Marja-Kaisa Koivula

AUTOANTIBODIES BINDING CITRULLINATED TYPE I AND II COLLAGENS IN RHEUMATOID ARTHRITIS
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Academic Dissertation to be presented with the assent of the Faculty of Medicine, University of Oulu, for public discussion in the Auditorium 9 of Oulu University Hospital, on June 9th, 2006, at 12 noon
Abstract

Rheumatoid arthritis (RA) is a systemic autoimmune disease with symmetrical articular manifestations. The etiology of the disease is unknown. The prevalence of RA is approximately 0.5–1.0% in adults. In Finland, the annual incidence is 39/100 000. RA is about three times more common in females than in males. Most commonly the disease affects first the joints of feet and fingers. Chronic inflammation leads to erosions of cartilage, bone and tendons and may destroy the whole joint. The diagnosis of RA is mainly based on the clinical features of the disease. The American College of Rheumatology (ACR) 1987 revised classification criteria of RA have commonly been used for diagnosis. No specific diagnostic test is available. Rheumatoid factor (RF) has traditionally been used in the diagnosis, but only 70 to 80% of RA patients have RF in their serum. Other antibodies found in RA are the antiperinuclear factor (APF), the anti-keratin antibody (AKA) and the antibodies to cyclic citrullinated peptide (CCP), which recognize the citrulline-containing antigenic filaggrin protein. Citrulline is an amino acid that is post-translationally formed from arginine by peptidylarginine deiminase enzymes (PADs). Autoantibodies to citrullinated proteins are more specific for RA than RF. There is no filaggrin in joints, which indicates that the autoantibodies reacting with this protein most probably only reflect immunological cross-reaction. It has been postulated that autoimmunity against collagens might be involved in the pathogenesis of RA. There are antibodies binding to collagen in cartilage (type II collagen) and in bone (type I collagen). They have been tested by using collagen preparations rendered soluble by pepsin digestion. This digestion removes the carboxyterminal (C-terminal) telopeptides of collagen.

Autoantibodies to the C-telopeptides of type I and II collagens were studied in this doctoral research. Autoantibodies to the citrullinated C-telopeptides of type I and II collagens were found in the serum of patients with RA. ELISA, CLIA and inhibition ELISA were used to detect these autoantibodies. Automatic CLIA gives a more than twofold number of positive findings compared to previous ELISA. Currently the best method for the detection of these autoantibodies is inhibition ELISA. These autoantibodies are specific for citrulline in the peptide sequence. Autoantibodies that bind the normal C-telopeptides of type I and II collagens were not inhibited by soluble normal or citrullinated telopeptides. However, the antibodies that bind only citrullinated telopeptides could be inhibited by corresponding citrullinated telopeptides. Autoantibodies binding the citrullinated telopeptides of type II collagen and anti-CCP predict synergistically the development of seropositive RA.

Keywords: citrullination, collagen, rheumatoid arthritis
Koivula, Marja-Kaisa, Sitrullinisoituneet tyypin I ja II kollageeniautovasta-aineet nivelreumassa
Lääketieteellinen tiedekunta, Kliinisen kemian laitos, Oulun yliopisto, PL 5000, 90014 Oulun yliopisto
Oulu

Tiivistelmä
Nivelreuma (arthritis rheumatoides) on krooninen autoimmunisairaus, jonka aiheuttajaa ei tunneta. Nivelreuman esiintyvyys aikuisilla on 0.5–1.0 prosenttia. Siihen sairastuu vuosittain 39/100 000 suomalaisista aikuista. Naiset sairastavat nivelreumaa kolme kertaa yleisemmin kuin miehet. Sairaus alkaa tavallisesti päkiöistä ja sormien nivelistä. Nivelreuma aiheuttaa ruston, luun ja nivelseteiden syöpymistä ja voi lopulta tuhota koko nivelen.


Asiasanat: kollageeni, nivelreuma, sitrullinisaatio
To my parents and siblings
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“...Fortune ever changeth here, Heavenly Father shield me e’er.” (SHZ 227:1)

Oulu, April 2006

Marja-Kaisa Koivula
Abbreviations

ACR  American College of Rheumatology (previously ARA)
AFA  anti-filaggrin antibody
AKA  antikeratin antibody
APF  antiperinuclear factor
ARA  American Rheumatism Association
BSA  bovine serum albumin
C/A (I)  ratio of citrullinated/arginine telopeptides of type I collagen
C/A (II)  ratio of citrullinated/arginine telopeptides of type II collagen
Ca²⁺  calcium ion
CCP  cyclic citrullinated peptide
CLIA  chemiluminescence immunoassay
C  Contingency Coefficient
Cit  Citrulline, C₆H₁₃N₃O₃ (a non-standard amino acid)
CIA  collagen-induced arthritis
C-terminal  carboxyterminal
CRP  (serum) C-reactive protein
CTX-I  C-terminal telopeptide of collagen type I
CTX-II  C-terminal telopeptide of collagen type II
DMARD  disease-modifying anti-rheumatic drug
DTT  dithiothreitol, C₄H₁₀O₂S₂
H₂O₂  hydrogen peroxide
EDTA  ethylenediaminetetraacetic acid, C₁₀H₁₈N₂O₈
EIA  enzyme immunoassay
ELISA  enzyme-linked immunosorbent assay
ER  endoplasmic reticulum
ESR  erythrocyte sedimentation rate
HLA  human leucocyte antigen
HRP  horseradish peroxidase
ICTP  carboxyterminal telopeptide of type I collagen
IIF  indirect immunofluorescence
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCP</td>
<td>metacarpophalangeal (joints of the fingers)</td>
</tr>
<tr>
<td>MMP</td>
<td>matrix metalloproteinase (an enzyme)</td>
</tr>
<tr>
<td>MTP</td>
<td>metatarsophalangeal (joints of the feet)</td>
</tr>
<tr>
<td>N-terminal</td>
<td>aminoterminal</td>
</tr>
<tr>
<td>PAD</td>
<td>peptidylarginine deiminase (an enzyme)</td>
</tr>
<tr>
<td>PADI</td>
<td>DNA encoding for PAD enzyme (a gene)</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PICP</td>
<td>carboxyterminal propeptide of type I collagen</td>
</tr>
<tr>
<td>PINP</td>
<td>aminoterminal propeptide of type I collagen</td>
</tr>
<tr>
<td>PIIANP</td>
<td>aminoterminal propeptide of type IIA collagen</td>
</tr>
<tr>
<td>PIICP</td>
<td>carboxyterminal propeptide of type II collagen</td>
</tr>
<tr>
<td>PIINP</td>
<td>aminoterminal propeptide of type III collagen</td>
</tr>
<tr>
<td>PIP</td>
<td>interphalangeal (joints of the fingers)</td>
</tr>
<tr>
<td>RA</td>
<td>rheumatoid arthritis</td>
</tr>
<tr>
<td>RF</td>
<td>rheumatoid factor</td>
</tr>
<tr>
<td>ROC curve</td>
<td>Receiver Operating Characteristic curve (a statistical test)</td>
</tr>
<tr>
<td>rs</td>
<td>Spearman’s rank correlation</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>SCW</td>
<td>Streptococcal cell wall (induced arthritis)</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SPSS</td>
<td>Statistical Package for Social Science (software)</td>
</tr>
<tr>
<td>TIINE</td>
<td>neoepitope of type II collagen</td>
</tr>
<tr>
<td>TMB</td>
<td>3,3',5,5'-tetramethylbenzidine (a chromogen)</td>
</tr>
<tr>
<td>TNFα</td>
<td>tumor necrosis factor alpha (a cytokine)</td>
</tr>
</tbody>
</table>
List of original articles

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


Some unpublished data are also presented in this thesis. The original publications have been reproduced with kind permission of the copyright holders.
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1 Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disease, whose etiology is unknown. Both genetic and environmental factors have a role in the etiopathogenesis of RA. (Feldmann et al. 1996.) Smoking is the only known avoidable risk factor (Heliövaara et al. 1993, Silman et al. 1996, Symmon et al. 1997). The familial clustering of the disease is known. The most important genes that influence the susceptibility to RA locate within the human leukocyte antigen (HLA) region of the sixth human chromosome (Lawrence et al. 1970, del Junco et al. 1984, Emery 1997, Jawaheer et al. 2002). The prevalence of RA is approximately 0.5–1.0% in adults (Gran 1987). In Finland, the annual incidence is 39/100 000 of the adult population (Kaipiainen-Seppänen et al. 1996). RA is about three times more common in females than in males (Lawrence 1970).

The early clinical features of RA are pain, morning stiffness and swelling of the joints. The disease usually affects first the metatarsophalangeal (MTP) joints of the feet and the metacarpophalangeal (MCP) and proximal interphalangeal (PIP) joints of the fingers (Tannenbaum H & Rosenthall L 1987). A chronic inflammatory condition leads to overgrowth of the synovium, pannus and effusion into the joint, which cause cartilage and bone erosions and cartilage thinning. Ultimately, the process may lead to total destruction of the joint and periarticular structures. The disease may also cause extra-articular manifestations such as vasculitis, rheumatoid nodules, pericarditis and neuropathy (Panayi 1995).

The diagnosis of RA is mainly based on the clinical features of the disease. The American College of Rheumatology (ACR) 1987 revised classification criteria of RA (Arnett et al. 1998) have commonly been used for diagnosis. No specific test is available for the diagnosis of RA. Rheumatoid factor (RF) has traditionally been used in the diagnosis of RA, but only 70 to 80% of RA patients are positive for it (Kroot et al. 2000, van Gaalen et al. 2004).

There are also other antibodies, such as the antiperinuclear factor (APF), anti-keratin antibody (AKA) and cyclic citrullinated peptide (CCP), that are more specific for RA than RF (Lee & Schur 2003, Saraux et al. 2003). These antibodies recognize the citrulline-containing antigenic filaggrin protein (Schellekens et al. 1998, Girbal-Neuhauser et al. 1999). Of these, only CCP has been in clinical use in recent years.
There is no filaggrin in joints, which indicates that the autoantibodies reacting with this protein most probably only reflect immunological cross-reaction. It has long been assumed that autoimmunity against collagens might be involved in the pathogenesis of RA. (Yamada et al. 2005, Suzuki et al. 2005.) Previously, anti-collagen antibodies formed against type II collagen in cartilage and type I collagen in bone were not citrullinated, and collagen autoantibodies were tested by using collagen preparations rendered soluble by pepsin digestion. This digestion removes the carboxyterminal telopeptides of collagens.

The primary aim of this study was to assess the possible presence of autoantibodies recognising the citrullinated peptides derived from type I and II collagens in patients with RA. A further aim was to develop sensitive methods for the detection of these autoantibodies.
2 Review of the literature

2.1 Rheumatoid arthritis (RA)

RA is a chronic inflammatory condition with symmetric articular manifestations. The etiology of the disease is not known, its prognosis is poorly predictable, and there is no definite cure for it. Both environmental and genetic factors have a role in the etiopathogenesis of the disease. RA affects females three times more frequently than males (Lawrence 1970). The disease manifests most commonly at the age of 40–70 years. RA occurs all over the world. In adults, the prevalence of RA is 0.5–1.0% in most Western countries (Gran 1987). The highest prevalence (3–6 %) has been reported among some North American Pima Indians (del Puente et al. 1989, Jacobsson et al. 1994). In tropical areas, the prevalence of RA is 0.7% in rural populations (McGill 1991). The lowest figures (0.35%) have been reported for the urbanized Chinese of Hong Kong (Lau et al. 1993). In Finland, the prevalence of seropositive RA is approximately 0.8% (Aho et al. 1989, Hakala et al. 1993a).

The annual incidence of RA with reference to the ACR criteria (Arnett et al. 1998) is 36/100000 for female and 14/100000 for male adults in the United Kingdom (Symmons et al. 1994). The incidence of RA has decreased during the last few decades (Kaipiainen-Seppänen et al. 1996, Shichikawa et al. 1999, Symmons et al. 2002).

A shortened life expectancy is associated with RA (Edwards 2005). The estimates of shortened life have varied from three (Vandenbroucke et al. 1987) to 18 years (Reah 1963). In Finland, the shortening of life span is 3.8 years for men and 3.4 years for women (Myllykangas-Luosujärvi et al. 1995a). Of the increased deaths, 40% were due to cardiovascular causes, 30% to infections, 15% to amyloidosis, 10% to anti-rheumatic medication and 5% to diverse causes (Myllykangas-Luosujärvi et al. 1995a, Myllykangas-Luosujärvi et al. 1995b).

The inflammation of RA may affect the whole synovial joint and, in severe cases, involve many other organs. The main point in the development of irreversible cartilage erosions is chronic synovitis, especially the pannus at the junction of the synovial membrane and cartilage.
The diagnosis of RA is mainly based on clinical features. There is no single objective diagnostic test. The ACR 1987 revised criteria for the classification of RA (table 1) have been generally used in the diagnosis of RA (Arnett et al. 1998).

Table 1. The revised classification criteria for rheumatoid arthritis (RA) by American College of Rheumatology (ACR). (Arnett et al. 1998).

<table>
<thead>
<tr>
<th>Criteria</th>
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<tbody>
<tr>
<td>1. Morning stiffness for at least one hour.</td>
</tr>
<tr>
<td>2. Soft tissue swelling (arthritis) of 3 or more joint areas (PIP, MCP, and MTP, wrist, elbow, knee or ankle) observed by a physician.</td>
</tr>
<tr>
<td>3. Swelling (arthritis) of the wrist, MCP or PIP areas.</td>
</tr>
<tr>
<td>4. Symmetric swelling (arthritis).</td>
</tr>
<tr>
<td>5. Rheumatoid nodules observed by a physician.</td>
</tr>
<tr>
<td>7. Radiographic changes (erosions and/or periarticular osteopenia) in the hand, wrist, PIP or MCP areas.</td>
</tr>
</tbody>
</table>

Four out of seven criteria must be fulfilled. The symptoms defined by the criteria 1–4 must have been present for at least 6 weeks.

The essential goal of treating RA is to induce complete remission and to prevent joint destruction. If remission is not attained, management should aim to control disease activity, to slow down the progression of tissue damage, to alleviate pain, to maintain functional capacity as well as the capacity for employment and to maximize the quality of life. (Möttönen et al. 1999.)

To achieve remission, disease-modifying anti-rheumatic drugs (DMARDs) are used nowadays. The best results have been obtained by combination therapy with DMARDs (Möttönen et al. 1999, O’Dell 2002). Aggressive initial treatment of early RA with a combination of 3 DMARDs for the first 2 years limits peripheral joint damage for at least
5 years (Korpela et al. 2004). A combination of DMARDs was shown to reduce occupational disability in the 5-year outcome (Puolakka et al. 2004).

Corticosteroids still have a controversial position in the treatment of RA (Morrison & Capell 1996). High doses of oral corticosteroids may cause significant side effects. Low-dose (7.5 mg/day) prednisolone has been claimed to slow down the erosive rate in newly diagnosed patients but, unfortunately, at two years, not to confer any other clinical benefit (Kirwan 1995).

