Mass-spectrometric identification of carbamylated proteins present in the joints of rheumatoid arthritis patients and controls

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Abstract

Objective

Antibodies targeting post-translationally modified proteins, such as anti-carbamylated protein antibodies (anti-CarP antibodies) are present in sera of rheumatoid arthritis (RA) patients. These autoantibodies associate with increased risk of RA development and with severity of joint destruction. It is not known which proteins in the RA joint are recognised by anti-CarP antibodies. Therefore, we investigated the presence and identity of carbamylated proteins in the human (inflamed) joint.

Methods

We obtained synovium, cartilage and synovial fluid from RA joints. Cartilage and synovium were obtained from controls. Samples were processed and used for immunohistochemistry or mass-spectrometric analysis to investigate the presence of carbamylated proteins. Anti-CarP antibody reactivity towards identified carbamylated proteins was tested by ELISA.

Results

Immunohistochemistry showed extensive staining of RA and control synovial tissue. Whole proteome analyses of the joint tissues revealed a large number of carbamylated peptidyllysine residues. We identified many carbamylated proteins in cartilage and were also able to detect carbamylation in synovial tissue and synovial fluid. Carbamylation was not exclusive to the RA joint and was also present in the joints of controls. Anti-CarP antibodies in the sera of RA patients were able to recognise the identified carbamylated proteins.

Conclusion

We conclude that numerous carbamylated proteins are present in the RA joint. These carbamylated proteins can be recognised by anti-CarP antibodies, substantiating the notion that anti-CarP antibodies may play a role in the pathogenesis of RA.

Key words

rheumatoid arthritis, autoimmunity, carbamylation, anti-CarP antibodies, post-translational modification

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Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterised by the presence of joint inflammation. Damage to cartilage and bone is observed in more severe cases. It is currently not known what causes this disease, even though many studies have been carried out to investigate potentially pathogenic pathways (1). RA is often associated with the presence of autoantibodies, which can be detected in serum and in the affected joints of RA patients. Well-known autoantibodies in RA are rheumatoid factor, anti-citrullinated protein antibodies (ACPA) and anti-Carbamylated protein antibodies (anti-CarP antibodies) (2, 3). The relationship between the presence of anti-CarP antibodies and clinical presentation and progression has been studied extensively and the observed associations with joint damage or disease development make these autoantibodies potentially interesting players in the pathogenesis of RA (2, 4-6). However, for autoantibodies to mediate direct pathogenic effects, binding to their cognate antigen locally in the joint is likely required.

During citrullination, arginine residues can be enzymatically modified into citrulline residues by peptidylarginine deiminases (PADs). ACPAs specifically recognise the citrullinated version of many peptides and proteins. Therefore, both targeted and full proteome analyses were carried out to investigate the presence of citrulline residues in the RA joint (7-10). A large number of citrullinated proteins was identified using these approaches (9). Examples of citrullinated proteins present in the joints of RA patients are fibrin, vimentin and enolase (7, 8). Many of these proteins are not exclusively present in the RA joint as they can also be identified in the non-RA joint (10).

Anti-CarP antibodies target homocitrulline residues that are derived from the post-translational modification of a lysine residue. This is a chemical reaction mediated by cyanate. Anti-CarP antibodies were discovered more recently compared to ACPA and RF. Therefore, little is known about carbamylated proteins in RA patients. Carbamylation has been studied in renal disease, in which uraemia (resulting in higher cyanate levels) increases carbamylation (11). Several carbamylated proteins were identified in renal disease, but many of the studies performed focused on (a selected number of) serum proteins, such as haemoglobin and albumin (11, 12). Carbamylation has also been studied in mice, in which carbamylation seems to increase with age (13). In RA patients, several proteins have been identified in the joint in carbamylated form, such as human serum albumin, alpha-1 antitrypsin and IgG (14-16). Also, the relative quantity of homocitrulline residues has been measured in several (inflamed) joints of one RA patient (17), showing that a similar amount of homocitrulline residues could be detected in both the affected and non-affected joints of this RA patient.

Importantly, it has not been described how many different proteins, and especially which proteins are present in carbamylated form in the RA joint. Therefore, we investigated the presence of carbamylated proteins in RA synovium, cartilage and synovial fluid, to provide a more complete overview of the "carbamylome".

