

Sanna Turunen

PROTEIN-BOUND
CITRULLINE AND
HOMOCITRULLINE IN
RHEUMATOID ARTHRITIS

*CONFOUNDING FEATURES ARISING
FROM STRUCTURAL HOMOLOGY*

UNIVERSITY OF OULU GRADUATE SCHOOL;
UNIVERSITY OF OULU,
FACULTY OF MEDICINE, INSTITUTE OF DIAGNOSTICS, DEPARTMENT OF CLINICAL CHEMISTRY;
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SANNA TURUNEN

**PROTEIN-BOUND CITRULLINE
AND HOMOCITRULLINE IN
RHEUMATOID ARTHRITIS**

Confounding features arising from
structural homology

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Abstract

Rheumatoid arthritis (RA) is an autoimmune disease causing inflammation of synovial joints, which may lead to permanent changes in cartilage and bone tissues. RA patients have antibodies binding to citrullinated and also to carbamylated proteins. Antibodies binding to citrulline are associated with more severe disease progression and may already appear years before clinical disease onset.

Citrulline and the lysine carbamylation product, homocitrulline, are similar in structure. Citrullinated proteins have been studied in RA and in neurological diseases, but researchers have been unaware of the effect of homocitrulline in citrulline detection methods. The purpose of the present study was to clarify the features of protein-bound citrulline and homocitrulline in relation to research done on citrullination and in immunological reactions related to rheumatoid arthritis.

In the first study of this thesis the confounding role of homocitrulline in citrulline detection was shown. In the first and second study the features of experimentally induced antibodies were assessed. The antibodies induced with citrulline- and homocitrulline-containing protein structures were shown to react both with the ureido groups and the protein structures. The antibodies were able to distinguish between citrulline and homocitrulline in the same sequence even though binding to both. In the third study the simultaneous presence of citrulline and homocitrulline in RA synovial tissue was shown.

In conclusion, considering the simultaneous presence of citrulline and homocitrulline and the binding features of the experimentally induced antibodies, homocitrulline could have a yet unsolved role in RA. Secondly, the existence of homocitrulline should be borne in mind in studies on citrullination.

Keywords: antibodies, citrulline, homocitrulline, immunization, post-translational protein processing, rheumatoid arthritis

Turunen, Sanna, Proteiineihin sitoutuneet sitrulliini ja homositrulliini nivelreumassa. Rakenteellisesta homologiasta johtuvat sekoittavat ominaisuudet

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Tiivistelmä

Nivelreuma on niveltulehduksen aiheuttava autoimmuunitauti, joka voi johtaa pysyviin muutoksiin nivelen rusto- ja luukudoksessa. Nivelreumaa sairastavilla esiintyy vasta-aineita sitrullinointuneita ja karbamyloituneita proteiineja vastaan. Sitrulliiniin sitoutuvia vasta-aineita voi esiintyä elimistössä jo vuosia ennen taudin puhkeamista, ja niiden esiintyminen on yhdistetty vaikeampaan taudinkuvaan.

Sitrulliini ja lysiinin karbamyylaatioaine homositrulliini ovat rakenteellisesti samankaltaisia. Proteiinien sitrullinaatiota on tutkittu nivelreumassa ja neurologisissa taudeissa, mutta homositrulliinin olemassaoloa tai sen vaikutusta tutkimusmenetelmiin ei ole huomioitu. Tämän tutkimuksen tarkoituksena oli selvittää proteiineihin sitoutuneiden sitrulliinin ja homositrulliinin ominaisuuksia aikaisempiin tutkimuksiin ja nivelreuman immunologisiin reaktioihin liittyen.

Tässä tutkimuksessa homositrulliinin osoitettiin häiritsevän sitrulliinin tunnistamista. Ensimmäisessä ja toisessa osatyössä aiheutettiin kokeellisesti vasta-aineita sitrulliinia ja homositrulliinia sisältävillä proteiinirakenteilla. Vasta-aineiden havaittiin reagoivan sekä ureidoryhmän että proteiinirakenteen kanssa. Vasta-aineet pystyivät erottamaan sitrulliinin ja homositrulliinin toisistaan samassa rakenteessa, vaikka sitoutuivat kumpaankin. Kolmannessa osatyössä osoitettiin, että sitrulliinia ja homositrulliinia esiintyy samanaikaisesti nivelreumapotilaan tulehtuneessa nivelkalvossa.

Tutkimus osoitti, että sitrulliinin ja homositrulliinin samanaikainen esiintyminen ja kokeellisesti aiheutettujen vasta-aineiden ominaisuudet huomioiden homositrulliinilla voi olla jokin tois-taiseksi selvittämätön rooli nivelreumassa. Homositrulliinin olemassaolo on syytä huomioida sitrullinaatiota tutkittaessa.

Asiasanat: homositrulliini, immunisaatio, nivelreuma, sitrulliini, translaation jälkeinen proteiinikäsittely, vasta-aineet

To our boys: May you find your own way.

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Abbreviations

ACR	American College of Rheumatology
ACPA	anti-citrullinated protein/peptide antibodies
AKA	anti-keratin antibody
APF	anti-perinuclear factor
AFA	anti-filaggrin antibody
AMC	anti-(modified)-citrulline (antibody)
BSA	bovine serum albumin
CCP	cyclic citrullinated peptide
CRP	C-reactive protein
DMARD	disease-modifying anti-rheumatic drug
EIA	enzyme immune assay
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
ESR	erythrocyte sedimentation rate
EULAR	European League Against Rheumatism
Fc	fragment crystallizable
F95	monoclonal antibody induced with decacitrullinated peptide
GFAP	glial fibrillary acidic protein
HCl	hydrochloric acid
HLA	human leucocyte antigen
HPLC	high performance liquid chromatography
HRP	horseradish peroxidase
HSA	human serum albumin
Ig	immunoglobulin
KCNO	potassium cyanate
KLH	keyhole limpet hemocyanin
MBP	myelin basic protein
MCP	metacarpophalangeal (joint)
MCV	mutated citrullinated vimentin
MPO	myeloperoxidase (enzyme)
MS	multiple sclerosis
MTP	metatarsophalangeal (joint)
NET	neutrophil extracellular trap
NSAID	non-steroidal anti-inflammatory drug
PAD	peptidyl arginine deiminase (enzyme)

PAGE	polyacrylamide gel electrophoresis
RA	rheumatoid arthritis
RF	rheumatoid factor
RT	room temperature
SDS	sodium dodecyl sulfate
SE	shared epitope
SNP	single-nucleotide polymorphism
TBS	Tris buffered saline
TFA	trifluoroacetic acid
TNF α	tumor necrosis factor alpha

List of original articles

This thesis is based on the following articles, which are referred to in the text by their Roman numerals:

- I Turunen S, Koivula MK, Risteli L & Risteli J (2010) Anticitrulline antibodies can be caused by homocitrulline-containing proteins in rabbits. *Arthritis Rheum* 62(11): 3345–3352.
- II Turunen S, Koivula MK, Risteli L & Risteli J (2014) Ureido group-specific antibodies are induced in rabbits with citrulline- or homocitrulline-containing antigens. Manuscript.
- III Turunen S, Koivula MK, Melkko J, Alasaarela E, Lehenkari P & Risteli J (2013) Different amounts of protein-bound citrulline and homocitrulline in foot joint tissues of a patient with anti-citrullinated protein antibody positive erosive rheumatoid arthritis. *J Transl Med* 11: 224.

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1 Introduction

Citrulline is an amino acid present in proteins in apoptosis and inflammatory situations. Citrullination has been studied in the context of rheumatoid arthritis (RA) and antibodies binding to citrullinated proteins are found in the sera of RA patients several years before the onset of the disease. Lately, also carbamylation has attracted attention as it has been associated with cardiovascular and renal diseases. The end product of lysine carbamylation is homocitrulline, a structural homolog of citrulline. These two amino acids are introduced to proteins posttranslationally. Citrulline is a product of enzymatic activity of peptidyl arginine deiminases while homocitrulline is formed from the spontaneous association of cyanate to the basic side chain of lysine. Homocitrulline may also be present in inflammatory situations as a side-product of inflammatory response assisted by myeloperoxidase.

In several studies antibodies binding to chemically modified citrulline have been used in identification of citrullinated proteins. These antibodies were reported to bind exclusively to citrulline irrespective of the surrounding protein sequence. In RA patients serum antibodies binding to citrullinated and recently also to carbamylated proteins have been found. The presence of homocitrulline in RA tissues has not been demonstrated. The homological structure of citrulline and homocitrulline and the colocalization of citrullination and carbamylation in inflammatory situations complicate the identification of the end-products and raise the question of the possible interference of homocitrulline in immunological reactions involving citrulline.

In this thesis the existence of these two independent but often simultaneous systems is reflected through methods of citrulline detection, immunization and analysis of protein-bound citrulline and homocitrulline in RA tissue.

2 Review of the literature

2.1 Rheumatoid arthritis (RA)

Rheumatoid arthritis is a progressive autoimmune inflammatory disease affecting 0.5 to 1.0% of the adult population of Western countries (Kaipiainen-Seppänen *et al.* 1996a). Smoking, obesity and genetic susceptibility are predisposing factors for RA (Conde *et al.* 2013, Stastny & Fink 1977, Stolt *et al.* 2003). Two out of three of those affected by the disease are women. In the beginning the symptoms vary from mild to severe; at this point permanent damage can be avoided by treating the inflammation effectively. Usual symptoms include tenderness and swelling of the joints, morning stiffness, weakness or mild fever, and symmetrical inflammation affecting multiple joints may appear accompanied by pain. Usually the metacarpophalangeal (MCP), metatarsophalangeal (MTP), wrist and knee joints are affected although any synovial joint could be affected. If untreated or if the chronic inflammation of the synovial tissue continues despite the treatment, irreversible erosions of cartilage and bone tissues will likely develop. The disease may also lead to extra-articular manifestations like rheumatoid nodules. The life expectancy of RA patients used to be shorter compared to general population. The increased deaths were due to cardiovascular disease, infections and pulmonary and renal disease (Sokka *et al.* 2008).

The disease activity is assessed clinically, often with the help of indices like DAS28, which summarize patients' and physicians' global assessments with a visual analog scale (VAS), the number of tender and swollen joints and systemic inflammation by C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR). The RA inflammation is treated by disease-modifying anti-rheumatic drugs (DMARDs) that are used for suppressing the pathological immune system activity and alleviating inflammatory symptoms and permanent destruction of the joints. Non-steroidal anti-inflammatory drugs (NSAIDs) are used for reducing inflammatory pain and stiffness and analgesics for reducing pain in general. DMARDs can be used as single medication or in combination with other DMARDs, glucocorticoids or biological drugs. Biological drugs refer to proteins produced in living organisms. These proteins are usually antibodies or fusion proteins directed to inhibit or augment immunological pathways. The most commonly used biological drugs are tumor necrosis factor alpha (TNF α) inhibitors. (Scott *et al.* 2010)

The medical treatment of RA has been studied intensively in recent years. The modern treatment goal is to achieve remission defined by absence of active joint inflammation and erosive and functional decline (Smolen *et al.* 2010). The FIN-RACo and NEO-RACo studies in Finland have covered ground on the combination treatments of RA (Leirisalo-Repo *et al.* 2012, Rantalaiho *et al.* 2012, Rantalaiho *et al.* 2013 Möttönen *et al.* 1999). RA patients can currently be treated effectively and permanent damages can usually be avoided, but on the other hand there are patients who have had RA before the modern medication was available, and sometimes their chronic symptoms need to be alleviated by more radical treatments like surgery.

The disease course is difficult to predict, but the presence of rheumatoid factor (RF) or anti-citrullinated protein antibodies (ACPA) in the patient serum have been connected to more erosive course of the disease (van Jaarsveld *et al.* 1999, Vencovsky *et al.* 2003). The incidence of RF-positive RA declined in Finland between the years 1980 and 2000 (Kaipiainen-Seppänen & Kautiainen 2006). The mean age of disease onset has also shifted from 40–50 years to closer to 60 years (Kaipiainen-Seppänen *et al.* 1996b). The reason for this shifting is unknown but the more efficient treatment of infections and the use of contraceptives may have contributed to it (Bhatia *et al.* 2007, Hannaford *et al.* 1990).

2.1.1 ACR/EULAR criteria for RA

In the 1950s there was no consensus on the definition of rheumatoid arthritis. Set criteria were needed for scientific research purposes to specify rheumatoid arthritis from other arthritides. The first diagnostic criteria for RA were launched in 1958 (American Rheumatism Association 1959). The criteria defined patients fulfilling the criteria in several subclasses of classical, definite, probable and possible RA and also defined a list of excluding symptoms. The criteria were updated in 1987 (Arnett *et al.* 1988). In these updated classification criteria the subclassification and the excluding symptoms were abandoned. In 2010 the American College of Rheumatology (ACR) and the European League Against Rheumatism (EULAR) presented new classification criteria for rheumatoid arthritis (Table 1). At this time, ACPA were for the first time included in the criteria and serology was emphasized in the grading. The criteria were aimed at classification of newly presenting patients, but patients who fulfilled the criteria before their publication should also be classified as having RA. Patients who have

at least 1 joint with definite clinical synovitis (swelling) and patients with the synovitis not better explained by another disease can be tested with the criteria. A disease score of $\geq 6/10$ in the criteria presented in Table 1 is needed to define a patient as having definite RA. When defining the synovitis status all joints may be assessed with the exception of distal interphalangeal joints, the first metatarsophalangeal joint and the first carpometacarpal joint. The aforementioned joints are typically affected in osteoarthritis and therefore inclusion of these joints could lead to misinterpretation. (Aletaha *et al.* 2010)

Table 1. The 2010 American College of Rheumatology (ACR)/European League Against Rheumatism (EULAR) classification criteria for rheumatoid arthritis (Aletaha *et al.* 2010).

Criteria	Score
Joint involvements	
1 large joint	0
2–10 large joints	1
1–3 small joints (with or without involvement of large joints)	2
4–10 small joints (with or without involvement of large joints)	3
> 10 joints (at least 1 small joint)	5
Serology (at least 1 test result is needed for classification)	
Negative RF and negative ACPA	0
Low-positive RF or low-positive ACPA	2
High-positive RF or high-positive ACPA	3
Acute-phase reactants (at least 1 test result is needed for classification)	
Normal CRP and normal ESR	0
Abnormal CRP or abnormal ESR	1
Duration of symptoms	
< 6 weeks	0
≥ 6 weeks	1

Scores of categories A-D are added; a score of $\geq 6/10$ is needed for classification of patient as having definitive RA. (RF, rheumatoid factor; ACPA, anti-citrullinated protein antibody; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate)

The new ACR/EULAR criteria have been compared to the earlier ACR 1987 criteria (Arnett *et al.* 1988) in several studies. The 2010 criteria have been shown to be slightly better at identifying erosive disease compared to the 1987 criteria (Mäkinen *et al.* 2012) although the ability to distinguish erosive patients in early arthritis remained low. Humphreys and Symmons (Humphreys & Symmons 2013) reviewed 17 studies on the new classification criteria and concluded that the new criteria recognize RA patients earlier in the disease, but may also result in some

false positive cases. They also concluded that seronegative patients are less likely to meet the new criteria. Patients presenting with early inflammatory arthritis meeting the ACR/EULAR 2010 criteria have increased mortality compared to early arthritis not fulfilling the criteria (Humphreys *et al.* 2013).

2.1.2 RA disease mechanism

The primary event in RA is not known and the cascade leading to the inflammatory symptoms has been studied but not yet solved. More effective ways of treating the disease have been developed based on these studies. Several sites in the body, such as the lungs and oral cavity, have been proposed as places for the primary RA antibody induction (Klareskog *et al.* 2006, Nesse *et al.* 2012, Rosenstein *et al.* 2009).

