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Calprotectin: from biomarker to biological function

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ABSTRACT

The incidence of inflammatory bowel diseases (IBD) emerged with Westernisation of dietary habits worldwide. Crohn's disease and ulcerative colitis are chronic debilitating conditions that afflict individuals with substantial morbidity and challenge healthcare systems across the globe. Since identification and characterisation of calprotectin (CP) in the 1980s, faecal CP emerged as significantly validated, non-invasive biomarker that allows evaluation of gut inflammation. Faecal CP discriminates between inflammatory and non-inflammatory diseases of the gut and portrays the disease course of human IBD. Recent studies revealed insights into biological functions of the CP subunits S100A8 and S100A9 during orchestration of an inflammatory response at mucosal surfaces across organ systems. In this review, we summarise longitudinal evidence for the evolution of CP from biomarker to rheostat of mucosal inflammation and suggest an algorithm for the interpretation of faecal CP in daily clinical practice. We propose that mechanistic insights into the biological function of CP in the gut and beyond may facilitate interpretation of current assays and guide patient-tailored medical therapy in IBD, a concept warranting controlled clinical trials.

INTRODUCTION

Inflammation describes an evolutionarily conserved process, which is characterised by the activation of innate and adaptive immune cells to protect the host against a wide range of potential threats. If such responses spiral out of control, dysregulated chronic inflammation turns into a detrimental condition underlying the pathophysiology of many human diseases.^{1,2} Recent advances fostered our cellular and molecular understanding of unresolved inflammation and consequently, anti-inflammatory targeted therapies, for example, for human inflammatory diseases of the skin, joints and gut have substantially changed clinical practice over the last decade. However, many inflammatory diseases are still poorly controlled, partly because the underlying disease trigger(s) remain enigmatic.

Inflammatory bowel diseases (IBD), including Crohn's disease (CD) and ulcerative colitis (UC), are characterised by chronic remittent episodes of inflammation in and beyond the GI tract.³ Recent insights have revealed the complex nature of IBD with a genetic underpinning that cannot explain the majority of cases.^{4,5} In line with this, a rising incidence and prevalence worldwide suggests that Westernisation of life style (with associated dietary and microbial cues) significantly contributes to the development and natural history of IBD.⁶ Specific dietary compounds that instigate or impact the

Summary box

What is already known about this subject?

- ▶ Calprotectin is an established clinical biomarker for inflammatory bowel diseases and harbours immune-regulatory functions.

What are the new findings?

- ▶ This review article summarises the extensive literature about the role of calprotectin in health and disease, covering a combination of clinical and basic research aspects.

How might it impact on clinical practice in the foreseeable future?

- ▶ Understanding the regulation and biological function of calprotectin in the gut might lead to novel diagnostic and therapeutic strategies in inflammatory bowel diseases.

Key messages

- ▶ Calprotectin concentration is an established biomarker that allows clinical decision-making in patients with suspected or confirmed inflammatory bowel disease.
- ▶ Faecal calprotectin levels correlate significantly with clinical or endoscopic disease activity in inflammatory bowel diseases.
- ▶ In health, calprotectin harbours immune-regulatory functions that are crucial for immune defence such as neutrophil chemotaxis and chelation of multiple divalent metal ions.
- ▶ In chronic inflammatory diseases calprotectin may fuel disease processes through cytokine receptor engagement and generation of reactive oxygen species.
- ▶ Better understanding of calprotectin biology in the gut may lead to novel diagnostic and therapeutic advances in inflammatory bowel diseases in future.

course of human IBD remain to be determined, while experimental studies in mice identified that nutritional compounds fuel gut inflammation partly by modulation of the intestinal microbiota.⁷⁻⁹

To accurately diagnose IBD, the entire spectrum of available tools should be exploited, including patient history, non-invasive and invasive imaging (endoscopy) and histological interpretation. In such a diagnostic algorithm, serum and especially faecal biomarkers help to select patients for an invasive diagnostic evaluation,¹⁰⁻¹² in that a specific threshold allows to discriminate between functional



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(non-inflammatory) diseases with a similar clinical presentation and an inherently high prevalence (such as irritable bowel syndrome).^{13–15} In IBD, but also in many other inflammatory conditions, clinicians increasingly employ calprotectin (CP) as well-studied (systemic and faecal) inflammatory biomarker due to its stability, assay reproducibility and low cost to guide diagnostic and therapeutic decisions.^{16–17} In contrast, the biological functions of CP in health and unresolved inflammation are poorly appreciated by most clinicians. In this review, we focus on emerging biological functions and clinical applications of CP in inflammatory diseases to facilitate interpretation in daily practice.

CP IN HEALTH

CP belongs to the family of calcium-binding S100 leucocyte proteins (with more than 24 members in vertebrates) that is evolutionary conserved and composed of two monomers in mammals (e.g. humans and mice): S100A8 and S100A9.^{18–19}

CP was first described in the 1980s and the protein complex was discovered independently in different inflammatory conditions, which was resolved in 1988.²⁰ Since then, the complex was termed calprotectin, emphasising the characteristic to bind Ca^{2+} and the antimycotic activity against *Candida albicans*.²¹

Cell-specificity, transcriptional regulation and assembly

CP is an abundant cytosolic protein complex (comprising S100A8 and S100A9) that is constitutively expressed in neutrophils, which represents ~45% of total cytosolic protein.²² The S100A8 and S100A9 genes are located on chromosome 1 (q21) in humans²³ and on chromosome 3 in mice.²⁴ CP is also constitutively expressed by monocytes,²² dendritic cells,²⁵ activated macrophages,²⁶ oral keratinocytes²⁷ and squamous mucosal epithelium.²⁸ Moreover, expression can be induced specifically during inflammation.^{29–30} As such, the expression of CP in health is restricted to a very limited amount of specialised cells and may be induced during inflammation.^{31–33}

