# Calprotectin in rheumatic diseases

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**Impact statement**

Calprotectin is an acute-phase protein produced by monocytes and neutrophils in the circulation and inflamed tissues. Calprotectin seems to be more sensitive than CRP, being able to detect minimal residual inflammation and is a candidate biomarker in inflammatory diseases. High serum levels are associated with some severe manifestations of rheumatic diseases, such as glomerulonephritis and lung fibrosis. Calprotectin levels in other fluids, such as saliva and synovial fluid, might be helpful in the diagnosis of rheumatic diseases. Of interest is also the potential role of calprotectin as a target of treatment.

## Abstract

Calprotectin is a heterodimer formed by two proteins, S100A8 and S100A9, which are mainly produced by activated monocytes and neutrophils in the circulation and in inflamed tissues. The implication of calprotectin in the inflammatory process has already been demonstrated, but its role in the pathogenesis, diagnosis, and monitoring of rheumatic diseases has gained great attention in recent years. Calprotectin, being stable at room temperature, is a candidate biomarker for the follow-up of disease activity in many autoimmune disorders, where it can predict response to treatment or disease relapse. There is evidence that a number of immunomodulators, including TNF-$\alpha$ inhibitors, may reduce calprotectin expression. S100A8 and S100A9 have a potential role as a target of treatment in murine models of autoimmune disorders, since the direct or indirect blockade of these proteins results in amelioration of the disease process. In this review, we will go over the biologic functions of calprotectin which might be involved in the etiology of rheumatic disorders. We will also report evidence of its potential use as a disease biomarker.

**Keywords:** Calprotectin, S100A8/A9, rheumatic diseases, inflammation, biomarker, rheumatoid arthritis

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## Introduction

Calprotectin (CLP) is a heterodimer formed by two proteins, S100A8 and S100A9. These proteins represent half of the cytosolic protein content of monocytes and neutrophils. They are constitutively expressed as an anti-microbial agent by these cells. CLP was initially named major leukocyte protein L1 or 27E10.[1,2] It was later identified as the combination of S100A8 and S100A9, which also have different synonyms: myeloid-related proteins-8 and -9 (MRP-8 and MRP-9); migration inhibitory factor-related protein of 8 and 14 kDa (MRP-8 and MRP-14); calgranulin A and B; alarmins.[1,2]

The implication of S100A8 and S100A9 in the inflammatory process was shown almost 20 years ago.[3,4] However, its role in the pathogenesis of rheumatic diseases has only gained great attention in recent years. Upcoming evidence shows that CLP might also be involved in cancer development, obesity, progression of dementia, and formation of the atherosclerotic plaque.[5–10] CLP, being stable at room temperature, is a candidate biomarker in inflammatory diseases and its levels in stools are routinely measured in the follow-up of inflammatory bowel diseases.[9] Serum levels are usually reported below 1 μg/ml in healthy subjects, but during inflammation they may increase by 100 times.[10]

The use of CLP in pediatric rheumatic diseases, such as juvenile idiopathic arthritis, Henoch-Schönlein purpura, Kawasaki disease, cryopyrin-associated periodic syndromes and familiar Mediterranean fever, has already been reviewed.[11] Here, we will go over the biologic functions of CLP which might be involved in the pathogenesis of rheumatic diseases. We will also report evidence of CLP use as a biomarker in the diagnosis and follow-up of adult rheumatic diseases (Tables 1 and 2).

## S100A8/9 Structure

S100A8 and S100A9 proteins belong to the S100 protein family[12] and share a common helix-loop-helix motif structure consisting of two α-helices bound by a central hinge region. Every monomer can bind two $Ca^{2+}$ ions and other divalent metal ions such as $Zn^{2+}$. The ion-binding properties of the S100 protein family modulate oligomerisation and consequently their function.[13–15] S100A8 and S100A9 can be found in the form of homodimers, heterodimers $(S100A8/A9)_2$, or heterotetramers $(S100A8/A9)_4$. The

**Table 1** Evidence supporting the use of serum calprotectin as a biomarker in rheumatic diseases

| | Diagnosis | Disease assessment | Treatment assessment | Association with specific disease features | Prognosis |
|---|---|---|---|---|---|
| Rheumatoid arthritis | Higher levels are found compared with HC and with OA, SpA, SLE, JIA, CPPD | Correlation with laboratory markers of inflammation<br>Correlation with disease activity measures<br>Correlation with ultrasound and radiographic damage scores | Reduction following effective treatment | High levels are associated with positive rheumatoid factor and ACPA | High levels are predictive of disease relapse<br>High levels are predictive of structural damage |
| Spondyloarthritis | Controversial results are available of higher levels compared with HC | Correlation with laboratory markers of inflammation<br>Modest correlation with disease activity measures | Reduction following effective treatment | High levels are associated with peripheral arthritis | NA |
| Psoriatic arthritis | Higher levels are found compared with HC | Correlation with laboratory markers of inflammation<br>Correlation with disease activity measures<br>Correlation with the extent of skin involvement<br>Correlation with radiographic damage scores | Reduction following effective treatment | NA | NA |
| Adult-onset Still's disease | Higher levels are found compared with HC and RA, OA, SLE, SS | Controversial correlation with laboratory markers of inflammation and ferritin<br>Correlation with disease activity measures | Reduction following effective treatment | High levels are associated with sore throat | NA |
| Gout | Higher levels are found compared with HC | Correlation with laboratory markers of inflammation | Reduction following effective treatment | NA | NA |
| Osteoarthritis | Higher levels are found in case of synovial inflammation | Correlation with the extent of structural damage at the histological level | NA | NA | High levels are predictive of structural damage |
| Systemic lupus erythematosus | Higher levels are found compared with HC | Correlation with disease activity scores and damage scores | NA | High levels associated with cerebrovascular events, acute myocardial infarction, proliferative glomerulonephritis, positive anti-dsDNA antibodies | NA |
| Sjögren syndrome | Higher levels are found compared with HC | No correlation with laboratory markers of inflammation<br>No correlation with the extent of inflammation at the histological level | NA | NA | NA |
| Systemic sclerosis | Higher levels are found in circulating polymorphonucleates and monocytes compared with HC | No correlation with laboratory markers of inflammation | NA | High levels are associated with lung fibrosis, arthritis, gastrointestinal involvement, presence of anti-Scl70, anti-hystone, or anti-U1RNP antibodies<br>High S100A8 levels are associated with kidney involvement High S100A9 are associated with myositis | High levels are predictive of reduced survival |

