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2017-07-24

https://doi.org/10.14989/doctor.k20611

Final publication is available at https://doi.org/10.1093/rheumatology/kex088. Shuichiro Nakabo, Motomu Hashimoto, Shinji Ito, Moritoshi Furu, Hiromu Ito, Takao Fujii, Hajime Yoshifuji, Yoshitaka Imura, Ran Nakashima, Kosaku Murakami, Nobuo Kuramoto, Masao Tanaka, Junko Satoh, Akihito Ishigami, Satoshi Morita, Tsuneyo Mimori, Koichiro Ohmura; Carbamylated albumin is one of the target antigens of anti-carbamylated protein antibodies, Rheumatology, Volume 56, Issue 7, 1 July 2017, Pages 1217-1226, https://doi.org/10.1093/rheumatology/kex088

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Carbamylated albumin is one of the target antigens of anti-carbamylated protein antibodies

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Short Title: Anti-carbamylated albumin antibody in RA

ABSTRACT

Objectives. Anti-carbamylated protein (anti-CarP) antibodies are detected in rheumatoid arthritis (RA) patients. Fetal calf serum is used as an antigen source in anti-CarP ELISA, and the precise target antigens have not been found. We aimed to identify the target antigens of anti-CarP antibodies.

Methods. Western blotting of anti-CarP antibodies was conducted. Anti-carbamylated
human albumin (CarALB) antibody was detected by in-house ELISA for 493 RA patients and 144 healthy controls (HCs). An inhibition ELISA of anti-CarP antibodies by CarALB and citrullinated albumin (citALB) was performed using 8 RA patients’ sera. Serum CarALB was detected by liquid chromatography-tandem mass spectrometry (LC/MS/MS), and the serum myeloperoxidase (MPO) level was measured by ELISA.

**Results.** We focused on carbamylated albumin because it corresponded to the size of the thickest band detected by Western blotting of anti-CarP antibodies. Anti-CarALB antibody was detected in 31.4% of RA patients, and the correlation of the titers between anti-CarALB and anti-CarP was much closer than that between anti-citALB and anti-cyclic citrullinated peptide (CCP) antibodies (ρ=0.59 and 0.16, respectively). The inhibition ELISA showed that anti-CarP antibodies were inhibited by CarALB, but not by citALB. CarALB was detected in RA patients’ sera by LC/MS/MS. The serum MPO level was correlated with disease activity and was higher in RA patients with anti-CarALB antibody than in those without.

**Conclusion.** We found that carbamylated albumin is a novel target antigen of anti-CarP antibodies, and it is the first reported target antigen that has not been reported as the target of anti-citrullinated protein antibody.

**Key words:** Rheumatoid arthritis, Autoantigens and Autoantibodies, Laboratory diagnosis, Neutrophils, Synovium

**KEY MESSAGES**

Anti-carbamylated human albumin antibody is detectable in 31.4% of rheumatoid arthritis patients.

Anti-carbamylated human albumin antibody is not the cross-reaction of ACPA.

The positivity of anti-carbamylated human albumin antibody was associated with high serum MPO level.
INTRODUCTION

Carbamylation is a post-translational modification of proteins introduced by cyanate, which is the non-enzymatic and irreversible conversion of lysine into homocitrulline [1]. Cyanate exists in equilibrium with urea in the blood and can be produced from thiocyanate by myeloperoxidase (MPO) [2]. Therefore, carbamylation occurs not only in uremic patients [3-5], but also at chronic inflammatory sites including those in atherosclerosis [2].

Recently, anti-carbamylated protein (anti-CarP) antibodies were reported to be new autoantibodies of rheumatoid arthritis (RA) [6]. They have drawn attention as new diagnostic biomarkers because they can be detected in RA patients without anti-citrullinated protein antibody (ACPA). Moreover, they have also been reported to be associated with disease severity [6, 7] and detected before the onset of RA development [8-10].

