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Auto-antibodies to post translationally modified type II collagen as potential biomarkers for rheumatoid arthritis

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Abstract

Objective: Collagen type II (CII), post-translationally modified by reactive oxygen species (ROS-CII), present in an inflamed joint, is an auto-antigen in rheumatoid arthritis (RA). In this study we investigated the potential use of anti-ROS-CII auto-antibodies as a biomarker.

Methods: CII was exposed to oxidants that are present in the rheumatoid joint. Auto-reactivity to ROS-CII was tested by ELISA in synovial fluid (SF) and serum samples taken from various phases of RA including: a) disease modifying anti-rheumatic drug (DMARD) naïve patients with early RA (n=85 serum); b) patients with established RA (n=80 serum and 50 SF), both DMARD responders (DMARD-R) and non-responders (DMARD-NR). As controls we used c) anti-citrullinated peptide antibodies (ACPA) positive individuals with arthralgia (n=58 serum); d) samples from patients with osteoarthritis (OA, n=49 serum and 52 SF) and e) healthy individuals (n=51 serum).

Results: Reactivity in DMARD naïve early RA to ROS-CII was significantly higher than in ACPA positive arthralgia, OA and healthy controls ($p<0.0001$), with 92.9% binders. There was no significant difference in anti-ROS-CII reactivity between ACPA positive and ACPA negative RA patients, with 93.8% and 91.6% binders, respectively. The sensitivity and specificity of binding to ROS-CII in early RA compared with HC was 92% and 98%, respectively. In established RA DMARD-NR serum reactivity was significantly higher than in DMARD-R ($p<0.0001$) with 58.3% and 70% binders compared to 7.6% and 60% in serum and SF, respectively. In longstanding RA, auto-reactivity to ROS-CII changed longitudinally.

Conclusion: Auto-antibodies to ROS-CII have the potential to become diagnostic biomarkers for RA.

Introduction

Rheumatoid arthritis (RA) is the most common autoimmune chronic arthritis and affects 0.5 to 1% of the population. This disease is characterized by chronic inflammation of the joints and is associated with synovitis and erosion of the cartilage and bone. The damage involves the action of pro-inflammatory cytokines (1), free radicals (2) and matrix metalloproteinases (MMP) (3). The high influx of metabolically active immune cells infiltrating the inflamed joints consumes increased amounts of oxygen, in association with respiratory burst and the generation of reactive oxidants. The key reactive oxygen species (ROS) present in inflamed joints are superoxide radical ($O2^{\bullet -}$), hydrogen peroxide ($H2O2$), hydroxyl radical ($^{\bullet}OH$), hypochlorous acid (HOCl), nitric oxide ($NO^{\bullet}$) and peroxynitrite ($ONOO^{-}$), which are involved in acute and chronic inflammation (4, 5). In addition, cartilage damage as a result of collagen oxidation by glycation and formation of advanced glycation end-products (AGE) are evident despite the absence of hyperglycemia (6).

Collagen type II (CII) is the principal component of human articular cartilage and thus a prominent target for chemical post-translational modification by ROS in inflamed joints. Native CII is a well-studied auto-antigen in RA (7, 8). Nevertheless, we have previously reported auto-immune reactivity against ROS-CII (9). Distinct from chemical post-translational modifications, the relevance of enzymatic post-translational modifications in modulating the immune response in RA has been demonstrated. Antibodies against cyclic citrullinated peptides (ACPA) and proteins have become important diagnostic and prognostic tools in RA (10). Notably, citrullinated CII is also part of the ACPA reactivity in many patients with RA (11-13). The origin of the citrullinated protein and its contribution to disease pathogenesis are, however, still incompletely understood. In addition, the

diagnostic sensitivity of ACPA is approximately 60%, with some centres reporting as low as 40% ACPA positivity at the time of diagnosis (14-16). In any case a significant percentage of RA patients are ACPA negative. Therefore, discovery of additional tissue-specific biomarkers that will improve diagnosis, prognosis or monitor response to therapy remains an important unmet clinical need (17).

In the present study, we explored the diagnostic potential of anti-ROS-CII in RA by testing RA samples from various stages of the disease continuum from DMARD naïve early RA to established RA. We show that anti-ROS-CII auto-reactivity is high in DMARD naïve early RA in both ACPA positive and negative patients and that it is significantly higher than in ACPA positive arthralgia, OA (either inflammatory or non-inflammatory) and healthy individuals. We further demonstrate a correlation between disease severity and ROS-CII auto-reactivity in established RA.

Patients and Methods

Patients and clinical samples

Serum samples were collected from the following centres: Karolinska Institute, Sweden; Leeds Division of Rheumatology and Musculoskeletal diseases, UK; Barts Hospital in London, UK; Kennedy Institute of Rheumatology, UK and University of Pavia School of Medicine, Italy. Patients were defined by ACR 1987 criteria and the diagnosis was made by a specialist rheumatologist. Ethical approval was obtained from all clinical centres involved, and informed consent was obtained from all individuals prior to collecting blood or synovial fluid (SF) samples. SF samples were collected during knee arthroscopy or directly by knee joint aspiration.

Tested samples were categorised as follows:

1) Disease Modifying Anti Rheumatic Drug (**DMARD)-naïve early RA** patients, with <12 months symptom duration. Bloods were taken at the first visit to a specialist clinic and before the use of any DMARD (n=85 serum samples). Patients were either ACPA positive (n=49) or ACPA negative (n=36).

