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REVIEW

Circulating Calprotectin as a Biomarker of COVID-19 Severity

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ABSTRACT

Introduction: Coronavirus disease 2019 (COVID-19) is an infectious disease caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection. Although demographic and clinical parameters such as sex, age, comorbidities, genetic background and various biomarkers have been identified as risk factors, there is an unmet need to predict the risk and onset of severe inflammatory disease leading to poor clinical outcomes. In addition, very few mechanistic biomarkers are available to inform targeted treatment of severe (auto)-inflammatory conditions associated with COVID-19. Calprotectin, also known as S100A8/S100A9, MRP8/14 (Myeloid-Related Protein) or L1, is a heterodimer involved in neutrophil-related inflammatory processes. In COVID-19 patients, calprotectin levels were reported to be associated with poor clinical outcomes such as significantly reduced survival time, especially in patients with severe pulmonary disease.

Areas covered: Pubmed was searched using the following keywords: Calprotectin + COVID19, S100A8/A9 + COVID19, S100A8 + COVID-19, S100A9 + COVID-19, MRP8/14 + COVID19; L1 + COVID-19 between May 2020 and 8 March 2021. The results summarized in this review provide supporting evidence and propose future directions that define calprotectin as an important biomarker in COVID-19.

Expert opinion: Calprotectin represents a promising serological biomarker for the risk assessment of COVID-19 patients.

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1. Introduction

Coronavirus disease 2019 (COVID-19) is an infectious disease caused by exposure to the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). First identified in December 2019 in Wuhan, Hubei, China, SARS-CoV-2 is one of 7 coronaviruses that can cause respiratory disease in humans. There are four subgroups of these viruses (alpha, beta, gamma, and delta) including SARS-CoV and Middle East respiratory syndrome CoV (MERS-CoV), which caused an outbreak of severe acute respiratory syndrome in 2002–2003 and 2012 respectively [1]. However, the world-wide case and fatality rates of the previous coronavirus outbreaks were associated with comparatively milder disease than that experienced in the current world-wide SARS-CoV2 pandemic. As of March 2021, more than115 million cases have been reported around 250 countries and while the vast majority of people have recovered, over2.5 million have died (https://coronavirus.jhu.edu) [2].

Common symptoms of COVID-19 include fever, cough, fatigue, shortness of breath, and loss of taste and smell [3,4]. Besides the pulmonary symptoms, several extrapulmonary symptoms have been reported in patients with COVID-19 including but not limited to diarrhea [5], nausea, and abdominal pain [6,7]. Recently, Lamers and colleagues provided evidence that SARS-CoV-2 not only interacts with the airway but also with human gut enterocytes which possibly explains the gastrointestinal symptoms observed in a portion of COVID-19 patients. In more detail, these interactions are mediated via highly expressed angiotensin converting enzyme 2 (ACE2) on differentiated enterocytes [8]. The interval from documented SARS-CoV2 exposure to onset of COVID-19 symptoms is typically 3–7 days but may range from 2 to 14 days [9]. A higher risk of more severe disease is associated with viral load [10], increased age and the presence of certain comorbidities such as diabetes, hypertension and obesity [11]. About 80% of cases have mild symptoms, about 15% may require hospitalization, and about 5% are classified as critically ill, possibly associated with a 'cytokine storm' and acute respiratory distress syndrome (ARDS), multi-organ failure, and septic shock [3,4,12]. However, more recent studies indicate that the 'cytokine storm' in COVID-19 is different from hemophagocytic lymphohistiocytosis (HLH) and macrophage activation syndrome (MAS) [13,14] which can be associated with autoimmune conditions such as systemic lupus erythematosus (SLE) [13–16]. Treatment of COVID-19 is largely supportive, although multiple treatment options are evolving as an understanding of the natural history of the SARS-CoV-2 virus and COVID-19 increases [17,18].

In order to estimate the risk of complications in COVID-19 patients, a number of risk scores have been developed and compared [19–21]. The sequential organ failure assessment score (SOFA score) or a modified version thereof (qSOFA) [19],

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Article highlights

- Calprotectin a heterodimer composed of S100A8 and S100A9 (or MRP8/14) represents a promising marker for disease severity in COVID-19 patients
- In independent studies, circulating calprotectin levels were associated with increased risk for mechanical ventilation/intensive care unit requirement, multi-organ failure as well as COVID-19 related death
- Circulating Calprotectin assays are available in certain geographies which allows testing of patients in the management of COVID-19
- Circulating Calprotectin might represent a novel companion or complementary diagnostic marker for management of COVID-19 severity. Studies are needed to evaluate the value.

previously known as the sepsis-related organ failure assessment score is frequently used for the assessment of COVID-19. Another risk score is the COVID-GRAM score [22] which is available online (http://118.126.104.170/). Other scores include, but are not limited to COVID severity index, A-DROP, National Early Warning Score 2 (NEWS2), CURB-65, systolic blood pressure, multi-lobar chest radiography involvement, albumin level, respiratory rate, tachycardia, confusion, oxygenation, and arterial pH (SMART-COP) and Pneumonia Severity Index (PSI). Although SOFA is an independent predictor for extended ventilation [23] additional prognostic factors and biomarkers are desired. Clinically, one of the glaring unmet needs are biomarkers that can predict sudden and unexpected clinical deterioration and disease severity [24].

