Antibodies to citrullinated peptides in patients with juvenile idiopathic arthritis and rheumatoid arthritis:

Shared expression of the inherently autoreactive 9G4 idotype

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Abstract

Objective

Antibodies to cyclic citrullinated peptides (CCP) in rheumatoid arthritis (RA) can express the inherently autoreactive gene, VH4-34, as detected using the rat monoclonal antibody, 9G4. Patients with the polyarticular sub-type of juvenile idiopathic arthritis (pJIA) share some but not all features of RA in adults. The aim of this study was to profile rheumatoid factor (RF), anti-CCP and 9G4-CCP serology of a large JIA cohort and compare to a cohort of adult RA patients.

Methods

Serum from 88 pJIA, 29 enthesitis-related arthritis (ERA), 38 extended oligoarthritis (EOA), 31 adolescent controls, 35 RA and 30 adult controls were tested for RF, IgG, IgA and IgM anti-CCP and 9G4-CCP by ELISA. Serum total 9G4+IgM was also measured.

Results

Of 65/88 pJIA (RF-ve) patients, 4 (4.6%) were IgG anti-CCP positive. In sera from pJIA (RF+ve) 20/23 (87.0%), RA 30/35 (85.7%) and one EOA patient contained IgG anti-CCP. IgA- and IgM anti-CCP levels were lower in the adolescent group (p<0.01). 9G4-CCP were higher in pJIA RF+ve vs RF-ve (p<0.0001). Median levels of 9G4-CCP in pJIA (RF+ve) and RA did not differ. 9G4 on serum total IgM was greater in pJIA (RF+ve) patients compared with adolescent groups (p<0.01), but similar to adult RA (RF+ve).

Conclusion

In healthy individuals, 9G4+B cells comprise 5-10% of peripheral blood pool but serum immunoglobulins utilising VH4-34 are disproportionately low. The idiotope recognised by 9G4 was detected on anti-CCP antibodies in >80% pJIA (RF+ve) patients. VH4-34 usage by anti-CCP in both JIA and RA patients suggest elicitation of these autoantibodies through shared pathogenic B cell selection processes.
Juvenile idiopathic arthritis (JIA), as defined by the updated International League Against Rheumatism (ILAR) classification criteria [1], is an umbrella term encompassing different sub-types of arthritis with onset in those under 16 years. Rheumatoid factor positive polyarticular JIA (pJIA (RF+ve)) is one of the more severe sub-types of JIA. It is associated with older onset (being more frequently seen in adolescents), more disability compared to other sub-types and more frequently progresses into adulthood, requiring continuing treatment [2]. Clinically, of all the sub-types of JIA, pJIA(RF+ve) most closely resembles that of adult onset rheumatoid arthritis (RA). However, there are subtle differences in the clinical pattern of disease as well as clearly the age of onset. Young people with pJIA(RF+ve) tend to have more frequent involvement of the temporomandibular joints and ankles. This is captured by the Juvenile Arthritis Disease Activity Score (JADAS) [3] but excluded from the DAS28 as used in routine practice in patients with RA. Hence it is uncertain to what extent pJIA(RF+ve) represents early onset RA, or whether this represents a type of inflammatory arthritis of childhood onset that has aetiopathogeneic pathways that are distinct from adult onset RA.

Consensus now is that pJIA(RF+ve) is associated with a high frequency of IgG-class antibodies to citrullinated proteins, as measured in the clinic using commercial cyclic citrullinated peptides (CCP) as substrate. The frequency of anti-CCP positivity in pJIA(RF+ve) has been shown to be comparable to those seen in adult seropositive RA [4-9]. However, most of these studies were on relatively small numbers of patients, did not include separate diagnostic cohorts within the global categorization of ‘JIA’ and very few compared with local adult cohorts of RA. Importantly, these studies have not investigated upstream pathways of autoantibody production in order to understand better the aetiopathogenesis of pJIA(RF+ve) and to what extent this overlaps with RA. One method to interrogate this research question is to define the genes that encode for anti-CCP antibody expression and whether these are shared across pJIA(RF+ve) and seropositive RA. Although the variable regions of the heavy chain of immunoglobulins are encoded by a total of 123 variable heavy (IGVH) genes, with nearly half expressed as VH segments, there is a degree of skewing toward the usage of certain VH genes in autoimmunity and B cell malignancy. The use of particular genes encoding IGVH has been associated with the development of autoantibodies [10-12], with those encoded by VH4-34 being the prototype in autoimmune diseases [13, 14]. Immunoglobulins derived from this gene, even in a germ-line configuration, are inherently autoreactive and can recognise a number of self-antigens in the absence of antigen driven
selection. The rat monoclonal antibody 9G4 binds a unique conformational epitope confined largely within framework (FR) 1 of the VH region of immunoglobulins derived from the VH4-34 gene. The availability of the 9G4 reagent thus allows the tracking of an autoimmune subset of B cells utilizing VH4-34 in the B cell receptor and the identification of soluble antibodies derived from this IGVH gene.

