

Anti-Citrullinated Peptide Antibodies in Sudanese Patients with *Leishmania donovani* Infection Exhibit Reactivity not Dependent on Citrullination

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Abstract

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African patients with *Leishmania donovani* infections have signs of strong systemic inflammation and high levels of circulating immune complexes (IC) and rheumatoid factor (RF), all serologic markers of rheumatic disease. As inflammation in general is associated with citrullination, we sought to investigate ACPA responses in Sudanese *Leishmania* patients. Serum samples were collected from Sudanese patients with visceral leishmaniasis (VL) and post-kala-azar dermal leishmaniasis (PKDL) as well as from ACPA-positive Sudanese rheumatoid arthritis patients and compared to healthy Sudanese controls. Levels of circulating C1q-binding IC and anticyclic citrullinated peptide 2 (CCP2) were investigated using ELISA, and RF was measured with nephelometry. C1q adsorption was carried out to investigate anti-CCP2 content in IC. Citrulline specificity was evaluated with control plates with cyclic arginine-containing control peptides. *Leishmania*-infected patients had elevated levels of RF and circulating IC but also a significant increase in anti-CCP2 (12%) as compared to healthy controls. Anti-CCP2-positive *Leishmania* patients displayed lower anti-CCP2 levels than Sudanese patients with rheumatoid arthritis (RA), and anti-CCP2 levels in *Leishmania* patients showed a continuum not resembling the dichotomous pattern seen in patients with RA. Whereas the anti-CCP reactivity of Sudanese RA sera was strictly citrulline dependent, anti-CCP2-positive *Leishmania* sera reacted equally well with ELISA plates containing arginine control peptides. There was a strong correlation between anti-CCP2 and circulating IC among the *Leishmania* patients, but IC depletion only marginally diminished anti-CCP2 levels. Our findings stress the importance to interpret a positive CCP test carefully when evaluated in non-rheumatic conditions associated with macrophage activation.

Introduction

Leishmaniasis is a parasitic disease caused by the *Leishmania* spp. parasites. Infection with *Leishmania donovani* causes an internal disease called visceral leishmaniasis (VL) or kala-azar, with an immunopathology characterized by a strong humoral immune response and high production of anti-*Leishmania* antibodies, circulating immune complexes (IC) and polyclonal activation of B-lymphocytes. Post-kala-azar dermal leishmaniasis (PKDL) is a complication of VL characterized by severe rashes in mostly young patients who have recovered from VL and who are otherwise well.

IgM rheumatoid factor (RF) is detected in the sera of a majority of patients with rheumatoid arthritis (RA) [1]. Even though the presence of RF in patients with RA is

associated with development of more severe radiological damage, RF has a low diagnostic specificity when compared to disease controls with other rheumatic diseases and infections where elevated RF levels are common [2–5].

Immune complexes exert central functions in RA inflammation through stimulation of monocytes/macrophage-mediated production of cytokines, and they are also implicated in the induction of RF in RA [6]. An association between IC and RF has also been demonstrated in other rheumatic diseases as well as in infectious diseases [7, 8]. Rheumatoid factor production in VL was reported years ago [9], and circulating IC are also increased in chronic leishmaniasis and many other tropical parasitic diseases [10–15]. Using the same technique as we utilized for evaluating TNF induction by IC in RA synovial fluid [6],

we have earlier demonstrated that IC isolated from *Leishmania*-infected patients induces both pro-inflammatory as well as immunosuppressive cytokine production. Furthermore, levels of C1q-binding IC in serum were closely correlated with IC-induced cytokine levels, notably GM-CSF [16].