Several cellular pathways and related transcription factors have been shown to (positively or negatively) control the expression of S100A8 and S100A9 in humans and/or mice (SPI/PU.1, SATB1, C/EBP α/β , HIF-1, Arnt, GLI1, BRCA1 and AP-1).³⁴ Conceptually, bacterial antigens and inflammatory mediators evoke expression of S100A8 and S100A9 by these transcription factors, as exemplified by the induction of CP expression with lipopolysaccharides (LPS), tumour necrosis factor- α (TNF- α) and interleukin 1- β (IL-1 β) in human monocytes.³⁵ Similarly, inflammation drives CP expression in human keratinocytes.³⁶ However, also *bona-fide* anti-inflammatory mediators, such as IL-10 facilitate S100A9 expression in myeloid-derived cells.³⁷ Moreover, eosinophils may be a source of CP during gut injury and inflammation in mice.³⁸ Notably, also nutritional deficiency and drugs may affect CP concentrations, as lack of zinc is described to increase CP levels and glucocorticoids positively regulate S100A8 expression.^{39–40}

S100A8 and S100A9 monomers are able to form heterodimers and tetramers in a Ca^{2+} -dependent manner.⁴¹ In 2007, the crystal structure of the Ca^{2+} -bound CP heterotetramer was resolved, showing transition metal-binding sites in each heterodimer.⁴² These sites allow binding of multiple divalent (first-row) metal ions in case of high concentrations (ie, Ca^{2+} , Mn^{2+} , Fe^{2+} , Ni^{2+} and Zn^{2+}).⁴³ In humans, S100A8 is composed of 93 amino acids (molecular weight 10.8 kDa), while S100A9 is composed of 113 amino acids (molecular weight 13.2 kDa).⁴⁴ S100A8 and S100A9 consist of two α -helix ('EF hand helix-loop-helix') motives

typically allowing Ca^{2+} -binding and CP complex formation.^{45–46} Upon Ca^{2+} binding, two heterodimers are able to self-associate to form a (S100A8/S100A9)₂ heterotetramer.⁴¹ Ca^{2+} -dependent formation of (S100A8/S100A9)₂ tetramers is considered to be fundamental for intracellular and extracellular biological function.^{45–47} This may be explained by the observation that Ca^{2+} binding and tetramerisation promotes resistance against proteases and increases binding affinities.^{48–49} The mechanism underlying the release of CP into the extracellular space may not require the classical endoplasmic reticulum-Golgi pathway. One study suggested that CP could be released via a novel tubulin-dependent mechanism of leucocyte activation, which is controlled by protein kinase C.⁵⁰ Notably, both monomers are vulnerable to oxidation on methionine and cysteine residues although functional consequences are poorly understood.⁵¹

Intracellular biological functions of CP

The S100A8/S100A9 complex controls intracellular pathways of innate immune cells and allows orchestration of an inflammatory response.¹⁸ CP modulates cytoskeletal rearrangements to allow leucocyte recruitment,⁴⁷ and facilitates the transport of arachidonic acid to sites of inflammation.⁵² Further, nuclear S100A9/CP modifies transcription as coactivator during inflammatory processes and malignant transformations.^{53–55}

Targeted genetic deletion indicated that mouse S100 proteins are required for transendothelial migration of phagocytes, likely by organisation of cytoskeletal metabolism and rearrangement.⁴⁷ For example, microtubule polymerisation and reorganisation (which controls leucocyte migration⁵⁶) requires the formation of the S100A8/S100A9 complex,⁴⁵ which is modulated by mitogen-activated protein kinase (MAPK)-mediated S100A9 phosphorylation⁴⁷ and depends on Ca^{2+} concentration.^{45,57} In line with this, S100A9-deficient granulocytes and mice exhibit poor neutrophil recruitment during inflammation in wound healing.⁴⁷ Rapid leucocyte recruitment from blood to inflammatory loci relies on a cascade of adhesion events that are triggered by selectins and β 2 integrins (CD11b/CD18).^{58–59} S100A8 and S100A9 control neutrophil adhesion to fibrinogen through the activation of the β 2 integrin Mac-1 (CD11b/CD18).⁶⁰ Similarly, S100A8/S100A9 influences transendothelial migration of monocytes via increased CD11b expression.⁶¹ These findings indicate that S100A9 is a regulatory subunit of the functional S100A8/S100A9 complex, which facilitates leucocyte trafficking.⁴⁷

In 1997, CP was identified as a fatty acid-binding protein.⁵² Another study indicated that the S100A8/A9 complex is the main arachidonic acid-binding protein in human neutrophils which is Ca^{2+} -dependent⁶² and appears unique to this specific S100 protein.⁶³ Arachidonic acid is a potent inflammatory lipid mediator as it is essential for the synthesis of leukotriene B4 that is described to favour inflammation and tissue damage during IBD.⁶⁴ Generally, polyunsaturated fatty acids modulate immune responses in various ways. For example, arachidonic acid derivatives fuel an inflammatory metabolite profile of innate immune cells and facilitate reactive oxygen species (ROS) production of neutrophils and potentially induce cell death.⁶⁵ In the context of IBD, arachidonic acid (and polyunsaturated fatty acids in general) induced production of chemokines from intestinal epithelial cells and evoked gut inflammation in genetically susceptible mice.⁹

