The humoral immune response to citrullinated proteins in patients with rheumatoid arthritis (RA):

Genetic, clinical, technical, and epidemiologic aspects

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Early synovitis in RA

Clinical aspects

- The disease progresses quickly from a predominantly immunoinflammatory to a destructive phase where established pannus erodes bone, tendons and joint capsule.
- The "therapeutic window" to get control of the early phase is very short (few months), and later therapy has little or no effect on the destructive phase.
Early diagnosis

- Clinical history
- Manifestations
- Physical examination
- Radiology
- Specialist evaluations
- Histopathology
- Immunopathology
- Laboratory tests to look for inflammation
- Immunoglobulin levels
- Complement activation
- Autoantibodies

Criteria-based early diagnostics

Criteria = rigorously defined items

Clinical symptom 1
Clinical symptom 2
Clinical symptom 3
Not present
Not found yet
Specific serologic result: Diagnosis and Prognosis
Particular importance: focus

Serological markers in RA

- Rheumatoid factors (Waaler’s sheep red blood cell agglutination test) (1939)*
- Rheumatoid factors (Wager’s Streptococcus agglutination test) (1950)*
- Anti-RA 33 antibodies (1995)*
- (Anti-p68/-BIP) (1998)

* Rather non-specific tests for RA
**Anti-perinuclear factor**

- Keratohyaline body
- Nucleus

**Rheumatoid arthritis**

- Esophagus tissue
- Antibodies to the dying epithelium
- An autoimmune response to modified (citrullinated) proteins

**Related serological markers for RA**

- Anti-perinuclear factor (1964) □
- Anti-keratin antibodies (1979) □
- Anti-filaggrin antibodies (1993) □
- Anti-Sa (1995) □
- Anti-citrullinated peptides (1998) □

¤=citrullinated antigens
Autoantibodies in RA which are related to anti-citrullinated protein antibodies

**Antibody system:**

- Anti-perinuclear factor
- Anti-keratin
- Anti-Sa
- Anti-(pro)filaggrin
- Anti-citrullinated peptides (α-CCP1) (α-CCP2)
- Anti-citrullinated proteins (human fibrinogen peptides)

**Citr. antigens:**

- Trichohyalin in mouth mucosal cells
- Keratin in oesophagus mucosal cells
- Filaggrin in epithelium
- Vimentin in MΦs
- Fibrin in RA joints
- Alpha-enolase
- Collagen I and II

Citrullination of arginines in proteins

![Citrullination diagram](image_url)

Peptide cfc1 and control peptides

- **cfc1** SHQESTXGRSGGRSGSGS
- **cfr** SHQESTRGRSGGRSGSGS
- **cfa** SHQESTAGRSGGRSGSGS
- **cfe** SHQESTEGRSGGRSGSGS
- **cfo** SHQESTQGRSGGRSGSGS
- **cfc2** SHQESTZGRSGGRSGSGS

N-Citrullin, aa 306-324 in filaggrin
R-arginine
A-valine
E-glutamic acid
Q-glutamine
Z-ornitine

*Schellekens et al., J Clin Invest 1998, 101:273-281*
Binding of RA antibodies to cfc1 and control peptides in ELISA

ELISA of a typical RA serum
Cfc 1 is recognized but unmodified control peptides are not

Citrullinated peptides of filaggrin
Amino acids 306-324

- cfc0 SHQESTGRSRSGRSGS
- cfc1 SQHESTXGRSRSGRSGS
- cfc2 SQHESTGRSXRGRSRSGRSGS
- cfc3 SQHESTGRSXGRSRSGRSGS
- cfc4 SQHESTGRSGXGRSRSGRSGS
- cfc5 SQHESTGRSGRGRSGXGRSRSGRSGS
- cfc6 SQHESTGRSGRGRSGXGRSRSGRSGS
- cfc7 SQHESTGRSXGRSRSGRSGS
- cfc8 SQHESTXGRSXRGRSRSGRSGS
- cfc9 SQHESTXGRSRSGRSGXGRSRSGRSGS

Reactivity of RA sera with citrullinated peptides

- Practically every patient serum was found to react differently with the various citrullinated peptides, and thus no particular peptide was found to carry a dominant epitope.
- Very often several citrullinated peptides were recognized by each RA serum.
- Hence, the first assays used several citrullinated peptides as antigen source.

Each RA serum has a unique pattern of recognition of citrullinated peptides

Cyclic Citrullinated Peptide: CCP

Cyclisation of the peptide enhances its recognition by RA autoantibodies


How do synovial antigens become modified: arginine to citrullin?