(continued)

**Table 1** Continued

| | Diagnosis | Disease assessment | Treatment assessment | Association with specific disease features | Prognosis |
|---|---|---|---|---|---|
| Behçet's disease | Higher levels are found compared with HC | No correlation with disease activity scores or quality of life indices | NA | NA | High levels are predictive of disease relapse |
| Antineutrophil cytoplasm antibody–associated vasculitis | Higher levels are found compared with HC | Correlation with disease activity scores<br>No correlation with laboratory markers of inflammation or ANCA levels | Reduction following effective treatment | Associated with proliferative glomerulonephritis | High levels are predictive of disease relapse |
| Polymyalgia rheumatica and giant cell arteritis | Higher levels are found compared with HC | Correlation with laboratory markers of inflammation | Reduction following effective treatment | NA | NA |

Note: No data as a clinical biomarker were available for idiopathic inflammatory myopathies.
HC: healthy controls; OA: osteoarthritis; SpA: spondyloarthritis; SLE: systemic lupus erythematosus; JIA: juvenile idiopathic arthritis; CPPD: calcium pyrophosphate dehydrate deposition disease; ACPA: anti-citrullinated peptide antibodies; SS: Sjögren syndrome; ANCA: antineutrophil cytoplasm antibody; NA: not available.

**Table 2** Evidence supporting the use of calprotectin as a biomarker in fluids/faeces other than serum in rheumatic diseases

| | |
|---|---|
| Bronchoalveolar lavage | Systemic sclerosis<br>High levels are associated with lung fibrosis. |
| Faeces | Rheumatoid arthritis, spondyloarthritis and psoriatic arthritis<br>High levels are associated with bowel inflammation.<br>Sjögren syndrome<br>Higher levels are found compared with HC; high levels are associated with bowel inflammation<br>Systemic sclerosis<br>Higher levels are found compared with HC, SS, RA; high levels are associated with gastrointestinal involvement and micronutrient deficiency. |
| Saliva | Sjögren syndrome<br>Higher levels are found compared with HC.<br>Systemic sclerosis<br>Higher levels of S100A8 and S100A9 are found compared with HC. |
| Synovial fluid | Rheumatoid arthritis<br>Higher levels are found compared with the serum; higher levels are found compared with OA, SpA, PSA; controversial results are available compared with SLE, CPPD.<br>Gout<br>Higher levels are found compared with the serum; higher levels are found compared with OA, RA, SpA, PSA; levels are similar to CPPD.<br>Osteoarthritis<br>Controversial results are available compared to HC; higher levels are found in the case of synovial inflammation, lower levels are found compared with gout and RA. |

SSc: systemic sclerosis; RA: rheumatoid arthritis; SpA: spondyloarthritis; PSA: psoriatic arthritis; SS: Sjögren syndrome; HC: healthy controls; OA: osteoarthritis; SLE: systemic lupus erythematosus; CPPD: calcium pyrophosphate dehydrate deposition disease.

heterodimer is the most stable form and is responsible for the majority of the protein biologic interactions.[16] S100A8 and S100A9 are expressed separately but S100A8 has a turnover higher than S100A9. In the absence of its binding partner, as in S100A9-knockout mice, S100A8 serum levels are almost undetectable.[17]

## Biology of CLP

S100A8 and S100A9 proteins are classically expressed by granulocytes, monocytes, and macrophages in an early differentiation stage, after their activation via-pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs).[18,19] Under specific conditions, other cell lines can express and secrete CLP such as endothelial cells, keratinocytes, osteoclasts, chondrocytes, and fibroblast-like synoviocytes.[20–24] (Figure 1).

The precise function of CLP in the immune process has not been unraveled yet. Some major functions have been acknowledged, such as the regulation of the cytoskeleton,

