IgA antibodies to oxidized collagen type II as a potential biomarker for the stratification of spondyloarthritis from rheumatoid arthritis

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ABSTRACT

Background

Spondyloarthropathies (SpA) comprise an inflammatory spectrum that can involve peripheral and axial joints, enthesial sites and extra-articular tissues. We hypothesized that oxidation of fibrocartilage matrix proteins collagen type II (CII) at enthesial sites might generate oxidative post-translational modification (oxPTM)-associated neoepitopes that provoke humoral autoimmunity. Our objectives were to test for the presence and clinical significance of antibodies to oxPTM-CII in patients with SpA.

Methods

Levels of antibodies specific to native CII and oxPTM-CII were assessed by enzyme-linked immunosorbent assays (ELISA). Reactivity in serum samples obtained from patients with axial SpA (axSpA, n=242) was compared to reactivity in samples from patients with predominantly peripheral arthritis such as psoriatic arthritis (PsA, n=69), undifferentiated arthritis (UA, n=48) and early rheumatoid arthritis (RA, n=60). Controls included psoriasis without musculoskeletal symptoms (Ps, n=35), fibromyalgia (FM, n=19) and healthy subjects (HC, n=178). 97^{th} percentile of the healthy individuals cut-off point absorbance units obtained for healthy control samples was used to construct a contingency table of positive binders to oxPTM-CII and tested it by Fishers Exact Test.

Results

IgG binding to oxPTM-CII was observed in serum samples from axSpA (52%) compared to RA (83%), PsA (28%), UA (35%), and FM (15%). Importantly, while strong IgA anti-oxPTM-CII was detected in axSpA and PsA patients, with 47% and 84% respective binders, no IgA anti-oxPTM-CII was detected in RA patients. IgA anti-oxPTM-CII reactivity in axSpA patients treated with biologics was higher and more frequent with 85% binders compared to 9% binders in patients treated with synthetic DMARDs. Sensitivity values for both IgG and IgA anti-oxPTM-CII for RA samples were

91% and 32%, respectively. IgG and IgA anti-oxPTM-CII for axSpA were 64% and 80%, respectively. Similartly, sensitivity for IgA anti-oxPTM-CII in PsA was doubled to 87%.

Conclusions:

Our data implies that axSpA is associated with the presence of antibodies specific for oxPTM-CII, suggesting that there may be a humoral component to disease-associated inflammation that may stratify patients with SpA from RA.

Keywords: post-translational modifications; collagen type II; reactive oxidants; spondyloarthritis; biomarker

Introduction

Spondyloarthropathies (SpA) comprise of inflammatory disorders that can involve peripheral and axial joints, enthesial sites and extra-articular tissues including the eye, skin and gut (1-3). SpA can be divided usefully into two main groups, namely axial SpA (axSpA) affecting predominantly the spine and sacroiliac joints, and peripheral SpA (primarily peripheral joints and/or entheses). Classification criteria for SpA currently include clinical symptoms and radiographic findings (4). Axial SpA is often misdiagnosed or undiagnosed for several years following the onset of clinical symptoms (5). The delay between the appearance of first symptoms and diagnosis of axial SpA is approximately 5 to 10 years (6, 7), meaning that an opportunity for early intervention may be missed.

SpA is not generally considered to be driven by humoral immune pathways and there is not yet a characteristic gold standard auto-antigen(s)/auto-reactivity described for SpA. This is in contrast to RA that is characterised by the presence of anti–citrullinated protein antibodies (ACPA), and other autoantibodies recognising post-translationally modified proteins (e.g. via acetylation or carbamylation) (8). Recent studies reported on elevated levels of antibodies to human leukocyte antigen class II-associated invariant chain peptide (anti-CD74) in axSpA (9, 10). There was, however, a lack of sufficient specificity for a significant diagnostic value of anti-CD74 IgA (11).

SpA involves cartilage and bone destructive processes along with new bone formation. Articular cartilage is formed primarily from collagen type II (CII) based fibrillar network complexes with the large proteoglycan aggrecan (12). Cartilage turnover in general and CII degradation in particular has therefore been investigated as possible biomarker for disease progression. A metalloproteinase-generated neoepitopes of collagen type II (C2M) and type III (C3M) have been shown to be elevated in axSpA patients (13). Vimentin, a type III intermediate filament protein that is expressed by various cells as an important part of the cytoskeleton was also shown to be elevated in patients with axSpA in correlation with CRP and spinal radiographic damage (14). Additional elevation in markers of

cartilage matrix synthesis and turnover has been demonstrated, including the 846 epitope of aggrecan and C-propeptide of collagen type II (CPII), as well as 2 markers of cartilage degradation (C2C and C1–2C) reflecting CII and collagen type I (CI) cleavage by collagenases (15). However, only low antibody activity was detected to both native and denatured CII in patients with SpA (16).

We hypothesized that oxidation of fibrocartilage matrix proteins at enthesial sites might generate oxidative post-translational modification associated neoepitopes that may provoke humoral autoimmunity. SpA comprises of progressive inflammation involving cartilaginous structures in the spine and peripheral joints. The inflamed articular environment is populated by resident immune cells likely exhibiting abnormal metabolic activities (17, 18). Notably, immune cells consume increased amounts of oxygen, leading to a respiratory burst and the generation of reactive oxidants (ROS) (19, 20). Conceivably, in the articular/entheseal inflammatory milieu in SpA, ROS over-production could lead to formation of neoantigens, as a result of oxidative post-translational modification (21, 22). Since CII is the principal component of human articular cartilage, it is a prominent target for oxidative post-translational modification by ROS (oxPTM) in the inflamed joints.

In the current study, we investigated whether antibodies to oxidized CII neoantigens, namely oxPTM-CII, were present in spondyloarthropathy (SpA): axial SpA (axSpA) affecting predominantly the spine and sacroiliac joints, and peripheral SpA (PsA). We compared such reactivity across a range of inflammatory conditions including rheumatic arthritis (RA), undifferentiated arthritis (UA), psoriasis (Ps), fibromyalgia (FM) and healthy volunteers (HC).