Antibodies against citrullinated proteins and/or peptides (ACPA/anti-CCP2) are serological markers with higher diagnostic specificity for RA, making them superior to RF in laboratory diagnostics. The event of citrullination of proteins and peptides occurs naturally during inflammation and represents a post-translational modification of arginine through deamination [17]. Antibodies towards several different citrullinated proteins have been associated with RA, and patients with anti-CCP2-positive RA develop more severe clinical manifestations than do anti-CCP2 negative patients [18, 19]. The presence of anti-CCP2 has also been demonstrated in a number of infectious diseases, for example pulmonary tuberculosis, hepatitis C infection and type I autoimmune hepatitis [20–24]. Importantly, anti-CCP2 positivity evident in non-RA disease sera seems to not always be citrulline dependent, as demonstrated by Vannini *et al.* [23]. Due to our interest in the ACPA response in RA [18, 25, 26] and our previous studies on IC-mediated inflammation in *Leishmania*-infected patients [16, 27], we wanted to investigate the occurrence of ACPA in relation to RF and circulating IC in African patients infected with *L. donovani*.

Methods

Patients and sampling. Serum samples were collected from 74 patients with VL; median 23 year, range 3–73, female/male ratio: 25/48 (data on sex missing for one individual), 46 patients with PKDL (median 11 year, range: 4–27, female/male ratio: 14/25 (data on sex missing for seven individuals), 93 healthy Sudanese controls (median age 23 year, range: 3–54, female/male ratio: 26/67). Patients with VL and PKDL mainly from the Tabarakalla and Barbar Elfogara villages were received at the Tabarakalla rural hospital in Gadarif state, along the lower Atbara River in the Gallabat Province, eastern Sudan. This area, where the main vector is *Phlebotomus orientalis*, is endemic for *L. donovani* [28]. A detailed clinical history was evaluated and thorough clinical examination was conducted by one of the co-authors (AIE) as previously described, with particular reference to hepatosplenomegaly, enlargement of lymph nodes and recurrent fever for more than 1 month [16]. Thick and thin blood films for detection of *Plasmodium* parasites were examined from all individuals who either had fever, looked ill or had splenomegaly, and those with positive blood films for malaria were excluded. An inguinal lymph node aspiration was performed on those clinically suspected of having VL (i.e. all individuals with fever for >2 months, left upper

quadrant pain, lymphadenopathy, splenomegaly or wasting). Those with a negative result underwent bone marrow aspiration from the superior posterior iliac crest. PKDL was diagnosed on clinical grounds, on the appearance and distribution of the rash after treatment in previously diagnosed patients with VL. There are no laboratory tests for diagnosing PKDL.

Nineteen patients with RA (median age 45 years, range: 22–60, female/male ratio: 17/2) were sampled from a larger RA cohort from the rheumatology unit at Alribat university hospital, Khartoum (A. I. Elshafie unpublished observations), where their diagnosis had been determined by a rheumatology specialist (MAMN) following the 1987 ACR criteria [29]. The patients with RA primarily served as positive controls in the anti-CCP2 specificity investigation, thus only anti-CCP2-positive patients were included. Healthy Sudanese controls were collected from both the Tabarakalla rural area and from Alribat university hospital. Data were compared with 100 Swedish healthy controls earlier investigated for RF and anti-CCP2 in the same laboratory, and a smaller Swedish control group ($n = 20$) investigated for C1q-binding circulating IC in conjunction to this study. Sera were separated by centrifugation within 2 h of collection, and samples of the Sudanese sera were separated and frozen in liquid nitrogen (countryside) or in $-70\text{ }^{\circ}\text{C}$ freezer (Khartoum) within 2 h of sampling, stored frozen at $-70\text{ }^{\circ}\text{C}$ and transported on dry ice to Uppsala, Sweden. Ethical approval for this study was obtained from the Ethical Committee of the Alribat university hospital, from the Ministry of Health at Gadarif State, from the Ethical Committee at Uppsala University and orally from the alderman in Tabarakalla village. Informed consent was obtained from all of the adults who participated in the study. For young children, consent was obtained from their parents.