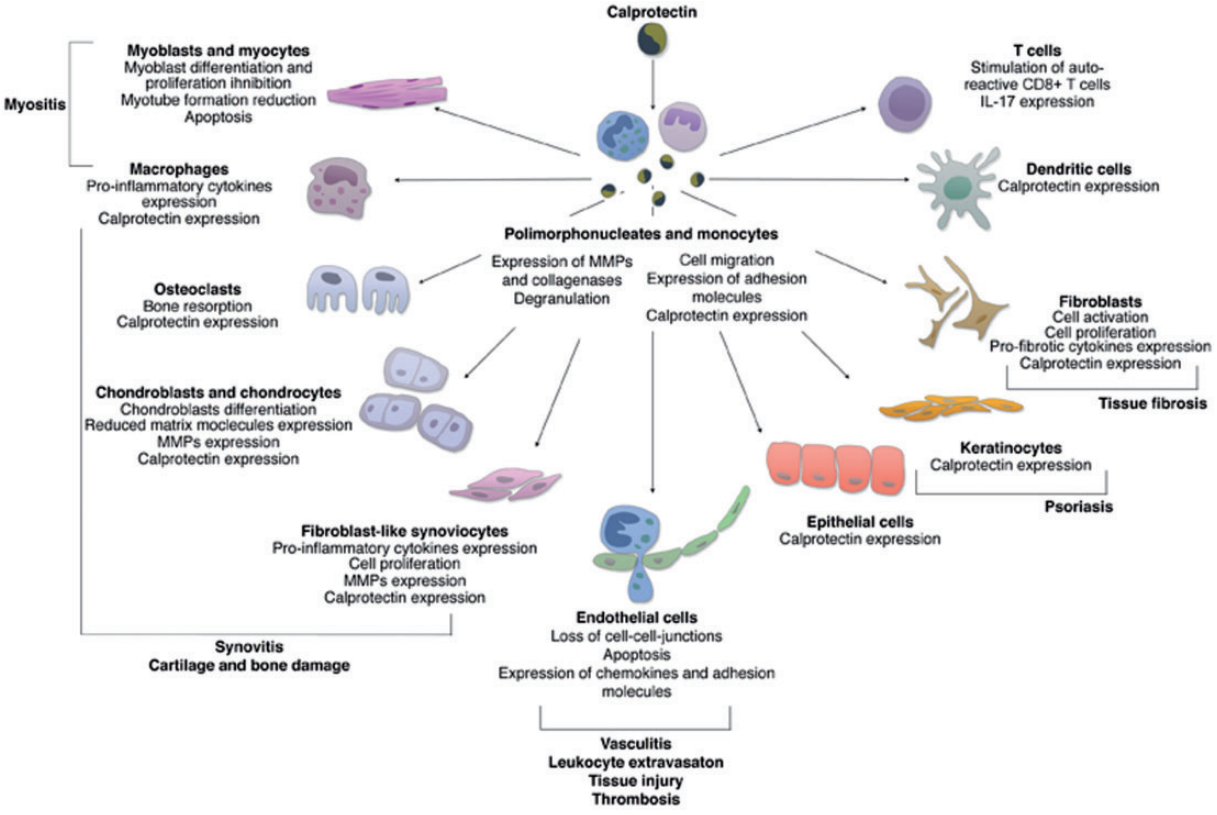


**Figure 1** Effects of calprotectin on the cells implicated in the pathogenesis of rheumatic diseases. CLP is mainly produced by activated monocytes and granulocytes and mediates the production of pro-inflammatory cytokines and chemokines, cell activation, and apoptosis in targeted cells. CLP induces the expression of further CLP with a consequent positive autocrine and paracrine feedback loop. (A color version of this figure is available in the online journal.)

leukocyte migration and trafficking, and amplification of inflammation, which are pivotal in the host response to infections. CLP also has a direct anti-microbial effect due to the chelation of $Mn^{2+}$ and $Zn^{2+}$, which are metal nutrients for bacteria.[25]

CLP is part of the innate immune response. It is recognized as a DAMP itself being an endogenous ligand of toll-like receptor (TLR) 4.[17] TLR4 belongs to the pattern recognition receptor family and it transduces the danger signal after interacting with PAMPs and DAMPs. CLP mediates the response to pathogen-derived factors, such as lipopolysaccharide (LPS), and contributes to the inflammatory process occurring in infections and sepsis.[7] It is involved in the inflammatory pathway upstream of tumor necrosis factor (TNF)-α and, like TNF-α, is crucial for LPS toxicity.[26] Like all DAMP molecules, CLP has a double role in the homeostasis of phagocytes. In a steady condition, it contributes to the regulation of the cytoskeleton, and it is released as a danger signal when phagocytes are activated.

## Intracellular functions of CLP

In polimorphonucleates (PMNs), CLP is implicated in the rapid rearrangement of tubulin-dependent cytoskeleton, which allows cell migration[1,13,14,19,27] (Figure 2). In the presence of calcium, S100A8 and S100A9 form heterotetramers and translocate to the cell membrane allowing tubulin polymerisation.[13,14] The evidence that S100A9-knockout mice have reduced granulocyte migration supports the idea that CLP has a role in cytoskeleton rearrangement.[14]

CLP is implicated in the activation of the respiratory burst. In the presence of calcium, S100A9 binds to arachidonic acid in the cytosol and transports it to the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex in the neutrophil plasma membrane through a PKC-dependent mechanism. S100A8 and S100A9 have multiple effects on NADPH oxidase. By interacting with gp91phox, p67phox, and rac-2 subunits of the NADPH oxidase complex, S100A8 and S100A9 induce the production of reactive-oxygen species, which are necessary for PMNs activity.[28]

CLP is secreted by granulocytes and other cells after a danger signal by PAMPs or DAMPs. It appears to be mostly released through a non Golgi-associated pathway with an active non-classical secretion, which requires again PKC activation.[14,19,29] An additional more recently discovered mechanism is the release of CLP bound to chromatin in neutrophil extracellular traps (NETs).[30,31] The protein can also be passively secreted from necrotic cells after tissue insult has occurred.[14,19]

## Extracellular functions of CLP

S100A8 and S100A9 bind their receptors with different affinity according to the protein conformation.[32]