Several studies have evaluated biomarkers that can help predict severe complications in COVID-19 patients including but not limited to virus load [10], interleukin (IL)-6 [25], D-dimer [26], C-reactive protein (CRP) [27], lactate dehydrogenase (LDH) [27], ferritin [27], serum amyloid A (SAA) [28,29] as well as components of the complement pathways [30,31] and others [32] (Table 1). Interestingly, combining biomarkers can increase the predictive value for COVID-19 outcomes [20]. It has been reported that the ratio of IL-6 to IL-10 linked to a 5-point linear score (Dublin-Boston score) informed prognosis by helping to determine when to revise care, such as mechanical ventilation, or determine considerations for therapies and clinical outcomes in hospitalized COVID-19 patients [21]. Along these lines, and as discussed in more detail below there is mounting evidence that a neutrophil mediated inflammatory protein called calprotectin might represent a strong candidate biomarker that can aid in risk stratification [33–37].

2. Link between COVID-19 and autoimmunity

Several studies have linked COVID-19 with autoimmune conditions [54] such as anti-phospholipid syndrome [55–60], Guillain-Barré [61–65] and Miller Fisher syndromes [66,67] (reviewed in [68]). In addition, some patients with COVID-19 develop signs and symptoms that are also observed in certain autoimmune conditions [54,69–73], but the number of patients reported to date tend to be small, lack immunoassay details and contemporaneous non-COVID-19 respiratory disease controls. A recent study reported autoantibodies to cytokines as a marker for disease severity in COVID-19 [74], which has interest because of previous associations of anti-cytokine antibodies to infectious disease susceptibility and outcomes. In addition, there is increasing evidence that several drugs, such as IL-6 antagonists, that have been used in patients with HLH, MAS and other autoimmune/autoinflammatory conditions were beneficial in COVID-19 [75–88]. Interestingly, autoimmune disease patients treated with anti-TNF alpha inhibitors do not show increased risk of COVID-19 infection or more severe disease cause [89]. Along those line, there is preliminary evidence of the usefulness of anti-C5 blocking monoclonal antibody (eculizumab) in COVID-19 which represents a standard treatment for some autoinflammatory disorders such as hemolytic uremic syndrome (HUS), thrombotic thrombocytopenic purpura or catastrophic APS [90].

3. Relevance of neutrophil extracellular traps in COVID-19

It is well-established that neutrophil extracellular traps (NETs) can mediate inflammation-associated lung damage, thrombosis, and fibrosis [91–93]. Consequently, to shed more light on the potential involvement of NETs in COVID-19, several studies have been initiated [94]. Further evidence came from a recent study that investigated postmortem lung specimens from four COVID-19 patients and four patients who died from a COVID-19-unrelated cause [91]. NETs were observed in the lungs of each COVID-19 patient, especially in the airway compartment and neutrophil-rich inflammatory areas of the interstitium. In addition, NET-primed neutrophils were present in arteriolar microthrombi. Preliminary data indicate that SARS-CoV-2 can activate NETosis in human neutrophils, potentially due to increased levels of intracellular Reactive Oxygen Species (ROS) [95]. Besides thrombosis, NETosis has also been associated with ST-elevated myocardial infarction (STEMI) in COVID-19 disease [96]. All these data point to a crucial role of NET formation leading to severe pulmonary complications of COVID-19. Based on the growing evidence about the involvement of NETs in the pathogenesis of COVID-19, NET inhibitors have been proposed and are being studied as a feasible way to prevent coronary thrombosis in patients with severe COVID-19 disease [95–98]. Several intervention trials are currently recruiting with a diversity of study designs and outcome measures. For example, nebulized dornase alfa, a common cystic fibrosis medication, is currently undergoing trials for COVID-19 treatment [99,100]. Its proposed protective effect relates to its clearance of neutrophil extracellular traps, which play a pathogenic role in SARS-CoV-2 infection [101]. Preliminary data also indicates that dornase alfa is effective in limiting the in vitro infection of green cell lines by SARS-CoV-2 (see also below under treatment) [99].

4. Calprotectin

Calprotectin a heterodimer composed of S100A8 and S100A9 proteins (also called myeloid related proteins (MRP) 8/14), belongs to the S100 family of calcium-binding proteins whose levels are increased in patients with various

Table 1. Overview of potential risk biomarkers for COVID-19.

