Unravelling the anti-carbamylated protein antibody response in rheumatoid arthritis

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Chapter 9

The identification of carbamylated alpha 1 anti-trypsin (A1AT) as an antigenic target of anti-CarP antibodies in patients with rheumatoid arthritis

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As published in: J Autoimmun. 2017 Jun;80:77-84

In 2011 a novel autoantibody system, anti-carbamylated protein (anti-CarP) antibodies, was described in rheumatoid arthritis (RA) patients. Anti-CarP antibody positivity associates with a more severe disease course, is observed years before disease onset, and may predict the development of RA in arthralgia patients. Although many clinical observations have been carried out, information on the antigenic targets of anti-CarP antibodies is limited. Most studies on anti-CarP antibodies utilize an ELISA-based assay with carbamylated fetal calf serum (Ca-FCS) as antigen, a complex mixture of proteins. Therefore, we analysed the molecular identity of proteins within Ca-FCS that are recognized by anti-CarP antibodies.

Ca-FCS was fractionated using ion exchange chromatography, selecting one of the fractions for further investigation. Using mass-spectrometry, carbamylated alpha-1-antitrypsin (Ca-A1AT) was identified as a potential antigenic target of anti-CarP antibodies in RA patients. A1AT contains several lysines on the protein surface that can readily be carbamylated.

A large proportion of the RA patients harbour antibodies that bind human Ca-A1AT in ELISA, indicating that Ca-A1AT is indeed an autoantigen for anti-CarP antibodies. Next to the Ca-A1AT protein, several homocitrulline-containing peptides of A1AT were recognized by RA sera. Moreover, we identified a carbamylated peptide of A1AT in the synovial fluid of an RA patient using mass spectrometry.
We conclude that Ca-A1AT is not only a target of anti-CarP antibodies but is also present in the synovial compartment, suggesting that Ca-A1AT recognized by anti-CarP antibodies in the joint may contribute to synovial inflammation in anti-CarP-positive RA.

Introduction

Anti-citrullinated protein antibodies (ACPA) and rheumatoid factor (RF) are important serological markers in the diagnosis of rheumatoid arthritis (RA)[1,2] and are therefore part of the American College of Rheumatology (ACR) / European League Against Rheumatism (EULAR) disease classification criteria for RA[3]. ACPA are mostly detected using anti-cyclic citrullinated peptide (CCP) antibody assays. In 2011, we described antibodies to carbamylated proteins (anti-CarP antibodies) in RA patients using an ELISA, based on carbamylated fetal calf serum (Ca-FCS)[4-6]. In contrast to citrullination, which is mediated by enzymes, carbamylation is a chemical reaction mediated by cyanate, converting lysine into a homocitrulline[7] which is very similar to citrulline (only one CH_2 longer side chain)[8].

Importantly, anti-CarP antibodies have been detected in both ACPA-positive and ACPA-negative RA patients, indicating a potential diagnostic value of anti-CarP antibody testing[9-11].

A recent meta-analysis estimated the sensitivity, specificity and odds ratio of anti-CarP antibodies as 42% (95% CI 38-45), 96% (95% CI 95-97) and 17 (95% CI 12-24), respectively when comparing RA patients to healthy controls[12]. Also, using a large cohort of longitudinal samples, it was demonstrated that anti-CarP antibodies predict joint damage as assessed by total Sharp van der Heijde Score[4]. Additionally, anti-CarP antibodies are present in individuals that developed RA already many years prior to disease onset[13-15]. The presence of anti-CarP antibodies has a prognostic value in arthralgia regarding the future development of RA[5]. Similar to ACPA and RF, anti-CarP antibodies have been reported in first degree relatives of RA patients and could help to identify individuals that might develop RA[16]. Even though anti-CarP antibodies are predominantly found in RA patients, they can also present in other inflammatory conditions at considerably lower frequencies[17-19].
Studies have also shown that carbamylation can occur *in-vivo*[20,21]. However, little is known about the antigens that can be recognized by anti-CarP antibodies. Although it is wel understood that Ca-FCS is recognized by anti-CarP antibodies, it is yet unknown which antigens within the complex mixture of Ca-FCS are targeted by anti-CarP antibodies. The identification of the molecular targets by anti-CarP antibodies in RA patients could aid in the etiological understanding of RA. Therefore, the goal of the current study was to characterize the protein(s) present in carbamylated FCS that are targeted by anti-CarP antibodies, where we, as a proof of concept, focus on one of the identified proteins.

Materials and methods

Patients and sera

We selected 80 ACPA-positive and 80 ACPA-negative RA patients from the Leiden Early Arthritis Clinic (EAC; Leiden, The Netherlands). Serum samples from disease controls were also derived from the EAC cohort; patients with gout (n=51), psoriatic arthritis (n=40), osteoarthritis (n=40), sarcoidosis (n=36) and spondylarthropaty (n=40).

In addition, serum samples were collected from 80 healthy controls from the Leiden area[4]. ACPA, RF and anti-CarP antibody status were acquired previously for the samples from RA patients and healthy controls[4]. Patient identity was not disclosed and the data were used anonymously in accordance with the Helsinki Declaration of human research ethics.