However, the precise target antigen of anti-CarP antibodies has not been elucidated. Anti-CarP antibodies are detected by ELISA and Western blotting in which carbamylated fetal calf serum (FCS) is used as a complex of the protein mixture [6]. Fibrinogen and vimentin have been reported as target antigens [6, 11-13], but they are also known as the target antigens of ACPA. Since homocitrulline closely resembles citrulline, the cross-reaction between anti-CarP antibodies and ACPA has been a concern. Actually, anti-CarP antibodies and ACPA co-exist frequently [6, 14], and show cross-reaction to some extent [15].

Therefore, it is desired to find new target antigens of anti-CarP antibodies that are not targets of ACPA in order to resolve the concern regarding cross-reaction and evaluate the role of anti-CarP antibodies.

In this study, we focused on carbamylated human albumin (CarALB) as a target antigen of anti-CarP antibodies, and attempted to identify the factors that are related to anti-CarALB antibody.
METHODS

Patients and clinical information

A total of 493 RA patients were recruited from Kyoto University Hospital and their clinical information was obtained from the KURAMA (Kyoto University Rheumatoid Arthritis Management Alliance) database, which was established in 2011 and contains detailed clinical information added yearly. One-hundred-and-forty-four control sera being unlinkably anonymized were obtained from healthy donors. Written informed consent was obtained from the participants to enroll in this database. All the data were analyzed anonymously. This study was designed in accordance with the Helsinki Declaration, and was approved by Kyoto University Graduate School and Faculty of Medicine, Ethics Committee (approval number: E1308).

Protein preparation

Fetal calf serum (FCS; Biowest, Nuaille, France), bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, USA) and human albumin (ALB; Sigma-Aldrich) were carbamylated by potassium cyanate (KOCN; NACALAI TESQUE, Kyoto, Japan) following the method previously reported [6, 16]. In short, equal volumes of 2 mol/L of KOCN in distilled water and 4 mg/mL of FCS, 2 mg/mL of BSA or 2 mg/mL of ALB were mixed and incubated overnight at 37°C.

ALB was also citrullinated by incubating with rabbit skeletal peptidylarginine deiminase (Sigma-Aldrich) for 3 hours at 50 °C, as previously described [17].

Successful protein carbamylation was confirmed by mass spectrometry or Western blotting using rabbit anti-carbamyl-lysine polyclonal antibody (anti-CBL antibody; Cell Biolabs, San Diego, USA). Citrullination was confirmed by the AMC-Senshu method [18].
Western blotting

Carbamylated FCS (CarFCS) and unmodified FCS (UmFCS), or carbamylated BSA (CarBSA) and unmodified BSA (UmBSA) were electrophoresed and transferred onto nitrocellulose membranes. Membranes were incubated for 5 minutes in Bullet Blocking One for Western Blotting (NACALAI TESQUE) or for 30 minutes in 5% skimmed milk in phosphate-buffered saline with 0.05% tween 20 (PBS-T); the former was for patients’ sera and the latter was for anti-BSA antibody, described below.

Then, they were incubated for one hour in patients’ sera or rabbit anti-BSA antibody (Sigma-Aldrich) diluted 1,000-fold by each blocking buffer followed by three washes in PBS-T. Sera were obtained from 7 RA patients with anti-CarP antibodies. Subsequently, they were incubated for one hour in horseradish peroxidase-conjugated anti-human or anti-rabbit IgG (Promega, Madison, USA) diluted 10,000-fold by each blocking buffer. Finally, they were washed three times and antibodies were detected by SuperSignal™ West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Waltham, USA). All the procedures were conducted at room temperature.