Group	Marker	Reference	Level of Evidence	Comments
Hemostasis	D-dimer	[20,26,38–41]	High	Heart involvement
Acute phase reactant	Procalcitonin	[39,42–44]	Moderate	
	Ferritin	[27,35,76]	Moderate	Marker of HLH, MAS
	CRP	[26,27,44]	High	Low specificity, in combination with diagnosis of COVID-19 (currently) strongly predictive
Cytokine	IL-6	[21,25,44]	Moderate	Associated with 'cytokine storm' in COVID-19
COVID-19 related markers	Anti-SARS-CoV-2 antibodies (isotypes)	[45,46]	Limited	Ratio between isotypes might indicate severity; IgA might play important role in COVID-19 severity
	Viral load in RT-PCR	[10]	Moderate	Long turn-around time
Inflammatory protein	Amphoterin, HMGB1	[34,47]	Limited	Released during NETosis, also discussed as therapeutic target
	Calprotectin, S100A8/S100A9, MRP8/14	[33,37]	Limited	More global/specific indicator of PMN and monocyte activation
Autoantibodies	Anti-phospholipid antibodies	[56,59,60]	Limited	Controversial findings; further studies required
Complement system	Complement activation	[48]	Limited	SC5b-9 and C5a
Serum protein	Serum amyloid A (SAA)	[28,29,49]	Evolving	Might be useful in cases which have low CRP levels; potentially combined with IL-6
	Fibrinogen (Fib)	[50]	Moderate	Potentially combined with Albumin in ratio
	Albumin (Alb)	[41,50]	High	Potentially combined with Fibrinogen in ratio
Cardiac biomarkers	Cardiac troponin I (cTn1)	[43,51,52]	Moderate	Might predict death related to myocardial injury
Liver enzyme	Lactate dehydrogenase (LDH)	[41,53]	High	Routinely used
	Alanine aminotransferase (ALT)	[41,53]	High	Routinely used ALT might have the highest value among liver markers
	Aspartate aminotransferase (AST)	[41,53]	High	Routinely used

Abbreviations: CRP, C-reactive protein; HMGB1, high mobility group protein B1; HLH, hemophagocytic lymphohistiocytosis; IL, interleukin; MAS, macrophage activation syndrome; RT-PCR, real time primer chain reaction.

inflammatory and autoimmune conditions [102–104]. Calprotectin is particularly abundant in the cytoplasm of neutrophils and is expressed on the membrane of monocytes and has been recognized as a valid functional biomarker of inflammation as it is involved in recruiting inflammatory cells upon interacting with endothelial cells [5]. Fecal calprotectin represents a reliable biomarker in the context of inflammatory bowel disease [105], which might gain additional value during the COVID-19 pandemic [106,107]. Further, elevated fecal calprotectin has been studied in COVID-19 [107–109] where it is associated with intestinal inflammation [110]. Although these observations are intriguing, this review focuses on the measurement of calprotectin in blood matrices (serum, plasma).

4.1. Measurement in blood

Although fecal calprotectin measurement represents a well – established and reliable biomarker in the diagnosis of inflammatory bowel disease, the role of serum (blood/plasma/circulating) calprotectin in the pathogenesis of diseases is less established. Recently, it has gained increasing attention because it may be a novel biomarker of (auto)inflammatory disorders such as rheumatoid arthritis [102,103,111–117], psoriatic arthritis [117,118], and systemic lupus erythematosus [102,119–123]. It has been reported that calprotectin measurement in blood-derived matrices could be affected by preanalytical factors such as sample type, collection and storage conditions (temperature), and the presence and type (if any) of anticoagulant used; all potentially leading to inconsistent measurement of calprotectin levels [124–127]. In different clinical studies, calprotectin has been measured either in plasma or in serum as interchangeable sample matrix despite lacking consensus. Although it is widely known that neutrophils are highly labile in-vitro (calprotectin is a major component of these cells) and platelet activation and coagulation may induce release of intracellular calprotectin into the extracellular medium, pre-analytical sample processing of serum calprotectin has not been thoroughly studied. According to Rammes et al. [128], monocytes release calprotectin after activation of protein kinase C (PKC), an enzyme that requires calcium ions for activation. Ethylenediaminetetraacetic acid (EDTA), which inhibits coagulation by binding calcium, reduces PKC availability and consequently calprotectin derived from monocytes. Clark et al. showed that the activation of platelets via Toll-Like receptor 4 during coagulation induces neutrophils to bind platelets with the activation and production of NETs [129]. In the 1990's, Dale showed that serum calprotectin levels were about twofold increased in plasma, which was attributed to its release from cells activated during in-vitro coagulation [125]. Dale also reported that calprotectin levels showed very small changes in plasma when blood was collected in EDTA, acid citrate dextrose, and acid phosphate dextrose [125]. Despite both citrate and EDTA being calcium chelators that inhibit the coagulation process, EDTA plasma was found to have more reliable quantification of calprotectin levels. According to Pedersen et al., higher calprotectin levels were found in lithium heparin and serum samples compared to EDTA plasma when stored at high temperatures. In line with these observations, Nordal et al. [126] reported that calprotectin levels measured in EDTA plasma were lower

compared to serum and concluded that calprotectin measurements are most reliable in EDTA plasma. Van Hoovels et al. [124], also reported lower calprotectin levels in EDTA plasma compared to serum, confirming that blood calprotectin levels were matrix dependent with higher values in serum as compared to plasma. Most recently, we performed additional pre-analytic studies related to the measurement of circulating calprotectin in serum/plasma. Our data is consistent in terms of time sensitivity of processing after blood draw. In addition, our data showed that Citrate plasma might represent a reliable matrix for the measurement of circulating calprotectin [130]. Other pre-analytical factors can impact the test result for markers of inflammation such as cytokines [131], and also calprotectin [35]. For example, heavy exercise can lead to transient inflammation and increases in IL-6 and calprotectin levels [132]. Consequently, additional studies of pre-analytical parameters are warranted to define the specimen requirements for accurate and reliable measurement of circulating calprotectin. Based on the current knowledge, this will likely lead to a limitation for certain sample types (e.g. serum) and/or the definition of matrix specific cutoff values. As reviewed below, several studies have established a correlation between calprotectin and disease severity of COVID-19.