VH4-34 derived 9G4+B cells are present across all ethnic groups, comprising up to 10% of peripheral blood B cells. Their phenotype is consistent with being predominantly within naïve B cell populations (IgD+CD27-). In contrast, except for transient rises in the context of infection (especially with EBV, CMV and pneumococcus), serum levels of predominantly IgM-class VH4-34 derived immunoglobulins are disproportionately low in normal individuals. 9G4+B cells are also not commonly seen within germinal centres, but are capable of some degree of class-switch recombination as small amounts of 9G4-IgG can be detected in serum [15, 16].

In autoimmunity however, VH4-34 derived sequences are over-represented in autoantibodies. VH4-34 usage is for example obligatory for most cold agglutinins [17], utilized by some IgG-anti-dsDNA antibodies in serum and also found deposited in renal biopsies from patients with systemic lupus erythematosus (SLE)[18] and used by anti-myeloperoxidase antibodies in systemic vasculitis [19]. More recently we have described 9G4+ autoantibodies specific for CCP in patients with early (<6 weeks history of joint symptoms) and also established RA [20]. Investigating the isotype distribution and idiotope of anti-CCP antibodies in pJIA(RF+ve) and comparing this to a local seropositive adult RA population may therefore further our understanding as to potential common pathways for autoreactive B cell selection in pJIA(RF+ve).

Methods

Patients

Demographic data for adolescent and adult patients are shown in Table 1, all of whom were under the care of University College London Hospital (UCLH), London, UK. A total of 88 pJIA, 29 enthesitis related arthritis (ERA), 38 extended oligoarticular (EOA) JIA, 31 age and gender matched healthy individuals (median 21 years; range 13-23), 35 gender-matched adult RA and 30 (74% female; median 38 years (range 24-72)) adult healthy controls (HC) were included. None of the patients had received Rituximab at or before times of sampling. In the
juvenile cohorts, approximately two thirds of pJIA (53/88) and EOA patients (22/38) were receiving methotrexate (MTX), with equal proportions (36%) in each group receiving biologic therapies (TNF inhibition). Two thirds of ERA patients (19/29) were on MTX, six on additional or alternative DMARDS (sulfasalazine (SSZ) or hydroxychloroquine (HCQ)) with 17% (n=5) on biologics. These were mainly TNF inhibitors with one patient on abatacept and two patients on tocilizumab. Of 35 adult RA patients, two were not on treatment, 33 were receiving DMARDS, usually MTX (n=18), SSZ (n=8) and HCQ (n=7), 13 were on biologics (all anti-TNF) and nine were receiving oral prednisolone (all <20mg/day). Sera were obtained from collections within The Centre for Rheumatology, and the Arthritis Research UK Centre for Adolescent Rheumatology, University College London, UK and selected on the basis that they fulfilled the ILAR classification criteria for JIA [1] or the American College of Rheumatology (ACR) and European League Against Rheumatism (EULAR) classification criteria for Rheumatoid Arthritis [21]. All subjects donated blood after informed consent and the study was approved by the local ethics committee (REC 11/LO/0330 (adolescent cohorts)) and (REC 08/H0714/18 (adult cohorts)).

Measurement of anti-cyclic citrullinated peptide antibodies

Anti-CCP antibodies in patient and control serum diluted 1/200 were measured using a commercial 96-well enzyme-linked immunosorbent assay (ELISA), using plates pre-coated with second-generation citrullinated peptides (CCP2) (FCCP600, Axis Shield Diagnostics, Dundee, UK). IgG anti-CCP were measured according to kit protocol with the cut-off for positivity being 5U/ml.