2) Samples from established RA patients with disease duration of more than one year were grouped into: 2a) **DMARD responders (DMARD-R,** n=26 serum and n=10 SF**)**: achieving low disease activity (LDAS<3.2) after treatment with DMARD: mono-therapy (methotrexate or sulfasalazine) or a combination of MTX with sulfasalazine or prednisolone; and 2b) **DMARD non-responders** (DMARD-NR, n=24 serum and n=10 SF) with DAS remaining ≥3.2 despite treatment. At the time of sampling, patients had already failed escalation therapy with DMARDs (n=16) or had been already treated with anti-TNF (n=7) or rituxan (n=1).

3) Longitudinal follow up of established longstanding RA: matched SF and serum from 30 established RA patients who were followed longitudinally for up to forty three years (2-11 samples per patient). 15 patients were ACPA positive and 15 were ACPA negative. Patients were grouped into individuals with high disease activity with DAS28≥5.1; medium with 3.2≤DAS28≤5.1 and low disease activity with DAS28≤3.2. All patients except n=2 had medium and low disease activity with DAS28≥3.2. Most patients were already treated with biologics at the time of sampling.

Blood samples and DAS28 measurement were taken in the clinic at the time of visit for group 2 and 3.

Control groups included:

4) ACPA positive individuals presenting with arthralgia but with no clinical evidence of synovitis (n=58 serum samples). Individuals were recruited from patients with new musculoskeletal pain complaints (usually involving 1 or 2 joint) or arthralgia. When patients were ACPA positive by the CCP-2 test, they were seen by an experienced rheumatologist, who established the absence of clinical evidence of synovitis. Normal levels of CRP<10 mg/L (according to local range) was also confirmed. 11 out of 58 individuals had signs of OA but were followed for 36 months with no sign of synovitis.

5) OA serum samples (n=49) and OA SF (n=52) from patients with ACR criteria of OA knee (18). When detailed clinical information was available, OA patients were classified as inflammatory or not according to the symptoms and the presence or absence of clinical synovitis and/or joint effusion. Patients with severe knee pain during any level of physical activity and which disturbed sleep on a daily basis, or who had persistent joint effusion despite intra-articular steroid and oral anti-inflammatories were classed as inflammatory. Patients with only intermittent mild knee pain

and/or swelling which responded to quadriceps strengthening exercises and/or paracetamol were classed as non-inflammatory OA.

6) Sex and age matched serum samples from healthy individuals (HC, n=51) were collected from a range of volunteers with no inflammatory joint disease reported. OA may be present in a few older individuals but with no required medication to alleviate their symptoms. CCP-2 test was negative in all HC individuals (rheumatoid factor (RF) data was not available).

Enzyme-linked immunoabsorbent assay (ELISA)

CII was chemically modified as previously described to generate CII post-translationally modified by HOCl and ribose (9). Bovine serum albumin (BSA, Sigma) and human serum albumin (HSA, Sigma) were similarly modified and were used as control antigens. The results section shows the data for CII modified by glycation/glycoxidation: glycated CII (Gly-CII) and CII modified by HOCl (HOCl-CII) as an example for ROS-CII in comparison to native CII (NT-CII). ROS-CII encompasses glycated CII and HOCl-CII.

An ELISA was performed using the ROS-CII or native CII as targets as described previously (9). Briefly, ELISA plates were coated with 10µg/ml of ROS-CII or native CII as bait to bind auto-antibodies from SF or serum samples. The ELISA optical density measurements (O.D) obtained for BSA, ROS-modified BSA (ROS-BSA), HSA, and ROS-modified HSA (ROS-HSA) were used as background controls to normalise the respective ELISA-OD for native and ROS-CII. In addition, to control the assay fluctuation we performed the ELISA using the same batch of modified-CII for any groups that were going to be compared. Each assay included a known reference positive or negative control sample. Longitudinal samples from the same individual (serum and SF) were tested on the same day using the same batch of ROS-CII.

In the absence of absolute standards (as for the CCP2 kits), titres could not be measured by the ELISA. Arbitrary OD values were therefore used. Patients positive for anti-ROS-CII auto-antibodies (later abbreviated as "binders") were therefore defined using the mean of the ELISA ODs from the healthy controls plus 3 standard deviations (SDs) as a cut-off, set to O.D.=0.24.

When paired serum and SF were tested, arbitrary ELISA OD units were normalised to their respective IgG levels. IgG levels were measured using Human IgG ELISA Quantitation set (Cambridge Bioscience, Cambridge, UK) following the manufacturer's instructions.

An ACPA ELISA was performed using the anti-CCP2 test kit and according to the manufacturer's instructions using Axis-Shield Diagnostics Limited, (Dundee, UK) for the UK samples or Eurodiagnostica, (Malmö, Sweden), for the Swedish samples. The cut-off was set according to the manufacturers' instructions (5 IU/ml and 25 IU/ml for Axis-Shield and Eurodiagnostica, respectively).