In recent years, new biological drugs, especially tumour necrosis factor alpha (TNFα) blockers, have been introduced into clinical use. In combination with methotrexate, they have been able to retard considerably or even stop the radiographically detected destruction of joints in RA (Navarro-Sarabia et al. 2005).

### 2.2 Markers used in the diagnosis and assessment of RA

Erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) are useful indicators of disease activity (Banks et al. 1998). The ACR criteria include only one serological parameter, rheumatoid factor (RF). There are also other serological markers, such as antiperinuclear factor (APF), antikeratin antibody (AKA) and antibodies against cyclic citrullinated peptide (CCP), of which only CCP has been in clinical use.

#### 2.2.1 Rheumatoid factor (RF)

Traditionally, rheumatoid factor (RF) has been the most important marker for RA. RF was described 1940 in patients with RA (Waaler 1940). RF is a circulating antibody binding against multiple antigenic determinants on immunoglobulins. Several techniques of measurement are available. The traditional agglutination and immunoturbidimetric techniques measure immunoglobulin IgM class RF. Radioimmunoassay and enzyme-linked immunosorbent assay (ELISA) allow the measurement of RF in all major immunoglobulin classes (IgM, IgG and IgA). (Lemm et al. 1988.)

The presence of IgM RF is one of the ACR criteria for the diagnosis of RA. Seventy to eighty percent of patients with RA have elevated serum RF levels (Kroot et al. 2000, van Gaalen et al. 2004). The IgG RF antibody, which reacts with the Fc fragment of the IgG heavy chain, is present in about 75% of RA patients. RA has been divided into two categories, seropositive and seronegative, on the grounds of the absence or presence of IgM RF (Masi & Feigenbaum 1983). The presence of RF and its serum level correlate with radiologic erosions, poor outcome and extra-articular manifestations (e.g. rheumatoid nodules and vasculitis) in RA (Paimela et al. 1995, Richardson & Emery 1996, Saraux et al. 1997).

Male patients have been found to be more often RF-positive than female patients (Meyer et al. 1996, Weyand et al. 1998, del Rincón et al. 2002). RF often precedes the onset of RA by several years (Aho et al. 1994).

IgM RF is not very specific for RA because it can be found in other autoimmune diseases and in 3–5% of the healthy population, which percentage increases up to 10–
30% with ageing (Mikkelsen et al. 1967, Shmerling & Delbanco 1991, Mageed 1996). RF may be also present in patients with some non-autoimmune conditions, including chronic infections (Tighe & Carson 1997).

### 2.2.2 Specific autoantibodies

APF, AKA and CCP are known antibodies that react against citrullinated filaggrin. Citrulline (Cit, C₆H₁₃N₃O₃) is a non-standard amino acid which is not incorporated into proteins during translation. However, it can be generated post-translationally from arginine by peptidylarginine deiminase enzymes (PADs, EC 3.5.3.15) (Vossenaar et al. 2003b, Vossenaar et al. 2004a, Yamada et al. 2005). Citrullination of proteins occurs in normal individuals late in the protein existence, e.g. in vimentin and filaggrin during apoptosis. Citrullination has recently aroused interest in rheumatology, since the specific autoantibodies found in RA are mainly directed to proteins where certain arginine residues have been modified to citrulline (Nijenhuis et al. 2004, Yamada et al. 2005). Autoantibodies to citrullinated proteins can be detected with several methods.

These autoantibodies are more specific for RA than RF (Lee & Schur 2003, Saraux et al. 2003). They are detectable in early RA and may predict the clinical disease outcome. These antibodies will probably become progressively more valuable for clinicians. (Vossenaar et al. 2004a.)

Not every citrulline residue will provide a good epitope in proteins, because the amino acids flanking the citrulline residue are important for the presentation of the antigenic citrulline residue (Schellekens et al. 1998, Lapointe et al. 1999). Proteins with high arginine content (such as filaggrin, fibrinogen, vimentin, histones and myelin basic protein) are more likely to include reactive epitopes upon citrullination than proteins with low arginine content (Lapointe et al. 1999).

RA-specific antibodies can be detected by using either citrullinated protein or citrullinated peptide(s) as an antigen. In a diagnostic test, the use of citrullinated protein as an antigen involves serious disadvantages, particularly the difficulty of standardizing the in vitro citrullination process. In contrast to proteins, a citrullinated peptide contains only one (or a few) epitope(s). This can limit the sensitivity of measuring the polyclonal response of a patient. (Nijenhuis et al. 2004.)

#### 2.2.2.1 Antiperinuclear factor (APF)

These autoantibodies were discovered as early as 1964, when Neinhuis and Mandema described APF. This antigen is present in the keratohyaline granules surrounding the nucleus of human buccal mucosa cells. The localisation of APF in the keratohyalin granules of buccal mucosa cells was verified by immunoelectron microscopy (Hoet et al. 1991). The sensitivity of APF is about 50 % and specificity about 80 % (Saraux et al. 2003). Positive reactions in RA patients have been detected by indirect immunofluorescence (IIF). The presence of APF correlates with RF positivity (Cassani et al. 1983), and they may sometimes cross-react, but many IgM RF-containing fractions do
not show APF activity (Nesher et al. 1991). APF-positive, RF-negative patients with RA seem to have a poor prognosis (Janssens et al. 1988, Westgeest et al. 1987). The results of Munoz-Fernandez et al. (1999) suggested APF as a possible marker of poor prognosis in RA. Kerstens et al. (1994) reported that the APF titre showed no sustained change during a 48-week follow-up period with DMARD treatment.

2.2.2.2 Antikeratin antibody (AKA)

In 1979, Young et al. discovered AKA by using IIF to study sections of rat oesophagus. AKA is a highly specific (92–99%), but not very sensitive (40–60%) marker of RA (Hajitroussou et al. 1981, von Essen et al. 1993). A highly significant correlation was found between AKA and the presence of rheumatoid hand deformity (Kirstein & Mathiesen 1987, Kurki et al. 1997). The presence of AKA showed a marked correlation with IgM RF (Johnson et al. 1981) but not with IgA RF (Berthelot et al. 1994).

The test for AKA is more specific but less sensitive than that for APF or RF (Miossec et al. 1982). Cordonnier et al. (1996) pointed out that AKA and APF have an independent and statistically significant diagnostic value for early RA.

Girbal et al. (1993) reported that AKA does not react with cytokeratins. They suspected that reacting proteins may be related to (pro)filaggrin. In 1995, Sebbag et al. and Simon et al. obtained results indicating that AKA and APF are largely identical autoantibodies. They recognized human epidermal filaggrin and (pro)filaggrin-related proteins in buccal epithelial cells. Similar results were also reported by others (Schellekens et al. 1998, Smolen & Steiner 1998, Girbal-Neuhauser et al. 1999, Vincent et al. 1999). It was shown that in vitro epidermal keratinocytes expressed various molecular forms of (pro)filaggrin that carried epitopes targeted by AKA and APF of RA sera (Girbal-Neuhauser et al. 1997). Nogueira et al. (2001) found that the diagnostic performance of the test was similar to that of AKA and antibodies to human epidermis filaggrin. Nowadays, AKA and APF are designated as anti-filaggrin antibodies (AFA) (Girbal-Neuhauser et al. 1999).

2.2.2.3 Cyclic citrullinated peptide (CCP)

Schellekens et al. (1998) used filaggrin-derived linear citrulline-containing peptide and showed that the citrulline-containing peptides were recognized by IgG antibodies in RA sera. Sensitivity was rather low (<50%), however. To optimize this method, cyclic variants of the peptides were developed. These cyclic citrullinated peptides were used as antigen in the first-generation CCP test with sensitivity of 68% for RA. There is no filaggrin in the synovium. It has been suggested that other citrullinated proteins exist in the joint. A library of citrullinated peptides was screened with a pool of RA sera. The result was a number of highly reactive peptides. A highly reactive cyclic peptide from library screening is used in the second-generation CCP test (CCP2). This CCP2 assay has a better sensitivity of about 82% for RA and 98–99% specificity. (Nijenhuis et al. 2004.)
Anti-CCP2 ELISA was developed to determine the presence of antibodies directed towards citrullinated peptides, using a synthetic peptide designed for this purpose (Schellekens et al. 2000). The sensitivity of anti-CCP antibody is about 50%, with a high specificity (97%) for RA (Bizzaro et al. 2001, Zeng et al. 2003, Nijenhuis et al. 2004). In spite of that, the prevalence of false positive RF reactions increases with advancing age, but the test for anti-CCP has good specificity among the aged (Palosuo et al. 2003, Samanci et al. 2005). Anti-CCP antibody is more specific than RF for RA (Lee & Schur 2003, Girelli et al. 2004). Anti-CCP testing combined with IgM-RF testing has additional value over IgM-RF testing alone in patients with early RA (Jansen et al. 2002). The data of Kroot et al. (2000) showed that almost 70% of RA patients have anti-CCP antibody present at the early stages of the disease. Anti-CCP-positive patients developed significantly more severe, radiologically detectable damage than patients who were anti-CCP negative, although the additional predictive value was rather moderate in multiple regression analysis.

Samanci et al. (2005) found that anti-CCP can appear in cases of both early and advanced RA with the same prevalence, sensitivity and specificity. The presence of antibodies to anti-CCP predicts the development of RA in apparently healthy individuals (Rantapää-Dahlqvist et al. 2003). Anti-CCP also predicts the development of RA in patients with undifferentiated arthritis (van Gaalen et al. 2004).

The role played by anti-CCP autoantibodies in the pathogenesis of RA is unclear. Possible clues are the association of citrullination with apoptosis, the appearance of anti-CCP antibodies before the occurrence of clinical symptoms, their specificity for RA and the fact than a genetic risk factor that leads to increased citrullination is associated with RA (van Gaalen et al. 2004).

### 2.3 Other markers in the assessment of RA

#### 2.3.1 Other antibodies in RA

Other autoantibody systems that may sometimes be associated with RA are anti-RA33, anti-calpastatin, anti-neutrophil cytoplasmic antibodies (pANCA), anti-glucose-6-phosphate isomerase (GPI), anti-heavy chain binding protein (BIP) and anti-phospholipid antibodies. These antibodies are not specific for RA but they can occasionally be used in the screening and monitoring of the disease and in the prediction of disease development (van Boekel et al. 2002, Steiner & Smolen 2002, Nijenhuis et al. 2004). For example, in patients with early RA, pANCA is associated with specific serologic markers of RA and predicts rapid radiographic joint destruction (Mustila et al. 2000). These antibodies may be present in early disease stages, particularly in seronegative sera (Cordonnier et al. 1996).

Recent studies suggest that the prevalence of antinuclear antibodies (ANA) in serum in early RA is 31 – 63% (Paulus et al. 2002, Jacobsen 2004). In women, ANA positivity was reported to be lower with disease onset at older age, whereas no such association was found among men (Jacobsen 2004). The findings in 200 RA patients, 143 women and 57
men, showed age at onset to be related to the presence of ANA in serum at presentation. The patients were stratified into age brackets at onset: <40 years, 40 – 60 years and >60 years. In women, the prevalences of ANA were 63.0%, 45.5% and 31.1% in the respective age brackets (p=0.002). The prevalences of ANA among men were, respectively, 35% with no association with age at onset. The findings may indicate interactive effects between gender and various pathogenetic factors. (Jacobsen 2004.)

Antibodies against citrullinated vimentin (anti-Sa) were discovered in 1994 (Despres et al. 1994, Steiner & Smolen 2002). The anti-Sa assay has specificity and positive predictive value of nearly 100% in RA. Its sensitivity is 21–43% (Nijenhuis et al. 2004). Anti-Sa antibodies are present from disease onset onwards and are predictive of disease severity. (Hayem et al. 1991, Menard et al. 2000.) Immune reactants are abundant in the target tissue. Antigen is present in the synovium and IgG antibody in synovial fluid. Immunologically, Sa is a hapten-carrier antigen, in which vimentin is the carrier and citrulline is the hapten. (Menard et al. 2000.) Anti-Sa antibodies occur independently of RF (Despres et al. 1994). The citrullination of vimentin is closely related to apoptosis (Menard et al. 2000). The antigen, a doublet of protein bands of about 50 kDa, is present in the placenta, spleen and synovial tissue of RA patients (Despres et al. 1994, Vossenaar et al. 2004a).

Profilaggrin and filaggrin are not present in pannus but Sa is (Menard et al. 2000). According to Vossenaar et al. (2004a), citrullinated vimentin is a candidate autoantigen in RA. However, Masson-Bessiere et al. (2001) showed that deposits contain citrullinated proteins. One of these proteins was identified as citrullinated fibrin. The anti-Sa assay is not in clinical use.

2.3.2 Genetic markers

Genetic factors play a role in the etiopathogenesis of RA. Familial clustering of the disease has been shown previously (Lawrence 1970, del Junco et al. 1984). It has been estimated that two to three percent of RA patients have first-degree relatives with the same disease, and positive family history is one of the risk factors for RA (de Blécourt et al. 1961, Deighton & Walker 1991, Weyand & Goronzy 1995).


An association between RA and human leukocyte antigens (HLA) was first demonstrated in the 1970s (Jawaheer et al. 2002). The availability of serological typing showed a genetic association of RA with the complex HLA-DR4 antigen (Stastny 1976, Stastny 1978). The presence of HLA-DR4 specificity could be an important factor in determining RA concordance (Jawaheer et al. 1994).

HLA-DR4 is the only widely validated genetic association in RA. The doctoral dissertation of Laivoranta-Nyman (2003) showed familial RA patients to have an increased frequency of HLA-DR4 and a lower mean age at disease onset compared to non-familial RA patients. Familial male RA patients turned out to have more genetic background affected by HLA region genes than familial female patients.
HLA-DR status, RF seropositivity (Lawrence 1970, Barrera et al. 1999) and early age at onset or long disease duration (Wasmuth et al. 1972, del Junco et al. 1984, Sanders et al. 1987, Kwoh et al. 1996) have shown an association with familial RA (Barrera et al. 1999).

It is known that there is a stronger HLA-DR4 association with RA in familial than in sporadic cases (Khan et al. 1983, Sanders et al. 1987, Deighton & Walker 1992). It was shown that citrullinated peptides can be bound more efficiently by HLA-DR4 molecules than by corresponding non-citrullinated peptides (Hill et al. 2003).

Post-translational modifications of proteins occur very frequently. One of the post-translational modifications of proteins is citrullination by peptidylarginine deiminase (PAD). PAD activity is under strict genetic control. It is known that very sensitive and specific markers for serum antibodies are reactive with citrullinated proteins/peptides. The genes encoding for PAD enzymes (PADs) have been investigated in RA. (Migliorini et al. 2005.)