ELISA

We established an in-house anti-CarP-antibody ELISA system following the original report [6] with a few modifications. UmFCS or CarFCS was diluted to 0.02 mg/mL with pH 9.6 0.1 mol/L carbonate-bicarbonate buffer and then used to coat Nunc Maxisorp plates (Thermo Fisher Scientific) at a volume of 50 μL overnight at 4°C. Then, plates were washed 3 times in PBS-T followed by blocking with phosphate-buffered saline containing 2% bovine serum albumin (BSA-PBS) for 6 hours at 4°C. All the washing procedures were conducted 3 times using PBS-T. Then, patients’ sera (150-fold dilution by BSA-PBS) were applied to the ELISA plates in 50 μL. After overnight incubation at 4°C and washing, the wells were reacted for 3
hours with 50 μL of rabbit anti-human IgG antibody (Thermo Fisher Scientific), which was
diluted 10,000-fold with BSA-PBS. After washing, 50 μL of alkaline phosphatase-conjugated
anti-rabbit IgG (Promega) diluted 2,000-fold with BSA-PBS was added and incubated for 3
hours at 4°C followed by washing, and finally 3,3’-diaminobenzidine (Sigma-Aldrich) was
added. The optical density of each well was read at 405 nm.

A standard curve was drawn using one patient’s standard serum. Arbitrary units (AUs) of
the anti-CarP titer were calculated by subtracting the titer against UmFCS from that against
CarFCS. Negative values were regarded as zero. The cut off value was set at the titer which
makes the specificity higher than 0.95 based on the analysis of the receiver operating
characteristic curve of our data.

Anti-carbamylated ALB (anti-CarALB) and anti-citrullinated ALB (anti-citALB) antibody
ELISA systems were established by modifying anti-CarP-antibody ELISA. Points differing from
the anti-CarP-antibody ELISA were as follows: While anti-CarP ELISA consisted of three types
of antibodies (primary, secondary, and labeled tertiary), anti-CarALB and anti-citALB ELISAs
involved two steps (primary and labeled secondary antibodies). The secondary antibody
was alkaline phosphatase conjugated anti-human IgG (Promega). After the blocking step,
each procedure was performed at room temperature. Reaction steps of patients’ sera and
anti-human IgG were for 2 and 1.5 hours, respectively.

Inhibition assay

We chose 8 anti-CarP-strongly-positive patients’ sera and incubated them overnight at
4°C in each concentration of UmFCS (negative control), CarFCS (positive control), citALB, or
CarALB. Subsequently, they were analyzed by the anti-CarP-antibody ELISA system.

In the same manner, inhibition assays of anti-CarALB and anti-citALB antibodies were
performed using 8 other RA patients’ sera with both anti-CarALB and anti-citALB antibodies.
They were incubated overnight at 4°C in each concentration of CarALB, citALB, or unmodified ALB (UmALB).

**Calculation of carbamylation index of serum albumin based on data obtained by liquid chromatography-tandem mass spectrometry**

Sera from 4 anti-CarALB-antibody-strongly-positive RA patients, 3 anti-CarALB-antibody-negative RA patients, 3 healthy controls, and a chronic kidney disease (CKD) patient were diluted, electrophoresed (SDS-PAGE), and dyed by Rapid Stain CBB Kit (NACALAI TESQUE). Purified albumin was recovered from gel pieces corresponding to the band for albumin. The in-gel digestion and extraction of albumin peptides were carried out using In-Gel Tryptic Digestion Kit (Thermo Scientific) according to the manufacturer’s instruction.

The tryptic digests were resuspended in 0.1% formic acid and separated using Nano-LC-Ultra 2D-plus equipped with cHiPLC Nanoflex (Eksigent, Dublin, CA, USA) in the trap-and-elute mode, with a trap column (200 μm x 0.5 mm ChromXP C18-CL 3 μm 120 Å (Eksigent)) and an analytical column (75 μm x 15 cm ChromXP C18-CL 3 μm 120 Å (Eksigent)). The separation was carried out using a binary gradient with solvent A (98% water, 2% acetonitrile, 0.1% formic acid) and solvent B (20% water, 80% acetonitrile, 0.1% formic acid). The gradient program was as follows: 2 to 40% of solvent B for 125 min, 90% of solvent B for 5 min, and 2% of solvent B for 19 min, at a flow rate of 300 nL/min. The eluates from the analytical column were directly infused into a mass spectrometer (TripleTOF 5600+ System with NanoSpray III source and heated interface (SCIEX, Framingham, MA, USA)).

