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Chapter 11

Usefulness of Faecal Markers in Cow's Milk Protein Immunomediated Reactions

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/62544

Abstract

Cow's milk protein allergy (CMPA) affects children most commonly than adults, with symptoms usually developing before 1 year of age and within 1 week after the intake of cow's milk. Food allergies can be divided into: IgE mediated and non-IgE mediated. Some reactions may include both mechanisms (mixed type). The most studied faecal Mar-kers, so far, are calprotectin, Tumor necrosis factor-alpha (TNF-α), beta-defensin and eosinophil cationic protein (ECP). Calprotectin belongs to the S-100 family of calcium-binding proteins and seems to be involved in the regulation of inflammation. Faecal calprotectin (FC) values are significantly higher in infants suspected of having CMPA than in a comparison group of healthy infants. Moreover, there is a significant decrease in FC in infants with CMPA after a period of dietary antigen elimination, although levels use to remain higher than in age- and diet-matched comparisons. TNF-α is a cytokine involved in systemic inflammation and is one of the cytokines that make up the acute phase reaction. TNF-α expression in the epithelial cells and mononuclear cells in the lamina propria is markedly increased in FPIES patients. TNF-α is also increased in the stools of patients with gastrointestinal milk allergy after milk challenge. Defensins are small (~29 to 42 amino acid) cationic arginine and cysteine rich, amphipathic peptides with a molecular weight of 3–5 kDa. They can be classified into three groups: α-, β- and θ-defensins. Among them, only α- and β-defensins are expressed in humans. Defensins display various functions, including antimicrobial activity and also act as chemoattractant. They contribute to host immunity and to maintain the balance between pathogens and normal flora. Beta-defensins values detected in infants with a previous diagnosis of CMPA prior to the oral food challenge, and during each provocation do not seem to show significant changes. ECP is a single-chain, zinc-containing protein with a molecular weight ranging from 16 to 22 kDa and is one of the most important proteins in the granules of eosinophil granulocytes. Infants with atopic eczema exhibit a specific faecal protein pattern characterized by an increase in both ECP and TNF-α. The faecal concentration of ECP enhances particular-

ly in patients with immediate-type reactions to the cow's milk challenge whereas faecal TNF-α enhances in those with delayed-type reactions, confirming the different pathogenesis (IgE mediated and non-IgE mediated) of these two types of reactions.

Keywords: Cow's milk protein allergy, faecal markers, faecal calprotectin, human β-defensins, TNF-α, eosinophilic cationic protein

1. Introduction

Food allergy is an abnormal immune response to components of the diet, in particular to proteins. Food allergy can manifest itself by configuring different clinical entities, including atopic dermatitis, gastrointestinal or respiratory symptoms, and anaphylaxis.

The clinical history and sometimes allergen-specific serum IgE tests, skin tests and/or elimination diets can help you achieve the diagnosis.

The therapy is mainly based on the elimination of the food that triggers the reaction.

The prevalence of true food allergy ranges from <1 to 3% and varies by geography and method of assessment.

It is important not to confuse food allergy with non-immune reactions to food (e.g. lactose intolerance, irritable bowel syndrome, infectious gastroenteritis) and reactions to food contaminants (e.g. latex dust in food handled by workers wearing latex gloves) or additives (e.g. monosodium glutamate, metabisulphite, tartrazine), which cause most food reactions [1].

Milk allergy is one of the most common food allergies in infants and children. It consists in an abnormal immune response towards milk and its products.

There are two main groups of proteins in cow's milk that can cause an allergic reaction:

- Casein, found in the solid part (curd) of milk that curdles
- Whey, found in the liquid part of milk that remains after milk curdles

Cow's milk is the most usual cause—as it is the most consumed milk worldwide—but milk from sheep, goats, buffalo and other mammals can cause a reaction as well. Less commonly, people allergic to cow's milk are also allergic to soya milk.

Food allergy can be mediated by IgE, T cells or both. IgE-mediated allergy (e.g. asthma, urticaria or anaphylaxis) presents acute onset, usually starts during infancy, and generally occurs in people with a strong family history of atopy. On the other hand, T-cell-mediated allergy (e.g. celiac disease or dietary protein gastroenteropathies) develops gradually and is chronic; it is common among infants and children [1]. Allergies mediated by both IgE and T cells (e.g. atopic dermatitis, eosinophilic gastroenteropathy) tend to be delayed in onset or chronic.