- Peptidyl-arginine deiminase enzymes (PAD2 and PAD4) are richly represented in monocytes, macrophages and neutrophils
- When these cells undergo apoptosis Ca++ ions are transferred into the cells and activate PADs
- Ca++ concentration in normal cells ~10^{-7}M
- Threshold for PAD enzyme activity ~10^{-5}M
- PAD enzymes most likely act on the enzyme-containing cells themselves (Monos, M\(\Phi\)s, PMNs)
Citrullinated proteins are richly represented in human inflammed synovia

- Citrullinated proteins are present both intra- and extracellularly in synovial membranes from patients with various forms of arthritis. (Vossenaar E et al. Arthritis Rheum 2004)
- Citrullinated peptides fit perfectly into the P4 pocket of shared epitope on APC, but the homologous arginine peptides do not. (Hill JA et al. J Immunol 2003)

Candidate citrullinated proteins in Non-RA and RA tissues.

- Type I and II collagens (Koivula MK et al. Ann Rheum Dis 2005).

Association between anti-CCP production and shared epitope

HLA DR typing and anti-CCP2 antibodies were studied in 268 RA patients from an early arthritis clinic cohort in Leiden. Radiographic disease progression was measured over 4 years. Carriers of shared epitope DRB1 alleles were more commonly anti-CCP positive than non-carriers (OR 13.3) and also showed the most pronounced radiographic progression. (van Gaalen FA et al.:Arthritis Rheum 50:2113-2121,2004).

Figure 2 Relative risk (RR) for development of rheumatoid arthritis (RA) in current smokers (with different numbers of copies (0-2) of the shared epitope (SE) of HLA-DR) compared with never smokers. (A) RR for seropositive RA and (B) RR for seronegative RA. These graphs are schematic representations of the original data from a case-control study of RA reported in reference 9.

Risk factors in RA.
Recent study in 515 Danish RA patients and 769 sex- and age-matched controls

Risk factors in anti-CCP positive RA patients:
- menarche at = or >15 years of age (OR 1.87)
- tobacco smoking (both sexes): confirmed, both former and current smokers, dose dependent effect
- coffee consumption > 10 cups/day (OR 2.75)
- alcohol consumption > 15 drinks/week (OR 0.58)
- moderately demanding exercise (OR 0.51)
- pets as adult (ever) (OR 0.73)

Risk factors in anti-CCP negative RA patients:
The strongest risk factor was increased body mass index 10 years before the study:
- obese (BMI = or >30 kg/m²) (OR 9.79*)
- moderate (BMI 25-30 kg/m², OR 3.53*)
  *compared to underweight (<18.5 kg/m²)
- menarche = or >15 years of age (OR 2.27)
- pets (ever) (OR 0.65)
- moderately demanding exercise (OR 0.69)
Local production of anti-filaggrin antibodies in the rheumatoid pannus.

- Anti-filaggrin (AFA) antibodies were studied at equal IgG concentrations in paired syn. membrane extracts, syn. fluids and sera from 31 RA patients.
- No difference was found between syn. fluid and serum levels of AFA, whereas extracts contained 7.5 fold higher AFA levels.

Studies done on anti-citrulline antibodies are difficult to compare

- The citr. antigens are very different
- The cut-offs used are different
- The RA populations studied are different
- The differential diagnostic populations studied for comparison with RA patients are different
- Some studies include undifferentiated arthritis, palindromic syndrome, RF+JRA, RF+psoriatic arthritis etc. all of which may actually become RA.

Commercially available assays for anti-CCP (now CCP2)

The most recent version of anti-CCP2 ELISAs from Euro-Diagnostika, Axis-Shields and INOVA give identical results (identical coating of peptide)

Beads, multiarrays, and ELIA solid phase assays have been coated in basically the same way and are thus expected to give identical results.
Nosographic sensitivity  
(sensitivity in RA patients)

- Anti-CCP2, anti-filaggrin and APF show very similar sensitivities:  
  - at diagnosis < 6 months: around 50%  
  - at diagnosis 1 year: around 60%  
  - at diagnosis >2 years: around 70%  
- AKA and anti-Sa: usually show lower sensitivity than the above methods

Diagnostic specificity

- When compared with healthy controls the specificity is around 99% for anti-CCP2, anti-filaggrin, anti-citrullinated human fibrin peptide, and anti-Sa, while the specificity is somewhat lower for APF and AKA  
- When compared with different immuno-inflammatory CTDs the specificity is around 95 to 98%  
- 1-3% of acute and chronic infectious disease sera are anti-CCP2 positive (at low levels)

Prediction of erosive disease

- and many others!!
Summary of data on anti-CCP2 in adult rheumatoid arthritis 2006

<table>
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<tr>
<th></th>
<th>No.</th>
<th>Pos.</th>
<th>%</th>
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<tr>
<td>Rheumatoid arthritis</td>
<td>7769</td>
<td>5465</td>
<td>70%</td>
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<tr>
<td>-early</td>
<td>2936</td>
<td>1752</td>
<td>60%</td>
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<tr>
<td>-established</td>
<td>4833</td>
<td>3713</td>
<td>77%</td>
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<td>Healthy donors</td>
<td>2855</td>
<td>26</td>
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<tr>
<td>Non-RA controls</td>
<td>7978</td>
<td>420</td>
<td>1%</td>
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van Venrooij W.J. et al., Autoimmunity Rev 2006

Prediction of RA in patients with undifferentiated arthritis (UA)

• RF and anti-CCP1 in patients with undifferentiated arthritis predicted later onset of RA with 55% sensitivity and 97% specificity. (Jansen A. et al., 2002: J Rheumatol 29:2074-2076,2002)

• 318 patients with undifferentiated arthritis followed for 3 years. 40% of these developed RA. 93% of anti-CCP2 positive and 25% of anti-CCP2 negative patients developed RA. (Van Gaalen F et al.: Arthritis Rheum 50:709-715,2004)

Production of anti-citrullin antibodies before onset of clinical RA symptoms.

