# *Chlamydophila psittaci* subclinical infection in chronic polyarthritis

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## Abstract

### Objective

*Recent evidence indicates that Chlamydophila psittaci (Cp) may establish chronic infections, which may promote autoimmunity and/or B cell lymphoproliferation.*

### Methods

*The presence of a subclinical Cp infection was investigated in 293 patients with chronic inflammatory polyarthritis, including 175 patients with rheumatoid factor (RF)-positive and/or anti-CCP-positive rheumatoid arthritis (RA) and 118 with seronegative polyarthritis (46 RF-negative/anti-CCP-negative RA, 36 psoriatic arthritis and 36 undifferentiated spondyloarthritis). One hundred and eighty-five healthy controls were also investigated. The presence of Cp infection was assessed in peripheral blood mononuclear cells using several PCR protocols targeting different regions of the Cp genome (16S-23S spacer rRNA, OMP-A, and Gro-EL). The DNA of other Chlamydia species (C. Pneumoniae and C. Trachomatis) was also investigated. Amplicons were sequenced to confirm the specificity of PCR products.*

### Results

*The presence of a subclinical chronic Cp infection was observed in a significantly higher percentage of patients with chronic polyarthritis (38/293; 13%) compared to healthy controls (1/185, 0.5%; OR=27.4, 95%CI:3.73-201.6, p<0.0001). Furthermore, the prevalence of Cp was higher in seronegative polyarthritis (23/118; 19.5%) than in seropositive RA patients (15/175; 7.4%; OR=2.58, 95%CI: 1.28-5.19, p=0.0078). The highest prevalence of Cp infection was found in RF/anti-CCP double-negative RA patients (13/46, 28.3%), followed by patients with psoriatic arthritis (6/36; 16.7%). No differences in age, sex, disease duration and undergoing therapies were noticed between Cp-positive and Cp-negative patients; nor between seropositive and seronegative patients.*

### Conclusion

*Cp may be an infectious trigger possibly involved in the pathogenesis of a fraction of inflammatory polyarthritis, particularly in seronegative patients.*

**Key words**

chlamydophila psittaci, autoimmunity, rheumatoid arthritis, pathogenesis, rheumatoid factor, anti-citrullinated peptide antibodies

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## Introduction

Our understanding of the pathogenic mechanisms involved in systemic inflammatory diseases, such as rheumatoid arthritis (RA) and spondyloarthritis (SpA), has considerably improved in the last decade. The relationship between infection and seronegative spondyloarthritis is well-established. Conversely, whether infection is implicated for the development of rheumatoid arthritis is still unknown. In the past, several rheumatic diseases were hypothesised to be linked to viral or bacterial infections, and several potential bacterial and viral agents may induce rheumatic manifestations (1-3). On the other hand, infections may also protect from autoimmunity (4) and autoimmune patients may have an increased susceptibility to infections, favoured by immunosuppressive drugs (5, 6) or biologic agents (7). Thus, infections and autoimmune diseases have complex relationships, which have been only partially elucidated so far (8).

There are several mechanisms through which pathogens can initiate or perpetuate autoimmunity. In most cases, infectious agents may persist and trigger autoimmunity by mimicking host epitopes, which normally do not elicit self-directed immune responses due to tolerance mechanisms (9-11). Pathogen persistence is typically found in reactive arthritis (ReA) (12, 13), but active infections sustained by viruses or by other infectious agents have been also documented in the synovium of RA patients (14-17). The classical microbial organisms linked to ReA are *Chlamydia Trachomatis* (Ctra) and enterobacteriacee, but recently, several other non-classical infections were recognised, such as *Chlamydophila pneumoniae* (Cpneu) and *Borrelia Burgdoferi* (18). Co-infection by different microorganisms may be also detected by RT-PCR studies, such as Ctra and Cpneu or Ctra and *Borrelia Burdgoferi*. In the joints, these organisms may evade the immune system by down-regulating membrane antigens, a process that may be induced by antibiotic treatments. The hypothesis that RA might be also caused by infection with mycoplasma or similar organisms (19) provided the rationale to perform several clinical trials with tetracyclines in RA patients, with encouraging, even if not conclusive, results (20-22).