Currently there are two alternative hypotheses for RA induction. The more generally accepted outside-in hypothesis suggests that ACPA attack the proteins of the synovia, causing inflammation, and the progression of the inflammation leads to erosions of cartilage and bone. (Lundberg *et al.* 2005, Masson-Bessiere *et al.* 2000, Vossenaar & van Venrooij 2004).

A recently suggested theoretical hypothesis challenges the earlier opinions as it describes the disease as progressing outwards from the inside of the bone. In this hypothesis the ACPA cause bone loss before clinical onset of the joint symptoms by affecting the osteoclast differentiation. The bone loss leads to widening of the cortical bone channels and fenestration of the cortical bone. Through these channels the inflammatory cells from the bone marrow could enter the joint space. The primary induction of ACPA could be anywhere in the body, possibly in the lungs or gingiva. (Harre *et al.* 2012, Kleyer *et al.* 2013)

2.1.3 Genetic factors

The susceptibility to RA has been connected to human leukocyte antigen (HLA) class II alleles (Stastny & Fink 1977). This susceptibility is in genes coding epitope-recognizing parts on the HLA-DRB1. The susceptibility is defined by the shared epitope (SE), an amino acid sequence that is located in the third hyper-variable region coded by the gene (Gregersen *et al.* 1987). The sequence of the antigen-binding pocket in SE is QR(K)RAA (de Vries *et al.* 2005). SE alleles have been indicated as a risk factor for ACPA (van der Helm-van Mil *et al.* 2006) and have also been reported to influence the fine specificity of the ACPA response

(Verpoort *et al.* 2007). Genetic variation in protein tyrosine phosphatase gene (*PTPN22*) has been suggested to have an interaction with HLA alleles in developing ACPA positive RA (Kallberg *et al.* 2007).

Genetic susceptibility to RA has also been proposed with a genetic variant of a serine protease, granzyme B (Wellcome Trust Case Control Consortium 2007), and SNP located in this gene has been connected to more severe joint destruction in RA patients (Knevel *et al.* 2013). Certain SNPs in genes coding PAD4 have been connected to susceptibility to ACPA-positive RA in Asian populations (Yamada *et al.* 2003) but the results in Caucasian populations are not unanimous (Caponi *et al.* 2005, Plenge *et al.* 2005)

2.1.4 Environmental factor - Smoking

The RA-predisposing nature of cigarette smoking was recognized by chance in the 1980s while investigating the effects of oral contraceptives in RA (Vessey *et al.* 1987). It is unclear how smoking acts in the development of RA, but it is likely that the mechanism of action is multifactorial. Smoking acts as a mediator for inflammation and increases the production of IL-6 and CRP (Yanbaeva *et al.* 2007). The concentration of thiocyanate in the serum of smokers is about triple the concentration of non-smokers (Zil-a-Rubab & Rahman 2006), and smokers are at increased risk of infections (Huttunen *et al.* 2011). Smoking is the only environmental factor connected to the development of RF-positive RA (Costenbader *et al.* 2006, Stolt *et al.* 2003). The development of ACPA in genetically susceptible (HLA-DRB1) individuals has also been associated with smoking (Klareskog *et al.* 2006, Linn-Rasker *et al.* 2006). Smoking has been shown to increase PAD2 expression and citrullination in bronchoalveolar lavage fluid cells (Makrygiannakis *et al.* 2008). The effect of smoking is not restricted to the predisposition to RA but also to disease severity (Manfredsdottir *et al.* 2006). Smoking predicts the development of RF positive RA and radiographic bone erosions in dose-dependent manner (Saag *et al.* 1997). The risk of RA increases with the duration of smoking and remains high years after smoking cessation (Costenbader *et al.* 2006, Stolt *et al.* 2003).

2.1.5 Serum antibodies found in RA

ACPA and RF are frequently found in RA patient sera and are included in ACR/EULAR criteria, but not all RA patients possess these antibodies. The

clinical disease in the seronegative subpopulation is usually milder than in the seropositive patients. ACPA and RF antibodies may precede clinical onset of the disease by several years. The prevalence of ACPA two years before onset of the disease is nearly the same as in the established disease (Aho & Heliövaara 2004). Before outbreak of RA ACPA and RF may fluctuate from positive to negative (Barra *et al.* 2013), but in established disease the positive status to each antibody remains quite constant regardless of disease activity (Mjaavatten *et al.* 2011, Rönnelid *et al.* 2005).

Even though rheumatoid arthritis is described as one disease in the ACR/EULAR criteria there are differences in the disease characteristics connected to the serological status of the patient. Genetic factors (HLA-DR) have been linked to both RF- and ACPA- (van der Helm-van Mil *et al.* 2006) positive disease, the disease progression in seropositive patients is more severe and smoking seems to be a predisposing factor only in the seropositive subgroup (Linn-Rasker *et al.* 2006, Manfredsdottir *et al.* 2006). Because of these characteristics even the treatment of the disease may be affected.

2.1.6 Rheumatoid factor (RF)

RF was first identified in 1939 as a factor potentiating agglutination of sheep cells pre-treated with rabbit anti-sheep serum (Waalder 1939). The method was redescribed by Rose in 1948 (Rose & Ragan 1948). The Waaler-Rose test was followed by a latex-based agglutination test introduced in 1956 by Plotz and Singer (Plotz & Singer 1956). Today, RF is clinically tested with automated methods measuring IgM class RF.

RF antibodies can be found both in RA sera and synovial fluid. These antibodies recognize the IgG Fc region. These antibodies exist as IgG, IgM and IgA class antibodies. 70–85% of RA patients are positive for RF, but the etiology and the role of RF in RA pathogenesis is unknown. RF in the serum may precede the RA symptoms by several years (Aho *et al.* 1994). Positivity for RF has been connected to disease severity and RF positive patients have extra-articular manifestations like subcutaneous nodules, pulmonary disease or vasculitis more frequently than RF negative patients (Aho & Heliövaara 2004). However, RF is not specific for RA, but false positive results can be found in other autoimmune diseases, temporarily in infectious diseases and also in some healthy subjects (Dörner *et al.* 2004, Korpilähde *et al.* 2003). This has led to the search of better markers for RA diagnosis.

2.1.7 Anti-citrullinated protein antibodies (ACPA)

History of anti-citrullinated protein antibodies

ACPA are a relatively recent finding in the history of rheumatoid arthritis. In 1964 anti-perinuclear factor (APF) antibodies (Nijenhuis and Mandema 1964) and in 1979 anti-keratin antibodies (AKA) (Young *et al.* 1979) were found rather specific to rheumatoid arthritis. APF and AKA were found to recognize filaggrin of the human epidermis (Sebbag *et al.* 1995, Simon *et al.* 1995). At this time the presence of citrulline in the antigenic determinants of these antibodies was not yet known. Fifteen years ago Schellekens and co-workers reported the presence of antibodies binding to citrullinated antigens (Schellekens *et al.* 1998). Both APF and AKA were found to recognize citrullinated filaggrin epitopes and the two groups of antibodies were combined as anti-filaggrin antibodies (AFA) (Girbal-Neuhauser *et al.* 1999).

Specific antibodies binding to intermediate filament protein vimentin were found by Osung and co-workers in 1982 (Osung *et al.* 1982). In 1994 Despres and co-workers presented the anti-Sa autoimmune system (Despres *et al.* 1994) which was later shown to recognize citrullinated vimentin (Vossenaar *et al.* 2004a). Antibodies binding to citrullinated α -enolase (Lundberg *et al.* 2008), fibrin (Masson-Bessiere *et al.* 2001) and type I and type II collagen, major constituents of bone and cartilage (Burkhardt *et al.* 2005, Koivula *et al.* 2005, Koivula *et al.* 2006), have also been found in RA patient sera, but the true antigens of the anti-citrulline antibodies are still unknown. There are probably several different citrullinated proteins that may act as triggers to ACPA. The presence of citrullinated proteins is not exclusive to RA, but the immunological response to these proteins distinguishes RA from other diseases.

New insights into the role of ACPA in other diseases arise from the fact that ACPA-positive RA patients are at increased risk for cardiovascular disease. The presence of ACPA has been found in a sub-population of coronary heart disease patients without RA (Cambridge *et al.* 2013), but whether they have a role in disease development or severity remains to be elucidated.

Features of anti-citrullinated protein antibodies

Antibodies binding to citrullinated proteins may appear in the serum of RA patients' even years before the onset of clinical symptoms (Nielen *et al.* 2004).

After the disease onset the antibody status remains fairly constant (Rönnelid *et al.* 2005). ACPA antibodies are found in IgG, IgM and IgA subclasses (Kokkonen *et al.* 2011). Indications of the varying origins of ACPA are presented in several studies. Epitope spreading between citrullinated antigens before disease onset has been reported (van de Stadt *et al.* 2011, van der Woude *et al.* 2010a). ACPA have been shown to cross-react between several citrullinated antigens and even with carbamylated antigens (Shi *et al.* 2011). These antibodies have also been shown to have some overlapping specificities (Ioan-Facsinay *et al.* 2011, Shi *et al.* 2013).

ACPA seem to have low avidity compared to antibodies produced against inoculation antigens (Suwannalai *et al.* 2011). The avidity of ACPA goes trough maturation before disease onset and is then stabilized. This maturation leads to increased avidity but is pronounced in only a limited group of patients (Suwannalai *et al.* 2012).

The ACPA development is different from the traditional antibody response also in respect to antibody isotypes. While traditionally B-cells go through isotype switching from primary IgM response to IgG, in rheumatoid arthritis the IgM antibodies are present even after IgG switching (Suwannalai *et al.* 2011a). The fine-specificity of the IgM class antibodies is also different from the specificity of the IgG class (Suwannalai *et al.* 2011b). When ACPA profiles between RA patients and their healthy relatives were compared the antibody isotype profiles were different, especially IgM antibodies being rare in healthy relatives (Ioan-Facsinay *et al.* 2008). In RA patients the wide isotype distribution has been linked to radiographic progression (van der Woude *et al.* 2010b).

Clinical testing of anti-citrullinated protein antibodies

The determination of ACPA status is part of the ACR/EULAR 2010 classification criteria for RA (Aletaha *et al.* 2010) but the method used for this is not restricted in the criteria. Several commercially available tests for citrullinated protein binding antibodies have been developed and many are already in clinical use.

Anti-cyclic citrullinated peptide (anti-CCP) is a test widely used in RA diagnostics. The first version of this test, anti-CCP1, is based on fillagrin peptides (van Jaarsveld *et al.* 1999) with a sensitivity of 47% in RA (Schellekens *et al.* 2000). The second version, anti-CCP2, is based on citrulline-containing peptides selected from a library by their ability to distinguish between RA patients and controls. The origin of these antibodies is irrelevant in the tests since these are

based on the cross-reaction of anti-citrullinated protein antibodies. The sensitivity of CCP2 is 70–75% and specificity 95–99%. (van Venrooij & Zendman 2008)

A third-generation test, CCP3, has also been introduced (van der Linden *et al.* 2009), and it has been suggested as a diagnostic marker in RF-negative RA patients (Szekanecz *et al.* 2013). Antibodies binding to citrullinated proteins can also be measured with anti-mutated citrullinated vimentin (anti-MCV) test with a sensitivity of 70.7% and specificity of 95% (Mathsson *et al.* 2008). Most commercially available tests for ACPA are based on EIA methods and measure IgG class antibodies. No internationally approved standard for ACPA measurements exists, which is why results are expressed as arbitrary units defined by each manufacturer.

2.1.8 Antibodies binding to carbamylated proteins

First antibodies binding to carbamylated proteins in RA patient sera were reported in 2011 (Shi *et al.* 2011). Carbamylation and antibodies binding to carbamylated proteins in RA are a recent discovery and have been only little studied. The antibodies binding to carbamylated proteins seem to cross-react with citrullinated antigens to some extent (Shi *et al.* 2013) and in arthralgia they predicted the development of RA (Shi *et al.* 2012).

2.2 Ureido groups in proteins

Ureido group is a chemical functional group with a structure derived from urea (Fig. 1). Ureido group can be formed in protein indirectly without the influence of urea by citrullination or derived from urea through the spontaneous incorporation of isocyanate to free aminogroup in carbamylation.

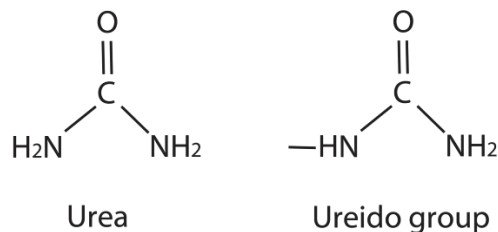


Fig. 1. Chemical structures of urea and ureido group.

Citrulline and homocitrulline are amino acids that can be considered as substituted ureas. They are present in proteins as products of posttranslational modifications. Citrulline is formed enzymatically from arginine and homocitrulline from lysine either spontaneously or in an enzyme-assisted reaction. These two amino acids have common features in immunogenicity, charge and reactions with chemical substances. Both feature in several, but mostly different diseases.

Citrulline and homocitrulline are structural homologs. This means that they are similar in structure and separated only by a repeating unit, one additional methylene group (-CH₂-), in the side chain of homocitrulline (Fig. 2). Arginine and lysine, precursors of citrulline and homocitrulline, are basic amino acids and often located on the surface of the protein. The loss of charge caused by carbamylation (Kraus & Kraus 2001, Kraus *et al.* 2001) or citrullination distorts the protein structure (Kraus & Kraus 2001, Tarcsa *et al.* 1996) resulting in a potentially antigenic structure (Mydel *et al.* 2010). Citrullination has been studied in RA (Kinloch *et al.* 2008), neurological disorders (Moscarello *et al.* 2007, Nicholas 2013) and periodontitis (Nesse *et al.* 2012). Homocitrulline has lately been connected to poorer prognosis of end-stage renal disease patients (Koeth *et al.* 2013) and patients with chronic systolic heart failure and renal impairment (Tang *et al.* 2013).

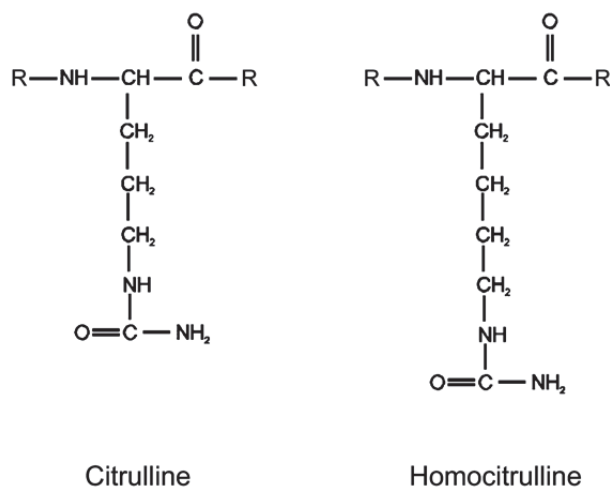


Fig. 2. The structures of citrulline and homocitrulline.

2.2.1 Citrullination

The amino acid citrulline was first isolated in 1930 (Wada 1930) from watermelon (*citrullus vulgaris*). Citrulline is present in free form in mammalian tissue in the urea cycle (Krebs and Henseleit 1932) and as a by-product in nitric oxide synthesis (Mori 2007). When citrulline is found attached to a protein it is a product of citrullination also known as deamination (Fig. 3), a posttranslational modification of protein-bound arginine. The presence of citrulline in proteins was already reported in the 1930s (Fearon 1939).