Statistical analysis

Variables were not normally distributed, therefore non-parametric tests were used. The Wilcoxon signed rank sum test was used to compare the reactivity between native and ROS-CII, while Mann-Whitney tests were used to compare between the various groups. Correlation was measured using Spearman test, a nonparametric correlation test. To determine diagnostic discrimination between early RA, arthralgia, OA and HC, we used the cut-off point of 0.24 OD units to construct a contingency table of positive autoantibodies against clinical diagnosis (early RA versus healthy control; early RA versus arthralgia; early RA versus OA) and tested it by Fisher's Exact Test. A nonparametric Wilcoxon-type test was used to test the longitudinal follow up of anti-ROS-CII reactivity across time for each individual (19). A mixed model was also fitted to the longitudinal

data to investigate whether time since diagnosis was a significant predictor of anti-ROS-CII reactivity. Statistical analysis was performed using the GraphPad Prism software package (GraphPad Software, San Diego, CA) and Stata 12 (StataCorp. 2011. Stata Statistical Software: Release 12. College Station, TX: StataCorp LP).

RESULTS

Binding to ROS-CII in samples from early RA versus HC, arthralgia and OA

Reactivity in DMARD naïve early RA to ROS-CII was significantly higher than in ACPA positive arthralgia, OA and HC (Fig.1, $p<0.0001$) and irrespective of ACPA status with 92.9% binders to HOCl-CII and 64.7% to Glycated CII (Table 1). In contrast, binding to native CII was significantly lower than binding to ROS-CII ($p<0.0001$) with only 18.8% binders. There was no significant difference ($p>0.05$) in binding to ROS-CII between the DMARD naïve early RA ACPA positive (n=49) and ACPA negative (n=36) with 93.8% and 91.6% binders to HOCl-CII, respectively (Fig. 1, Table 1). Reactivity to glycated CII was however slightly higher in ACPA positive than in ACPA negative with 71.4% versus 55.5% binders ($p=0.024$). In ACPA positive arthralgia, we observed significantly higher binding to HOCl-CII than in healthy controls ($p<0.001$) with 6.8% binders, while only 3.4% bound to glycated and 1.7% to native CII (Fig 1). Although patients with OA had significantly lower reactivity in comparison to early RA ($p<0.001$), 34.6% bound to glycated CII (Fig.1), but only 20.4% bound to HOCl-CII (Table 1). There were no significant gender or age differences between the HC versus OA, arthralgia and early RA DMARD naïve patients (Table 1).

The sensitivity and specificity of the binding of autoantibodies to ROS-CII (both glycated CII and HOCl-CII) in early RA compared with HC was 92% and 98%, respectively (OR 671 [95% CI 78.4-5745], $p<0.0001$); for early RA compared with arthralgia sensitivity and specificity was 92% and 93%, respectively (OR 177.8 [95% CI 47.8 - 660.1], $p<0.0001$). In respect to OA, specificity and sensitivity for anti-CII-HOCl reactivity were 75% and 92% (OR 40.6 [95% CI 14.1-116,6], $p<0.0001$). However, the reactivity to glycated CII was less specific in early RA compared to OA

with sensitivity of and specificity of 64% and 65%, respectively. (OR 3.45 [95% CI 1.65-7.2], p=0.0011).

In order to examine whether the presence of auto-reactivity to modified collagen was a novel diagnostic biomarker, we investigated whether autoantibodies were associated with disease activity or levels of inflammation reflected by DAS28 values. There was no relationship between anti-ROS-CII ODs and DAS28 whether ACPA was negative or positive (ρ=0.03, ρ=-0.10, for ACPA+ or ACPA-, respectively; p>0.58, Fig.1B).

Anti-ROS-CII reactivity in patients with established RA

Despite the similarity in age and sex between the DMARD-R and DMARD-NR groups (Table 1), there was a striking difference in the observed auto-immune-reactivity towards ROS-CII in the tested serum samples (Fig. 2). The strongest reactivity was seen in DMARD-NR with 58.3% binders to HOCl-CII versus 7.6% in DMARD-R (p<0.01, Table 1). Similarly, SF samples categorised according to patients' response to DMARD displayed the same pattern of reactivity, but it was quite apparent that the levels of anti-ROS-CII auto-response in DMARD-NR and DMARD-R SF was significantly higher than in serum (p<0.05 and p<0.007, respectively), with 70% and 60% binders to HOCl-CII in DMARD-NR and DMARD-R, respectively (Fig. 2A). To confirm that the increased binding in SF was not an artefact related to the difference in levels of immunoglobulin in SF versus blood, we tested a set of paired SF and serum samples where binding to HOCl-CII was normalised according to their corresponding levels of IgG. Increased anti-ROS-CII reactivity in the SF compared to serum was further confirmed in the paired SF and serum samples (Fig. 2B, p=0.001).

Patients were both ACPA positive and negative. No association between anti-ROS-CII reactivity and ACPA status for either DMARD-NR or DMARD-R was observed (ρ=0.32, ρ=-0.22, for DMARD-NR or DMARD-R, respectively; p>0.134) or CRP (ρ=0.08, ρ=0.12, for DMARD-NR or

DMARD-R, respectively; p>0.74) (Fig2C). In addition, reactivity in RF positive patients was similar to the reactivity in the RF negative patients (p>0.05, Fig 2C).