Methods

Patients

Tissue samples from the joint (cartilage, synovium and synovial fluid) were obtained as anonymised leftover material from joint-replacement surgeries, at the departments of Orthopaedic Surgery, either at the LUMC (Leiden) or at the Alrijne Hospital (Leiderdorp). This procedure was approved by the local ethics committee nr. B15/003, 16-02-2015. Additional synovial fluid was acquired from patients visiting the outpatient clinic for the Rheumatology Department at the LUMC. An overview of the tissue samples used for mass spectrometry can be seen in Table I.

Serum samples from RA patients were selected as before (15) from 80 ACPA-positive and 80 ACPA-negative patients. Also, serum samples from 80 healthy controls were collected in the Leiden area (2).

Table I. Characteristics of samples used for mass spectrometric analysis.

ID	Group	Tissue	Gender	Age	Location
1	RA	Cartilage	Female	76	Knee
2	RA	Cartilage	Male	56	Hip
3	RA	Synovium	Female	76	Knee
4	RA	Synovium	Male	56	Hip
5	RA	Synovial fluid	Male	49	Knee
6	RA	Synovial fluid	Male	67	Knee
7	OA	Cartilage	Male	77	Knee, affected
8	OA	Cartilage	Male	75	
9	OA	Synovium	Male	75	
10	OA	Synovium	Female	70	
11	OA	Synovial fluid	Female	75	Knee
12	OA	Synovial fluid	Male	75	Knee
13	Other	Cartilage	Male	77	Knee, preserved
14	Other	Cartilage	Male	33	Shoulder

Multiple samples were derived from the same person (1 and 3, 2 and 4, 8 and 9, 7 and 13). Sample 13 and 14 were assigned to the control group, as they were deemed unaffected by rheumatoid arthritis or osteoarthritis. Missing data cannot be acquired, since patient identity was not disclosed.
ID; sample identification number; RA; rheumatoid arthritis; OA; osteoarthritis.

Patient identity was not disclosed and the data were used anonymously in accordance with the Helsinki Declaration of human research ethics.

Immunohistochemistry

Paraffin embedded synovial tissue samples were used for immunohistochemistry. Paraffin on the synovial tissue slides was removed in xylene, followed by ethanol rehydration. Antigen retrieval was carried out with EDTA pH9 (DAKO, S2367) at 96˚C for 30 minutes. The presence of carbamylated proteins was visualised with a polyclonal rabbit anti-carbamyl-lysine antibody (aCBL, Cell Biolabs, STA-078), which was compared to a matching isotype control. For some conditions, the aCBL antibody was pre-incubated for 60 minutes at room temperature with 0.2mg/ml of carbamylated fetal calf serum or non-modified fetal calf serum (Ca-FCS, FCS). The primary antibody was allowed to bind to the tissue for 2 hours. As a secondary antibody, polyclonal goat anti-rabbit Ig/HRP (DAKO, P0448) was incubated for 1 hour. The staining was finalised with DAB peroxidase (Vector Labs, SK4100) and combined with haematoxylin (Klinipath, 4085-9001) staining. Pictures of the stained slides were made on a Zeiss Axio ScopeA1 microscope.

Mass spectrometry

Mass spectrometry was carried out as described previously (14, 15, 18). Synovial fluid was centrifuged at 2000 rpm for 5 minutes. The supernatant was collected and stored in aliquots at -80°C until further use. Synovial fluid samples (500 µg protein) were first depleted for the top-12 most abundant serum proteins (Pierce/Thermo) according to the instructions of the manufacturer. The depleted sample (50 µg) was subjected to filter-aided sample preparation (FASP II) (19) using ^{13}C-urea instead of regular ^{12}C urea to distinguish artificial *in vitro* ^{13}C carbamylation during the FASP procedure from genuine *in vivo* ^{12}C carbamylation events. In vitro carbamylation events were not observed after FASP II procedure.