Recent findings indicate that *Chlamydophila psittaci* (Cp), the etiologic factor of psittacosis, may also establish extra-nodal chronic infections, which may promote autoimmunity or B cell lymphoproliferation. In particular, several lines of evidence indicate that Cp infection is strictly associated with the development of ocular adnexal lymphomas (23, 24). Notably, in these patients the DNA of Cp is often detected also in peripheral blood mononuclear cells (PBMCs), indicating the occurrence of a systemic infection (23). In the present study, the presence of a subclinical Cp infection was investigated in a large cohort of patients with inflammatory polyarthritis, including RA, psoriatic arthritis (PsA) and undifferentiated spondyloarthritis (uSpA). Considering that available serological tests for Cp suffer from cross-reactive responses with other *Chlamydiae*, DNA detection by PCR analysis is the current gold standard for chlamydial infection recognition (23, 24). We therefore analysed the presence of Cp infection by DNA analysis in peripheral blood mononuclear cells (PBMCs) from patients and healthy controls. The same series of samples was also analysed for the presence of Cpneu and Ctra.

## Patients and methods

*Study populations*

A series of 293 consecutive unselected patients with inflammatory polyarthritis were enrolled in the study. Among these patients, 175 cases with RF-positive and/or anti-CCP-positive rheumatoid arthritis (RA) and 118 patients with seronegative inflammatory chronic polyarthritis (46 RF-negative/ anti-CCP-negative RA, 36 PsA and 36 uSpA) were analysed. Patients were all Caucasian and resident in Northern Italy. Rheumatoid factor (RF) and anti-CCP antibodies were assessed by standardised diagnostic methods: RF by turbidimetric assay (positive when >20 IU/ml) and anti-CCP antibodies by ELISA (Eurodiagnostica, Malmö, Sweden; positive when >5 IU/ml). A

written informed consent, according to the Declaration of Helsinki 1975/83, was obtained for all the enrolled subjects and the study was approved by the Insititutional Review Board. Two hundred and twenty-five (127 males, 98 females; mean age 44.4±7, range 18-70 yrs) blood donors resident in the north of Italy were analysed as controls. The main epidemiological and clinical features of patients and controls are illustrated in Table I.

**Table I.** Epidemiological and clinical features of study patients and controls.

| | Seropositive RA (175) | Seronegative polyarthritis (118) | HBDs (225) |
|---|---|---|---|
| Age (yrs) | 61.0 ± 12.9 | 54.9 ± 14.4 | 44.4 ±7 |
| range (yrs) | 23–85 | 20–85 | 18–70 |
| Sex M/F | 36/139 | 43/75 | 127/98 |
| Disease duration (yrs) | 15.7 ± 16.3 | 11.7 ± 10.1 | |
| Undergoing therapies | | | |
| NSAIDs n(%) | 4 (2.3%) | 5 (4.2%) | |
| DMARDs n(%) | 58 (33.1%) | 26 (22%) | |
| anti-TNFs n(%) | 108 (61.7%) | 81 (68.7%) | |
| RTX n(%) | 5 (2.9%) | 6 (5.1%) | |

Seropositive RA comprised all rheumatoid arthritis' patients with RF-positive and/or anti-CCP-positive antibodies while seronegative polyarthritis comprised 46 patients with RA and without RF and anti-CCP antibodies, 36 patients with psoriatic arthritis and 36 patients with undifferentiated spondyloarthritis. NSAIDs: non-steroidal anti-inflammatory drugs; DMARDs: disease modifying anti-rheumatic drugs; anti-TNF: anti-tumour necrosis factor agents (*i.e.* etanercept, infliximab, adalimumab); RTX: rituximab.