Anti-ROS-CII binding in inflammatory OA versus non-inflammatory OA

The presence of anti-ROS-CII antibodies was examined in more details in OA with respect to the presence of clinical evidence of synovitis, severe pain or persistent effusion (Table 1, Fig.3A). Reactivity to HOCl-CII in serum samples from severe inflammatory OA and mild non-inflammatory OA was low with 28.6% and 13.8% binders, respectively. Reactivity to glycated CII was however higher with 50% and 30% binders to glycated CII in severe inflammatory OA and mild non-inflammatory OA, respectively (Fig 3A, Table 1). In addition, the pattern of binding to ROS-CII in OA was different from RA with a tendency for higher reactivity in the serum than in the SF, the opposite of the situation in RA. In contrast to RA, matched OA SF and serum samples showed no tendency towards higher reactivity in SF and samples displayed either higher or lower reactivity (p=0.272, Fig 3B).

Longitudinal study of ROS-reactivity in patients with chronic longstanding disease

To further study the correlation between anti-ROS-CII reactivity and disease evolution, we analysed matched SF and serum samples from 30 patients with chronic RA collected longitudinally. As seen in Fig. 4A-B the anti-HOCl-CII reactivity varies considerably, similar variability was shown also for glycated CII (data not shown). A trend of higher reactivity was observed in the SF of the ACPA positive group compared to the SF of the ACPA negative group. Anti-ROS-CII reactivity in the serum of both groups was, however, similar (Fig. 4B). Nevertheless, no correlation between levels of ROS-CII auto-reactivity and disease activity (DAS28) at the time of sampling was found,

(ρ=0.3006; p=0.2173 and ρ=0.31718; p=0.1736 for SF and serum from ACPA negative patients and ρ=-0.04581; p=0.8134 and ρ=0.0696; p=0.5346 for serum and SF from ACPA positive patients, DAS28 was not available for all tested samples, Fig.4C).

A nonparametric test for trend stratified by individual showed no evidence of a trend in anti-ROS-CII reactivity over time (p=0.634). Similarly, when a mixed model was fitted to the data, time since diagnosis was not a significant predictor of anti-ROS-CII reactivity, neither in the unadjusted model (p=0.834) nor when adjusted for ACPA status and DAS category (p=0.631).

Discussion

A common biomarker for RA diagnosis is the presence of ACPA (10) and to a lesser extent, RF. ACPA and/or RF in combination with elevated levels of cytokines/chemokines, ESR and CRP are predictive biomarkers for RA development (20). Only 60~70% of individuals with RA are ACPA positive (15, 21)with some centre reporting as low as ~40% sensitivity of an ACPA test in individuals awaiting a diagnosis for RA (22, 23). Furthermore, levels of ACPA do not change significantly during disease progression even after treatments, although a limited reduction (~20%) in ACPA levels can be observed after rituximab therapy (24-26). Therefore, RA diagnosis may benefit from the use of additional tissue and disease-specific biomarkers that may facilitate improved diagnosis and more accurate monitoring of treatment efficacy.

CII is the predominant cartilage collagen and a known auto-antigen (7, 8). Thus, antibodies to CII could potentially be the most relevant diagnostic test for RA. Unfortunately, anti-native CII antibodies occur only in 3 - 27% of patients with RA (27-30). Recent studies demonstrated that antibodies to citrullinated-CII are common in RA (11-13). We previously developed an approach that tests autoimmune reactivity towards CII neoepitopes that result from some of the pathogenic processes in the inflamed joint, namely collagen type II post-translationally modified by reactive oxidants: ROS-CII. We observed increased binding to ROS-CII in RA serum samples, but no anti-ROS-CII reactivity was detected in other inflammatory arthritis conditions such as psoriatic arthritis, systemic lupus erythematosus (SLE), ankylosing spondylitis, palindromic arthritis, scleroderma, Behçet's disease, primary Sjögren's syndrome, fibromyalgia, inflammatory arthritis, tendonitis and reactive arthritis (9).

In this study, we analysed the anti-ROS-CII auto-reactivity in patients at various stages of the disease and studied the potential of anti-ROS-CII as a diagnostic/prognostic biomarker for RA. In early RA, before treatment with DMARD, only 18% of patients bound to native CII. In contrast, a high proportion of DMARD naïve early RA patients had auto-reactivity to ROS-CII (92.9% binders, Fig 1A) suggesting that a routine test would have high detection power. Importantly, in the group of patients who were ACPA negative, we observed a similar proportion of binders as in the ACPA positive patients with 91.6% versus 93.8% binders to HOCl-CII, respectively, suggesting the potential use of anti-ROS-CII as an ACPA independent serum biomarker. To investigate the specificity of anti-ROS-CII reactivity for RA we have used, apart from healthy control individuals, two control disease groups: ACPA positive arthralgia individuals that had no clinical evidence of synovitis and OA patients with or without sign of knee inflammation. Reactivity in DMARD naïve early RA to ROS-CII was significantly higher than in ACPA positive arthralgia, OA and healthy controls (Fig.1, $p<0.0001$). Hence anti-HOCl-CII reactivity is highly specific and sensitive with specificity and sensitivity >75%. For glycated CII sensitivity and specificity against OA was reduced to 64% and 65% respectively due to background anti-glycated CII reactivity in OA, thus suggesting a potential development of anti-HOCl-CII test for further validation as an RA diagnostic.

