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Circulating Calprotectin (cCLP) in autoimmune diseases

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Abstract

Background and aim: Calprotectin (CLP) is a heterodimeric complex formed by two S100 proteins (S100A8/A9), which plays a pivotal role in innate immunity. Due to its intrinsic cytotoxic and proinflammatory properties, CLP controls cell differentiation, proliferation and NETosis and has been associated with a wide range of rheumatic diseases.

Our review summarizes the widespread interest in circulating CLP (cCLP) as a biomarker of neutrophil-related inflammation, in autoimmune rheumatic disease (ARD) and non-ARD.

Methods: A through literature review was performed using PubMed and EMBASE databases searching for circulating calprotectin and synonyms S100A8/A9, myeloid-related protein 8/14 (MRP8/MRP14), calgranulin A/B and L1 protein in addition to specific ARDs and autoimmune non-rheumatic diseases. We selected only English-language articles and excluded abstracts without the main text.

Results:

High cCLP serum levels are associated with worse structural outcomes in rheumatoid arthritis and to a lesser extent, in spondyloarthritis. In addition, cCLP can predict disease relapse in some autoimmune diseases including systemic lupus erythematosus (SLE), anti-neutrophil cytoplasmic antibodies-associated vasculitis (AAV) and some severe manifestations of connective tissue diseases, such as glomerulonephritis in SLE, AAV, juvenile idiopathic arthritis, adult-onset Still's disease and lung fibrosis in systemic sclerosis. Therefore, cCLP levels enable the identification of patients who need an accurate and tight follow-up.

The clinical usefulness of cCLP as an inflammatory marker has suggested for non-rheumatic diseases, and especially for the monitoring of the inflammatory bowel diseases patients. Currently, there are only a few studies that evaluated the cCLP efficacy as a clinical biomarker in autoimmune non-rheumatic diseases with controversial results. Future studies are warranted to better clarify the role of cCLP in relation to the disease severity in myasthenia gravis, multiple sclerosis, chronic inflammatory demyelinating polyneuropathy, Graves' orbitopathy, autoimmune bullous diseases and uveitis.

Conclusion:

Our literature review supports a relevant role of cCLP as potential prognostic biomarker mirroring local or systemic inflammation, especially in chronic inflammatory rheumatic diseases.

1. Introduction

The role of circulating Calprotectin (cCLP) in the inflammatory process and pathogenesis of various autoimmune diseases has already been documented (1). In addition, as cCLP is stable at room temperature and easily detectable in serum or plasma, it has recently gained major interest as a promising biomarker for the diagnosis, prediction of treatments' response or disease relapse of some autoimmune disorders (2), (3).

2. Methods

The scientific literature involving cCLP in autoimmune diseases has been reviewed thoroughly. The research was carried out using PubMed and EMBASE databases, searching the following keywords:, circulating calprotectin (including synonyms as S100A8/A9, myeloid-related protein 8/14 (MRP8/MRP14), calgranulin A/B and L1 protein), Rheumatoid Arthritis, Spondyloarthritis, Psoriatic Arthritis, Gout, Juvenile idiopathic arthritis, Adult-Onset Still's Disease, Anti-neutrophil cytoplasmic antibodies-associated vasculitis, Granulomatosis with polyangiitis, Microscopic polyangiitis, Eosinophilic granulomatosis with polyangiitis, Systemic Lupus Erythematosus, Idiopathic Inflammatory Myopathy, Polymyositis, Dermatomyositis, Immune-mediated necrotizing myopathy, Anti-synthetase syndrome, Sporadic inclusion body myositis, Sjögren's Syndrome, Systemic Sclerosis, Inflammatory Bowel Diseases, Crohn's disease, Ulcerative Colitis, Myasthenia Gravis, Multiple Sclerosis, Chronic Inflammatory Demyelinating Polyneuropathy, Graves' Orbitopathy, Autoimmune Bullous Diseases, Uveitis, Behcet's syndrome, Psoriasis, Diabetes Mellitus. We selected only English-language articles excluding abstracts without the main text. In detail, the manuscript has been structured as a narrative review.

3. Biology of Calprotectin (CLP)

CLP is a heterodimeric complex formed by two S100 proteins, S100A8 and S100A (4), also known as myeloid-related protein 8 and 14 (MRP8/MRP14), calgranulin A and B and L1 protein (5), (1), (3). In 1965, Moore *et al.* (6) first isolated S100 proteins from the bovine brain; up to now, more than twenty S100 proteins are known. S100A8 and S100A9, which are both encoded by highly conserved genes on human chromosome 1 (q21), share a common helix-loop-helix structure characterized by two α-helices bound by a central hinge region that can bind two divalent ions such as Ca^{2+} and Zn^{2+} (7). The most commonly used MeSH term 'cal-protectin' emphasizes the protein capacity to bind divalent ions; activity related to the antimycotic property against *Candida albicans* (8), (9). In blood, the heterodimer (S100A8-S100A9) is the most stable CLP form and accounts for

greatest of its biological functions, but homodimers and heterotetrameric $(S100A8\text{-}S100A9)_2$ forms can also be found (10), (10). In the presence of Ca^{2+} excess, heterodimers aggregate to heterotetramers which are more resistant to proteolysis (11), (12).