The PADI4 gene seemed to confer susceptibility to RA in Japanese patients (Suzuki et al. 2003, Ikari et al. 2005), but not in Caucasians (Migliorini et al. 2005). Another study indicated that intracellular citrullinated proteins co-localized with PADI2, which was found in 59% of RA samples versus 17% of control samples (de Rycke et al. 2005).

PADI4 mRNA, although absent from healthy synovium, was readily transcribed and translated by polymorphonuclear neutrophils infiltrating synovial tissue during inflammation. (Vossenaar et al. 2003a.)

2.3.2.1 Comparison of RA markers


Hoet et al. (1991) found a positive correlation between the presence of APF and the presence of AKA but no correlation with the presence of RF. However, Aho et al. (1993) showed that AKA and APF appear to be linked markers of an immunological process which, in RF-positive subjects, predicts the development of clinical arthritis. They also found that the assays for AKA and APF, compared with RF testing, yield greater specificity rather than an ability to define any subgroup with particularly severe disease. (Aho et al. 1994). A positive correlation between APF and AKA is obvious because AKA and APF are nowadays designated as anti-filagrin antibodies (AFA).

Anti-CCP, APF and AKA did not correlate perfectly with one another (Saraux et al. 2003). Zeng et al. (2003) obtained different results; anti-CCP correlated with APF, AKA, RF and the HLA-DR4 gene complex. According Kamali et al. (2005), sensitivity and specificity were 43% and 44% for RF, 65% and 98% for anti-CCP and 58% and 100% for AKA in RA patients. In the RF-negative RA group, AKA was found to have a high frequency (55%) in comparison to anti-CCP (38%). Vencovsky et al. (2003) confirmed that the measurement of anti-CCP, AKA, APF and individual isotypes of RFs was useful for the prediction of structural damage early in the disease course.
AFA and anti-Sa were detected in community cases of very early RA (Vittecoq et al. 2001). Vossenaar et al. (2004) reported the anti-CCP titres of anti-Sa-positive patients to be more than threefold compared to anti-Sa-negatives. Goldbach-Mansky et al. (2000) showed that anti-Sa, AFA and anti-CCP were all rather specific for early RA, but overall, had little additional diagnostic value compared to RF alone. These antibodies may prefentially recognize citrullinated antigens.

Table 2. The autoantibodies binding citrullinated proteins in RA. A summary of the different assays used for the detection of these autoantibodies. Profilaggrin was obtained from human buccal mucosa cells and rat filaggrin from rat oesophagus. Total means of all patients (RA, healthy control and other rheumatic disease) in the study. Other rheumatic diseases could have joint synovitis but not RA. IB means immunoblotting. (See Nijenhuis et al. 2004.)

<table>
<thead>
<tr>
<th>Protein</th>
<th>Antibody</th>
<th>Material</th>
<th>Method</th>
<th>Sensitivity (%)</th>
<th>Specificity (%) Healthy control / Other diseases</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Pro)-filaggrin</td>
<td>APF</td>
<td>RA / Total</td>
<td>IIF</td>
<td>49–91</td>
<td>71-99 / 82</td>
<td>Hoet et al. 1991</td>
</tr>
<tr>
<td>Human filaggrin</td>
<td>AFA</td>
<td>190 / 670</td>
<td>IB</td>
<td>41</td>
<td>– / 99</td>
<td>Vincent et al. 1998</td>
</tr>
<tr>
<td></td>
<td></td>
<td>55 / 55</td>
<td>ELISA</td>
<td>47</td>
<td>99 / –</td>
<td>Palosuo et al. 1998</td>
</tr>
<tr>
<td>Rat filaggrin</td>
<td>AKA</td>
<td>96 / 316</td>
<td>IIF</td>
<td>40</td>
<td>98 / 98</td>
<td>Miossec et al. 1982</td>
</tr>
<tr>
<td></td>
<td></td>
<td>55 / 55</td>
<td>ELISA</td>
<td>51</td>
<td>99 / –</td>
<td>Palosuo et al. 1998</td>
</tr>
<tr>
<td></td>
<td></td>
<td>179 / 229</td>
<td>IIF</td>
<td>46</td>
<td>– / 94</td>
<td>Bas et al. 2002</td>
</tr>
<tr>
<td>Citrullinated</td>
<td>Anti-Sa</td>
<td>206 / 482</td>
<td>IB</td>
<td>43</td>
<td>100 / 99</td>
<td>Despres et al. 1994</td>
</tr>
<tr>
<td>vimentin</td>
<td></td>
<td>154 / 489</td>
<td>IB</td>
<td>40</td>
<td>100 / 79</td>
<td>Hayen et al. 1999</td>
</tr>
<tr>
<td></td>
<td></td>
<td>957 / 2737</td>
<td>IB</td>
<td>37</td>
<td>– / 98</td>
<td>Menard et al. 2000</td>
</tr>
</tbody>
</table>

2.4 Citrullinisation of proteins

Citrulline has been named after the Latin word for watermelon, Citrullus vulgaris, which contains large amounts of this amino acid. Citrulline is an amino acid (Cit, C₆H₁₃N₃O₃) that is post-translationally formed from arginine in peptides or proteins by the enzyme peptidylarginine deiminase (PAD; EC 3.5.3.15). (Curis et al. 2005.) (See figure 2.) Calcium ions (Ca²⁺) are needed for the activation of PAD enzymes (Keilhoff et al. 2003.) One molecule of the PAD protein can bind five Ca²⁺ (Arita et al. 2004). The intracellular Ca²⁺ concentration in normal cells is lower (10⁻⁷ mol/l) than the threshold Ca²⁺ concentration for PAD activity, which is approximately 10⁻⁵ mol/l (Vossenaar & Venrooij 2004.) During cell death, the solidity of the plasma membrane is lost (Schwab et al. 2002, Tombal et al. 2002) causing a flow of Ca²⁺ from the extracellular space. This makes the

The enzymes and proteins that require Ca\(^{2+}\) for their activities are thought to be part of the apoptotic processes (McConkey & Orrenius 1996). Ca\(^{2+}\) ionophores (for example ionomycin) are known potent inducers of apoptosis (Lennon et al. 1991, Whyte et al. 1993, Hagiwara et al. 2005), suggesting that the Ca\(^{2+}\) influx may itself be a trigger for apoptosis. Asaga et al. (1998) found out that deiminated proteins increase during ionomycin treatment. Mizoguchi et al. (1998) discovered a significant degree of immunoreactivity to citrulline around the nucleus after ionomycin treatment in cultured cells from a rat epidermal keratinocyte cell line. Alternatively, PAD enzymes may leak out from dying cells and become activated because the extracellular Ca\(^{2+}\) concentration is about 10\(^{-3}\) mol/l, which is adequate for PAD activity (Takahara et al. 1986). Citrullination occurs during embryonic development already (Nijenhuis et al. 2004).

Conversion of arginine to citrulline results in a mass increase of 0.984 and a loss of the positive charge from the side chain. This causes a significant acidic shift in the two-dimensional electrophoretic migration. In addition, the pattern of peptides produced on tryptic digestion is altered because the modified amino acids are unsuitable for trypsinolysis and yield peptides containing citrulline rather than C-terminal arginine. (Kinloch et al. 2005.)

![Fig. 2. Irreversible hydrolysis of L-arginine to citrulline and ammonia catalyzed by PAD (See Galkin et al. 2005). PAD is a calcium-dependent enzyme. Citrullination of proteins results in a loss of the positive charge of arginine. (Modified from Nijenhuis et al. 2004.)](image)

### 2.4.1 Citrullinisation in different mammalian tissues

Five different isotypes of PADs (PAD1–PAD4, PAD6) have been found in mammalian tissues (Vossenaar et al. 2003b, Vossenaar & Venrooij 2004, Dong et al. 2005), and two of the five PAD isotypes are known to be expressed in RA synovial tissue (Yamada 2005). The isotypes share 50–55% sequence identity (Arita et al. 2004). Theoretically, every protein that contains an arginine residue in its sequence can be citrullinated. (Vossenaar et al. 2003b.) Every citrulline residue can constitute an antigenic epitope together with its neighbouring amino acids (Nijenhuis et al. 2004). PAD2 and PAD4 are
the most likely candidate PAD isotypes for the citrullination of synovial proteins in RA (Vossenaar et al. 2004b).

Citrullination occurs only in specific tissues (Watanabe et al. 1988, Terakawa et al. 1991). The best known role of PAD1 is the modification of the proteins involved in epidermal keratinization, i.e. the final stage of keratinocyte differentiation. The main targets are trichohyalin, keratin and filaggrin. (Curis et al. 2005.) PAD2 is the most widely expressed isotype. PAD2 occurs in many different tissues, including skeletal muscle, brain, spleen, secretory glands and macrophages (Vossenaar et al. 2003b). Despite the broad expression pattern, only filaggrin and keratin in the stratum corneum of the epidermis (Ishigami et al. 2002), myelin basic protein (Moscarello et al. 1994) and vimentin (Vossenaar et al. 2004a, Asaga et al. 1998, Vossenaar et al. 2004b) have been identified as its substrates. Trichohyalin is modified by the isotype 3 of PAD, which in humans is found in the hair medulla and in the capillary follicle of the hair root sheath (Kanno et al. 2000) and, to a lesser extent, in other specialized epithelia (Rogers et al. 1997, Rogers et al. 1999). PAD isotype 4 (previously also referred to as PAD5) is localized in the nucleus. The N-terminal part of the PAD protein was found to contain the nuclear localization signal of PAD4. (Nakashima et al. 2002.) PAD4 occurs mainly in peripheral blood granulocytes, monocytes and macrophages (Vossenaar et al. 2003b). PAD6 is known to be expressed in leukocytes (Chavañas et al. 2004).

PAD2 and PAD4 are candidates for autoimmunogen, since they take part in protein citrullination during the RA disease process. PAD2 and PAD4 are expressed in monocytes and macrophages, which occur in inflamed synovium and in granulocytes, mainly in synovial fluid (Chapuy-Regaud et al. 2003). The hypothesis is that, during rheumatoid synovitis, PAD is released into the extracellular space and could induce citrullination locally in several proteins, such as vimentin, fibrin and fibrinogen and fibronectin (Nijenhuis et al. 2004, Chang et al. 2005, Yamada et al. 2005). Schellekens et al. (1998) found citrullinated peptides, which are deiminated arginines identified in filaggrin and in fibrin. Citrullinated filaggrin was recovered in 1999 and citrullinated fibrin in 2001 (Steiner & Smolen 2002). Recently, Chapuy-Regaud et al. (2005) found out that fibrin deimination in synovial tissue is not specific for RA but commonly occurs in other kinds of synovitides.

The existence of five isoforms of PAD is encoded by five different genes, which are distinct in their substrate specificities and tissue-specific expression. PADI4 has been found in synovium, and there is a polymorphism related to RA (Worthington & John 2003, Yamada et al. 2003). The gene encoding PADI4 (PADI4) has been identified as linked to increased RA susceptibility in Japan (Suzuki et al. 2003). However, this finding could not be confirmed in a cohort of RA patients (Barton et al. 2004) or patients with other kinds of inflammatory polyarthritis (Barton et al. 2005) in the United Kingdom.

PADs and citrullinated proteins are also associated with some other human diseases, such as psoriasis and multiple sclerosis. Psoriasis is characterized by the absence of citrullinated keratin in the epidermis (Ishida-Yamamoto et al. 2000). It is known that anti-CCP antibodies are more prevalent in patients with psoriatic arthritis than in patients with psoriasis without arthritis, but less prevalent than in patients with early RA (Alenius et al. 2006). Multiple sclerosis is characterized by the highly citrullinated forms of myelin basic protein in the brain (Wood et al. 1996).
Trichohyalin (APF), filaggrin (AFA), keratin (AKA), vimentin (anti-Sa), myelin basic protein and fibrin can be citrullinated in vivo. All these proteins can also be citrullinated in vitro. (Asaga et al. 1998.) The citrullinated protein product of PAD was detected in some synovial cells. Citrullinated fibronectin occurs in synovial tissue and plasma of RA patients (Chang et al. 2005).

RA is about three times more common in females than in males (Lawrence 1970). It is interesting that enzyme induction by estradiol increased mRNA coding for PADI in mouse uterus (Takahara et al. 1992).

Table 3. Tissue specificity of PAD expression. PAD4 has previously also been designated as PAD5.

<table>
<thead>
<tr>
<th>Isotype</th>
<th>Protein expression sites</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAD1</td>
<td>Bovine / rodent epidermis</td>
<td>Kubilus et al. 1980, Guerrin et al. 2003</td>
</tr>
<tr>
<td></td>
<td>Mouse epidermis and uterus</td>
<td>Terakawa et al. 1991</td>
</tr>
<tr>
<td></td>
<td>Rat pituitary</td>
<td>Akiyama et al. 1989, Senshu et al. 1989</td>
</tr>
<tr>
<td></td>
<td>Mouse salivary gland, pancreas, uterus</td>
<td>Takahara et al. 1989</td>
</tr>
<tr>
<td></td>
<td>Rat skeletal muscle, glia cells, uterus</td>
<td>Akiyama et al. 1990</td>
</tr>
<tr>
<td></td>
<td>Rat / mouse skeletal muscle</td>
<td>Nagata &amp; Senshu 1990</td>
</tr>
<tr>
<td></td>
<td>Mouse uterus</td>
<td>Urano et al. 1990, Terakawa et al. 1991</td>
</tr>
<tr>
<td></td>
<td>Rat skeletal muscle</td>
<td>Takahara et al. 1992, Tsuchida et al. 1993</td>
</tr>
<tr>
<td></td>
<td>Mouse yolk sac (leukocytes)</td>
<td>Vincent et al. 1992</td>
</tr>
<tr>
<td></td>
<td>Mouse macrophages</td>
<td>Koike et al. 1995</td>
</tr>
<tr>
<td></td>
<td>Rat skeletal muscle, brain, spinal cord, submaxillary gland</td>
<td>Asaga et al. 1998</td>
</tr>
<tr>
<td></td>
<td>(Rat) myelin basic protein</td>
<td>Watanabe et al. 1988</td>
</tr>
<tr>
<td></td>
<td>Human skin</td>
<td>Akiyama et al. 1999, Gould et al. 2000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ishigami et al. 2002</td>
</tr>
<tr>
<td>PAD3</td>
<td>Mouse hair follicles</td>
<td>Terakawa et al. 1991</td>
</tr>
<tr>
<td></td>
<td>Sheep hair follicles</td>
<td>Rogers et al. 1997</td>
</tr>
<tr>
<td></td>
<td>Human skin</td>
<td>Kanno et al. 2000</td>
</tr>
<tr>
<td></td>
<td>Rat epidermis and hair follicles</td>
<td>Nishijyo et al. 1997</td>
</tr>
<tr>
<td>PAD4</td>
<td>Human eosinophils, neutrophils, granulocytes, monocytes, macrophages</td>
<td>Asaga et al. 2001</td>
</tr>
<tr>
<td>PAD6</td>
<td>Embryonic stem cells, oocytes</td>
<td>Nakashima et al. 2002</td>
</tr>
</tbody>
</table>

2.4.2 Citrullinisation in vitro

Rabbit PAD is commercially available (e.g. from Sigma). Rabbit PAD is a counterpart of human PADI2 and highly homologous to human PADI4 (Chang et al. 2005). PAD activity can be up-regulated by modulation of disulfide bonds. Optimal PAD2 activity
was observed at pH 7.5 in the presence of 10 mM CaCl₂ and 2 mM dithiothreitol (DTT) (Fujisaki & Sugawara 1981).