Data acquisition was carried out with an information-dependent acquisition method and the acquired datasets were imported to the platform of Progenesis QI for proteomics
software version 2.0 (Nonlinear Dynamics, Newcastle upon Tyne, UK) for label-free, global quantification of peptides. The MS/MS spectrum corresponding to the peaks found by Progenesis QI for proteomics were exported for identification by Mascot Server version 2.4.1 (Matrix Science, London, U.K.) with SwissProt database for human (July 2016). Carbamidomethyl (C) as fixed modification, and Oxidation (M), acetyl (protein N-term), carbamyl (K), and carbamyl (N-term) as variable modifications, were taken into consideration. The relative abundance of each peptide was calculated using the method to normalize to all proteins provided by the Progenesis QI for proteomics software.

The carbamylation index (CI) was calculated by the following equation: CI = Sum of normalized abundance of peptide whose lysine is carbamylated / Sum of normalized abundance of peptide containing lysine residues. Peptides with Mascot peptide scores above the identification level were used for this calculation. All LC/MS/MS analyses were performed twice in differently-randomized injection orders, and the mean CI was calculated based on the average of the two trials for each sample.

Analysis of serum MPO

Seventy RA patients’ sera whose titers of the anti-CarALB antibody were the first to the 35th highest and the first to the 35th lowest, and 11 HCs’ sera were chosen to test the serum MPO level. Serum MPO was quantified using Quantikine ELISA Human Myeloperoxidase Immunoassay (R&D Systems, Minneapolis, USA) following the manufacturer’s instructions.

Statistical analysis

All statistical analysis were conducted using R version 3.1.1 [19]. Correlations between the titers of each antibody were analyzed using Spearman’s rank correlation coefficient.
Significance was calculated by the Mann-Whitney U test with Bonferroni’s correction in MPO ELISA. Correlations between the MPO titer and DAS28 were analyzed using the Pearson product-moment correlation coefficient. On clinical evaluation, the Mann-Whitney U test was used for continuous variables and the Chi-squared test or Fisher’s exact test was used for categorical variables. Binary logistic regression was used to assess odds ratios and 95% confidence intervals for the presence of the anti-CarALB antibody. Under the assumption that the missing data occurred completely at random, pairwise and listwise deletions were used in uni- and multivariate analyses, respectively.

RESULTS

In order to identify the target antigens of anti-CarP antibodies, we first performed Western blotting. Unmodified FCS (UmFCS) and carbamylated FCS (CarFCS) were blotted onto the membrane and then incubated with seven RA patients’ sera containing high titers of anti-CarP antibodies. The band commonly detected by the seven RA patients’ sera corresponded to a 70-kDa protein (Fig. 1A). Since the size of the bands was similar to that of albumin detected by anti-bovine serum albumin (BSA) antibody and albumin was also the most abundant protein in FCS, we considered that the main target antigen of anti-CarP antibodies is albumin. As expected, the Western blotting using unmodified BSA (UmBSA) and carbamylated BSA (CarBSA) showed the similar results (Fig. 1B). These results strongly suggest that the antibody against CarBSA is a major component of anti-CarP antibodies.

We next established in-house ELISA systems of anti-carbamylated human albumin (CarALB), anti-citrullinated human albumin (citALB), and anti-CarP antibodies. Sera from 493 RA patients and 144 healthy controls were assayed. Anti-CarP was positive in 344 RA patients (69.8%). When stratified by the positivity of anti-CCP antibody, anti-CarP antibody was mainly detected in CCP-positive RA (Fig. 2A), which is consistent with the previous
reports [6, 14]. Anti-CarALB antibody was detected in 155 RA patients (31.4%), and was also detected mainly in the CCP-positive RA group (Fig. 2B). Sensitivities of anti-CarALB in CCP-positive RA and CCP-negative RA were 34.2 and 18.4%, respectively. On the other hand, the prevalence of anti-citALB antibody was comparable to that of anti-CarALB antibody (38.3%) (Fig. 2C).

