



(REVIEW ARTICLE)



Analytical characteristics of faecal and serum calprotectin or calprotectin assay: What clinical interest?

Raihane Bahri ^{1,2,*}, Fadoua Elfarssani ^{1,2}, Siham Khayati ^{1,2}, Saida Eddyb ^{1,2}, Hajar Saffour ^{1,2}, Abderrahman Boukhira ^{1,2} and Saliha Chellak ^{1,2}

¹ Department of Biochemistry-Toxicology, Avicenne military hospital in Marrakech, Marrakech 40000, Morocco.

² Faculty of medicine and pharmacy of Marrakesh, Morocco.

GSC Advanced Research and Reviews, 2021, 06(03), 156–163

Publication history: Received on 12 February 2021; revised on 08 March 2021; accepted on 10 March 2021

Article DOI: <https://doi.org/10.30574/gscarr.2021.6.3.0052>

Abstract

Calprotectin is a calcium binding protein expressed at the inflammatory site by neutrophils and monocytes to activate the innate immune system. Thanks to this unique characteristic, it is a good indicator of local inflammation in chronic inflammatory rheumatism.

All of the data highlight the key role that calprotectin plays in the inflammatory process in several inflammatory rheumatism. The value of serum and faecal calprotectin assays has been widely studied in recent years, in particular in rheumatoid arthritis, spondyloarthritis, juvenile idiopathic arthritis and vasculitis associated with ANCA but also for chronic inflammatory bowel disease. calprotectin has been identified as a powerful biomarker that allows the evaluation and monitoring of the activity of disease but also to predict structural evolution or response to treatment. Calprotectin is also a predictive biomarker of risk relapse. In this review, we discuss the value of calprotectin in chronic inflammatory affections as a diagnostic, potency or prognostic biomarker.

Keywords: Calprotectin; IBD; rheumatoid arthritis; ELISA

1. Introduction

Polynuclear neutrophils (PNN) and monocyte / macrophage are the first cells to be recruited to the site of inflammation. Their recruitment is active, and is regulated by many factors such as adhesion molecules (selectin, integrin and their ligands) and cell activating factors (chemokines, cytokines and their receptors).

Calprotectin plays an important role during the inflammatory response and is a non-injurious biomarker

Calprotectin is mainly expressed in cells of myeloid origin, namely neutrophils and monocytes (40% and 5% of the protein cytosolic content, respectively), as well as in the early stages of differentiation of macrophages. Keratinocytes and epithelial cells can also express it in an inflammatory environment.

1.1. History

1980: 1st description in the literature Calcium binding leucocyte, L1 protein then variable names: MRP-8/14; calgranulin A / B; S100A8 / S100A9 protein. 2000: optimization of the dosing method. The normality cut-off goes from 10 mg / kg to 50 µg / g 2010: 1st meta-analysis validating the use of calprotectin in the differential diagnosis between IBD and IBS.

* Corresponding author: Raihane Bahri

Department of Biochemistry-Toxicology, Avicenne military hospital in Marrakech, Marrakech 40000, Morocco.

1.2. Physiopathological data

1.2.1. Genetics and expression

Twenty-one members of the S100 family have been described to date and the genes encoding 16 of them are located on a cluster located on chromosome 1q21. This cluster comprises in particular the genes for the S100A8 and S100A9 proteins. The organization of this locus is remarkably conserved during evolution, this organization being able to allow the coordinated expression of these genes.

The S100A8 and S100A9 genes are both located on chromosome 1 at 1q21.3 and each have 3 exons separated by 2 introns, like most genes of the S100 family. Exon 1 encodes the untranslated 5' part, exons 2 and 3 encode the N-terminal portion and C-terminal EF motifs, respectively. Currently, no pseudogenes have been described for S100A8 or S100A9. The coding sequence of S100A8 comprises 279 nucleotides, encoding a 93 aa protein. The cDNA of the S100A9 gene corresponds to a reading frame of 352 nucleotides and codes for a protein of 114 aa. Several regulatory regions have been identified upstream of the coding region of these two genes, the transcription factors and regulatory mechanisms still remain relatively poorly understood.