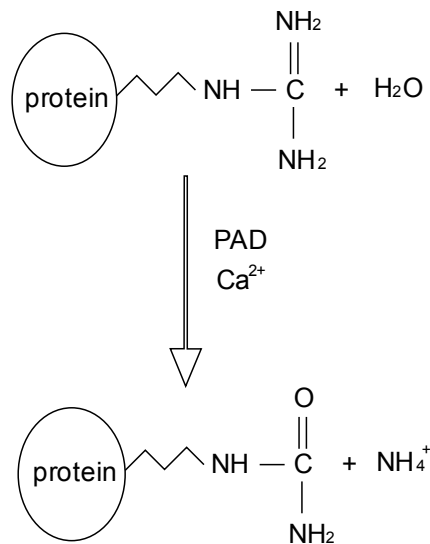


Fig. 3. Citrullination: deamination of peptidyl arginine to peptidyl citrulline by peptidyl arginine deiminase. (PAD, peptidyl arginine deiminase enzyme; Ca²⁺, calcium ion; H₂O, water molecule).

Citrullinating enzymes peptidyl arginine deiminases (PADs, EC 3.5.3.15) were first discovered in the hair follicles (Rogers & Taylor 1977, Rogers *et al.* 1977) and later isolated and characterized in more detail in rabbit skeletal muscle (Sugawara *et al.* 1982). PADs react almost exclusively with peptide-bound arginine and not with free arginine (Sugawara *et al.* 1982) and are dependent on calcium concentrations higher than present in intact cells (Vossenaar & van Venrooij 2004). Five isoforms of PADs have been described in humans (PAD1, PAD2, PAD3, PAD4 and PAD6). PAD1 is found in epidermis, PAD2 in muscle tissue and central nervous system, PAD3 in hair follicles, PAD4 in hematopoietic

cells and PAD6 in embryonic cells. PAD enzymes have five calcium-binding sites. The binding of calcium results in considerable conformational changes and activates the enzyme. Each PAD isoform has its specific substrates. (Baka *et al.* 2012, Bicker & Thompson 2013)

Citrullination in physiological processes

Citrullination takes place in several physiological processes. Citrullination is a significant event in apoptosis. The skin proteins keratin, filaggrin and trichohyalin are citrullinated in the terminal differentiation stages of skin keratinocytes. Citrullination of central nervous system myelin basic protein (MBP) is also an example of the physiological citrullination that undergoes changes during brain development. (Baka *et al.* 2012, György *et al.* 2006) Citrullination of histones is involved in gene regulation, and hypercitrullination of histones is used as neutrophil defense mechanism, neutrophil extracellular traps (NETs), for trapping and killing bacteria (Brinkmann *et al.* 2004).

Citrullination in disease

Citrullination is also present in several diseases. Citrullination has been found in cancer (Chang & Han 2006, Chang *et al.* 2009), prion diseases (Jang *et al.* 2010), neurological disorders (Ishigami *et al.* 2005), periodontitis (Nesse *et al.* 2012) and RA (György *et al.* 2006). Neurological disorders with the involvement of citrullination include citrullination of glial fibrillary protein (GFAP), which is characteristic for Alzheimer's disease, and overcitrullination of MBP, which is considered the cause of multiple sclerosis (MS) (Moscarello *et al.* 2007).

Citrullination of human proteins by bacterial peptidylarginine deiminases has also been studied. PAD from *Porphyromonas gingivalis*, a bacterium present in periodontitis, has been found to citrullinate human fibrinogen and α -enolase (Wegner *et al.* 2010). The *Porphyromonas gingivalis* PAD is different from human PADs. It is not calcium-dependent, the optimum pH is higher and it mostly citrullinates C-terminal arginines and is also autocitrullinated. (McGraw *et al.* 1999, Rodriguez *et al.* 2009).

In RA, citrullination has been in the focus of interest as antibodies against several citrullinated proteins have been found in RA patients' sera and synovial fluids. PAD2 and PAD4 expression has been shown in RA patient tissues: synovial membrane (Chang *et al.* 2005, De Rycke *et al.* 2005, Foulquier *et al.*

2007, Nakayama-Hamada *et al.* 2005), synovial fluid cells (Vossenaar *et al.* 2004b), and extracellularly in synovial fluid (Kinloch *et al.* 2008). PAD2, PAD3 and PAD4 each have their specific substrates that are not restricted by their subcellular localization and only some of the proteins citrullinated by PADs are recognized by RA autoantibodies (Darrach *et al.* 2012). Antigenicity of citrullinated proteins in experimental animals has also been demonstrated (Lundberg *et al.* 2005).

2.2.2 Carbamylation

The first publications on carbamylation date back to the 1960s. Carbamylation can affect the free aminotermius or lysine residues in a protein. The spontaneous reaction of isocyanic acid with the free amino terminus leads to N-carbamylated protein product, or in the case of lysine, to the formation of homocitrulline (ϵ -carbamyl lysine). The reaction of cyanate with α -amino groups is 100 times faster than for the ϵ -amino group of lysine. (Stark 1965)

Cyanate is in equilibrium with urea and is always present in physiological conditions. The plasma concentrations of isocyanate in healthy individuals are around 50 nmol/L and in uremic patients about 150 nmol/L (Nilsson *et al.* 1996). In addition to urea decomposition cyanate may be gained from cigarette smoke or as recently suggested, from the smoke of biomass combustion (Roberts *et al.* 2011).

Carbamylation is covalent post-translational modification accumulating in proteins during aging and in various diseases (reviewed in (Jaisson & Gillery 2010)). At protein level the effects of lysine carbamylation are similar to those of citrullination. Lysine is a basic amino acid and often located on the surface of the protein enhancing the protein solubility in water environment. The modification to homocitrulline causes loss of positive charge and a change in protein conformation (Kraus & Kraus 2001). The carbamylation of the free amino terminus does not change the protein structure similarly.

The health effects related to carbamylation have recently raised interest. Carbamylation found in uremic patients (Kraus & Kraus 2001) and inflammatory situations (Wang *et al.* 2007) has attracted attention. Carbamylation of hemoglobin has been studied in respect to assessing the adequacy of hemodialysis. In hemoglobin the site of carbamylation is a valine residue (Stim *et al.* 1995). The carbamylation of histones (Ramponi *et al.* 1971) and actin (Kuckel *et al.* 1993) has been shown *in vitro*. Carbamylation changes the structural stability of serum

albumin (Fazili *et al.* 1993) and alters the clearance of lipoproteins from serum in experimental animals (Hörkkö *et al.* 1992). Carbamylation is implicated in atherogenesis (Roxborough & Young 1995) and was found to accelerate the growth of mesengial cells and increase type I and IV collagen synthesis (Shaykh *et al.* 1999). Carbamylation-dependent activation of T cells in the pathogenesis of autoimmune arthritis has been suggested (Mydel *et al.* 2010) and the interaction of carbamylated albumin and collagen with the immune response cells was found (Jaisson *et al.* 2006, Jaisson *et al.* 2007).

Carbamylation has been widely studied *in vitro*. Proteins containing homocitrulline *in vivo* have been detected in serum preparations from end-stage renal disease patients (Koeth *et al.* 2013) and patients with renal impairment related to chronic systolic heart failure (Tang *et al.* 2013) where the presence of homocitrulline was connected to poorer outcome of the diseases. In the case of heart failure patients the simultaneous presence of myeloperoxidase was relevant to the outcome.

Carbamylation assisted by myeloperoxidase

In addition to the spontaneous carbamylation, there is carbamylation route assisted by myeloperoxidase (MPO) enzyme (Fig. 4). MPO is present in inflammatory cells and catalyzes the oxidation of thiocyanate to cyanate in the presence of hydrogen peroxide, enabling protein carbamylation (Wang *et al.* 2007).

MPO is a heme peroxidase present in phagocytic cells, granulocytes and monocytes. In monocytes MPO is lost when they differentiate to macrophages (Deimann 1984). MPO is part of the defense mechanism against outside pathogens. It acts in the phagosome or is released in the extracellular space if the pathogen is too large to be ingested by the phagocytic cell. MPO functions in the NETs (Brinkmann *et al.* 2004) where it is important during NET formation (Papayannopoulos *et al.* 2010) and actively mediates bacterial killing in the formed NET (Parker *et al.* 2012). MPO uses hydrogen peroxide (H₂O₂) to react with halides (iodide, bromide, chloride) (Klebanoff 1968) or with thiocyanate (Klebanoff *et al.* 1966). Thiocyanate, gained from diet or tobacco smoke, is turned into active cyanate leading to carbamylation, thus linking inflammation, smoking and uremia to carbamylation (Wang *et al.* 2007). The presence of active MPO in extravascular tissue is one of the first markers of neutrophil infiltration to inflammation sites (Prokopowicz *et al.* 2012).

Carbamylation in urea solutions

Urea is a chaotrope that is used for solubilizing proteins in solution. In this context urea is used in high concentrations (6–8 M). When handling tissue or purified protein samples for citrulline or homocitrulline detection the use of urea should be avoided. The chemical balance between urea and ammonium and cyanate ions is present in solution and carbamylation is significant already at low temperatures. Even though cyanate can be removed from urea solutions by de-ionization and somewhat controlled by using scavengers (tris or glycine), the chemical dissociation of urea restores cyanate to the solution in a few days (Stark 1965). The carbamylation by isocyanic acid is pH-dependent and pH 8.5–9.5 is optimal for carbamylation of lysine; in lower pH the carbamylation of protein-free α -amino groups is favored (Righetti 2006).

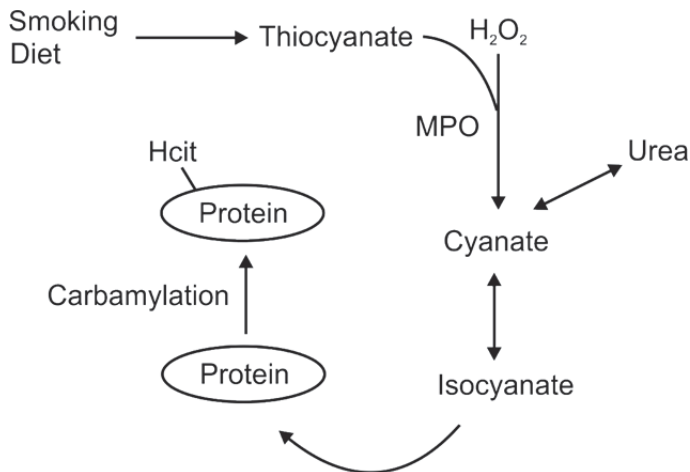


Fig. 4. Carbamylation assisted by myeloperoxidase. (H_2O_2 , hydrogen peroxide; MPO, myeloperoxidase; Hcit, homocitrulline)

2.2.3 Detection of ureido groups

The need for the detection of ureido groups in proteins has risen since the presence of several citrullinated and carbamylated proteins have been connected to aging and various diseases. Methods for detecting ureido groups are described below.

Chemical modification of ureido groups

The chemical modification of ureido groups with 2,3-butanedione (also known as diacetyl) creates an adduct with yellow/orange color. This characteristic can be used to detect free citrulline and homocitrulline in a solution. The chemical modification method has been used in absorbance measurements of ureido group-containing molecules since the 1930s (Fearon 1939), but it can also be used as part of other techniques like high performance liquid chromatography (HPLC) or mass spectrometry.

Mass spectrometry

Citrullinated proteins and peptides can be analyzed using mass spectrometry. The mass shift caused by arginine deamination to citrulline is 1 dalton. Such a small mass shift is difficult to detect and the chemical modification of citrulline residues with antipyrine and 2,3-butanedione has been introduced also in mass spectrometric analysis (De Ceuleneer *et al.* 2011). The modification reaction with only 2,3-butanedione results in a mass shift of +50 Da, or the reaction can be taken further with antipyrine to result in a mass shift of +238 daltons (Holm *et al.* 2006) to make citrulline easier to detect (De Ceuleneer *et al.* 2011, Ling *et al.* 2013). Protein-bound citrulline and homocitrulline can also be detected from hydrolyzed samples using mass spectrometry. A method for quantitative measurements of protein bound homocitrulline has been introduced (Tang *et al.* 2013).

Anti-(modified)-citrulline antibodies

Anti-(modified)-citrulline (AMC) antibodies are rabbit polyclonal antibodies induced with enzymatically citrullinated histones where citrulline residues were chemically modified with antipyrine and diacetyl monoxime (2,3-butanedione monoxime). The antibodies were introduced as a detection method for chemically modified citrulline in proteins blotted on polyvinylidene difluoride membrane irrespective of the protein backbone. (Senshu *et al.* 1992)

This method has been used in detection of citrullinated proteins in several studies related at least to RA (Chang *et al.* 2005, Klareskog *et al.* 2006, Matsuo *et al.* 2006), cancer (Chang & Han 2006), neurological disorders (Raijmakers *et al.* 2005) and cardiovascular disease (Sokolove *et al.* 2013).

F95

F95 is a monoclonal antibody produced in hybridoma cells of mouse origin (Nicholas & Whitaker 2002). The immunogen for this antibody clone was a decacitrullinated peptide linked to maleimic acid activated KLH. When characterized by the discoverers it was found to recognize citrullinated forms, but not native myelin basic protein (Nicholas & Whitaker 2002). There was staining of the myelin, as expected by the investigators, but also staining in fibers associated with ventricular and vascular structures and stellate cells in the proximity of small blood vessels.

F95 has later been used in a wide variety of studies on citrullinated proteins. In RA-related studies intracellular F95 staining was found to be connected to the presence of ACPA and HLA-DR (SE). The extracellular F95 staining co-localized with high numbers of polymorphonuclear cells and was not found to be correlated with either ACPA or SE. (De Rycke *et al.* 2005)

2.3 Collagens

Collagens are a group of extracellular matrix proteins encompassing 28 proteins in five different functional categories: Fibril-forming collagens, fibril-associated collagens with interrupted triple helices (FACITs), membrane-associated collagens with interrupted triple helices (MACITs), network forming collagens and MULTIPLEXINs (multiple triple-helix domains and interruptions). (Shoulders & Raines 2009)

Collagens compose about 30% of the extracellular matrix proteins. All collagens have a common structure of three α chains. The chains can be identical, forming a homotrimer, or different, forming a heterotrimer. All collagens contain one or more triple helical domains, called collagenous domains. The triple helix is formed in a region of repeating glycine-X-Y triplets where X is often proline and Y hydroxyproline. Collagenous domains are flanked or interrupted by non-collagenous domains. (Gordon & Hahn 2010, Ricard-Blum 2011, Shoulders & Raines 2009)

Type I and II collagens are major components of bone and cartilage and belong to fibrillar collagens. In RA, the environment of these two collagens is targeted by inflammation and erosion. The properties of type I and II collagens are described below in more detail.