Methods

Patients and serum samples. Samples were collected across two sites: Rheumatology Unit of San Giovanni di Dio Hospital, Florence (Italy) and the University of Glasgow, Institute of Infection, Immunity and Inflammation. We tested 242 samples from patients with longstanding axSpA with disease duration of over 2 years. Patients with axSpA were defined according to Assessment of Spondyloarthritis International Society (ASAS) criteria (23). Patients were receiving a range of different drugs including synthetic and biologic DMARDs (including infliximab, adalimumab, etanercept, certolizumab pegol or secukinumab). Among the samples tested, 10 patients had axSpA in association with inflammatory bowel disease (IBD); 7 had axSpA and oligoarthritis and 10 patients had axSpA with enthesitis. Anti-oxPTM-CII reactivity was compared to early rheumatoid arthritis (RA, n=60), undifferentiated arthritis (UA, n=48), 69 psoriatic arthritis (PsA) and 35 psoriasis (Ps, n=35) samples. As controls, 19 samples from patients with fibromyalgia (FM) and 178 healthy controls (HC) were investigated (Table 1). Disease activity was assessed in axSpa, PsA and UA via the Bath Ankylosing Spondylitis Disease Activity Index (BASDAI, (24)), American College of Rheumatology (ACR 20, (25)) and Clinical Disease Activity Index (CDAI, (26)) respectively.

The Institutional Review Board, the Health Director of San Giovanni di Dio Hospital in Florence, reviewed and approved this research and the use of clinical and laboratory data of common clinical practice, in the respect of Privacy Law, for clinical and scientific studies and publications. Ethical approvals for the collection and analysis of samples at the University of Glasgow was obtained from the West of Scotland Research Ethics Service (Institute of Infection, Immunity and Inflammation Research Tissue Bank, REC: 11/S0704/7). All patients and all healthy controls gave their written informed consent. Line

Chemical modifications of collagen type II and type III. *In vitro* chemical modifications were performed as previously described (27, 28). Briefly, 1mg/ml of bovine CII (CII is very conserved between species with 98% similarity between bovine and human CII) in phosphate buffered saline

(PBS) was chemically modified by HOCl modification by overnight incubation at 37^0C with sodium hypochlorite (NaOCl) (VWR, Leicestershire UK). As an antigen control, collagen type III (Sigma-Aldrich, Gillingham UK) was similarly modified.

Enzyme-linked immunosorbent assay (ELISA). oxPTM-CII and native CII (Nt-CII) were used as targets in ELISA as previously described (27, 28). ELISA plates were coated and incubated overnight at 4˚C with 100µl of 10µg/ml per well of oxPTM-CII or Nt-CII. Following blocking using 2% (w/v) powdered milk (Marvel) in PBS + 0.05% Tween20 (PBS-T) a 1:200 dilution of serum sample in 2% milk in PBS-T was added to each well and incubated for 2 hours. Plates were washed 3 times with PBS-T, followed by the addition of a 1:1000 anti-human IgG or 1:2500 dilution of anti-human IgA horseradish peroxidase conjugated (Sigma-Aldrich Gillingham UK) in 2% milk in PBS-T and incubation for 2 hours, respectively. After washing 3-3', 5-5' tetramethylbenzidine substrate (Sigma Gillingham UK) was added. The reaction was stopped using 0.5M sulfuric acid. The optical density (O.D.) was measured at 450 nm using a Thermofisher multiskan fc plate reader. Competition ELISA was conducted as above, except that serum samples were pre-incubated at room temperature with and without 100µl of 50µg/ml Nt-CII or oxPTM-CII or control Nt-CIII and oxPTM-CIII for 2 hours before addition to the assay. An ACPA ELISA was performed with an anti–CCP-2 test kit according to the manufacturer's instructions (Axis-Shield). The cutoff was set according to each manufacturer's instructions (5 IU/ml).

Statistical analysis. In the absence of absolute standard, titre could not be measured by ELISA and we therefore used an ELISA O.D. cut-off that was determined arbitrarily as the 97^{th} percentile of the oxPTM-CII antibodies levels detected in healthy individuals (O.D. = 0.2845 for the IgG ELISA and 0.245 for the IgA ELISA). Data analysis was performed using Prism Software version 8 (GraphPad, San Diego, CA, USA). The Mann-Whitney test was used to test and compare antibody binding between two groups or Kruskal-Wallis test to compare multiple groups. Pearson correlation

coefficients was used to test correlation between oxPTM-CII antibodies levels and markers of inflammation. To determine predictive discrimination between axSpA and healthy control groups, we used the 97th percentile of the healthy individuals as cut-off point absorbance units to construct a contingency table of positive binders to oxPTM-CII and tested it by Fishers Exact Test.

Results

Reactivity of serum samples from patients with axSpA to CII modified by oxidants (oxPTM-CII) was significantly higher than reactivity to native CII (Nt-CII), with 13% of samples with IgG anti Nt-CII and 52% of samples with IgG anti-oxPTM-CII antibodies ($p<0.0001$, Figure 1A, Table 2). Strong IgA anti-oxPTM-CII antibodies binding was, however, observed in 47% compared to 25% IgA anti-Nt-CII (Figure 1A, Table 2, $p<0.0001$). Hence, samples that responded to both Nt-CII and oxPTM-CII had stronger reactivity toward oxPTM-CII ($p<0.0001$, Figure S1). Similar to our previous studies (28), 83% RA samples revealed IgG anti-oxPTM-CII binders (Figure 1A, Table 2). Surprisingly, in RA very low IgA anti-oxPTM-CII were detected in only 7% of samples, and no IgA anti-native CII was observed (Figure 1A, Table 2). Binding to ox-PTM-CII appeared specific, as no reactivity to oxPTM collagen type III (or native CIII) was detected (Figure S2). Moreover, only oxPTM-CII but not oxPTM-CIII was competing with the binding in both axSpA and RA samples (Figure S3).