*Molecular studies*

DNA was extracted from PBMCs using an automated system (Maxwell 16, Promega) in all cases. The presence of Cp DNA was analysed by several PCR approaches targeting different regions of bacterial genome and protocols specific for 16S-23S spacer rRNA, OMP-A, and Gro-EL (hsp-60) (23, 24). A multiplex touchdown, enzyme time-release polymerase chain reaction (PCR) assay, designed to simultaneously detect Ctra, Cpneu and Cp DNA at bacterial loads lower than 1 infection-forming unit was performed according to a previously published protocol with few modifications (25). Cp infection was also detected in single PCR assays using different primers: PSIF 5'CGT TGA CTC AAC CTG CAA AG 3'and PSIR 5'CAA CCT AGT CAA ACC GTC CT 3'. Amplified DNA was from the end of the 16S rRNA gene and the beginning of the 16S-23S spacer region in the ribosomal genes. The primer pairs used specifically for Ctra and Cpneu were located entirely in the 16S rRNA gene; for Cp, one primer was located in the 16S rRNA gene and one primer was located in the 16S-23S spacer region. The omp-A nested PCR and Gro El were done according published protocols (26, 27) Blank reactions filled with 50 µL of PCR mixture were interspersed every ten samples to monitor possible contamination of PCR reagents by chlamydial DNA and to rule out any false-positive results. Amplicons were sequenced to confirm the specificity of PCR products. More than 75% of samples were analysed using three different PCR protocols (16S-23S spacer rRNA, OMP-A, and Gro-EL) and a 100% of concordance between the results obtained with the different methods was observed. The remaining cases were analysed only by 16S-23S spacer rRNA and Gro-EL protocols, the completion of the analysis was prevented by the lack of available DNA for the third protocol. In this subset of cases, we also obtained a 100% concordance.

*Statistical analysis*

Statistical analysis was performed using the GraphPad and Instat softwares. The difference in the prevalence of Cp infection in the different autoimmune diseases were analysed by 2x2 contingency tables using the Fisher's exact test with Woolf's approximation. Differences in means were analysed by the non-parametric Mann Whitney *t*-test .

## Results

The prevalence of Cp DNA in the peripheral blood of polyarthritis' patients resulted significantly higher than in controls (38/293, 13% in chronic arthritis patients *versus* 1/225, 0.4% in healthy donors; OR=33.4, 95%CI: 4.54–245.2, $p<0.0001$; Table II).

Of note, the prevalence of Cp DNA was significantly higher in seronegative polyarthritis (RF/anti-CCP double-negative RA plus psoriatic arthritis plus undifferentiated spondyloarthritis), than in patients with seropositive (RF-positive and/or anti-CCP-positive) RA, (23/118, 19.5% *vs*. 15/175, 8.6%; OR=2.58, 95%CI: 1.28–5.19, $p=0.0078$; Table II). The highest prevalence of Cp DNA was found among the RF/anti-CCP double-negative RA (13/46, 28.3%; *vs*. HBDs: OR=88.24, 95%CI: 11.17–697.2, $p<0.0001$), followed by patients with psoriatic arthritis (6/36, 16.7%; *vs*. HBDs: OR=44.8, 95%CI: 5.21–385.2, $p<0.0001$). Of note, the prevalence of Cp DNA still remained significantly more elevated in RF-positive and/or anti-CCP-positive RA patients (15/175, 8.6%) than in HBDs (OR=21; 95%CI: 2.75–160.7, $p<0.0001$; Table II).

Cp-positive and Cp-negative patients did not differ significantly in terms of age, sex, and disease duration (Table III). When considering the pharmacological treatments of patients, some differences were noticed between Cp-positive and Cp-negative patients (Table III); in particular, Cp-positive patients were equally treated with DMARDs (17/38, 44.7%) and anti-TNF agents (17/38, 44.7%), while Cp-negative patients were largely treated with anti-TNF therapies (172/255, 67.4%; OR=0.39, 95%CI: 0.19–0.78; $p=0.01$). Since anti-TNF therapies are usually introduced after DMARDs' failure, present results suggest that introducing anti-TNF agents did not increase Cp infection prevalence in inflammatory polyathritis. Of note, 12/13 of Cp-positive RA patients who were tested for Cp when under treatment with DMARDs, successively started biological therapies (all in association with DMARDs) due

**Table II.** Cp prevalence in patients, overall and according to the autoantibodies status. [A]Patients with chronic polyarthritis *vs*. HBDs; [B]Seronegative polyarthritis (see text) *vs*. seropositive RA (RF-positive and/or anti-CCP-positive RA); [C]Seropositive RA *vs*. HBDs.