2.3.1 Type I collagen

Type I collagen is a major constituent of bone comprising 90% of the organic matrix of mineralized bone and about 70% of the body collagens. Two type I collagen $\alpha 1$ chains and one $\alpha 2$ chain are synthesized in the endoplasmic reticulum (ER), and after series of enzymatic posttranslational modification these are assembled to procollagen molecules in the cisternae of the ER. The procollagen molecule is cleaved from both aminoterminal and carboxyterminal ends, releasing the propeptides (measured as PINP and PICP respectively), to allow the mature collagens to aggregate spontaneously to fibrils outside the cell. The large propeptide sequences cleaved before fibril formation can be used as markers of collagen synthesis. (Koivula *et al.* 2012)

When type I collagen is degraded, covalently cross-linked cleavage products from both aminoterminal (NTX) and carboxyterminal ends (CTX and ICTP) are released in the circulation and can be used as markers of collagen type I degradation. (Koivula *et al.* 2012, Risteli *et al.* 2012)

2.3.2 Type II collagen

Type II collagen is predominantly expressed in cartilage and constitutes 90% of the adult cartilage collagen. Type II collagen is synthesized as three α chains. The type II collagen formation resembles the cascade of type I collagen formation. The synthesis of the chains is followed by hydroxylation and glycosylation reactions before assembly of procollagen molecules in ER. The amino- and carboxyterminal propeptides are cleaved by extracellular proteases to allow helix formation and aggregation to fibrils. Type II collagen synthesis can be assessed by measuring the amino- (PIINP/PIIANP) and carboxyterminal propeptides (PIICP). (Eyre *et al.* 2006, Garvican *et al.* 2010)

The breakdown of type II collagen is executed by matrix metalloproteases and can be measured by the cleavage products of carboxyterminal telopeptides. These are measured as cross-linked peptides CTX-II of the carboxyterminus. (Garvican *et al.* 2010)

2.3.3 Ureido groups in type I and II collagens

Posttranslational changes accumulate in proteins with a long half-life in the body. The half-life of cartilage collagen is approximately 117 years and that of skin

collagen 15 years (Verzijl *et al.* 2000). Citrullination and carbamylation of collagens *in vivo* has not been demonstrated to date, but the consequences of these events have been studied *in vitro*. Carbamylation affects the conformational structure of collagen and its ability to activate human polymorphonuclear neutrophils (Jaisson *et al.* 2006). Carbamylation also alters type I collagen sensitivity to collagenases (Jaisson *et al.* 2007). Antibodies binding to citrulline-containing type I and II collagen-derived sequences have been detected in RA patients' sera (Koivula *et al.* 2005). Antibodies binding to citrullinated and native collagen telopeptide sequences (Koivula *et al.* 2007) may precede the clinical onset of the disease by several years. Antibodies binding to type II collagen have been detected in RA patient sera, and in an animal model (murine collagen antibody-induced arthritis) antibodies binding to collagen type II were able to induce cartilage damage in joints without active inflammation (Croxford *et al.* 2013).

3 Outlines of the study

The purpose of the present study was to clarify the features of protein-bound citrulline and homocitrulline in relation to research done on citrullination and in immunological reactions related to rheumatoid arthritis. The thesis aims at answering the following questions:

1. Can homocitrulline interfere in methods designed for citrulline detection?
2. Is it possible to induce citrulline-binding antibodies with carbamylated immunogens?
3. Do the antibodies induced with citrulline-containing immunogens bind to homocitrulline-containing antigens?
4. Are the antibodies able to make a distinction between citrulline and homocitrulline in the same sequence?
5. Can homocitrulline be found in RA tissue?
6. Are citrulline and homocitrulline present simultaneously in RA tissue?

4 Materials and methods

4.1 Preparation of citrullinated proteins (I, II)

10 mg of human albumin (Octapharma AB, Stockholm, Sweden) was citrullinated with rabbit skeletal muscle PAD (Sigma, Steinheim, Germany) in 0.1 M Tris, 10 mM CaCl₂, 5mM dithiotreitol, pH 7.6 at 50°C for 2 h (Girbal-Neuhauser et al, 1999). The reaction was stopped by addition of sodiumdodecylsulphate (SDS) to a final concentration of 2%. The reaction mixtures were stored at -20 °C until use.

4.2 Preparation of homocitrulline containing proteins (I)

For immunizations 10 mg of human albumin (Octapharma AB) were carbamylated by incubation with 10 mL of 0.1 M KCNO in 150 mM phosphate buffer, pH 7.4, at 70°C for 16h. The insoluble organic matrix of human bone was prepared by demineralization of femoral heads removed during elective hip surgery (Department of Surgery, Oulu University Hospital), as described previously (Risteli *et al.* 1993). 1 g of human bone type I collagen was carbamylated by incubation with 100 mL of 0.1 M KCNO in 0.05M Tris buffer, pH 9, at 70°C for 16h. Both reaction mixtures were dialyzed at 4°C against distilled water for 24 h, freeze-dried and stored at -20 °C.

For the evaluation of carbamylation in different temperatures and timescales 10 mg of human albumin (Octapharma AB) was carbamylated by incubation with 10 mL of 0.1 M KCNO in 150 mM phosphate buffer, pH 7.4, at 37°C for 2, 6 and 17 h and at 70°C for 2, 6 and 17 h (Jaisson *et al.* 2007). All reaction mixtures were dialyzed at 4°C against distilled water for 36 h, freeze-dried and stored at -20°C.

4.3 SDS-PAGE and immunodetection (I)

Native, carbamylated and citrullinated albumins were reduced with 10% (v/v) β-mercaptoethanol and electrophoresed on a 10% (m/v) polyacrylamide gel (PAGE) with SDS (NuPAGE, Invitrogen, Carlsbad, CA, USA), and the gel was stained for protein using Bio-Safe Coomassie (Bio-Rad, Hercules, CA, USA) following the manufacturer's instructions. For immunoblotting, the proteins were transferred to a nitrocellulose membrane.

4.3.1 Anti-(modified)-citrulline detection (I)

The detection of modified citrullinated proteins was performed following manufacturer's instructions (Upstate Biotechnology, Lake Placid, NY, USA). In brief, proteins immobilized to nitrocellulose membrane were cross-linked with paraformaldehyde to improve protein retention. Ureido groups were modified with 2,3-butanedione monoxime and antipyrine in a strong acid solution to form a ureido group adduct. The modified proteins were localized by reacting with anti-(modified)-citrulline antibodies (Upstate Biotechnology, Lake Placid, NY, USA). The immunoreaction was visualized on x-ray film using a goat anti-rabbit IgG secondary antibody conjugated to horseradish peroxidase (HRP) and a chemiluminescence detection system (SuperSignal West Pico Chemiluminescent Substrate, ThermoScientific, Rockford, USA).

4.3.2 F95 (additional data)

The F95 antibody (Nicholas & Whitaker 2002) was a kind gift from Dr. A.P. Nicholas. F95 is an antibody claimed specific for unmodified citrulline irrespective of the surrounding sequence. The nitrocellulose membrane was blocked for 3 h in 5% BSA-TBS (5% bovine serum albumin, 50 mM Tris, 150 mM NaCl, pH 7.4)- 0.05% Tween 20 at RT, proteins immobilized on nitrocellulose were stained for 1 h with IgM F95 antibody diluted 1:500 in 5% BSA-TBS at RT. The nitrocellulose was washed twice with TBS-0.05% Tween 20 and the immunoreaction was visualized on x-ray film with HRP-conjugated goat anti-mouse IgM (μ specific) second antibody diluted 1:20,000 in 5% BSA-TBS-0.05% Tween 20 and a chemiluminescence detection system (SuperSignal West Pico Chemiluminescent Substrate, ThermoScientific, Rockford, USA).

4.3.3 KS350/1 (additional data)

We induced antibodies in rabbit against collagen type I telopeptide sequence containing chemically modified citrulline. The modification was done with antipyrine and 2,3-butanedione in acidic conditions as was the case with AMC antibodies. The modified peptides were dialyzed before injection to the rabbits. The immunization followed the protocol described later and the staining protocol was the same as described earlier for the anti-(modified)-citrulline detection.

4.4 Detection of citrulline and homocitrulline on high-performance liquid chromatography (I, III)

Native, carbamylated and citrullinated albumins or small tissue samples were hydrolyzed with 6 M HCl at 110 °C for 16 h and evaporated to dryness. 300 µL of 50 mM antipyrine, 200 µL of trifluoroacetic acid (TFA), 300 µL of 2,3-butanedione and as an internal standard 0.5 µg of albizzin (Bachem, Weil am Rhein, Germany) was added to the dried sample and incubated at 37 °C for 2 h covered from light. Fresh solutions of antipyrin and butanedione were prepared daily from 1 M stocks. The samples were filtered and 75% of the original volume was analyzed on HPLC. The HPLC protocol was adapted from Holm and co-workers (Holm *et al.* 2006). Analysis was performed on Agilent 1100 series instrument (Agilent, Germany) equipped with diode array spectrophotometer. The amino acids were separated over 250 x 4,6-mm Kromasil C18 column (5 µm particles) using a solvent gradient from 27 to 32% B in 10 min and 32 to 95% B in the next minute (solvent A: 0.1% TFA in H₂O, solvent B: 0.1% TFA in acetonitrile). The detection was performed at 464 nm. The values for citrulline and homocitrulline in the sample were read from the automatically integrated peak area.

4.5 Experimental animals and immunizations (I, II)

The animals used in the studies (n = 32) were 3-month-old male New Zealand White rabbits (Harlan, France). The rabbits were immunized at the Laboratory Animal Center of the University of Oulu. All antigens were suspended in 0.9% NaCl and emulsified with Freund's complete adjuvant in the primary immunization and with Freund's incomplete adjuvant in booster injections. Booster injections were given at 3-week intervals and blood samples were collected 7 to 10 days after booster injections. In study I two rabbits were immunized with 1 mg of carbamylated albumin, another two rabbits with 4 mg of carbamylated collagen, and two rabbits with a mixture of 1 mg of carbamylated albumin and 4 mg of carbamylated collagen. In study II rabbits were immunized with citrulline-containing KLH-linked synthetic peptides derived from collagen type I or collagen type I telopeptides digested with collagenase (Risteli *et al.* 1993), KLH-linked synthetic citrulline-containing vimentin derived sequences or with native, citrullinated or carbamylated human hemoglobin A0.

4.6 Detection of antibodies binding to ureido group-containing antigens (I-III)

Antibodies binding to citrulline- or homocitrulline-containing antigens were measured with three different methods which are described below: anti-CCP constituted of several citrulline-containing sequences (van Venrooij & Zendman 2008), anti-MCV based on mutated citrullinated vimentin sequence, and the inhibition enzyme-linked immunosorbent assay (ELISA) method based on type I and type II collagen carboxyteleopeptide sequences.

4.6.1 Anti-CCP ELISA

Anti-CCP (Immunoscan RA (Mark 2) from Euro Diagnostica, Malmö, Sweden) was used for human samples according to manufacturer's instructions. In brief, given controls, calibrators and patient sera diluted 1:50 in the dilution buffer were pipetted into the wells and incubated at room temperature (RT) for 60 minutes. Solutions were discarded and the wells washed three times. 100 μ L of anti-human-IgG-HRP conjugate was added to each well and incubated at RT for 30 minutes. Solutions were again discarded and the wells washed three times. 100 μ L of substrate solution was added to the wells and incubated at RT for 30 minutes. The reactions were stopped by adding 100 μ L of stop solution to each well and absorbances were read at 450 nm by Victor² instrument (Wallac, Turku, Finland). The result was considered positive if the absorbance measured from the sample exceeded the absorbance of the lowest kit calibrator that was equivalent to 25 arbitrary units defined by the manufacturer.

The method was adapted for rabbit sera by changing the kit's assay buffer to EIA buffer (20mM Tris-HCl, 150mM NaCl, 0.1% BSA, 0.05% Tween; pH 7.5) and the second antibody to anti-rabbit IgG conjugated to HRP (Jackson ImmunoResearch, West Grove, PA, USA) diluted 1:50,000 in EIA buffer. For rabbit sera the result was considered positive if the absorbance exceeded the mean + 2SD absorbance of 25 U calibrator of the human assay (0,421 AU).

4.6.2 Anti-MCV ELISA

Anti-MCV assays (ORG 546, from ORGANTEC Diagnostica GmbH, Mainz, Germany), was used for human samples according to manufacturer's instructions. Shortly, calibrators, controls and patient sera diluted 1:100 in the dilution buffer

were pipetted in to the wells and incubated at RT for 30 minutes. Solutions were discarded and the wells washed three times. 100 μ L of rabbit anti-human-IgG-HRP conjugate was added to each well and incubated at RT for 15 minutes. Solutions were again discarded and the wells washed three times. 100 μ L of substrate solution was added to the wells and incubated at RT for 15 minutes. The reactions were stopped by adding 100 μ L of stop solution to each well, incubated at RT for 5 minutes and absorbances were read at 450 nm by Victor² instrument (Wallac, Turku, Finland). The result was considered positive if the absorbance measured from the sample exceeded the absorbance of the lowest kit calibrator that was equivalent to 20 arbitrary units defined by the manufacturer.

The method was adapted for rabbit sera by changing the kit's assay buffer to EIA buffer and the second antibody to anti-rabbit IgG conjugated to HRP (Code 111-036-046, Jackson ImmunoResearch, West Grove, PA, USA) diluted 1:50,000 in EIA buffer. For rabbit sera the result was considered positive if the absorbance exceeded the mean +2SD absorbance of 20 U calibrator of the human assay (0.346 AU).

4.6.3 Inhibition ELISA

The RA patient serum was tested with ELISA with citrulline- and homocitrulline-containing type I collagen telopeptides under standard conditions and inhibited with several added soluble antigens (Table 2), the concentration of which was 100 μ g/mL. The synthetic, citrulline-containing peptides that were used as inhibitors in the ELISA represent parts of varying lengths related to the carboxyterminal halves of the telopeptide sequences. For each serum the differences without and with inhibition were calculated for all peptide pairs. For adaptation for rabbit serum, the second antibody was changed to anti-rabbit IgG conjugated to HRP and concentrations of soluble inhibitors were 100 μ g/mL.

All rabbit sera were tested by ELISA for binding to native, citrulline- and homocitrulline-containing type I and II collagen carboxytelopeptides, and the binding (A450 nm > 1.0) was inhibited by addition of soluble antigens to a final concentration of 200 μ g/mL in study I and 100 μ g/mL in study II. The four biotinylated peptides corresponding to the carboxyterminal telopeptide of the α 1 chain of human type I collagen (NeoMPS, Strasbourg, France) represent amino acids 1193–1218, and the four biotinylated peptides corresponding to the α 1 chain of human type II collagen represent amino acids 1217–1241, counted from the amino-terminus in the pro α 1 chains of type I or type II procollagen,

respectively. The synthetic peptides that were used as inhibitors in the ELISA (Table 2) represent varying lengths of these parent peptides. In each serum, the difference between binding with and binding without inhibition was calculated for all peptide pairs. Inhibition values > 20% were considered positive.

Table 2. List of synthetic peptides used in the studies.