	RA patients	Healthy controls	Disease controls
n	160	80	207
age (average, stdev)	54,9 (14,7)	41,8 (12,7)	48,5 (16,5)
gender (% female)	66.4	51.3	44.0
DAS (average, stdev)	3 (1.3)		
ACPA (% positive)	50	1.3	5.1
RF (% positive)	56.9	5	8.3
anti-CarP antibodies (% positive)	37.5	5	10,8

Table 1 – Biographical data on RA patients and healthy controls. The table shows the available characteristics for RA patients and healthy controls. RA = rheumatoid arthritis, stdev = standard deviation, DAS = disease activity score, ACPA = anti-citrullinated protein antibodies, RF = rheumatoid factor, anti-CarP = anti-carbamylated protein, measured using FCS as antigen.

Carbamylation of antigens

Carbamylated proteins were produced by allowing the protein to react with potassium cyanate (KOCN, Sigma-Aldrich, St. Louis, MO) as previously described[4]. In brief, a 2M solution of KOCN was prepared in PBS. Fetal calf serum (FCS, Bodinco, Alkmaar, the Netherlands) or Alpha-1-antitrypsin (A1AT, Lee Biosolutions, Maryland heights, USA) were mixed with the 2M KOCN solution in a 1:1 volume-by-volume proportion. The mixed solution was incubated overnight at 37°C to produce Ca-FCS and Ca-A1AT. Following the incubation period, the resulting carbamylated solution was dialyzed against PBS (2L) for 48hrs, during which the PBS was refreshed at least 5 times. The *in-vitro* carbamylation of A1AT and FCS was confirmed by MS (data not shown).

Immunoassays

Anti-CarP antibodies were detected using Ca-FCS or Ca-A1AT as previously described[4]. In brief, unmodified FCS and Ca-FCS were coated overnight on NUNC MAXISORP® plates (Thermo Scientific, Waltham, MA). Following washing and blocking, the wells were incubated with serum samples obtained from human RA patients and healthy volunteers. Bound human IgG was detected either indirectly using rabbit anti-human IgG (Dako, Glostrup, Denmark), followed by HRP-labeled goat anti-rabbit IgG antibody (Dako), or directly using an HRP-labeled rabbit anti-human IgG (Dako). Following additional wash steps, HRP enzyme activity was measured using ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)). The cut-off for a positive response was chosen as the mean plus two times the standard deviation (SD) of the anti-CarP reactivity of healthy controls.
For epitope mapping studies using peptide fragments of A1AT, anti-biotinylated peptide ELISAs were carried out. NUNC MAXISORP® plates were coated overnight with streptavidin (Invitrogen) followed by incubation of 10ug/ml of biotinylated peptide diluted in PBS with 0.1% BSA for a period of 1 hour at room temperature.

Serum samples were diluted 50x in PBS with 1% BSA and 0.05% Tween and also incubated for 1 hour at 37 °C. Reactivity was detected using the HRP-labeled goat anti-rabbit IgG antibody for 1 hour at 37°C. Again, HRP enzyme reactivity was measured using ABTS.

Identification of A1AT by ion exchange fractionation and mass spectometry
Ca-FCS was fractionated by ion-exchange HPLC (high-performance liquid chromatography) using a MonoQ column. HPLC fractions were analyzed by measurement of absorption for their overall protein content using a plate reader as well as by ELISA used to test anti-CarP antibody reactivity (not normalized for protein content). Five fractions, covering the entire spectrum were selected for further ELISA experiments. Antibody reactivity against these fractions was determined in six serum samples, now coating equal concentrations. The samples included two serum samples with anti-CarP antibodies and no ACPA (CarP+/ACPA-), two serum samples with ACPA antibodies and no anti-CarP antibodies (CarP-/ACPA+), as well as two negative control serum samples from healthy volunteers. The fraction that showed highest reactivity in ELISA was run on an SDS-PAGE. Coomassie stained bands were excised and subjected to chymotryptic digestion and mass spectrometry (MS).

Synthetic overlapping peptides
Synthetic 21-mer peptides were synthesized based on the human A1AT sequence. Each lysine within the human A1AT sequence was identified and used as the basis for designing a peptide. On each side, the 10 flanking amino acids were added. If multiple lysines were present within 21 amino acids, multiple peptides were made. All peptides contained a biotin at the N-terminal end for ELISA testing. The peptides were synthesized as partially overlapping fragments, covering all lysines in the human A1AT sequence.

Modeling of A1AT
The known protein sequence of A1AT was entered into RasMol to generate a 3 dimensional model of the protein. RasMol (www.openrasmol.org) is a molecular graphics program intended for the visualisation of proteins, nucleic acids and small molecules. The program is aimed at display, teaching and generation of publication quality images. The program reads in a molecule coordinate file and interactively displays the molecule on the screen in a variety of colour schemes and molecule representations. All lysin residues were marked on the A1AT molecule and illustrations from two different perspectives were generated.