It may be speculated that CP transports polyunsaturated fatty acids to inflammatory loci to fuel local immune responses.⁶⁶

Additionally, nuclear S100A9/CP was reported to have a possible transcription coactivator function. During sepsis,

S100A9 was described to migrate from the cytosol to the nucleus in distinct myeloid-derived suppressor cells to enhance the expression of immunosuppressive mediators.⁵³ Nuclear S100A8/A9 may trigger oncogenic pathways and amplify transformation in breast cancer.⁵⁴ We demonstrated a relationship between the S100A8/S100A9 protein complex and complement factor C3 expression, mediating disease course in a psoriasis mouse model, as S100A9 deficiency in mice was associated with weakened psoriasis-like disease and decreased amounts of C3. S100A9 was detected in the chromatin enriched fraction in keratinocytes, modulating C3 transcription most likely through chromatin remodelling.⁵⁵ Whether nuclear CP is important also in the gut and beyond remains to be demonstrated.

Extracellular biological functions of CP

The S100A8/S100A9 complex is readily secreted to allow extracellular CP functions mediated by Toll-like receptor 4 (TLR4) and receptor for advanced glycation end products (RAGE).⁶⁷ However, extracellular CP can form complex protein configurations with distinct biological functions and equivalent receptors, which may not be explained by these signalling pathways. For example, CP may interact with the cluster of differentiation 36 (CD36) receptor during formation with polyunsaturated fatty acids.⁶⁸ Likewise, dysregulated primary bone marrow expansion can be mediated by S100A9-induced CD33 signaling,⁶⁹ and S100A9 regulates TLR2/3 cascades.^{70–71} Thus, the S100A8/A9 complex activates innate immune responses and promotes inflammation likely by involvement of several receptors in a cell-type and tissue specific manner.

For example, CP is described to trigger neutrophil chemotaxis and endothelial adhesion. The injection of S100A8, S100A9, or S100A8/A9 into a mouse air pouch model caused fast accumulation of neutrophils, indicating that CP promotes neutrophilic inflammation.⁷² Moreover, CP exerts antimicrobial activity which has been extensively studied. Extracellular CP complexes allow chelation of diverse transition metal ions (see above),⁴³ which are crucial for invasive and commensal gut bacteria, as they allow bacterial enzymatic functions, cellular homeostasis and signalling cascades.^{73–74} Numerous microbes gained the ability to overcome and escape CP-induced metal starvation through the expression of high-affinity metal transporters or metabolic alterations.^{75–76} Nevertheless, S100A8/S100A9 deficiency in mice alters the intestinal microbiota,⁷⁷ and mice lacking S100A9 show susceptibility to *Streptococcus pneumoniae* infection.⁷⁸

CP also promotes the expression of pro-inflammatory and anti-inflammatory mediators. Human monocytes stimulated with S100A9 secreted IL-1 β , IL-6 and TNF- α in association with oxidative stress,^{79–80} which was also observed in gingival fibroblasts.⁸¹ In human neutrophils, it appears that S100A9 promotes cytokine expression.^{82–84} CP fuels IL-1 β secretion induced by crystals in gout which is initiated by TLR4 activation⁸⁵ and overexpression of S100A8 and S100A9 in macrophages induced expression of anti-inflammatory IL-10.⁸⁶ In turn, an inhibition of pro-inflammatory signalling by myeloid cells is reported after S100A9 blocking.⁸³ In mice with acute pancreatitis S100A9 gene silencing is associated with decreased release of pro-inflammatory cytokines.⁸⁴

Besides a role for CP in orchestrating an acute inflammatory milieu, CP also controls cell proliferation, differentiation and apoptosis.^{87–89} Several studies revealed that CP, especially S100A9, modulates proliferation of tumour, epithelial and smooth muscle cells, while CP concentrations may differ significantly.^{90–93} CP-induced proliferation was shown to be mediated

via RAGE ligation and NF- κ B activation in tumour cells, linking inflammation with tumorigenesis.⁹¹ S100A9 may also bind to TLR4 to promote MAPK signalling and monocytic cell differentiation.⁸⁹ Additionally, CP plays a role in regulatory T-cell (Treg) differentiation⁹⁴ that exert immunosuppressive effects and maintain self-tolerance.⁹⁵ CP is also described to activate natural killer (NK) cells and enhance interferon-gamma (IFN- γ) expression via RAGE signalling, linking inflammation with NK cell responses.⁹⁶ Distinct S100A8 and S100A9 concentrations were reported to inhibit the growth of murine embryonic and human dermal fibroblasts⁹⁷ and induce apoptosis of tumour cells.^{88–97} In human epidermal keratinocytes CP confers a survival signal at lower concentrations, while higher (μ M) concentrations evoked apoptosis.⁹⁸

Interestingly, a lack of S100A8/A9 in epidermal keratinocytes is associated with enhanced susceptibility for papillomas and squamous cell carcinomas. In the skin of S100A9-deficient mice elevated levels of Ki-67 were detectable during the formation of papillomas highlighting a potential regulatory function of S100A8/A9 during epidermal proliferation.⁹⁹ An additional study revealed that during inflammation and malignant transformation in the skin RAGE expression on immune cells mediates S100/RAGE-driven signalling cascades, while expression on keratinocytes or endothelial cells is not required.¹⁰⁰ We speculate that the function of each CP component depends on the expression level in specific cell types and tissues.