Then, we assessed the correlation of anti-CarALB and anti-CarP titers. They showed a good correlation ($\rho=0.59$, Fig. 2D), suggesting that albumin is the major constituent of target antigens of anti-CarP antibodies. In contrast, anti-citALB and anti-CCP titers showed almost no correlation ($\rho=0.16$, Fig. 2E), suggesting that albumin is not the target antigen of anti-citrullinated protein antibodies. The correlation of anti-CarALB and anti-citALB titers was modest ($\rho=0.48$, Fig. 2F), which may indicate that anti-CarALB antibody cross-reacts with citALB to some extent.

In order to clarify that CarALB and not citALB is the target antigen of anti-CarP antibody, we performed an inhibition ELISA assay. Sera with high titers of anti-CarP antibody from 8 RA patients were pre-incubated with different concentrations of CarFCS, CarALB, citALB, or UmFCS and then titers of anti-CarP antibody were measured. The titer of anti-CarP antibody was mildly inhibited by CarALB, but was not inhibited by citALB at all (Fig. 3A). Although the strength of the inhibition by CarALB varied among patients, all the samples were inhibited by CarALB to some extent (Fig. 3B), which indicates that CarALB is one of the target antigens of anti-CarP antibodies and that the repertoire of target antigens is different among patients.

Next, we assessed the cross-reactivity between anti-CarALB and anti-citALB antibodies using the similar inhibition ELISA. Sera from 8 RA patients with both anti-CarALB and anti-citALB antibodies were pre-incubated with different concentration of CarALB, citALB, and unmodified ALB (UmALB) and then titers of anti-CarALB and anti-citALB antibodies
were measured. The anti-CarALB and anti-citALB titers were hardly affected by citALB and CarALB, respectively (Fig. 3C, 3D). This shows that cross-reaction between anti-CarALB and anti-citALB antibodies are limited.

We then performed LC/MS/MS in order to clarify whether serum albumin is actually carbamylated in RA patients. Sera from 4 RA patients with anti-CarALB antibody, 3 RA patients without anti-CarALB antibody, 3 healthy controls (HCs), and a chronic kidney disease (CKD) patient were examined. The CKD patient’s serum was regarded as the positive control because the proportion of carbamylated albumin has been reported to be high in end-stage renal disease patients [20]. The carbamylation index (CI) was calculated based on the abundance of albumin peptides with homocitrulline over those with lysine. As shown in Table 1, we could detect some carbamylation of albumin even in the healthy people. CI of anti-CarALB-positive RA patients was slightly higher than that of anti-CarALB-negative RA patients or HCs, but the differences were modest compared with the CKD patients. Although the interpretation of these data requires further investigation, at least we can conclude that carbamylation of albumin occurs in vivo.

Since carbamylation occurs due to cyanate that is produced from thiocyanate enzymatically with MPO in vivo [2], we speculated that serum albumin was carbamylated by MPO released from inflammatory sites. Therefore, we measured serum MPO levels in the sera from 35 RA patients with high titers of anti-CarALB antibody, 35 RA patients without anti-CarALB antibody, and 11 healthy controls (HCs), using the MPO ELISA kit.

The serum MPO level was significantly higher in RA patients with anti-CarALB than in those without anti-CarALB and HCs (Fig. 4A). Notably, the serum MPO level was correlated with disease activity (Fig. 4B), supporting the idea that MPO released from inflammatory sites carbamylates serum albumin, which triggers anti-CarALB antibody production.

Finally, we assessed the clinical relevance of anti-CarALB using clinical data from the
KURAMA cohort (Table 2). The presence of anti-CarALB antibody was significantly correlated with a higher Steinbrocker stage, higher DAS28, biologic use, as well as anti-CCP and anti-CarP antibodies. In multivariate analysis, DAS28, history of cardiovascular disease, BUN, MTX use, biologic use, and anti-CarP antibodies were independently associated with the presence of anti-CarALB antibody while the Steinbrocker stage and anti-CCP antibody were not (Table 2).