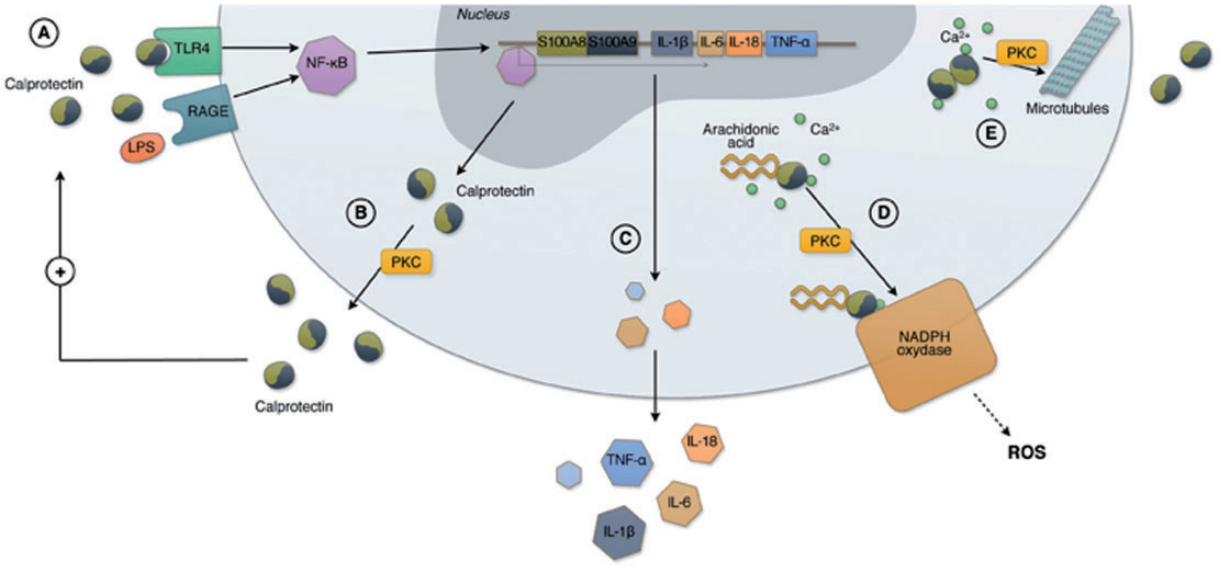


**Figure 2** Intracellular functions of calprotectin in polimorphonucleates and monocytes. CLP is a heterodimer composed of two proteins, S100A8 and S100A9. A danger signal molecule, such as LPS and CLP itself, can bind TLR4 and RAGE directly or through carboxylated glycans triggering inflammation via-NF-B which translocates into the nucleus (a). In the nucleus NF-B induces the expression of further S100A8 and S100A9. CLP is secreted through an energy dependent process, which requires PKC activation (b) and/or the interaction with microtubules (e). TLR4 or RAGE binding by CLP can induce the expression of proinflammatory cytokines and adhesion molecules, such as CD11b and CD18, contributing to the amplification of the inflammatory response and leading to leukocyte adhesion to the endothelium (c). In the presence of calcium, S100A9 subunit binds arachidonic acid and transports it to the NADPH oxidase complex expressed in the plasma membrane with a PKC-dependent mechanism. S100A9 transfers arachidonic acid to $gp91^{phox}$ subunit of the NADPH complex while S100A8 binds to $p67^{phox}$ and rac-2 subunits. Activated NADPH oxidase produces reactive oxygen species which are crucial for the inflammatory activity of granulocytes (d). In the presence of calcium, S100A8 and S100A9 also form heterotetrameters which translocate to the cell membrane and allow tubulin polymerization, microtubules bundling and stabilization of tubulin filaments. CLP regulates the cytoskeleton cell migration (e). (A color version of this figure is available in the online journal.)

CLP heterodimer binds different cell-surface proteins like heparan sulphate proteoglycan, carboxylated N-glycan, and, directly or through carboxylated glycans, TLR4 and receptor for advanced glycated end products (RAGE).[17,33]

TLR4 is the main CLP receptor[34] and is preferentially bound by the heterodimer.[17] The signal transduction cascade is mediated by MyD88 and NF-κB, which translocate into the nucleus[17,35,36] and promote the expression of pro-inflammatory cytokines, such as TNF-α, interleukin (IL)-1β, IL-6, IL-8, IL-23, chemokine (CXC)-8, and other CXCs[17,37,38] (Figure 2). RAGE is also bound by CLP[33] and activates inflammatory signaling pathways, including MAPK and NF-κB.[35] Notably, the expression of S100A8 and S100A9 increases following NF-κB activation, thereby generating a positive feedback loop which amplifies inflammation via an autocrine and paracrine stimulation of the cells responsible for S100A8 and S100A9 production.[17]

A major function of S100A8 and S100A9 is the regulation of leukocyte chemotaxis and tissue infiltration.[37] CLP induces the expression of integrin receptors on leukocytes, thus increasing their adhesion to fibrinogen, fibronectin, and endothelial cells.[37,39]

CLP causes a pro-inflammatory and thrombogenic response in the endothelium: it binds to endothelial cells through carboxylated glycans and TLR4 leading to cell activation. [40,41]Following CLP activation, endothelial cells express CXCs, such as IL-8 and MCP-1, and further S100A8 and S100A9. Endothelial cells also express VCAMs, ICAMs, and selectins on their surface which results in a chemotactic gradient, which then attracts PMNs and favors their binding to the endothelium.[37,39,42] CLP release leads to loss of cell–cell contacts and consequently alters the permeability of endothelium with leukocyte extravasation.[42] It also triggers endothelial cell apoptosis and necrosis which are responsible for vascular and tissue damage.[43]

## Putative role in adaptive immunity

Along with its classical role as an endogenous activator of innate immune response, CLP might represent a connection between inflammation and the adaptive immune response. CLP contributes to the induction of auto-reactive CD8+ T cells during the activation process by antigen-presenting cells.[44] This molecule is a costimulatory enhancer together with CD40/CD40 ligand signaling and leads to the loss of tolerance of T cells.[44] In a murine model of autoimmunity, the absence of S100A8 and S100A9 resulted in reduced IL-17 production by autoreactive CD8+ T cells and in lower autoantibody production.[44]In addition to its proinflammatory activity, CLP exerts also a regulatory function in the adaptive immune system. CLP overexpression in dendritic cells (DCs) is associated with an impaired T cell proliferation.[45] A study by Chih-Ru *et al.*,[46] showed that CLP is the endogenous ligand of CD69 expressed on regulatory T cells. The interaction between CLP and CD69 favors the differentiation of CD4+ T cells into regulatory T cells, thus hindering the exacerbation of T cell response. Furthermore, CLP regulates cytokine production,

particularly supporting the expression of transforming growth factor – ß which is an anti-inflammatory cytokine.[46]

## CLP in rheumatic diseases

### Rheumatoid arthritis

S100A8 and S100A9 are the most up-regulated proteins in rheumatoid arthritis (RA) synovial tissue and synovial fluid, [47–49] where they are produced by macrophages, PMNs, synovial fibroblasts, and chondrocytes.[24,47–51] S100A8 and S100A9 are involved in the amplification of the inflammatory process, neutrophil and monocyte recruitment, cartilage destruction, and bone resorption.

Macrophages producing S100A8 and S100A9[20,37,52] are present in the crucial sites of joint destruction, in the synovial membrane and the cartilage-pannus junction,[20,40,53,54] where they cause an imbalance in favor of bone resorption.[55]

The interaction of TLR4 with its ligands, including CLP, induces synovial fibroblast proliferation and the production of metalloproteinase (MMP)-1, IL-6, and further S100A8 and S100A9.[50,56] Activated chondrocytes also express CLP which, in turn, induces a catabolic effect on these cells via TLR4 signaling and the activation of NF-kB.[22,52] This activation leads to proteoglycan depletion, prevention of new cartilage formation and, eventually, chondrocyte death.[17,22,24,57,58] CLP also causes the degranulation of PMNs and the release of MMPs and collagenases, thus contributing to cartilage degradation.[59] Osteoclast differentiation is also affected by CLP primarily through a TLR4-mediated signal and S100A8-induced osteoclastic bone resorption in a murine model.[60]

CLP levels are higher in the serum of RA patients compared with healthy subjects or patients with osteoarthritis (OA), spondyloarthritis (SpA), systemic lupus erythematosus (SLE), and pseudogout.[47,61,62] Still, no cut-off levels have been identified to help in the diagnosis of RA. High levels of CLP are observed in RA synovial fluid, even >5 μg/ml, and might differentiate RA from OA and other inflammatory arthritides.[47,63–66] Notably, proteomic analysis has revealed the presence of S100A9 in synovial fluid from RA patients, but not in other disease controls.[67]