Transcription factors such as PU.1, C/EBP α and C/EBP β appear to regulate the expression of these genes in the myeloid lineage. The S100A9 gene has regulatory elements specific to the myeloid and epithelial lineage, as well as a powerful enhancer at position 153 - 361 bp of its first intron. Furthermore, the promoter of the S100A9 gene contains an MRP regulatory element, in position -400 - -374 bp, which controls its expression according to the cell type and the degree of differentiation.

The expression of these two proteins is constitutive at the level of the myeloid lineage, but it seems specific to certain stages of its differentiation. They are present in polynuclear neutrophils and monocytes, but not in macrophages residing in tissues. In circulating monocytes, their expression is reduced during transformation into macrophages. They are also expressed in epithelial tissues, in particular keratinocytes, under certain circumstances: cellular stress, irradiation by UV rays, exposure to pro-inflammatory cytokines (TNF α , IL1 β), rupture of the cell layer, etc. PARP-1 complexes (poly ADP-ribose polymerase) and Ku70 / Ku80 appear to be involved in the regulation of S100 protein expression in response to cellular stress. These two proteins are also strongly expressed in the keratinocytes of psoriasis lesions and in various types of cancer (skin, pulmonary and pancreatic adenocarcinomas, bladder, prostate and mammary cancers, thyroid carcinomas).

2. Structure

Calprotectin, also known as leukocyte protein L1, MRP8 / 14 (macrophage inhibitory factor related protein), protein S100A8 / S100A9, calgranulin A / B, antigen 27E10 is a calcium and zinc binding protein, belonging to the S100 family of proteins.

These proteins get their name "S100" from their solubility in saturated ammonium sulfate solution. Twenty-one members of this family have been described to date, and the genes encoding 16 of them are located on a cluster on chromosome 1q21.

Calprotectin is a 36.5 kDa heterotrimer, composed of one light chain (MRP8 or S100A8) and two heavy chains (MRP14 or S100A9).

In the presence of EDTA (ethylenediaminetetraacetic acid), calprotectin is anionic and migrates into the α 2-globulin zone, while in the presence of calcium, it becomes slightly basic and migrates into the γ -globulins. The two types of subunits that compose it could be demonstrated after dissociation, based on heating in the presence of SDS (sodium dodecylsulphate) and urea, followed by electrophoretic separation in SDS polyacrylamide gel or of two-dimensional electrophoresis. The S100A8 protein contains 93 amino acids (aa) and has a relative molecular mass of 10.8 kDa and an isoelectric pH of 6.6. The S100A9 protein contains 114 aa and has a relative molecular mass of 13.2 kDa. Next to the full-length form of S100A9, there is a truncated form resulting from an alternative translation. The pHi of these two forms of S100A9 is 5.7 for the full-length form and 5.5 for the truncated form, respectively.

Like all proteins of the S100 family, each subunit of calprotectin has two main EF motifs for binding calcium, separated by a hinge region. In the S100A8 and S100A9 proteins, the EF hand in the C-terminal position has a canonical 12 aa loop; the EF hand in the N-terminal position contains a 14 aa loop, which binds calcium by their side chains, this structure being specific to the S100 family (thus, the S100 proteins have a lower affinity for calcium than other proteins sensitive

to calcium such as calmodulin). The binding of a calcium atom to the EF domain causes a change in the conformation and electrical charge of the protein: a portion of the disordered region following helix 4 organizes into additional turns of the alpha helix; helices 2 and 3 rotate in the direction of helices 1 and 4. This change in secondary structure may reveal new sites of interactions at the calprotectin subunit level.