CLP plays a pivotal role in innate immunity. During an inflammatory response, the tissue damage release damage-associated molecular pattern molecules (DAMP) and pro-inflammatory cytokines. Stimulated by the upregulation of pattern recognition receptors (PRRs), neutrophils are rapidly recruited (13), (14), (15), (16), (17) and secrete a wide variety of chemokines, cytokines and leukotrienes, including CLP.

The CLP constitutes up to 60% of neutrophil and monocyte cytosolic proteins. Nonetheless, under specific scenarios, additional cell types including chondrocytes, myocytes, endothelial cells, epithelial cells, keratinocytes, fibroblasts, dendritic cells, osteoblasts and osteoclasts can express and secrete CLP as well. Besides active, calcium-dependent secretion by neutrophils, CLP is also excreted during neutrophil extracellular traps (NETs) formation (13), (14), (15), (16), (17), (18). NETs comprise a scaffold of DNA laced with histones and cytotoxic neutrophil-derived proteins and are released by neutrophils during infection and inflammation as a protective response, mainly targeting invading pathogens (19), (15). Of note, CLP was identified as one of the main components of NETs (19)(**Figure 1**).

As a DAMP, CLP binds the most important PRRs, e.g. Toll-like receptor 4 (TLR4) and receptor for advanced glycation end products (RAGE) (18), inducing the expression of proinflammatory cytokines and adhesion molecules, positively promoting the amplification of the inflammatory response and increasing leukocyte adhesion to the endothelium. Next, CLP binds arachidonic acid and activates NADPH oxidase to produce reactive oxygen species, crucial for the inflammatory activity of neutrophils. In the presence of Ca^{2+}, CLP allows tubulin polymerization, microtubule bundling, and stabilization of tubulin filaments, thereby regulating the cytoskeleton cell migration (1) (**Figure 1**).

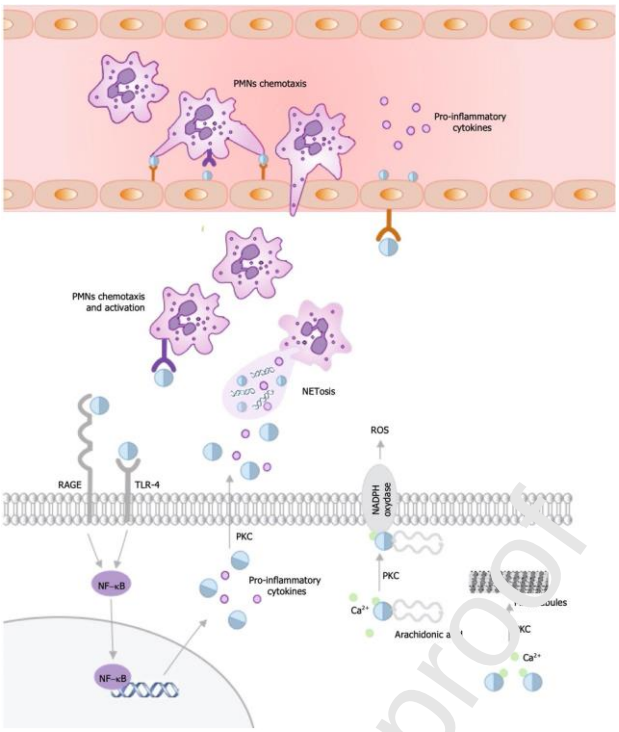

Figure 1. Calprotectin's functions. Extracellular CLP functions include endothelial cell activation, chemotaxis and activation of polymorphonuclear cells (PMNs), promoting adhesion of PMNs to the vascular endothelium, thrombi formation and antimicrobial activity. Intracellular functions are promoted by CLP binding to Toll-like receptor 4 (TLR4) and receptor for advanced glycation end products (RAGE), triggering the amplification of pro-inflammatory cytokines by the NF-kB pathway. In the presence of calcium (Ca^{2+}) and protein kinase C (PKC) CLP (i) regulates microtubule bundling attributing to cytoskeleton cell migration and (ii) binds arachidonic acid, thereby activating NADPH oxidase to produce reactive oxygen species (ROS). CLP is secreted actively by a Ca^{2+} and PKC-dependent pathway or is released bound to chromatin in neutrophil extracellular traps (NETs).

Due to its intrinsic cytotoxic and proinflammatory properties, CLP controls cell differentiation, proliferation (e.g. tumorigenesis) and NETosis and has been associated with a wide range of pathologies including autoimmune diseases, thrombosis, and cancer (17), (20).