4.2. Calprotectin as risk marker for COVID-19

In order to summarize the current knowledge on circulating calprotectin as a marker for disease severity in COVID-19 patients, PubMed was searched using the following keywords: Calprotectin + COVID19, S100A8/A9 + COVID19, S100A8 + COVID-19, S100A9 + COVID-19, MRP8/14 + COVID19; L1 + COVID-19. No publication time or language restrictions were applied. A total of twelve studies were identified that analyzed blood calprotectin (either serologically measured or via gene-expression analysis) in the context of COVID-19 severity (Table 2). Due to the relatively low number of studies (related to the recent onset of the pandemic) we have not attempted a systematic literature review and/or meta-analysis and present the information below as a narrative review. This is in contrast to a recent review article that provides preliminary meta data on circulating and fecal calprotectin [133]. Both approaches can be regarded as synergistic.

In the most comprehensive study of calprotectin in COVID-19 to date, Silvin et al. used a discovery approach to define signatures that were associated with disease severity in COVID-19 patients [33]. In this study of severe cases, high-dimensional flow cytometry and single-cell RNA sequencing of COVID-19 patient's peripheral blood cells detected disappearance of non-classical (CD14LowCD16High) monocytes, accumulation of HLA-DR, low classical monocytes (Human Leukocyte Antigen – DR isotype), and release of massive amounts of calprotectin (S100A8/S100A9). Calprotectin levels were positively correlated with neutrophil count ($r = 0.62$), fibrinogen (0.76), D-dimer ($r = 0.64$) and disease severity ($p < 0.001$). Immature (CD10LowCD101-CXCR4+/) neutrophils with an immunosuppressive profile accumulated in the blood and lungs, suggesting emergency myelopoiesis. Finally, they reported that plasma levels of calprotectin and a routine flow cytometry assay detecting decreased frequencies of non-classical monocytes could discriminate patients who develop a severe form of COVID-19, suggesting a predictive value that deserves prospective evaluation. Calprotectin levels showed excellent discrimination between controls and mild cases vs. moderate and severe cases with an AUC of 0.959 derived from ROC analysis.

In another recent study by Chen et al. [34] comparing the results from 40 COVID-19 patients admitted to the ICU with 81 in general wards, significant discrimination of calprotectin levels was observed using ROC with an AUC of 0.875 and a sensitivity and specificity of 83.3% and 83.5%, respectively. In the same study, the AUC for death vs. survivor was 0.860 with a sensitivity and specificity of 85.0% and 82.7%, respectively. Kaplan-Meier analysis showed a significant difference between individuals with high levels of calprotectin (cutoff 6195 ng/mL) vs. those with low levels. The resulting COVID-19 hazard ratio (HR) was 13.32 ($p < 0.0001$) and significantly higher compared to the COVID-GRAM score (HR 4.81, $p < 0.0001$). Calprotectin levels were significantly correlated with both, the COVID-GRAM ($r = 0.47$, $p < 0.001$) and qSOFA ($r = 0.50$, $p < 0.001$) sores.

In a small series of patients, de Guadiana Romualdo et al. [37] reported significant differences in calprotectin levels in COVID-19 survivors (3.1 µg/mL, 1.9–4.4 mg/mL) vs. non-survivors (7.1 mg/mL, 4.5–10.3 µg/mL) (adjusted $p < 0.001$). The corresponding AUC and odds ratio were 0.801 (95% CI 0.691–0.894), and 13.30 (95% CI 1.53–116) respectively. The results were compared to other risk markers such as ferritin, C-reactive protein, D-dimer and GDF-15. Although the OR were higher for CRP (15.56, 95% CI 1.78–136), D-dimer (38.11, 95% CI 4.17–-348) and GDF-15 (a member of the Tumor Growth Factor-beta family) (40.50, 95% CI 6.09–270) all 95% CI intervals significantly overlapped.

Shi et al. [35], studied 172 COVID-19 patients, 60 of which required mechanical ventilation and compared those to 47 healthy individuals. The ROC analysis showed good discrimination between COVID-19 patients and controls for calprotectin (AUC = 0.794) which was significantly higher when compared to LDH (AUC = 0.699), CRP (AUC = 0.614) and ferritin (AUC = 0.562). In addition, Shi et al. compared changes of circulating calprotectin levels among three groups: worsening, stable and improving clinical conditions (by oxygenation). Patients with a deteriorating clinical condition demonstrated increasing levels of serum calprotectin while stable or improving individuals had no significant change in circulating calprotectin levels.

In a more recent study, the gene expression in COVID-19 patients was studied in 48 subjects including 28 COVID-19 patients (8 severe/critical vs. 20 mild/moderate cases) and age/sex-matched 20 healthy controls [36]. Among the most highly increased inflammatory mediators in severe/critically ill patients, S100A9 (calprotectin component), an alarmin and toll-like receptor (TLR) 4 ligand, was found as a notable biomarker, because it inversely correlated with the serum

Table 2. Overview of studies on calprotectin in COVID-19 patients.