IgA anti-oxPTM-CII reactivity in axSpA samples treated with biologics (TNF or IL-17 inhibitors) were significantly higher ($p<0.001$) with 85% of binders compared to 9% of samples with IgA anti-oxPTM-CII activity in patients treated with synthetic DMARD ($p<0.0001$, Figure 1B, Table 2). The difference in IgG anti-oxPTM-CII reactivity between the different groups was not as striking (66% vs 34%, Table 2) although also statistically significant ($p<0.001$). Binding to oxPTM-CII in axSpA patients with active disease and treated with IL-17 inhibitors was similar to binding observed in patients after long term treatment with TNF blockade where disease was controlled. Clinical state was assessed according to Assessment of Spondyloarthritis International Society (ASAS) or previous diagnosis by an independent Consultant Rheumatologist. ASDAS lower than 2 was considered as remission. Similarly, patients in remission after treatment with synthetic DMARDs displayed similar reactivity to oxPTM-CII when compared to DMARD non-responsive patients (ASDAS>2, $p>0.05$, Figure 1B, Table 2). Further evaluation of axSpA groups revealed no significant difference in anti-

oxPTM-CII IgG or IgA reactivity in patients with 'pure' axSpA versus clinical subgroups e.g. axSpA-associated with oligoarthritis (axSpA-OA), inflammatory bowel disease (axSpA-IBD) or patients with enthesitis (axSpA-En), ($p>0.05$, Figure S4). Consistent with our prior data in RA (27, 28) markers of inflammation did not correlate with IgG or IgA anti-oxPTM-CII ($\rho<0.0408$, Figure 2).

Cross-examination of oxPTM-CII reactivity across a range of disease conditions revealed that the level of IgG anti-oxPTM-CII was lower in PsA compared to axSpA, observed in 28% of serum samples from patients with PsA (Figure 3, Table 2). IgA anti-oxPTM-CII binding was, however, detected in 84% PsA samples with comparable binding to axSpA samples ($p>0.05$) and significantly higher than IgG anti-oxPTM-CII ($p<0.0001$, Figure 3A, Table 2). Similar to axSpA samples, PsA samples that responded also to Nt-CII had a stronger reactivity to oxPTM-CII (Figure S5). Similar to axSpA, markers of inflammation did not correlate with anti-oxPTM-CII antibodies (Figure S6). Low IgG anti-oxPTM-CII activity was detected also in 35%, 20% and 15% of UA, Ps and FM, respectively. Higher IgA anti-oxPTM-CII was also detected in 38% serum samples from patients with UA (Figure 3B, Table 2).

Given the presence of different oxPTM-CII reactivity profiles in axSpA versus RA, ROC analysis was performed to obtain specificity and sensitivity values for both IgG and IgA anti-oxPTM-CII (Figure 4). We have previously observed high specificity and sensitivity of IgG anti-oxPTM-CII reactivity in RA (28), which are confirmed in the current study; AUC=0.961±0.018 for IgG anti-oxPTM of RA samples with 91% and 94% sensitivity and specificity, respectively (11.4 likelihood ratio). This was however significantly reduced to AUC=0.556±0.0548 for IgA anti-oxPTM-CII with 32% and 89% sensitivity and specificity, respectively. Among the non-RA patient groups, the highest sensitivity and specificity for IgG anti-oxPTM-CII was observed for axSpA reactivity against oxPTM-CII, namely 64% sensitivity and 80% specificity representing a 3.3 likelihood ratio; AUC=0.818±0.020. For IgG anti-oxPTM-CII in PsA, we observed 42% sensitivity and 86%

specificity representing a 3.2 likelihood ratio; AUC=0.727±0.034. In contrast to RA, sensitivity and specificity for IgA anti-oxPTM-CII for axSpA was increased to 80% and 85%, respectively; AUC=0.833±0.0345. Sensitivity for IgA anti-oxPTM-CII in PsA was doubled to 87% sensitivity and 92% specificity; AUC=0.944±0.0287. (Figure 4, Table S1).

Discussion

The results of our study suggest that (1) serum anti-oxPTM-CII IgG and IgA antibodies are elevated in patients with axSpA compared to healthy controls; (2) serum anti-oxPTM-CII IgG antibodies are lower in patients with axSpA compared to IgA anti-oxPTM-CII; (3) serum IgA anti-oxPTM-CII antibodies are higher than IgG anti-oxPTM-CII in PsA and (4) opposite to high IgG anti-oxPTM-CII no or very low IgA anti-oxPTM-CII is present in patients with RA.

The patho-immunobiological pathways responsible for SpA remain unknown. However, there are many shared features among the group, the most important of which include: (1) an asymmetric oligoarthritis predominantly of the lower extremities, (2) radiological evidence of sacroiliitis, (3) familial aggregation and (4) negative serology for rheumatoid factor (RF) and anti-citrullinated antibodies (ACPA) (29, 30). The estimated prevalence of axial spondyloarthritis is between 0.9 to 1.4% of the adult population, and is similar to that of RA (31, 32). While both RA and SpA feature joint inflammation, RA is defined as a systemic, inflammatory autoimmune disease with an overt humoral immune response. For instance, ACPA are the gold standard diagnostic tools for RA (33). There is still debate within the field as to whether SpA is an autoimmune or auto-inflammatory disease (34). Autoantibodies to gold standard neoantigens in general, or ACPA in particular is infrequent in SpA (33). Notably, of all the axSpA samples tested in this study only two were positive for ACPA (16, 19 and 45 units which all the other samples were negative).

Our data confirm previous reports where ACPA positivity was detected in about 1% to 13% of PsA and axSpA patients, depending if second or third generation anti-cyclic citrullination peptide antibody assay were used (35). This alone does not rule out the involvement of a humoral immune response, as it should be appreciated that back in the 1990s, a number of studies demonstrated reactivity to fibrocartilage proteins, including aggrecan, suggesting that an autoimmune antibody response may underlie enthesitis and spondylitis pathology (36). This was further supported by murine studies, which have shown that active immunisation with G1 globular domain of versican can lead to both

spondylitis and enthesitis pathology (37). In addition, the presence of antibodies to carbamylated proteins (anti-CarP), a non-enzymatic post-translational modification in which cyanate binds to molecules containing primary amine or thiol groups and forms carbamyl groups were detected in PsA patients (38).