Measurement of levels of circulating IC, anti-CCP2 and RF. C1q-binding assay for circulating IC. Levels of circulating IC were measured by a solid-phase C1q assay (Bindazyme C1q binding kit; Binding Site, Birmingham, UK). Levels above 10.8 Eq/ml were regarded as positive, as suggested by the manufacturer. The range of the assay is 1.23–100 Eq/ml.

Anti-CCP2. Anti-CCP2 was measured using the Immunoscan RA Mark 2 assay (Euro-Diagnostica, Malmö, Sweden). Anti-CCP2 positivity was determined in accordance with the manufacturer's instructions with 25 U/ml used as a cut-off. The assay does not yield quantitative levels below the company-defined cut-off. For that reason, we extended the standard curve by dilution to also obtain values below 25 U/ml (extended range 3.126–1600 U/ml). Values exceeding the range of the standard curve were given the value 1600 U/ml.

A control ELISA plate with cyclic peptides containing arginine instead of citrulline in the relevant peptide positions was kindly provided by Jörgen Wieslander at

Euro-Diagnostica and was used to evaluate citrulline-specific reactivity. The cut-off value for the arginine control was determined arbitrarily by the absorbance corresponding to 25 U/ml in the standard curve for the citrulline (CCP) variant. Results were then calculated as cut-off index (COI): observed OD₄₅₀/citrulline cut-off OD₄₅₀ as according to Vannini *et al.* [23].

To test if circulating IC present in the investigated samples influenced anti-CCP2 reactivity, we adsorbed C1q-binding IC from sera and evaluated CCP reactivity afterwards. Eight anti-CCP2-positive VL, four of the patients with PKDL and six of the patients with RA were tested. Sera were diluted 1:50 and incubated for 2 h on C1q coated plates (Bindazyme C1q binding kit; Binding Site). After incubation, the samples were directly transferred to the CCP plate and analysed for anti-CCP2 according to the manufacturer of the anti-CCP2 test kit. Levels of circulating IC remaining after adsorption were determined in anti-CCP2-positive *Leishmania* samples.

Rheumatoid factor. Rheumatoid factor was measured by nephelometry (Image; Beckman Coulter, Stockholm, Sweden) and expressed in international units/ml (IU/ml) with values >20 IU/ml regarded as positive. The analysis was standardized using the NIBSC 64/002 reference serum. The nephelometer does not yield quantitative data below 20 IU/ml, and RF negative samples were given the value 0 IU/ml when comparing different groups.

Statistics. The Mann–Whitney *U*-test was used to compare groups. For correlations between groups, Pearson's correlation test was used. *P*-values below 0.05 were considered significant.

Results

RF

Among patients with VL, 86% (64/74) were RF positive, with a median of 71 IU/ml among the positive subjects, range: 20–1440 IU/ml. For PKDL, patients 69% (29/42) were positive with a median of 34 IU/ml and range 20–165 IU/ml. Due to technical reasons, data on RF were not obtained from four individuals. In the anti-CCP2-positive RA group, 84% (16/19) were also positive for RF (median: 239 IU/ml, range: 69–3470 IU/ml). Among the Sudanese healthy controls, 11% (10/93) had RF levels above 20 IU/ml (median 23 IU/ml, range: 20–96 IU/ml). In a cohort of 100 healthy Swedish subjects investigated with the same equipment, two were weakly RF positive (20.4 and 21.6 IU/ml, respectively).

The VL group had higher RF levels as compared to patients with PKDL ($P < 0.0001$). There was no significant difference between patients with anti-CCP2-positive RA and patients with VL. However, patients with RA had higher levels compared to patients with PKDL ($P = 0.0004$). All three of the disease groups had signif-

icantly higher RF levels than did the healthy Sudanese controls. RF levels in the Sudanese groups are presented graphically in Fig. 1.