Identifier	Sequence	
Type I collagen		
SP133	biotin	SAG FDF SFL PQP PQE <i>Lys</i> AH DGG <i>Arg</i> YY <i>Arg</i> A
SP112	biotin	SAG FDF SFL PQP PQE <i>Lys</i> AH DGG <i>Arg</i> YY <i>Cit</i> A
SP122	biotin	SAG FDF SFL PQP PQE <i>Hcit</i> AH DGG <i>Arg</i> YY <i>Arg</i> A
SP132	biotin	SAG FDF SFL PQP PQE <i>Lys</i> AH DGG <i>Arg</i> YY <i>Hcit</i> A
SP4		SAG FDF SFL PQP PQE <i>Lys</i> Y
SP65		--- --- --- --- -- E <i>Lys</i> AH DGG <i>Arg</i> YY <i>Arg</i> A
SP66		--- --- --- --- -- E <i>Lys</i> AH DGG <i>Arg</i> YY <i>Cit</i> A
SP72		--- --- --- --- -- E <i>Lys</i> AH DGG <i>Cit</i> YY <i>Arg</i> A
SP73		--- --- --- --- -- E <i>Lys</i> AH DGG <i>Cit</i> YY <i>Cit</i> A
SP135		--- --- --- --- -- E <i>Lys</i> AH DGG <i>Arg</i> YY <i>Hcit</i> A
SP82		--- --- --- --- -- E <i>Lys</i> AH DGG
SP123		--- --- -- L PQP PQE <i>Hcit</i> AH D
SP124		--- --- -- L PQP PQE <i>Lys</i> AH D
Type II collagen		
SP134	biotin	GID MSA FAG LGP RE <i>Lys</i> GPD PLQ YM <i>Arg</i> A
SP86	biotin	GID MSA FAG LGP RE <i>Lys</i> GPD PLQ YM <i>Cit</i> A
SP125	biotin	GID MSA FAG LGP RE <i>Hcit</i> GPD PLQ YM <i>Arg</i> A
SP109	biotin	GID MSA FAG LGP RE <i>Lys</i> GPD PLQ YM <i>Hcit</i> A
SP40		--- --- --- --- - E <i>Lys</i> GPD PLQ YM <i>Arg</i> A
SP41		--- --- --- --- - E <i>Lys</i> GPD PLQ YM <i>Cit</i> A
SP136		--- --- --- --- - E <i>Lys</i> GPD PLQ YM <i>Hcit</i> A
SP54		--- --- --- --- - E <i>Lys</i> GPD PLQ YM <i>Arg</i>
SP55		--- --- --- --- - E <i>Lys</i> GPD PLQ YM <i>Cit</i>
SP56		--- --- --- --- - E <i>Lys</i> GPD PLQ YM
SP57		--- --- --- --- - E <i>Lys</i> GPD PLQ Y
SP58		--- --- --- --- - E <i>Lys</i> GPD PLQ
SP126		--- --- FAG LGP RE <i>Hcit</i> GPD

4.7 Patient tissue samples (III)

Tissues were collected in surgical debridement operations of a non-smoking, RF-positive female RA patient with a disease history of 10 years. At the time of the first operation described here the patient was 47 years old with persistent synovitis symptoms in right MTP1 and MTP5 joints. The synovitis tissues collected were operated on two separate occasions with a two-year timespan. The sites where tissues were removed are shown in Fig. 12 (first operation A and B, second operation 1–6). The first operation was targeted to remove rheumatoid synovitis. The second operation was scheduled due to synovitis relapse. A more radical operation was programmed in consensus with the patient due to extensive synovial tissue growth in multiple sites, the aim being to remove all active synovitis, and hence 6 separate cuts were made.

Six approximately 10 mg (wet weight) samples were cut from each collected tissue. The samples were dialyzed 16 h against 0.2 M NH_4HCO_3 , pH 7.4 to separate free amino acids from protein-bound ones, freeze-dried, then rehydrated in dH_2O , freeze-dried and hydrolyzed in 6 M HCl at 110 °C for 16 h and freeze-dried. The samples were chemically modified and analyzed for citrulline and homocitrulline on HPLC as described earlier.

4.7.1 Histology

Samples were cut from the tissues (A, B and 1–6) frozen at -70 °C, fixed in formalin and embedded in paraffin. 5- μm sections were mounted on glass slides and stained with hematoxyline-eosine.

4.7.2 Statistical analyses

In study III the differences in peak areas of citrulline and homocitrulline between tissues were assessed by independent samples Mann-Whitney U test for the first operation tissues ($n = 2$) and by Kruskal-Wallis test for the second operation tissues ($n = 6$) using Statistical Package for Social Science (SPSS) for Windows version 20 (IBM SPSS Statistics, Armonk, New York, USA). The differences were considered significant at $p < 0.05$.

4.8 Ethical considerations (I - III)

The use of animals for immunization was approved by the Southern Finland State Provincial Office. The Laboratory Animal Center of the University of Oulu complies with the “3R” principles (refinement, reduction, and replacement) of animal testing. The research plans were approved by the Ethics Committee of Oulu University Hospital. In study III involving human samples the guidelines of the Declaration of Helsinki were followed and informed consent was signed by the patient before the samples were taken.

5 Results

5.1 Homocitrulline as a confounding factor in citrulline detection (I)

The structural homology of citrulline and homocitrulline is obvious, yet this has not been accounted for in assays developed for citrulline detection. This unheeded issue was addressed in this study.

5.1.1 Immunological detection: AMC, F95 and KS350/1 (I and additional data)

As is shown in Fig. 5, all tested antibodies induced with d) citrulline-peptide (F95, additional data) or b) and c) proteins containing chemically modified citrulline (AMC, study I and KS350/1, unpublished data) bound to carbamylated human serum albumin. AMC and F95 antibody binding increased with increasing time and temperature of the carbamylation reaction. This suggested that the binding was mostly affected by the number of ureido groups and the loosening of the three-dimensional structure of the protein. As the structure became dissociated more homocitrulline was available for binding. The KS350/1 antiserum behaved differently as the binding decreased as the protein structure collapsed. Native human serum albumin was not stained by any of the antibodies while citrullinated albumin was stained by AMC and KS350/1 as expected. To define the ureido group-containing amino acids in the proteins and to verify that the antibodies were actually binding to homocitrulline we also analyzed the samples used here by HPLC.

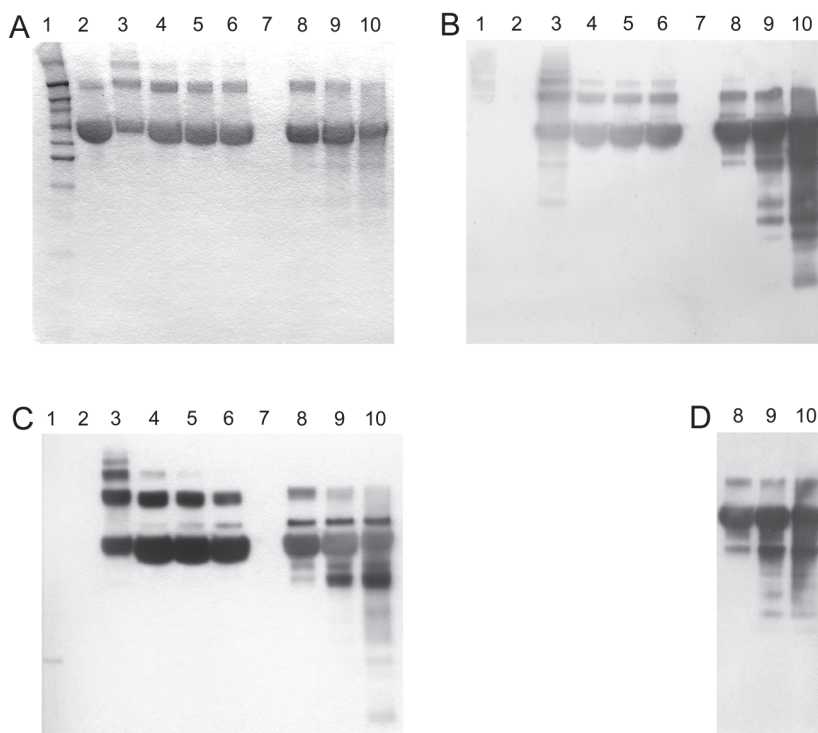


Fig. 5. a) Protein staining (Coomassie), b) Anti-(modified)-citrulline, (study I) c) KS350/1 rabbit polyclonal antibody against modified citrullinated type I collagen telopeptide (citrullinated peptide modified by 2,3-butanedione and antipyrine in acidic conditions, unpublished data) and d) F95 staining (additional data) on molecular weight standard (lane 1), native (lane 2), PAD treated (lane 3) and a series (lanes 4–6 and 8–10) of carbamylated human serum albumins. KCNO-treatment was done at 37 °C (lane 4: 2 h, 5: 6 h and 6: 17 h) and at 70 °C (lane 8: 2 h, 9: 6 h and 10: 17 h). Urea in solution is known to be in equilibrium with cyanate/isocyanate, the latter of which readily carbamoylates the amino groups of proteins. As we demonstrated with KCNO treatment of human albumin (lanes 4–6 and 8–10), the degree of carbamoylation is significant even at 37 °C. The immunoreaction with anti-modified-citrulline antibody from Upstate Biotechnology and KS350/1 was carried out after chemical modification of the ureido groups present in the separated and blotted proteins by antipyrine and 2,3-butanedione monoxime. (Combined from Study I, unpublished data and additional data (Turunen et al. 2013)).

5.1.2 High performance liquid chromatography

As could be expected, both citrulline and homocitrulline were modified and could be detected at wavelength 464 nm. The peaks were clearly separated in the chemically modified standard solution containing albizzine, citrulline and homocitrulline (Fig. 6) and the sample peaks could be easily identified. No other peaks than the internal standard albizzine was detected in the hydrolyzed native albumin sample. Albizzine and citrulline peaks were found in the hydrolyzed citrullinated albumin sample and albizzine and homocitrulline peaks were detected in the hydrolyzed carbamylated albumin sample. This verified that AMC antibodies and F95 and KS350/1 stained both citrulline and homocitrulline and that chemically modified citrulline and homocitrulline could be separated on HPLC and detected separately at wavelength 464 nm.

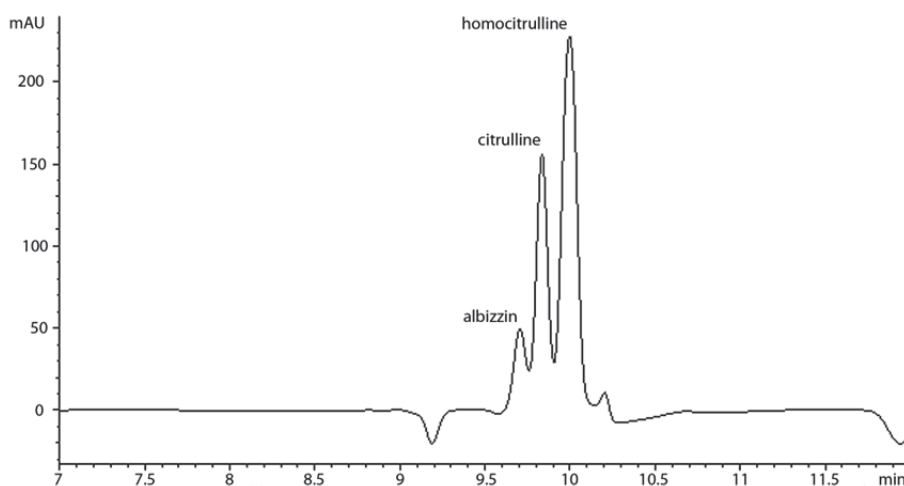


Fig. 6. Albizzine, citrulline and homocitrulline separated on HPLC after modification.

5.2 Immunization studies (I, II)

Rabbits were immunized with several citrulline- or homocitrulline-containing immunogens, but antibodies binding to ureido group-containing antigens did not develop in all of the rabbits. Rabbits immunized with native, citrulline-containing and homocitrulline-containing human hemoglobin A0 did not develop antibodies binding to any of the tested antigens and only anti-CCP and anti-MCV binding

antibodies were induced in rabbits immunized with citrulline-containing vimentin sequences. Other immunogens used induced antibodies with different specificities that are described in more detail in the following sections.

5.2.1 Immunization with citrulline-containing immunogens

Eight rabbits were immunized with citrulline-containing type I collagen telopeptides. Binding of the rabbits' end-point sera to anti-CCP and type I and II collagen telopeptides was defined by ELISA (Table 3). Six out of the eight rabbit sera bound to CCP antigens. The citrulline-containing immunization antigen (SP112) was strongly bound, as expected, but so was the homocitrulline-containing variant (SP132). There was moderate binding to the corresponding peptide with native sequence (SP133). The peptides related to the corresponding citrulline- and homocitrulline-containing sequences of type II collagen (SP86 and SP109) were only weakly or moderately bound. The native type II collagen sequence (SP134) was not bound, except for weak binding by two of the eight rabbit sera.

The first tests were done on the end-point sera of the rabbits, but since we wanted to study the development of the antibodies in more detail, we measured the binding of two rabbit sera from the primary and secondary samples in addition to the end-point samples. We selected two rabbits (rabbits 4 and 6) that were behaving differently at the end-point with respect to CCP and type II collagen telopeptide antigens (table 3). Four additional serum samples were available from rabbit 4 and two from rabbit 6. However, the sera from the two rabbits behaved alike when all samples were tested; rabbit number 6 serum binding is shown in Fig. 7 as an example.

Binding to type I telopeptides (Fig. 7 A) was sequence-specific. There was negligible difference between binding to citrullinated (SP112), homocitrulline-containing (SP132) and native (SP133) peptide. Binding to type II collagen telopeptides (Fig. 7 B) was ureido group-specific. Distinct binding to citrullinated (SP86) and homocitrulline-containing (SP109) forms of the peptide was observed while there was no binding to native peptide (SP134). The rabbit 4 serum behaved alike, the only difference being in the titers of the antibodies.

Table 3. Binding of all rabbit sera to CCP, MCV and collagen telopeptides.

Rabbit	Immunization antigen	anti- CCP	anti- MCV	CitI SP112	HcitI SP132	NatI SP133	CitII SP86	HcitII SP109	NatII SP134
1	Cit ICTP	+	-	+++	+++	++	+	+	-
2	Cit ICTP	+	-	+++	+++	++	++	++	+
3	Cit type I collagen telopeptide	+	-	+++	+++	++	++	+	-
4	Cit type I collagen telopeptide	-	-	+++	+++	++	++	++	-
5	Cit type I collagen telopeptide	-	-	+++	+++	++	+	+	-
6	Cit type I collagen telopeptide	+	-	+++	+++	+++	+	+	-
7	Cit type I collagen telopeptide	+	-	++	++	++	++	++	-
8	Cit type I collagen telopeptide	+	-	+++	+++	+++	+++	+	+
9	Cit type II collagen telopeptide	+	-	++	++	+	++	+++	++
10	Cit type II collagen telopeptide	+	-	+	-	-	++	++	++
11	Cit vimentin	+	+	-	-	-	-	-	-
12	Cit vimentin	+	+	-	-	-	-	-	-
13	Cit vimentin	+	+	-	-	-	-	-	-
14	Cit vimentin	+	+	-	-	-	-	-	-
15	Cit vimentin	+	+	-	-	-	-	-	-
16	Cit vimentin	+	+	-	-	-	-	-	-
17	Hb A0	-	-	-	-	-	-	-	-
18	Hb A0	-	-	-	-	-	-	-	-
19	Cit Hb A0	-	-	-	-	-	-	-	-
20	Cit Hb A0	-	-	-	-	-	-	-	-
21	Hcit Hb A0	-	-	-	-	-	-	+	-
22	Hcit Hb A0	-	-	-	-	-	-	-	-
23	Hcit-HSA	+	+	+	+++	-	++	++	-
24	Hcit-HSA	+	+	-	++	-	+	++	-
25	Hcit HSA+ human bone collagen	+	+	++	++	-	+	++	-
26	Hcit HSA+ human bone collagen	+	+	+	++	-	-	++	-
27	Hcit human bone collagen	+	+	+	++	+	-	++	-
28	Hcit human bone collagen	+	+	++	++	+	+	++	-
29	Hcit type I collagen telopeptide	+	-	-	+	-	-	+	-
30	Hcit type I collagen telopeptide	+	-	-	-	-	-	-	-
31	Hcit type II collagen telopeptide	+	-	-	+	-	++	++	++
32	Hcit type II collagen telopeptide	+	-	-	-	-	++	+	+

Binding to antigens related to type I and type II collagens was semiquantified according to the serum dilution required to obtain an optical density of 1 at A450nm. - serum dilution below 1:50, + from 1:50 to 1:999, ++ from 1:1,000 to 1:9,999, +++ serum dilution over 1:10,000. Cit, citrulline-containing; Hcit, homocitrulline-containing, Hb A0, human hemoglobin A0, HSA, human serum albumin

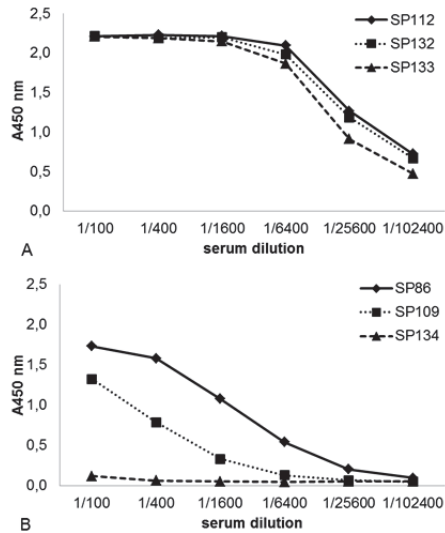


Fig. 7. Binding of rabbit 6 first sample serum to type I and II collagen telopeptides. A) Binding to type I telopeptides was sequence specific. There was negligible difference between binding to citrullinated (SP112), homocitrulline-containing (SP132) and native (SP133) peptide. B) Binding to type II collagen telopeptides was ureido group-specific. Distinct binding to citrullinated (SP86) and homocitrulline-containing (SP109) forms of the peptide was observed while there was no binding to native peptide (SP134).