CMPA produces a range of symptoms and clinical entities (**Figure 1**). The principal symptoms are gastrointestinal (GI-CMPA) and dermatological and are represented by atopic

http://dx.doi.org/10.5772/62544

dermatitis, vomiting and gastrointestinal distress such as infantile colic, gastroesophageal reflux, esophagitis, diarrhoea (typically in the very young), constipation (typically in older children), stomach pain or flatulence. In some cases, particularly in infants, it may also cause proctocolitis, with the presence of blood and/or mucus in the stools.

According to the main clinical manifestations, organic GI-CMPA in infants can be usefully classified [2] into:

- food protein-induced enterocolitis syndrome (FPIES),
- food protein-induced proctocolitis (FPIP),
- food protein-induced enteropathy.

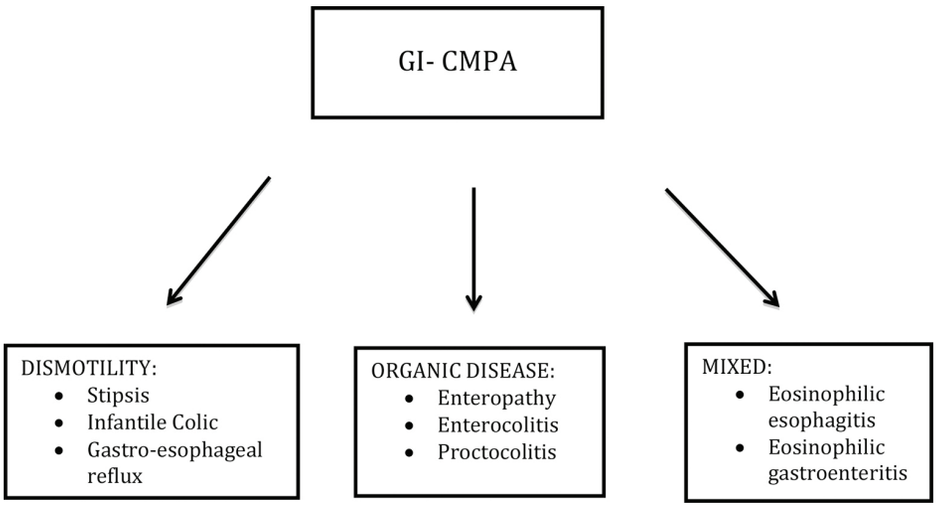

Figure 1. GI-CMPA clinical manifestations in infants can be classified into three main groups: gastrointestinal dysmotility, organic disease and mixed forms characterized by eosinophilic mucosal infiltration.

Patients with FPIES experience repetitive vomiting, starting 1 or 2 hours after the ingestion of offending foods, followed by diarrhoea. However, these patients do not develop acute cutaneous or respiratory symptoms, which commonly accompany IgE-mediated food allergy. FPIES can manifest with systemic symptoms such as lethargy, hypotension, hypothermia, pallor, ileus, bloody stools, methemoglobinemia, thrombocytosis and, sometimes, high temperature with neutrophilia. Therefore, the first diagnoses to be excluded for these patients are sepsis or surgical abdominal emergency.

Patients with FPIP typically develop grossly blood-streaked stools with mucus in the first few months of life. In contrast to FPIES, almost all patients with FPIP develop no systemic symptoms and seem to be well except for the bloody stools. They have no growth delay or poor weight gain. Mild anaemia is seen in rare cases. Many patients with FPIP are breast fed, and the cause is thought to be mainly cow's milk proteins passed through the breast milk (**Table 1**).

It is still unclear when the sensitization phase of allergic proctocolitis occurs. It is thought that dietary antigens can cross the placental barrier or enter the amniotic fluid, which is swal-

lowed by the foetus, causing in utero sensitization. Another possibility is that variations in the concentration of immunomodulatory substances in human milk can alter the protective effect of breastfeeding against allergy. In particular, maternal leukocytes contained in human milk may play a role in antigen processing and presentation to neonatal lymphocytes in the intestine. Thus, it is possible that the ingestion of dietary food proteins excreted in the mother's milk, in case of particular physiologic conditions favouring immunogenic responses (in the neonate or maternal milk), may result in allergic sensitization. However, there are insufficient data to recommend dietary restriction during pregnancy and/or lactation in order to prevent allergy onset [3].