Study	COVID-19	Mild	Moderate	Severe	Survivor	Non-survivor	ICU	Non-ICU	Controls	Survival Kaplan Meier	ICU	MV vs. non-MV	Method for calprotectin	Sample matrix	Comments/Key findings
de Guadiana Romualdo et al. [37]	66	N/R	N/A	N/A	58	8	N/R		N/A	AUC = 0.801 (0.691–0.894) OR 13.3 (1.53–116)	Yes	N/R	Particle enhanced turbidimetric immunoassay (PETIA, GenyianAS, Norway	Blood (not specified further)	Discrimination between death/survival
Chen et al. [34]	121	N/R	N/A	N/A	83	36	40	81	N/A	HR 13.32 AUC = 0.875 Sen/Spe 83.3/83.5 c/o 6284	AUC = 0.860 Sen/Spe 85.0/82.7 c/o 6195	N/R	Human S100A8/S100A9 Heterodimer DuoSet ELISA (DY8226-05, R&D Systems)	Serum (processed within 24 hours)	Discrimination between ICU/non-ICU and death/survival
Shi et al. [35]	172*	N/R	N/A	N/A	N/A	N/A	N/A	N/A	50#	N/R	N/R	AUC = 0.794	Human S100A8/S100A9 Heterodimer DuoSet ELISA (DY8226-05, R&D Systems)	Plasma/serum (stability study in supplement)	Discrimination between patients in need of MV vs. no MV Monitoring
Silvin et al. [33]	86	27	16	43	76	10	N/R	N/R	72$	N/R	N/R	N/R	R-plex Human Calprotectin Antibody Set (Meso Scale Discovery, ref: F21YB-3 + MESO QuickPlex SQ120 reader + MSD's Discovery Workbench 4.0). Average of duplicates	EDTA plasma	Discovery work revealed calprotectin Controls + Mild vs. Moderate + severe AUC = 0.959
Sohn et al. [36]	28	20	N/A	8	N/A	N/A	N/A	N/A	20#	N/R	N/R	N/R	N/A, Gene expression of S100A8 and S100A9	N/A	Gene expression of S100A8 and S100A9 Link between TLR4 and inflammation in COVID-19
Wu et al. [145]	N/R	N/R	N/A	9	N/A	9	9	N/A	N/A	N/R	N/R	N/R	Gene expression	Lung tissue	Low virus load, high calprotectin expression Also other S100 proteins overexpressed
Shu et al. [136]	120	40	N/A	40	80	40			40	N/R	N/R	N/R	Gene expression/ELISA CUSABIO (Cat#CSB-E12149h)	EDTA Plasma	Combination with CRP, CETP accurately identifies severe COVID-19
Shaath et al. [137]	8	3	N/A	5	N/A	N/A	N/A	N/A	2	N/R	N/R	N/R	Single cell analysis	N/A	
Bauer et al. [138]	19	N/R	N/R	N/R	17	2	8	11	N/A	AUC = 0.85 (0.54–1.00)	AUC = 0.70 (0.42–0.99)		turbidimetric method, Gentian AS, Norway	Serum, centrifuged within 30 min	Predictor of multi-organ failure

(*Continued*)

Table 2. (Continued).

Study	COVID-19	Mild	Moderate	Severe	Survivor	Non-survivor	ICU	Non-ICU	Controls	Survival Kaplan Meier	ICU	MV vs. non-MV	Method for calprotectin	Sample matrix	Comments/Key findings
Kaya et al. [134]	80	N/A	N/A	N/A	N/A	N/A	38	42	N/A	N/R	AUC = 0.64 (0.52–0.76)		ELISA (Elabscience, Bioassay Technology Laboratory, China)	Serum	Associated with ICU requirement (p = 0.031)
Ren et al. [135]	171	N/A	122	134	N/A	N/A	N/A	N/A	25	N/R	N?R	N?R	Single cell analysis	blood	Calprotectin highly upregulated in severe COVID-19 immune cells
Abers et al. [139]	175		30	145	142	33	N/A	N/A	60	N/R	N/R	N/R	Custom multiplex assay (R&D Systems)	Serum/plasma	Slope of ± 40% excepted between serum and plasma Calprotectin as one of the most significant predictors of severity

Abbreviations: AUC = area under the curve; c/o = cutoff; CETP = Cholesteryl ester transfer protein; HI = healthy individuals; ICU = intensive care unit; N/R = not reported; OR = odds ratio; S100A8 and S100A9 = components of calprotectin complex; MV = mechanical ventilation; Sen = sensitivity; Spe = specificity; TLR = toll-like receptor # healthy controls, $ disease controls, * Room air oxygen = 41, noninvasive supplement oxygen = 71, mechanical ventilation = 60

albumin levels. It was also observed that recombinant S2 and nucleocapsid proteins of SARS-CoV-2 significantly increased pro-inflammatory cytokines/chemokines and S100A9 in human primary peripheral blood mononuclear cells (PBMCs). These data supported a link between TLR4 signaling and pathological inflammation during COVID-19 and may be used to inform therapeutic approaches through targeting TLR4-mediated pathways.

A more recent study on calprotectin by Wu et al. [145] describes S100A9 as being highly upregulated in lung tissue of individuals that died from COVID-19. In addition to S100A9, several other members of the S100 protein family were also overexpressed. Interestingly, all of the patients had low viral load indicating that the cause of death is related to the hyperinflammatory process rather than to the virus itself. Another key observation of the study is that IL-6 was not highly expressed in the lung of severe COVID-19 cases which is in keeping with lack of effectiveness of IL-6 inhibition in late-stage COVID-19 pneumonia.