Collectively, these studies demonstrate that CP may shape the cellular and molecular inflammatory niche at a site of inflammation. However, CP has also been reported to maintain a chemo-repulsive effect on peripheral leukocytes i.e. movement of leukocytes away from CP. This chemorepulsive effect was reversed by oxidation of methionine at position 63 and 83 in S100A9. It may be speculated that CP function is adjusted during oxidative i.e. inflammatory conditions.¹⁰¹

CP IN UNRESOLVED INFLAMMATION AT MUCOSAL SURFACES

CP may initially be released by myeloid cells upon danger signalling,²⁹ while tissue inflammation perpetuates the release of S100A8 and S100A9 by transcriptional induction in epithelial cells.^{32–33} The initial culprit for an inflammatory response may be an infection at the mucosal surface, a trauma or environmental stress. During unresolved inflammation, however, CP contributes to mucosal injury, inflammation and disease, for example in the skin, lung and gut.^{55–100–102–105}

In epidermal keratinocytes S100A8 and S100A9 promoted chemotaxis and psoriasis in mice.^{55–106} Similarly, S100A8/A9 induction during inflammatory skin disease is associated with enhanced tissue damage, reduced skin integrity and increased pro-inflammatory pathways.^{104–106–107} Lung injury induced by influenza virus infection is partially mediated by S100A9-driven lung inflammation.¹⁰³ Additionally, S100A8/A9 was shown to play a major role during tissue damage in tuberculosis.¹⁰² Vice versa, anti-S100A8 and anti-S100A9 antibodies impair migration of phagocytes to alveoli in mice by \sim 75% in a *S. pneumoniae* model.¹⁰⁸ In gout, S100A8 and S100A9 drive neutrophil migration initiated by monosodium urate crystals and the production of IL-1 β fuelling joint inflammation.^{85–109} In gut inflammation, the biological function of CP is poorly understood. As inferred by experiments with pharmacological inhibition of S100A9 by antibody treatment, CP appears to drive dextran sodium sulfate (DSS)-induced colitis and inflammation-associated gut tumorigenesis in mice.¹⁰⁵ In conflict with these observations,

CP treatment protected against experimental colitis in mice induced by DSS.¹¹⁰ Collectively, various studies indicate that S100A9 promotes tissue inflammation at mammalian inner and outer mucosal surfaces. Recent studies indicate that CP drives inflammation beyond mucosal surfaces. For example, S100A8 and S100A9 modulate the tumour microenvironment of a broad spectrum of tumours.^{111–117} S100A8/A9 triggers tumorigenesis by RAGE mediated NF- κ B activation at low concentration⁹¹ and mice that lack S100A9 were protected against gut tumorigenesis and inflammation.¹¹⁸

Most reports do not explore functional variability among the CP subunits. S100A8 and S100A9 are considered to function mainly synergistically. Especially, S100A8 was described to rely on S100A9, as *in vivo* studies implicated that S100A8 is unstable without S100A9.¹¹⁹ For a long period, S100A9 knockout mice were described to lack S100A8 protein as well. However, constitutively active S100A8 homodimers were reported to be present during TNF- α signalling in the absence of S100A9.¹²⁰ Interestingly, mice lacking S100A9 are viable and healthy,^{119,121} while the disruption of S100A8 in mice is associated with lethality during embryo development.¹²² This outcome revealed a previously unknown function of S100A8 in early embryonic preimplantation phase and S100A8 was shown to be important for placenta maturation.¹²³ For more than two decades the disruption of the S100A8 gene in mice was reported to be lethal during embryogenesis. However, recently Cesaro *et al* published the first viable and fertile S100A8-deficient mice.^{124,125} Future studies with conditional S100A8 alleles will be essential to explain this discrepancy. Collectively, the biological function of the S100A8 subunit may be opposed to that of S100A9 in tissue inflammation. For example, S100A8 diminished mast cell degranulation, the expression of pro-inflammatory mediators and eosinophil infiltration during acute asthma in mice.¹²⁶

Interestingly, distinct CP subunit configurations were shown to trigger different cellular pathways especially in the context of inflammation and tumorigenesis.^{120,127,128} S100A8 and S100A9 usually organise as heterodimeric complexes that form heterotetramers in case of calcium availability.⁴¹ Recently, homodimeric complexes were reported to play a crucial role in various inflammatory conditions. Active S100A9 homodimers were found in the spleen of mice with present inflammation or after tumour inoculation.¹²⁷ It might be speculated that distinct CP subunit configurations may be responsible for the amplification and maintenance of inflammatory processes.^{120,128} Recently, Vogl *et al* revealed that tetramer formation may limit local inflammatory properties of both subunits, as S100A8/S100A9 is only transiently active in the extracellular environment, since calcium-dependent tetramerisation of S100A8/S100A9 may lead to an autoinhibition of both subunits. In contrast, homodimers were shown to constitutively trigger inflammatory pathways through TLR4 activation.¹²⁰ However, the exact molecular functions of the individual protein configurations remain unclear.