Circulating immune complexes

Among the patients with VL, 23/74 (31%) had elevated IC levels (median: 7 Eq/ml, range: 0.0–100.6 Eq/ml), whereas among patients with PKDL 2/42 (7%) had elevated IC levels (median: 2.0 Eq/ml, range 0–21 Eq/ml). One of 20 patients with anti-CCP2-positive RA had IC level above 10.8 Eq/ml (median 1.5 Eq/ml, range 0–10.8). In the Sudanese control group, 2/93 were positive for circulating IC. In the Swedish control group, all individuals were negative for circulating IC. The patients with VL had significantly higher IC levels than all other investigated groups, $P < 0.0001$ for all comparisons. Levels of circulating IC in the Sudanese groups are graphically depicted in Fig. 2.

Anti-CCP2

Among the patients with VL, 12.2% (9/74) were determined to be anti-CCP2 positive (median for anti-CCP2-positive patients: 30.86 U/ml, range: 25–148). Anti-CCP2 reactivity was also present in 4.2% of the patients with PKDL (2/

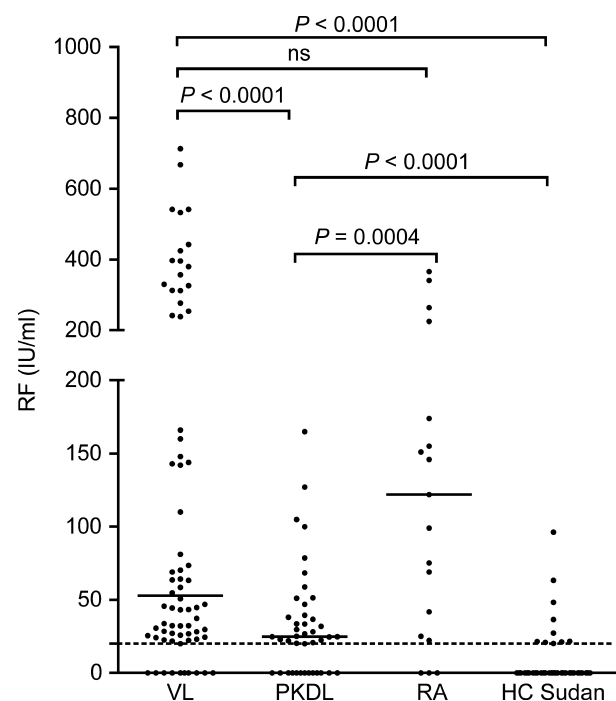


Figure 1 Levels of rheumatoid factor. Information is shown separately for patients with visceral leishmaniasis (VL), post-kala-azar dermal leishmaniasis (PKDL), Sudanese anti-CCP2-positive rheumatoid arthritis patients (RA) and Sudanese healthy controls (HC Sudan). The dotted line represents the lowest measurable level (20 IU/ml) for the nephelometer, which is also the clinically established cut-off. Solid lines depict the median in each group.