Shu et al. conducted a proteomic approach on plasma samples to identify biomarkers associated with COVID-19 pathogenesis using 160 samples from 40 fatal, 40 severe and 40 mild cases as well as 40 healthy controls [136]. The study utilized a discovery and validation cohort and machine learning to evaluate the clinical and pathogenic importance of proteins identified. The final validation using ELISA assays, confirmed calprotectin levels as being significantly increased in severe vs. mild cases. Especially the combination of S100A9 with CRP and cholesterol ester transfer protein (CETP) showed excellent discrimination between severe and non-severe COVID-19 patients.

Shaath et al. [137] employed iterative clustering and guide-gene selection 2 (ICGS2) as well as uniform manifold approximation and projection (UMAP) dimensionality reduction computational algorithms to decipher the complex immune and cellular composition of bronchoalveolar lavage (BAL), using publicly available datasets from a total of 68,873 single cells derived from two healthy subjects, three patients with mild, and five patients with severe COVID-19. The data revealed the presence of neutrophils and macrophage cluster-1 as a hallmark of severe COVID-19. Among the identified gene signatures of the neutrophil cluster, S100A8 was identified among others. Transcriptome data from a cohort of COVID-19-derived peripheral blood mononuclear cells (PBMCs) validated the data from BAL from patients with severe and mild COVID-19 (including S100A8).

In one of the smallest series of patients studied for calprotectin levels, Bauer et al. [138] compared Calprotectin levels in 19 COVID-19 patients based on ICU requirement, multi-organ failure (MOF) after 72 hours (in alignment with SOFA assessment), MOF in total as well as 90-day mortality. Calprotectin levels were determined in serum samples using a turbidimetric assay in addition to standard of care biomarkers for COVID-19 (lactate CRP, PCT). High calprotectin levels were associated with MOF after 72 hours as well MOF in general with AUC values of 0.87 (95% CI 0.63–1.00) and 0.91 (95% CI 0.77–1.00), respectively. Although the AUC values for calprotectin were higher compared to most other markers, due to the small number of patients, differences were not significant (strongly overlapping 95% CI). However, the data strongly supports the value of calprotectin as biomarker for risk stratification, in particular with regard to subsequent MOF. Indeed, measurement of calprotectin might add to the biomarker repertoire in the emergency department or ICU since it seems to perform better than traditional markers such as lactate, CRP and PCT. Compared to CRP and PCT, calprotectin might add value in early management of COVID-19 patients.

Abers et al. described a immune based-biomarker signature that is associated with mortality in COVID-19 patients [139]. The authors used multiplex testing using serum or plasma samples. Out of 66 biomarkers tested, circulating calprotectin was one of the few markers that remained significantly associated with mortality after full adjustments in multi-variate models.

Kaya et al. conducted a study to compare individuals hospitalized but not referred to the ICU (n = 38) with critical patients in the ICU setting (n = 42). Calprotectin levels were determined in serum using an ELISA system. The calprotectin levels in ICU patients were higher compared to the non-ICU group ($p = 0.031$).

Very compelling data were derived from comprehensive single-cell immune landscape mapping using samples from 171 COVID-19 patients. Virus RNA was found in epithelial and immune cells leading to systemic upregulation of calprotectin potentially contributing to the mechanisms observed in severe COVID-19 patients. This data might indicated that calprotectin can function as a mechanistic biomarker and fit into the concept of precision medicine [135].

Although the data reported for circulating calprotectin in COVID-19 patients is promising, some limitations of the studies have to pointed out. One limitation that is common for most studies is the relatively small number of cases and the imbalance among the comparator groups. For example, the study by de Guadiana Romualdo et al. included 58 survivors and only 8 non-survivors. In addition, the endpoints of the studies are heterogenous (see Table 2). Lastly, different assays have been applied to measure calprotectin levels and no commutability data is available, especially around the decision point .

4.3. Calprotectin levels for monitoring and cutoff

Only one study utilized longitudinally collected samples to explore the utility for monitoring. Although this study provided promising data, several limitations were observed (including samples size, time difference between sequential samples etc.) [35]. In addition, the cutoff value needs to be established. It is very likely that different thresholds provide the best performance depending on the clinical scenario (e.g., prediction of severity or mortality). In addition, as pointed out above, different cutoff values might be required for different sample types (i.e., serum vs. plasma).

4.4. Calprotectin and other biomarkers

Calprotectin levels showed significant correlation with several other biomarkers of inflammation including, but not limited to, IL-6 [33,34], CRP [34,35], neutrophil count [33] as well as other markers of disease severity such as D-dimer [33,34] (Table 1, Table 3). In addition, other members of the S100 protein family might correlate/overlap with calprotectin levels [136,140]. Future studies using multivariate analyses are needed to identify independent predictors of severe disease and to define the best combination of markers to aid in the risk stratification and management of COVID-19 cases.