The hinge domain separating the two EF motifs varies depending on the protein and, in some cases, followed by a C-terminal chain: the S100A9 protein has an extended C-terminal region, unlike S100A8 and most of the other proteins of the S100 family. This region contains two additional functional motifs: the first contains a Thr113, which can be phosphorylated by protein kinase C and MAP kinase p38, in particular after activation of the neutrophils; the second contains histidine residues (His103 - His105), involved in binding to arachidonic acid.

Like several S100 proteins (S100A2, S100A5, S100A12, S100A13, S100B), calprotectin contains two additional transition metal binding sites, according to a tetrahedral coordination, these sites being created on the surface of the dimer by the juxtaposition of 4 ligands, of which 2 are provided by each monomer. One such site is created by His83 and His87 of the S100A8 subunit, in conjunction with His20 and Asp30 of the S100A9 subunit. The other site involves the His17 and His27 residues of the S100A8 subunit, and His91 and His95 of S100A9. These transition metal binding sites are brought together when calprotectin binds calcium, which organizes its secondary structure. This transition metal binding causes additional conformational changes and confers additional functions on calprotectin: the chelation of zinc and magnesium on extracellular calprotectin gives it its ability to inhibit bacterial and fungal growth; zinc binding also gives it the ability to regulate immuno-inflammatory responses and pro-apoptotic functions.

3. Biological functions

The extracellular secretion of calprotectin by myeloid cells occurs by two mechanisms:

- Passive release as a result of cell death by necrosis or by the phenomenon of NETosis.
- An active release dependent on calcium.

The active secretion of calprotectin occurs mainly during the inflammatory process. Therefore, this protein acts as a warning signal (alarmin) to the host. TNF-alpha or IL-1beta, pro-inflammatory cytokines, are known to induce the transcription of S100A8 and / or S100A9.

Extracellular calprotectin helps induce rapid recruitment of neutrophils from the bone marrow. This protein also activates P2Y6 causing increased synthesis of pro-inflammatory cytokines as well as alpha 2 integrin expression leading to slowing of P2Y6 rolling along endothelial cells.

Thus, P2Y6 can adhere to the endothelial cell which allows it to be extravasated to the inflammatory site.

Calprotectin has been shown to positively increase the surface expression of CD11b on monocytes, thereby promoting increased adhesion to fibrinogen contributing to the accumulation of monocytes at the site of inflammation.

Extracellular calprotectin also exhibits pro-inflammatory effects on other cells, such as lymphocytes and endothelial cells, by binding to receptors for advanced glycosylation end products (RAGEs) and TLR-4 receptors. The effect of calprotectin on endothelial cells leads to a pro-thrombotic inflammatory response, endothelial cell death induced by caspase activation and increased vascular permeability allowing extravasation of immune cells.

Calprotectin by binding divalent metal ions of Ca²⁺, Zn²⁺, Mn²⁺ and Cu²⁺ can alter the intracellular and extracellular concentrations of these ions. These changes in ionic concentration influence the activity of other proteins. Part of the antimicrobial properties would come from its ability to chelate Zn²⁺ and Mn²⁺ ions from the extracellular environment, leading to a deficiency in these nutrients in bacteria, responsible for their death.

Calprotectin also plays an intracellular role. It is involved in the phagocytosis process by causing conformational changes in the tubulin of the cytoskeleton. The intracellular S100A8 and S100A9 proteins are also involved in the defense against infections by intracellular pathogens but also confer increased cellular resistance to bacterial infections such as *Porphyromonas gingivalis*. Finally, calprotectin potentiates the activity of NADPH oxidase causing the release by this route of oxygenated free radicals participating in the anti-bacterial defense but also which may be responsible for the degradation of the extracellular matrix.

3.1. Exploration of calprotectin

3.1.1. Preanalytical step

It should be noted that proton pump inhibitors and nonsteroidal anti-inflammatory drugs (NSAIDs) such as aspirin can induce an increase in the fecal concentration of calprotectin, to levels close to the cut-off, even higher, in relation to the probable development of enteropathy. It will therefore be important to have information on possible patient treatments for the interpretation of the results.