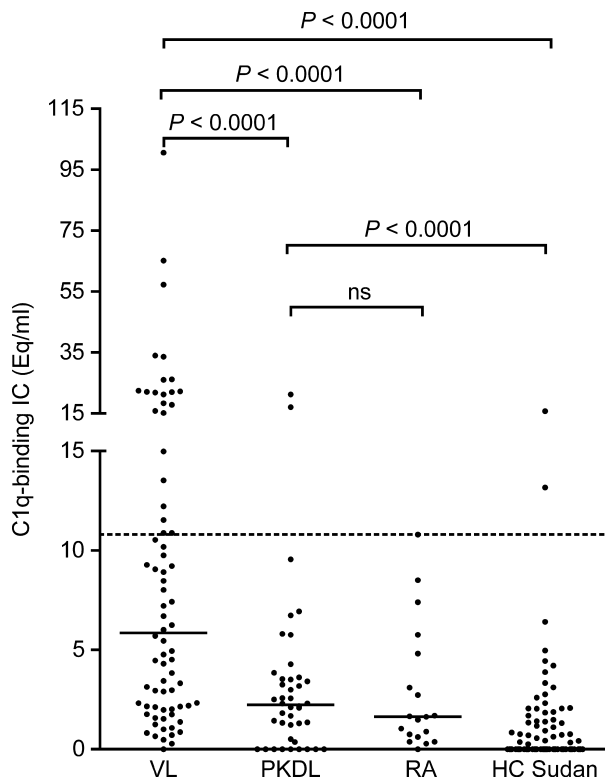


Figure 2 Levels of circulating immune complexes. Data are shown separately for patients with visceral leishmaniasis (VL), post-kala-azar dermal leishmaniasis (PKDL), Sudanese anti-CCP2-positive rheumatoid arthritis patients (RA) and Sudanese healthy controls (HC Sudan). The dotted line represents the cut-off (10.8 Eq/ml) as recommended by the manufacturer, and solid lines depict the median in each group.

42, 34 and 95 U/ml). Among the Sudanese patients with anti-CCP2-positive RA, anti-CCP2 levels were much higher: median 1265 U/ml, range: 50–>1600 U/ml). In the Sudanese healthy control group, one anti-CCP2-positive subject was identified (51 U/ml). In the Swedish control group investigated earlier, 3/100 were positive, two of them in the borderline region (30, 42 and 1643 U/ml, respectively).

Data for the Sudanese groups are shown in Fig. 3. Anti-CCP2 levels in patients with VL and PKDL represented a continuum encompassing both the rather low anti-CCP2-positive and the negative subjects (Fig. 3). These results diverged from those for Swedish patients with RA [18] and our recently collected cohort of Sudanese patients with RA (A. I. Elshafie, unpublished observations) where anti-CCP2 levels showed a clear dichotomy between very high levels in anti-CCP2-positive subjects and clearly negative levels in anti-CCP2 negative subjects.

The levels of anti-CCP2 among patients with VL correlated well to the observed IC levels ($r = 0.6586$, $P < 0.001$), whereas no significant correlation was found for any other patient group (Table 1). In the anti-CCP2-positive RA group, no association was determined between IC levels and anti-CCP2; instead, there was a correlation

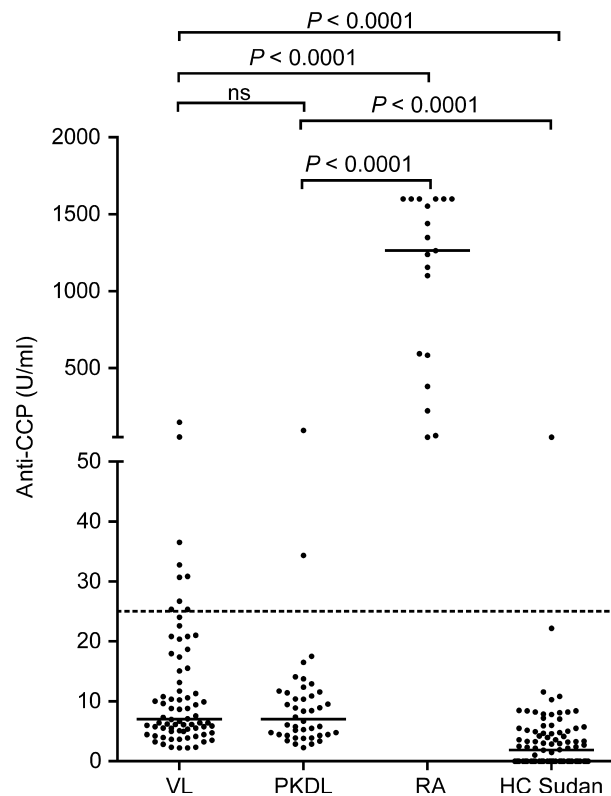


Figure 3 Anti-CCP2 levels. Data are shown separately for patients with visceral leishmaniasis (VL), post-kala-azar dermal leishmaniasis (PKDL), Sudanese anti-CCP2-positive rheumatoid arthritis patients (RA) and Sudanese healthy controls (HC Sudan). The dotted lines represent the cut-off (25 U/ml) as described by the manufacturer, and solid lines depict the median in each group.