	IgE mediated	Non–IgE mediated
Calprotectin	++	+++
Beta-defensins	–	–
TNF-α	+	+++
ECP	+++	+/–

Table 1. Faecal markers values in IgE-mediated and non–IgE-mediated CMPA.

Cow's milk and soya-based formulas are the major causative foods in the remaining cases.

Patients with enteropathy typically develop chronic diarrhoea and show poor weight gain in the first several months of life. Mild-to-moderate anaemia and hypoproteinemia were seen in some patients with enteropathy. Enteropathy has to be distinguished from celiac disease (CD), that is associated with sensitivity to wheat protein, and is characterized by similar symptoms (diarrhoea, poor weight gain, sideropenic anaemia) usually occurring when the infant is 7–8 months old, as this is the common period of gluten introduction in the diet.

2. Diagnosis of CMPA and faecal markers

Cow's milk protein allergy (CMPA) affects children most commonly than adults [4], with symptoms usually developing before 1 year of age and within 1 week after the intake of cow's milk [5, 6]. During infancy, symptoms suggesting CMPA are observed in 5–15% of the population; however, when specific diagnostic criteria are used, the incidence of CMPA is approximately 2–5% [7].

Skin prick test and serum-specific IgE tests are helpful for diagnosis, mainly in IgE-mediated forms. On the other hand, in cases of CMPA with gastrointestinal chronic signs and symptoms (GI-CMPA), skin prick test and specific IgE test are usually negative, because these forms are mostly non-IgE mediated. This is the reason why the only reliable method of diagnosis in such cases is a food challenge, double blind and placebo controlled [7]. The food challenge needs to be done in hospital, and it could be also dangerous. This easily explains why there is a growing interest to find any faecal biomarker for diagnosis and follow-up (**Table 2**).

A National Institutes of Health working group defined a *biomarker* as 'a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacological responses to a therapeutic intervention'. A biomarker has to be reproducible, accurate, easy to interpret by the clinician, acceptable for the patient, sensitive and specific for the outcome it is expected to identify [8].

Faecal inflammatory markers are represented by different molecules that leak from or are generated by the inflamed mucosa of the bowel. Therefore, these markers could represent a non-invasive means of evaluating objectively mucosal inflammation [9].

The most studied faecal markers, so far, are calprotectin, tumor necrosis factor-alpha (TNF-α), β-defensin and eosinophil cationic protein (ECP).

	GA	Birth weight	Delivery	Antibiotic	Diet	Microbiota	NEC	Gender
FC	None	− (only in term)	+ (only for C-section in preterm)	− (in very low birth weight (VLBW))	Not agree on	−		
HBD2	+		−		−		+	
TNF-α	−		−		−			
ECP								−

Table 2. Relations between faecal markers values in term and preterm newborns and different variables.

3. Calprotectin

Calprotectin was first discovered as a protein with antibacterial activity [9]. It can be found in the cytoplasm of neutrophil granulocytes where it forms about 60% of cytosolic proteins [10], but it is also expressed on the cell membranes of monocytes and in some mucosal epithelial cells [11, 12]. It is a 36.5 kDa heterodimer composed of one light (MRP8) and two heavy (MRP14) chains (8 and 14 kDa) and belongs to the S-100 family of calcium-binding proteins [13, 14]. Calprotectin also contains histidine-based zinc-binding sequences (His-X-X-X-His motif) involved in its antibacterial activity [15]. Although its exact biological function is not known, calprotectin was shown to have bactericidal and fungicidal properties [16]. It is thought that, binding calcium and zinc, calprotectin deprives microorganisms of zinc and additionally inhibits many zinc-dependent enzymes [17–21].