FAECAL CP IN HEALTH AND DISEASE

Faecal CP concentrations found in healthy individuals somewhat range between ~10 and 50 μ g/g stool which depends on study cohorts and the used assay.^{129–131}

However, various studies proved that faecal CP concentrations are physiologically elevated in infants younger than 4 years when compared with adults.¹³² A recent study reported that faecal concentration of S100A8 and S100A9 are physiologically higher in healthy term neonates after vaginal delivery compared with caesarean section. Labor-associated stress was determined

as the decisive factor leading to elevated CP concentration after secondary caesarean section and vaginal delivery.⁷⁷ Additionally, high levels of S100A8 and S100A9 are contained in breast milk, indicating a role for CP in shaping the immune system of newborns.¹³³ Indeed, Willers *et al* demonstrated that S100A8 and S100A9 regulate programming of intestinal immunity and high faecal CP levels are associated with intestinal colonisation by a favourable microbiota in neonates.⁷⁷ As a result, CP concentrations in paediatric patients should be interpreted with caution.¹³⁴

Faecal CP as biomarker of inflammatory diseases in the gut

Before the possibility to detect CP in the stool in 1992,¹³⁵ clinicians relied on serological markers to assess the possibility (or severity) of gut inflammation. However, erythrocyte sedimentation rate and serum C-reactive protein (CRP) are elevated in response to various non-inflammatory processes and poorly correlate with patient symptoms and intestinal disease activity.¹³⁶ In contrast, faecal CP is able to discriminate between non-inflammatory and inflammatory disease of the intestine,^{13,14} can be retrieved non-invasively, is inexpensive and remains stable at room temperature in stool for at least 3 days (with 30% inadequacy after 7 days).¹³⁷ Notably, studies indicated that faecal CP represents a more sensitive marker than CRP in the context of IBDs, while it remains unclear if the combination of these biomarkers improves diagnostic accuracy.^{138,139} These features make faecal CP an excellent (i.e. sensitive) biomarker to detect gut inflammation in IBD—which may be some reasons for its worldwide use today. In contrast, specificity of this biomarker is relatively low, opening a range of differential diagnoses.

Considering the plethora of biological functions of CP in health and disease, high sensitivity but low specificity of faecal CP to detect gut inflammation may not be surprising. Faecal CP correlates with the number of neutrophils present in the intestinal lumen and thus allows to detect an acute inflammatory response in the gut,^{140,141} but faecal CP does not allow to discriminate distinct aetiologies. For example, human faecal CP concentrations are substantially elevated during *Salmonella* infection (median of 765 μ g/g), *Campylobacter* infection (median of 689 μ g/g) or *Clostridioides difficile* infection (median of 740 μ g/g) and correlate with disease severity.^{142–144} In contrast, viral infections for example, rotavirus or norovirus are usually present with lower (but elevated) concentrations (~90 μ g/g) when compared with healthy controls. Notably, most data on faecal CP levels during GI infections are based on paediatric patients.¹⁴² Likewise, elevated faecal CP concentrations were reported for HIV infection (regardless of antiretroviral therapy status)¹⁴⁵ and corona virus disease 2019 induced by SARS-CoV2 infection.¹⁴⁶ Further, also intestinal malignancies are associated with increased faecal CP concentration as observed for example in colorectal cancer, probably because of a local inflammatory response.¹⁴⁷ Typically, chronic inflammatory diseases of the gut also demonstrate increased faecal CP concentrations, partly because neutrophilic inflammation is an aspect of the disease¹⁴¹ and partly because gut inflammation may induce intestinal epithelial CP expression (figure 1).^{32,33,148} Faecal CP is elevated (and correlates with disease activity) in IBD,^{149–153} in necrotising enterocolitis,¹⁵⁴ graft-versus-host disease,¹⁵⁵ and drug-induced enteropathy (e.g. non-steroidal anti-inflammatory drugs (NSAIDs)).¹⁵⁶ Diagnostic precision to detect gut inflammation in the lower GI tract is better when compared with the upper GI tract.¹⁵⁷ A study which included patients with IBD (and patients with

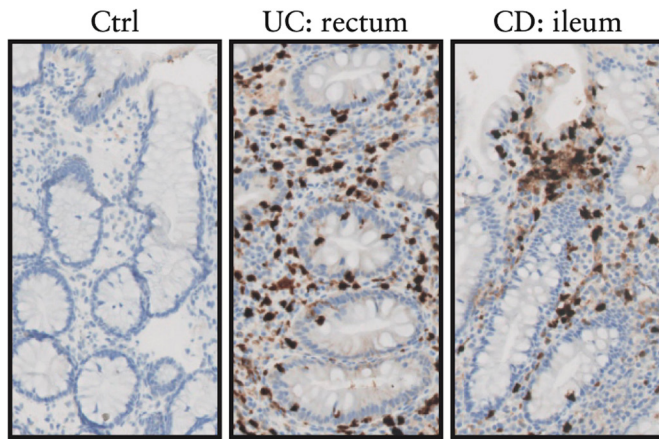


Figure 1 Calprotectin expression in the gut, adapted from reference 148. Mucosal calprotectin expression indicated by brown immunohistochemical staining is visualised in lamina propria immune cells and to a lesser extent the intestinal epithelium in ulcerative colitis (UC) and Crohn's disease (CD), but not healthy controls (Ctrl).

GI malignancies) undergoing colonoscopy indicated that bowel preparation and upper or lower endoscopy did not affect faecal CP concentration after the procedure.¹⁵⁸

Collectively, faecal CP can be used to virtually exclude a wide range of intestinal diseases characterised by gut inflammation, while it does not allow to discriminate potential triggers. As such, other causes of elevated faecal CP concentration must be ruled out prior to IBD diagnosis. Strongly elevated faecal CP concentration is frequently observed during bacterial infections. Interpretation of slightly elevated faecal CP concentrations should be made with care, as viral infections and drugs (e.g. NSAIDs,¹⁵⁶ proton pump inhibitors,¹⁵⁹ glucocorticoids,⁴⁰ and levodopa¹⁶⁰) may induce S100A8 and/or S100A9 expression, and GI bleeding is associated with moderately elevated CP levels (i.e. 50–200 µg/g).¹⁶¹