between RF levels and levels of circulating IC ($r = 0.6398$, $P = 0.0032$; Table 1). To rule out the possibility that anti-CCP2 positivity was due to cross-reactions with IC or that the CCP reactivity might be primarily bound to IC, we adsorbed C1q-binding IC from sera and evaluated anti-CCP2 reactivity afterwards. This procedure did not diminish the anti-CCP2 reactivity in either the VL group or among patients with anti-CCP2-positive RA. The median anti-CCP2 reactivity remaining after IC adsorption was 97% in all groups, range: VL 61–175%, RA 77–107%, PKDL 85–102% (data not included). When anti-CCP2-positive (range: 37–42 U/ml) VL samples on the other hand were investigated concerning the efficiency of IC removal, only 23% (median) of the circulating IC remained (data not shown).

Citrulline specificity among anti-CCP2-positive patients was investigated using a control plate containing non-citrullinated (arginine-containing) cyclic peptides as target antigen (Fig. 4). Among the anti-CCP2-positive samples in the VL group, there was no difference in reactivity against CCP2 and the non-citrullinated control peptide (Fig. 4A). The same pattern was apparent for the two patients with anti-CCP2-positive PKDL (Fig. 4B)

Table 1 Correlations between levels of rheumatoid factor (RF), anti-CCP2 and circulating immune complexes (IC) in the different groups of investigated subjects. Due to technical reasons, data on RF were not obtained from four of the patients with post-kala-azar dermal leishmaniasis (PKDL) and accordingly levels of IC and anti-CCP2 from those individuals are excluded in the analysis.

| | Visceral leishmaniasis (VL) + PKDL (<i>n</i> = 116) | VL (<i>n</i> = 74) | PKDL (<i>n</i> = 42) | Patients with Anti-CCP2-positive rheumatoid arthritis (<i>n</i> = 19) | Sudanese healthy controls (<i>n</i> = 95) | All groups together (<i>n</i> = 228) |
|---------------------|--|------------------------------|-----------------------------|--|--|---------------------------------------|
| RF versus IC | 0.1985 (<i>P</i> = 0.0327) | 0.1225 (<i>P</i> = 0.2983) | 0.1055 (<i>P</i> = 0.5061) | 0.6398 (<i>P</i> = 0.0032) | -0.0735 (<i>P</i> = 0.4836) | 0.1966 (<i>P</i> = 0.0029) |
| RF versus anti-CCP2 | -0.0087 (<i>P</i> = 0.9260) | -0.0394 (<i>P</i> = 0.7388) | 0.0133 (<i>P</i> = 0.9334) | 0.2638 (<i>P</i> = 0.2751) | -0.0031 (<i>P</i> = 0.9766) | 0.3008 (<i>P</i> < 0.0001) |
| IC versus anti-CCP2 | 0.5598 (<i>P</i> < 0.0001) | 0.6586 (<i>P</i> < 0.0001) | 0.0687 (<i>P</i> = 0.6655) | 0.3395 (<i>P</i> = 0.1550) | -0.0204 (<i>P</i> = 0.9766) | -0.0153 (<i>P</i> = 0.8183) |

This non-citrulline-specific reactivity was in strict contrast to the Sudanese patients with anti-CCP2-positive RA in whom anti-CCP2 reactivity was specific for CCP and with very low reactivity with the arginine-containing control peptides (*P* < 0.0001; Fig. 4C).

Discussion

In this study, we found that sera from patients with VL were often RF positive, had elevated levels of circulating IC and that a rather substantial amount (12.2%) exhibited seroreactivity towards CCP2. However, contrary to what was evident in Sudanese RA sera, the CCP reactivity was not restricted to citrulline-containing peptides, as there was an equal reactivity against cyclic arginine-containing control peptides, both in patients with the acute form of VL as well as with the post-treatment PKDL form.