Various data also suggest that it may be involved in the regulation of inflammation. Calprotectin is secreted extracellularly from stimulated neutrophils [22] and monocytes [23], or is released by cell disruption or death. Once released, calprotectin may be detected in serum, body fluids and faeces [24]. A high level of calprotectin was found in extracellular fluid during several inflammatory diseases, such as rheumatoid arthritis [17], cystic fibrosis [25] and active

multiple sclerosis. Once released in extracellular fluids, soluble calprotectin provides both bacteriostatic and cytokine-like effects in the local environment. When calprotectin metabolism is affected on a systemic level, the zinc-binding properties of the protein may induce severe dysregulation of zinc homeostasis causing severe clinical symptoms. Only monocytes and immature macrophages present the membrane form of calprotectin; therefore, the presence of calprotectin-positive infiltrating cells is related to the influx of mononuclear phagocytes to the site of inflammation. On the other hand, the intracellular distribution of calprotectin is influenced by the activation state of macrophages. In non-activated macrophages, the protein complex is in the cytosolic fraction; once stimulated, the complex moves towards the cell membrane, thus localizing with proteins of the cytoskeleton. Therefore, calprotectin may be related to phagocytosis, cell movement or signal transduction. Although calprotectin is classified as a specific marker for neutrophils and macrophages, it was also found in other cell types, such as in keratinocytes in inflammatory dermatoses [26] and squamous cell carcinoma. Moreover, the 14 kDa subunit of calprotectin is expressed in a subset of microglia in brain tissue of patients affected by Alzheimer's disease [27]. Since the expression of calprotectin in these cell types seemed to be up-regulated by the inflamed state of the tissue, the functional relevance of the factor to each inflammatory process was suggested [25–27]. In synthesis, calprotectin expression and release seem to be of particular importance in immune and immunopathological reactions. However, the exact biological role(s) of the factor is now under investigation.

Moreover, calprotectin presents growth-inhibitory and apoptosis-inducing activities against various cell types, even including tumour cells and normal fibroblasts. Calprotectin seems to induce apoptosis through a dual mechanism. One is the zinc exclusion from the target cells, and the other is the binding of the factor to target cell surface, possibly in a ligand-receptor fashion [28].

Several gastrointestinal diseases, inflammatory bowel diseases (IBD) among them, can cause a higher release of leukocytes in stools [29, 30].

In adults, a strong correlation between 4-day faecal excretion of 111-indium leukocytes, considered the standard criterion faecal marker of inflammation [31] and faecal calprotectin levels, raised the interest in calprotectin as a marker for intestinal mucosa inflammation [9].

Calprotectin can be easily measured in stools. In fact, the calcium saturated form of calprotectin is highly resistant to proteolysis and colonic bacterial degradation, allowing faeces sample to be kept for up to 1 week at room temperature without any significant degradation [9, 20, 32–34]. Moreover, calprotectin is stable at −20°C for at least 6 months. Several enzyme-linked immunoabsorbent assays (ELISA) using small stool samples (0.1 g) are commercially available. The reference value is 50 μg/g faeces for healthy adults and children aged from 4 to 17 years, regardless of sex [35].

Less agreement has been shown in regard of cut-off values in neonatal age, for both term and preterm newborns. This is mostly due to the high interindividual variations of calprotectin values in this population. Calprotectin is already present at high levels in the first passed meconium, indicating the capability of the foetal GI tract to produce and secrete this protein [9].

http://dx.doi.org/10.5772/62544

Several factors can influence faecal values of calprotectin in newborns, such as gestational age, postnatal age, delivery mode, antibiotic treatment, diet and gut microbiota.

In term infants, there is a negative correlation between calprotectin levels in meconium and gestational age or birthweight [9]. Such correlation was not confirmed in preterm infants, without any obvious reason for this discrepancy [36, 37]. Healthy full-term and preterm infants, especially younger than 3 months old, present high faecal calprotectin levels, comparable to those seen in children or adults affected by IBDs. Full-term and preterm neonates at the same postnatal age do not show significant differences in calprotectin values [38].

Calprotectin values in full-term newborns seem to slightly but significantly increase at day of life 7 compared with values at day of life 3, the levels then remaining similar for the first month of life [24]. Then, a decrease occurs between 6 weeks and 6 months of life [39].

The mode of delivery was not found to influence faecal calprotectin in full-term neonates [24, 40], even if a positive correlation was found with caesarean delivery in preterm infants [37]. No relationships were found with gestational age [37, 41, 42].