Mass spectrometric identification of carbamylated A1AT
MS analysis was used to identify the protein(s) present in the selected protein band in SDS-PAGE and to confirm full conversion to homocitrulline in the in vitro carbamylated A1AT preparations used in our studies. MS was also used to identify

the presence of carbamylated A1AT in the joint of an RA patient. The details of the MS procedures are described in the supplementary information.

Statistical evaluation

Data were statistically evaluated using Analyse-it software (Version 4.51; Analyse-it Software, Ltd., Leeds, UK). Comparative descriptive analyses were carried out to analyze the discrimination between different patient groups. Since values were not normally distributed according to Kolmogorov-Smirnov, non-parametric statistics (median and percentiles) have been used. The spearman rank test was used to analyze the correlation between biomarker results and disease activity as well as joint erosions. Mann Whitney tests were used to investigate the differences between two groups. Receiver operating characteristic (ROC) analyses were used to assess the discriminatory power of the different assays. *P* values < 0.05 were considered as significant.

Results

Ca-A1AT is one of the antigens in Ca-FCS targeted by antibodies in sera of RA patients

To identify possible antigens of anti-CarP antibodies within Ca-FCS, the protein mixture was first separated into fractions using ion exchange chromatography. Both protein content and antibody reactivity against each of these fractions were determined using ELISA measurements (Figure 1A). While the antibody reactivity with a healthy serum sample was minimal, reactivity with an anti-CarP-positive serum sample was evident in most of the fractions. Next, five fractions, namely 6, 19, 24, 29 and 35 were selected for further investigation. Each of these fractions was coated at an equal protein concentration and subsequently tested for antibody reactivity, using six different serum samples. All five fractions were able to bind to antibodies from two anti-CarP-positive serum samples while the signal for the four negative control serum samples was barely detectable (Figure 1B). The fraction with the highest ratio between the positive and the negative controls, fraction 19, was used for a Coomassie staining (Figure 1C) and protein identification using mass spectrometry (MS). MS analysis of the main protein band in fraction 19, resulted in the identification of 4 different carbamylated proteins. These proteins were carbamylated alpha-1-antitrypsin (A1AT), Alpha-2-HS-glycoprotein, Alpha-1-acid glycoprotein and Fetuin-B of which carbamylated alpha-1 antitrypsin (A1AT) was the most pronounced candidate, since most high-quality carbamylated peptides identified were derived from this protein (Supplementary figure 1).

Following the identification of bovine Ca-A1AT in Ca-FCS as a target of anti-CarP antibodies we analysed the protein for the presence of lysine residues and their surface exposure. In order to localize lysine residues exposed on the surface of A1AT a 3 dimensional theoretical model was generated. 32 lysine residues were identified in the bovine protein sequence (34 in the human sequence) of which a significant number is exposed on the surface and which therefore represent potential sites for carbamylation (Figure 2).