Horseradish peroxidase (HRP)-conjugated sheep anti-human IgA or IgM (The Binding Site, UK) was used to detect anti-CCP antibodies of the given isotype using the same ELISA plates as for IgG anti-CCP.

Levels of IgA anti-CCP antibodies were calculated following reference to an in-house standard (representing 100 Arbitrary Units AU) included on each ELISA test plate as well as negative controls. Results were expressed as a proportion of the positive control following subtraction of background binding of HRP-conjugate and normalization between different ELISA plates. IgA anti-CCP are rarely observed in healthy control sera [20] and cut-off was therefore based on calculations using mean+3SD of binding by 60 sera from HC across all age groups (giving a value of 11AU/ml) [20]. In contrast, IgM class antibodies to CCP are
commonly observed in sera from adult HC, and as we had no reference for a cut-off level to cover both adolescent and adult IgM anti-CCP levels in samples, results are expressed as optical density (x1000) given by serum samples following subtraction of background binding of conjugate alone to CCP-coated wells.

**Determination of IgM Rheumatoid Factor**

For JIA patients, IgM-RF positivity status was obtained from historical clinical data from standard laboratory tests (Rheumatoid arthritis particle agglutination (RAPA) test). In-house protocol was used to determine RF in adult and adolescent healthy controls using affinity purified rabbit IgG (Sigma Aldrich, St Louis, USA) as substrate. Briefly, binding of sera (diluted 1/200) to affinity purified rabbit IgG coated and to uncoated wells was measured using goat-anti-human IgM-HRP conjugate (The Binding Site, Birmingham, UK). After subtracting background binding (to uncoated wells), arbitrary units (AU) of binding were calculated by reference to a standard curve constructed from a commercial source (Cambridge Life Sciences) with values above 23AU/ml regarded as positive.

**Detection of 9G4 expression on anti-CCP and on serum total IgM**

For detection of 9G4 expression on antibodies to CCP, sera (diluted 1/50 in RD6Q diluent, R & D Systems, Abingdon, UK) were added to antigen-coated wells of ELISA plates. Following incubation, the 9G4 reagent (IGM Bioscience, Palo Alto, USA) was added at a concentration of 2μg/ml to one side of the plate, the duplicate serum-incubated wells receiving diluent buffer containing equivalent affinity purified normal rat IgG1 (Sigma-Aldrich, Dorset, UK) instead of 9G4. An affinity purified HRP-conjugated goat anti-rat-IgG reagent (Amersham, UK) was used to detect 9G4 recognition of CCP2-binding antibodies. Results were calculated and presented as optical densities (OD x 100 at 450nm) following the subtraction of any background binding in wells in the absence of the 9G4 reagent. To assess 9G4 binding to total serum IgM, sera diluted 1/250 were added to each side of ELISA plates coated with either 2μg/ml murine Fab2 anti-human IgM (eBioscience, San Diego, USA) or left uncoated. Following blocking with 1%BSA, the rat 9G4 reagent was added at 2μg/ml. Detection was subsequently with goat anti-Rat HRP conjugate (Abcam) and development with TMB. Background binding to the uncoated side of the plates was subtracted and results expressed as OD at 450nm.
**Statistical Analysis**

GraphPad Prism (GraphPad, San Diego, USA) was used for all statistical analysis. Non-parametric statistics for populations not following a normal distribution (Mann-Whitney U test) were used to compare groups. For determination of relationships between variables, linear regression (Pearson’s correlation) was used. Differences with a p<0.01 were considered significant.