In autoimmunity context, the NETs can serve as a source of self-antigen in autoimmune diseases, in fact, an abnormal or dysregulated NETs formation has been associated with several autoimmune diseases (13), (14), (15), (17), (21). In addition, CLP might represent a connection between inflammation and the adaptive immune response, contributing to the induction of autoreactive CD8+ T cells and in association with CD40/CD40 ligand signalling, leads to the loss of T cells' tolerance (22). Its overexpression in dendritic cells (DC) is related to impaired T-cell proliferation (22). The subsequent DC activation triggers a massive release of interferon-gamma and the release

of B cell activating factor (BAFF) that supports the proliferation of B cells inside germinal centers (23).

Furthermore, neutrophil activation (including NETs formation) is shown to be detrimental to the development of endothelial damage and subsequent atherosclerotic plaque formation (24), (25), (26), (27), likely through easing extravasation and RAGE–mediated inflammation (28). Elevated CLP levels have been associated with cardiovascular (CV) disease (30-32) and revealed to be an early and sensitive marker for unstable angina and CV events (29), (30).

In contrast to C-reactive protein (CRP), the classic acute phase protein produced by the liver, CLP is released locally at the site of inflammation. Therefore, cCLP, seems to be a good systemic proxy of local inflammation in different chronic neutrophil-related inflammatory processes (1), (3), (18).

Our review summarizes the widespread interest in cCLP as a biomarker of neutrophil-related inflammation, in autoimmune rheumatic diseases (ARD) and in autoimmune non rheumatic diseases.

4 cCLP in autoimmune rheumatic diseases

4.1 Rheumatoid Arthritis (RA)

Among the different ARDs, the link of cCLP and Rheumatoid Arthritis (RA) is the most extensively studied (3). RA is the most common chronic inflammatory joint disease, characterized by the presence of rheumatoid factor (RF) and antibodies to cyclic citrullinated peptides (ACPA) (31). Both ACPA and RF are with inflammatory markers such as erythrocyte sedimentation rate (ESR) and CRP included in the current 2010 ACR/EULAR classification criteria for RA (32).

A high cCLP concentration is found in the synovial fluid obtained from the joints of RA patients (33). Owing to its low molecular weight (36.5 kDa), cCLP diffuses into the circulation, and a good correlation between cCLP serum and synovial levels is found (34).

Already in 2017, two metanalyses were available confirming the significantly higher cCLP concentration in active RA patients and the correlation with disease activity (35), (36). cCLP revealed a positive correlation with RA activity measured by Disease Activity Score for 28 joints **(**DAS28) and CRP levels, showing a positive correlation with synovitis detected with ultrasound (37), (38), (39). Interestingly, cCLP levels correlated with RF and ACPA titers (37), (40), (41), (42), and higher cCLP levels were found in seropositive patients (37), (41), (43). Furthermore, cCLP levels were independently associated with radiographic progression in RA and high baseline cCLP levels predicted future erosive damage (38), (39), (43).

A greater incidence of myocardial infarction has been attributed to the major types of arthritis, including RA (44). Is worth exploring whether cCLP might play a role in rushing atherosclerotic disease secondary to systemic inflammation in RA as it could identify patients needing a close CV follow-up.

Next, in RA patients, cCLP levels predict response to both methotrexate and biological disease-modifying antirheumatic drugs (bDMARDs) therapy (1), (45), (46), (47), (48). In one of the first studies performed by Choi *et al.*, responders showed significantly higher cCLP levels compared to non-responders before the initiation of bDMARD therapy, and cCLP levels decreased in line with the successful treatment (45). Hurnakova *et al*. described cCLP as a more sensitive biomarker for monitoring RA disease activity than ESR and CRP, routinely used in indexes like DAS (49), (50). In addition, the use of cCRP as an inflammatory biomarker is jeopardized in patients treated with interleukin-6 (IL-6) blocking agents (e.g. tocilizumab, TCZ), as CRP production by the liver is stimulated by IL-6, making cCLP a useful alternative biomarker.

Finally, the evidence of cCLP as a RA biomarker in TCZ-treated patients is increasing (51) and recently Gernert *et al*., quantified the CLP in the serum of 114 RA patients, of whom 69 were treated with TCZ and 45 with tumor necrosis factor alfa (TNF-α)-inhibitors (TNFi) (52). The authors demonstrated that cCLP is suitable to detect RA activity in both treatment groups, being higher in RA patients with active disease compared to RA patients with stable disease. This was not the case for ESR and CRP in the TCZ-treated groups. In TNFi-treated patients, all three biomarkers were significantly higher for RA patients with active disease, with cCLP outperforming ESR and CRP in diagnostic performance.

More recently, Bettner *et al.* showed that adding elevated cCLP levels to RF and ACPA positivity resulted in a high positive predictive value (i.e. 53%) for the RA development within 3 years or less, which could be helpful for preventing RA (53).