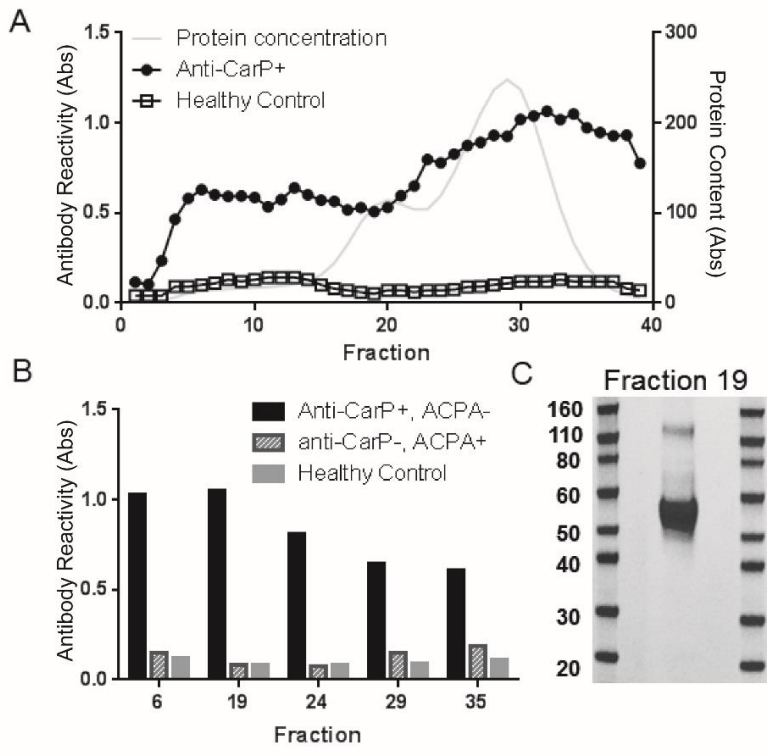

Figure 1 - Identification of alpha 1 anti-trypsin (A1AT) as a major carbamylated protein recognized by anti-CarP antibodies. In a.) the characteristics of the protein fractions obtained by ion exchange chromatograpy are shown. On the left Y-axis, antibody reactivity against each of the fractions is shown for a patient sample positive for anti-Carbamylated protein (anti-CarP) antibodies (solid squares) and for a healthy serum sample (solid circles). The protein content of the individual HPLC fractions as measured by ELISA is shown on the right Y-axis (grey line). Abs; absorbance In b.) the antibody reactivity of 5 HPLC fractions (all coated at the same concentration) with serum samples from rheumatoid arthritis patients healthy controls (two samples in each category) are displayed. For each serum sample group, the average of the two samples is shown. Abs; absorbance Furthermore, c.) illustrates the analysis of the 19th HPLC-fraction on a SDS-PAGE gel (4-12% SDS Page; simply blue safe stain). The main protein band was excised and subjected to chymotrypsin digests.

Serum samples from RA patients display autoreactivity against human Ca-A1AT

After the identification of bovine Ca-A1AT as a possible antigen for anti-CarP antibodies the autoantibody reactivity against human A1AT and human Ca-A1AT was investigated using sera from 160 RA patients and 80 controls. As a comparison and to show the anti-CarP antibody status of these patients and controls, Ca-FCS and FCS were also included as antigen in ELISA. Analysis of autoantibody binding using 240 sera revealed a strong binding to Ca-A1AT in RA patients and only minimal background binding to non-modified A1AT (Figure 3A), similar as to what was

observed for FCS (figure 3E). The levels of antibodies that target Ca-A1AT and Ca-FCS is higher in sera of RA patients when compared to sera of healthy controls (Mann-Whitney test, p<0.001) (Figure 3B and 3E).

We also observed a correlation between the levels of antibodies against Ca-FCS and Ca-A1AT (Spearman's rho = 0.58; p <0.001) (figure 3C). Correlations were also found between anti-Ca-A1AT antibodies and RF or ACPA (figure 3D). ROC analysis showed similar discrimination between RA and controls for Ca-FCS and Ca-A1AT as expressed by similar AUC values (0.73 for Ca-A1AT and 0.69 for Ca-FCS) (Figure 3F). A detailed comparison of the diagnostic properties of anti-Ca-FCS and anti-Ca-A1AT antibodies is shown in Table 1. The ROC curve for anti-Ca-A1AT antibodies discriminating between RA patients and disease controls is shown in figure 3G (AUC = 0,652). Combined these data indicate that Ca-A1AT, a single protein derived from Ca-FCS can be recognized in a similar manner as the complex protein mixture FCS. Also, these data show that a possible self-antigen, carbamylated human A1AT, can also be recognized by anti-CarP autoantibodies.

<u>Alpha 1 antitrypsin as in vivo target of carbamylation</u>
To investigate whether Ca-A1AT could indeed serve as an autoantigen in RA patients, an unbiased, proteome-wide MS analysis was carried out. As input material we have used a synovial fluid sample from an RA patient. Using MS we could identify a carbamylated peptide, derived from A1AT (supplementary figure 2). The peptide sequence is AVH**K**AVLTIDEK, where the bold k indicates the site of carbamylation. This indicates that A1AT can be found in carbamylated form at the site of inflammation in RA patients.

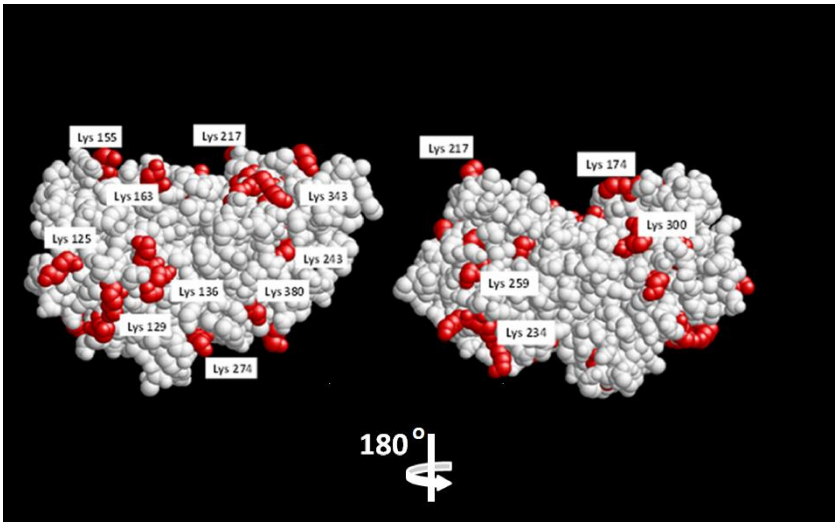

Figure 2 - Theoretical three dimensional structure model of human alpha 1 anti-trypsin (A1AT). The lysine residues of A1AT were mapped in a three dimensional structure, showing the samples mapped at the surface of the molecule.