Cartilage and synovium samples (20 mg) were washed with PBS to remove adherent body fluids such as synovial fluid and blood. Samples were incubated in ST lysis buffer (4% SDS in 0.1 M Tris-Cl pH 7.6) for 15 min at 70°C. Initially, SDS lysates were subjected to FASP II as described above, but yielding low numbers of carbamylated peptide hits. In contrast, subsequent trypsinisation of the cartilage samples yielded much more hits. Therefore, the cartilage and synovium samples (after their extraction with hot SDS to remove adherent and easily soluble proteins) were digested with trypsin using the following procedure: samples were incubated in 100 µl 100 mM DTT in 25 mM NH_4HCO_3 for 20 min at 54°C. After a 5-min centrifugation at max speed, the supernatant was saved and the pellet incubated in 150 µl 15 mM iodoacetamide in 25 mM NH_4HCO_3 for 30 min at room temperature. After a 5-min centrifugation at max speed, the supernatant was saved and the pellet incubated in 200 µl 25 mM NH_4HCO_3

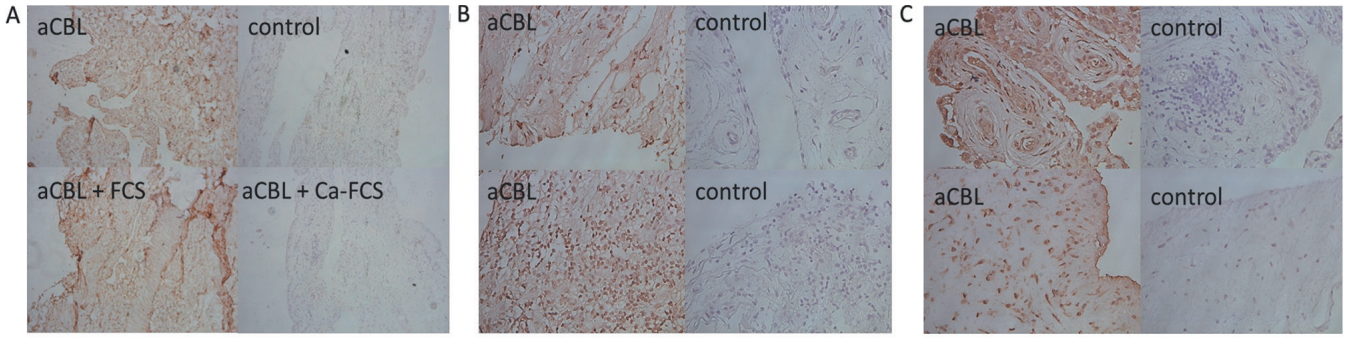
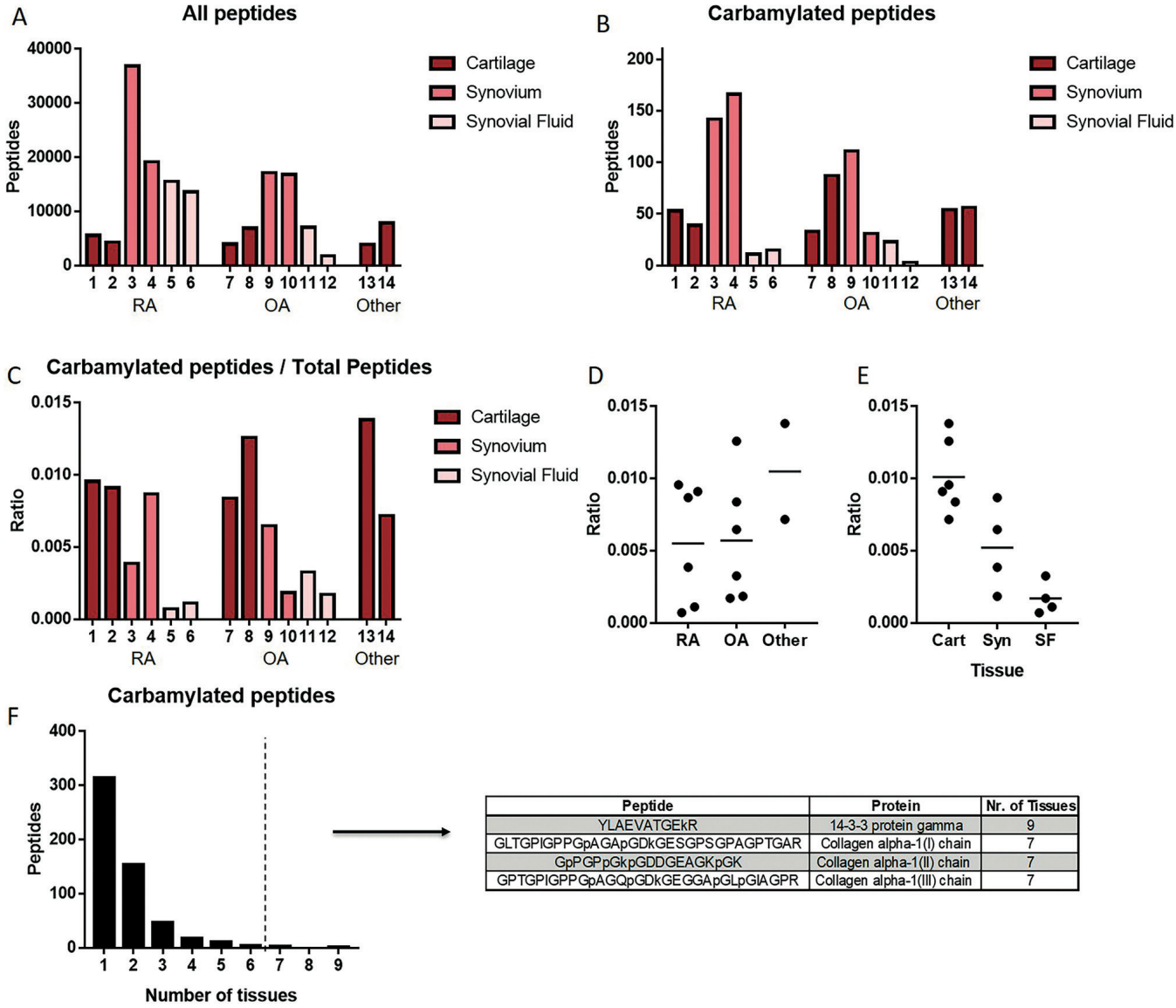