4.2 Spondyloarthritis and Psoriatic Arthritis

Besides RA, CRP and ESR are also routinely used for the evaluation of disease activity and treatment response in Spondyloarthritis (SpA) and Psoriatic Arthritis (PsA). However less than half of the SpA or PsA patients shows elevated CRP levels (54), (55). Therefore, novel inflammatory biomarkers may have additional applications for these clinical entities.Both SpA and PsA are characterized by the infiltration of monocytes and neutrophils in the synovial tissue and CLP is typically localized in perivascular areas in the synovial tissue (56). As cCLP levels reflect local synovial inflammation, several reports found higher cCLP levels in SpA and PsA patients compared to the healthy control (HC) group, but especially in peripheral and polyarticular disease (57), (58) or

in large joint involvement (59). A recent metanalysis confirmed higher cCLP levels in SpA patients and correlation with disease activity scores (DAS) (60). For PsA, this observation is further supported by the correlation of cCLP with ultrasound DAS measures and the ultrasound power Doppler score (61), with cCLP outperforming CRP (59).

Conflicting results have been reported regarding the association between cCLP with clinical DAS, such as the PsA Disease Activity Index for PSoriatic Arthritis (DAPSA) and SpA Bath Ankylosing Spondylitis Disease Activity Index (BASDAI), the Bath Ankylosing, Spondylitis Functional Index (BASFI) and the Ankylosing Spondylitis Disease Activity Score (ASDAS) (59). A probable explanation could be the difference in the number of the swollen joint count and the distribution of the joint involvement of the patients involved (59). Next, in both PsA and SpA, cCLP is not only secreted in the joints, but respectively also in the skin by keratinocytes and in the gastrointestinal mucosa. Up to 50% of SpA patients have subclinical bowel inflammation (62), which is associated with increased serum cCLP levels (57). This extra-synovial production of CLP may partially explain the weak correlation between cCLP levels and disease activity.

As for RA, cCLP levels in PsA patients were independently associated with the presence of carotid plaque, probably involving platelet and endothelial cell activation (63).

In both SpA and PsA, cCLP was a sensitive and specific indicator of treatment response, decreasing rapidly upon effective treatment (63), (64). Moreover, higher baseline cCLP amounts were found to be an independent predictor of radiographic spinal progression in axial SpA (64), (2), (65), and peripheral radiographic abnormalities in PsA, indicating more erosive disease.

4.3 Juvenile idiopathic arthritis (JIA) and Adult-Onset Still's Disease

Still's syndrome includes systemic juvenile idiopathic arthritis (JIA) and Adult-Onset Still's Disease (AOSD). Except for age, there are many similarities between JIA and AOSD. Recurrent fever, a fleeting, salmon-colored rash, generalized lymphadenopathy, hepatosplenomegaly, leukocytosis and arthralgia/arthritis are common signs and symptoms of both JIA and AOSD. Early initiation of targeted therapy with cytokine blockade can prevent disease progression to chronic destructive arthritis and life-threatening complications. However, diagnosis of JIA and AOSD is difficult because of the broad spectrum of disease manifestations and relies on the combination of clinical and (non-specific) laboratory findings, as well as the exclusion of other inflammatory conditions (15), (66), (67), (68). Besides (glycosylated) ferritin, several laboratory biomarkers, including acute phase reactants, inflammatory cytokines and chemokines, are intended to be used for diagnosis in routine practice; however, their clinical utility is still unclear and non-specific for diagnosis or follow-up (67). Furthermore, the course of the disease is characterized by alternating periods of

flares and remission. Some predictors of poor outcome have been identified (presence of RF, early radiographic changes, symmetrical disease, extension of arthritis at onset, positivity of antinuclear antibodies), but the lack of reliable prognostic markers remains a major problem in JIA (69).

Recently, there is growing evidence of NETs involvement in the pathogenesis of JIA and AOSD (70) and in line with these findings, cCLP has been raised as a reliable biomarker for diagnosis and disease activity in JIA and AOSD, outperforming CRP in specificity (69), (70), (71), (72), (73), (74), (75), (76). cCLP was significantly elevated in JIA/AOSD patients versus HD and increased with disease activity (69), (70), (71), (72), (73), (74), (75), (76), differentiating forms with active oligoarthritis from polyarthritis and systemic forms as CRP (69). However, cCLP strongly correlates with the evolution of disease activity according to Juvenile Arthritis Disease Activity Score (JADAS10-CRP) and DAS28 (69) contrary to CRP.

In a recent study (69), significant higher cCLP levels were found for JIA patients with active disease reaching remission after 6 months of therapy. For JIA patients with a subsequent flare, constant and higher cCLP levels were documented, suggesting residual disease activity, even in the absence of clinical or biological signs of persistent inflammation. These findings are in concordance with previous reports, showing encouraging results of cCLP as a biomarker by predicting disease relapse after stopping of non-steroidal anti-inflammatory drugs, methotrexate or bDMARD treatment (76), (77), (78), (79).