CLP serum levels correlate with disease activity and are associated with markers of more severe RA in many studies.[48,49,61,64,66,68–76] CLP has been found to be higher in the serum of rheumatoid factor[48,49,69,70,71,73] and anti-citrullinated peptide positive patients[23,49,64,72] compared with seronegative ones.[23] CLP correlates with inflammatory indices, including C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), and serum amyloid A protein (SAA) and inversely with TNF-α inhibitors through levels.[74,76,77] CLP serum levels seem to perform even better compared to CRP and ESR. In fact, CLP has showed to correlate better with all clinical indices, such as 28 joint-disease activity score and simplified disease activity index.[48,74–78]

CLP serum levels can decrease in response to treatment with both conventional and biologic disease modifying anti-rheumatic drugs.[72,74–80] Indeed, CLP serum levels have shown a prompt and more marked response to inflammatory changes compared with classic inflammatory indices, thus representing a sensitive biomarker for assessing treatment response.[72,74] This finding is supported by evidence of S100A8 and S100A9 expression in PMNs decreasing after treatment with TNF-α inhibitors.[79] Baseline CLP serum levels were higher in responders to treatment compared with non-responders and their decrease was predictive of treatment response in some studies,[74,75,80] but not in all.[73,80]

A controversial effect of corticosteroids on CLP serum levels has been observed. Effective corticosteroid treatment decreases CLP serum levels in autoimmune diseases.[81] However, Klingberg *et al*.[82] reported an increase in CLP levels along with the increase in white blood cells (WBC) count after corticosteroid administration. There is evidence that corticosteroids can induce the expression of S100A8 in monocytes, DCs, and synovial macrophages in RA.[83] Corticosteroids might therefore promote the expression of CLP also in circulating PMNs.

CLP has been independently associated with ultrasonography assessment scores, particularly in large joints.[72] A recent study by Inciarte-Mundo *et al*.[84] showed that CLP was associated with synovitis at the ultrasonography assessment in RA and psoriatic arthritis (PSA) patients in remission. CLP serum levels had the highest discriminatory capacity compared to CRP and ESR and a cut-off of 1.66 μg/ml for synovitis presence was also identified.[84] Notably, CLP is the only biomarker which has been shown to correlate with radiographic scores[48,49] and there is some evidence that baseline CLP might be predictive of radiographic damage.[48,49,85] In a recent study by Vogl *et al*.,[86] CLP was tested as a marker of inflammation in a murine model of arthritis by optical molecular imaging. It appeared sensitive in the identification of sub-clinical disease activity in the joints.

Notably, CLP might have a role as a therapeutic target in RA. In an arthritis murine model, the blockade of S100A9 through a monoclonal antibody was effective.[87] This finding supports the putative role of CLP as a treatment target in RA and, possibly, in other immune-mediate arthritides.

### Spondyloarthritis

Heterogeneous studies on CLP in SpA are available. Some include all subtypes of SpA and others are focused on ankylosing spondylitis (AS) alone. Although the results in SpA are similar to those obtained in PSA, studies regarding PSA will be separately discussed.

CLP is highly expressed in the synovial tissue of SpA patients, and it mirrors the presence of PMNs and monocytes which are responsible for its production.[88] The distribution of S100A8 and S100A9 in the synovial tissue is characteristic of SpA, being mainly localized in perivascular areas of the synovial sub-lining layer.[54,89] In a study by De Rycke *et al*.,[89] CLP was increased in the synovial fluid of inflamed joints where it positively correlated with local markers of inflammation, such as SAA and WBC.

Some authors have reported increased CLP serum levels in SpA patients,[54,89–91] while others have found similar levels in AS and controls.[92,93] Other studies have described

lower or similar CLP serum levels in patients with SpA compared with RA.[88,94] One study by Cypers *et al*.[94] found high CLP serum levels associated with peripheral involvement in SpA, which could explain the almost normal levels observed in patients with only axial involvement. However, this observation was not confirmed in another study by De Rycke *et al*.[89] and needs to be supported by further evidence. It was shown that serum CLP positively correlates with CRP, ESR, WBC, and platelet count in SpA patients.[54,89,94,95] However, CLP does not seem to be a reliable disease activity biomarker in SpA. Indeed, almost no correlation was found between CLP and Ankylosing Spondylitis Disease Activity Score,[82]Bath AS disease activity index, or Bath AS functional index.[89,91,92,94–96]

Notably, CLP is secreted not only in the joints, but also in other inflamed tissues such as the gastrointestinal mucosa. Up to 50% of patients with SpA have subclinical bowel inflammation, which is associated with increased serum levels of CLP.[97] The mucosal contribution to the increase in circulating CLP levels may explain the modest correlation with SpA disease activity.[94]

CLP decreases rapidly upon effective treatment with TNF-α-inhibitors and secukinumab in both axial and peripheral SpA and it has been suggested as a useful biomarker for disease monitoring, where it performs even better than high-sensitivity CRP.[91,95] Baseline CLP was also found to be an independent predictor of radiographic spinal progression in axial SpA in a cohort of German patients.[95] CLP concentration >0.5 μg/mL was associated with worsening of the modified Stoke Ankylosing Spondylitis Spine Score and the development or progression of syndesmophytes at the two year follow-up.[95]

### Psoriatic arthritis

Serum levels of S100A8, S100A9, and CLP are increased in PSA patients compared with healthy controls. Like other inflammatory arthritis, PSA is characterized by monocytes and PMNs infiltration in the synovial tissue,[98]which are responsible for CLP production. In PSA, CLP is produced also in the skin by keratinocytes[21] and CLP serum levels correlate with the extent of skin involvement.[99] However, high circulating CLP levels appear to be associated with articular rather than skin involvement, since they are higher in PSA patients compared with psoriatic patients. The activation of the monocyte/macrophage system might be responsible for the increase in CLP in patients with arthritis compared with psoriasis.[54,100]

As in other SpA, in PSA, CLP is typically localized in perivascular areas in the synovial tissue.[54] Such distribution might be related to the distinctive synovial macro-vascular changes found in PSA, which are not observed in RA.[101]

CLP seems to be a useful biomarker since it correlates with clinical measures, including the number of involved joints,[100] the Ritchie Articular Index, and systemic inflammatory indices.[54] In a study by Kane *et al*.[54] in PSA patients, CLP was a sensitive and specific indicator of treatment response, as previously reported in SpA.[54] CLP was even more sensitive than clinical joint scores in assessing response to MTX treatment.[54] Notably, Madland *et al*.[70] found that high CLP serum levels were associated with peripheral radiographic abnormalities thereby suggesting a more erosive disease.