5. Treatment

Although it is of value to be able to predict complications in patients with COVID-19 (such as with any other disease), it is of utmost importance to have actionable biomarkers. Calprotectin levels have been shown to predict treatment responses in patients with RA [111], especially patients treated with TNF-alpha inhibitors who showed different treatment effects when stratified according to the calprotectin levels [111]. In addition, several biological disease modifying anti-rheumatic drugs currently used in RA have shown benefits in patients affected with COVID-19 [18,141,142]. Using biomarker prediction models that include calprotectin levels, could open new precision medicine approaches in COVID-19 (Figure 1). Efforts are underway to study whether anti-TNF alpha therapy is effective in COVID-19 (CATALYST, ISRCTN40580903) [143,144]. Such trials should ideally include testing for calprotectin levels. In addition, considering that calprotectin levels are significantly elevated in more severe COVID-19 combined with the promising outcomes in treating inflammatory diseases with using S100A8/S100A9 inhibitors (e.g. quinoline-3-carboxamide compounds) [145,146], might represent a promising strategy for treating severe or critically ill COVID-19 cases. Several other treatments have been proposed based on biological roles of calprotectin. One of them, alpha-1-antitrypsin (AAT) represents an established treatment for AAT deficiency which is manifest in lung damage. A recent paper outlines several reasons why AAT might be an effective treatment in COVID-19 including (among others) the inhibitory effect of AAT on neutrophil elastase [147,148]. Consequently, several clinical trials have been approved to study the efficacy of AAT in COVID-19 related lung disease. Lastly, in murine models of autoimmune disorders, the direct or indirect blockade of S100A8 or S100A9 exerted a beneficial effect (reviewed in [103]).

6. Conclusion

In conclusion, evidence to date has suggested that calprotectin is a potentially valuable addition to biomarker panels to assess the risk of disease severity in COVID-19. The link of calprotectin to the inflammatory pathway might open new opportunities to improve the management and outcomes of COVID-19. It has yet to be clarified if other variables such viral load, SARS-CoV2 antibodies, the use of corticosteroids, anti-coagulants (i.e., low dose heparin) or biologics (i.e., tocilizumab, anakinra) could affect neutrophil function and circulating calprotectin levels. Additional studies are required to elucidate these issues. However, the notion that different targeted approaches [34,35,37] and discovery research [33,136,145]) indicate that calprotectin represents an intriguing and promising biomarker for COVID-19 severity.

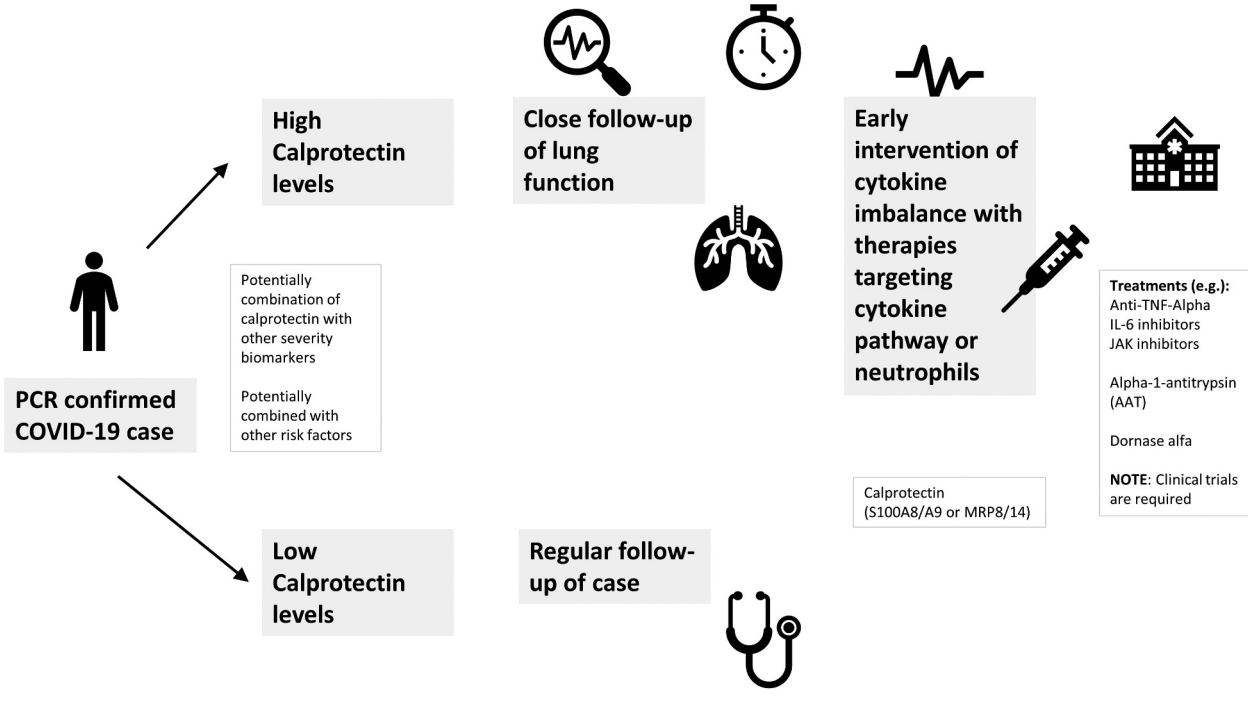

Figure 1. Potential application of Calprotectin measurement in precision medicine model for severe lung disease in COVID-19. Confirmed COVID-19 with high baseline calprotectin levels might require close follow-up of lung function and potentially are candidates for early interventions to reduce cytokine imbalance.

Table 3. Correlation of calprotectin with other biomarkers in COVID-19 patients.