4.4 Anti-neutrophil cytoplasmic antibodies-associated vasculitis

Anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) comprise granulomatosis with polyangiitis (GPA), microscopic polyangiitis (MPA) and eosinophilic granulomatosis with polyangiitis (EGPA), that share features of pauci-immune small-vessel vasculitis and the positivity of ANCA targeting proteinase-3 (PR3-ANCA) or myeloperoxidase (MPO-ANCA) (80). In AAV patients, the genetic associations are stronger with ANCA specificity (PR3- versus MPO-ANCA) than with the clinical diagnosis, supporting an ANCA-based re-classification of these disorders, e.g. PR3-positive AAV (PR3-AAV), MPO-positive AAV (MPO-AAV) and for EGPA, by the presence or absence of ANCA (ANCA+ or ANCA-, respectively) (81).

In the AAV pathogenesis, ANCAs produced by plasma cells directly activate neutrophils, eventually leading to NETs formation and the production of ROS, resulting in tissue damage, granulomatosis and necrosis (13). Although any tissue can be involved in AAV, the upper and

lower respiratory tract and kidneys are most commonly and severely affected. Discovering of AAV biomarkers is crucial to identify patients at higher risk of poorer outcomes, to modulate immunosuppressive treatment and to balance the risk of relapse and treatment adverse effects (82), (83), (84).

Although the value of ANCAs as diagnostic markers for AAV is irrefutable (85), (86), their role as a disease activity marker has been the topic of frequent studies with different outcomes (87). In line with the recent insights on AAV pathogenesis (13), several independent groups considered cCLP as a promising biomarker of disease activity in AAV (88). cCLP is released and is elevated in patients in the acute AAV phase compared to patients in remission and its level of expression is increased with the activity of the vasculitis (89), (90), (91), (92). Growing cCLP levels positively correlates with the clinical Birmingham Vasculitis Activity Score (BVAS) and represents an independent risk factor for AVV relapse (93). The increase of cCLP levels seems to predict a disease relapse with a higher likelihood than ANCAs (87), (89), (93). Besides disease activity markers, cCLP also revealed a promising renal function outcome biomarker in AAV. Higher cCLP levels at recruitment were positively correlated with the worsening of renal function, hematuria, rising proteinuria and non-decreasing ANCA titers (90).

4.5 Systemic Lupus Erythematosus

Systemic Lupus Erythematosus (SLE) is a chronic, systemic autoimmune disease with multiple clinical phenotypes (94). Despite the availability of recently updated SLE classification criteria (95), the final diagnosis is left to the clinicians' judgment (96). Lupus nephritis is the most common target-organ manifestation and damage is strongly associated with morbidity and mortality (96). Significant progress in the management of SLE patients has been described, due to treatment advances and earlier diagnosis (96). Nevertheless, early SLE diagnosis and monitoring of associated renal disease remains challenging (94), (97). Various new diagnostic markers for SLE have been proposed, however, only a few disease activity biomarkers such as ESR, anti-dsDNA antibodies or complement protein levels are currently used in clinical care (98), (99). Therefore, there is an unmet need for specific, early diagnostic, sub-classifying, monitoring and predictive biomarkers for routine clinical use {Kiriakidou, 2020 #5938} (99).

Recent studies elucidated a central role of neutrophils and NETs in the SLE pathogenesis (21) showing that in these patients, the process of NETs degradation is lower than in healthy people (100), (101). In parallel, in SLE patients, an increase of cCLP levels and its correlation with disease

activity (e.g. particularly with SLE activity index (SLEDAI) score) has been described (99), (102), (103), (104), (105), (106), (107), (108). cCLP levels are higher in SLE patients than in HC (99), (103), (106) and lower in patients with milder (e.g. skin or inactive renal) disease manifestations than in SLE patients with active glomerulonephritis (99),(102), (103), (104), (105). However, not all studies agree that cCLP correlates with global disease activity {Davies, 2020 #5946}, {Sumova, 2019 #5951}. In addition, cCLP levels positively correlated with the presence of anti-dsDNA antibodies {Tyden, 2017 #5950}, (108) and negatively with C3 protein levels (108). Moreover, elevated cCLP in SLE could be helpful in identifying patients at risk of cardiovascular events who might benefit from preventive treatment (1). Tyden *et al*. showed that cCLP levels were high in SLE patients with acute myocardial infarction and cerebro-vascular events (105).

Finally, cCLP may be used to monitor the treatment efficacy, as its levels decrease over time after a successful treatment (99), (105).

4.6 Idiopathic Inflammatory Myopathy

The Idiopathic Inflammatory Myopathies (IIMs), known as myositis, represent a heterogeneous group of chronic autoimmune muscle diseases that are classified into main clinical entities, such as polymyositis (PM), dermatomyositis (DM), immune-mediated necrotizing myopathy (IMNM), anti-synthetase syndrome and sporadic inclusion body myositis (IBM) (109), (110). Testing of myositis-specific or associated autoantibodies, the use of Magnetic Resonance Imaging (MRI) and extensive investigation of muscle biopsy samples significantly improved diagnostic and prognostic capabilities in myositis (111). Although the different clinical subsets are characterized by dissimilar pathogenic mechanisms, they share some common features, including a similar cytokine expression pattern and the central role of macrophages in muscle lesions (112). Multiple inflammatory mediators, including cCLP, exert a pathogenic impact within the myositis muscle and their levels may reflect the clinical myositis course (111), (112), (113), (114). Elevated cCLP levels after neutrophils activation have been described in the sera of JDM patients and are correlated with disease activity and the muscle enzyme creatine kinase levels (112), (114).