Dosage in stool

A fecal sample of 50 to 100 mg is sufficient for the assay.

However, it remains desirable to monitor any result obtained in this particular therapeutic context. Conversely, contamination of the sample with blood (and therefore with leukocytes) does not seem to have a significant influence on the dosage as long as the blood volume remains below 100 ml.

The collection of stools must be carried out on a tube or a dry pot (of the stool culture pot type), containing neither additive nor preservative, or on a specific collection system of the kit used.

This sample does not need to be performed under sterile conditions. The width of the pot must be sufficient to allow correct homogenization of the saddle,

Avoid too hard stools, Avoid liquid stools

- less dry matter → possibility of false negative
- possibly infectious diarrhea → possibility of false positive

Avoid stool containing many pieces of poorly digested food

Avoid bloody stools

During the 8 days preceding and during the collection, it is necessary to avoid radiological examinations involving digestive opacification, as well as preparations for colonoscopy.

In the event of particularly severe diarrhea, a dilution of the sample could lead to an “artefactual” decrease in the measured concentration of calprotectin.

In fact, it is interesting to know the patient's fecal flow at the time of the dosage, in order to correctly interpret the result and return it with possible reservations. It may therefore be necessary to check any borderline results obtained during a period of profuse diarrhea on a new sample taken after normalization of the transit.

Dosage in the serum

Dry tube, avoid EDTA.

Centrifugation after 30 min of coagulation.

Storage at -20 ° C.

Stability of the molecule

When bound to calcium, calprotectin undergoes a conformational change, allowing its binding to proteins and making it resistant to leukocyte or bacterial lysis. This provides calprotectin with remarkable stability in the intestinal proteolytic medium. The native stool sample can thus be stored for 7 days at room temperature without significant degradation. These pre-analytical characteristics make it possible to envisage performing the assay on samples taken at home from patients. In addition, calprotectin is stable for 1 year at -20°C, if the sample is stored in a large volume (10-20 g), or 6 months, if it is stored in a low volume (50-100 mg).

3.2. Assay techniques and performance of these techniques

3.2.1. Extraction step

Different methods are available for the determination of calprotectin in the stool, but all require a preliminary extraction step.

Initially, this step was carried out by weighing a mass of approximately 100 mg of stool and diluting it in a suitable volume of extraction buffer. This long method remains the reference method.

More recently, various calibrated extraction devices, similar to those used for the detection of occult blood in the stool, have been proposed. However, these devices remain unsuitable for very liquid stools or heavily loaded with mucus, leading to a risk of underdosing of the analyte.

Calprotectin epitope mapping, using a series of 7 amino acid peptides, overlapping each other, and spanning the light and heavy chain sequence of the molecule, showed that the rabbit could produce antibodies against 3 linear antigens on each of these chains. Numerous monoclonal antibodies directed against calprotectin have been produced and are reported in the literature (Mac387, 27E10, S 36.48...). The use of 6 mouse monoclonal antibodies has thus made it possible to demonstrate 4 epitopes on calprotectin. The binding of calprotectin to calcium, resulting in a change in the conformation of the protein, also alters its reactivity towards monoclonal antibodies.

3.2.2. Assays by EIA technique

Several techniques of enzyme-linked immunosorbent assay (enzyme-linked immunosorbent assay or Elisa) or tube (chemiluminescent immunoassay or CLIA), or immuno-fluorimetric (time resolved fluorimetric immunoassay or TRFIA) are available for assaying calprotectin. The techniques use for coating either monoclonal antibodies or polyclonal antibodies. Regardless of the technique used.

3.2.3. Rapid methods (For fecal calprotectin)

Rapid immunochromatographic techniques have been developed in recent years as an alternative solution to Elisa techniques, in order to allow analyzes to be carried out "piecemeal". Certain tests allow a semi-quantitative estimation of the calprotectin concentration, by integrating two test bands, each reacting to a different threshold in analyte.