Anti-CarP antibodies also recognize A1AT-derived carbamylated peptides

We have observed that Ca-A1AT as full-length protein can be recognized by antibodies from RA patients. However, the *in vitro* carbamylated A1AT might potentially involve other modifications besides the creation of a homocitrulline. To investigate the recognition of A1AT containing homocitrulline residues, synthetic 21-mer peptides, derived from A1AT, were designed. In these peptides, homocitrulline residues were incorporated at the location of the lysine residue in the original sequence. The peptides were tested in ELISA using samples from RA patients (n=24) [anti-Ca-A1AT positive (n=9) and negative (n=15)] and healthy controls (n=6).

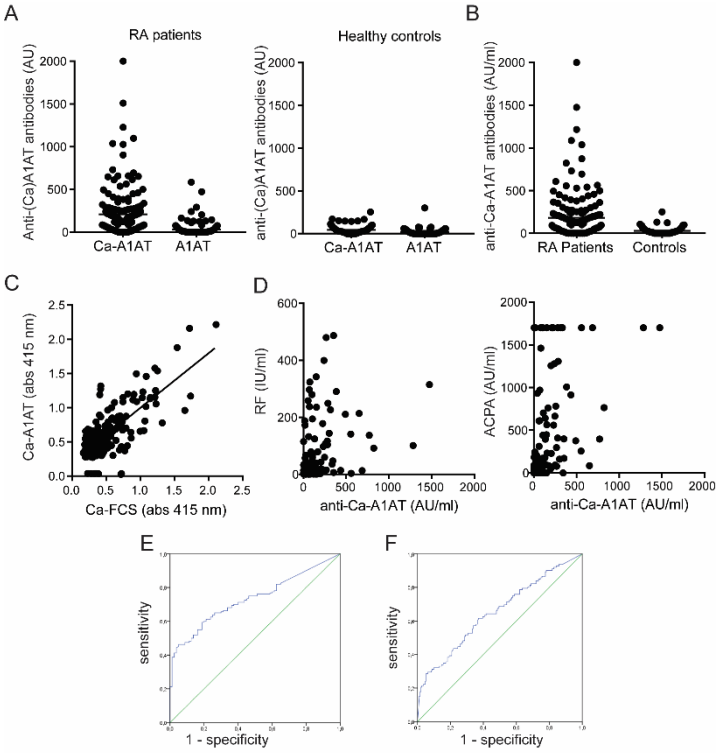

Figure 3 - Analysis of alpha 1 anti-trypsin (A1AT) as potential antigen by ELISA. In a.) antibody reactivity against Ca-A1AT and A1AT as measured by ELISA is shown for rheumatoid arthritis patients (RA, n = 160, left) and healthy controls (n = 80, right). b.) shows a complete overview of the anti-Ca-A1AT antibodies in which the A1AT signal has been subtracted from the Ca-A1AT signal for both RA patients and healthy controls. In c.) the correlation between anti-Ca-FCS and anti-Ca-A1AT antibodies is analysed and a clear correlation was observed (spearman correlation, rho=0.58; p<0.001). In d.) the correlation between anti-Ca-A1AT antibodies and RF or ACPA are shown (rho = 0.56 and 0.61 respectively, both p<0.001). e) shows the receiver operating characteristics (ROC) Ca-A1AT (green) as the antigen for discrimination between RA patients and healthy controls (AUC = 0,733). The diagonal reference line indicates no discrimination. In f) the ROC for Ca-A1AT as the antigen for discrimination between RA patients and disease controls (n=207) is shown (AUC = 0,652). The diagonal reference line indicates no discrimination.

The reactivity against the carbamylated peptides is virtually absent the serum samples from healthy controls, especially when compared to a non-carbamylated control peptide (Figure 4A and a cluster analysis in supplementary figure 3).In the sera of RA patients, an increased reactivity towards the carbamylated peptides can be observed. The sera of the RA patients that were positive in the anti-Ca-A1AT ELISA displayed a much more pronounced reactivity towards the peptides as compared to the RA samples that were negative in the anti-Ca-A1AT ELISA. We observed that many different peptides are recognized and that there is not one peptide that is recognized by the majority of the anti-CarP positive serum samples (Figure 4A). Out of these peptides, 6 peptides with a high reactivity in the RA patients were selected and a non-carbamylated control peptide was used in ELISA (figure 4B). Also in this situation, an increased reactivity was seen in anti-Ca-A1AT-positive RA patients when compared to anti-Ca-A1AT-negative RA patients or healthy controls.Furthermore, a trend is observed that the presence of multiple homocitrulline residues results in increased antibody reactivity in RA patients (supplementary figure 3B). Peptide 22-25 contain the homocitrulline residue that was found to be carbamylated *in vivo*. Interestingly, this region of the A1AT molecule is also recognized by anti-CarP antibodies.
Combined, these data indicate that A1AT peptides containing a homocitrulline can also be recognized by anti-CarP antibodies from RA patients.