RA and axSpA are distinct diseases with unique pathobiology, however both are associated with active inflammation and cartilage damage. In our previous studies we showed that RA patients have anti-oxPTM-CII antibodies (27, 28). Based on this we hypothesised that the overt inflammatory and metabolically active nature of the joint in SpA would be analogous enough to RA to result in the generation of oxPTM-CII neoepitopes that would lead to an auto-inflammatory response and the generation of an autoimmune response to oxPTM-CII in SpA. We further reasoned that the chronic inflammation may further enhance the immune response against oxPTM-CII neoepitopes, which are formed as a result of the high levels of oxidants in the inflamed enthesis. In the current study, we observed a strong and specific IgG reactivity to oxPTM-CII in 52% of axSpA patients compared to 83% RA patients (Figure 1, Table 2), supporting the concept that axSpA does contain auto-reactivity as part of its immuno-pathobiology. We confirm in the current study our previous observation that IgG anti-oxPTM-CII is very prevalent in RA, and may provide a novel serologic biomarker that can facilitate the diagnosis of RA, especially in ACPA-negative patients where we previously observed 92% sensitivity (28). It should be appreciated that IgG anti-oxPTM-CII reactivity in the axSpA samples was as strong, and in some samples even stronger, than in RA samples (Figure 1 and 3).

Total IgA levels have previously been shown to be elevated in patients with axSpA (39, 40). Francen et al investigated the possible association between serum IgA, IgM, and IgG and disease activity in a one year longitudinal study in patients with active SpA receiving regular DMARD treatment with either phenylbutazone or diflunisal. Throughout the study changes in IgA, but not in IgM and IgG, correlated with changes in disease activity. Similar to our result, changes in serum IgA and ESR showed no consistent correlation, suggesting that both parameters reflect different aspects of disease

(41). Previous studies detected IgA autoantibodies against Cluster of Differentiation 74 (CD74) with high prevalence in patients with established spondoloarthritis (9, 10). CD74 plays a role in preventing premature binding of peptides to MHC class II. In addition, CD74 has an impact on B cell differentiation. A multi-center study conducted by the same group compare sensitivity and specificity of anti-CD74 and HLA-B27 in patients with non-radiographic axSpA and found that IgA anti-CD74 may help to improve the diagnostic value of HLA-B27 to diagnose axSpA and the identification of IgA anti-CD74 antibodies without and particularly with the simultaneous presence of HLA-B27 (42). This, however, was conflicted in a follow up study that reported a lack of sufficient specificity to yield significant diagnostic value of anti-CD74 IgA (11).

In the current study, axSpA display strong IgA anti-oxPTM-CII in contrast to no or very low IgA anti-oxPTM-CII reactivity that was observed in RA (Figure 1, 3). We observed high IgA response regardless of disease duration, whether one year or long standing disease. The sensitivity and the specificity of IgG anti-oxPTM-CII reactivity in axSpA patients were 64% and 80%, respectively, but increased to 80% and 85% sensitivity and specificity, respectively for IgA anti-oxPTM-CII (Table S1). In our studied cohorts we tested patients that were treated with conventional synthetic DMARDs or biological DMARD that include TNF or IL-17 inhibitors, commonly used to treat patients with SpA (43-45). While there was no difference in response to oxPTM-CII between patients in remission and patients with active disease, there was a significantly higher IgA anti-oxPTM-CII reactivity in axSpA patients are treated with biologics compared to patients that were treated with synthetic DMARD regardless of disease activity (Figure 1). These different results regarding reactivity during treatment reflect either a possible role of drugs in modifying the immune response or that those patients are at different stages of the disease. In regard to this interesting data, we think that further studies are needed, in larger cohorts and with longitudinal follow up. Within the group of axSpA patients that had in addition to axSpA IBD, OA or enthesitis, we saw similar reactivity as the patients with axSpA only (Figure S4). Similar to our previous observation for RA (28), we did not see a

correlation between anti-oxPTM-CII reactivity and ESR, CPR or DAS28 (Figure 2). Therefore, anti-oxPTM-CII reactivity is not a marker of inflammation.

Interrogation of oxPTM-CII reactivity across a range of disease conditions revealed lower IgG anti-oxPTM-CII in PsA compared to axSpA, with only 28% in PsA serum samples. IgA anti-oxPTM-CII binding was, however detected in 84% PsA samples with comparable binding to axSpA samples ($p>0.05$) and significantly higher than IgG anti-oxPTM-CII (Figure 3 and Table 2). An increase in sensitivity was observed for IgA anti-oxPTM-CII with 87% and 92%, sensitivity and specificity compared to 42% and 86% sensitivity and specificity for IgG anti-oxPTM-CII in PsA. IgA-containing circulating immune complexes was previously found in 80% patients with PsA with significantly higher levels of these complexes in the patients with more severe arthritis but only 37% had IgG-containing circulating immune complexes. Hence, a significant correlation between the level of IgA-containing circulating immune complexes and the severity of the arthritis was revealed. It was therefore suggested that IgA-containing circulating immune complexes may play a role in the pathogenesis of psoriatic arthritis (46).

We observed a striking difference in IgA anti-oxPTM-CII response between RA and SpA. In contrast to axSpA and PsA, we observed very low IgA anti-oxPTM-CII binding in RA patient with 32% sensitivity and 89% specificity compared to over 90% specificity and sensitivity for IgG anti-oxPTM-CII (Figure 1, 3, 4, and Table S1). This interesting observation may reflect the different pathobiology of axSpA and RA. Although both diseases lead to inflammation and damage of cartilage tissue, and although further confirmation with longstanding RA samples is needed, it confirms antibody species reactivity observed for other auto-antigens in RA. Of note, about 50% of the samples analysed in this study are ACPA negative with confirm our previous observation. In RA, ACPA and rheumatoid factor (RF) are predominantly of the immunoglobulin IgM (RF) or IgG (ACPA) isotype. IgA isotypes and other autoantibodies—such as RA33 antibodies—have been repeatedly reported but their

diagnostic value is still not been fully elucidated. While IgG-ACPA, and IgG-RF specificity >98%, IgA-RF and IgA-ACPA sensitivity was found to be 50.7% and 35% respectively (47)

Although the aetiology of SpA remains obscure, it has demonstrated a strong association with environmental factors including pathogenic intestinal microbes (48). Mucosal surfaces serve as a protective barrier against most pathogens. These surfaces are protected by a first-line defence mediated by IgA (49). Moreover, it is also known that T helper T_H17 cells are more abundantly present at the mucosal surface of the intestine, compared with other T-cell subsets (50). Accumulating evidence has demonstrated that T_H17 cells contribute to intestinal homeostasis by regulating intestinal IgA secretion supporting a link between intestinal T-cell function and IgA production. Less is known about the potential role of T_H17 cells for IgA induction in the joint, though chronic activation of T_H17 was shown to induce hyperactive IgA synthesis in many types of inflammatory joint diseases. Surprisingly, in the current study we did not observe a significant difference in IgG versus IgA anti-oxPTM-CII reactivity in patient that had IBD associated with axSpA. Thus, the involvement of IgA anti-oxPTM-CII might be a more complex mechanism and possibly further studies of T_H17 and IL-17 levels in the various group will shed light on these mechanisms.