• Anti-CCP1 were found in 39% of donors at a median of 5.3 years before onset of RA symptoms. Nielen M et al., Arthritis Rheum 50:380-386,2004.

• Anti-CCP2 were found in 25% of donors 1.5 to 9 years before onset of the first RA symptoms. Rantapää-Dahlqvist S et al. Arthritis Rheum 48:2741-2749,2003.

Anti-CCP were developed together with increased CRP and sPLA2 and IgM RF in blood donors developing RA. Nielen MM et al. Ann Rheum Dis 2005.
Production of anti-CCP in RA

ANNA level

Onset of first clinical symptoms

Cut-off value

Detection limit

Diagnosis

Time (Years)

AW 2006

Decrease of anti-CCP after therapy?

• Decrease was seen after 6 months infliximab/MTX in parallel with IgM RF and clinical improvement.

• 17 RA patients followed over many years of therapy showed decrease of anti-CCP during remission and increases with disease activity in parallel with CRP and ESR.

Decrease of anti-CCP after therapy?

• 5 year follow-up of early RA cohort showed that anti-CCP is a stable phenotype. The first year anti-CCP levels fell after sulfasalazine therapy, not other DMARDs. After that time little changes were seen.

• 52/90 RA patients who failed DMARD treatment were given etanercept for 3 months. Mean anti-CCP levels fell 31% and IgM RF 36% during this time in parallel with CRP and disease activity.
**Prediction of RA in palindromic rheumatism**

- Salvador G et al. showed that 56% of palindromic rheumatism patients (pre-RA patients?) harboured anti-CCP. This compared with a frequency of 55% positive sera in early RA patients studied simultaneously. In contrast, only 2.5% of spondylarthropathy patients sera were positive. (Rheumatology (Oxford) 42:972-975, 2003)

**Anti-CCP in juvenile chronic arthritis**

- Anti-CCP shown in only 2 patients among 109 JCA patients from Slovenia and Italy, 1 polyarticular and 1 oligoarticular onset JCA. Avcin T et al.: Ann Rheum Dis 61:608-611, 2002.
- Anti-CCP found in 5% of JCA patients from The Czech Republic. Hromadnikova I et al. Autoimmunity, 2002.
- Anti-CCP shown in 6 of 59 JCA patients from Hong Kong, 4 of which were RF-positive with a polyarticular onset and 1 was oligo-articular onset JCA. Kwok S et al.: Scand J Rheumatol 34:359-366, 2005.

**Anti-CCP in Psoriatic arthritis**

- Anti-CCP found in 7% of 160 patients with PsA and 1 of 146 Swedish non-arthritis patients. Alenius GM et al. Ann Rheum Dis, 2005
- Anti-CCP found in 5.6% of 126 English patients with PsA. Korendowych E et al. Rheumatology, 2005.
- Anti-CCP found in 16% of 102 PsA patients from Italy. Bogliolo L et al. J Rheumatol 2005.
- Anti-CCP found in 8% of 192 Belgian PsA patients, using a chosen specificity level of 98.5% for RA. Van der Cruysen B et al. Ann Rheum Dis 2005.
Use for differential diagnostics towards RA-mimicking conditions

Towards erosive polyarthritis in SLE:

Towards hepatitis C-associated polyarthritis

All studies showed a high differential diagnostic specificity for RA!

Conclusions

• Anti-citrullinated protein/peptide antibodies (ACPA) are very specific markers for RA.
• Cyclic citrullinated peptide 2 (CCP2) acts as a sensitive artificial mimotope for ACPA antigens in solid phase assays.
• Anti-CCP antibodies are present very early in disease, sometimes before clinical onset.
• Anti-CCP levels decrease with remission induction and increase with disease exacerbation.

Conclusions ctd.

• High levels of anti-CCP antibodies are prognostic for an erosive disease course, not only in adult RA.
• Anti-CCP antibodies prevail in RA patients carrying the HLA-DR4 shared epitope, most of which are RF-positive.
• Several environmental factors influence the onset of anti-CCP positive RA (tobacco smoking, coffee consumption, alcohol consumption, exercise).
Some references

- Vossenaar E et al.: Arthritis Rheum Ther B142:R130,2004

AW 2006