Development of antibody specificities

The development of the antibody specificities induced with citrulline-containing type I collagen telopeptides was assessed by titration of the binding of rabbit 4 and 6 sera to type I and II collagen telopeptides at different time points. The results for rabbit 6 sera are shown in Fig. 8.

Throughout the immunization, there was strong binding to the citrulline- (SP112, Fig. 8A) and homocitrulline- (SP132, Fig. 8B) containing peptides related to type I collagen. Contrary to this, the binding to peptides related to type II collagen (citrulline-containing SP86, Fig. 8C; homocitrulline-containing SP109, Fig. 8D) declined from moderate to low during the immunization. The decline in the type II collagen peptide binding and the maintained type I peptide binding seem to indicate increasing sequence specificity during the development of the immune response.

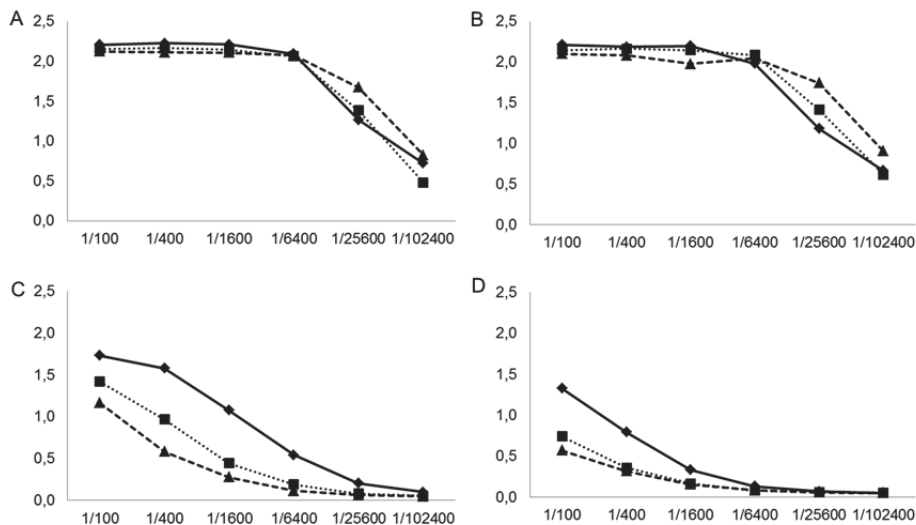


Fig. 8. Development of the antibody specificity against collagen type I and type II telopeptides in rabbit serum. Titration of binding of rabbit 6 sample 1 (diamonds), sample 2 (boxes) and final bleeding (triangles) to A) citrullinated type I collagen telopeptide (SP112), B) homocitrulline-containing type I collagen telopeptide (SP132), C) citrullinated type II collagen telopeptide (SP86) and D) homocitrulline-containing type II collagen telopeptide (SP109). Binding to type I collagen telopeptides is sequence-specific and maintained at high titer throughout the immunization. The binding to type II collagen telopeptides is ureido group-specific and the affinity declines during the immunization.

This hypothesis was challenged by inhibiting the binding of midpoint and endpoint serum samples of both rabbits (Table 4). In the midpoint sample, marked inhibition was observed as the homocitrulline-containing peptide SP135 inhibited antibody binding to the native (SP133) and homocitrulline-containing (SP132) peptides and also moderately inhibited binding to the citrullinated (SP112) peptide. As the immunization proceeded, the binding of the antibodies to any variant of the type I collagen peptides could no longer be inhibited by the soluble homocitrulline-containing peptide. Binding to all variants could be either markedly or moderately inhibited by the citrulline-containing soluble peptide. The type I collagen telopeptide inhibition tests results suggested the development of specificity towards the recognition of native and citrullinated peptides.

Table 4. Inhibition of the citrullinated antigens immunized rabbit sera binding to type I collagen telopeptides at different time points. Values in the table indicate per cent (%) of inhibition. M = midpoint sample, E= endpoint sample.

Rabbit	Binding and inhibitory antigens								
	SP112 (Cit)			SP132 (Hcit)			SP133 (Nat)		
	SP65	SP66	SP135	SP65	SP66	SP135	SP65	SP66	SP135
	(Nat)	(Cit)	(Hcit)	(Nat)	(Cit)	(Hcit)	(Nat)	(Cit)	(Hcit)
4/Mid	22	87	38	53	88	66	74	78	62
6/Mid	26	66	29	53	66	47	53	72	57
4/End	14	78	1	22	59	-	80	94	4
6/End	12	35	1	30	35	-	44	76	2

The results obtained from the inhibition tests with type II collagen-related antigens (Table 5) were similar to type I results. Binding to citrulline-containing (SP86) antigen was markedly inhibited by the corresponding citrulline-containing peptide in all midpoint and endpoint samples. Also homocitrulline-containing peptide could moderately inhibit the binding to citrulline-containing antigen in both rabbit no. 4 samples. The binding to homocitrulline-containing type II collagen related antigen (SP109) could be markedly inhibited by the homocitrulline-containing peptide (SP136) in the midpoint samples. In later samples, however, such inhibition was no longer seen for rabbit no. 6.

Table 5. Inhibition of the citrullinated antigens immunized rabbit sera binding to type II collagen telopeptides at different time points. Values in the table indicate per cent (%) of inhibition. M = midpoint sample, E= endpoint sample.

Rabbit	Binding and inhibitory antigens								
	SP86 (Cit)			SP109 (Hcit)			SP134 (Nat)		
	SP40	SP41	SP136	SP40	SP41	SP136	SP40	SP41	SP136
	(Nat)	(Cit)	(Hcit)	(Nat)	(Cit)	(Hcit)	(Nat)	(Cit)	(Hcit)
4/Mid	-	83	37	5	66	76	-	-	-
6/Mid	3	75	2	4	62	61	-	-	-
4/End	-	83	30	31	77	56	-	-	-
6/End	-	71	4	43	47	13	-	-	-

Basically, binding to citrulline-containing peptides was only inhibited by citrulline-containing peptides and binding to any other type I or II collagen telopeptide was at least moderately inhibited by the corresponding citrulline-containing peptide. Preferential specificity to citrullinated antigens and

specificities towards the peptide sequence of the immunogen thus developed during the immunization.

5.2.2 Immunization with homocitrulline-containing immunogens

Six rabbits were immunized with carbamylated antigens. Two rabbits were immunized with carbamylated collagen (27, 28) and two with carbamylated albumin (23, 24), and two with both of them in combination (25, 26). All six rabbit sera bound to anti-CCP originally designed for measuring antibodies binding to citrullinated proteins in RA patients (Fig. 9.A). All sera also showed binding to another citrulline-containing antigen, anti-MCV, a commercial method for detection of antibodies binding to mutated citrullinated vimentin (Fig. 9.B). The binding to MCV antigen was distinct but not as extensive as binding to CCP.

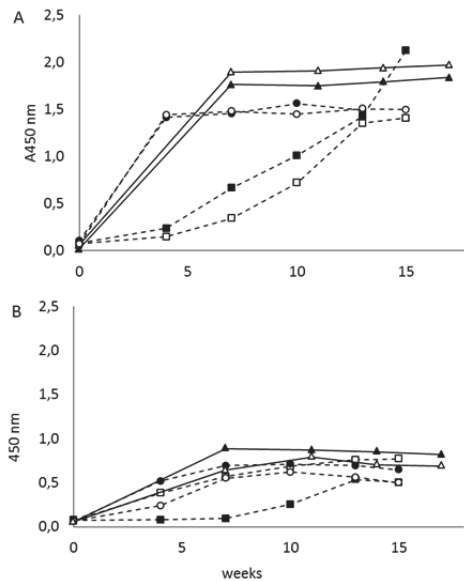


Fig. 9. Binding of six rabbit antisera [squares carbamylated collagen immunized rabbits (27, 28), circles carbamylated albumin immunized rabbits (23, 24), triangles carbamylated albumin and collagen immunized rabbits (25, 26)] to: A. anti-CCP; B. anti-MCV. Samples are different takes of the rabbits after immunizations.

The binding to citrulline-containing type I collagen telopeptide differed between rabbits (Fig. 10A), carbamylated albumin and collagen immunized rabbit sera binding the antigen most prominently. All rabbit sera bound extensively to homocitrulline-containing type I collagen telopeptide (Fig. 10B).

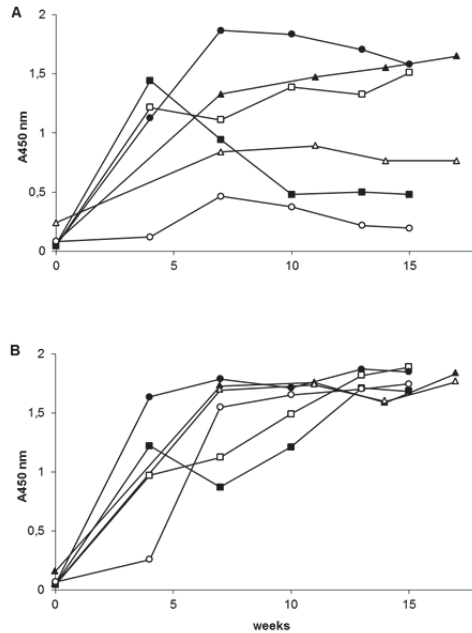


Fig. 10. Binding of six rabbit antisera [squares carbamylylated collagen immunized rabbits (27, 28), circles carbamylylated albumin immunized rabbits (23, 24), triangles carbamylylated albumin and collagen immunized rabbits (25, 26)] at different time points after immunization to the following immobilized antigens: A. citrulline-containing telopeptide of type I collagen; B. homocitrulline-containing telopeptide of type I collagen.

Inhibition tests were conducted to find out if the above-mentioned binding was specific. Both carbamylylated albumin-immunized rabbits and one of the carbamylylated albumin- and collagen-immunized rabbits (25) generated antibodies that seemed generally ureido group-specific when binding specificity to immobilized citrullinated type I collagen was tested, presented in Table 6. The ureido group-specific nature was shown as inhibition by all ureido group-containing soluble antigens (SP66, SP72, SP73 and SP123), even though the inhibition with SP72 was very low. The antibodies of the other carbamylylated albumin- and collagen-immunized rabbit seemed citrulline- or sequence-specific as their binding was only inhibited by a sequence identical to the citrulline-containing carboxyterminal part of the immobilized antigen. The binding of carbamylylated collagen-immunized rabbit's antibodies to citrullinated type I peptide was only inhibited by the phenylalanine-rich domain of type I collagen,

the aminoterminal part of the immobilized antigen. When antibodies were bound to homocitrulline containing antigen all (except for one of the carbamylated albumin- and collagen-immunized rabbit sera, rabbit 25) were inhibited by the homocitrulline-containing soluble peptides.

Table 6. Inhibition of the carbamylated antigen-immunized rabbit sera binding to type I collagen telopeptides. Values in the table indicate per cent (%) of inhibition.

Type I binding antigens	Rabbit	Inhibitory antigens						
		SP4 (Nat)	SP65 (Nat)	SP66 (Cit)	SP72 (Cit)	SP73 (2xCit)	SP123 (Hcit)	SP124 (Nat)
SP112 (Cit)	23	5	2	48	13	62	85	2
	24	3	3	43	22	45	47	-
	25	2	-	50	9	29	86	-
	26	5	-	78	-	2	5	-
	27	78	-	-	-	-	-	-
	28	90	2	-	2	4	7	6
SP122 (Hcit)	23	2	3	12	-	16	61	6
	24	1	4	3	5	9	59	4
	25	2	-	20	3	16	70	-
	26	-	-	1	2	3	75	-
	27	4	-	2	6	8	57	-
	28	8	5	-	6	9	71	5
SP132 (Hcit)	23	2	3	2	-	8	48	-
	24	2	2	3	1	4	34	-
	25	1	2	-	-	-	2	-
	26	5	4	-	3	4	85	-
	27	6	3	-	5	6	48	-
	28	8	6	3	4	7	40	-

The ureido-specific nature of antibodies induced with carbamylated immunogens was also shown when binding to anti-CCP and anti-MCV was inhibited with citrulline- and homocitrulline-containing inhibitors shown in Fig.11. Carbamylated human serum albumin inhibited the binding almost totally in both assays, and also homocitrulline-containing type I collagen telopeptide sequence could inhibit the binding to anti-MCV noticeably.

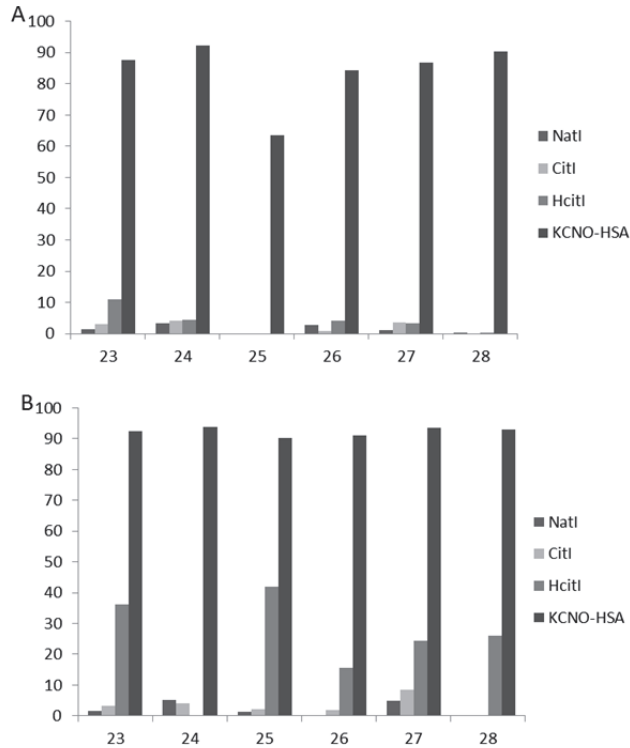


Fig. 11. Inhibition (%) of A) CCP binding and B) MCV binding antibodies induced with homocitrulline-containing immunogens. The inhibition tests were performed only to rabbit sera that showed binding in Table 3 and the numbers below correspond to the rabbit numbers presented in the same table. NatI = SP65, CitI = SP66, HcitI = SP135, peptide sequences can be found in Table 2 and KCNO-HSA refers to carbamylated human serum albumin.