### Adult-onset still's disease

Adult-onset still's disease (AOSD) is characterized by high levels of inflammation, and ferritin is the most commonly used laboratory biomarker, although it is not specific for AOSD diagnosis and follow-up. Two studies are available on CLP in AOSD patients.[102,103] In the study by Guo *et al*.,[102] serum levels of CLP were higher in AOSD patients than in controls or other autoimmune disorders, including RA, SLE, and Sjögren's syndrome (SS), thereby supporting CLP use as a diagnostic biomarker.[102] In both studies, CLP showed to have a positive correlation with laboratory biomarkers particularly ferritin[102,103] and to a lesser extent leukocyte count, CRP, and liver enzymes.[103]A negative correlation was found with haemoglobin.[102] CLP was also associated with disease activity scores and treatment response.[102,103] No association was found between CLP and clinical manifestations, apart from sore throat, which is one of the early disease manifestations.[102]

## Gout

A study by Holzinger *et al.*[104] demonstrated that S100A8 and S100A9 proteins are actively secreted by monocytes at moderate monosodium urate monohydrate (MSU) crystal concentrations. At high MSU crystal concentrations, these proteins are also passively released following neutrophil and monocyte death.[104,105]

IL-1β plays a pivotal role in MSU crystals-induced inflammation. CLP provides a danger signal which induces the expression of IL-1β precursor in vitro. IL-1β is activated through cleavage by the inflammasome after a second signal from MSU crystals.[104] This function was further confirmed in a murine model of MSU-induced inflammation, where S100A9-knockout mice had lower IL-1β secretion and moderately reduced recruitment of neutrophils and monocytes compared with wild-type mice.[104] The moderate impairment of IL-1β secretion suggests that other endogenous triggers may be involved and that S100A8 and S100A9 proteins have a role in maintaining and amplifying rather than in inducing the inflammatory process.

In the study by Holzinger *et al.*, CLP serum levels ≥2000 ng/ml were found in patients with active gout; similar levels were found in patients with RA, SpA, PSA, and calcium pyrophosphate dehydrate deposition disease (CPPD). A correlation between CLP in the serum and in the synovial fluid was observed. In the synovial fluid, CLP levels were significantly higher in gout compared with RA, SpA, PSA, and OA, but similar to those observed in CPPD.[104] CLP correlates with disease activity in gout, being high in the acute phase, and decreasing after treatment with non-steroidal anti-inflammatory drugs and betamethasone. Serum levels were higher in gouty patients compared with controls during the inter-critical phase of the disease suggesting the persistence of subclinical inflammation. A potential source of CLP production could be the

S100A8 and S100A9 positive cells found in the tophi of gouty patients.[104,106] Thus, CLP seems to be a better biomarker of gout than IL-1β, which is unstable in serum and therefore not suitable for use in clinical practice.[107]

### Osteoarthritis

There is a lot of evidence of CLP being involved in cartilage damage in OA. Synovial lining macrophages play a crucial role in the OA process and in cartilage damage.[108] For example, they release IL-1β, which is pivotal in cartilage destruction.[109] S100A8 and S100A9 are the most expressed proteins by macrophages[14] and they have been shown to induce a specific inflammatory response in human chondroblasts, mediated by TLR4.[52] Furthermore, they cause a catabolic phenotype in murine chondrocytes, characterized by the release of MMP-1, MMP-3, MMP-9, and the inhibition of matrix molecule production.[22,57,110] S100A8 and S100A9 stimulate other cells, such as fibroblast-like type B cells to produce MMPs.[111] In a murine OA model, S100A9-knockout mice had reduced damage compared with wild-type controls, which further confirms the role of these proteins in cartilage destruction.[24] Furthermore, in synovial biopsies from OA patients, levels of S100A8 and S100A9 correlated with markers of tissue damage, such as synovial lining thickness, sub-intima cellularity, and cartilage destruction.[57]

Synovial CLP expression seems to be increased only if synovial inflammation is present. Vogl *et al*.[57] demonstrated that in a murine model of collagenase-induced OA, which is characterized by synovial inflammation, the expression of S100A8 and S100A9 was higher compared with another model of OA, without synovial inflammation. Also, CLP serum levels were found higher in OA patients with synovial inflammation compared with those without.[112]

CLP levels have been found at high levels also in the synovial fluid (up to 5–7 μg/ml).[42] A moderate CLP increase has been observed in the serum of OA patients compared with healthy controls, particularly in early OA.[37,57,113] Notably, in the study by Vogl *et al*.[57], high CLP serum levels were also predictive of cartilage damage: a cutoff value of 0.6 μg/ml was associated with an odds ratio of 7.5.

### Systemic lupus erythematosus

In SLE, CLP is expressed by monocytes, PMNs and also plasmocytoid DCs upon immune complex stimulation.[114] SLE plasmocytoid DCs and leukocytes, except T cells, express the CLP complex on their cell surface[114] bound to heparan sulphate proteoglycans and carboxylated glycans.[40] Along with active secretion, CLP is released in NETs[30,31] and passively by necrotic PMNs[19] CLP might stimulate the adaptive immune system in SLE through TLR4 signaling in auto-reactive CD8+ T cells leading to the increase in IL-17 expression.[44]

CLP expression is up-regulated in SLE kidneys and skin. It has been found in renal tissue from SLE patients with glomerulonephritis, especially with proliferative lesions, and in the epidermis where it is produced by keratinocytes lesions.[4,44,115] Skin stressors, including UV exposure,[116]lead to an inflammatory response, which is usually part of the physiological mechanism of wound healing, but might also be responsible for autoimmune disregulation in skin lesions.[21]