	Ca-A1AT	**Ca-FCS**
Sensitivity at ~99% specificity	35.0 (28.0-42.7)	31.3 (24.2-38.8)
Likelihood ratio +	28.0 (5.1-159.7)	25.0 (4.6-142.8)
Likelihood ratio -	0.66 (0.58-0.73)	0.70 (0.62-0.77)
Odds ratio	42.5 (7.2-248.6)	35.9 (6.1-210.1)

Table 2 - Overview of the diagnostic performance of ELISAs using carbamylated Alpha-1 antitrypsin Ca-A1AT or carbamylated fetal calf serum (Ca-FCS) as antigen.

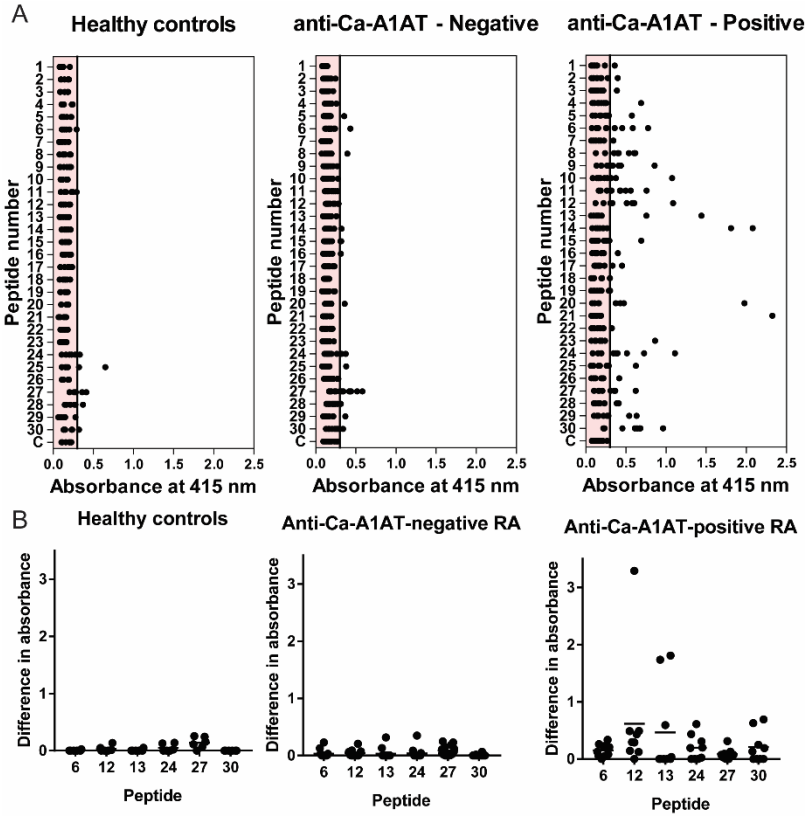

Figure 4 - Anti-CarP reactivity against alpha 1 anti-trypsin (A1AT) derived peptides. a.) A1AT peptides were generated synthetically and lysines were replaced by homocitrulline residues. The reactivity of anti-CarP antibodies against these peptides was tested by ELISA, using both samples from rheumatoid arthritis (RA, n=24) [anti-Ca-A1AT positive (n=9) and negative (n=15)] as well as from healthy controls (n=6). Significant reactivity was found with several of the A1AT peptides, mostly in the RA patient samples that were positive for anti-Ca-A1AT antibodies. C indicates a control peptide that does not contain a homocitrulline residue. b.) shows the difference in antibody reactivity between 6 carbamylated and the 6 matching non-carbamylated peptides for samples from RA patients (n=23) [anti-Ca-A1AT-positive (n=9) and negative (n=14)] as well as for healthy controls (n=6)

Discussion

Here we have described the identification of Ca-A1AT as one of the antigenic targets of anti-CarP antibodies present in Ca-FCS and showed that anti-CarP antibodies are able to recognize human Ca-A1AT as well. In addition, we have identified a carbamylated peptide derived from A1AT in the synovial fluid of an RA patient. Moreover, anti-CarP antibodies are able to recognize homocitrulline containing peptides that are derived from A1AT.

The peptide sequence as identified in synovial fluid is recognized by a substantial portion of the anti-CarP positive sera. Collectively this demonstrates that human Ca-A1AT serves as an autoantigen for anti-CarP antibodies from RA patients. Other protein antigens that have been described previously are mainly carbamylated fibrinogen[4,5,13,19,22,23] as well as enolase[24] and vimentin derived peptides[25]. However, to our knowledge these particular antigens have not yet been described in carbamylated form in RA patients. In other conditions, such as renal disease, many different carbamylated proteins have been identified[20,21], indicating that it is unlikely that A1AT is the only carbamylated protein in RA patients.

Our approach to identify linear B-cell epitopes on A1AT identified several epitopes that are recognized by antibodies targeting Ca-A1AT. However, none of the peptides were recognized by the majority of the patient samples containing anti-Ca-A1AT antibodies. Additionally, we did not find a correlation between the reactivity against the individual A1AT peptides and the complete protein (data not shown). Consequently, it is highly likely that conformational epitopes are the main target of the anti-CarP antibodies. However, it is possible that patients positive for antibodies to specific carbamylated A1AT derived peptides represent important subsets of RA patients with specific clinical characteristics[4,26-30]. Studies are needed to analyse if antibodies to these peptides show correlation to disease severity, disease activity and / or treatment response in certain RA patient subsets. Larger cohorts will have to be tested on Ca-A1AT to verify the clinical utility of the antibodies. A desirable outcome could be that anti-Ca-A1AT antibodies show similar characteristics as anti-Ca-FCS antibodies in terms of erosive disease, but yield higher disease specificity.