Binding to citrulline-containing peptides was ureido group-specific as the binding could be inhibited by both citrulline- and homocitrulline-containing soluble peptides. When antibodies were bound to the homocitrulline containing-peptides only homocitrulline-containing soluble peptides inhibited the binding. Homocitrulline in the sequence inhibited the binding even if it was in a different location in the sequence.

The rabbit sera were also tested for binding to type II collagen telopeptides that presented antigens not present in the immunization mixtures. A similar pattern of binding and inhibition could be detected in the type II assays shown in Table 7. As seen in type I telopeptide assays the binding to citrulline-containing

telo peptide could be inhibited ureido group-specifically in carbamylated albumin- and collagen and carbamylated albumin-immunized rabbit sera, but not in the carbamylated collagen-immunized rabbit sera. Only one of the two carbamylated collagen-immunized rabbit sera bound to type II telopeptides. When the antibodies were bound to homocitrulline-containing type II telopeptides the binding could only be inhibited by homocitrulline-containing soluble peptides.

Table 7. Inhibition of the carbamylated antigen-immunized rabbit sera binding to type II collagen telopeptides. Values in the table indicate per cent (%) of inhibition.

Type II binding antigens	Rabbit	Inhibitory antigens							
		SP40 (Nat)	SP41 (Cit)	SP54 (Nat)	SP55 (Cit)	SP56 (Nat)	SP57 (Nat)	SP58 (Nat)	SP126 (Hcit)
SP86 (Cit)	23	-	68	-	22	-	-	-	90
	24	-	62	-	53	-	-	-	-
	25	1	65	-	36	-	-	-	33
	26	11	49	1	43	-	-	3	51
	27	10	66	6	15	-	-	-	-
SP125 (Hcit)	23	2	17	3	6	4	7	3	61
	24	-	8	-	-	1	2	2	64
	25	6	8	11	13	10	12	8	12
	26	3	-	-	2	1	2	3	65
	27	1	1	-	3	-	-	-	61
	28	-	-	1	-	-	-	-	88

5.3 Citrulline and homocitrulline in tissues (III)

In study III one single RA patient was studied for the citrulline and homocitrulline content of the affected tissues and for the serum antibodies binding to citrulline- or homocitrulline-containing peptides. In the immunoassays the patient was found positive for antibodies binding to CCP (800.5 U/mL) and MCV (55.9 U/mL), but no antibodies binding to type I or II collagen telopeptides containing either citrulline or homocitrulline could be detected.

The tissue specimens were collected in two separate surgical operations in 2009 and 2011. In the first operation tissues from the hallux and little toe (A and B in Fig. 12) were collected. In the second operation, performed after two years of follow-up, six samples were collected (Fig. 12: 1–6).

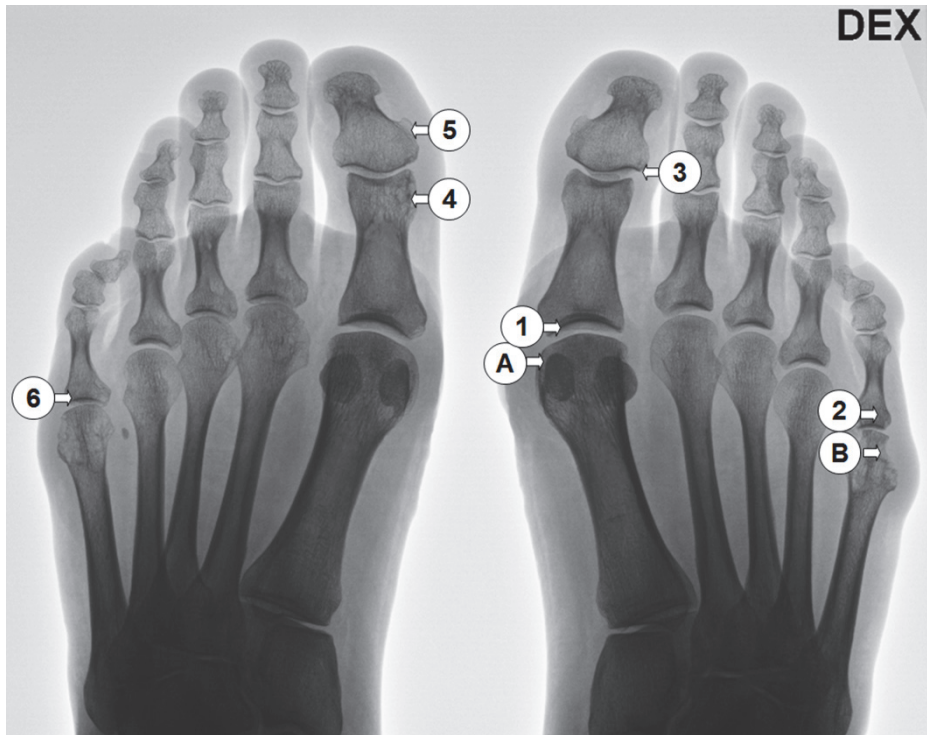


Fig. 12. The patient radiograph taken before the first surgery. Tissues were collected in two separate operations. Tissues collected in the first operation are marked with letters A and B and tissues collected in the second operation are numbered 1 to 6 in the figure.

5.3.1 Histology

The histological analysis was performed by Dr. Jukka Melkko. In histological studies the tissues were classified as rheumatoid nodules characterized by necrotic tissue surrounded by palisading histiocytes (Fig. 13). RA tissue consists of a mixture of various tissues, such as vascular, epithelial and different types of fibrotic and necrotic tissues. All samples were adjacent to synovium, hence some synovial fragments were present, as shown in Fig. 13. The presence of macrophages was localized. Sections made at 2 mm interval from the same piece of tissue were dramatically different. Fibrotic tissue was present in all specimens. There were more macrophages and blood vessels in the deeper tissue compared to the surface of the nodules. Macroscopically all samples looked the same and the

erosive tissue looked similar to the non-erosive tissue. The bone defect was also replaced by similar mixed tissue.

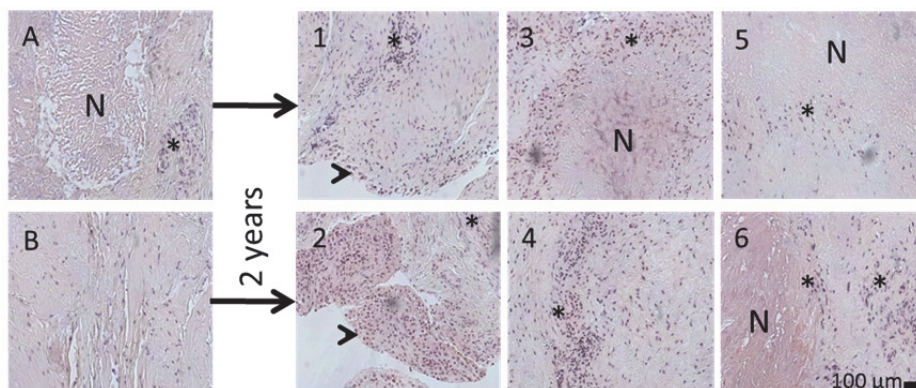


Fig. 13. Histological images of removed tissues. Numbers correspond to those shown in Figure 10. Interval between operations was 2 years, A and 1, B and 2 are samples from the same anatomical site, respectively. Arrowheads indicate epithelial tissue, * indicates inflammatory cells, mostly macrophages, N indicates necrosis in the rheumatoid nodule. RA tissue always consists of a mixture of various tissues, such as vascular, epithelial, different types of fibrotic and necrotic tissues. All samples were adjacent to synovia, hence also some synovial fragments were present.

5.3.2 Analysis of citrulline and homocitrulline content

All tissues were analyzed for citrulline and homocitrulline content in the dialyzed and hydrolyzed samples using HPLC. Significant differences were detected between citrulline content of the tissues A and B (Fig. 12: A and B), collected in the first operation. In the non-erosive tissue A the mean level of citrulline was lower than in the visually and radiologically eroded tissue B. The level of homocitrulline was greater in tissue A than in tissue B, but the difference in citrulline content was more pronounced (Fig. 14). In the Mann-Whitney independent samples U test both the differences in citrulline content and in homocitrulline content between tissues A and B were statistically significant ($p < 0.001$).

In the second operation six tissue specimens were collected from metatarsal joints (Fig. 12: 1, 2, 6) and from interphalangeal joints (Fig. 12: 3, 4, 5), and also these were analyzed for citrulline and homocitrulline content. In HPLC analysis homocitrulline and citrulline were present in all samples. The level of

homocitrulline remained more constant between the different samples compared to citrulline (Fig. 14.). In the Kruskal-Wallis independent sample tests both the differences in citrulline and in homocitrulline levels between tissues of the second operation were statistically significant, $p = 0.001$ and $p = 0.003$, respectively. In the pairwise comparison tissue number 5 differed statistically from tissues 1 and 2 with a greater amount of homocitrulline ($p = 0.003$ and 0.022). The amount of citrulline in tissue number 4 was statistically greater than in tissues 1, 2 and 6 ($p = 0.020$, 0.024 and 0.029 , respectively).

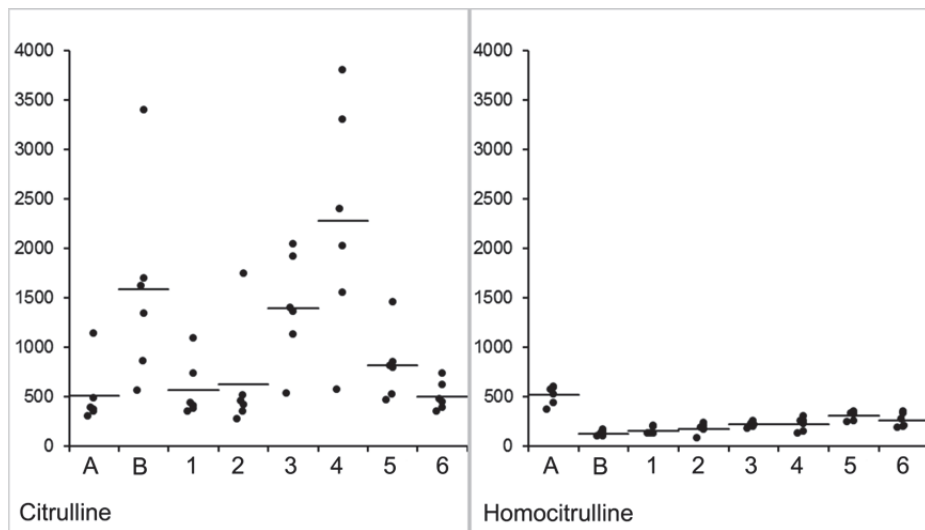


Fig. 14. The auto-integrated HPLC peak areas of citrulline and homocitrulline in the six samples taken from each of the removed foot joint tissues. Sample A was non-erosive and sample B was from an erosive location.

6 Discussion

6.1 Methodology: detection of ureido groups (I)

6.1.1 Immunoblotting ureido group-containing proteins

Citrullination has been studied in respect to several physiological processes and diseases, but research related to carbamylation and homocitrulline is only beginning. On many occasions citrullination of proteins has been demonstrated using antibodies induced against a chain of citrullines or against chemically modified citrulline (Nicholas & Whitaker 2002, Senshu *et al.* 1992). As we have shown in this study these antibodies recognize the ureido group of both citrulline and homocitrulline irrespective of the amino acid or the sequence. This is not surprising as the antibodies were designed to identify citrulline regardless of the surrounding sequence. In this function the antibodies perform splendidly, but the existence of the citrulline homolog, homocitrulline, has been overlooked. Since the antibodies do not discriminate between citrulline and homocitrulline, either native or chemically modified, this makes protein-bound homocitrulline a confounder of protein-bound citrulline detection.

A recent example of the use of AMC antibodies (Senshu *et al.* 1992) in tissue sections is seen in the study of Sokolove and co-workers. In their report Sokolove and co-workers state that even though citrullinated proteins and PAD-4 colocalized, citrullinated proteins were outside the cells and PAD-4 inside the cells (Sokolove *et al.* 2013). This could be explained by the fact that citrullination and MPO colocalize in extracellular traps found to be created by inflammatory cells. In response to pathogens histones in the inflammatory cells are hypercitrullinated, which makes the chromatin structure open to a web-like structure that is released to extracellular space (Wang *et al.* 2009). Bacteria are trapped in this web of chromatin that is armed with active myeloperoxidase (Parker *et al.* 2012). In this respect in inflamed tissue the staining of both PAD and MPO and analysis of the amino acid content of the tissue would be more appropriate before making further conclusions on the origin of the ureido groups. MPO has also been connected to Alzheimer's disease (Reynolds *et al.* 1999) and multiple sclerosis (Nagra *et al.* 1997). Both of these diseases are also studied in respect to citrullination (Moscarello *et al.* 2007, Nicholas 2013).

Here, it should be noted, that not only the carbamylation *in vivo*, but also possible carbamylation during sample preparation should be acknowledged. As could be observed in our study, protein carbamylation is efficient already at low temperature and in urea-containing solutions it can affect the result of the analysis. As an example of this, the results of identification of citrullinated proteins in RA synovium using AMC antibodies by Matsuo and co-workers (Matsuo *et al.* 2006) should be interpreted with caution, as 8 M urea was used in the sample preparation.

Because the mapping of citrullinated proteins has often involved antibodies produced against citrullinated proteins or modified ureido groups, further work is needed for identifying citrulline and homocitrulline separately in tissues, thus excluding the possibility of homocitrulline confounding citrulline detection or vice versa. Attention should be paid to the choice of method in sample treatment and analysis while studying ureido group-containing samples. Nevertheless, even the ureido group-specific detection methods AMC or F95 can be used in the primary work regarding citrullination and carbamylation as these are easy to use, but further characterization and the distinction between the two amino acids is needed before conclusions can be drawn.

6.1.2 Detection of citrulline and homocitrulline using HPLC

The chemical modification of ureido groups can be used in identification of citrulline and homocitrulline HPLC. The modification reaction creates a chemical adduct that can be identified reading absorbance at 464 nm. We adapted a method for citrulline and homocitrulline separation and detection from hydrolyzed samples on HPLC and it performed well. We analyzed the native, PAD-treated and carbamylated albumin on HPLC and found the PAD-treated albumin containing citrulline, carbamylated albumin containing homocitrulline and the native albumin containing neither. This confirmed the results of the aforementioned antibody detection methods as there was only one of the ureido groups containing amino acids present in each sample.

Chemical modification reaction with 2,3-butanedione and antipyrine is common to both citrulline and homocitrulline so the identification of these amino acids separately in intact proteins or peptides after modification is not possible on HPLC. However, chemically modified citrulline and homocitrulline can be separated on HPLC in hydrolyzed samples and detected as separate absorbance peaks at 464 nm. A downside of our method is that the sample is lost in the

analysis and the location of citrulline or homocitrulline can only be determined by sample purification and identification before this analysis. Even then the location of citrulline or homocitrulline in the sequence can be only hypothesized if there are more than one available arginines or lysines in the sequence.

6.2 Immunization studies (I, II)

The structural homology of citrulline and homocitrulline gives a reason to study the possible cross-reaction of antibodies induced with immunogens containing either of them. Genetic susceptibility for developing citrulline antibodies has been established in RA patients. The susceptibility in RA is defined by the sheared epitope possessed by the susceptible individuals. The sequence of the peptide binding pocket in SE is QR(K)RAA, which would favor the binding of an uncharged residue (de Vries *et al.* 2005) like citrulline or homocitrulline in the sequence. It is interesting that in our experimental model antibodies binding to ureido group-containing antigens were induced only by some of the introduced immunization antigens. Human hemoglobin A0 did not induce ureido group-specific antibody development in citrullinated or in carbamylated form. This just shows the regulation of antibody development to avoid life-threatening pathology. In humans antibody development is also strictly regulated to avoid autoimmunity, but since we did not have the opportunity of adding all the environmental and genetic factors to rabbits the next best thing is to raise the antibody response with selected epitopes injected with adjuvant. Citrullination is present in several processes and most people never develop antibodies binding to citrullinated proteins, so in RA there are also other factors involved.