One study has previously demonstrated anti-CCP2 reactivity in a small group of ten Brazilian patients infected with the South American parasite *Leishmania major* [30]. However, citrulline dependency of CCP-reactive sera was not evaluated in that study. We have now extended these findings to encompass a larger cohort of Sudanese patients infected with the African parasite *L. donovani*, both the acute VL form as well as the post-treatment PKDL form not occurring in South America. We demonstrate that patients with both these diagnoses might have reactivity in anti-CCP2 ELISA that is not dependent on citrullination of the peptide antigen, that is representing a false-positive ACPA reactivity. This was in contrast to Sudanese patients with RA for whom a strict dependency for citrullination of the arginine residues was needed for the high anti-CCP2 reactivity.

Besides our study, only two reports of ACPA reactivity in non-rheumatic conditions have investigated their sera using proper arginine-containing control ELISA wells. In the study by Vannini *et al.* [23] of autoimmune hepatitis type I patients, appropriate controls were used both for the commercial and proprietary CCP2 assay, where the peptide composition has not been disclosed to the scientific community, as well as for the publicly described CCP1 peptide [5]. They demonstrated that whereas the majority (87%) of CCP-positive sera from rheumatological patients with other diagnoses than RA were citrulline specific, this was the case for only half of the investigated hepatitis sera.

In the tuberculosis study by Kakumanu *et al.* [20], the reactivity against the CCP1 peptide was citrulline specific in 94% of RA sera and in 22% of sera from pulmonary tuberculosis patients. The authors also reported that soluble CCP1 peptide inhibited reactivity with RA sera but not with TB sera. In the present investigation, a parallel protocol could not be employed as the proprietary CCP2 peptides are not available in soluble form.

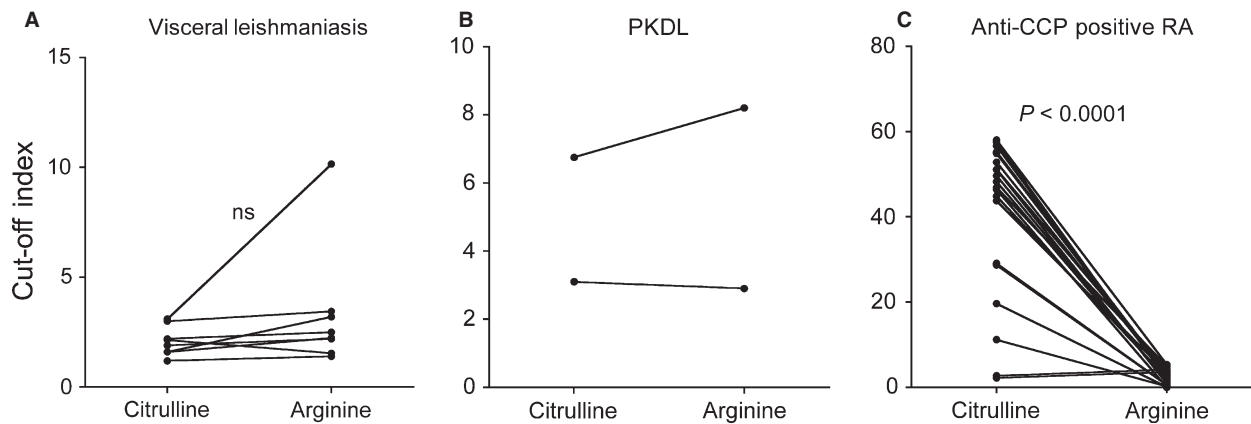


Figure 4 Citrulline specificity among anti-CCP2-positive patients. Information is shown for (A) visceral leishmaniasis patients, (B) post-kala-azar dermal leishmaniasis patients (PKDL) and (C) Sudanese patients with anti-CCP2-positive rheumatoid arthritis. Results are calculated as cut-off index (COI): observed $OD_{450}/\text{citrulline cut-off } OD_{450}$.