The TLR4 (the main receptor of cCLP) is shown to be highly expressed in muscle tissue from IIM patients and was more expressed in those with PM and DM than in juvenile DM, IBM and controls (112), (115).

The Interstitial Lung Disease (ILD) is considered a common systemic complication of DM, causing increased morbidity and mortality. In the study of Lou *et al*. the cCLP levels significantly correlated with disease activity in DM-ILD patients, showing also a positive correlation with high-resolution

computed tomography and a anti-correlation with diffusing capacity of the lung for carbon monoxide (DLCO%) and forced vital capacity (FVC%). Multivariate logistic regression demonstrated that only cCLP levels were a significant independent risk factor associated with ILD development. Therefore, cCLP represents a promising predictor for assessing the occurrence and evaluating clinical severity of DM-ILD patients (116).

4.7 Sjögren's Syndrome

The Sjögren's Syndrome (SS) is a chronic autoimmune disease characterized by xerostomia and keratoconjunctivitis sicca, caused by lympho-plasmocytic infiltration of the exocrine glands. In 30-50% of the patients, the disease symptoms stretch beyond the classical sicca syndrome and systemic manifestations such as arthritis, interstitial lung disease, neurological manifestations, lymphadenopathy and even an increased risk of lymphoma (117), (118). In general, the diagnosis is based on oral and ocular sicca symptoms and their evaluation (e.g. Schirmer's test), a labial biopsy showing a focal lymphocytic infiltration as well as the presence and detection of autoantibodies (e.g. anti-SSA/Ro60) in serum (118). Recent evidence suggest that innate immunity plays a primary role in the pathogenesis of primary SS (pSS) (119).

cCLP could represent a reliable biomarker as an up-regulation of cCLP in serum (120), (121), (122) and in salivary gland epithelial cells, together with a higher level of TLR-4 and RAGE expression (123), (124). In fact, cCLP was found in saliva (120), (121), (122), (125), (126) and serum (120), (121), (122), (126) from SS patients at higher concentration than in HD. Nordal *et al*. (122) revealed a cCLP correlation with SSA/Ro60 and SSB antibody levels and with some indicators of disease activity, e.g. the fatigue severity scale, which could not be shown for ESR and CRP (122). A proteomic analysis of parotid saliva suggested that levels of cCLP might act as a biomarker for the development of mucosa-associated lymphoid tissue (MALT) lymphoma (126).

Independent of traditional CV risk factors, higher cCLP levels were associated with a higher incidence of carotid atherosclerosis, suggesting cCLP is a biomarker of subclinical atherosclerosis in SS patients (127).

4.8 Systemic Sclerosis

The cardinal pathological features of Systemic Sclerosis (SSc) are autoantibodies, vasculopathy, and fibrosis (128), (129), (130). Due to the heterogeneous disease nature, it is hard to predict disease progression and complications. Despite the discovery of novel autoantibodies associated

with SSc (130), there is an unmet need for biomarkers for diagnosis, disease progression and response to treatment (131).

cCLP levels have been associated with specific and severe SSc manifestations, including lung fibrosis. cCLP triggers the pro-fibrotic response and its main receptors TLR4 and RACE are highly expressed on the surface of fibroblasts, keratinocytes, and endothelial cells (1).

In a cohort of Asian patients, cCLP levels were higher in those with diffuse cutaneous SSc than in those with limited SSc (132), while in Caucasian patients cCLP levels were increased only in limited SSc associated with lung fibrosis (133). Therefore, it has been shown that cCLP could identify patients who need accurate screening and tight follow-up (1), associating higher cCLP concentrations with mortality in patients with idiopathic pulmonary fibrosis (IPF) (129), (134), (135).

5 cCLP in inflammatory/autoimmune non-rheumatic diseases

5.1 Inflammatory Bowel Diseases

The Inflammatory Bowel Diseases (IBD), including Crohn's disease (CD) and Ulcerative Colitis (UC), are chronic inflammatory disorders of the gastrointestinal tract affecting people of any age. A recent systematic review showed that IBD has become a global disease with an increasing incidence in newly industrialized countries (136).

Topical studies have greatly improved knowledge of the IBD pathophysiology. Host genetic predisposition, gut microbiota, environmental factors, and abnormal innate and adaptive immune responses are all involved (137), (138). IBD patients should be frequently monitored to evaluate disease activity, treatment efficacy and prevent complications. The optimal method for monitoring disease activity in IBD patients is still being defined. Endoscopic evaluation represents the gold standard for this purpose, but it is an invasive, unpleasant, and high-cost procedure. For this reason, an alternative marker capable of mirroring intestinal inflammation and which can be used as a surrogate for endoscopy, is required (139).