Fig. 1. Immunohistochemistry shows the presence of carbamylation in synovial tissue. Synovial tissue was stained with an aCBL antibody, targeting carbamylated proteins and is compared to an isotype control.
A: Additonally, the staining with the aCBL antibody is pre-treated with either FCS or Ca-FCS, to investigate specificity.
Both RA synovial tissue (**B**) and OA synovial tissue (**C**) were used for further immunohistochemistry stainings with the aCBL and control antibody.
aCBL; anti-carbamyl-lysine; FCS; fetal calf serum; Ca-FCS; carbamylated fetal calf serum; RA; rheumatoid arthritis; OA; osteoarthritis.



Peptide	Protein	Nr. of Tissues
YLAEVATGEkR	14-3-3 protein gamma	9
GLTGPIGPPGpAGApGDkGESGPSGPAGPTGAR	Collagen alpha-1(I) chain	7
GpPGPpGkpGDDGEAGKpGK	Collagen alpha-1(II) chain	7
GPTGPIGPPGpAGQpGDkGEGGApGLpGIAGPR	Collagen alpha-1(III) chain	7

Fig. 2. Carbamylated peptides in the joint. Joint tissues samples, as described in detail in Table I, were subjected to mass spectrometry.
A: the total number of peptides identified in each of the tissues is shown.
B: the number of carbamylated peptides.
C: the ratio between the carbamylated and the total number of peptides, separated for each of the samples.
D: the ratio as in **C** is shown while organising the samples based on original disease state (RA, OA or other).
E: the ratio as in **C** is shown by organising the samples based on tissue (cartilage, synovium or synovial fluid).
F: the number of tissues that carbamylated peptides were identified in, with an additional table containing the peptides that were identified in carbamylated form in seven or more tissues. No peptide was identified in more than 9 out of the 14 samples.
RA; rheumatoid arthritis; OA; osteoarthritis.

containing 10 µg trypsin for 4 hours at 37°C. The combined supernatants from DTT and iodoacetamide incubation were concentrated on a 30 kDa filter (Microcon, Millipore), washed 3 times with 100 µl 25 mM NH_4HCO_3 and also incubated with 1 µg trypsin for 4 hours at 37°C. Next the supernatant containing digested protein from the pellet was added to the digest on the filter. The filter was washed once with 100µl 0.5M NaCl. Peptides were recovered from the filtrate and subjected to solid phase extraction on C18 cartridges (Oasis HLB Waters).