Only low levels of anti-ROS-CII reactivity were detected in 6.8% of ACPA positive arthralgia individuals, thus suggesting that anti-ROS-CII may appear closer to the clinical synovitis onset, possibly with different dynamics when compared to ACPA that in many cases appears years before the clinical onset of RA (31, 32). In the absence of full information as to whether and when the onset of clinical symptoms may occur in all of these individuals (this cohort is still being followed),

it is difficult to estimate precisely the predictive value of the low ROS-CII auto-reactivity in ACPA positive arthralgia group. So far, 2 of the 4 positive participants progressed to RA within one month of the follow up and a third positive patient developed RA three months later. In contrast 18 anti-ROS-CII negative individuals progressed to RA over longer periods of time (median 7 months). In any case, our data showed that anti-ROS-CII reactivity is independent of ACPA, and suggests the need to perform a more comprehensive study to establish when the anti-ROS-CII reactivity appears and whether it provides additional insight into the clinical development of RA.

Although OA is not a systemic inflammatory disease, synovial inflammation is highly prevalent albeit not as severe as in RA (33). Chondrocytes, synoviocytes and infiltrating immune cells produce similar inflammatory mediators in an OA joint to those present in inflamed RA joints. OA chondrocytes are metabolically active and produce high levels of ROS (33, 34). In fact, in both OA and RA, cartilage damage as a result of collagen oxidation by glycation and formation of advanced glycation end-products (AGE) is evident (35). Therefore, although OA is not considered an autoimmune inflammatory disease, it was interesting to study whether post-translational modification of CII and thus formation of neoepitopes could stimulate an immune response in OA. Although binding of OA samples to ROS-CII was significantly lower than in RA, reactivity to ROS-CII in serum samples from inflammatory OA was higher compared to non-inflammatory OA (Fig 3A, Table 1). The elevated reactivity to glycated CII is interesting and might represent a screening test for present of arthropathy as opposed to chronic pain/arthralgia. This will be addressed in a future study. ACPA-positivity was also observed in some of these OA patients, more frequently in SF (21%) than in serum (6%). In fact, the presence of ACPA in OA and in other joint diseases (36) is slowly being acknowledged.

In our cohort of established RA patients on DMARD (despite small numbers), it appears that most patients without reactivity to ROS-CII have a better outcome to DMARD treatments. The strongest reactivity toward ROS-CII was in patients who were DMARD-NR (Fig. 2A) independently of ACPA, CRP and RF status (Fig.2C). In addition, 34% of ACPA and RF double negative patients were anti-ROS-CII positive.

Interestingly, although displaying the same pattern, anti-ROS-CII reactivity in SF was higher than in serum (Fig. 2A-B, $p<0.007$ and $p=0.04$ for DMARD-R and DMARD-NR, respectively). The fact that higher levels of anti-ROS-II are present in SF may be explained by the local presence of the target CII neoepitopes in the inflamed joint and thus local production and/or local retention of these antibodies (37). Due to high infiltration of immune cells in the inflamed RA joint (including B-cells), as well as the formation of ectopic lymphoid structures in RA (38), local production of anti-ROS-CII in RA joints is possible. In addition, the presence of cartilage debris, possibly modified by the high local levels of ROS (33, 39), may trigger localisation of anti-ROS-CII antibodies in the inflamed joints. Interestingly, reactivity in OA SF was similar/lower than in OA serum (Fig.3, $p>0.05$). The lower reactivity in OA SF may reflect the lack of local ectopic lymphoid formation and local anti-ROS-CII formation. Probably systemic auto-immunity to ROS-CII is stimulated by the breach of tolerance as a result of post-translational modification and formation of neoantigens. This process spreads to the inflamed OA joint but is intensified only in RA joints due to the formation of ectopic germinal centres in the RA joints.

To investigate the changes in anti-ROS-CII auto-reactivity, samples collected longitudinally over 1-43 years after the onset of RA were analysed showing that anti-ROS-CII reactivity changed over

time with at least one peak (Fig4. A-B). This is in contrast to anti-native CII antibodies that occur in some patients with RA at the time of diagnosis and thereafter the levels tend to decrease during the first few years of disease (40). In our hands, only 2 patients displayed low anti-native CII reactivity at this late stage of the disease. Once again, in this group of patients we observed anti-ROS-CII reactivity in both ACPA positive and ACPA negative patients. There was no correlation between serum levels of ROS-CII auto-reactivity and DAS28 (Fig. 4C). Nevertheless, we had only 2 patients with low disease activity and only 5 measures with DAS28≤3.2 at some time points in patients with high or medium disease activity. Therefore we were unable to carry out a statistical analysis to compare patients with low disease activity (DAS28≤3.2) with those with high/medium disease activity (DAS28≥3.2) in this cohort. Furthermore, higher levels of anti-ROS-CII in SF were observed in ACPA positive patients with high disease activity suggesting that ROS-CII auto-antibodies in SF may reflect tissue/cartilage damage more closely.

Our data suggest a lack of direct correlation between anti-ROS-CII reactivity and DAS28 in active disease (DAS28≥3.2). A potential interpretation of this observation is that once the disease was active (DAS28≥3.2) the redox state was unbalanced (i.e. oxidative stress) such that there was a burst in oxidative reactions that result in the formation of ROS-CII neoepitopes. In turn, this perhaps lead to an auto-immune response regardless of further fluctuations in DAS28. In those patients who respond to DMARDs, achieving DAS28≤3.2, the redox state was re-balanced with a consequent reduction of ROS-CII neoepitopes production and thus a lowering of anti-ROS-CII autoimmunity (Fig.2). Therefore, anti-ROS-CII reactivity does not simply reflect the degree of inflammation. This is suggested by the fact that anti-ROS-CII reactivity did not have a direct

correlation with either DAS28 or CRP at any stage of the disease whether early, established or longstanding RA.