Faecal calprotectin was also found to correlate negatively with antibiotic treatments in this population of very low birthweight infants [37].

The influence of diet on calprotectin levels is not agreed on. Some researchers [24, 40] found no differences between breast fed and formula-fed infants during the first month of life in full-term infants, while in another study [43], faecal calprotectin concentrations were found to be significantly lower in breast fed than in formula-fed infants during the 'preweaning' period. Finally, some other researchers [44, 45] described opposite results with higher calprotectin levels in exclusively breast fed infants compared with mixed-fed or formula-fed ones. However, the comparison between the different studies is difficult because infants were recruited at various ages. As calprotectin levels change during the first year of age, the different inclusion criteria could explain the obtained results. Moreover, most of these studies gave poor information about the infants' background and the composition of the formulas, which could affect calprotectin levels [9].

Gut microbiota has been shown to affect faecal calprotectin values. Oral supplementation by Bifidobacterium lactis Bb12, which modified the equilibrium of the gut microbiota, led to a significant decrease in calprotectin levels in the probiotic group compared with the placebo one [46].

As high faecal calprotectin values in newborns show an increment of granulocytes in the intestinal lumen due to enhanced intestinal permeability and/or development of the bowel-associated lymphoid tissue, interindividual variations should be related to environmental factors (e.g. mode of feeding, intestinal colonization or response to dietary antigens) which could individually alter this process [9].

In the light of this, we can now analyze calprotectin usefulness in the management of CMPA.

4. Calprotectin as a predictive factor in CMPA

It was hypothesized that alterations in newborns faecal calprotectin (FC) could be associated with specific disorders in infancy such as atopic dermatitis, cow's milk intolerance, severe infantile colic and gastroesophageal reflux [47], so that calprotectin was proposed as a predictive marker in these pathological conditions.

The predictive value of calprotectin measured at birth as a possible marker of allergic predisposition in the first 2 years of age was tested, but the comparison of calprotectin concentration at birth did not lead to any statistically significant result in allergic vs. non-allergic children at 2 years of age. The levels of FC in the first month of life are not influenced by a possible individual predisposition to atopy [48].

5. Calprotectin, diagnosis and management of CMPA

No single laboratory test is either sensitive or specific enough to be diagnostic of allergic colitis, but the finding of either peripheral eosinophilia or eosinophils in stool samples is often considered suggestive of this condition. It was widely demonstrated that faecal calprotectin (FC) values are significantly higher in infants suspected of having CMPA than in a comparison group of healthy infants. Moreover, there is a significant decrease in faecal calprotectin in infants with CMPA after a period of dietary antigen elimination, although levels use to remain higher than in age- and diet-matched comparisons [7, 49].

FC levels before the CMP elimination diet seem to be higher both in the IgE-mediated CMPA group and in the non-IgE-mediated CMPA group compared with the control group. However, the difference seems to be statistically significant only in the non-IgE-mediated CMPA group (**Table 3**).

Calprotectin	**Useful for diagnosis and treatment follow up**
	Not susceptible to degradation
HBD2	Not useful
TNF-α	Useful for diagnosis and treatment follow up
	Highly susceptible to degradation
ECP	Usefulness not agreed on

Table 3. Usefulness of faecal markers in CMPA.

On the other hand, a statistically significant difference was found between FC levels before and those after the cow milk's proteins elimination diet both in the IgE-mediated CMPA group and in the non-IgE-mediated CMPA group. According to these findings, FC levels may be useful only in treatment follow-up for the IgE-mediated group, while, in the non-IgE-mediated group, FC may be useful both for the follow-up of treatment and recurrence determination [49].

Moreover, a comparison of the IgE-mediated and non-IgE-mediated groups revealed significantly higher FC levels in the non-IgE-mediated group.

It was supposed that FC levels within the non-IgE-mediated group are higher because gastrointestinal symptoms and colitis are predominant in these patients. Therefore, faecal calprotectin may be more useful to detect relapses during the follow-up of patients in the non-IgE-mediated group, as gastrointestinal involvement is more common in this patients' population.

Given that FC can increase in case of several inflammatory bowel conditions, FC can be useful only to determine relapses and follow ups after diagnosing patients as CMPA particularly with gastrointestinal involvement [7].