Proteome analysis and mass spectrometric identification of carbamylation

Peptides were analysed via on-line C18-nano-HPLC-MS with a system consisting of an Easy nLC 1000 gradient HPLC system (Thermo, Bremen, Germany), and a Q-Exactive mass spectrometer (Thermo). Fractions were injected onto a homemade precolumn (100 µm × 15 mm; Reprosil-Pur C18-AQ 3 µm, Dr. Maisch, Ammerbuch, Germany) and eluted via a homemade analytical nano-HPLC column (15 cm × 50µm; Reprosil-Pur C18-AQ 3 µm). The gradient was run from 0% to 50% solvent B (100/0.1 water/formic acid (FA) v/v) in 120 min. The nano-HPLC column was drawn to a tip of 5µm and acted as the electrospray nee-

Table II. Identified carbamylated peptides that were confirmed with synthetic peptides.

Peptide	Protein
AEFAEVSkLVTDLTK	Serum albumin
AGEkGLPGAPGLR	Collagen alpha 1(II)
AVAEPGIQLkAVK	Thrombospondin 4 / Cartilage oligomeric matrix protein
cDckPGYR	Fibrillin-1
DGPkGARGDSGPpGR	Collagen alpha 1(II)
EGcYGDkDEFPGVR	Aggrecan Core Protein
EIQcSGYTLPTkVAK	Cartilage intermediate layer protein 1
EkEVVLLVATEGR	Aggrecan Core Protein
EkMAEPEKLTAR	Sushi-repeat containing protein X-linked 2 (precursor)
ESkPLTAQQTTK	Fibronectin
GADGSPGkDGVR	Collagen alpha 1(I)
GDVGEkGPEGApGKDGGR	Collagen alpha 1(II)
GFpGADGVAGPkGPAGER	Collagen alpha 1(I)
GLEPGQEYNVLLTAEkGR	Tenascin
GLLGpkGPpGIpGPpGVR	Collagen alpha 2(XI)
GLpGTAGLpGmkGHR	Collagen alpha 1(I)
GPAGPNGIpGEkGPAGER	Collagen alpha 1(III)
GPAGPSGPAGkDGR	Collagen alpha2(I)
GpPGPpGkpGDDGEAGKpGK	Collagen alpha 1(II)
GPQGALGEpGkQGSR	Collagen alpha 2(VI)
HkVYAcEVTHQGLSSPVTK	Ig Kappa chain
HLGVkVFSVAITPDHLEPR	Collagen alpha1(VI)
IGDQWDkQHDMGHMmR	Fibronectin
IGILITDGkSQDDIIPPSR	Collagen alpha1(XIV)
ISEAkLTGIPK	Biglycan
KVESLQEEIAFLkK	Vimentin
kVPQVSTPTLVEVSR	Serum Albumin
LAkEIAIGR	Cartilage intermediate layer protein 2
LFAVAPNQNLkEQGLR	Collagen alpha 2(VI)
NGDkGHAGLAGAR	Collagen alpha 2(I)
NIDSEEVGkIASNSATAFR	Collagen alpha 3(VI)
NLkYLPFVPSR	Fibromodulin
NTDEDkWGDAcDNcR	Cartilage oligomeric matrix protein
QYNVGPSVSkYPLR	Fibronectin
TLLIkTVETR	Vimentin
TLVkVIPQGScR	Cartilage intermediate layer protein 1
VFDEFkPLVEEPQNLIK	Serum Albumin
VGVVQFSNDVFPEFYLkTYR	Collagen alpha 3(VI)
VkIVGPLEVNVR	Cartilage intermediate layer protein 1
VSKEkEVVLLVATEGR	Aggrecan Core Protein
VVQcSDLGLkSVPK	Biglycan

The carbamylated lysine is indicated with a lower case "k". The lowercase "p" represents a hydroxyproline. The lowercase "c" represents a carbamidomethylated cysteine.