Several protocols have been published for *in vitro* citrullinisation (e.g. Tarcsa *et al.* 1997, Girbal-Neuhauser *et al.* 1999, Masson-Bessiere *et al.* 2001, Vossenaar *et al.* 2003a, Vossenaar 2004a, Kinloch *et al.* 2005, Suzuki *et al.* 2005). For example, Chang *et al.* 2005 proposed a protocol which takes protein at a final concentration of 0.5 mg/ml and incubates it with 5 units/ml of PAD from rabbit skeletal muscle (Sigma) in incubation buffer (100 mM Tris-HCl, 5 mM CaCl₂, pH 7.4) for 24 h at 37°C. The protein concentrations in the different protocols range between 0.5 and 1 mg/ml. The buffer contains 50–100 mM Tris-HCl and 5–10 mM CaCl₂. 1–5 mM DTT is added to the buffer. The reaction time is reported to be from 15 min to 3 h at 50°C or 2–24 h at 37°C. Deimination is usually stopped by addition 0.5 M ethylenediaminetetraacetic acid (EDTA), which chelates calcium.

### 2.5 Collagens

The adult human body contains 3–4 kg of collagens, which is more than of any other proteins. Nowadays, 28 distinct proteins are known that meet the structural and functional criteria of collagen (Veit *et al.* 2005). The different collagen types have been numbered with Roman numerals in the order in which they were discovered (Risteli 1993). Collagens have been defined as structural, extracellular proteins that are basically present in all connective tissues of multicellular animals. Collagens have a major role in maintaining the structural integrity of tissues and organs, but they also have many other important functions in cell adhesion, chemotaxis, migration, development and organogenesis. The distribution and amount of collagens vary greatly between different tissues. (Byers 2000, Kielty 2002, Gelse *et al.* 2003, Myllyharju & Kivirikko 2004.)

Every collagen molecule is composed of three polypeptide chains, called α chains. These chains can be either identical or different, depending on collagen type. The collagen molecule is called homotrimer if the three chains are identical and heterotrimer if the chains are different. Collagen molecules contain at least one collagenous domain and at least two non-collagenous domains. The collagenous domain has the characteristic triple helical structure. The domains that are not collagenous are referred to as non-collagenous domains. (Kielty 2002, Ottani *et al.* 2002, Gelse *et al.* 2003, Myllyharju & Kivirikko 2004.)

A protein is defined as a collagen if it meets certain structural and functional criteria. The structural criterion is fulfilled if a large part of the molecule consists of repetition of the basic sequence (Gly–X–Y)n, where Gly denotes glycine and X and Y can be almost any other amino acid. The functional criterion is met if the protein forms aggregates that act as supporting elements in tissues. The primary structure (Gly–X–Y)n defines the formation of a tertiary structure typical of collagens. The α chains of collagen coil into a left-handed α helix (minor helix), and the three chains are wound around each other along a central axis to create a superhelix, i.e. a helical molecule (Cremer *et al.* 1998). (See figure 5A.) The triple helix is stable at 37°C only if it is stabilized by the presence of
hydroxyproline residues. The triple helical parts of collagens cannot be destroyed by ordinary proteolytic enzymes (e.g. pepsin, trypsin) in their native state. (Risteli 1993.)

2.5.1 Collagen biosynthesis

Biosynthesis of collagen is a multistep process from the intracellular co- and post-translational modification to the extracellular formation of collagen fibrils. The steps of biosynthesis can be best studied in fibril-forming collagens, especially type I collagen. (Kivirikko 1995, Kielty 2002, Gelse et al. 2003.)

The expressions of collagen genes are strictly regulated. The genetic information about the structure of procollagen chains is split into a large number of exons in DNA, separated by large intron areas. This arrangement requires excessive processing of RNA before the mature mRNA is ready to be used in protein synthesis. The procollagen polypeptide chains are assembled into ribosomes. A pro-α chain is 1.5 times larger than the final collagen α chain. The polypeptides are transported into cisternae of the endoplasmic reticulum (ER) because of the signal sequence at their amino terminus. In the lumen of the ER, a number of enzymes modify the polypeptide. As a result of these modifications, at least 20% of the amino acids in an extracellular collagen molecule are different from what they were in the original translation product. (Risteli 1993.)

The triple helical procollagen gives rise to the final collagen molecules after secretion into the extracellular space. Large extra domains, known as procollagen propeptides, are enzymatically removed from both ends (N- and C-propeptides, respectively) of the protein by N- and C-proteinases. Despite their names, the propeptides are not small peptides but rather proteins. The propeptides are removed en bloc. After these proteolytic reactions, the collagen molecules are rapidly and spontaneously assembled into collagen fibres. (Risteli 1993, Canty & Kadler 2002, Kielty 2002, Gelse et al. 2003.)

Triple helix formation is initiated from the C-terminus and progresses towards the N-terminus in a zipper-like fashion. Intermolecular covalent cross-links stabilize these fibrils. Cross-links are derived from specific lysine/hydroxylysine residues in both the non-helical (telopeptide) and helical domains, where the molecules are stacked with respect to one another approximately one-quarter of their length. (Risteli 1993, Canty & Kadler 2002, Kielty 2002, Gelse et al. 2003.)

The biosynthesis of non-fibrillar collagens differs in some respects from the above description (Prockop & Kivirikko 1995). In many of these collagens, the N- and/or C-terminal propeptides are not removed. Moreover, the N-propeptides are not necessarily always removed from fibrillar type IIA collagen (Reardon et al. 2000).

2.5.2 Collagen degradation

In normal physiological processes, such as morphogenesis, growth, wound healing and physiological bone turnover after calcium release, collagens are degraded together with other extracellular matrix molecules. In addition, the extracellular matrix is degraded
during pathological processes, such as arthritis, osteolysis or spreading of tumour cells. Cell-cell and cell-matrix interactions also include extracellular matrix proteolysis.

The enzymes capable of degrading collagens are usually manufactured by the same cells that also produce collagens. The enzymes are secreted as inactive proenzymes, which are later activated by proteolytic cleavage. The enzymes participating in the degradation of collagen fibres are intestinal collagenases, stromelysins, gelatinases, telopeptidases, cathepsins and plasmin. (Risteli 1993.) Intestinal collagenase, stromelysin and gelatinase are members of the matrix metalloproteinase (MMP) family, which function at neutral pH. MMPs can be categorized into 3 main classes. Group 1 contains MMP-1 (intestinal) and MMP-8 (neutrophil) collagenase, whose major substrates are collagen types I, II and III. Group 2 includes gelatinases/type IV collagenases. The third group is composed of stromelysins: stromelysin 1 (MMP-3), stromelysin 2 (MMP-10) and pump-1 (MMP-7). A broad spectrum of substrates, e.g., proteoglycans, laminin, fibronectin and some collagens, are cleaved by stromelysins (Vincenti et al. 1994). Stromelysin 1 is more abundant than either gelatinase or collagenase, and stromelysin is found more consistently in samples of rheumatoid synovium (Harris 1997).

Degradation begins when active collagenase attaches to individual triple-helical molecules approximately three fourths of the way from the amino-terminal end of the molecule and cleaves through all of the three polypeptide helical chains. The fragments lose their helical structure, uncoil into gelatin and either are degraded by extracellular proteases (e.g. stromelysin, gelatinase) or are endocytosed and degraded within phagolysosomes (Harris 1997). Sassi et al. 2000 reported that recombinant human cathepsin K cleaved the structure of the trivalently cross-linked carboxyterminal telopeptide of type I collagen (ICTP) at two sites between the phenylalanine-rich region and the cross-link, destroying the reactivity with ICTP antibodies. On the contrary, the treatment of isolated ICTP by the matrix metalloproteinases MMP-9 (gelatinase B), MMP-1 (collagenase 1) or MMP-13 (collagenase 3) had no effect on the immunoreaction. Garnero et al. 1998 also showed that the activity of cathepsin K alone is sufficient to dissolve completely the insoluble collagen of adult human cortical bone.

The initial step in the breakdown of the connective tissue matrix is an extracellular process. Mechanical disruption and the presence of free radicals may also boost the degradative process. Matrix fragments undergo phagocytosis for processing intracellularly within the lysosomal system.

These processes are normally strictly regulated by a complex interplay of cell-cell and cell-matrix interactions involving the production of proteinases, activators, inhibitors and other regulatory molecules (Murphy & Hembry 1992). A mixture of small peptides and amino acids is formed when collagen is completely degraded by collagenolytic enzymes (Risteli 1993).

### 2.5.3 Type I collagen

In the human body, the most abundant protein is type I collagen. Type I collagen accounts for about 70% of total collagen. Most of this collagen is present in bones. Type I collagen accounts for about 90% of the organic matrix. The type I collagen molecule is a long and
rigid rod. The normal type of I collagen molecule is heterotrimeric with two identical α1(I) chains, while the third chain is a different but homologous α2(I) chain. These chains form a triple helical structure. A variant form of type I collagen contains three α1(I) chains, which can be synthesized in pathological situations. (Risteli 1993, Risteli & Risteli 1999.)

The whole type I collagen consist of 1464 amino acids. The α1(I) chains of type I collagen are synthesised as procollagen molecules containing N- and C-terminal propeptides, which are removed by site-specific endopeptidase. The central triple helical domain is formed by 338 repeats of a Gly-X-Y triplet, where X and Y are often a proline. Type I collagen is the most abundant protein in vertebrates and a constituent of the extracellular matrix in the connective tissue of bone, skin, tendon, ligament and dentine. It is mostly produced and secreted by fibroblasts and osteoblasts. Two pro-α1(I) chains associate in trimers with one pro-α2(I) chain to form the type I procollagen. (http://www.infobiogen.fr/services/chromcancer/Genes/COL1A1ID186.html.)

Type I collagen is encoded by the same genes in all parts of the human body; COL1A1 on chromosome 17 and COL1A2 on chromosome 7. In soft tissues, type I collagen is mainly synthesized by fibroblasts. Bone collagen is synthesized by osteoblasts. The formation of type I collagen fibrils is different in bones and skin, and this arrangement influences the cross-linking of collagen molecules in collagen fibers, in their C-termini. The mineralization of bone also influences the maturation of bone collagen cross-links. The best known and most abundant form of type I collagen is a heterotrimer. The two bulky domains at both ends of the procollagen molecule are usually called the amino (N) -terminal (PINP) and carboxy (C) -terminal (PICP) propeptides of type I procollagen (figure 3). (Risteli & Risteli 1993.)

![Fig. 3. Construction of type I collagen protein. The light grey colour denotes a signal peptide. The white colour stands for the von Willebrand factor domain. Black illustrates the Col 2 domain. The helical domain is coloured dark grey and C-propeptide is striped. The lines between the helical domains and propeptides denote telopeptides. (See http://www.infobiogen.fr/services/chromcancer/Genes/COL1A1ID186.html)](http://www.infobiogen.fr/services/chromcancer/Genes/COL1A1ID186.html)

### 2.5.4 Type II collagen

The main fibrous collagen component of cartilage is type II collagen. But type II collagen is also found in intervertebral discs, vitreous humour and other tissues during development. Its function is to provide tensile strength, and it confers to cartilage the ability to resist shearing forces. Type II collagen represents 80–90% of the collagen in cartilage tissue (Harris 1997). Hyaline cartilages contain mainly type II collagen, which is arranged in a fibrillar network (Poole et al. 1989). Other collagens thought to exist in the
growth plate are the types V, VI, IX, X and XI (Gibson & Flint 1985, Mayne 1986). Cartilage has been reported to contain some type I collagen (Wardale & Duance 1993). Type II collagen forms the backbone of cartilage heteropolymeric fibrils, and type I collagen plays this role in non-cartilaginous fibrils (Cremer et al. 1998). Cartilage collagen degradation represents an irreversible step in metabolism (Shingleton 2003).

Type II collagen is synthesized as a homotrimeric procollagen consisting of three identical proα1(II) chains (Vikkula et al. 1994). Type II collagen is produced by chondrocytes, and its fibres make up 40–50% of cartilage dry weight. Type II is structurally highly similar to type I collagen. Type II collagen may cause arthritis in rats and mice immunized with it. (Harris 1997.)

Cartilage can be classified into three types: hyaline, elastic and fibrocartilage. Hyaline cartilage is a fibre-reinforced gel produced and maintained by chondrocytes. Articular cartilage consists of 70–80% water, which is held in the structure by negatively charged proteoglycans. The water creates a swelling force that is resisted by a scaffold of type II collagen fibers. Proteoglycans are held within this scaffold. (Shingleton 2003.) Hyaline cartilage is found in epiphyses, joint cartilages, costal cartilages, nose, larynx, trachea and bronchi. Elastic cartilage can be discovered mainly in ears and epiglottis. Fibrocartilage is found in symphysis joints, intervertebral discs and articular discs. The amount of fibrocartilage increases with age as hyaline cartilage transforms into fibrocartilage.

2.5.5 Markers of type I and II collagen synthesis

During the synthesis of type I collagen, specific by-products are produced that provide a possibility to assess the rate of synthesis. The bulky domains at both ends of the type I collagen propeptide are usually called the N-terminal (PINP) and C-terminal (PICP) propeptides. These parts are removed by the N- and C-proteinases once the molecule has reached the extracellular space. The PICP and PINP concentrations increase during wound healing and decrease during local treatment with glucocorticoids. (Risteli 1993, Risteli & Risteli 1999.)

There are only a few reports on measurements specific to the synthesis of type II collagen. Mansson et al. (1995) measured elevated levels of the serum C-propeptide of type II procollagen, also known as chondrocalcin. Type II collagen synthesis markers include the C-terminal propeptide of type II collagen (PIICP) and the N-terminal propeptide of type IIA collagen (PIIANP) (Elsaid & Chichester 2005).

PIICP undergoes specific enzymatic cleavage before incorporating type II collagen into mature fibrils. The PIICP half-life is relatively short, i.e. about 16h. Two assays have been developed for PIICP detection. (Elsaid & Chichester 2005.) Hinek et al. (1987) developed a solution-phase competitive radioimmunoassay, and Shinmei et al. (1993) designed a one-step sandwich immunoassay.

PIIANP is the product of an alternatively spliced variant of the type II collagen gene COL2A1. PIIANP is later removed by specific proteases to yield the mature triple helical collagen type II. (Elsaid & Chichester 2005.) Rousseau et al. (2004a, 2004b) reported a competitive ELISA for PIIANP.
The synovial fluid measurements of type II collagen markers reflect the turnover in cartilage more directly than either serum or urine measurements (Elsaid & Chichester 2005).