Conclusions

our study implies that axSpA is associated with the presence of antibodies specific for oxPTM-CII, suggesting that there may be a humoral component to SpA. The exact immunopathology that leads to this antibody reactivity is not clear yet. Although in both axSpA and RA there is a similar lack of correlation between markers of inflammation, the humoral component in SpA might reflect an alternative autoimmune mechanism. The striking difference in IgA anti-oxPTM-CII between RA and SpA may indicate such an alternative pathogenic autoimmune pathway. This proof-of-concept study

needs to be validated in a larger, longitudinal follow up to establish whether anti-oxPTM-CII antibodies are present at the onset, early stages or at later stage of disease when inflammation is substantial and patients are treated with immunosuppressive drugs. Prospective studies are required to validate the potential of antibodies to oxPTM-CII and particularly IgA anti-oxPTM-CII as a biomarker that can stratify SpA from RA.

List of abbreviations

ACR20, American College of Rheumatology 20;

ASAS, Assessment of Spondyloarthritis International Society;

axSpA, axial SpondyloArthritis;

BASDAI, Bath Ankylosing Spondylitis Disease Activity Index;

CII, collagen type II;

CIII, collagen type III;

CDAI, Crohn's Disease Activity Index;

CRP, C-reactive protein;

DAS28, Disease Activity Score 28 joints;

ESR, erythrocyte sedimentation rate;

FM, FybroMyalgia;

HC, Healthy Control;Nt-CII, native CII

oxPTM, oxidative post-translational modifications;

oxPTM-CII, oxidative post-translationally modified collagen type II;

Ps, Psoriasis;

PsA, Psoriatic Arthritis;

RA, Rheumatoid Arthritis;

ROS, reactive oxidants;

UA, Undifferentiated Arhtitis.

Declaration

Ethics approval and consent to participate

The Institutional Review Board, the Health Director of San Giovanni di Dio Hospital in Florence, reviewed and approved this research and the use of clinical and laboratory data of common clinical practice, in the respect of Privacy Law, for clinical and scientific studies and publications. Ethical approvals for the collection and analysis of samples at the University of Glasgow was obtained from the West of Scotland Research Ethics Service (Institute of Infection, Immunity and Inflammation Research Tissue Bank, REC: 11/S0704/7). All patients and all healthy controls gave their written informed consent.

Consent for publication

Not applicable

Availability of data and materials

All data generated or analysed during this study are included in this published article [and its supplementary information files].

Competing Interest

All authors have declare no conflicts of interest.

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Authors' contributions

AN, MI, CV, RS contributed to the conception and study design, CV, SR contributed to the acquisition of the data. AN analysed the data. AN, MI, CV, RS, IM, CG, MB, AT, LT, SG, VG, MM, IR, FB, FG, AD ontributed to the interpretation of the data. AN, CV and MI wrote the first version of the manuscript, and MB, HA, PP, CG and IM revised it critically.

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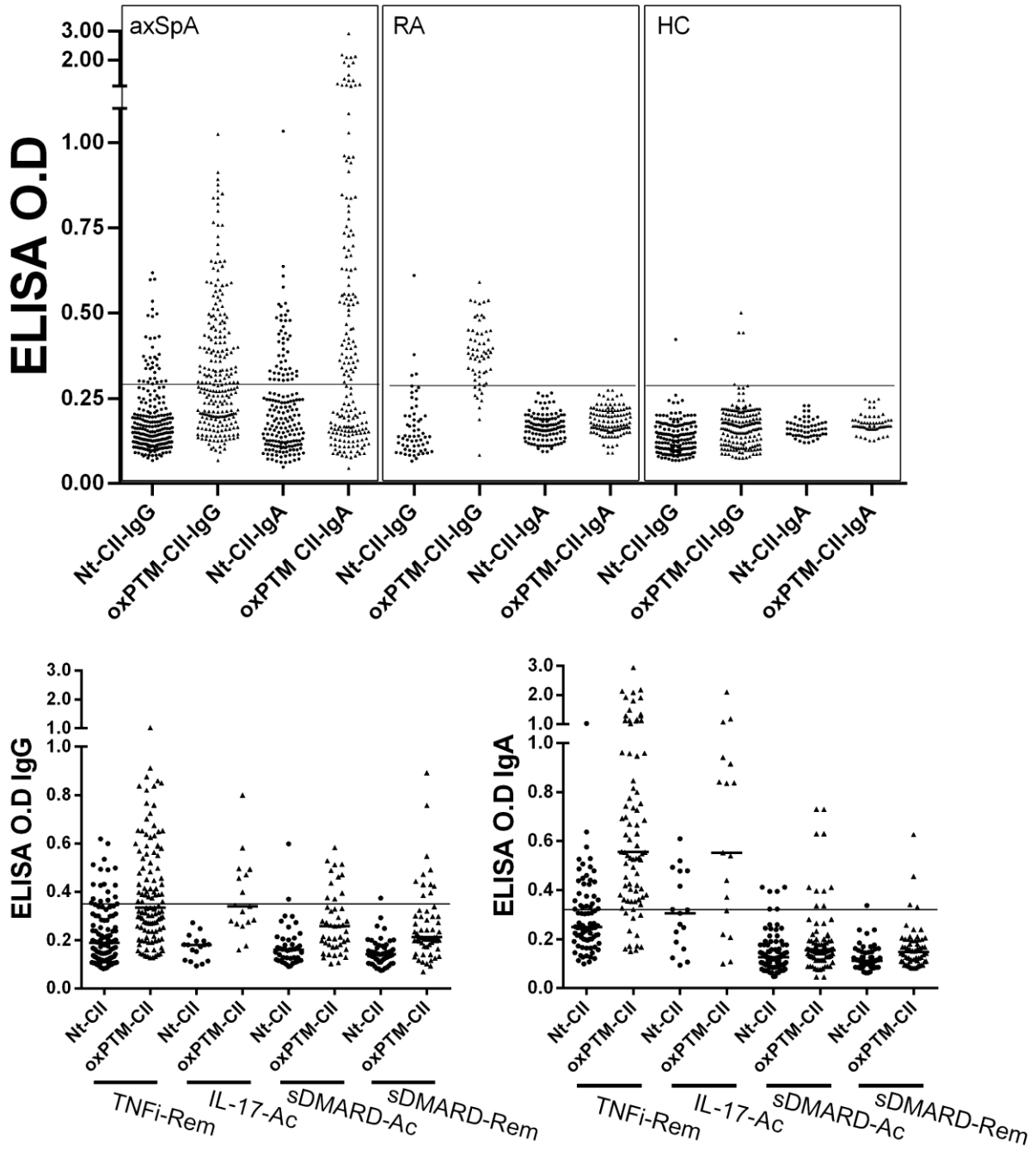