The lack of association between anti-ROS-CII and inflammation is supported by our previous study (9) that detected no anti-ROS-CII reactivity in other inflammatory arthritis conditions. In addition, in a parallel recent study we have detected anti-ROS-CII reactivity in type 1 autoimmune diabetes patients who were HLA-DRB1*4+ and carried the shared epitope alleles associated with susceptibility to both type 1 diabetes and RA. Interestingly, anti-ROS-CII reactivity in HLA-DRB1*4 negative and type 2 diabetes was low/absent (41). Our finding will need to be addressed in greater depth in future studies using much larger cross-sectional and longitudinal cohorts versus large numbers of disease control patients with inflammatory arthritis distinct from RA to exclude the possibility that the current study lacked the power to detect a meaningful biological association between anti-ROS-CII and inflammatory markers.

In conclusion, our results imply that anti-ROS-CII auto-antibodies may provide a novel, serological biomarker that can: a) facilitate RA diagnosis, particularly in the ACPA negative patients; b) lead to better RA subgrouping and c) possibly may provide an additional criterion to define remission. The identification of the exact ROS-CII neo-epitope(s), the mechanism which results in the anti-ROS-CII reactivity and validation of its potential value as a novel RA specific biomarker will require further studies. It appears to be as a promising new member of the family of post-translationally modified protein which are able to elicit auto-immune responses.

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	Group	n	Age	Gender (% F)	Binders (%)		
					NT-CII	GLY-CII	HOCl-CII
RA serum	**Early RA**	85	51 (22-70)	72	18.8	64.7	92.9
	Early RA ACPA-	36	53 (29-70)	66	19.4	55.5	91.6
	Early RA ACPA+	49	48 (22-69)	78	18.3	71.4	93.8
	DMARD-R	26	55 (23-84)	76	7.6	7.6	7.6
	DMARD-NR	24	56 (31-81)	75	37.5	58.3	58.3
RA SF	**DMARD-R**	10	46 (21-75)	80	30.0	50.0	60.0
	DMARD-NR	10	48 (48-71)	100	60.0	70.0	70.0
Controls							
Arthralgia serum	**ACPA positive**	58	53 (27-76)	83	1.7	3.4	6.8
OA serum	**OA**	49	61.5 (27-81)	72	8.1	34.6	20.4
	no synovitis	35	61 (27-81)	67	0.0	30.0	13.8
	with synovitis	14	65 (29-75)	78	21.4	50.0	28.6
OA SF	**no synovitis**	36	60 (39-89)	50	0.0	0.0	0.0
	with synovitis	16	58 (43-78)	50	4.1	25.0	16.6
Healthy serum	**Healthy**	51	48 (26-70)	66	0.0	0.0	0.0

Table 1. Distribution of binders to ROS-CII

DMARD-R: established RA patients that respond to DMARD; DMARD-NR: established RA patients that did not respond to DMARD; SF: synovial fluid; Inflammatory OA is sever OA and non-inflammatory OA is mild OA.

oxidant	Chemical reaction	Key amino acids modified	Resulting "ROS-CII" modification
HOCl	Oxidation	Lysine Methionine Cysteine	a) Modified amino acids within the polypeptide backbone of CII, including lysine chloramines, methionine sulfoxide, cysteine sulfinic acid and disulfides b) Fragmentation c) Crosslinking d) Aggregation
Ribose	Glycation and glycoxidation	Lysine Arginine	**a**) Modified amino acids within the polypeptide backbone of CII, in the form of advanced glycation end-products (AGEs)and glycoxidation products, e.g. N^{ϵ}-carboxymethyllysine, N^{ϵ}-carboxyethyl-lysine and pentosidine **b**) Fragmentation **c**)Crosslinking **d**) Aggregation

Table 2. Chemical post-translational modifications of CII. The main chemical reactions resulting from the exposure of proteins to the strong oxidant, HOCl, are the oxidation of methionine, cysteine and lysine residues (42) A minor reaction is the chlorination of aromatic amino acids, in particular tyrosine residues, generating modified forms of this amino acid – including 3-chlorotyrosine - within the polypeptide backbone (42) Exposure of CII to ribose results in the formation of advanced glycation end-products (AGEs) and glycoxidation products within the target protein (43). These products – which include pentosidine - are formed through Maillard reactions between reactive carbonyl groups and basic amino acid side chains such as lysine and arginine (4).

Figure legends

Figure.1. Binding to ROS-CII in serum samples from early RA versus control arthralgia, osteoarthritis (OA) and healthy control (HC).

Early RA samples were taken from patients less than 12 months after diagnosis and before the use of any DMARD. Samples were grouped according to the presence of antibodies to citrullinated peptide (ACPA): ACPA positive (ACPA+, n=49) and ACPA negative (ACPA-, n=36). Reactivity in the early RA patients was significantly higher than in arthralgia, OA and HC ($p<0.0001$). In addition, regardless of ACPA + or ACPA-, reactivity to ROS-CII was significantly higher than to native CII ($p<0.001$). NT-CII is native CII, GLY is CII modified by ribose and HOCl is CII modified by HOCl.