Even though the CCP2 test is the most commonly used ACPA test in clinical diagnostic settings, the proprietary and secluded nature of the CCP2 antigen represents an obvious hurdle in studies of non-rheumatic conditions such as autoimmune hepatitis [23] and tuberculosis [20], where the use of proper controls has shown the CCP reactivities to be much less citrulline dependent than in parallel studies of patients with RA. The citrulline dependency of ACPA reactivity has been unequivocally proven in numerous studies of patients with RA. The fact that only a small minority of commercial ACPA tests is designed with proper control wells with non-citrullinated antigens is therefore no major drawback in clinical rheumatology practice. When ACPA are recorded in non-rheumatic conditions, the findings should, however, be preferably investigated concerning citrulline dependency of the reactivity.

Our finding that CCP reactivity in patients with VL was not restricted to citrulline argues that this anti-CCP2 reactivity is an effect of extensive inflammation and immune activation more than a sign of shared pathogenic characteristics with anti-CCP2-positive arthritis. In RA, anti-CCP2 reactivity exhibits a bimodal distribution with mostly either totally negative or very high-positive levels, where the anti-CCP2-positive patient group is defined by certain genetic characteristics and the impact of environmental triggers, notably smoking [31]. In the present study, the mean anti-CCP2 reactivity among positive *Leishmania* subjects was 49 U/ml, representing 1.96 times the cut-off value, data on low-positive anti-CCP2 reactivity in agreement with the findings in Brazilian leishmaniasis [30]. This is in stark contrast to Swedish patients with anti-CCP2-positive RA with a mean level of 1128 U/ml (45.1 times the cut-off) using the same anti-CCP2 test [18]. This hypothesis is also supported by the fact that the CCP2 reactivity in patients with VL and PKDL displayed a continuum between the anti-CCP2-positive sera and the 'negative' interval below the cut-off, whereas anti-CCP2

negative Sudanese controls had lower reactivity (Fig. 3). This is also in accordance with our experience of Swedish patients with RA (J Rönnelid).

Although it seems that some infections might yield falsely positive anti-CCP2, there is a possibility that infections really are associated with the appearance of ACPA and that citrulline specificity of the ACPA response might develop over time. One example is immunity to the bacteria *Porphyromonas gingivalis* associated with periodontitis [32, 33].

An intriguing finding is that two groups of infections where a non-citrulline-specific and non-arthritis-associated ACPA response has been reported represent agents localizing intracellularly in tissue macrophages. Both *Leishmania* parasites (this paper and [30]) as well as tuberculosis [20, 24] represent intracellular infections in tissue macrophages. The findings of ACPA responses in hepatitis C [21, 22, 34] where the infection primarily resides in hepatocytes might not seem in accordance with such a hypothesis. There is, however, a debate on whether macrophages/Kupffer cells in addition to hepatocytes are infected by hepatitis C virus, as reviewed in [35]. Additionally, autoimmune hepatitis type 1 in which arthritis-independent ACPA reactivity has been reported [23] is also associated with macrophage activation both in the early [36] as well as in later fibrotic stages [37].

Herein, we have for the first time demonstrated that Sudanese patients infected with *L. donovani* exhibit false-positive anti-CCP2 reactivity similarly with patients with RA from Sudan. Contrary to what is seen in Sudanese patients with RA, the ACPA reactivity in leishmaniasis patients is not dependent on citrullination of the antigenic target and might be associated with extensive activation of macrophages. Our results stress the importance of including appropriate assay controls when defining ACPA reactivities in new patient groups, especially in non-rheumatic patient cohorts.

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Competing interests

The authors declare no conflict of interests.

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