It is to say that the addition of Lactobacillus GG (LGG) to an extensively hydrolysed casein formula significantly improves the recovery of the inflamed colonic mucosa as indicated indirectly by greater decreases in faecal calprotectin and in the number of infants with the persistence of occult blood in stools after 1 month. The mechanisms of this beneficial effect are not well known but may be linked to the effects that LGG has on enhancing the intestinal mucosa's barrier function, cooperating in breakdown of protein antigens, competing with bacterial pathogens and fostering early immune system development towards nonallergy, as well as easing symptoms of eczema attributed to CMPA [49–53].

6. Beta-defensins

The sterile amniotic fluid fills the foetal gut and delivery triggers a rapid transition to bacterial colonization, a crucial challenge for the immune system of the newborn. Despite a naive adaptive immune system, infants rarely become infected, suggesting strong innate defence mechanisms [54]. Several peptides have been identified in meconium and faeces from neonates during the first weeks of life, suggesting their participation in the gut barrier against infection [55].

Defensins are small (~29 to 42 amino acid) cationic arginine and cysteine rich, amphipathic peptides with a predominantly β-sheet structure stabilized by 3 disulfide bonds and a molecular weight of 3–5 kDa [56]. They can be classified on the basis of structure and disulfide bond organization into three groups: α-, β- and θ-defensins. Among them, only α- and β-defensins are expressed in humans. In particular, humans express six α-defensins and up to 31 β-defensins [57]. The α-defensins can be further subdivided into myeloid (HNP1-4) and enteric [human defensin (HD) 5 and 6] peptides on the basis of both expression patterns and genetic organization [58]. HNP (from 1 to 4) are mainly expressed by neutrophils but can also be expressed by B cells some T cells, natural killer (NK) cells, monocyte/macrophages and immature dendritic cells (DCs) [58, 59]. HD5 and HD6 can be found in epithelial Paneth cells belonging to the small bowel [60, 61]. HD5 is also expressed by epithelial cells in the genitourinary tract [62–65].

Human β-defensins (HBDs) are largely expressed by skin epithelial cells and at mucosal surfaces in contact with the environment [66, 67]. They are also expressed by monocytes, macrophages, and certain DCs, and a subset of β-defensins are only expressed in the male reproductive tract [68, 69].

Defensins display various functions, including antimicrobial activity towards Gram-positive and Gram-negative bacteria as well as towards enveloped viruses and fungi [70], and also act as chemoattractant. They also trigger histamine release, wound repair and apoptosis. Defensins contribute to host immunity and to maintain the balance between pathogens and normal flora [71], creating small micropores in the bacterial membranes: this causes a damage to the cell structural integrity and the consequent breakdown of the bacterial cell. Therefore, defensins, thanks to this antimicrobial quality, protect the host epithelium and stem cells from virulent pathogens and also contribute to regulate the number and composition of commensal microbiota [72]. It seems that α-defensins influence the composition of the small intestinal commensal microbiota and the presence of interleukin-17-producing T cells in the lamina propria [73]. In the intestine, α-defensins are highly expressed by Paneth cells and largely confined to the small intestine, whereas β-defensins are expressed by epithelial cells at multiple sites. In general, α-defensins and β-defensin 1 are constitutively expressed, whereas β-defensin 2 (HBD2) to 4 are inducible at sites of infection or inflammation [74, 75]. Recent findings have suggested that, in addition to genetic factors, IBD pathogenesis may result from a breach in the effective mucosal barrier to constituents of the commensal microbiota, thus eliciting pathologic responses from the normal mucosal immune system. In particular, Crohn's disease may, at least in part, be due to a relative defensin deficiency related to reduced expression of Paneth cell α-defensin in disease of the ileum and reduced secretion of inducible β-defensin, namely due to lower HBD2 gene copy number in colonic Crohn's disease [76], allowing intestinal microbes to invade the mucosa and stimulate uncontrolled pro-inflammatory immune responses [77].