Patients with SLE have higher serum CLP levels compared with healthy individuals and also SS patients.[117,118] Serum CLP has been found correlated with disease activity,[3,114,118] particularly with SLE activity index (SLEDAI) score.[3,118] CLP serum levels have been also found higher in patients with inactive disease compared with controls and correlating with the systemic lupus international collaborating clinics/American College of Rheumatology (SLICC/ACR) damage index.[119] This association supports the idea that the persistence of subclinical inflammation can be responsible for disease progression.[120]

CLP seems associated with some clinical manifestations in SLE. Haga *et al*.[3] described high CLP serum levels associated with arthritis and positive anti-double strand DNA antibodies.[3] In a study by Tyden *et al*.,[119] CLP serum levels were high in SLE patients with acute myocardial infarction and cerebro-vascular events, probably due to the involvement of CLP in the initiation and progression of atherosclerosis through RAGE and TLR4.[121] However, CLP circulating levels were not increased in patients with venous thromboembolism,[119] despite CLP being implicated in the thrombogenic response of the endothelium. Vessel inflammation might be an additional source of CLP[122]and could perpetrate a positive feedback loop maintaining both inflammation and atherogenesis in SLE patients. Thus, elevated CLP serum levels in SLE could be helpful in identifying patients at risk of cardiovascular events who might benefit from preventive treatment.

Interestingly, there is evidence that CLP might be an effective treatment target in SLE. Quinoline-3-carboxamides, which bind S100A9 inhibiting CLP interaction with RAGE and TLR4, are about to enter a phase II study for the treatment of SLE.[123]

### Sjögren syndrome

Increased serum levels of CLP in SS patients compared with healthy subjects were first reported by Kuruto *et al*.[117] and subsequently confirmed by other studies,[102,124]except one study which did not specifically refer to primary SS.[61] Nordal *et al*.[125] showed that CLP serum levels correlated with some indices of disease activity, especially with the fatigue score, but not with ESR and CRP; no association between CLP and arthritis was observed. CLP was found in saliva from SS patients at a concentration higher than in healthy subjects, and CLP levels were higher in the saliva than in the serum of SS patients.[61,124,126] Notably, faecal CLP is increased in SS patients compared with normal subjects.[127] Indeed, CLP is also produced by exocrine glands of the gastrointestinal mucosa in SS patients.

CLP was also found in salivary gland biopsies from SS patients.[124,125] CLP might be locally released by inflammatory cells, as well as actively secreted by epithelial cells.[128]

The evidence that RAGE, a receptor of CLP, is overexpressed by myoepithelial cells in labial salivary glands of SS patients confirms the implication of CLP in the development of local inflammation.[129] No correlation has been found between CLP expression in the salivary glands and the lymphocytic focus score,[61,125] CLP concentration in the saliva might be a promising marker of local inflammatory activity in SS.

### Systemic sclerosis

CLP plays a central role in skin injury by maintaining persistent inflammation and triggering the pro-fibrotic response. S100A8 and S100A9 are produced by injured keratinocytes[21] and by plasmocytoid DCs via TLR4.[114] In addition, they are highly expressed in infiltrating mononuclear cells in the early stages of systemic sclerosis (SSc).[130]CLP is able to activate fibroblasts, which proliferate and produce pro-fibrotic cytokines as well as further CLP.[21,130] CLP receptors, TLR4, and RAGE are highly expressed on the surface of fibroblasts and probably mediate CLP signal in these cells.[130,131] RAGE is implicated in the development of lung fibrosis[132] and its expression on the fibroblast cell surface has been correlated with SSc severity.[133] TLR4 signaling has been found to induce fibroblast proliferation[134] and the activation of plasmocytoid DCs.[114] Notably, TLR4 inhibition hindered the fibrotic process in a model of LPS-induced lung fibrosis.[135]

High concentrations of S100A8 and S100A9 have been found in the skin, bronchoalveolar lavage (BAL), saliva[130,131,136–138] and also in the peripheral blood cells of SSc patients.[117,127,139] In a cohort of Asian patients, serum CLP levels were higher in those with diffuse cutaneous SSc compared with limited SSc,[130] while in Caucasian patients CLP levels were increased only in limited SSc associated with lung fibrosis.[139] Although no clear correlation with disease activity was found, CLP serum levels seem to be associated with specific and severe manifestations of SSc, including lung fibrosis, kidney involvement (only S100A8), myositis and myalgia (only S100A9), and arthritis and arthralgia.[130] Xu *et al*.[130] reported high S100A8 and S100A9 serum levels in diffuse SSc with anti-Scl70, anti-hystone, or anti-U1RNP antibodies. Van Bon *et al*.[139] confirmed the association between high S100A8 serum levels and anti-Scl70, which is a marker of severe disease. The production of CLP in the gastrointestinal mucosa of SSc patients was also shown. Indeed, CLP was increased in the faeces of SSc patients compared with other autoimmune disorders and was correlated with the severity of digestive symptoms.[127,140]

Importantly, the association between CLP serum levels and lung fibrosis is supported by higher CLP and S100A9 concentrations in the BAL of SSc patients with lung involvement compared with patients without lung involvement and healthy controls.[136,138] However, this result was not confirmed by other authors.[141] Furthermore, high CLP concentrations in the BAL were associated with extensive fibrosis by high-resolution computed tomography and were correlated with BAL eosinophil count.[138] Thus, since CLP is a marker of severe disease, and especially of lung fibrosis, it might be useful in identifying patients who need a tight follow up.

### Idiopathic inflammatory myopathies

Although the major idiopathic inflammatory myopathies (IIM) subsets, polymyositis (PM), dermatomyositis (DM), inclusion body myositis (IBM), and juvenile dermatomyositis (JDM) are characterized by different pathogenic mechanisms, they share some common features, including a similar cytokine expression pattern and the important role of macrophages in muscle lesions.[142]

In fact, S100A8 and S100A9 proteins are expressed by macrophages in muscles where they induce the release of inflammatory mediators.[143–145] A clear association between S100A8 and S100A9 expression and the degeneration of muscular fibers was shown in muscle biopsies from PM, DM, and IBM patients.[145] CLP inhibits the differentiation and proliferation of myoblasts in vitro through the reduction of myotube formation and expression of myocyte differentiation markers.[145,146] At high levels, CLP induces apoptosis of myoblasts by caspase-3 activation.[145]

Some indirect evidence of the role of CLP in IIM derives from the observation that TLR4 is involved in the pathogenesis of IIM[147,148] and in the promotion of autophagy,[149] which is implicated in myositis, particularly in IBM.[13,19] TLR4, the main CLP receptor, is highly expressed in muscle tissue from patients with IIM and was more expressed in PM and DM than in JDM, IBM, and controls.[150]

In a study by Nistala *et al*.,[144] S100A8 was shown to induce the secretion of MCP-1 and IL-6 from skeletal muscle cells in vitro. Indeed, MCP-1 mediates the activation of monocytes and memory T cells and the differentiation of local B cells into plasma cells in the muscles of DM patients.[151] Once recruited and activated, myeloid cells amplify the inflammatory signal by producing CLP.[145] Thus, CLP could represent a link between the innate immune stimulation and activation of cells of the adaptive immune system.