**DISCUSSION**

In the present study, we showed that albumin is one of the target antigens of anti-CarP antibodies. This is the first target antigen that has not been reported as a target of ACPA. We also showed that CarALB exists in RA patients’ sera. The serum MPO level was higher in RA patients with anti-CarALB antibody than in those without it, and the MPO level was clearly correlated with the disease activity. Furthermore, the presence of anti-CarALB antibody was associated with more severe disease activity. All these results suggest that the involvement of anti-CarALB or anti-CarP antibodies in the pathophysiology of RA.

Previously reported target antigens of anti-CarP antibodies are fibrinogen and vimentin [6, 11-13], which are also known as target antigens of ACPA. Because cross-reaction between anti-CarP antibody and ACPA has been a concern, the identification of antigens that are recognized only by anti-CarP antibodies has been desired. We showed that inhibition ELISA of anti-CarALB antibody by citALB hardly changed the titer of anti-CarALB antibodies, which indicates that anti-CarALB antibody is not the cross-reaction of ACPA and strongly supports the notion that anti-CarP antibody exists independently of ACPA.

The mechanism of anti-CarALB antibody production in RA has yet to be clarified. *In vivo*, protein carbamylation naturally occurs by cyanate which exists in equilibrium with urea [21], as we found in healthy control sera (Table 1). However, cyanate is also synthesized from
thiocyanate by MPO enzymatically at inflammatory sites [2]. The plasma thiocyanate level was reported to be higher in smokers [22], which is interesting because smoking is thought to be a risk factor of RA [23, 24]. Previous studies demonstrated that the serum or plasma MPO level was higher in patients with RA than patients with OA or healthy controls [25, 26], and higher-level protein carbamylation was detected in the blood and synovial fluid of RA patients [27, 28]. These reports are also consistent with our results showing that the serum MPO level was correlated with disease activity (DAS28). Taking them together, the carbamylation of albumin naturally occurs, but is enhanced by MPO from inflammatory sites including the RA synovium, which may trigger the disruption of tolerance to produce anti-CarALB antibody.

Currently, we do not have data on how the tolerance against carbamylated albumin is disrupted. However, Mydel et al. reported that the severest arthritis was induced after the intra-articular injection of citrullinated peptides in mice immunized with carbamylated peptides or carbamylated mouse albumin [27]. This suggests that the immunodominance of carbamylated peptide and protein. The co-existence of carbamylated and citrullinated proteins may be important for the pathogenicity of RA. We believe that the key players are neutrophils since MPO is an enzyme released from activated neutrophils, and neutrophil extracellular traps (NETs) are thought to be important for ACPA induction [29-31]. The fact that anti-CarALB antibody frequently coexisted with anti-CCP antibody supports this idea. However, further research is required to clarify the relationship between neutrophils and anti-CarALB antibody.

On the other hand, it is noteworthy that a correlation of anti-CarALB antibody with cardiovascular disease (CVD) incidence was shown. A high plasma homocitrulline level [2] and carbamylated low-density lipoprotein (LDL) [32] were reported to be risk factors of CVD. It is also well-known that the risk of CVD in RA patients is increased [33-35]. Although we
did not examine whether other proteins are carbamylated, our data suggest that protein carbamylation is one reason for a high CVD risk in RA patients.

Conclusions

We found that carbamylated albumin is a novel target antigen of anti-CarP antibodies, and it is the first reported target antigen that has not been reported as the target of anti-citrullinated protein antibody.

Acknowledgements

We would like to thank Mr. Wataru Yamamoto at Kurashiki Sweet Hospital for his excellent support to establish and maintain the KURAMA database, and Makoto Teramoto and his colleagues at Shin-Kawabata hospital, and Tsuyoshi Torii and his colleagues at Torii Clinic Medical Corp. Hospitals & Clinics for collecting the blood samples from healthy volunteers.