2.5.6 Markers of type I and II collagen metabolism

Bone metabolites share a special feature. Their by-products can be directly released into circulation, whereas most tissues are first drained via lymphatics into larger vessels. Several immunoassays have been developed for structures involving the C-terminal telopeptides, and they give different results. The C-terminal telopeptide of type I collagen (ICTP) antigen is a trivally cross-linked structure that was isolated from human femoral bone after trypsin or bacterial collagenase digestion. The ICTP antigen contains the C-terminal telopeptides of two α1(I) chains and material from the helical part of the third chain. (Risteli & Risteli 1993). Bone resorption may also generate collagen fragments, such as the C-terminal telopeptide of collagen type I (CTX, commercial name CrossLaps). CTX can be quantified in serum and/or urine by using specific immunoassays and used as clinical markers. However, the relative abundance of ICTP and CTX varies according to the type of bone pathology, suggesting that these two fragments are generated through distinct collagenolytic pathways. It was shown that ICTP and CTX are released from bone collagen by the proteinases reported to play a role in the solubilization of bone matrix. Cathepsin K released large amounts of CTX, but did not allow detectable release of ICTP. (Garnero et al. 2003b.)

Markers of collagen metabolism both in serum and in synovial fluid can be measured to provide an assessment of the disease process in patients with RA. ICTP and the aminoterminal propeptide of type III collagen (PIIINP) are the most informative. (Hakala et al. 1995.)

Sassi et al. (2003) reported that serum ICTP correlates with the other markers of collagen metabolism as well as with CRP and ESR. Serum CrossLaps correlated only with PINP and ICTP, but not with serum PIIINP, CRP or ESR. The CrossLaps assay is able to detect the same ICTP antigen, but not vice versa. Collagen metabolism markers could be used as methods to monitor RA because, during the months following the institution of anti-rheumatic treatment, the mean ICTP levels decreased in parallel with the clinical and laboratory variables measuring disease activity. (Paimela et al. 1994.) Kotaniemi et al. (1994) showed serum ICTP to be a valuable marker of tissue destruction in patients with early RA, and Hakala et al. (1993b) found out that elevated serum concentrations of ICTP are associated with signs of aggressive disease.

Type II collagen’s degradation markers include neoepitope of type II collagen (TIINE), C-terminal telopeptide of collagen type II (CTX-II) and helix-II. These markers differentiate between the early stages of osteoarthritis, RA and reference controls. TIINE is formed only when type II collagen is digested by collagenases and is absent in native triple helical type II collagen. The C-terminus of the three-quarter fragment can be identified in urine. Garnero et al. (2003a) developed an ELISA for the urinary levels of CTX-II as a marker of cartilage degradation. CTX-II is small in size and freely filtered by the renal system, and it is concentrated in urine. The CTX-II assay is a competitive
ELISA using the EKGPDP sequence. Helix-II assay is for a helix-II epitope 642ERGETPOGTS652 (where O is hydroxyproline), a sequence in the helical part of the type II collagen α chain, and it is released during proteolytic degradation. The antibodies did not cross-react with intact, denatured type II collagen. (Elsain & Chichester 2005.)

2.5.7 Treatment of type I and II collagens in vitro with PAD

Human acid-soluble type I and II collagens have been treated in vitro with PAD, and several arginine residues appear to be citrullinated (Suzuki et al. 2005). In rats, citrullination can break the tolerance against the rat serum albumin and to increase the arthrogenic properties of the cartilage type II collagen (Lundberg et al. 2005). Burkhardt et al. (2005) showed that type II collagen, without C-telopeptide because cleaved with pepsin, as a cartilage-specific extracellular matrix component can serve as a substrate for PAD-catalyzed conversion of arginine residues to citrulline. This post-translational modification by PADs has been demonstrated for the purified native protein as well as for synthetic triple helical collagen peptides.

2.6 Autoantibodies binding type I and type II collagens

It has long been suspected that autoimmunity against collagens could be involved in the pathogenesis of RA. Autoimmunity to native type II collagen in RA was reported in 1976 (Andriopoulos et al. 1976). In certain animal species, immunisation with native type II collagen results in the development of polyarthritis that resembles human RA. Type II collagen is post-translationally modified, and some of these modifications, e.g. glycosylations of lysine at position 264, may be recognised by T cells (Holmdahl et al. 2003). It was reported that B cells in rheumatoid synovium and synovial fluid produce antibodies to type II collagen (Cremer et al. 1998). A subset of patients also have antibodies binding native type II collagen, especially in early disease (Clague 1981). Anti-collagen antibodies are not formed only against cartilage collagen, but also against type I collagen in bones and soft tissue collagens, such as type III and V collagens (Stuart et al. 1983).

It has been suggested that antibodies binding native collagen are primary and those binding denatured collagens are secondary. Antibodies against denatured collagens are more frequent and present in higher concentrations in RA sera than antibodies against native collagens (Nomura 1992, Nijenhuis et al. 2004). These anti-collagen antibodies are by no means specific for RA, and their formation could be secondary to the destruction of connective tissues rather than a cause of the disease. There are reports of 5–15% prevalence of autoantibodies to type I and II collagen in RA (Clague et al. 1981, Rowley et al. 1986). Autoimmunity of type II collagen represents an early or late event in the disease (Choi et al. 1988, Cook et al. 1996). Antibodies to native type II collagen in RA were predominantly of the complement-fixing subclasses of IgG that are potentially damaging (Cook et al. 1997). There are also antibodies binding normal type II collagen in healthy persons (Cremer et al. 1998).
Burkhardt et al. (2005) showed that, in a cohort of early RA patients, IgG antibodies directed towards a synthetic citrullinated type II collagen peptide were detectable in 40.4% of cases. These autoantibodies are specifically bound to the citrullinated triple helical collagen type II amino acid residues 359-369.

Suzuki et al. (2005) studied human type I collagen peptides as a substrate of PAD and autoantibodies for type I and II citrullinated collagens using a RA synoviocyte cDNA library and immunoscreening. They showed that the levels of anti-citrullinated type I collagen were significantly higher in RA sera (N=117) than in patients with non-RA diseases (N=37) with high specificity (99 %) and positively correlated with the levels of anti-CCP antibodies.

Autoantibodies have mainly been tested by using collagen preparations rendered soluble by pepsin digestion. Pepsin protease removes the C-telopeptides of collagens. The C-telopeptides of collagens remain intact if collagens are treated with trypsin or bacterial collagenase.

2.6.1 Animal models

Type II collagen-induced arthritis (CIA) is a widely used model of arthritis in mice and rats (Staines & Wooley 1994, Luross & Williams 2001, Bajtner et al. 2005, Kannan et al. 2005). The first time a cartilage-specific molecule was used to cause autoimmune arthritis was in 1976 (Trentham et al. 1977). When rats were immunized with type II collagen, they developed destructive polyarthritis. It was found that mice and monkeys are susceptible to type II collagen-induced arthritis. Type XI collagen is arthritogenic in rats but not in mice, and type IX induces autoimmunity in both species but not arthritis. Interesting differences exist between the immunization protocols. Rats develop arthritis when administered incomplete and mice when given complete Freund’s adjuvant. (Cremer et al. 1998.)

Experimental arthritis models have so far failed to demonstrate arthritogenicity of citrulline-directed immunity in murine models of joint inflammation or to provide convincing evidence for the induction of autoimmunity to deiminated arginines (Vossenaar et al. 2004d).

On the other hand, much more is known about the potential of autoantibodies directed towards collagen type II epitopes, which are recognized in the cartilage-specific autoimmune responses of RA patients, to induce erosive arthritis in naïve mice upon antibody transfer (Burkhardt et al. 2002, Nandakumar et al. 2003)

Lundberg et al. (2005) examined the responses of rat T and B cells to citrullinated rat serum albumin (Cit-RSA) in comparison with unmodified rat serum albumin (RSA). They found that Cit-RSA leads to a breakdown of immunological tolerance, since antibodies were produced against both Cit-RSA and RSA antigens. However, RSA alone did not induce any antibodies. Citrullinated type II collagen induced arthritis at a higher incidence and with earlier onset than did the native counterpart. By injecting type II citrullinated collagen into rats, Lundberg et al. (2005) demonstrated that the amount of citrullinated proteins and PADI4 enzyme correlated with the severity of inflammation, and that neither were detectable in healthy joints.
Steroid hormones and aging appear to affect PADI expression in rats (Senshu et al. 1989) and mice (Takahara et al. 1989, Takahara et al. 1992, Akiyama et al. 1995). Vossenaar et al. (2003a) reported on the synovial expressions of PAD4 but not PAD2 and the presence of citrullinated proteins in collagen-induced arthritis (CIA) and streptococcal cell wall (SCW)-induced arthritis in murine models. At first, no autoantibodies to citrullinated proteins were found in murine models. However, Burkhardt et al. (2005) reported a humoral immune response to citrullinated collagen type II in mice.
3 Purpose of the present study

The primary purpose of present study was to assess the possible presence of autoantibodies recognising citrullinated peptides derived from type I and II collagens in RA patients. A further aim was to develop sensitive methods for the detection of these autoantibodies. The specific aims of the study were to answer the following questions:

1. Are there autoantibodies binding citrullinated peptides derived from human type I and II collagens in the serum of patients with RA?
2. What methods can be used for the detection of these autoantibodies?
3. Are these autoantibodies specific for citrulline in the peptide sequence, and can they bind both normal and citrullinated peptides?
4. Could these autoantibodies predict the onset of RA?
4 Subjects and methods

4.1 Patients and controls

In the studies I–II and IV, the serum samples from 120 RA patients were from the Division of Rheumatology of Oulu University Hospital. The controls consisted of 81 sera from age- and sex-matched healthy persons (table 4). Experienced rheumatologists made the clinical diagnosis of RA on clinical grounds. The patients were in the early stages of the disease. No signs of RA were seen in the control group.

Table 4. Characteristics of the RA patients and controls studied in studies I – II and IV.

<table>
<thead>
<tr>
<th>Variable</th>
<th>RA patients</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of subjects</td>
<td>120</td>
<td>81</td>
</tr>
<tr>
<td>Women / men</td>
<td>89 / 31</td>
<td>58 / 23</td>
</tr>
<tr>
<td>Mean age (years) range</td>
<td>(19–84)</td>
<td>(14–83)</td>
</tr>
<tr>
<td>Women mean age (years)</td>
<td>53</td>
<td>54</td>
</tr>
<tr>
<td>range</td>
<td>(21–84)</td>
<td>(14–83)</td>
</tr>
<tr>
<td>Men mean age (years)</td>
<td>57</td>
<td>55</td>
</tr>
<tr>
<td>range</td>
<td>(19–81)</td>
<td>(21–74)</td>
</tr>
</tbody>
</table>

In study III, the serum samples were from National Public Health Institute, Helsinki. Serum samples were collected from 10 199 individuals during 1973–1977 in conjunction with a community-oriented epidemiological study (Aho et al. 1997). From this series, the sera of 124 subjects who later developed rheumatoid arthritis were drawn, and three sex-, age- and location-matched controls were chosen for each (372 sera from controls).
4.2 Methods

4.2.1 Enzyme-linked immunosorbent assay (ELISA)

The biotinylated peptide (see figure 5B and 5E) from NeoMPS, Strasbourg, France was coupled to streptavidin-coated 96-well assay plates (BioBind Assembly, Thermo Labsystems Oy, Vantaa, Finland) at a concentration of 10 µg/well. The coupling was performed at room temperature (RT), pH 7.5, for two hours. The streptavidin-coated wells had been blocked by the manufacturer to prevent unspecific binding. The sera to be tested were diluted in enzyme immunoassay (EIA) buffer, which contains 20 mM Tris-HCl, 150 mM NaCl, 0.1% bovine serum albumin (BSA) and 0.05 % Tween; pH 7.5. The sera were also diluted in assay buffer, which contains 10 mM Tris-HCl, 350 mM NaCl, 1% BSA, 1% (vol/vol) Triton X-100, 0.5 % (wt/vol) Na-deoxycholate and 0.1 % sodium dodecyl sulphate (SDS) in pH 7.6 supplemented with 1% rabbit serum. Diluted sera were incubated at 100 µl/well for an hour at RT. After washing 3 times with phosphate-buffered saline (PBS) with 0.05% (vol/vol) Tween, 100 µl of antihuman IgG conjugated to horseradish peroxidase (HRP) (product No 31412, Pierce, Rockford, IL, USA) diluted 1:7500 in EIA buffer was added. After incubation for an hour at RT, the plates were washed 3 times with PBS-Tween. The bound antibodies were detected with 3,3’,5,5’-tetramethylbenzidine (TMB) (Sigma-Aldrich, St Louis, MN, USA) as a chromogen 0.01 mg/100 ml per well. TMB was diluted in 100 mM sodium acetate trihydrate with 1.5 mM citric acid monohydrate and 0.0015% hydrogen peroxide (H₂O₂). After 30 minutes, the reaction was stopped by adding 100 µl of 2 M sulphuric acid/well. The absorbances at a wavelength of 450 nm were read in a Victor² instrument (Wallac, Turku, Finland). The principle of ELISA is presented in figure 4.