B. No correlation between levels of anti-ROS-CII reactivity and levels of DAS28 were observed whether ACPA positive (black triangle) or ACPA negative (white square) ($\rho=0.03$, $\rho=-0.10$, for ACPA+ and ACPA-, respectively; $p>0.58$).

Figure 2. Anti-ROS-CII reactivity in samples from established RA patients.

A. Serum and synovial fluid (SF) samples from patients with established RA were grouped into those patients who responded to DMARD (DMARD-R) and patients who did not respond to DMARD (DMARD-NR). Higher reactivity was observed in DMARD-NR ($p<0.01$) in both serum and SF samples. Higher ROS-CII auto-response in SF was also observed compared to serum ($p<0.05$). NT-CII is native CII, GLY is CII modified by ribose and HOCl is CII modified by HOCl.

B. Anti-ROS-CII reactivity was tested in matched SF and serum samples. Levels of anti-ROS-CII reactivity were normalised to total levels of IgG in matched serum and SF. Higher reactivity in SF was seen ($p=0.001$).

C. No correlation between levels of anti-ROS-CII reactivity and levels of ACPA (ρ=0.32, ρ=-0.22, for DMARD-NR or DMARD-R, respectively; $p>0.134$) and CRP (ρ=0.08, ρ=0.12, for DMARD-NR or DMARD-R, respectively; $p>0.74$) were observed whether DMARD-R (black triangle) or DMARD-NR (white triangle). In addition, reactivity in RF positive patients (black circle, RF+) was similar to reactivity in RF negative samples (white diamond, RF-, $p>0.05$).

Figure 3. Anti-ROS-CII reactivity in serum and synovial fluid (SF) from patients with osteoarthritis (OA).

A. The binding to ROS-CII in OA was distinct from RA with tendency for higher reactivity in the serum. Samples from inflammatory (INF) OA had higher reactivity to ROS-CII compared to non-inflammatory (NON) OA ($p<0.005$ for SF and $p=0.05$ for serum).

B. Matched OA SF and serum samples displayed no significant difference in reactivity ($p=0.272$), with samples displaying either higher or lower reactivity in SF versus serum. Levels of anti-ROS-CII reactivity in matched serum and SF were normalised to their respective total levels of IgG.

Figure 4. Longitudinal follow up of anti-ROS-CII auto-reactivity in chronic longstanding RA.

A. Anti-ROS-CII reactivity in four different patients is shown as examples to demonstrate the longitudinal changes. Reactivity in SF (black square) is higher than in serum (white circle) in the ACPA positive samples but similar or lower compared to serum in the ACPA negative samples. High and medium are patients with high or medium disease activity, respectively.

B. ELISA O.D for all tested samples is displayed as a gradient from black to light grey colour representing the highest and lowest OD, respectively. Matched SF and serum samples collected from each patient are shown as pairs (F represents the SF and S the serum). In the SF a trend for higher OD was observed in the ACPA positive group but not in ACPA negative group.

C. No correlation between DAS28 and anti-ROS-CII reactivity was observed in both serum (white circle) and SF (black square) in both ACPA positive and ACPA negative patients (ρ=0.3006; p=2173 and ρ=0.31718; p=0.1736 for SF and serum from ACPA negative and ρ=0.04581; p=0.8134 and ρ=0.0696; p=0.5346 for serum and SF from ACPA positive patients).

Patients were grouped into high (DAS28>5.1), medium (3.2<DAS28<5.1), low (2.6<DAS28<3.2) disease activity and with 2 unknown.