Furthermore, A1AT has interesting characteristics and the protein has been suggested to be relevant in RA as A1AT is a protease inhibitor belonging to the serpin superfamily, and is also known as serum trypsin inhibitor or alpha-1 proteinase inhibitor. In addition, A1AT protects tissues from enzymes of inflammatory cells, especially neutrophil elastase, and has a reference range in blood of 1.5 - 3.5 gram/liter, and the concentration can rise manifold upon acute inflammation. In its absence, neutrophil elastase is free to break down several molecules including elastin, which contributes to e.g. the elasticity of the lungs, resulting in respiratory complications such as emphysema, or chronic obstructive pulmonary disease (COPD) in adults and in cirrhosis in adults or children. Besides these actions, A1AT might also exert other anti-inflammatory or tissue-protecting effects via dendritic cells or regulatory T cells[31,32]. A1AT is a 46 kDa (394 amino acids) protein with 34 lysine residues, most of them exposed on the surface of the protein which makes the protein a suitable target for carbamylation. Compared to the average amino acid content of mammalian proteins, the content of lysine in A1AT is higher (11.9% vs. 7.2%).

Interestingly, anti-A1AT IgA complexes have been previously described in RA patients[33,34], but our study is the first to show that Ca-A1AT represents an immunogenic target of autoantibodies. It has also been suggested that there might be a genetic association between certain A1AT phenotypes or A1AT heterozygosity[35] with the presence of RA[31], although a smaller study was not able to replicate this finding[32].

On the basis of preclinical and clinical studies, A1AT therapy for non-deficient individuals may prevent disease progression in several inflammatory and immune-mediated diseases. In animal models for example, A1AT might be able to prevent arthritis[36,37]. In RA, patients with carbamylated and therefore possibly enzymatically inactive A1AT or with anti-Ca-A1AT antibodies might consequently lack the protective effect of this enzyme and would benefit from A1AT treatment. This approach is in line with recent concepts in precision medicine and companion diagnostics in RA[9,38].

We therefore conclude that Ca-A1AT might be one of the *in vivo* antigens for anti-CarP antibodies in RA patients. The presence of Ca-A1AT within the synovial fluid of an RA patient, suggests that carbamylation of A1AT might play a role in the development of synovial inflammation in anti-CarP positive RA patients.

References

1 van Venrooij, W. J., van Beers, J. J. & Pruijn, G. J. Anti-CCP antibodies: the past, the present and the future. *Nature reviews. Rheumatology* **7**, 391-398, doi:10.1038/nrrheum.2011.76 (2011).

2 Wiik, A. S., van Venrooij, W. J. & Pruijn, G. J. All you wanted to know about anti-CCP but were afraid to ask. *Autoimmunity reviews* **10**, 90-93, doi:10.1016/j.autrev.2010.08.009 (2010).

3 Aletaha, D. *et al.* 2010 rheumatoid arthritis classification criteria: an American College of Rheumatology/European League Against Rheumatism collaborative initiative. *Annals of the rheumatic diseases* **69**, 1580-1588, doi:10.1136/ard.2010.138461 (2010).

4 Shi, J. *et al.* Autoantibodies recognizing carbamylated proteins are present in sera of patients with rheumatoid arthritis and predict joint damage. *Proc Natl Acad Sci U S A* **108**, 17372-17377, doi:10.1073/pnas.1114465108 (2011).

5 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).

6 Shi, J. *et al.* Recognition of citrullinated and carbamylated proteins by human antibodies: specificity, cross-reactivity and the 'AMC-Senshu' method. *Annals of the rheumatic diseases* **72**, 148-150, doi:10.1136/annrheumdis-2012-201559 (2013).

7 Trouw, L. A. & Mahler, M. Closing the serological gap: promising novel biomarkers for the early diagnosis of rheumatoid arthritis. *Autoimmunity reviews* **12**, 318-322, doi:10.1016/j.autrev.2012.05.007 (2012).

8 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).

9 Jorgensen, J. T. Companion diagnostics: the key to personalized medicine. Foreword. *Expert Rev Mol Diagn* **15**, 153-156, doi:10.1586/14737159.2015.1002470 (2015).

10 Scinocca, M. *et al.* Antihomocitrullinated fibrinogen antibodies are specific to rheumatoid arthritis and frequently bind citrullinated proteins/peptides. *The Journal of rheumatology* **41**, 270-279, doi:10.3899/jrheum.130742 (2014).

11 Verheul, M. K. *et al.* Anti-carbamylated protein antibodies in rheumatoid arthritis patients of Asian descent. *Rheumatology* **54**, 1930-1932, doi:10.1093/rheumatology/kev250 (2015).