Faecal CP discriminates between inflammatory and non-inflammatory gut disease

Although faecal CP values >600 µg/g are strongly associated with IBD (or food-borne infections), no consistent CP cut-off is established that would allow to diagnose IBD with high accuracy.¹⁶² Thus, clinicians rely on probabilities to rule in or to rule out IBD (or functional gut diseases) based on faecal CP concentration. The threshold of what is considered an 'elevated' faecal CP (or vice versa what is considered healthy) is still debated. As already mentioned earlier, faecal CP concentrations found in healthy individuals mainly range between ~10–50 µg/g stool, which depends on study cohorts and the used assay.^{129–131} A meta-analysis including 12 studies (comprising 491 healthy controls, 595 patients with IBS and 1059 patients with IBD) generally indicated that faecal CP concentrations of ≤40 µg/g (using sensitive assays) rule out IBD (i.e. provides ≤1% probability of having IBD).¹⁴ Several studies implicated that faecal CP allowed differentiation between non-IBD and IBD at a cut-off between 100–200 µg/g.^{14 149 163 164} However, an exact faecal CP cut-off value for the discrimination between IBD and functional bowel diseases is not established.¹¹ Clinical decision-making to discriminate inflammatory from non-inflammatory disease with relative accuracy and the need for endoscopy is indicated in figure 2A.

Faecal CP allows evaluation of IBD course

Most clinical studies indicate a strong correlation between faecal CP concentration and clinical or endoscopic disease activity.^{150 152 165 166} For example, one study associated faecal CP concentrations, i.e. grade 0 (≤16 (10–30) µg/g), grade 1 (~35 (25–48) µg/g), grade 2 (~102 (44–159) µg/g), grade 3 (~235 (176–319) µg/g) and grade 4 (~611 (406–868) µg/g), with endoscopic UC activity.¹⁶⁷ Zollner *et al* compared clinical and endoscopic activity-related correlations between faecal CP and faecal lipocalin-2 in a cohort of 132 patients (72 patients with CD, 40 controls) and confirmed the diagnostic equivalence of both biomarkers in IBD. Analysis by confocal microscopy and immunocytochemistry implicated strong expression of CP in granulocytes, macrophages and to a lesser extent the intestinal epithelium in patients with IBD (see also figure 1).¹⁴⁸ Data generally indicate that faecal CP is significantly decreased in patients with clinical and endoscopic remission and that values of >150 µg/g are mainly associated with disease relapse.^{168–170} However, other studies reported higher cut-off values of >200–300 µg/g to implicate disease relapse.^{171–173} Recently published Selecting Therapeutic Targets in Inflammatory Bowel Disease (STRIDE-II) recommendations by the International Organization for the Study of Inflammatory Bowel Diseases (IOIBD) announced faecal CP values of <150 µg/g as goal reflecting remission, stating that faecal CP values ranging from 150 to 250 µg/g are a grey zone.¹⁷⁴ In contrast, a patient population from China demonstrates a cut-off of 50 µg/g to be suitable for the differentiation between active and inactive UC,¹⁵³ such that studies suggest different cut-off values for active disease which may be explained by the definition of remission, assay variability and ethnic differences of faecal CP concentration.^{129–131} Prior to clinically based diagnosis of a flare, faecal CP is reported to be elevated ~8 weeks in advance. In contrast, patients who maintain remission usually present faecal CP concentrations of <60 µg/g.^{175 176} Notably, Theede *et al* reported that faecal CP evaluation is sufficient to determine risk of relapse 6 and 12 months in advance and to evaluate mucosal healing in UC.¹⁷⁷ Based on a plethora of studies, clinical decision-making in IBD is indicated in figure 2B. Faecal CP is also used as biomarker to identify postoperative disease recurrence in adult¹⁷⁸ and paediatric patients with IBD.¹⁷⁹ A meta-analysis including nine studies indicated that a faecal CP cut-off of >150 µg/g is likely to have the best overall accuracy to predict postoperative endoscopic recurrence with a sensitivity of ~70%.¹⁸⁰ Notably, current recommendations suggest endoscopy (rather than sole biomarker screening) to detect postoperative disease recurrence. Clinical trials are necessary to establish faecal CP (rather than endoscopy) to guide therapeutic decisions and to establish treatment algorithms postoperatively. Only then, clinical guidelines may recommend specific faecal CP concentrations to indicate disease activity and the requirement for therapeutic intervention, which is currently being avoided.^{181 182}

SERUM CP AS BIOMARKER OF INFLAMMATORY DISEASES IN AND BEYOND THE GUT

Recently, serum CP has gained attention as a potential biomarker for the evaluation of IBD and other inflammatory diseases with heterogeneous results. For example, one study comprising 156 patients (82 IBD and 74 non-IBD) suggested that serum CP may represent a promising marker for the assessment of inflammatory status and disease course¹⁸³ and further studies confirmed the observation in adult and paediatric patients with IBD.^{184 185}