Figure 1. Binding to oxPTM-CII in samples from patients with axial spondylitis arthritis (axSpA). **A**. IgG anti-oxPTM-CII binding in axSpA was higher than to native CII ($p<0.0001$) and was similar to rheumatoid arthritis (RA) patients with mean O.D of 0.189±0.103, 0.335±0.189, 0.164±0.0.0916, 0.381±0.0959, 0.135±0.0471 and 0.167±0.0629 for native and oxPTM-CII in

axSpA, RA and healthy controls (HC), respectively. IgA anti-oxPTM-CII was high in axSpA ($p<0.0001$, O.D=0.474±0.482) but not present in RA (O.D=0.185±0.0388). **B.** IgA anti-oxPTM-CII reactivity in axSpA that were treated with biologics, TNF or IL-17 inhibitors (respectively TNFi and IL-17i), was higher compared to the ones treated with synthetic DMARDs (sDMARD with mean O.D=0.398±0.122 and 0.376±0.161 vs 0.275±0.129 and 0.261±0.152,respectively; $p<0.001$) regardless if disease is active (Ac) or in remission (Rem). Line indicate the ELISA O.D. cut-off that was determined arbitrarily as the 97th percentile of the oxPTM-CII antibodies levels detected in healthy individuals (O.D=0.285). Nt-CII and oxPTM-CII mark rispectevely native and modified by oxidants CII.

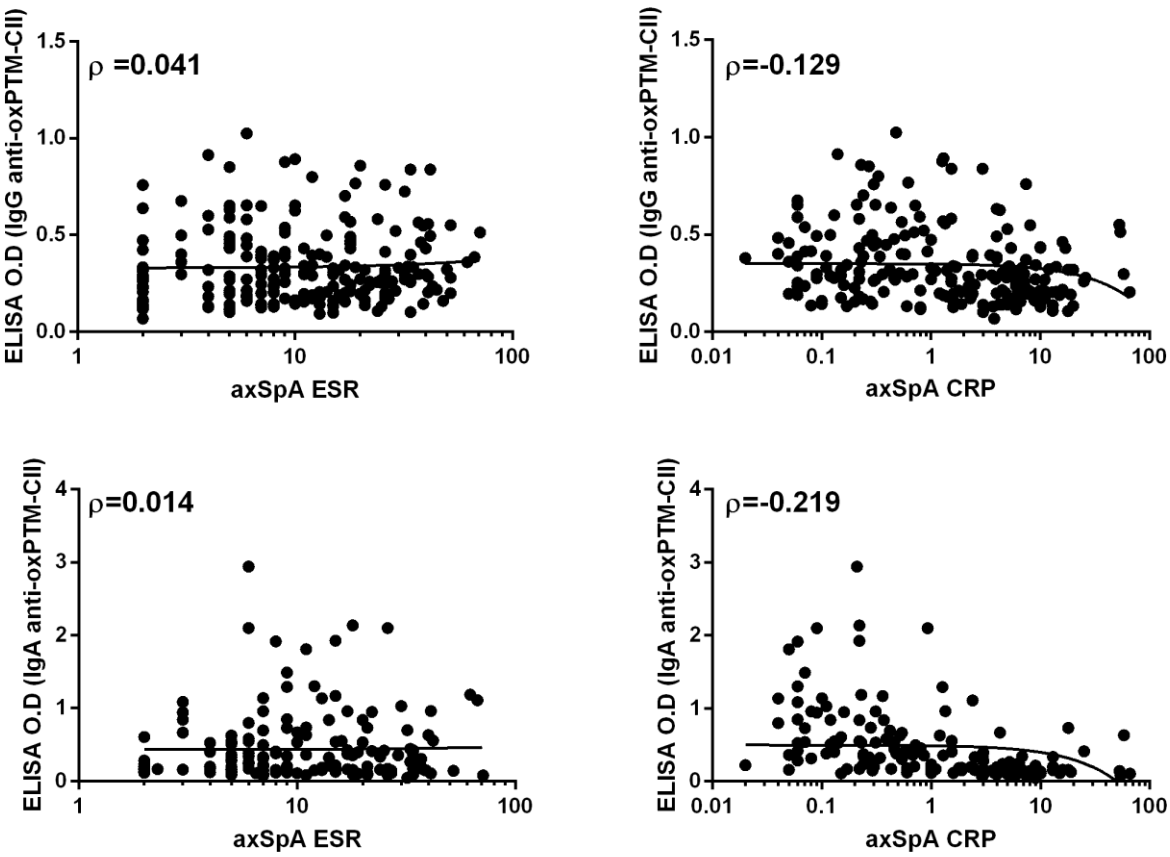

Figure 2. Anti-oxPTM-CII reactivity is not associated with markers of inflammation. There was no association in axial Spondyloarthritis (axSpA) patients between IgA and IgG anti oxPTM-CII reactivity with erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP).