Suppliers offer optical readers making it possible to quantify the intensity of the bands on the wafers, which makes it possible not only to overcome the subjectivity of reading with the naked eye, but also to have a quantitative reading of the intensity of the reaction and ensure increased traceability of the analysis. These methods are most often well correlated with Elisa kits from the same manufacturer. They have a good specificity allowing both:

- Exclusion of the diagnosis of IBD, when the concentration remains undetectable, and
- Identification of active disease, in patients with elevated levels. In the event of moderate intestinal inflammation or for the long-term follow-up of the patients, the interpretation of the results can prove to be more delicate and it seems reasonable to confirm any positive result, obtained by rapid technique, by a "classic" assay in Elisa.

Recently, a self-test method, which can be used directly by the patient, was proposed: the reading of the intensity of the reaction band is carried out automatically by a smartphone.

3.2.4. Immunoturbidimetric methods

A new automated assay kit has recently been developed for the immunoturbidimetric assay of calprotectin. This kit, based on a Petia (particle-enhanced turbidimetric immunoassay) technique, is adaptable to most clinical biochemistry analyzers.

It uses the same antibodies as an Elisa test developed by the same supplier, and the results of the two techniques appear to correlate well. This complete automation offers the possibility of a very wide distribution of this assay, with a routine implementation and very fast results delivery times (of the order of 10 minutes).

The reference technique is represented by the ELISA (sandwich) after extraction of fecal proteins, although it requires a lot of handling and calibration to be carried out for each series of samples.

4. Results interpretation

Normal values

20 to 30 µg / g of stools Usual values: <50 µg / g of stools (threshold applicable from 4 years).

5.3 mg/ml

4.1. Clinical interest

4.1.1. Interest of the dosage of serum calprotectin

- In rheumatoid arthritis, calprotectin has been identified as an interesting biomarker. The level of concentration of this protein in the synovial fluid helps differentiate rheumatoid arthritis from other rheumatologic diseases.
- There is growing evidence that calprotectin plays a role in several chronic inflammatory rheumatic diseases such as juvenile arthritis, rheumatoid arthritis, Behcet's disease and ANCA vasculitis. In rheumatoid arthritis, for example, there is a significant correlation between markers of inflammation and disease activity.
- Some authors even report that serum calprotectin would make it possible to estimate subclinical joint inflammation, as shown by the correlations found between calprotectin and the presence of B mode synovitis or power Doppler ultrasound.
- Finally, in this disease, calprotectin can predict radiological progression at 3 years.

Calprotectin plays a role in the local inflammation of spondyloarthritis:

Nevertheless, serum calprotectin does not appear to be useful in discriminating axial SpA from patients with chronic inflammatory low back pain not meeting ASAS criteria.

Calprotectin is also of interest in evaluating the activity of spondyloarthritis

4.1.2. Interest of the determination of fecal calprotectin

- Identify an outbreak of IBD
- Predict response and help scale up treatment
- Identify patients with mucosal scarring
- Identify patients at risk of relapse
- Help decide on therapeutic de-escalation

5. Conclusion

Calprotectin is a calcium binding protein expressed at the inflammatory site by neutrophils and monocytes to activate the innate immune system. Thanks to this unique characteristic, it is a good indicator of local inflammation in chronic inflammatory conditions.

All of the data highlight the key role that calprotectin plays in the inflammatory process in several inflammatory rheumatism. The value of serum and faecal calprotectin assays has been widely studied in recent years, in particular in rheumatoid arthritis, spondyloarthritis, juvenile idiopathic arthritis and vasculitis associated with ANCA but also for chronic inflammatory bowel disease.

Calprotectin has been identified as a powerful biomarker that makes it possible to assess and monitor disease activity as well as predict structural evolution or response to treatment. Calprotectin is also a predictive biomarker of the risk of relapse.

Compliance with ethical standards

Acknowledgments

I would like to thank everyone who contributed to the success of this work.

Disclosure of conflict of interest

Authors declare no conflict of interest.

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