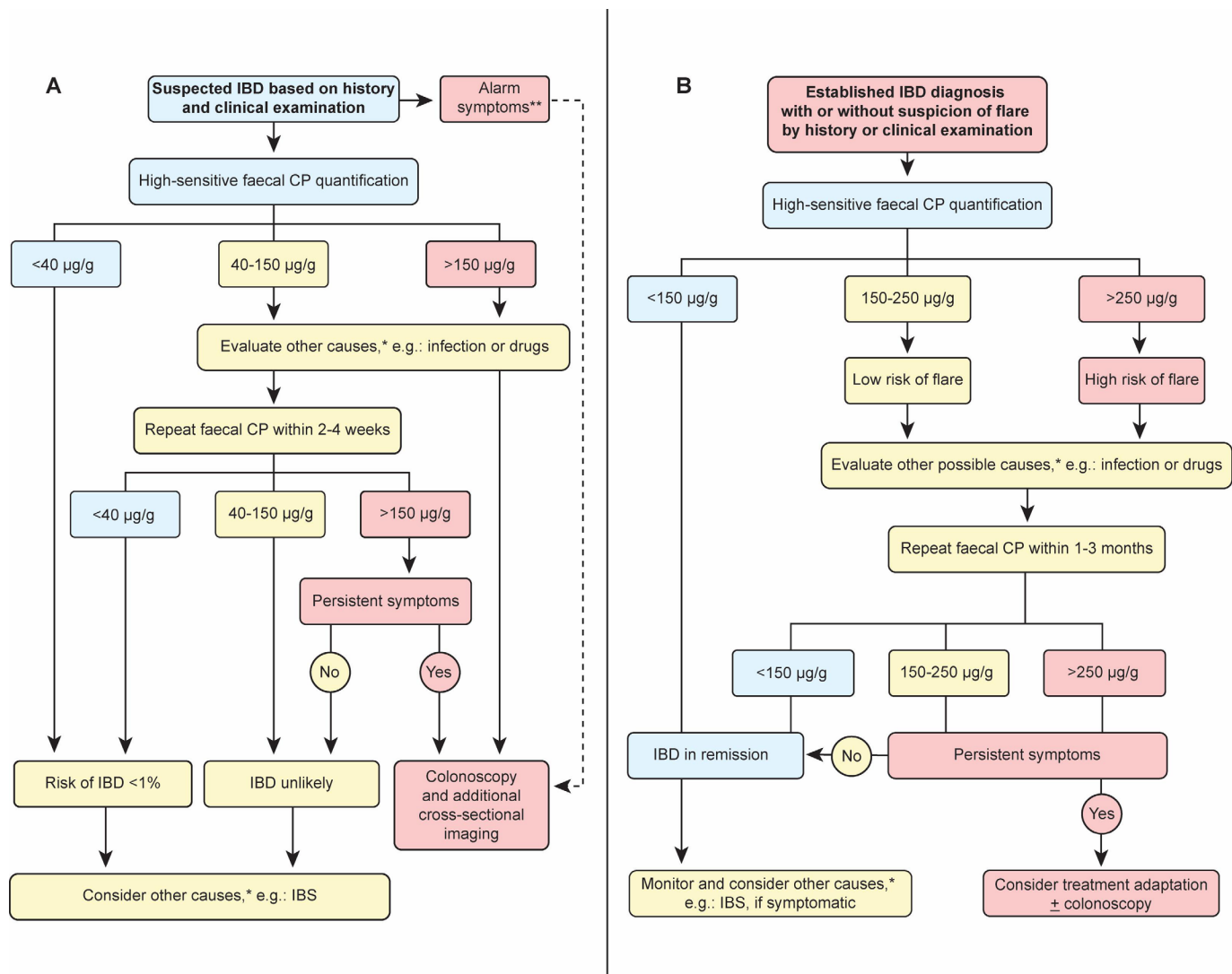


Figure 2 Proposed algorithm for the interpretation of faecal calprotectin (CP) concentration. No evidence-based algorithm has been established by controlled clinical trials. This algorithm is based on literature research and clinical experience. Note that faecal CP concentration reflects only one aspect of disease evaluation and should always be interpreted in conjunction with history and clinical and/or endoscopic examination. Panel A: In suspected inflammatory bowel diseases (IBD) (e.g. GI symptoms such as diarrhoea, bleeding or abdominal pain >4 weeks), faecal CP and a stool (pathogen) culture should be obtained. Repeated elevated faecal CP concentration (>40 µg/g) or persistent symptoms require further investigations. Faecal CP is also elevated during gastroenteritis (viral and bacterial), diverticular disease, drug-induced enteropathy and intestinal malignancies. IBS may also present with elevated CP but its probability decreases with higher concentrations (>150 µg/g). Panel B: In patients with an established diagnosis of IBD, faecal CP supports the diagnosis of active disease or remission. Faecal CP concentration <150 µg/g usually indicates remission with high accuracy. Elevated faecal CP concentration (>150 µg/g) raises suspicion for disease activity as elevated CP concentration is associated with increased risk of a clinically apparent disease flare in the following months. Based on the recently published Selecting Therapeutic Targets in Inflammatory Bowel Disease-II (STRIDE-II) recommendations values of 150–250 µg/g are considered as grey zone.¹⁷⁴ In symptomatic patients with IBD, other causes for elevated faecal CP should be excluded (such as infection and drug-induced enteropathy) and endoscopy should be performed before switching medical therapy. *In immunocompromised patients, serological testing for cytomegalovirus (CMV), HIV and herpes simplex virus (HSV) may be obtained and history should exclude drug-induced enteropathy associated with non-steroidal anti-inflammatory drugs, proton-pump inhibitors, sartans, levodopa and rarely other drugs. **Alarm symptoms are defined by unintentional weight loss, night sweat, bloody diarrhoea and/or vomiting indicating upper and/or lower endoscopy.