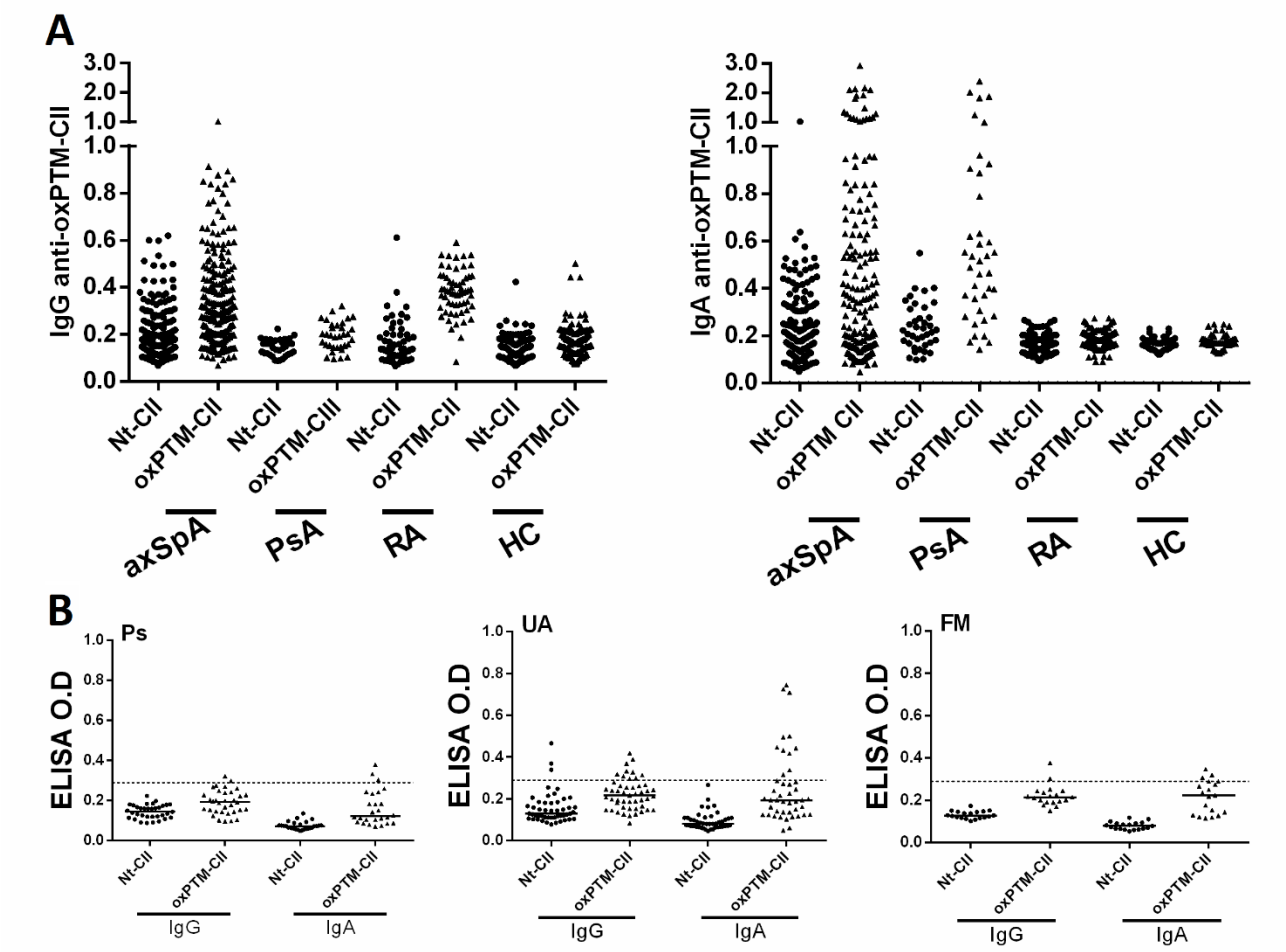

Figure 3. Binding to oxPTM-CII in samples from patients with PsA in comparison to axSpA and RA. A. IgG anti-oxPTM-CII in psoriatic arthritis (PsA, n=60) was lower compared to axial spondyloarthritis (axSpA) while IgA anti-oxPTM-CII binding was comparable to axSpA samples ($p>0.05$) and significantly higher than IgG anti-oxPTM-CII ($p<0.0001$). B. IgG and IgA reactivity in serum samples from patients with psoriasis (Ps, n=35), undifferentiated arthritis (UA, n=50 and fibromyalgia (FM, n=19). While significant higher IgA-anti-oxPTM-CII binding in 38% patients from UA low/no binding in Ps, and FM was observed. Gray line in B indicate the ELISA O.D. cut-off that was determined arbitrarily as the 97th percentile of the oxPTM-CII antibodies levels detected in healthy individuals. RA indicates rheumatoid arthritis and HC indicates healthy controls; Nt-CII and oxPTM-CII mark native CII and CII modified by oxidants, respectively.