Very few data are yet available on innate defence in neonates. In this population, defensins should provide a first-line defence against infection, promoting interactions between the innate and adaptive immunity in newborn infants [78]. Low levels of two enteric α-defensins, that is HD-5 and HD-6, were found in foetus at a gestational age corresponding to preterm infants suggesting that an immaturity of local defence could predispose infants born prematurely to infection from intestinal microorganisms [79]. Besides, these α-defensins were up-regulated during necrotizing enterocolitis (NEC) [80]. Moreover, the expression of mucosal HBD2 mRNA seems to be increased in colonic inflamed mucosa in adults [81].

HBD2 can be easily measured in faeces of adults and children using a commercially available ELISA [82–84].

HBD2 is always detectable in the faeces of full-term and preterm neonates and provides a first kinetic analysis throughout the first weeks of life. The levels are higher than those observed in healthy children and adults and are positively correlated with gestational age at birth [85]. Mode of delivery and mode of feeding do not seem to influence HBD2 values in healthy infants. After 2 weeks of post-natal age, the levels are identical in full-term and preterm infants. These time course mimic partially the one previously described with faecal calprotectin [9, 24],

http://dx.doi.org/10.5772/62544

suggesting that birth is associated with a 'physiological' inflammation, which might represent a trait of the gut neonatal adaptation to the various encountered antigens.

A reduction in β-defensin production, as seen in preterm newborns, is associated with an alteration of the colonic microbiota that can determine a colonic inflammation [77] and may be related with a high risk of NEC [80, 86].

Moreover, HBD2 is up-regulated in infants suffering from severe intestinal distress due to NEC, indicating an activation of the mucosal innate defence. As HBD2 has a chemoattractant activity for cells expressing the chemokine receptor CCR-6, such as DCs, defensin could serve as a bridge between the innate immunity at the intestinal mucosa and subsequent adaptive immune responses during NEC [87].

7. Beta-defensins and CMPA

Few studies have been performed to evaluate the correlation between faecal β-defensins values in infants affected by CMPA. In particular, β-defensins values detected in infants with a previous diagnosis of CMPA prior to the oral food challenge, and during each provocation period (3–5 days after the start of either the active or placebo provocation) do not seem to show significant changes [88].

Therefore, nowadays β-defensins are not considered useful in the management of CMPA.

8. Faecal Tumor necrosis factor-alpha

TNF-α is a cytokine involved in systemic inflammation and is one of the cytokines that make up the acute phase reaction.

TNF is an endogenous pyrogen, as it can provoke fever, but it also shows other properties such as apoptosis, cachexia and inflammation induction. It inhibits tumorigenesis and viral replication and responds to sepsis through IL1 and IL6 producing cells. Several human diseases including Alzheimer's disease [89], cancer [90], major depression [91] and IBD [92] seem to be linked to an impaired TNF production. Increased faecal TNF-α levels have been found in Shigella enteritis [93] and in children with IBD and correlate with the severity of colitis in the latter [94].

TNF is primarily produced as a 212-amino acid-long type II transmembrane protein arranged in stable homotrimers [95, 96]. Then, metalloprotease TNF-alpha converting enzyme (TACE, also called ADAM17) generates the soluble homotrimeric cytokine (sTNF) by cleaving the membrane-integrated form [97]. Both the secreted and the membrane bound forms are biologically active, although the specific functions of each are controversial.

TNF was thought to be produced primarily by macrophages [98], but it is produced also by a broad variety of cell types including lymphoid cells, mast cells, endothelial cells, cardiac myocytes, adipose tissue, fibroblasts and neurons.

It has a number of actions on various organ systems, generally together with interleukin (IL)-1 and IL-6.

Among these actions, one of the most important is stimulating the acute phase response, leading to an increase in C-reactive protein and a number of other mediators.

It is a potent chemoattractant for neutrophils and promotes the expression of adhesion molecules on endothelial cells, helping neutrophils migrate.

A local increase in concentration of TNF causes the cardinal signs of inflammation to occur: heat, swelling, redness, pain and loss of function.

The overexpression of TNF causes many of the clinical problems associated with autoimmune disorders such as rheumatoid arthritis, IBD, psoriasis. Thus, these disorders are sometimes treated by using a TNF inhibitor, such as infliximab (Remicade), adalimumab (Humira), certolizumab pegol (Cimzia) or etanercept (Enbrel).