**Results**

*Levels of IgG anti-CCP are elevated in pJIA(RF+ve) patients and comparable to those in RA(RF+ve) patients*

Table 2 summarises the RF status and frequency of class-switched anti-CCP antibodies in adult RA, JIA, EOA and ERA patient groups. In the adult RA patients, 20/26 (77%) RF+ve sera also contained IgG anti-CCP. Of 88 patients diagnosed with pJIA, there were 65 in the pJIA(RF-ve) group. Only 4 (6%) of these patients had levels of IgG anti-CCP above the cut-off for a positive result (>5U/ml). In the pJIA(RF+ve) group, 20/23 (87%) contained IgG anti-CCP. These findings validate the suggestion that IgG anti-CCPs are frequently seen in the pJIA(RF+ve) subset of JIA, with a specificity of 96.9% (95% CI: 92.99% - 99.00%). Only 1 patient in another adolescent patient group (in the EOA cohort) had a positive result in the IgG anti-CCP test. Figure 1A shows the comparative levels of IgG-anti-CCP antibodies seen across all disease and control groups, and illustrates that pJIA(RF+ve) patients had significantly higher levels of IgG-anti-CCP antibodies than those who were RF-ve, (p<0.0001), and also in the other diagnostic categories within JIA, namely EOA (p<0.0001) and ERA (p<0.0001) and as compared with adolescent healthy controls (p<0.0001). No significant difference in IgG-anti-CCP antibody levels was seen between the adolescent pJIA(RF+ve) and adult RA(RF+ve) patient cohorts (44.1 [IQ range 15.4-84.1] Vs 25.7 [IQ range 4.6-56.0]; p=0.21).

Adult RF+ve RA patients had significantly higher levels of IgG anti-CCP than (RF-ve) RA patients and adult age/sex-matched controls (both p<0.0001).

*IgM- and IgA anti-CCP antibodies are present in pJIA(RF+ve) patients*

Figures 1B and 1C show the comparative levels of IgM- and IgA-anti-CCP, respectively, across all patient and control groups. Although levels of IgG anti-CCP were similar between
adolescent pJIA(RF+ve) and adult RA(RF+ve), both IgM- and IgA anti-CCP levels were significantly lower in the adolescent group.

The differences in IgA anti-CCP levels were mirrored when sera from healthy adolescent -3.4 [IQ range 3.6-3.9], and adult - 6.3 [5.2-7.7] samples were compared (p<0.0001), but most were well below the cut-off for positive in the samples from adolescents. Healthy adults also had significantly higher median levels of IgM-anti-CCP (59.0 [IQ range 38.5-73.6] vs 42.4 [IQ range 28.40-52.69]; p=0.001), than healthy controls of ≤23 years.

Although adult HC and RA were not matched for age there was no correlation between levels of any isotype of anti-CCP with disease duration or age within either juvenile or adult cohorts (data not shown), so therefore was unlikely to be a contributory factor.

**Anti-CCP antibodies in pJIA(RF+ve) and RA(RF+ve) share an inherently autoreactive germline gene as detected by 9G4 binding**

Usage of the VH4-34 immunoglobulin gene by autoantibodies recognising CCP was tracked using binding of the anti-idiotype rat monoclonal antibody, 9G4 (Figure 2A). 9G4 expression on anti-CCP antibodies in adult patients with RA was significantly higher in RF+ve compared with RF-ve RA patients (median, 25.7 [IQ range 6.0-105.0]) vs 5.2 [5.0-8.0] p=0.01). Similarly, binding of 9G4 antibodies to anti-CCP was significantly higher in the pJIA(RF+ve) vs pJIA(RF-ve) (median 9.8 [IQ range 3.3-41.8]) vs 1.2 [0.7-2.0]p= <0.0001), and also significantly higher than in EOA (p<0.01) and ERA ( p<0.0001). Similar median levels of 9G4-CCP were present in pJIA(RF+ve) and adult RA(RF+ve) (p=0.13).

One explanation for detecting 9G4 expression on CCP in pJIA(RF+ve) patients is that there is a general expansion of usage of this VH gene in adolescents. As serum IgM contains virtually all 9G4-expressing immunoglobulin species, we compared levels of 9G4-IgM between pJIA(RF+ve) patients and other adolescent cohorts (Figure 2B). 9G4 expression on serum total IgM was significantly higher in pJIA(RF+ve) patients compared with adolescent HC and JIA control groups (EOA, ERA and pJIA(RF-ve) all p<0.01), but similar to that found in adult RA(RF+ve). However, only 5/19 samples exceeded the upper limit of the range given by sera from HC≤23 years (indicated by shaded area in Figure 2B) and there was no correlation between 9G4 anti-CCP and 9G4 on serum total IgM by capture ELISA (Figure 3A) (Pearson’s correlation coefficient: $R^2=0.11$; p=0.15) suggesting that despite a possible increase of 9G4-IgM, 9G4-B cells committed to anti-CCP antibody production may have
undergone differentiation by a different pathway of activation. In adult RA patients there was also no correlation between 9G4 anti-CCP and 9G4 binding to serum total IgM (Figure 3B).