dle of the MS source. The Q-Exactive mass spectrometer was operated in top10-mode. Parameters were: resolution 70,000 at an AGC target value of 3 million maximum fill time of 100ms (full scan), and resolution 17,500 at an AGC target value of 100,000/maximum fill time of 60 cms for MS/MS at an intensity threshold of 17,000. Apex trigger was set to 1 to 5 seconds, and allowed charges were 2-5. For peptide identification, MS/MS spectra were submitted to the uniprot Homo Sapiens database (UP000005640; Jan 2015; 67911 entries) using Mascot version 2.2.04 (Matrix Science) with the following settings: 10 ppm and 20 millimass units deviation for precursor and fragment masses, respectively; trypsin was set as enzyme. Fixed modification was carbamidomethyl on Cys. Variable modifications were carbamylation on K and protein N-terminus, oxidation on M and acetylation on the protein N-terminus. A decoy database was used during the search and only peptides above the false discovery rate (FDR) threshold of 1% are reported.

Protein carbamylation

Carbamylation was carried out directly in the ELISA plate (Nunc Maxisorp, Thermo Scientific). First, proteins were coated overnight at a concentration of 10 ug/ml, incubating the plate at 4 degrees. This was followed by an overnight incubation with 1M of KOCN or PBS at 37 degrees. The KOCN solution was removed by washing with PBS 0.05% Tween20. Using the 1M of KOCN on empty wells showed results comparable to the blank (data not shown).

The following proteins were used for in-plate carbamylation: Fibronectin from human plasma (Sigma, F2006), Fibrinogen from human plasma (Sigma, F3879) and human transferrin (Sigma, T8158).

Anti-CarP antibody ELISAs

ELISAs to measure the presence of antibodies towards carbamylated proteins were carried out as described previously (2), with some minor adjustments. Here we coated non-modified proteins and used in-plate carbamylation, followed by a blocking step with PBS 1% BSA. As a secondary antibody, rabbit anti-human IgG was used (DAKO, P0214). Finally, HRP enzyme activity was detected using ABTS (2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonic acid)).

Results

Immunohistochemistry staining shows carbamylation in RA and OA synovium

To investigate whether carbamylation is detectable in synovium, immunohistochemistry was carried out. We have previously tested the used aCBL antibody and confirmed its specificity for carbamylated proteins by ELISA (18). Here, the specificity of the antibody used for detection of homocitrulline in synovium was verified by blocking the antibody staining with either Ca-FCS or FCS (Fig. 1A). A clear staining of the synovium was observed when using the aCBL antibody alone or the aCBL antibody pre-incubated with FCS. Pre-incubation of the aCBL antibody with Ca-FCS reduced the staining intensity. The fact that the aCBL antibody can be blocked by Ca-FCS, but not by unmodified FCS, indicates that the antibody indeed detects carbamylated proteins present in the synovium. Next we compared synovium samples of 2 RA (Fig. 1B) and 2 OA (Fig. 1C) patients. We did not observe major differences in the staining intensity or pattern between these two patient groups with regards