Study	Neutrophil count	D-dimer	IL-6	CRP
Chen et al. [34]	N/R	0.51 $p < 0.001$	$p < 0.0001$	N/R
Shi et al. [35]	0.50 $p < 0.0001$	N/R	N/R	0.44 $p < 0.0001$
Silvin et al. [33]	0.62 $p = 2.8$ e10	0.64 $p = 1.7$ e7	0.43 $p = 0.00013$	N/R

Abbreviations: N/R = not reported

7. Expert opinion

The research discussed in this review article provides further insights into the pathogenesis of COVID-19 and risk biomarkers. In addition, the compiled data serves as a call to facilitate additional clinical trials, regulatory submission (and approvals) as well as clinical adoption of circulating calprotectin levels in the management of COVID-19 patients. The desired outcome is to benefit healthcare utilization, health economics as well as patient morbidity and mortality. Considering the mounting evidence of circulating calprotectin as risk marker for COVID-19 severity, it is conceivable that calprotectin might become part of risk stratification panel combined with, for example, CRP, PCT, D-dimer, IL-6, and/or ferritin. This approach is intended to be evidence-based. First, several independent discovery studies with thousands of analyzed expressed genes identified calprotectin as one or the strongest predictors of COVID-19 severity. In addition, several validation cohorts and different methods for the measurement of circulating calprotectin were used. However, additional studies are warranted to better define the potential role of calprotectin as part of a risk panel and to define how many days prior to the onset of severe complications this biomarker is detectable. Data derived from RA indicate that elevated calprotectin levels are detected before clinical escalation, a feature which also needs to be extended in COVID-19. In this context, longitudinal evaluation will also provide valuable insights into the utility of measuring circulating calprotectin as a potential monitoring parameter that objectively informs the pathogenesis of the disease. In addition to clinical studies, pre-analytical aspects are of utmost importance when measuring calprotectin levels. Although some pre-analytical studies have been conducted and published [124,127,130], larger studies that address all aspects of sample processing (including blood draw, hold time as well as shipping simulation studies) are warranted. All these studies will be crucial in defining the intended clinical use of calprotectin measurements. The long-term utility of calprotectin as a marker for autoinflammation causing multi-organ failure goes far beyond COVID-19 as several other diseases are mediated via similar mechanisms. Lastly, the implication for evidence-based treatment selection is less clear and could benefit from retrospective analysis of ongoing or completed clinical trials using bio-banked serum or plasma samples. Examples for such clinical trials include but are not limited to trials on TNF-alpha, IL-6, C5 (complement), dornase alpha or JAK inhibitors. If calprotectin levels predict treatment response in COVID-19 as demonstrated in RA, calprotectin might be considered as a companion or complementary diagnostic test, where the regulatory requirements differ significantly. While for companion diagnostics, the biomarker must appear in the drug labeling and therefore has to be included in early phases of the clinical trials, for complementary diagnostic tests, this is not a requirement. From our viewpoint it is conceivable that circulating calprotectin will be used clinically to assess a number of autoinflammatory conditions. Although speculative, calprotectin might be considered as the CRP of autoinflammation as it is primarily released from neutrophils (not from the liver) and therefore more closely linked to the autoinflammatory process. The role of calprotectin testing in COVID-19 is somewhat unsettled because much is to be learned on the effectiveness of vaccines and the impact of herd-immunity as the spread of the virus and COVID-19 becomes endemic [148]. However, knowing that there have been previous outbreaks of coronavirus, it is likely that new viruses will emerge, and some might also trigger such autoinflammatory reactions. To translate the promising data into clinical practice, regulatory hurdles need to be overcome which strongly depend on the geography and target markets. While in the US, emergency use authorizations (EUA) are used by the FDA to allow clinical applications, in other geographies the CE-mark still allows for self-declaration depending on the indented use of the proposed product. Close collaborations between clinical sites, regulatory agencies and diagnostic companies are need to advance circulating calprotectin from bench to bedside.

Abbreviations

ARDS, acute respiratory distress syndrome; AUC, area under the curve; BAL, bronchial alveolar lavage; CETP, Cholesteryl ester transfer protein; CI, confidence interval; COVID-19, corona virus infections disease 19; CRP, C-reactive protein; EDTA, ethylenediamine tetracetic acid; GDF-15, growth differentiation factor; HLH, hemophagocytic lymphohistiocytosis; ICGS2, iterative clustering and guide-gene selection; LDH, lactate dehydrogenase; ICU, intensive care unit; IL, interleukin; MAS, macrophage activation syndrome; MOF.MRP, myeloid related protein; NETs, neutrophil extracellular traps; OR, odds ratio; PKC, protein kinase C; ROC, receiver operator characteristic; SOFA, sequential organ failure assessment; TLR, toll-like receptor; TMA, thrombotic microangiopathies; UMAP, uniform manifold approximation and projection, VWF, von Willebrand factor.

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Declaration of interest

Michael Mahler is an employee of the Research department of Inova Diagnostics, a diagnostic company commercializing diagnostic assays (holds no stocks or equity in the company). Marvin J. Fritzler is a consultant to Inova Diagnostics. Pier Luigi Meroni has been a consultant to Inova Diagnostic in the past. Maria Infantino has been providing webinars on Circulating Calprotectin on behalf of Inova Diagnostics. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

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