With respect to the Ig-class distribution of 9G4-CCP antibodies in adult RA patients, results from our previous experiments suggested that 9G4-expression was associated with IgM anti-CCP but that a small proportion may also be of IgG class [20]. We have yet to undertake similar experiments using adolescent patient samples, but analysed possible correlations between the levels of the different classes of anti-CCP in adult RA and pJIA (RF+ve) and 9G4 anti-CCP, using linear regression analysis (Figure 4). Interestingly, differences were found between pJIA and adult RA patients. 9G4+CCP binding was strongly correlated with levels of IgM anti-CCP in adolescent but not adult patients (Figure 4A and D respectively). IgG anti-CCP antibody levels however were similarly correlated, albeit weakly, with 9G4+CCP expression in both diseases (Figure 4B and E). The strongest correlation was between IgA anti-CCP and 9G4 anti-CCP in pJIA with a much weaker correlation in adult RA patients (Figure 4C and F). However, IgA anti-CCP levels were only positive in 5/20 (25%) of samples from pJIA compared with 20 adult RA.

**Discussion**

Class switched (IgG) anti-CCP antibodies, whilst rare in JIA patients overall, were found to occur predominantly in the subset of polyarticular patients who also tested positive for RF, supporting studies of smaller cohorts [4-9]. We also confirmed that the IgG anti-CCP antibody profile of pJIA(RF+ve) patients was significantly distinct from both age/sex-matched HCs, and other sub-types of JIA. Only 3.1% (5/163) of all disease controls or age/sex matched HCs tested positive for IgG anti-CCP antibodies. A direct comparison in IgG anti-CCP isotype serology was then made between adolescent pJIA(RF+ve), and adult RA(RF+ve) serum samples. Although the prevalence of anti-CCP antibodies in RA has been widely studied, for the purposes of this investigation we included a random sample of our own adult cohort, to eliminate discrepancies in methodology or positivity cut-offs. No significant difference was found between levels of IgG anti-CCP antibodies in pJIA(RF+ve) and RA(RF+ve). This suggests that the IgG anti-CCP antibody phenotypes of the two diseases are similar, consistent with the parallels seen in other diagnostic criteria between the two conditions.

IgG anti-CCP antibodies are routinely tested upon clinician’s request, but do not feature in the current diagnostic criteria for pJIA(RF+ve) [1]. The confirmation of their IgG anti-CCP
specificity for pJIA(RF+ve), in contrast to both healthy and disease JIA controls, suggests that IgG anti-CCP levels could also prove a worthwhile formal addition to existing criteria for pJIA(RF+ve).

Levels of IgA anti-CCP antibodies were significantly higher in pJIA(RF+ve) patients than all control groups, but values were very low with medians and most samples (14/19, 74%) within the normal range in our assay. Adult RA patients also had significantly higher levels of IgM anti-CCP compared with the adolescent pJIA(RF+ve) patients, despite their IgG anti-CCP levels being comparable. Most patients across all diagnoses were receiving DMARDS and many also biologic agents, so it was difficult to attribute any significant effects of treatment on autoantibodies between patient groups. It was also not as a result of disease duration, and thus presents the interesting question as to whether anti-CCP antibodies follow the same pathway of class-switching that is seen in adult RA patients. IgA anti-CCP antibody levels being lower in JIA may also indicate that the class switch to IgG anti-CCP precedes the possible accumulation of switched B cells of IgA-isotype in juvenile vs adult patients. As we and others have shown, IgM autoantibodies can persist at high levels alongside class-switched species and therefore do not follow the patterns that would be expected in a normal humoral response to immunization or some infectious insults[22].