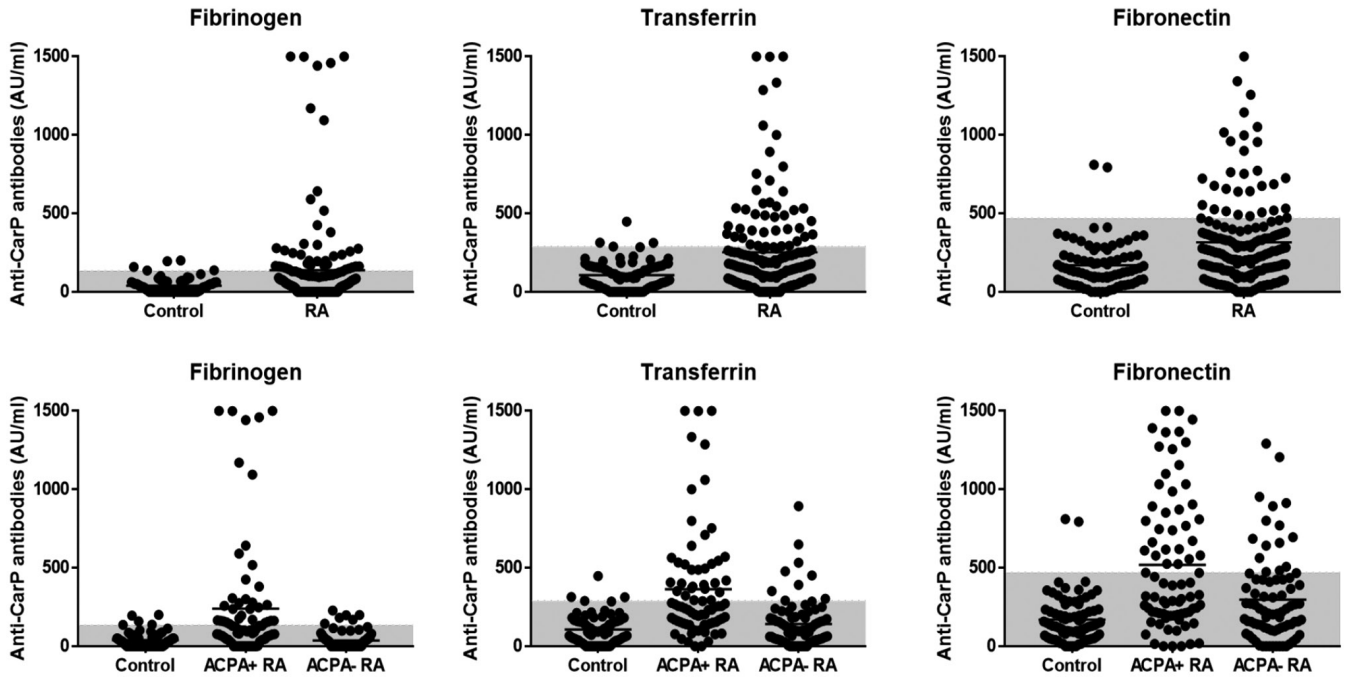


Fig. 3. Antibody reactivity towards carbamylated proteins in sera of RA patients compared to healthy controls. Reactivity of 160 RA serum samples (80 ACPA-positive, 80 ACPA-negative) and 80 healthy control serum samples against fibrinogen, fibronectin and transferrin are shown, either with all samples combined or split by ACPA-status. The grey area indicates the samples that are below the cut-off.
Anti-CarP; anti-carbamylated protein; AU/ml; arbitrary units per millilitre.

to aCBL binding. Combined, these data suggest that carbamylation is present in synovial tissue of RA patients as well as in synovial tissue of OA patients.

Carbamylated proteins are present in the joints of RA patients, OA patients and healthy controls

To further investigate the nature of the carbamylated proteins present in the joint, mass spectrometry was carried out. We studied synovium (n=4), cartilage (n=6) and synovial fluid (n=4) from RA patients, OA patients and other controls. An overview of these 14 samples can be seen in Table I. A large number of unique peptides were identified in the different joint tissues (Fig. 2A). Carbamylated peptides could be identified in each of the studies tissues (Fig. 2B), confirming the results obtained by immunohistochemistry. To ensure that the carbamylated peptides were correctly assigned by the software (18) a total of 45 of the identified peptides were analysed in synthetic format as well. The synthetic peptides with exactly the same sequence as identified in the tissue by MS were analysed with the same methods. Out of these peptides, the spectra from 42 matched the initial spectra acquired from the complex mixture. Table II shows an overview of the identified peptides and their corresponding proteins. A complete overview of the observed spectra from the 42 confirmed peptides are depicted in Supplementary data 1. To obtain an estimation on the relative abundance of carbamylated peptides present in the joint we next divided the number of unique carbamylated peptides by the total number of unique peptides detected in a particular sample (Fig. 2C).

When comparing the results obtained with MS between RA patients to OA patients, we observed no differences with regards to the calculated ratio (Fig. 2D). Upon further examination of the data obtained we noted that the ratio of carbamylated peptides over total peptides was higher in cartilage tissue as compared to synovium or synovial fluid (Fig. 2E). Although the method used is not quantitative, these data may suggest that carbamylated proteins are relatively more abundant in cartilage, a site that predominantly consists of long-lived matrix proteins.

More detailed analysis of the individual tissues revealed that many of the identified modified peptides were unique for a particular tissue. However, some of the carbamylated peptides could be identified in up to 9 out of the 14 investigated samples (Fig. 2F). A complete overview for each of the individual peptides and samples can be found in Supplementary data 2.