### Behçet's disease

Serum levels of CLP were found higher in a cohort of 47 patients with Behçet's disease (BD) compared with healthy controls.[152] CLP was also tested as a disease biomarker, but no correlations were found with disease activity scores or quality of life indices in BD patients.[152]

Uveitis and oral aphthous ulcerations might be potential sources of S100A8 and S100A9 production in BD. In fact, raised CLP serum levels have been found in patients with uveitis[153–155] and it has been shown that mucosal squamous epithelial cells can express CLP under inflammatory conditions.[156–158]

CLP might have a role in the pathogenesis of vascular involvement in BD.[42,43,159] CLP is implicated in neutrophil recruitment and extravasation leading to tissue injury.[160] The thrombogenic phenotype, induced by CLP-activated endothelial cells, could contribute to the

thrombotic manifestations of BD, reported in up to 40% of the patients.[161]

### Antineutrophil cytoplasm antibody-associated vasculitis

In two studies including antibody-associated vasculitis (AAV) patients, CLP was found highly expressed on the surface of circulating leukocytes along with high serum levels of this protein in patients with AAV compared with controls.[162,163] CLP serum levels correlated with disease activity but not with antineutrophil cytoplasm antibodies (ANCA), CRP or WBC.[162,163] CLP decreased during treatment but did not get back into the normal range, suggesting the persistence of subclinical inflammation.[162,163] Importantly, the increase of CLP serum levels seems to predict a disease relapse with a higher likelihood than ANCA serum levels.[162,164]

CLP is expressed in the kidneys of patients with AAV-associated glomerulophritis. CLP positive leukocytes and macrophages are present in glomeruli and their concentration correlates with the severity of histological inflammation. A prominent CLP expression has been observed in areas of focal necrosis and active crescents, but not in sclerotic glomeruli.[4,162,165] A pathogenetic role of CLP is endorsed by an experimental vasculitis model with S100A9-knockout mice showing a significant decrease in the inflammatory vascular lesions.[166] Furthermore, TLR4, the major CLP ligand, is expressed in glomerular endothelial cells and has been involved in the development of renal injury in animal models.[41,162]

### Giant cell arteritis and polymyalgia rheumatica

A few studies with a low number of patients were focused on CLP in giant cell arteritis (GCA) and polymyalgia rheumatica (PMR).[79,167,165] In all these studies, serum levels of CLP were higher in PMR and GCA than in controls. As expected, serum levels correlated with inflammatory biomarkers and decreased in response to treatment.[81,167,168] Notably, CLP lowered after prednisone initiation and the decrease was inversely correlated with oral prednisone dose.[81]

In a study by Brun *et al*.,[81] CLP was found abundantly expressed in the adventitia and media of affected arteries. Like in other vasculitis, CLP probably mediates the activation of endothelial cells in an early disease stage, contributes to the maintenance of vessel inflammation, and leads to leukocyte extravasation from the vasa vasorum into the adventitia, which is pivotal in GCA.[167] CLP might also damage arterial smooth muscle cells in the adventitia and media. In fact, infiltrating macrophages produce CLP[168] which can induce the apoptosis of myoblasts.[145] In addition to the production in the arterial wall, in PMR other inflamed tissues, such as bursae and synovial tendon sheaths, might be responsible for the release of CLP and the increase in serum levels.

## Conclusions

CLP is considered an acute-phase protein which is not synthesized in the liver but produced by activated PMNs in the circulation and inflamed tissues. In contrast to cytokines such as IL-6, TNF-α or IL-1β, CLP is relatively stable and easily measurable in the serum, which makes this protein a candidate biomarker in inflammatory diseases. Nevertheless, a comparison of CLP levels between the studies is not possible. In clinical studies, CLP levels have been detected with either commercial kits or home-made ELISAs. Every test has a different sensitivity and different limits to define a positive CLP, ranging from 3 ng/ml and 2.9 μg/ml.[76,119] A standardized method to measure CLP concentration is required. A potential diagnostic role of CLP has not been clarified yet. CLP serum levels are particularly high in AOSD, but cut-off levels need to be identified and tested in larger populations. CLP levels in other fluids, such as saliva and synovial fluid, might be helpful in the diagnosis of SS and gout or RA, respectively. However, concomitant non-autoimmune inflammatory conditions must be considered, particularly infections, which can be associated with rheumatic diseases and can also raise CLP serum levels.[118]

Of interest is the possible role of S100A8 and/or S100A9 as a target of treatment. There is evidence that a number of immunomodulators, including TNF-α inhibitors or JAK/STAT inhibitors,[169] may reduce CLP expression. In murine models of autoimmune disorders, the direct or indirect blockade of S100A8 or S100A9 exerted a beneficial effect.

CLP seems to be more accurate than CRP, as it is able to detect minimal residual inflammation and can predict disease relapse in some autoimmune diseases including SLE, BD, and AAV. High CLP serum levels are also associated with worse structural outcomes in RA and, to a lesser extent, in SpA. Thus, they might have a role in the therapeutic decision, especially in the treatment tapering.[170] Furthermore, high CLP serum levels are associated with some severe manifestations of connective tissue diseases, such as glomerulonephritis in SLE and AAV and lung fibrosis in SSc, so can identify patients who need an accurate screening and tight follow-up. Although its pathogenic role remains to be elucidated, CLP has demonstrated to be a highly sensitive biomarker of inflammation. In future, once cut-off levels will be identified in the different rheumatic diseases, CLP might replace classical markers of systemic inflammation.

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