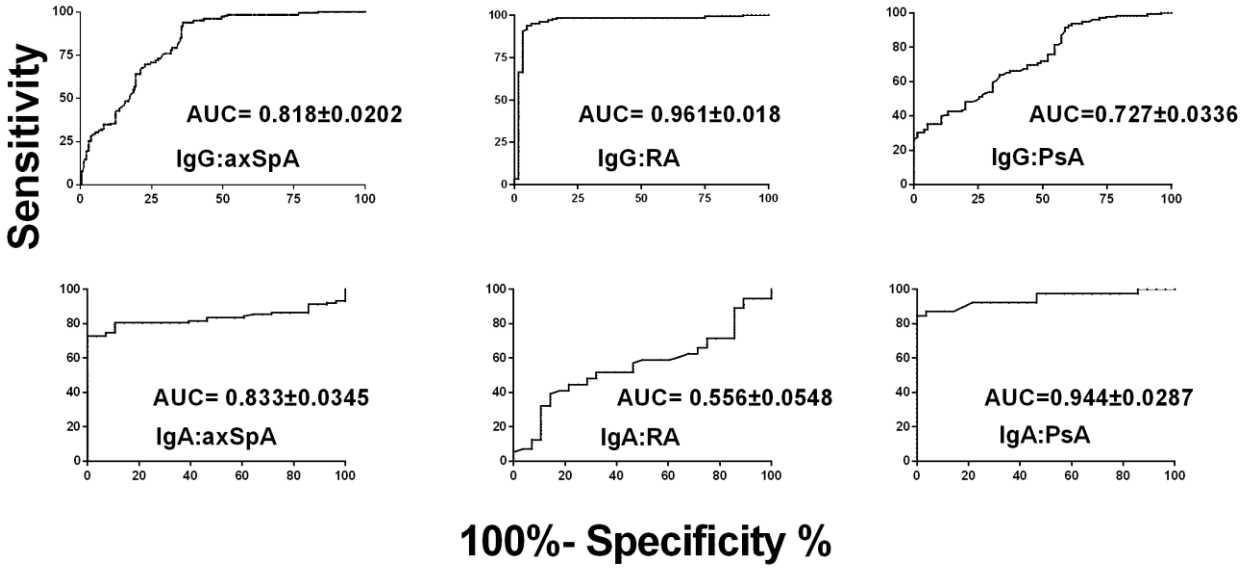

Figure 4. ROC analysis for sensitivity and specificity of IgG and IgA anti-oxPTM-CII. High specificity and sensitivity of IgG antibodies to CII modified by oxidants (oxPTM-CII) reactivity in rheumatoid arthritis (RA) with AUC=0.961±0.018; 91% and 94% sensitivity and specificity is reduced to AUC=0.556±0.0548 for IgA anti-oxPTM-CII with 32% and 89% sensitivity and specificity, respectively. For axial spondyloarthritis (axSpA) IgG anti-oxPTM-CII AUC=0.818±0.020; 64% sensitivity and 80% specificity which increase to AUC=0.833±0.0345 and 80% and 85%, respective sensitivity and specificity. For IgG anti-oxPTM-CII in psoriatic arthritis (PsA) AUC=0.727±0.034 we observed 42% sensitivity and 86% specificity that was doubled to 87% sensitivity and 92% specificity; AUC=0.944±0.0287.

Table 1

Patients and serum samples.

	axSpA	*RA*	*PsA*	*Ps*	*UA*	*FM*	*HC*
Number	242	60	69	35	48	19	178
Gender(F/M)	0.5	2	1.4		1.9	1.4	0.4
Age (y)	54 (28-80)	50 (28-63)	56 (31-76)		60 (33-84)	51 (40-64)	47 (21-58)
Duration (y)	2-64	<1	1-25	19 (1-51)	<1	4 (3-11)	-
BASDAI	0.6-9.4	-	-		-	-	-
ACR20	-	-	15.5 (10.4-24.6)		-	-	-
CDAI	-	-	-		18.3 (6-62)	-	-
ESR	14.9 (2-71)		10.9 (2-29)		23.6 (1-55)		
CRP	7.7 (0.2-54)		4.9 (1-15)		1 (0.2-4.4)		
DAS28		5.3 (3.7-7.4)			3.36 (0.9-6.2)		

We tested 242 samples from patients with longstanding axial spondyloarthritis (axSpA) with disease duration of over 2 years. In addition, early rheumatoid arthritis (RA, n=60), undifferentiated arthritis (UA, n=48), 69 psoriatic arthritis (PsA) and 35 psoriasis (Ps, n=35) samples, 19 samples from patients with fibromyalgia (FM) and 178 healthy controls (HC) were investigated. Disease activity was assessed for axSpa, PsA and UA calculating respectively Bath Ankylosing Spondylitis Disease Activity Index (BASDAI, (24)), American College of Rheumatology (ACR 20, (25)) and clinical disease activity index (CDAI, (26). For axSpA, PsA and UA were evaluated also with erythrocyte

sedimentation rate (ESR) and C-reactive protein (CRP). For RA and UA the Disease Activity Score 28 joints (DAS28) was evaluated.

Table 2. Reactivity of serum samples from patients to native CII (Nt-CII) and CII modified by oxidants (oxPTM-CII).

	IgG		IgA	
	Nt-CII	**oxPTM-CII**	**Nt-CII**	**oxPTM-CII**
axSPA*^{T}	13% (33/242) 0.189±0.103	52% (128/242) 0.335±0.189	25% (42/165) 0.224±0.143	47% (79/165) 0.474±0.482
axSpA*^{B}	21% (29/136) 0.214±0.117	66%(91/136) 0.395±0.203	45% (38/83) 0.297±0.150	85%(71/83) 0.729±0.536
axSpA*^{D}	3% (3/106) 0.159±0.074	34% (37/106) 0.272±0.155	4%(4/82) 0.137±0.071	9%(8/82) 0.181±0.118
RA	5% (3/60) 0.164±0.092	83% (50/60) 0.381±0.096	0% (1/56) 0.167±0.039	7% (4/56) 0.185±0.034
PsA	0% (0/69) 0.146±0.034	28% (20/69) 0.193±0.060	35% (14/39) 0.238±0.100	84% (33/39) 0.667±0.540
Ps	2% (1/35) 0.145±0.034	20% (7/35) 0.193±0.060	0% (0/26) 0.076±0.021	19% (5/26) 0.162±0.087
UA	6% (3/48) 0.157±0.076	35% (17/48) 0.224±0.075	2% (1/44) 0.095±0.043	38% (17/44) 0.256±0.173
FM	0% (0/19) 0.131±0.018	15% (3/19) 0.222±0.050	0% (0/19) 0.080±0.018	31% (6/19) 0.216±0.078
HC	0% (0/178) 0.135±0.047	1.6% (3/178) 0.168±0.063	0% (0/28) 0.163±0.026	0% (0/28) 0.176±0.028

For each group % of positive responders, numbers of positve on the total number of samples in brackets and mean plus/minus standard deviation. IgG and IgA anti oxPTM-CII was significantly higher than reactivity to Nt-CII ($p<0.0001$). Higher IgA anti-oxPTM-CII antibodies were observed in axial Spondyloarthritis (axSpA) and psoriatic arthritis (PsA) but were low in rheumatoid arthritis (RA). Total axSpA (axSpA*^{T}) were split into axSpA samples treated with biologics (axSpA*^{B}),

which had a high reactivity compared to axSpA patient treated with synthetic DMARD (axSpA*^{D}). Ps indicates psoriasis, UA is undifferentiad arthritis, FM is fibromyalgia and HC indicates healthy controls.