12 Li, L. *et al.* Meta-Analysis: Diagnostic Accuracy of Anti-Carbamylated Protein Antibody for Rheumatoid Arthritis. *PloS one* **11**, e0159000, doi:10.1371/journal.pone.0159000 (2016).

13 M. Brink, M. K. V., J. Rönnelid, E. Berglin , R. Holmdahl, R.E. Toes ,L. Klareskog, L.A. Trouw , S. Rantapää-Dahlqvist. 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 research & therapy* **17** (2015).

14 Gan, R. W. *et al.* Anti-carbamylated Protein Antibodies Are Present Prior to Rheumatoid Arthritis and Are Associated with Its Future Diagnosis. *The Journal of rheumatology*, doi:10.3899/jrheum.140767 (2015).

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

16 Alessandri, C. *et al.* Anti-carbamylated protein antibodies in unaffected first-degree relatives of rheumatoid arthritis patients: lack of correlation with anti-cyclic citrullinated protein antibodies and rheumatoid factor. *Clinical and experimental rheumatology* **33**, 824-830 (2015).
17 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).
18 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).
19 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).
20 Berg, A. H. *et al.* Carbamylation of serum albumin as a risk factor for mortality in patients with kidney failure. *Science translational medicine* **5**, 175ra129, doi:10.1126/scitranslmed.3005218 (2013).
21 Desmons, A. *et al.* Homocitrulline: a new marker for differentiating acute from chronic renal failure. *Clin Chem Lab Med* **54**, 73-79, doi:10.1515/cclm-2015-0398 (2016).
22 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).
23 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).
24 Reed, E. *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 research & therapy* **18**, 96, doi:10.1186/s13075-016-1001-6 (2016).
25 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).
26 Ajeganova, S. *et al.* The association between anti-carbamylated protein (anti-CarP) antibodies and radiographic progression in early rheumatoid arthritis: a study exploring replication and the added value to ACPA and rheumatoid factor. *Annals of the rheumatic diseases* **76**, 112-118, doi:10.1136/annrheumdis-2015-208870 (2017).
27 Akdemir, G. *et al.* Predictive factors of radiological progression after 2 years of remission-steered treatment in early arthritis patients: a post hoc analysis of the IMPROVED study. *RMD Open* **2**, e000172, doi:10.1136/rmdopen-2015-000172 (2016).
28 Humphreys, J. H. *et al.* Anticarbamylated protein antibodies are associated with long-term disability and increased disease activity in patients with early inflammatory arthritis: results from the Norfolk Arthritis Register. *Annals of the rheumatic diseases* **75**, 1139-1144, doi:10.1136/annrheumdis-2015-207326 (2016).
29 Montes, A. *et al.* Anti-Carbamylated Protein Antibodies as a Reproducible Independent Type of Rheumatoid Arthritis Autoantibodies. *PloS one* **11**, e0161141, doi:10.1371/journal.pone.0161141 (2016).
30 Yee, A. *et al.* Anti-CarP antibodies as promising marker to measure joint damage and disease activity in patients with rheumatoid arthritis. *Immunologic research* **61**, 24-30, doi:10.1007/s12026-014-8560-x (2015).

31 Cox, D. W. & Huber, O. Rheumatoid arthritis and alpha-1-antitrypsin. *Lancet* **1**, 1216-1217 (1976).
32 Karsh, J., Vergalla, J. & Jones, E. A. Alpha-1-antitrypsin phenotypes in rheumatoid arthritis and systemic lupus erythematosus. *Arthritis and rheumatism* **22**, 111-113 (1979).
33 Scott, L. J., Dawes, P. T. & Mattey, D. L. Comparison of IgA-alpha 1-antitrypsin levels in rheumatoid and reactive arthritis. *Biochem Soc Trans* **25**, 370S (1997).
34 Scott, L. J., Evans, E. L., Dawes, P. T., Russell, G. I. & Mattey, D. L. Comparison of IgA-alpha1-antitrypsin levels in rheumatoid arthritis and seronegative oligoarthritis: complex formation is not associated with inflammation per se. *Br J Rheumatol* **37**, 398-404 (1998).
35 Cox, D. W. & Huber, O. Association of severe rheumatoid arthritis with heterozygosity for alpha 1-antitrypsin deficiency. *Clin Genet* **17**, 153-160 (1980).
36 Grimstein, C. *et al.* Combination of alpha-1 antitrypsin and doxycycline suppresses collagen-induced arthritis. *J Gene Med* **12**, 35-44, doi:10.1002/jgm.1409 (2010).
37 Grimstein, C. *et al.* Alpha-1 antitrypsin protein and gene therapies decrease autoimmunity and delay arthritis development in mouse model. *J Transl Med* **9**, 21, doi:10.1186/1479-5876-9-21 (2011).
38 de Jong, T. D., Vosslamber, S. & Verweij, C. L. Moving towards personalized medicine in rheumatoid arthritis. *Arthritis research & therapy* **16**, 110, doi:10.1186/ar4565 (2014).