37:328–336.

[58] Turunen S, Koivula M-K, Nicholas AP, et al. Homocitrulline: an analog and confounder related to citrulline. In: Nicholas AP, Bhattacharya SK, editors. Protein Deimination in Human Health and Disease. Springer; 2014. p. 367–376.

[59] Verheul MK, van Veelen PA, van Delft MAM, et al. Pitfalls in the detection of citrullination and carbamylation. Autoimmun Rev. 2018;17:136–141.

[60] Slade DJ, Subramanian V, Fuhrmann J, et al. Chemical and biological methods to detect post-translational modifications of arginine. Biopolymers. 2014;101:133–143.

[61] Turunen S, Koivula MK, Risteli L, et al. Anticitrulline antibodies can be caused by homocitrulline-containing proteins in rabbits. Arthritis Rheum. 2010;62:3345–3352.

[62] Mydel P, Wang Z, Brisslert M, et al. Carbamylation-dependent activation of T cells: a novel mechanism in the pathogenesis of autoimmune arthritis [Research Support, Non-U.S. Gov’t]. J Immunol. 2010;184:6882–6890.