| | Cp prevalence | Statistics |
|---|---|---|
| All the patients with chronic polyarthritis | 38/293 (13%) | [A]OR=33.4<br>95%CI: 4.54–242.2; $p<0.0001$ |
| HBDs | 1/225 (0.4%) | |
| *Subanalyses* | | |
| Seronegative polyarthritis | 23/118 (19.5%) | [B]OR=2.58<br>95%CI: 1.28–5.19; $p=0.0078$ |
| Seronegative RA | 13/46 (28.3%) | |
| Psoriatic arthritis | 6/36 (16.7%) | |
| Undifferentiated Spondyloarthritis | 4/36 (11.1%) | [C]OR=21 |
| Seropositive RA | 15/175 (8.6%) | 95%CI: 2.75–160.7; $p<0.0001$ |

**Table III.** Demographic and clinical features of Cp-positive and Cp-negative patients.

| | Cp-positive (38) | Cp-negative (255) | Statistics |
|---|---|---|---|
| Age (yrs) | 56.3 ± 17.7 | 58.4 ± 13.8 | $p$=ns |
| Sex (F/M) | 27F/11M | 188F/67M | $p$=ns |
| Disease duration (yrs) | 15.7 ± 16.3 | 11.7 ± 10.1 | $p$=ns |
| *Therapies* | | | |
| NSAIDs n (%) | 2 (5.3%) | 7 (2.7%) | $p$=ns |
| DMARDs n (%) | 17 (44.7%) | 67 (26.3%) | OR=2.27; 95%CI=1.13–4.56, $p=0.033$ |
| anti-TNFs n (%) | 17 (44.7%) | 172 (67.4%) | OR=0.39; 95%CI=0.196–0.779, $p=0.010$ |
| RTX n (%) | 2 (5.3%) | 9 (3.5%) | $p$=ns |
| Seropositive n (%) | 15 (39.5%) | 160 (62.7%) | OR=0.39; 95%CI=0.193–0.779, $p=0.008$ |

NSAIDs: non-steroidal anti-inflammatory drugs; DMARDs: disease modifying anti-rheumatic drugs; anti-TNF: anti-tumour necrosis factor agents (*i.e*. etanercept, infliximab, adalimumab); RTX: rituximab.

to unsuccessful response to DMARDs alone; after 6 months the new biological therapies provided a EULAR good response in 2/12, a moderate response in 6/12 and failed in 4/12 (data not shown).

All patients and controls were also screened for the presence of the other *Chlamydia* spp by specific PCR protocols: Cpneu DNA was detected in 7/293 (2.4%) of patients, while no Cpneu DNA positive samples were found in HBDs. Finally, all patients and controls were negative for CTra DNA in the study samples.

## Discussion

Infection may be related to the development of autoimmunity, but the underlying pathogenic mechanisms are still largely unknown (1-3). In the present study, we report a new association between a subclinical, chronic infection by Cp and inflammatory polyarthritis in Northern Italy, as indicated by a significantly higher prevalence of Cp DNA in the PBMCs of these patients as compared to healthy individuals.

Notably, among the study patients, a higher prevalence of Cp infection was observed in seronegative polyarthritis, mainly in RF/aCCP double negative RA patients, followed by psoriatic arthritis. These findings then suggest a possible pathogenic role of Cp in a subset of inflammatory polyarthritis.

Cp-positive and Cp-negative patients did not differ in terms of age and sex and the lack of differences concerning these variables supported the significant difference in Cp-DNA prevalence between patients and controls, even if controls were not exactly matched for age and sex.

Chlamydia-induced arthritis (*i.e*. by Ctra and Cpneu) is the most frequent form of ReA in western countries (18). The DNA of both Ctra and Cpneu was detected in the synovium, supporting an active role of these agents in joint inflammation (14, 15, 17). To the best of our knowledge, no data have been so far reported about Cp detection in ReA and other rheumatic diseases.

Ramos *et al*. (28) demonstrated that chlamydial peptides generated following proteolysis and presented on the surface of chlamydia-infected cells are natural ligands of arthritis-associated HLA-B27 haplotypes. Molecular mimicry is probably one of the major mechanisms by which *Chlamydia* infection may trigger autoimmune disease in the predisposed individual: the microbial epitope is able to elicit a lymphocyte response that is cross-reactive to self-epitopes. Several homologous peptides can be expressed by the three known *Chlamydia* spp involved in autoimmune reactions (29). The evidence herein provided stimulates further studies to assess whether Cp may also induce molecular mimicry and whether any Cp pathogenic epitopes may be distinct or similar to those identified for Ctra and Cpneu.