![Fig. 4. ELISA for an autoantibody of a RA patient by coating biotinylated peptide as antigen on a microplate (black). The autoantibody of the patient with RA (black) immobilized by coated antigen is detected based on a reaction with an enzyme-labelled second antibody against human IgG (grey).](image-url)
Fig. 5. The sequences and locations of the chosen peptides in the primary structures of human type I and type II collagens. A: Localisation of the peptides CC1 – CC4 in human type II collagen; the numbers in brackets refer to arginine residues. B: Sequences of the peptides CC1–CC4. C: Detailed structure of the carboxyterminal telopeptide of type II collagen; the 12 most carboxyterminal amino acids represent the peptide CC1. D: Detailed structure of the carboxyterminal telopeptide of type I collagen. The third chain can be either α1 or α2. E: Sequences of the peptides related to the carboxyterminal telopeptides of the α1 and α2 chains of human type I collagen.
4.2.2 Chemiluminescence immunoassay (CLIA)

The principle of automatic assay that was developed is schematically presented in figure 6. In brief, the measurements were done with two-site chemiluminescence immunoassays (CLIA), which detect IgG antibodies binding the synthetic C-telopeptides of the α1 chain of human type I and II collagens (EKAHDGGHYYRA and EKGPDPLQYMRA or EKAHDGGYYXA and EKGPDPLQYMXA, where X stands for citrulline, from NeoMPS, Strasbourg, France). The serum samples were diluted in assay buffer and incubated with the above biotinylated peptide and magnetic particles for 10 minutes at 37°C. The antibody-antigen complex bound to the magnetic particles was separated from unbound biotinylated antigen and antibodies by precipitation with a magnet and subsequent washing. Thereafter, acridinium-labelled antihuman IgG antibodies were added to the reaction mixture, followed by another 10 minute incubation to produce a sandwich complex. The unbound label was separated by aspiration of the reaction mixture and subsequent washing. The wells containing the washed magnetic particles were transferred into the system luminometer, which automatically injected the triggers 1 and 2, initiating the chemiluminescence reaction. The trigger 1 solution contained H2O2 in diluted acid, and the trigger 2 solution contained diluted sodium hydroxide. Light was quantified by a luminometer and expressed as relative luminescence units (RLU). The amount of bound, labelled antibody was directly proportional to the titre of the antibodies in the sample.
Fig. 6. Two-site chemiluminescence immunoassays (CLIA) used in this study. A: The serum sample containing IgG autoantibodies (light grey) is incubated with a biotinylated peptide (small striped ball with a sequence) and magnetic particles (big black ball with dotted streptavidin). B: The unbound biotinylated peptide and the other antibodies are separated from the complex. C: Acridinium-labelled antihuman IgG antibodies (dark grey with a white star) are added to produce a sandwich complex. The chemiluminescence of acridinium is measured with a luminometer.
4.2.3 Inhibition ELISA

Since there are also antibodies binding normal and citrullinated collagens, two assays should be performed using both arginine and citrulline-containing peptides. To detect specific antibodies binding citrullinated forms, the differences between the absorbance results of citrulline and the respective arginine peptides were calculated. Preferentially, it is also possible to use only the citrullinated peptide assay version, where this assay is performed with standard conditions and by adding similar soluble peptide into the assay solution. The sera were diluted 1:100 in the assay buffer and/or inhibiting buffer containing soluble citrullinated peptide 200 µg/ml. The inhibition time was 30 minutes at RT. After the inhibition reaction, the sera were transferred into the wells of streptavidin-coated 96-well assay plates, to which the biotinylated citrulline-containing peptides had been coupled. After washing 3 times with PBS-Tween, 100 µl of antihuman IgG conjugated to HRP diluted 1:40000 in Guardian™ Peroxidase Conjugate Stabilizer/Diluent (Product No 37548, Pierce) was added. After incubation for an hour at RT, the plates were washed 3 times with PBS-Tween. The bound antibodies were detected with TMB as a chromogen 0.01mg/100µl per well. After 30 minutes, the reaction was stopped by adding 100µl of 2 M sulphuric acid/well. The plates were read at a wavelength of 450 nm in a Victor instrument. The principle of the inhibition ELISA that was developed is schematically presented in figure 7. The principle of TMB-degradation in ELISA is presented in figure 8.

Fig. 7. Inhibition ELISA by coating biotinylated peptide as antigen on a microplate and by adding similar soluble peptide into the assay solution. The autoantibody of a patient with RA can bind to the coated or inhibiting peptide. Autoantibody binding to coated peptide is detected based on a reaction with an enzyme-labelled second antibody against human IgG.
Fig. 8. TMB degradation in ELISA. TMB is a soluble chromogen substrate for horseradish peroxidase detection systems. TMB yields a soluble blue end product that can be analyzed kinetically using the microtitre plate reader at a wavelength of 650 nm. The reaction can be stopped by adding sulfuric acid (H₂SO₄), which yields a deep yellow colour, and read at 450 nm. When evaluating the end point reaction visually, consider deep yellow colour as positive and clear as negative. TMB is much more sensitive and less hazardous than benzidine or the other derivatives traditionally used as HRP chromogen.

4.2.4 Inhibition CLIA

In the CLIA format it is also possible to use only the citrullinated peptide assay version, where the assay is performed in standard conditions and by adding a similar soluble peptide into the assay solution. Sera were diluted 1:10 in assay buffer and/or inhibiting buffer containing soluble citrullinated peptide 200 µg/ml. The inhibition time was 30 minutes at RT. After inhibition, the sera were incubated with the biotinylated peptide and magnetic particles. The unbound biotinylated antigen and antibodies were separated from the complex bound to the magnetic particles. After washing, acridinium-labelled antihuman IgG antibodies were added to the reaction mixture to produce the sandwich complex. The unbound label was separated. After washing, the wells containing the washed magnetic particles were transferred into the system luminometer, which automatically injected the triggers, initiating the chemiluminescence reaction. Light was quantified by a luminometer and expressed as RLU. The amount of bound, labelled antibody was directly proportional to the titre of the antibodies in the sample.

4.2.4.1 Sequences and lengths of inhibitory peptides

Different lengths of synthetic, biotinylated arginine or citrulline-containing peptides derived from the carboxytelopeptide of type I and type II collagens (NeoMPS Strasbourg, France) were used. Their sequences (nomenclature: SP + number) are shown in the table.

Sera were diluted in assay buffer to such antibody dilutions that, in each assay, the initial binding could be noticeably inhibited by different soluble peptides. Serial dilutions of competitive (different lengths) peptides (the arginine and citrulline forms of the C-telopeptide of the α1(I) and α1(II) collagen antigens) were added into the assay buffer.
Table 5. Synthetic peptides used in this study derived from the α1 chains of the carboxytelopeptides in type I and II collagens.

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<thead>
<tr>
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<td>EKA HDG G Arg Y Y Cit  A</td>
<td></td>
<td>EKG PDP LQY M Cit  A</td>
</tr>
</tbody>
</table>

4.2.4.2 Anti-CCP ELISA

Anti-CCP ELISA (anti-CCP Mark2 assay) was used according to the procedure described by the manufacturer (Euro-Diagnostica, Malmö, Sweden). All sera, calibrators and controls were measured in duplicate, and the results were averaged.

4.3 Statistical analysis

The raw data were recorded using Microsoft Excel software. After that, the data were analyzed by using the Statistical Package for Social Science (SPSS Inc, Chicago, IL, USA) for Windows version 12 and version 13.

In study I, the variance of the differences between the absorbance results of the citrulline and the respective arginine peptides in the control samples was calculated for each collagen peptide pair. In study II, the variance of the ratio between the citrulline and the respective arginine peptides in the control samples was calculated for both peptide pairs. Based on these variances, the RA patients' samples were identified as positive whenever this difference/ratio exceeded the mean ± 2 x standard deviation (SD) of the controls. Correlations were expressed as Spearman’s rank correlation coefficient (r_s). The positive/negative results of the anti-CCP and collagen autoantibody assays were compared by cross-tabulation, using 2 x 2 contingency tables tested on the Contingency Coefficient (C, which is always 0 ≤ C < 1).
In study III, the associations between the different autoantibodies, antibody ratios, sex and age were assessed with a general linear model. The significance of the differences in the mean levels of serum antibodies between the RA cases and their matched controls were tested by the paired t-test. The conditional logistic model was used to estimate the associations between the serum antibody levels and the risk of RA. Antibody levels were also entered as potential confounding factors into the model. The relative risks, estimated as odds ratios with 95% confidence intervals, were computed for tertiles of serum anti-CCP and the ratio of the citrullinated/arginine C-telopeptides of type I collagen (C/A (I)) and the ratio of the citrullinated/arginine C-telopeptides of type II collagen (C/A (II)). The tertile divisions were based on the antibody distributions among the controls. Effect modification of each antibody was tested by entering into the model the interaction terms “tertile A * tertile B” in addition to the tertiles A and B. The statistical significance of the interaction was tested with the likelihood ratio test based on the model and expressed as exact p-value.

In study IV, the signal at the wavelength of 450 nm obtained with human serum only (initial binding) was defined as 0% inhibition, and the signal of the blank (no serum, but competitive peptide) was defined as 100% inhibition. For each serum, the different values without and with inhibition with soluble antigens, at a concentration of 200 µg/ml, were calculated and compared to the control sera. On the basis of the inhibition percentages, those samples of RA patients were identified positive which exceeded the mean ± 2 x SD of the controls.

4.4 Ethical considerations

The present study was carried out in accordance with the provisions of the Declaration of Helsinki. The studies I – II and IV were approved by the ethical committee of Oulu University Hospital.

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I Annals of the Rheumatic Diseases
II Clinical Chemistry and Laboratory Medicine
IV Rheumatology (Oxford)

The figures 12, 13, 16 and 19 were published in the original article I. The figures 14 and 15 were published in the original article II.
5 Results

5.1 Different buffers and peptide lengths (additional data)

Before serum analysis, we tested the dilution buffers. It turned out that the binding of antibodies from the test sera diluted in the EIA buffer was considerable for both the arginine and citrulline peptides. The general binding of antibodies decreased in all peptide pairs when the assay buffer was used, which contains Triton, Na-deoxycholate and SDS (Schellekens et al. 2000). When the assay buffer was used in ELISA, the specific binding of citrullinated peptides compared to arginine peptides was better than when the EIA buffer was used (CC1 in figure 9). All the samples were continually diluted 1:100 in the assay buffer in ELISA. In CLIA, all the samples were diluted 1:10 in the assay buffer.

In anti-CCP ELISA, sera from two RA patients and two controls were also used. The anti-CCP ELISA assay contains only citrullinated peptide, but it was tested with both buffers, i.e. the EIA and assay buffers. When the assay buffer was used, binding was better in the patients, but no difference was seen in the controls. There was thus a difference between the patients and controls (figure 10). Anti-CCP ELISA was used in accordance with the manufacturer’s instructions, and the samples were diluted 1:50 in the assay buffer. The dilution buffer of the anti-CCP kit was similar to the assay buffer.

In inhibition ELISA, the dilution buffers were also tested using sera from two RA patients and two controls. When the assay buffer was used, the inhibition percentage was higher than with the EIA buffer. We also used the buffer test to test the lengths of the inhibitory peptides (figure 11). In the inhibition ELISA with short $\alpha$1(II) as antigen and SP41 (short) as inhibitory peptide, the upper limit of inhibition was 13.8 (mean+2SD) for healthy controls. When short $\alpha$1(I) was used as antigen and the inhibitory peptide was SP66 (short), the upper limit of inhibition was 17.6 for healthy controls. When long $\alpha$1(II) was antigen and SP85 (long) was used as inhibitory peptide, the upper limit of inhibition was 22.9. When long $\alpha$1(I) was antigen and inhibition was induced with with SP86 (long), the upper limit of inhibition was 23.1. Please, see chapter 5.3.
Fig. 9. Effect of dilution buffer on the binding of two control sera and sera from two RA patients to peptide CC1 in ELISA. The closed symbols stand for the assay buffer and the open symbols for the EIA buffer. The squares denote binding of the citrullinated CC1 peptide and the triangles that of the normal CC1 peptide.
Fig. 10. Effect of dilution buffer on the binding of two control sera and sera from two RA patients in anti-CCP ELISA. The squares stand for the EIA buffer and the triangles for the assay buffer.
Fig. 11. Effect on dilution buffer in inhibition ELISA. The black columns represent the assay buffer and the grey columns the EIA buffer. A: Inhibition ELISA with short \( \alpha_1(II) \) as antigen and SP41 as inhibitory peptide. B: Short \( \alpha_1(I) \) was antigen and inhibitory peptide was SP66. C: Long \( \alpha_1(II) \) was antigen and SP85 was used as inhibitory peptide. D: Long \( \alpha_1(I) \) was antigen and inhibition was made with SP86.
5.2 Autoantibodies binding citrullinated peptides derived from human type I and II collagen in the serum of patients with RA (I, II)

ELISA assay was able to identify some RA patients whose serum antibodies bound citrullinated peptides related to the carboxytelopeptides of the α1 and α2 chains of type I collagens. The sequences were YYXA and FYXA, where X stands for citrulline. 20 RA sera (out of 120) bound the citrullinated carboxytelopeptide from the α1(I) telopeptide more strongly than the respective arginine peptide. 42–53% of the RA sera showed increased binding of normal arginine peptides related to type II collagen. However, 12 RA sera bound the citrullinated form of the α1(II) telopeptide more strongly than the corresponding arginine peptide (figure 12). The correlation between the autoantibodies to type I and II collagen telopeptides was $r_s=0.576$ ($p<0.001$) (figure 13).

In the automatic CLIA, 38% of the RA patients (45/120) showed increased binding of synthetic C-telopeptide derived from the α1 chain of type I collagen. The difference compared to the controls was significant ($p=0.003$). For the corresponding C-telopeptide pair from the α1 chain of type II collagen, 35 patients’ sera bound the citrullinated peptide more strongly than the arginine peptide. The difference compared to the controls was not significant ($p=0.074$) (figure 14). The correlation between the two collagen carboxytelopeptides was $r_s=0.473$ ($p<0.001$). There was no linear correlation between the anti-CCP and collagen autoantibodies binding C-telopeptides.

We were able to detect autoantibodies to citrullinated filaggrin with anti-CCP assay in 71 out of 120 (59%) patients with RA. Anti-CCP assay detects a different subgroup of citrullinated antibodies than collagen C-telopeptide assays. The peptide sequence detected (-YYXA- and -YMXA-) was different from that based on the cyclic filaggrin antigen (-STXG-, where X represents citrulline). There was no linear relationship between the anti-CCP results and the antibodies binding collagen C-telopeptides, but both were increased in RA patients (figure 14 and 15). In anti-CCP ELISA and anti-α1(I) and anti-α1(II) ELISAs, the specificity of the autoantibodies was tested by analysing serum samples and by inhibiting the binding in each case with all normal and citrullinated antigens. Soluble citrullinated telopeptide antigens inhibited binding in both type I and type II telopeptide assays (figures 16A and B). Thus, it seems that the same antibody species are involved in the binding of both collagen-related peptides. However, the binding of CCP cannot be inhibited by collagen telopeptides (figure 16C).
Fig. 12. Differences in absorbance between the corresponding citrulline and arginine peptides in the RA patients and controls (C) for all peptide pairs tested in ELISA assays. Each circle represents one serum sample. The circles in the upper part indicate the sera that bind citrullinated peptide more strongly than arginine peptide and those in the lower part vice versa. The figures in the upper and lower parts indicate the numbers of cases with preferential binding to either citrullinated or arginine peptide, respectively (exceeding ± 2SD of the differences of control sera).