Funding: This work was supported by Grants-in-aid from Japan Agency for Medical Research and Development and from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Disclosure statement: M.F. reports grants from Mitsubishi Tanabe Pharma Corp., grants from Chugai Pharmaceutical Co., Ltd., grants from Bristol-Myers Squibb Co., Ltd., grants from Eisai Co, Ltd., grants from AbbVie GK, grants from Pfizer Japan Inc. , grants from Astellas Pharma Inc., grants from AYUMI Pharmaceutical Corp., grants from Taisho Toyama Pharmaceutical Co., Ltd. , outside the submitted work. M.T. belongs to the Department of the Control for Rheumatic Disease, which is financially supported by four pharmaceutical companies (Mitsubishi-Tanabe, Chugai, Bristol-Myers Squibb, and Eisai); five pharmaceutical companies (Pfizer, Astellas, AbbVie GK, Ayumi, Taisyo Toyama, and Eisai) provided scholarship donations for the department. M.H. is affiliated with the department
that is financially supported by five pharmaceutical companies (Mitsubishi Tanabe Pharma Co., Bristol-Myers K.K., Chugai Pharmaceutical Co., Ltd., AbbVie GK., Eisai Co., Ltd.) and has received grant and research support from Astellas Pharma Inc. All other authors have declared no conflicts of interest.

References

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### Table 1: Background information and carbamylation index of patients and healthy subjects

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<sup>a</sup>Mean of carbamylation index scores from two independent experiments. HC: healthy control; CKD: chronic kidney disease; BUN: blood urea nitrogen; CRE: serum creatinine; Carbamylation Index: Sum of normalized abundance of peptide whose lysine is carbamylated/Sum of normalized abundance of peptide containing lysine residues; anti-CarALB: anti-carbamylated human albumin.
### Table 2: Comparison of clinical characteristics between rheumatoid arthritis patients with and without anti-CarALB antibody

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<tr>
<td></td>
<td>n=155</td>
<td>n=338</td>
<td></td>
</tr>
<tr>
<td>Age, years, mean (SD)</td>
<td>62.8 (12.8)</td>
<td>62.2 (13.6)</td>
<td>0.85</td>
</tr>
<tr>
<td>Disease duration, years, mean (SD)</td>
<td>17.5 (19.7)</td>
<td>15.6 (18.3)</td>
<td>0.12</td>
</tr>
<tr>
<td>Women, %</td>
<td>81.9</td>
<td>85.2</td>
<td>0.36</td>
</tr>
<tr>
<td>Steinbrocker stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>21</td>
<td>63</td>
<td>0.01</td>
</tr>
<tr>
<td>II</td>
<td>29</td>
<td>82</td>
<td></td>
</tr>
<tr>
<td>High stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>30</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>66</td>
<td>114</td>
<td></td>
</tr>
<tr>
<td>DAS28(ESR), mean (SD)</td>
<td>3.24 (1.28)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.85 (1.12)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Smoking</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>current+ former</td>
<td>18+34</td>
<td>26+62</td>
<td>0.14</td>
</tr>
<tr>
<td>never</td>
<td>80</td>
<td>187</td>
<td></td>
</tr>
<tr>
<td>Cardiovascular disease, %</td>
<td>4.9&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.3&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.15</td>
</tr>
<tr>
<td>BUN, mg/dL, mean (SD)</td>
<td>15.9 (5.2)</td>
<td>16.2 (6.1)</td>
<td>0.94</td>
</tr>
<tr>
<td>Anti-CCP antibody-positive, %</td>
<td>89.7</td>
<td>79.0</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Anti-CarP antibody-positive, %</td>
<td>92.3</td>
<td>59.5</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>MTX use, %</td>
<td>61.6&lt;sup&gt;e&lt;/sup&gt;</td>
<td>70.1&lt;sup&gt;f&lt;/sup&gt;</td>
<td>0.07</td>
</tr>
<tr>
<td>Biologic use, %</td>
<td>41.1&lt;sup&gt;g&lt;/sup&gt;</td>
<td>28.6&lt;sup&gt;h&lt;/sup&gt;</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>
Missing data for the Steinbrocker stage (n=38), DAS28 (ESR) (n=85), smoking (n=86), cardiovascular disease (n=42), MTX use (n=36), and biologic use (n=36). Pairwise deletion was used in univariate analysis. On the other hand, 151 cases were excluded by listwise deletion in multivariate analysis.  