Likewise, several inflammatory diseases beyond the gut display elevated serum and/or tissue CP concentrations, as observed in psoriasis,¹⁸⁶ rheumatoid arthritis,¹⁸⁷ systemic lupus erythematosus,¹⁸⁸ ankylosing spondylarthritis,¹⁸⁹ periodontitis,¹⁹⁰ and human malignancies (e.g. myelodysplastic syndrome,¹¹¹ head and neck squamous cell carcinoma,¹⁹¹ bladder cancer,¹¹² non-small cell lung cancer,¹¹³ breast cancer,¹¹⁴ pancreatic cancer,¹¹⁵ prostate cancer¹¹⁶ and hepatocellular carcinoma¹¹⁷). Interestingly, systemic inflammation is detectable through serum CP in

the majority of patients with psoriatic disease especially with joint manifestation, even in case of low CRP levels, highlighting high diagnostic sensitivity of CP.¹⁹² However, systemic low grade inflammation, as observed in type II diabetes and obesity, is also linked with elevated blood CP concentration,^{193–195} which could be a significant confounder in CP studies beyond metabolic diseases.

Recently, plasma and serum CP concentrations were implicated as valuable prognostic biomarkers for the assessment

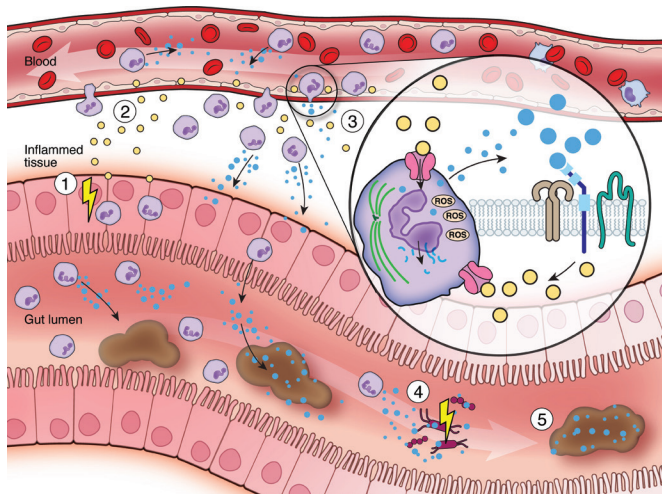


Figure 3 Calprotectin in the control of gut inflammation. (1) Inflammatory bowel diseases arise from a disrupted host-microbe interplay resulting in chronic remittent gut inflammation. (2) Tissue inflammation is driven by cytokines (yellow) activating innate and adaptive immunity. (3) Acute inflammation leads to the mucosal recruitment of neutrophil granulocytes (violet) which partly migrate into the gut lumen. Neutrophils constitutively express and release calprotectin (blue) which may fuel mucosal inflammation, as calprotectin drives neutrophil chemotaxis, induces the expression of endothelial adhesion molecules and activates pattern recognition receptors (e.g. Toll-like receptor 4 or receptor for advanced glycation end products) on innate and adaptive immune cells. Calprotectin in neutrophils modulates tissue adherence by microtubule rearrangements (green) and cytotoxicity by generation of reactive oxygen species via NADPH oxidase. (4) Calprotectin also harbours antimicrobial functions. Calprotectin allows chelation of essential divalent metal ions (e.g. calcium, iron or zinc) limiting growth of invasive and commensal gut bacteria. (5) Calprotectin represents a well-studied non-invasive (systemic and faecal) inflammatory biomarker in inflammatory bowel diseases due to its stability at room temperature, assay reproducibility and low costs. However, faecal calprotectin is also elevated during gut infection, other gastrointestinal diseases and in drug-induced enteropathy. ROS, reactive oxygen species.

of disease course and outcome in hospitalised patients with COVID-19, reflecting current and future disease severity.¹⁹⁶ In *Klebsiella pneumoniae* sepsis patients displayed increased serum CP concentrations which predicted 28-day mortality.¹⁹⁷ Collectively, these studies indicate the potential of CP to guide management (and potentially therapy) in many clinical scenarios, a concept that warrants disease-specific trials.

CONCLUSION

Major advances over the last decade have fostered our understanding of the molecular functions of CP in mammals. Translation of biological insights by mechanistic studies of mucosal surfaces in mice to human disease is scarce, but would evolve our understanding of CP as inflammatory biomarker. Both subunits, S100A8 and S100A9, harbour a broad spectrum of intracellular and extracellular immunomodulatory properties (figure 3).^{18 47 67 72} However, it is still debated whether the biological function of the S100A8 subunit may be opposed to that of S100A9 in tissue inflammation and tumorigenesis. Analysis of tissue-specific knock-out mice of either subunit and/or blocking distinct binding sites will help to delineate divergent intracellular or extracellular biological functions. Considering

the transcriptional regulation and biological functions of CP during inflammation, clinicians should be aware that other conditions, most commonly GI (bacterial or viral) infections, but also malignancies, drugs and graft-versus-host disease, are paralleled by increased faecal CP concentrations. Faecal CP represents an extensively validated biomarker for the diagnosis and longitudinal evaluation of IBD reflecting endoscopic disease activity reasonably well. Lack of guidelines and data regarding optimal faecal CP cut-offs renders intermediate faecal CP concentrations of 150–250 µg/g (declared as grey zone by STRIDE-II recommendations¹⁷⁴) frequently challenging to interpret in IBD, while <40 µg/g rules out IBD and >250 µg/g should prompt evaluation for IBD or raise suspicion for an IBD flare (figure 2).^{14 149 167} We propose that a better understanding of the biological functions of CP and particularly the S100A8 and S100A9 subunits will lead to novel diagnostic and therapeutic advances in humans in the future.

Contributors All authors contributed equally.

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