Fig. 13. Correlation between the bindings (absorbance units) of the C-terminal telopeptides of the α1 chains of type I and II collagens to the sera of RA patients in ELISA assays.
Fig. 14. Autoantibodies to the C-telopeptide of the α1(I) chain and of the CCP peptide in individual serum samples. A: The results of 120 RA sera. The dark grey columns denote the citrulline-containing peptide, while light grey stands for the arginine-containing peptide. B: The result of 81 controls. C: The anti-CCP results of 120 sera are shown in the grey columns.
Fig. 15. Results of CLIA and anti-CCP ELISA. Chi-square analysis of the antibodies binding α1(I) C-telopeptide, α1(II) C-telopeptide and anti-CCP. No linear correlations between the reactivities of autoantibodies against citrullinated C-telopeptides and CCP (r_s=0.113 and r_s=0.053 for type I and type II collagen antigens, respectively). Although it seems that the specificities of the antibodies are clearly different, both anti-CCP and anticollagen C-telopeptide assays behaved similarly in 2 x 2 contingency analysis.
Fig. 16. Competition assays. One human serum sample was tested in three different ELISAs: anti-carboxytelopeptides of the α1 chain of type I (A) and II collagens (B) and anti-CCP assays (C). The inhibitors were EKAHDGGRYYRA (open triangles), EKAHDGGRYYXA (closed triangles), EKGPDPLQYMRA (open squares) and EKGPDPLQYMXA (closed squares).
5.3 Autoantibodies binding citrullinated type I and type II telopeptides are specific for citrulline in the peptide sequence (IV)

Autoantibodies that bind normal C-telopeptides were not inhibited with soluble normal or citrullinated telopeptides (figure 17). However, the antibodies that bind only citrullinated telopeptides could be inhibited with corresponding citrullinated telopeptides (figure 18). Thus, it is not necessary to study the autoantibodies to normal collagens if the specificity of the binding is assessed in addition to immunologic inhibition. For type I telopeptide there are two arginines, the latter of which, when citrullinated, is important for binding (table 6). For type II telopeptide there is one arginine, which is important when citrullinated. The other amino acids, e.g. the last alanine, have only a slight effect on binding (table 7).

Figure 19 shows a summary of the results of both α1 telopeptide chains of type I and type II collagens when the sera were inhibited with corresponding soluble antigens (one concentration of 200 µg/ml). In the case on type I collagen assay, there were 48 sera of RA patients (40%) who showed increased specific binding, and three control sera were also positive (inhibition ≤ 17.6%; the mean + 2 SD of controls). The mean of the controls was 6.9% and that of the RA patients 22.6%. In the case of type II collagen assay, the corresponding figures were 36 positive sera of RA patients (30%) and one positive control serum sample (inhibition ≤ 13.8%). With type II collagen, the mean of the controls was 5.4% and that of the RA patients 14.5%.

![Figure 17](image.png)

Fig. 17. Inhibition ELISA of the binding of RA autoantibodies to arginine-containing type I and II collagen. A. Type I collagen carboxytelopeptide: white column is standard for an assay without inhibition, light grey denotes the same assay containing 200 µg/ml of soluble peptide SP65, and black represents the same assay containing 200 µg/ml of soluble peptide SP66. B. Type II collagen carboxytelopeptide: white is standard for an assay without inhibition, light grey represents the same assay containing 200 µg/ml of soluble peptide SP40, and black indicates the same assay containing 200 µg/ml of soluble peptide SP41.
Fig. 18. Inhibition ELISA of the binding of RA autoantibodies to citrulline-containing type I collagen carboxyteleopeptide. The white column is standard for an assay without any inhibition, light grey represents the same assay containing 200 µg/ml of soluble peptide SP65, and black denotes the same assay containing 200 µg/ml of soluble peptide SP66.

Table 6. Inhibitory characteristics of citrullinated carboxyteleopeptides of type I collagen in 10 patients’ sera with different inhibitory peptides from the same type I collagen sequence (concentration of each 200 µg/ml). The counts are percentages. Please, see the sequences in table 4.

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Table 7. Inhibitory characteristics of citrullinated carboxyteleopeptides of type II collagen in five patients’ sera with different inhibitory peptides from the same type II collagen sequence (concentration of each 200 µg/ml). The counts are percentages. Please, see the sequences in table 4.

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Fig. 19. Inhibition ELISA showing percentages of inhibition among 81 controls (C) and 120 patients with RA (RA). A. Inhibition obtained with type I collagen C-telopeptide and B. inhibition obtained with type II collagen C-telopeptide. The means are indicated with solid lines and the means ± 2SD of the controls are indicated with dashed lines.
5.4 Modification of methods that can be used for the detection of autoantibodies binding citrullinated collagen carboxyterminal telopeptides with RA (I, II, IV)

The automatic CLIA gives an over twofold number of positives compared to the ELISA method, which included two assays (for arginine and citrulline). The reason might be that the automatic CLIA involves less steric hindrance, since the antigen is bound in magnetic particles. Thus, the distinction of autoantibodies reacting to both citrullinated and normal peptides is better in the automatic method than in the ELISA method. The other reasons may be that the antigen concentration is higher in CLIA than in ELISA, and the chemiluminesce label is more sensitive than HRP.

For diagnostic purposes, the measurements of single autoantibodies with two different assays provide additional tests and introduce larger variance, in which case the results of the two assays should be combined. In the case of inhibition ELISA, there is no need to test the antibodies binding arginine-containing peptides, since these sera are also positive for citrulline-containing peptides. Some of the autoantibodies could not be inhibited with the corresponding soluble antigen, possibly representing unspecific binding.

We tested the modified inhibition ELISA method using previously tested sera and found superior discrimination compared to the earlier assays (table 8). In addition, there were more positives compared to the two ELISA assays.

Table 8. Performance characteristic of inhibition citrullinated ELISA, two ELISAs with arginine and citrulline-containing peptides and CLIA assay of the latter. P values and Student’s test values (t) between patients with rheumatoid arthritis (N=120) and controls (N=81). Positives indicate how many RA patients had positive results in certain assay modifications.

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<th>Antigen</th>
<th>Inhibition % mean ± SD in Inhibition ELISA</th>
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<td>Controls RA patients</td>
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<td>α1(I) C-telopeptide</td>
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<td>t = -7.313</td>
<td>t = -2.114</td>
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</tr>
<tr>
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<td>positives 36 p &lt; 0.001</td>
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<td>p = 0.074</td>
<td>positives 35</td>
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</table>

5.5 Development of methods for the detection of autoantibodies recognising citrullinated telopeptides from type I and II collagens

We found more autoantibodies to citrullinated C-telopeptides from human type I and II collagens in the serum of patients with RA compared to healthy controls. We developed
three different methods for their detection. (See table 8.) The same patients with RA (N=120) and controls (N=81) were used in the ELISA, CLIA and inhibition ELISA tests. The comparison between the methods is illustrated in figure 20. The Receiver Operating Characteristic (ROC) curves were made by the SPSS software. The area under the ROC curves indicates the accuracy of the test. The accuracy of the test depends on how well the test differentiates the group being tested into those with and without the disease or, in this case, the characteristic in question. Inhibition ELISA was the best method. Additionally, inhibition ELISA allows measurement of the autoantibodies binding specifically to the citrullinated telopeptides of type I and II collagens. When only one assay is involved, variance decreases and overall performance is easier than previously.

The best inhibiting peptide for type I collagen telopeptide seems to be SP66. The last citrulline (SP66) is important, since antibodies seem to bind less to the second citrulline residue (SP72) than to the first. The best inhibiting peptide for the type II collagen telopeptide seems to be SP41. (See tables 6 and 7 and figure 11.)

5.6 Autoantibodies binding the citrullinated telopeptide of type II collagen and anti-CCP predict synergistically the development of seropositive RA (III)

The mean levels of citrullinated peptides and the citrullinated/arginine peptide ratios of type I and II collagens and anti-CCP were higher in the subjects who later developed RF-positive RA. In the highest tertiles of C/A (I), C/A (II) ratios and anti-CCP, the relative risk of RF-positive RA was significantly increased. The three antibody predictors, however, tended to confound their mutual effects, and only anti-CCP retained its statistical significance in the multifactorial model. However, the interaction term of C/A (II) ratio and anti-CCP proved statistically significant (p=0.02). The subjects ranked into the highest tertiles of both C/A (II) ratio and anti-CCP had a relative risk of 20.06 (95% confidence interval, 4.37–92.06) to develop RF-positive RA compared to those in the lowest tertiles of these antibodies.
Fig. 20. ROC curves of the different methods for detecting autoantibodies to A: citrullinated C-telopeptides from human type I collagen and B: citrullinated C-telopeptides from human type II collagen in serum.
Table 9. A Relative odds (OR's), with 95% confidence intervals (CI's), of rheumatoid factor positive rheumatoid arthritis between the tertiles* of citrulline/arginine containing (C/A) α1 (I) C-telopeptide antibody ratios in the tertiles* of antibodies to cyclic citrullinated peptide (anti-CCP).

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<td>N † OR (95% CI)</td>
<td>N † OR (95% CI)</td>
<td>N † OR (95% CI)</td>
<td></td>
</tr>
<tr>
<td>&lt; 15.0</td>
<td>4/25</td>
<td>1.00</td>
<td>3/25</td>
</tr>
<tr>
<td>15.0-16.2</td>
<td>3/26</td>
<td>0.79 (0.12-5.25)</td>
<td>8/17</td>
</tr>
<tr>
<td>≥ 16.3</td>
<td>8/27</td>
<td>2.26 (0.41-12.59)</td>
<td>6/33</td>
</tr>
</tbody>
</table>

p-value for interaction = 0.28. * The tertiles are based on the distributions among the controls. † n = number of cases/controls

Table 9. B Relative odds (OR's), with 95% confidence intervals (CI's), of rheumatoid factor positive rheumatoid arthritis between the tertiles* of citrulline/arginine containing (C/A) α1 (II) C-telopeptide antibody ratios in the tertiles* of antibodies to cyclic citrullinated peptide (anti-CCP).

<table>
<thead>
<tr>
<th>anti-CCP</th>
<th>&lt; 0.85</th>
<th>0.85-0.94</th>
<th>≥ 0.95</th>
</tr>
</thead>
<tbody>
<tr>
<td>N † OR (95% CI)</td>
<td>N † OR (95% CI)</td>
<td>N † OR (95% CI)</td>
<td></td>
</tr>
<tr>
<td>&lt; 15.0</td>
<td>5/27</td>
<td>1.00</td>
<td>8/25</td>
</tr>
<tr>
<td>15.0-16.2</td>
<td>1/22</td>
<td>0.31 (0.03-3.26)</td>
<td>6/31</td>
</tr>
<tr>
<td>≥ 16.3</td>
<td>9/34</td>
<td>1.76 (0.36-8.69)</td>
<td>4/31</td>
</tr>
</tbody>
</table>

p-value for interaction = 0.02. * The tertiles are based on the distributions among the controls. † n = number of cases/controls
6 Discussion

RA is a common, systemic autoimmune disease of unknown etiology. The ACR classification is generally used for the diagnosis of RA. Five of the seven criteria are based on clinical parameters. The goal of treatment is remission and prevention of joint destruction. This goal can be best reached at the early stages of the disease. (Möttönen et al. 1999, O’Dell 2002, Landewe et al. 2002). Since the treatment may have major side effects, it is important to diagnose RA as early as possible. Based on what we know, citrullinated proteins, which are highly specific for RA, may participate in the etiopathogenesis of the disease, and their occurrence at the early phase of the disease is likely to have an important role. Future studies will show whether new therapeutic possibilities to treat RA at the level of citrullination may be feasible. (Nijenhuis et al. 2004.)

The seropositive (on the grounds of the presence of IgM RF) RA cases meet the ACR classification criterion of RA. In study III, we did not find any difference in antibody levels between cases with RF-negative RA (according to ACR criterion) and their matched controls. However, the mean antibody levels for RF-positive RA and total RA were higher in the cases than the controls. The studies I, II and IV included both seropositive and seronegative RA patients. That might have contributed to the low number of positives in these studies. We could have had more positives if only seropositive RA sera had been included.

We have shown autoantibodies binding citrullinated carboxytelopeptides to α1 chains from human type I and II collagens in the serum of patients with RA. It was very interesting to discover the autoantibodies of citrullinated C-telopeptides of type I and II collagens since RA patients have autoantibodies against normal collagens, especially cartilage collagens (Clague et al. 1981, Rowley et al. 1986). We have also found autoantibodies of non-citrullinated C-telopeptides. Nijenhuis et al. (2004) pointed out that epitope spreading during the inflammation process may possibly give rise to the production of autoantibodies directed to non-citrullinated proteins. In our own experiments with rabbits immunized with citrulline containing type I carboxytelopeptide, there were at first antibodies binding citrullinated type I carboxytelopeptides, but very soon there were also antibodies binding against arginine containing type I C-telopeptide.
Lundberg et al. (2005) reported that citrullinated collagen II induced arthritis in rats with a higher incidence and earlier onset than did the native counterpart.

Vossenaar et al. (2004c) found that, in RA patients, anti-citrullinated protein antibodies are produced in a 1.4-fold proportion of IgG in synovial fluid compared to serum. They took these findings to indicate that anti-CCP antibodies are also produced locally in the inflamed synovial compartment. Our unpublished experiments yielded similar results. We detected a larger amount of IgG autoantibodies to citrullinated C-telopeptides of \( \alpha_1 \) chains from human type I and II collagens in synovial fluid than in serum.

In the detection of autoantibodies to citrullinated C-telopeptides of \( \alpha_1 \) chains from human type I and II collagen in RA serum, it is possible to use the ELISA, CLIA and inhibition ELISA methods. We started by using ELISA to show the existence of antibodies to citrullinated type I and II collagens. We developed automatic assays using CLIA because we suggested that ELISA was not sensitive enough for the detection of autoantibodies to citrullinated telopeptides. With CLIA, we obtained a more than twofold number of positives compared to the previous ELISA method. The reason might be that automatic CLIA involves less steric hindrance, since the antigen is bound in magnetic particles. Thus, the distinction of autoantibodies reacting to both citrullinated and normal peptides is better in the automatic method than in the ELISA method. Other reasons may be that the antigen concentration used is higher in CLIA than in ELISA, and the chemiluminescence label is more sensitive than the HRP label.

After CLIA, we developed inhibition ELISA to detect specific antibodies against citrullinated collagens instead of using two assays using both arginine- and citrulline-containing type I and type II C-telopeptides. The specificity of the binding of these antibodies was tested by inhibition with corresponding soluble identical antigens. Based on these experiments, it was found unnecessary to measure the binding to normal, arginine-containing peptides. When the positive sera were inhibited in the arginine telopeptides assays with corresponding soluble peptides, no inhibition was found.

However, the antibodies that bind only to citrullinated telopeptides could be inhibited with corresponding citrullinated telopeptides. When only one assay is involved, variance is decreased and overall performance is easier than in our previous methods. Inhibition studies have shown shorter peptides to be better than longer, since overall inhibition was lower with the former peptides in the controls.

During the development of the CCP tests, corresponding non-citrullinated peptides were also tested. But the authors omitted arginine-containing peptides from the final assay. They used ELISA plates coated with citrullinated peptides, but specificity was not assessed. (Schellekens et al. 2000.) The studies of van Venrooij et al. (2002) indicated that arginine-containing counterparts were not recognized with autoantibodies of RA patients. However, Vittecoq et al. (2004) discovered that non-RA sera, which show increasing binding to citrullinated and/or non-citrullinated rat filaggrin, could be inhibited with increasing soluble citrullinated or non-citrullinated filaggrin, but only in citrullinated filaggrin-coated plates. The anti-citrullinated filaggrin activity of RA sera was inhibited only by citrullinated filaggrin in the rat filaggrin ELISA. Vittecoq et al. (2004) reported that it is possible that human sera contain antibodies to the non-citrullinated parts of the antigen. This may cause false positivity in a diagnostic test involving citrullinated and non-citrullinated filaggrin. The reliability of these tests can be increased by using corresponding non-citrullinated protein or peptide as a negative control.
Our studies included two patients with negative results in type I and type II inhibition ELISA but positive results in anti-CCP ELISA (table 2 in the original article IV). It is too early to speculate that human sera might contain antibodies to the non-citrullinated parts of the CCP antigen.