Among blood markers, the CRP is widely used in clinical practice for the evaluation of IBD patients but it is not specific for intestinal inflammation and may be elevated for other reasons such as infection or other inflammatory conditions. Studies have demonstrated a modest correlation between CRP and clinical and endoscopic findings in IBD patients. In addition, CRP can be falsely low despite active mucosal inflammation and is more reliable in cases of transmural inflammation (140).

Fecal CLP is a sensitive and specific marker of intestinal inflammation, and its analysis is universally accepted and applied for IBD diagnosis and follow-up (141). More recently, the cCLP has been proposed as a potential biomarker in monitoring IBD patients. Mori *et al*. demonstrated that cCLP levels were higher (i) in CD and UC patients than in HD and (ii) during active disease than during inactive disease in CD but not in UC. In UC a correlation was found between cCLP levels and serum CRP but not with other laboratory parameters or disease activity. On the contrary, in CD patients, the cCLP levels were positively correlated with disease activity assessed by Harvey-Bradshaw Index (HBI), serum CRP and platelet count. A correlation between cCLP levels and serum CRP was found in the UC group but not with other laboratory parameters or disease activity. The time course of cCLP and serum CRP followed longitudinally in two patients with UC and two patients with CD, showed increased levels during the acute phase and decreased levels towards remission (142).

Kalla *et al*. documented a strong correlation of cCLP with CRP but a moderate correlation between cCLP and fecal CLP that currently represents the strongest predictor of IBD diagnosis, treatment escalation and surgery (143).

Okada *et al*. confirmed higher cCLP concentration in IBD patients than that in HD, barely correlating with CRP and inflammatory cytokines. (144)(169).

Meuwis *et al*. demonstrated that cCLP was significantly higher in patients with active disease than in patients in remission and that cCLP correlated with Crohn's Disease Activity Index (CDAI). The cCLP was represented as a useful biomarker for relapse prediction and response to therapy after Infliximab treatment (145). In a recent study, Mortensen *et al.* proposed to evaluate a specific cCLP neo-epitope [CPa9-HNE] in the serum of UC and CD patients. The serum CPa9-HNE levels were highly associated with CD and UC patients and correlated with the Simple Endoscopic Score (SES)-CD score and the full Mayo score, showing high accuracy in identifying IBD patients with moderate/severe disease activity (146).

5.2 Myasthenia Gravis

The Myasthenia Gravis (MG) forms the largest disease group of neuromuscular junction disorders. It is an autoimmune disease caused by pathogenic autoantibodies to components of the post-synaptic muscle endplate (147). In a recent cross-sectional and prospective study by Stascheit *et al*., significantly higher cCLP levels were found in the cohort of MG patients compared with HD and subjects with non-inflammatory neurological diseases. An cCLP cut-off value of 1.55 µg/ml was able to discriminate patients with MG from controls with a sensitivity of 90.4% and specificity of 45.1%. The cCLP values also appeared to correlate with disease activity: higher values were found in patients with generalized disease than in those with ocular disease or in remission (according to MGFA classification). The Quantitative Myasthenia Gravis (QMG) severity score values also correlated with baseline cCLP levels. Finally, the cCLP could represent a reliable marker to predict response to treatment and patients treated with monotherapy revealed to have higher cCLP levels than subjects treated with eculizumab. However, in a longitudinal analysis from the same study, conducted in 58 patients after 3 years of follow-up, individual disease activity did not show any significant correlation with cCLP levels (148).

5.3 Multiple Sclerosis

The role of cCLP in Multiple Sclerosis (MS) was firstly evaluated by Bogumil *et al.* in 1998 (149). The authors demonstrated that median serum levels of cCLP were significantly higher in MS patients compared to healthy controls and in relapsing compared to patients with stable disease. Wu

et al. documented that cCLP induces the activation, proliferation, migration of the BV-2 microglial cells and the conversion from an anti-inflammatory to a pro-inflammatory activated phenotype, confirming cCLP as a good marker for monitoring disease activity in MS patients. In fact, cCLP induced the release of pro-inflammatory factors (IL-1β, TNF-α, MMP-9) and chemokines (CCL2, CCL3, CXCL10) by activating the NF-κB signalling pathway, which further exacerbates the damage of oligodendrocyte precursor cells (OPCs). This process results in the apoptosis of OPCs, implicated in both MS development and progression (150).

On the other hand, Floris *et al*. concluded that although cCLP expression is a good histopathological marker for monocyte activation, serum levels of this marker do not correlate with disease activity in relapsing-remitting MS (178).

Olsson *et al*. confirmed that cCLP could also be used for monitoring the effect of treatment, since 12 months after initiation of disease modifying therapy, cCLP levels reduced by 29% (179). As confirmed by the study of Stascheit *et al*. (151), cCLP is increasingly considered as a potential biomarker to monitor ongoing disease activity in MS.