Several different events are involved in the development of RA. The as yet unknown primary event of ACPA-positive RA precedes the development of the autoantibodies and is followed by a latent period of undefined length before the onset of the clinical disease. In immunization studies the object is to try to mimic the antibody development in RA patients. The characteristics of antibodies binding to citrullinated proteins are hard to copy since no single consensus of RA patient antibodies exists; every patient has a unique antibody profile that is divided into several immunoglobulin subclasses (IgM, IgA and IgG). Type I and II collagen telopeptides are interesting since antibodies binding to these have been found in RA sera (Koivula *et al.* 2005) so we wanted to find out if we could induce antibodies in rabbits with the same sequences. In study II antibodies were induced with citrulline-containing type I collagen telopeptides. The developed

antibodies bound all variants of the immunization antigen, type I collagen telopeptide, irrespective of the ureido group in the sequence. Type II collagen telopeptides were bound only when these contained either citrulline or homocitrulline in the sequence. In addition, CCP antigen, not related to the immunization antigen, was strongly bound. This suggests that the antibodies possessed specificities both to the ureido groups and to the peptide sequence of the immunization antigen. The inhibition tests confirmed the sequence specificity as the immunogen, citrulline-containing type I collagen telopeptide, was the strongest inhibitor, but the corresponding native peptide also had some inhibitory effect. The ureido group specificity was confirmed as the antibodies were only bound to and inhibited by ureido group-containing peptides in type II collagen telopeptide assays.

In rheumatoid arthritis epitope spreading to several different types of citrullinated antigens has been found to take place prior to RA onset in humans (van der Woude *et al.* 2010a) and epitope spreading from citrullinated to native antigens has been found in experiments with rats (Lundberg *et al.* 2005). In our experimental model the affinity of the antibodies induced with citrulline-containing immunogens was probably strongest to the citrulline-containing sequences, but the affinity to the native sequence was adequate for recognition. The antibodies induced with citrulline-containing peptides related to type I collagen cross-reacted with homocitrulline, another ureido group-containing amino acid. In both assays the antibodies favored citrulline over homocitrulline. The induced antibodies recognized the sequence of the immunization antigen and the ureido group in the sequence. As the immunization proceeded, the ureido group specificity became more restricted to citrulline.

In study I we demonstrated that homocitrulline-containing proteins injected with adjuvant were immunogenic and that the antibodies induced bound to homocitrulline-containing and also to citrulline-containing antigens. The developed antibodies bound to several citrulline-containing antigens: type I collagen telopeptides, type II collagen telopeptides, CCP and MCV. This suggested ureido group-specific binding of the antibodies, since the sequences of CCP and MCV were not related to the immunogens. Despite the general ureido group specificity, similarly to antibodies induced with citrulline-containing immunogens, also antibodies induced with carbamylated immunogens preferred the peptide containing the amino acid that had been introduced in the immunogen. The inhibition of CCP and MCV binding by homocitrulline-containing type I collagen telopeptide or by carbamylated human serum albumin was a good

example of this. The specificity to the original amino acid was not as strict as was the case for antibodies induced with citrulline-containing peptides. The formation of citrulline and homocitrulline differ in the process that is regulated by the substrate specificity of PAD enzymes in citrullinisation but is uncontrolled in carbamylation. This difference makes the citrullinisation sites more constant and enables more specific development of response antibodies. Carbamylation can in theory affect any lysine residue (or free amino group) in any protein, so the repertoire of carbamylation products and thus the assortment of available immunogens is wider. This feature in specificity was thus probably due to the fact that several different epitopes containing homocitrulline were presented in the carbamylated proteins during immunization.

It is tempting to speculate on the possible role of homocitrulline in the development of antibodies binding to citrullinated proteins. In RA patients the antibody repertoire widens prior to disease onset and the development is characterized by epitope spreading between antigens and the tuning of the fine specificity of these antibodies (van de Stadt *et al.* 2011, van der Woude *et al.* 2010a). When the patient has an already established disease the antibody repertoire remains somewhat stable (van der Woude *et al.* 2010b). In our animal model, in the early stages of antibody development the antibodies seem to have partly overlapping recognition of citrulline and homocitrulline in the same sequence. Later the specificity develops towards the ureido group and the sequence of the immunization antigen. The simultaneous presence of antibodies binding to both citrullinated and carbamylated antigens with separate and partly overlapping specificities has also been demonstrated in RA patients (Shi *et al.* 2013). Some of these features might be explained by the simultaneous presence of citrulline and homocitrulline during immunization. The recognition of several citrullinated epitopes and the low avidity of the antibodies might be a consequence of the presence of carbamylated antigens simultaneously with the citrullinated antigens. This could boost the antibody production and widen the repertoire of the antibodies since carbamylation is not sequence-specific like citrullination.

Citrulline and homocitrulline are closely linked in several ways. The formation of the ureido group of these amino acids changes protein charge and structure and in these studies we showed that antibodies can be induced with citrulline- and homocitrulline-containing sequences injected with adjuvant in rabbits. The induced antibodies react both with the ureido groups and the sequence of the immunogen and are able to distinguish between citrulline and

homocitrulline in the same sequence even though they bind to both. Antibodies binding to CCP developed both with citrulline- and homocitrulline-containing antigens, but antibodies binding to MCV only developed in rabbits immunized with citrulline-containing vimentin sequences and homocitrulline-containing immunogens. This difference is probably not related to the ureido group-containing amino acid of the immunogen, but is more likely due to the more restricted sequence specificity of the collagen telopeptide-induced antibodies. The citrulline-containing immunization antigens were peptides and the carbamylated immunogens were whole proteins. Whole proteins present a wide variety of possible antigenic epitopes and because of the uncontrolled nature of carbamylation, any lysine or free amino group could be carbamylated and thus presented, whereas the epitopes presented in the citrulline-containing immunogens were tightly restricted to the selected peptide sequence. It should also be noted that some citrullination or carbamylation might be present at the injection site in the rabbit since inflammatory cascade is activated, and this could affect the outcome of the antibody repertoire.

6.3 Citrulline and homocitrulline in RA tissues (III)

In study III the synovial tissues of a single RA patient were studied for the presence of citrulline and homocitrulline since a systemically ACPA positive patient could have citrullinated proteins locally in the symptomatic tissue. The tissues were histologically defined as rheumatoid nodules. Both citrulline and homocitrulline were found in all tissues but the amount of the amino acids varied between samples and tissues.

Rheumatoid nodules are tissue formations consisting of connective tissue produced by fibroblasts. Histologically the tissues were characterized by necrotic areas surrounded by palisading histiocytes, i.e., tissue macrophages. When analyzed visually the tissues had little difference between them and the appearance of the slide was not well correlated to the HPLC analysis outcome, but this could be expected because the HPLC analysis could not be executed from the exact location of the histological analysis.

The amount of citrulline varied significantly between tissues and there were marked differences also between the samples of the same tissue specimen. Citrullination in rheumatoid nodules has been shown earlier (Bongartz *et al.* 2007), but the method used was the anti-modified citrulline antibody. Because of the method chosen those results should be interpreted with caution as stated

earlier. Despite that, extracellular staining by the anti-modified citrulline antibody in the rheumatoid nodule tissue was localized mostly in the necrotic areas (Bongartz *et al.* 2007). Our samples were handled and analyzed separately and the 10 mg tissue samples were taken side-by-side from the tissue, so a piece that contained a large necrotic area could contain significantly more citrulline than a sample from an area of fibrotic tissue.

Extracellular traps have been suggested as the source for citrullinated antigens in RA (Khandpur *et al.* 2013). Recently extracellular trap-like structures generated by macrophages were identified in mammary gland tissue (Mohanani *et al.* 2013). The presence of extracellular trap-like structures was not determined in our study, but would be justified in further investigation.

The presence of homocitrulline, the product of lysine carbamylation, has not been demonstrated earlier in RA tissues. Homocitrulline was present in all samples but the level of homocitrulline was low compared to citrulline and remained more constant between both operations and different tissues. The highest homocitrulline value was from tissue A, which came from the most symptomatic site that was the primary cause for the first surgical intervention. Tissue B from the most erosive site had the lowest level of homocitrulline and high level of citrulline. This would suggest increased citrullination in newly formed tissue.

Low amount of homocitrulline was present in each sample. So what is the role of homocitrulline in these tissues? Homocitrulline might be a simple consequence of aging of the tissue (Jaisson & Gillery 2010). Another alternative is that homocitrulline is a product of the chronic inflammation (Wang *et al.* 2007). The extracellular trap structures have been shown to contain active MPO (Brinkmann *et al.* 2004) that could explain the high homocitrulline content of tissue A. Homocitrulline could also be gained by lysine carbamylation over time as a result of aging of the tissue. The lowest homocitrulline content of the erosive tissue B could thus be due to new tissue formation that creates the RA synovitis tissue as the newly formed tissue has not gained age-related posttranslational modifications. At this stage the role of homocitrulline in RA is difficult to determine. Until more results from different tissues are obtained the relevance of homocitrulline to the pathogenesis of RA is not known.

Osteoclastogenesis and bone loss induced by antibodies binding to citrullinated vimentin was recently demonstrated (Harre *et al.* 2012). Our results support the pathogenic relevance of ACPA as high levels of citrulline were found from the synovial tissue (B) of erosive joint. ACPA alone are not enough to cause

classical bone erosions (Kleyer *et al.* 2013) so the local presentation of the citrullinated antigens in synovitis could be a cofactor in the development of erosions. Considering the high number of macrophages in rheumatoid nodule tissue, it is interesting that the PAD expression pattern in osteoclasts and in the macrophages of the inflamed RA synovium has been found to be similar (Chang *et al.* 2005, Foulquier *et al.* 2007). Taken together, although the primary induction of the ACPA might be elsewhere, at least here the removal of the citrulline- and homocitrulline-containing tissues seemed to affect the local disease activity as the patient reported alleviation of RA symptoms.

As a drawback it should be noted that the samples prepared for HPLC analysis could not be subjected to microscopy. Rheumatoid nodules are not uniform in structure as they consist of various amounts of necrotic tissue surrounded by different amounts of mainly macrophages, so even the same tissue contains areas that have different levels of each amino acid. For this reason the appearance of the tissue (inflammation and necrosis) did not correlate well with the results of the HPLC analysis.

Secondly, at this time we do not know whether the citrulline and homocitrulline levels found in this one single patient are common to all RA patients or whether they are just features of this particular patient, or maybe features of ACPA-positive RA patients. More extensive studies on citrulline and homocitrulline content in RA tissues are needed before the results can be generalized in any way.

6.4 About the origin of the ureido groups

The main issue in studying citrullinated proteins related to RA is that there could be a wide variety of citrullinated proteins present in the inflamed synovium and surrounding tissue. The antibodies present in RA sera seem to recognize several proteins either individually or by over-lapping specificities (Ioan-Facsinay *et al.* 2011). The possibility of carbamylation cannot be excluded at this point, since antibodies binding to carbamylated protein with partly overlapping specificities have also been detected in RA sera (Shi *et al.* 2013).

It is unclear how smoking acts in the development of RA, but it is likely that the way of action is multifactorial. The concentration of thiocyanate in the serum of smokers is about triple the concentration seen in non-smokers (Zil-a-Rubab & Rahman 2006) and smokers are at increased risk of infections (Huttunen *et al.* 2011). Thiocyanate can be used as an alternate substrate for myeloperoxidase.

The end product is a protein or peptide containing homocitrulline. This way the elevated thiocyanate concentration during infection could give the time and the place for carbamylation through MPO, or the inflammation caused by infections could increase apoptosis and citrullination.

We showed that in inflamed synovial tissue both citrulline and homocitrulline are present simultaneously. Citrullinated proteins and possibility to carbamylation coincide also in the neutrophil extracellular traps where hypercitrullinated histones and myeloperoxidase are present simultaneously. NETs are present in periodontitis (Vitkov *et al.* 2009) and as they contain citrullinated proteins may be involved in the ACPA formation in RA (Nesse *et al.* 2012). The chronic inflammation in periodontitis or synovitis would be a hospitable environment for presenting citrullinated or carbamylated antigens. In addition to these, in smokers the thiocyanate released from tobacco smoke could favor carbamylation at the site of inflammation. This could give the opportunity for the development of cross-reacting antibodies.

NETs have already been suggested as the source of citrullinated antigens in RA synovial fluid and rheumatoid nodules (Khandpur *et al.* 2013). In our RA tissue samples no neutrophils could be detected, but the presence of citrulline was evident. Of course the infiltration of neutrophils could be local, but since tissue sections of a total of 8 samples were studied and no neutrophils were found, the NETs are probably not the source of citrulline in these tissues. Tissue macrophages were present in the sections surrounding the necrotic tissue of the nodule and the PAD expression pattern in macrophages of the inflamed RA synovium and in osteoclasts has been shown to be similar (Chang *et al.* 2005, Foulquier *et al.* 2007). Recently, indications of extracellular trap-like structures derived from macrophages were shown in mammary gland adipose tissue (Mohanam *et al.* 2013). It would be interesting to know if such structures are also present in the rheumatoid nodule.

But what are the original antigens behind antibodies binding to citrullinated proteins? Where, when and why is the immunological tolerance broken? And what are the events that launch the clinical symptoms of RA? The multifactorial mechanism behind RA induction has not been solved yet.

7 Conclusions

From these studies the following is concluded:

1. Homocitrulline is a confounding factor in immunological citrulline detection methods that are based on antibodies recognizing either native or chemically modified citrulline. In studies involving citrulline and homocitrulline the existence of these two structurally related amino acids should be taken into account and attention should be paid to the choice of method in sample preparation.
2. Protein-bound citrulline and homocitrulline can be separated and identified after chemical modification in hydrolyzed samples using HPLC. Caution in sample preparation is justified to avoid carbamylation during protein purification.
3. Antibodies binding to citrulline-containing antigens can be induced in rabbits immunized with carbamylated immunogens. The induced antibodies bind ureido-group specifically to both citrulline- and homocitrulline-containing peptides, but have different affinities to citrulline and homocitrulline in the same sequence.
4. Antibodies induced with citrulline-containing immunogens can bind to homocitrulline-containing antigens. The developed antibodies recognize the ureido group but can also bind to the native sequence.
5. Immunization with citrulline- or homocitrulline-containing immunogens induces antibodies that contain separate sequence-related and partly overlapping ureido group-related specificities. The induced antibodies can distinguish and prefer the amino acid presented in the immunogen sequence. Ureido group-binding antibodies are not induced with all citrulline- or homocitrulline-containing proteins.
6. Both citrulline and homocitrulline are present in rheumatoid nodule tissues. Homocitrulline was present in all analyzed synovial tissue samples. The role of homocitrulline in RA is unclear. The significant variation between citrulline levels in the samples is related to the mixed tissue structure of rheumatoid nodules. The highest citrulline values were probably from the necrotic areas and less citrulline was found from the intact fibrotic tissue.
7. RA synovial tissue could present surroundings for development of cross-reacting antibodies since citrulline and homocitrulline are present simultaneously. We showed that even though the specificities of the

antibodies induced with citrulline- and homocitrulline-containing immunogens were different, the binding to both citrulline- and homocitrulline-containing antigens was significant. This kind of cross-reactive binding could boost antibody development and widen the repertoire of antibody specificities.

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