It should be considered that the prevalence of Cp infection observed in the present series might be underestimated. In fact, a proportion of patients may have a Cp infection without evidence of Cp DNA in PBMCs, as suggested by the detection of Cp DNA only in 40% of PBMCs of Cp-associated ocular adnexal lymphomas (23). Our findings are in any case consistent with the presence of a systemic, chronic Cp infection in a sizeable subset of RA and SpA patients, a finding up to now reported only in patients with Cp-associated ocular adnexal lymphomas (23, 30).

Geographical issues should also be taken into account. The present study was performed in Northern Italy, and the same higher prevalence of Cp infection in seronegative polyarthitis was noticed in patients coming from the different Centres involved in the study (data not shown). Whether this occurs also in other geographical areas has to be verified. A systematic collection of the history of enviromental exposures possibly associated with increased risk of Cp infection in the present series of patients was not available, but Cp-positive patients did not remember or present significant exposures to birds.

Indeed, Cp may also induce a persistent infection that may be totally asymptomatic for several years (23) and other unrecognised sources of Cp infection may exist.

Cp infection could be also a risk factor for possible lymphoma development in systemic rheumatic diseases, where an increased risk of haematological neoplasms is well established (31). A preliminary study in patients with other systemic autoimmune diseases revealed a Cp infection prevalence similar to that of controls (unpublished data), but allowed to identify one Cp-positive patient with systemic lupus erythematosus affected by ocular adnexal lymphoma.

Intriguingly, it has been recently reported that ocular, not genital, serotypes of Ctra are associated with arthritis (32). These findings are consistent with the possible role of an ocular pathogen, such as Cp, in the triggering of autoimmune diseases.

One major issue is whether immune suppression secondary to the treatment administered may account for a subsequent increased susceptibility to Cp infection. In this case, a pathogenic role of Cp infection would be very unlikely. High doses of glucocorticosteroids administered for prolonged time periods are significantly associated with increased risk of infections (31), but none of the study patients was submitted to this treatment regimen. Data about the association between the RA treated anchor drug MTX and an increased frequency of infections remain controversial, whereas it has been conclusively demonstrated that hydroxychloquine and sulphasalazine are not associated with such an increased risk (33). In the present study, the majority of patients were under treatment with disease modifying anthireumatic drugs and/or biologics, in particular with anti-TNF agents, at time of blood collection and Cp DNA analysis (34). The present DNA series derived from studies aimed to discover new markers of response to biological agents in RA and SpA; this is the reason why the majority of them were under treatment with anti-TNF agents, while looking at the general population of patients with RA and SpA, they are prevalently treated with DMARDs.

Of note, present results suggest that introducing anti-TNF agents did not increase Cp infection prevalence in inflammatory polyathritis, since Cp-positive patients were equally treated with DMARDs and biological agents, while Cp-negative patients were mostly treated with biological therapies. If the introduction of biological therapies may reduced Cp chronic infection in such patients remains on open issue.

No increase in the prevalence of Cpneu and CTra infection was found in the whole series of patients compared to controls and the prevalence of Cp infection among healthy individuals is far less common than that of other *Chlamydia* spp (*i.e.* Cpneu and Ctra), which were detected at very low frequency in our series of patients.

In the past, there has been sustained interest in the use of antibiotics for RA treatment, with conflicting results due to the lack of controlled trials. Recently, controlled trials with mynocycline alone or in combination with clyndamicine, demonstrated significant efficacy compared to placebo and to DMARDs (22). Whether the anti-rheumatic effect of this therapeutic approach was due to the immunomodulatory and anti-inflammatory properties of these drugs or to their broad range of antibacterial activity remains to be investigated, and early cases of arthritis remain poorly studied (35, 36). As in the case of reactive arthritis and HCV-related mixed cryoglobulinemia (10), infection might elicit the onset of the autoimmune disease but may be no longer required for disease persistence. A small trial is ongoing with doxycycline in our Cp-positive cases.

In conclusion, the results of this study highlight for the first time the presence of a chronic subclinical infection by *Chlamydophila Psittaci* in inflammatory polyarthritis, with particular regard on the subset of seronegative cases. Studies in larger series and in other geographical areas are ongoing.

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