Figures

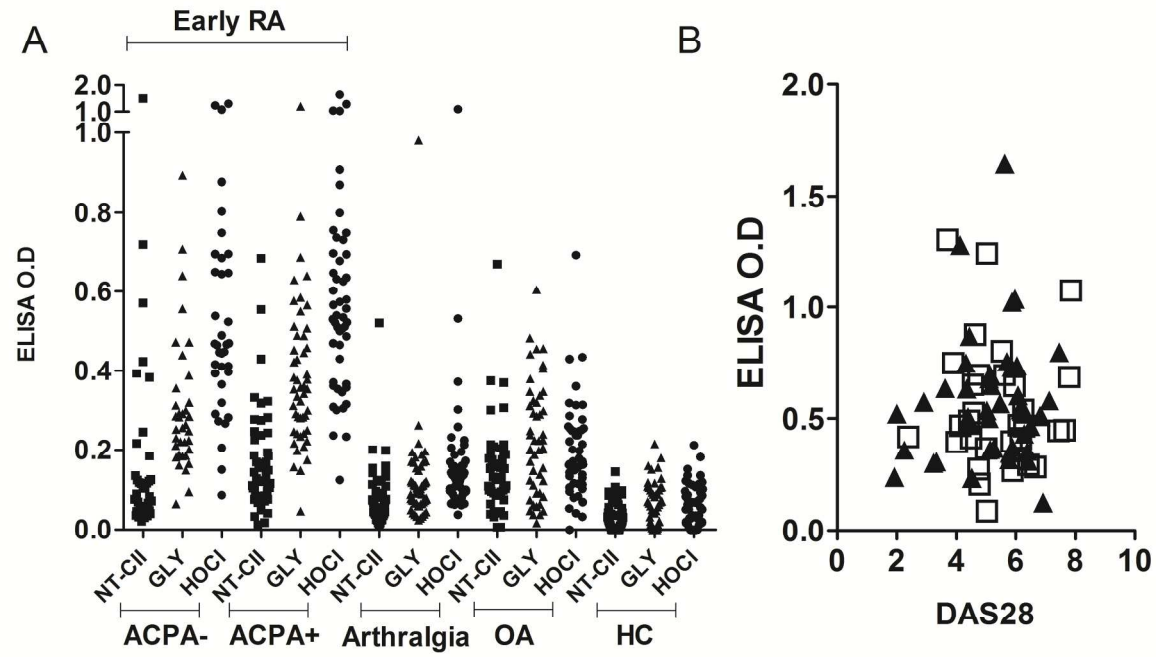

Figure.1.

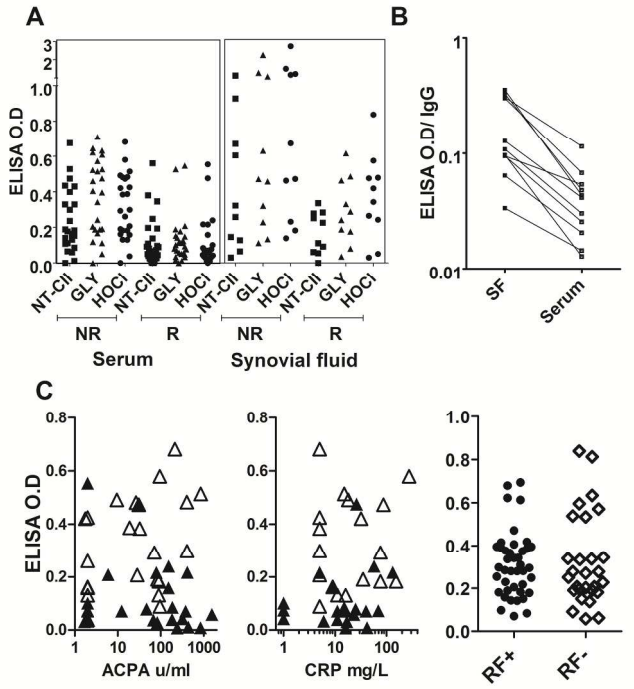

Figure 2.

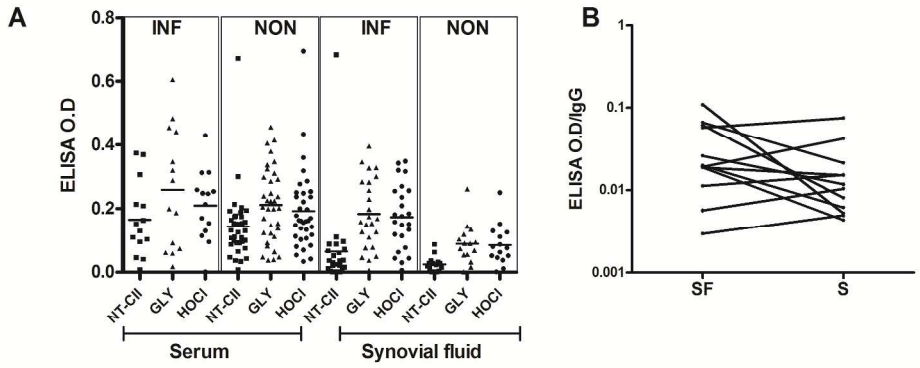

Figure 3.

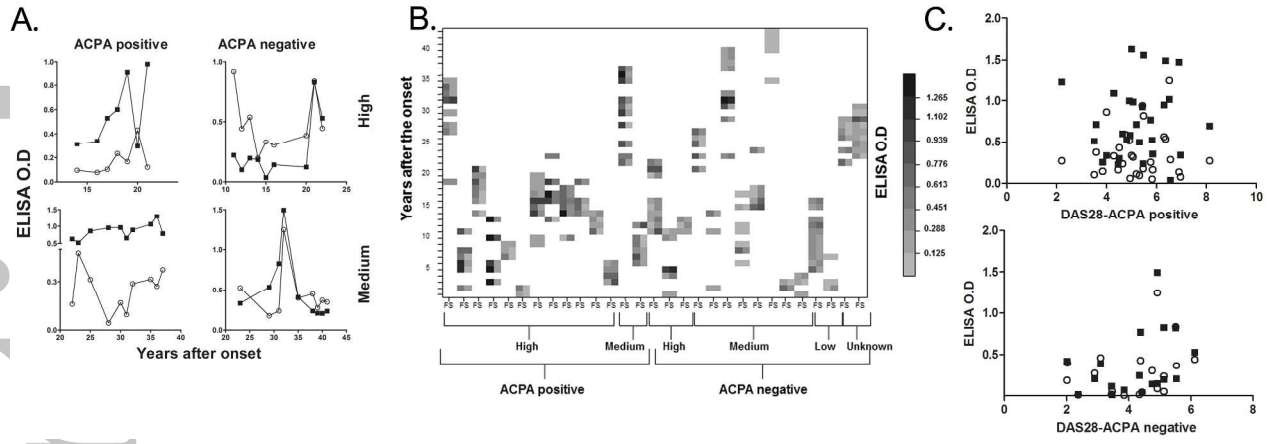

Figure 4.