The combination of immunohistochemistry and MS results indicate that carbamylation is present in the joint of RA patients. However, carbamylation does not seem to be specific for RA and can be detected in OA and control samples as well. In addition we observed that the number of carbamylated peptides identified with MS is higher in cartilage when compared to other compartments of the RA joint.

Carbamylated proteins in the RA joint can be recognised by anti-CarP antibodies in sera of RA patients

After the identification of carbamylated proteins in the joint, we next wished to investigate whether these proteins can be recognised in carbamylated form by antibodies that are present in the sera of RA patients. To this end we selected a set of 3 proteins (fibrinogen, fibronectin and transferrin), for these

experiments. These proteins, were selected based on their commercial availability in purified format. As depicted in Figure 3, the RA sera showed increased reactivity towards all the tested carbamylated proteins when compared to healthy controls. These data indicate that proteins present in carbamylated form in the joint of RA patients can act as target for anti-CarP antibodies in RA patients.

Discussion

Immunohistochemical stainings showed that carbamylated antigens are present in all areas of the synovium. To our knowledge, this is the first time that this particular aCBL antibody has been used to stain synovial tissue and we were surprised to see such an extensive staining. Previously, we have tested this antibody in ELISA to specifically detect carbamylated proteins, as proteins harbouring other post-translational modifications were not recognised (18). The use of Ca-FCS or FCS to block the IHC staining further supports the specificity of the currently used antibody.

Our observations in immunohistochemistry were supported by mass spectrometry, which showed that a large variety of carbamylated "neo-epitopes" can be present in the joint. Importantly, proteins that were identified previously as carbamylated in the joint, such as alpha-1 antitrypsin, could also be identified in our current dataset (15). However, the presence of carbamylated proteins is not specific for RA as similar findings were obtained upon analyses of synovial tissue from OA patients and controls. These data were in line with a previous study analysing material from one patient. It was shown that the amount of homocitrulline present in an affected joint was similar to the amount of homocitrulline present in an unaffected joint (17). The presence of inflammation is thought to increase carbamylation, through conversion of thiocyanate into cyanate by myeloperoxidase (20). Our data suggest that carbamylation is not substantially increased at an inflammatory site, although our method was not quantitative.

Interestingly, we observed that the number of identified carbamylated peptides compared to the total number of identified peptides in each of the tissues was increased in cartilage when compared to synovium and synovial fluid. These data are interesting as they point to the notion that especially cartilage will be targeted by anti-CarP-antibodies and/or anti-CarP-antibody expressing B-cells. It is not clear why carbamylation of cartilage would be more prominent than carbamylation of synovial tissue or fluid, but it is tempting to speculate that this relates to the longer half-life of proteins in cartilage. The longer life span of such proteins may therefore allow accumulation of carbamylated sites over time, thereby increasing the total carbamylation in this tissue. However, as MS is not considered to be quantitative, future methods development to determine the extent of carbamylation in different joint compartments would be of interest.

From the set of carbamylated peptides we selected three different proteins, *ex-vivo* carbamylated these proteins and confirmed that these carbamylated proteins are indeed targets of anti-CarP antibodies present in sera of RA patients. Clearly there are differences between these carbamylated proteins and their capacity to bind anti-CarP antibodies. Also the previously identified carbamylated alpha-1-antitrypsin binds anti-CarP antibodies very well (15).

Besides the presence of carbamylated proteins in the RA joint, other proteins with posttranslational modification can also be identified in the joint. Citrullination in the RA joint has been described, but acetylation, malondialdhyde adducts and other modifications may also be present in the joints of RA patients (21, 22). Antibodies targeting some of these additional modifications have been reported previously (21) and each of these antibodies could serve as a biomarker on their own. However, the combination of ACPA, anti-CarP antibodies and rheumatoid factor so far provides the strongest association with the development of RA (23).

This is the first study to investigate carbamylation in different joint tissues of RA patients. We identified a large number of carbamylated residues, especially in cartilage. In RA patients that are positive for anti-CarP antibodies, these antibodies recognise the carbamylated proteins that have been identified in the joint. Therefore it is conceivable that anti-CarP antibodies bind to carbamylated proteins in the joint contributing to the development or continuation of RA.

Key messages

- Numerous carbamylated proteins are present in the joints of both RA patients and controls.
- Carbamylated proteins in the RA joint are recognised by anti-CarP antibodies.

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