Early in the development of the CCP mark 1 assay, the problem of the test was its sensitivity. However, the selection of citrullinated peptides from synthetic libraries resulted in sensitive antigenic peptides, as in the anti-CCP mark 2 assay. (Van Venrooij et al. 2002.) The use of these peptides in a cyclic configuration improved the diagnostic performance of the assay (Schellekens et al. 2000). One explanation for the low sensitivity could be the fact that antibodies are a heterogeneous group (polyclonal) directed against different epitopes on the citrulline-containing molecule. Each patient serum contains different subsets of antibodies, and the synthetic peptide used in the assays represents a relatively small set of antigenic determinants present on the as yet unknown antigenic molecule in the joint. (See Bizzaro et al. 2001.) Van Venrooij et al. (2002) concluded that the residues flanking the citrulline introduce an additional level of epitope specificity, and a particular peptide may therefore be recognized by only a subset of anti-CCP-containing sera.

In our own experiments on rabbits immunized with citrulline-containing type I C-telopeptide, there were at first antibodies binding citrullinated type I C-telopeptides, but very soon there were also antibodies binding against arginine-containing type I carboxytelopeptide. We simultaneously had autoantibodies binding filaggrin (anti-CCP) and citrullinated collagen C-telopeptides. It is unknown why we will first find antibodies binding citrullinated antigen and then antibodies binding both citrullinated and normal (arginine containing) antigen. Vossenaar et al. (2004d) investigated the presence of anti-CCP antibodies in numerous animal models of arthritis. Some animals indeed showed antibody reactivity against the citrullinated peptide, but these animals also showed reactivity against the non-citrullinated control peptide.

Sverdrup et al. (2005) studied agents that induce arthritis in the human body. Adjuvants include bacteria, yeast, viruses and mineral oils. Their study showed that persons exposed to mineral oil have an increased risk to develop RF-positive and anticitrulline-positive RA. The findings are of particular interest since the same mineral oils can induce polyarthritis in rats. Wooley et al. (1985) found that the humoral responses to type II collagen in mice were transiently depressed 14 days after immunization, but the antibody levels did not differ after 28 days.

The immune system acts under strict homeostatic control. The T-cell responses to self-antigens and foreign antigens are regulated in the majority of individuals. Antigenic stimulation induces rapid expansion of antigen-specific T-cells to large clonal sizes. T-cell homeostasis was not intact in RA patients, and these patients thus carried large clonally expanded populations of T-cells. These T-cells were not specific for a common antigen. Expanded T-cell clones were also present in the circulation as well as in inflamed tissues. (Goronzy et al. 2003.)

Several groups have reported about the sensitivity and specificity of the test they have used (see table 2 in page 28). Sensitivity and specificity are the statistics most widely used to evaluate a diagnostic test. It should be noted that these indicators describe the accuracy of the test, not the prevalence of autoantibodies among patients with RA.
The sensitivity of the test determines the probability of a positive test among patients with autoantibodies. Sensitivity is calculated as true positive / (true positive + false negative). Sensitivity alone does not tell us all about the test, because 100% sensitivity can be trivially achieved by labelling all test cases as positive. Therefore, we also need to know the specificity of the test. The specificity of the test determines the probability of a negative test among patients without autoantibodies. Specificity is calculated as true negative / (false positive + true negative). Efficacy indicates the average of specificity and sensitivity. Positive predictive value refers to the chance that a positive test result will be correct. Positive predictive value can be calculated as true positive / (true positive + false positive). On the other hand, negative predictive value is concerned only with negative test results. Negative predictive value can be calculated as true negative / (true negative + false negative). (Loong TW 2003.)

Since the prevalence of autoantibodies among RA patients is not known, we did not calculate the sensitivity or specificity of the different tests used in this study. We studied the phenomenon of citrullination, and we did not have a standardized test for it. Citrullination consists of the formation of autoantibodies to citrullinated type I and II collagens.

Nijenhuis et al. (2004) suggested a hypothetical model of the development of chronic inflammation in RA. At the first step, inflammatory cells with PAD are activated. Then these cells die via apoptosis, and the intracellular Ca²⁺ concentration increases, resulting in PAD activation and citrullination of intracellular proteins. After that, peptides from proteins are presented by certain HLA molecules (e.g. HLA-DR4) to the immune system. This may trigger the immune system to generate a T cell and, next, B cell response to citrullinated antigens. As a consequence, local production of antibodies to the citrullinated peptides begins. The resulting antibodies induce the formation of immune complexes, which stimulate the inflammatory process. The repetition of this process for years finally leads to a chronic inflammation that may develop into RA.

Vossenaar et al. (2004c) showed that the presence of citrullinated proteins is not specific for rheumatoid synovial tissues, since these proteins can be detected in synovial tissues from patients with various inflammatory arthritides. The expression of citrullinated proteins in the non-RA group was not associated with circulating anti-CCP antibodies. Chang et al. (2005) discovered that fibronectin was citrullinated when immunoprecipitated from RA synovial tissue, whereas that from osteoarthritis synovial tissue, placenta and tumour tissue was not.

Vossenaar & van Venrooij (2004) and Vossenaar et al. (2004c) have given two explanations for the high specificity of autoantibodies to citrullinated antigens for RA. One of them is that there is RA-specific overexpression of citrullinated antigens in rheumatoid synovium, which leads to an immune response. Alternatively, the presence of citrullinated proteins may be a common event in any inflamed tissue, but RA patients may have an abnormal humoral response to them. Thus, the high specificity of anti-citrullinated protein antibodies for RA appears to be the result of an abnormal humoral response rather than representing disease-specific expression of citrullinated proteins.

Menard et al. (2000) speculated that several micro-organisms could have arginine-to-citrulline deiminase activities. It is possible that they may enter the human body and citrullinate its proteins. Such an environmental post-translational modification might be a fresh illustration of the ‘hit-and-run’ theory of autoimmunity.
Rosenstein et al. (2004) presented a hypothesis that the humoral response to bacteria provides a stimulus for RA development. There is some evidence that one oral pathogen implicated in the pathogenesis of periodontal disease possesses a unique microbial enzyme, PAD, which has been identified as a susceptibility factor for RA.

Suzuki et al. (2005) indicated that human type I collagen (not including C-telopeptide) is a substrate of PADs, and that citrullinated type I collagen strongly correlates with RA. They noticed that type I collagen can become an autoantigen of RA via citrullination by PADs. They further speculated that autoantibodies for citrullinated collagens react or cross-react with other citrullinated proteins that are locally produced at the site of rheumatoid inflammation of synovial tissue.

During the last few decades, it has been recognized that most autoantibodies are not specific to a clinical syndrome. In autoimmune diseases, autoantibodies may be the actual pathogenic agents of the disease, a secondary consequence of tissue damage or footprints of an etiologic agent. Although it has been difficult to link autoantibodies to pathogenesis, they can be used to predict disease progression and outcome. (Griesmacher & Peichl 2001.)

The result of study III was that autoantibodies to the citrullinated telopeptide of type II collagen and filaggrin predict synergistically the development of seropositive RA. The serum material in study III was different from that used in the other studies (I, II and IV). In study III, pre-illness serum specimens were analyzed, while the patients in the other studies had early RA. At the beginning of RA, there may appear autoantibodies to type II collagen. The reason could be that cartilage is destroyed earlier than bone.

Our hypothesis is that collagens may be destroyed if antibodies to the citrullinated collagens are produced. These antibodies would be harmless if there were no citrullination of connective tissues. If autoantibodies are produced, they induce the formation of immune complexes, which stimulates the chronic inflammatory process.

RA synovial membrane contains large numbers of activated macrophages (Cutolo et al. 1993). Cells described as expressing PAD enzymes and under suitable conditions contain citrullinated proteins (Asaga et al. 1998, Mizoguchi et al. 1998). PAD2 and PAD4 are the most likely candidate PAD isotypes for the synovial proteins in RA because PAD4 was present in monocytes and PAD2 both in monocytes and in macrophages.

The presence of PAD enzymes does not necessarily mean that citrullinated proteins exist in cells because calcium and other factors are needed for their activation. The citrullination of proteins by PAD enzymes is a process regulated at three levels: transcription, translation and activation by Ca²⁺ influx. (Vossenaar et al. 2004b).

Although citrullinated proteins were present during synovial inflammation in mice, no antibodies binding to synthetic CCP or in vitro citrullinated fibrinogen could be detected, either in sera or in synovial fluid. There are two possible explanations for this. First, there is a difference in the duration of the human disease compared to mouse models. Even symptomless humans can become anti-CCP-positive years before the manifestation of the first clinical symptoms of RA. Secondly, the role of genetic factors has been established. The HLA-DR4 phenotypes are especially important. The mice used in the CIA and SCW models did not have the arthritis-prone alleles. (Vossenaar et al. 2003a.)

It was shown that RA-susceptible haplotypes increase PADI4 mRNA stability. In theory, this could result in more PAD4 enzyme being produced and, subsequently, lead to
increased citrullination of proteins and a greater chance of developing (citrullinated) antibodies. (Vossenaar & Venrooij 2004.)

PAD2 occurs in many different tissues (Vossenaar et al. 2003b). Despite the broad expression pattern, only filaggrin and keratin in the stratum corneum of the epidermis (Ishigami et al. 2002), myelin basic protein (Moscarello et al. 1994, Wood et al. 1996) and vimentin (Vossenaar et al. 2004a, Asaga et al. 1998, Vossenaar et al. 2004b) have been identified as its substrates.

However, multiple sclerosis patients had no antibodies to citrullinated myelin basic protein in their serum or cerebrospinal fluid (de Seze et al. 2001). Citrullination seems to be related to psoriasis, an epidermic disease that induces abnormally fast epidermic turnover associated with itching and inflammations, and which can affect a large part of the total skin surface. The level of citrullination of proteins is much lower in the affected epidermic areas than in healthy areas, and there is hardly any citrullinated keratin (Ishida-Yamamoto et al. 2000). In patients with Alzheimer’s disease, abnormal accumulation of citrullinated proteins and abnormal activation of PAD2 in hippocampi have been seen. However, no citrullinated proteins were detected in the normal hippocampus, but PAD2 immunoreactivity was ubiquitous both in Alzheimer’s disease and in normal hippocampal areas. (Ishigami et al. 2005.)

Research on the structural basis of Ca\(^{2+}\)-induced activation of human PAD4 is important for understanding the mechanism of protein citrullination and for developing PAD-inhibiting drugs for the treatment of RA (Arita et al. 2004). Recently, Stone et al. (2005) found that 2-chloroacetamidine irreversibly inhibits human PAD4 in a time- and concentration-dependent manner. According Hidaka et al. (2005), methylation of the guanidino group of arginine prevented citrullination by PAD4.

Proteins with high arginine content (such as filaggrin, fibrinogen, vimentin, histones, myelin basic protein and \(\alpha\)-enolase) are more likely to include reactive epitopes upon citrullination than proteins with low arginine content (Lapointe et al. 1999). Kinloch et al. (2005) recognised the citrullinated \(\alpha\)-enolase protein as a candidate autoantigen in RA. \(\alpha\)-Enolase was detected in the synovial membrane in a joint of a RA patient, where it co-localised with citrullinated proteins. Antibodies have been found that co-occur with the expression of citrullinated antigen.

Other antibodies, especially APF and AKA, recognize the citrulline-containing antigenic filaggrin proteins (Schellekens et al. 1998, Girbal-Neuhauser et al. 1999). Rheumatoid synovium contains Sa antigen, but has not been seen to contain profilaggrin or filaggrin (Menard et al. 1996). Vossenaar et al. (2004a) suggested that citrullinated vimentin, Sa antigen, is a candidate autoantigen in RA.

We immunized rabbits with synthetic citrullinated vimentin. The sequence of this vimentin peptide resembles the collagen telopeptides sequence (SSSYXXMFGGPGGTAC, where only one the X’s is citrulline and the other arginine.) These rabbits had antibodies binding to citrullinated vimentin but not to citrullinated collagens. We also immunized rabbits with citrullinated collagens. Both groups had antibodies binding to the CCP antigen, which is based on the filaggrin sequence. This disproves the assumption that citrullinated vimentin or collagen can produce autoantibodies of different specificities.

In these studies, we described for the first time autoantibodies to citrullinated C-telopeptides of type I and II collagens in the serum of RA patients. Joints contain
cartilage (type II collagen) and bone (type I collagen), suggesting that the discovered autoantibodies reacting with these proteins do not reflect immunological cross-reaction in RA patients. The discovered autoantibodies are specific to citrulline in the peptide sequence. According to our results, the most accurate method for the detection of these autoantibodies is inhibition ELISA. Additionally, autoantibodies to the citrullinated telopeptide of type II collagen and filaggrin predict synergistically the development of seropositive RA.

Our results give a good starting point for future studies. What is the prerequisite of the specific binding of autoantibodies to citrullinated peptides, and what is the affinity of the bound antibodies? Are there in vivo citrullinated human type I and II collagens? Finally, what is the predictive value of different autoantibodies in the development of RA in asymptomatic patients and the onset of the disease.
7 Conclusions

1. Patients with RA have more autoantibodies binding to citrullinated carboxytelopeptides of α1 chains from human type I and II collagens than healthy controls. The significance of the finding depends on the methods. Clinical significance is uncertain.

2. It is possible to use ELISA, CLIA and inhibition ELISA for the detection of these autoantibodies. Automatic CLIA gives an over twofold number of positive findings compared to the traditional ELISA method. According to these studies, the best way to detect autoantibodies binding the citrullinated α1(I) and α1(II) telopeptides of collagen is inhibition ELISA. Anti-CCP ELISA detects a subgroup of antibodies different from the autoantibodies binding the citrullinated α1(I) and α1(II) telopeptides of collagen.

3. These autoantibodies are specific for citrulline in the peptide sequence. Autoantibodies that bind the arginine carboxytelopeptides of type I and II collagens were not inhibited with soluble arginine or citrullinated telopeptides. However, the antibodies that bind only citrullinated telopeptides could be inhibited with corresponding citrullinated telopeptides.

4. Autoantibodies binding to citrullinated telopeptide of type II collagen and to cyclic citrullinated peptides predict synergistically the development of seropositive rheumatoid arthritis
References


Original articles


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AUTOANTIBODIES BINDING CITRULLINATED TYPE I AND II COLLAGENS IN RHEUMATOID ARTHRITIS