In RA, it is well established that the presence of anti-CCP antibodies can pre-date clinical symptoms by up to 10 years [23-25]. If the case is put forward that pJIA(RF+ve) does represent very early onset RA, following these patients into adulthood should result in patients with pJIA(RF+ve) developing the same profile as RA(RF+ve). Although it would be very difficult to ascertain whether anti-CCP positivity antedates symptoms in children with pJIA(RF+ve), the question arises as to why these patients develop symptoms at a much younger age, while adult RA patients may be anti-CCP positive for many years before the onset of disease. The time at which biologics are initiated can influence the duration and severity of symptoms, as well as a patient’s chances of remission [26]. Both the TREAT trial [27] and the ACUTE study [28] have demonstrated that in pJIA, early and aggressive treatment induced significantly higher clinical remission rates and significantly reduced joint erosion and narrowing [29, 30]. Early detection of autoimmunity may therefore aid in exploiting such a window of opportunity in patients with pJIA.

It has been postulated that what begins as an abnormal immune response, with anti-CCP positivity; requires a ‘second event’ in order to convert this response into active disease [31].
Indeed, the number of citrullinated epitopes recognised by patient antibodies was shown to spread over time, and anti-CCP levels markedly increased ~2-4 years prior to a RA diagnosis, suggestive of a second stage in disease development [25], but then plateaus after onset. We also found that few new epitope specificities arose after B cell depletion with Rituximab in adult RA patients [32]. In future studies, it could prove interesting to explore whether this process is accelerated in pJIA(RF+ve), and if so; what drives this occurrence.

In addition to defining antibodies by the antigens that they detect, immunoglobulins may also be distinguished by hypervariable region structures known as idiotypes. The idiotope recognized by the 9G4 rat monoclonal antibody forms a hydrophobic patch which binds to N-acetylatedlactosamine (NAL) residues present on a number of self- and microbial glycoproteins and glycolipids [33, 34]. The ability to recognise NAL is potentially advantageous in assisting the clearance of damaged, apoptotic or neoplastic cells but may also risk autoimmunity if excessive mutation in the antigen combining site, located predominantly within the complementarity determining regions (CDR), confers additional binding to a self-specificity. If a particular idiotype is found in different patients, but on a particular group of antibodies, (e.g. anti-CCP); this is a strong indication that the unrelated individuals share usage of the same immunoglobulin-encoding gene [18]. The VH4-34 gene, which is strongly associated with autoimmunity [14] has been demonstrated to be utilized by anti-CCP antibodies in patients with (adult onset) RA[20]. This was seen in both early and established RA patients, but not in those with early polyarthritis not evolving into RA. It also suggests a notable restriction in VH gene usage that biases the development of their immunoglobulin repertoire which may be instrumental in the production of autoantibodies to citrullinated proteins [14]. We have here confirmed that this inherently autoreactive idiotope is also found on anti-CCP antibodies in rheumatoid factor positive pJIA patients.

In conclusion, this study has demonstrated the novel finding that adolescent pJIA(RF+ve) patients are distinct in their anti-CCP antibody phenotype from other JIA clinical patterns and age/sex matched healthy controls; but are comparable to adult RF-positive patients in their levels of IgG anti-CCP and shared expression of the same inherently autoreactive 9G4 idiotype. However, it was found that the adult group had significantly higher levels of IgA- and IgM anti-CCPs than the adolescent group, suggesting that further investigation is needed to fully elucidate the extent to which these two conditions may be seen as one.
1 References


<table>
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<tr>
<th>Patient groups</th>
<th>pJIA (n=88)</th>
<th>ERA (n=29)</th>
<th>EOA (n=38)</th>
<th>RA (n=35)</th>
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<tr>
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<td>17 (14-27)</td>
<td>18 (15-29)</td>
<td>56 (36-80)</td>
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<td>31</td>
<td>55.7</td>
<td>77.1</td>
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<td>Disease duration (years)</td>
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<td>4.5 (1.3-20.9)</td>
<td>13.4 (1.9-27.5)</td>
<td>13.2 (1.1-52.8)</td>
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*Median and range unless otherwise indicated. Abbreviations: pJIA, Polyarticular JIA; ERA, Enthesitis Related Arthritis; EOA, Extended Oligoarthritis; HC <23, Healthy control <23 years; RA, Rheumatoid Arthritis; HC >23, Healthy control >23 years; RF: Rheumatoid Factor.
Table 2. Autoantibody profiles of patient and control groups