- Data from 22 patients not available.
- Data from 63 patients not available.
- Data from 12 patients not available.
- Data from 30 patients not available.
- Data from 9 patients not available.
- Data from 27 patients not available.
- Data from 27 patients not available.

BUN: blood urea nitrogen; Biologic: abatacept, adalimumab, certolizumab pegol, etanercept, golimumab, infliximab, and tocilizumab; anti-CarALB: anti-carbamylated human albumin
Figure 1. Western blot analysis of anti-carbamylated protein antibodies
Carbamylated and unmodified fatal calf serum (FCS) were electrophoresed and blotted onto a nitrocellulose membrane, and then incubated with seven RA patients’ sera containing a high titer of anti-CarP antibodies, or anti-bovine serum albumin (BSA) polyclonal antibody. The common band detected by RA patients’ sera corresponded to carbamylated albumin (A). Western blotting was also performed using unmodified and carbamylated BSA (B).

Figure 2. Positivity and correlation of anti-carbamylated protein, anti-carbamylated albumin, and anti-citrullinated albumin antibodies in RA patients
ELISAs of anti-carbamylated protein (anti-CarP) antibody (A), anti-carbamylated human albumin (anti-CarALB) antibody (B), and anti-citrullinated human albumin (anti-citALB) antibody (C) were performed and titers of these antibodies are plotted in anti-CCP antibody-positive RA, anti-CCP antibody-negative RA, and healthy controls (HCs). Horizontal lines represent cut-off values of each antibody, set by the data from HCs. Correlations of antibody titers in RA patients are shown by the plots between anti-CarP and anti-CarALB antibodies (D), between anti-CCP and anti-citALB antibodies (E), and between anti-CarALB and anti-citALB antibodies (F). Spearman’s rank correlation coefficient ($\rho$) was used to identify the relationships between them.

Figure 3. Inhibition ELISA of anti-carbamylated protein antibodies, anti-carbamylated albumin antibody, and anti-citrullinated albumin antibody
Anti-carbamylated protein (Anti-CarP) antibodies were inhibited by different concentrations of carbamylated fetal calf serum (CarFCS), carbamylated albumin (CarALB), citrullinated albumin (citALB), or unmodified FCS (UmFCS). Percent inhibitions of the anti-CarP titer were plotted using 8 RA patients’ sera containing a high titer of anti-CarP antibodies. Data represent the mean ± S.E.M. (A). Percent inhibition of anti-CarP antibody by CarALB exhibited by individual patients’ sera (B). In the similar way, anti-CarALB antibody (C) and anti-citALB antibody (D) were inhibited by different concentrations of CarALB, citALB, and unmodified albumin (UmALB). The sera used were from 8 RA patients with both anti-CarALB and anti-citALB antibodies.

Figure 4. Serum MPO levels
Serum MPO levels of 35 RA patients with anti-carbamylated albumin (anti-CarALB) antibody, 35 RA patients without anti-CarALB antibody, and 11 healthy controls. Serum MPO levels were significantly different between the groups. The median with IQR is shown (A). The serum MPO level and DAS28 (ESR) in all RA patients showed a good correlation (B).
### A

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Carboxymethylation</th>
<th>RA01</th>
<th>RA02</th>
<th>RA03</th>
<th>RA04</th>
<th>RA05</th>
<th>RA06</th>
<th>RA07</th>
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<tbody>
<tr>
<td>Anti-BSA antibody</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>kDa</td>
<td>120</td>
<td>100</td>
<td>80</td>
<td>60</td>
<td>50</td>
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</tbody>
</table>

### B

181x127mm (300 x 300 DPI)
A

B

180x119mm (300 x 300 DPI)