5.4 Chronic Inflammatory Demyelinating Polyneuropathy

The Chronic Inflammatory Demyelinating Polyneuropathy (CIDP) is a rare, acquired immune-mediated demyelinating neuropathy, characterized by strong heterogeneity in terms of clinical presentation, prognosis, and treatment response. Diagnosis is based on progressive or relapsing course over 2 months, electrophysiological evidence of peripheral demyelination, and response to immune-modulating therapies (152).

cCLP levels were significantly higher in all CIDP patients compared to HD. Multivariate analysis adjusted for age and gender revealed that cCLP acts as an independent predictor for CIDP. cCLP was significantly associated with active disease course according to CIDP Disease Activity Scale and correlated with medical research council-sum score (MRC-SS) (151). Therfore, cCLP could represent an additive inflammatory biomarker to guide better CIDP patient care.

5.5 Graves' Orbitopathy

Graves' Orbitopathy (GO) is an autoimmune, organ-specific inflammatory disease. *Ji Won Kim et al*. evaluated the cCLP impact on orbital fibroblast inflammation and GO pathogenesis. The cCLP levels were significantly increased in GO patients and positively correlated with seven-point clinical activity score and serum thyroid-stimulating immunoglobulin levels. They suggest that cCLP could be used as an adjuvant biomarker to assess disease activity in GO patients (153).

5.6 Autoimmune Bullous Diseases

Autoimmune Bullous Diseases (AIBD) are a group of autoimmune disorders characterized by the presence of autoantibodies against distinct structural components of the dermal–epidermal junction, including epidermolysis bullosa acquisita, characterized by autoantibodies against type VII collagen, and bullous pemphigoid, characterized by autoantibodies against two hemidesmosomal proteins, BP180 and BP230. Schmidt *et al.* found higher cCLP levels in patients with bullous pemphigoid and epidermolysis bullosa acquisita than in HD (154). Their findings indicate that the role of cCLP as biomarkers can be also extended to AIBD (155).

5.7 Uveitis

The term "Uveitis" describes a heterogeneous set of conditions that feature intraocular inflammation, frequently leading to severe vision loss and blindness. Idiopathic Acute Anterior Uveitis (IAAU) is the most common form of uveitis in this pathologic group, which shares an underlying immune etiology and may be associated with systemic disease or confined primarily to the eye. Unfortunately, there is a lack of a serum marker for detecting the degree of inflammation in uveitis (156). Song *et al.* demonstrated that cCLP levels were higher in IAAU group than in HD. Moreover, cCLP levels were positively correlated with uveitis activity grading and macular thickness, indicating a potential cCLP role in the pathogenesis of IAAU (157). Finally, Wang *et al.* confirmed that cCLP can be used to monitor inflammatory activity in AAU and they found that protein levels were elevated in IAAU compared to control (158).

5.8 Gout

Gout is the most prevalent inflammatory arthritis in developed countries (159). In clinical practice, specific biomarkers for disease activity in gout are lacking (160). The serum level of rate is associated with the risk of acute arthritis and the decrease of serum urate levels lead to a reduction of tissue deposits and risk of flares (161), (162).

In gout, the deposition of the needle shaped monosodium urate crystals in joints and soft tissues can cause an acute, inflammatory joint disease, usually referred to as gouty arthritis (163). The crystals activate neutrophils to release cytokines and induce infiltration of further neutrophils to form NETs (164), (165), leading to acute, extremely painful, and tissue-damaging inflammation in joints. The cCLP levels are elevated in the synovium, tophi and serum of patients with gout (166), (167), especially in poly-articular gout (160). cCLP levels are higher in the acute phase and decreased after treatment with non-steroidal anti-inflammatory drugs and betamethasone (1). cCLP levels were higher in gouty patients compared with HD during the inter-critical disease phase (166). Hammer *et*

al., showed that cCLP levels correlate directly with the crystal load, assed by ultrasound and dual emission commuted tomography (DECT), both included in the classification criteria for gout.

Finally, increased cCLP levels have been suggested to be a link between chronic inflammation and risk of cardiovascular disease in gout patients (166), (168).

6 Conclusion

Our literature review supports a relevant role of cCLP as a key molecule of inflammation, which is not synthesized in the liver but produced by activated neutrophils in damaged tissues. Especially in chronic inflammatory rheumatic diseases, the cCLP represents a potential prognostic biomarker mirroring (residual) local or systemic inflammation. High cCCL serum levels are associated with worse structural outcomes in RA and to a lesser extent, in SpA. In addition, cCLP can predict disease relapse in some autoimmune diseases including SLE, AAV and some severe manifestations of connective tissue diseases, such as glomerulonephritis in SLE, AAV, JIA, AOSD and lung fibrosis in SSc. Therefore, cCLP levels enable the identification of patients who need an accurate and tight follow-up. The clinical usefulness of cCLP as an inflammatory marker has suggested for non-rheumatic diseases, and especially for the monitoring IBD patients. Currently, there are only a few studies that evaluated the cCLP efficacy as a clinical biomarker in non-ARD diseases, and the results are contrasting, but surely promising. Future studies are warranted to better clarify the role of cCLP in relation to disease severity in MC, MS, CIDP, GO, AIBD and uveitis.

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The authors declare no conflict of interests

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