<table>
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<tr>
<th>Patient groups</th>
<th>n</th>
<th>RhF+ve</th>
<th>IgG CCP+ve</th>
<th>IgA CCP+ve</th>
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<td>23 (100%)</td>
<td>19 (82.61%)</td>
<td>7 (30.43%)</td>
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<td>RhF-ve pJIA</td>
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<td>0 (0.00%)</td>
<td>4 (6.15%)</td>
<td>0 (0.00%)</td>
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<td>ERA</td>
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<td>0 (0.00%)</td>
<td>1 (3.45%)</td>
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<tr>
<td>EOA</td>
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<td>2 (5.26%)</td>
<td>1 (2.63%)</td>
<td>2 (5.26%)</td>
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<tr>
<td>RhF+ve RA</td>
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<td>26 (100%)</td>
<td>20 (76.92%)</td>
<td>17 (65.37%)</td>
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<td>0 (0.00%)</td>
<td>4 (44.44%)</td>
<td>2 (22.22%)</td>
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**Figure Legends:**

**Figure 1.** In A) IgG anti-CCP antibodies, in (B) IgM anti-CCP and (C) IgA anti-CCP antibodies across disease and healthy control groups. In (A) and (C) dotted lines depict upper limit of normal range as described in text.

Abbreviations: HC ≤23: Healthy control 23 years of age or less (n= 31); ERA: Enthesitis Related Arthritis (n= 29); EOA: Extended Oligoarthritis (n= 38); pJIA(RF+ve) : Rheumatoid Factor positive Polyarticular Juvenile Idiopathic Arthritis (n= 23); RF-ve pJIA: Rheumatoid Factor negative Polyarticular Juvenile Idiopathic Arthritis (n= 65); HC >23: Healthy Control over 23 years of age (n= 26); RF+ve RA: Rheumatoid Factor positive Rheumatoid Arthritis (n= 26); RF-ve RA: Rheumatoid Factor negative Rheumatoid Arthritis (n= 9). Shaded area on graphs denote 25th and 75th percentiles. Comparison of Groups was made using Mann-Whitney U test where *** p<0.0001, **p<0.001 and *p<0.01. Only selected comparisons were shown to preserve clarity.

**Figure 2.** In A) binding of the rat monoclonal antibody 9G4 to anti-CCP antibodies and in (B) to serum total IgM present in sera from patients and controls.

Abbreviations: HC ≤23: Healthy control 23 years of age or less (n= 31); ERA: Enthesitis Related Arthritis (n= 29); EOA: Extended Oligoarthritis (n= 38); pJIA(RF+ve) : Rheumatoid Factor positive Polyarticular Juvenile Idiopathic Arthritis (n= 23); RF-ve pJIA: Rheumatoid Factor negative Polyarticular Juvenile Idiopathic Arthritis (n= 65); HC >23: Healthy Control over 23 years of age (n= 26); RF+ve RA: Rheumatoid Factor positive Rheumatoid Arthritis (n= 26); RF-ve RA: Rheumatoid Factor negative Rheumatoid Arthritis (n= 9). Shaded area on A denotes the mean +SD of adolescent (HC≤23) samples. In B, shaded area encompasses the minimum and maximum OD of results of binding given by adolescent HC. Comparison of Groups was made using Mann-Whitney U test where *** p<0.0001, **p<0.001 and *p<0.01. Only selected comparisons were shown to preserve clarity.

**Figure 3:** Relationships between levels of 9G4-CCP and 9G4 expressed on serum total IgM for pJIA(RF+ve) patients (A) and sera from adult RA patients (B) containing IgG anti-CCP
antibodies. Linear regression (Pearson’s correlation coefficient) and significance at 5% level are indicated for each result.

Figure 4: Relationships between levels of 9G4-CCP and IgM anti-CCP (A), IgG anti-CCP (B) and IgA anti-CCP (C) for pJIA(RF+ve) patients and between levels of 9G4-CCP and IgM anti-CCP (D), IgG anti-CCP (E) and IgA anti-CCP (F) for adult patients with RA are shown. All pJIA and RA patients included were seropositive for IgG anti-CCP antibodies. Linear regression (Pearson’s correlation coefficient) and significance at 5% level are indicated for each result.