TNF-α time course was evaluated in preterm and term neonates. Mean values at day of life 15 were negatively related with GA with significantly higher values in preterm than in term newborns. The difference was more evident at day of life 30. Neither type of delivery nor type of feeding seem to influence TNF-α values, both in term and preterm neonates.

TNF-α is released after the contact of the macrophages with Gram-positive and Gram-negative bacteria. Gastrointestinal tract of preterm newborns is poorly colonized, harbouring no more than seven bacterial species [99], with high level of staphylococci and clostridia and low level of probiotic strains Lactobacilli and Bifidobacteria that demonstrated high capacities to reduce TNF-α concentrations in the gut [100]. However, the higher levels of TNF-α in preterm newborns may promote the increase of inducible HBD2 production: this agrees with the lower HBD2 levels demonstrated in preterm neonates, increasing over time. Defensins' expression, in fact, increases in response to TRL ligand, TNF-α, IL-1ß, INF-γ [101].

Faecal TNF-α can be determined using an ELISA kit adapted for faecal samples. In normal control cohorts of children, TNF-α levels are considered normal if they are inferior to 90 pg/g [102].

9. TNF-α and CMPA

TNF-α seems to be involved in the pathogenesis of FPIES through an alteration of intestinal permeability [2, 103, 104] that can lead to an aberrant increased absorption of luminal antigens.

TNF-α expression in the epithelial cells and mononuclear cells in the lamina propria is markedly increased in FPIES patients. In addition, TNF-α is highly secreted, antigen specifically, by peripheral blood mononuclear cells from patients with FPIES [103, 105, 106] and is

also increased in the stools after milk challenge of patients with gastrointestinal milk allergy [107, 108].

Infants with atopic eczema exhibit a specific faecal protein pattern characterized by an increase in both ECP and TNF-α [109]. The faecal concentration of ECP was enhanced particularly in patients with immediate-type reactions to the cow's milk challenge, whereas faecal TNF-α was enhanced in those with delayed-type reaction, confirming the different pathogenesis (IgE mediated and non-IgE mediated) of these two types of reactions.

TNF-α is a helpful faecal marker to discriminate two main types of persistent diarrhoea with onset within the first weeks or months of life: constitutive intestinal epithelial disorders, such as epithelial dysplasia (ED) or microvillus atrophy (MVA), and immune-inflammatory disorders, such as inflammatory colitis (IC) or autoimmune enteropathy (AIE) [102, 110–112].

Only in inflammatory disorders, an increase in TNF-α levels appears, whereas it remains undetectable/normal in constitutive epithelial disorders [102]. Moreover, TNF-α levels closely correlate with the inflammatory activity of the intestinal mucosa [94].

TNF-α increase reflects that previously seen for calprotectin. Calprotectin and TNF values are dramatically increased in neonates and small infants with immune-inflammatory disorders. The main difference between TNF-α and calprotectin is that the first is highly susceptible to degradation [113]. Therefore, calprotectin measurement might be preferable in the diagnostic work-up of diarrhoea and more appropriate to the clinical setting [102].

10. Eosinophil cationic protein (ECP)

ECP is one of the most important proteins in the granules of eosinophil granulocytes together with the major basic protein (MBP), eosinophil peroxidase (EPO) and eosinophil-derived neurotoxin/eosinophil protein X (EDN/EPX). Very small amounts of ECP can also be found in neutrophil granulocytes and monocytes [114, 115].

ECP is a single-chain, zinc-containing protein with a molecular weight ranging from 16 to 22 kDa. The heterogeneity of the molecule is partially due to differences in glycosylation of three potential sites in its amino acid chain [116].

The gene that codes for ECP has been located on chromosome 14q11.2 and three polymorphisms have been identified [117].

ECP synthesis in eosinophil granulocytes begins already at the stage of promyelocytes in primary granules; from the myelocyte stage, ECP is present only in the matrix of specific granules of eosinophil granulocytes [118, 119]. Mature eosinophil granulocytes contain 13.5 mcg ECP/10^6 cells [120]. Unstimulated neutrophil granulocytes cannot produce ECP themselves, but these granulocytes can take ECP from the local environment [121]. After cellular stimulation, ECP mRNA can be found in neutrophil granulocytes [122]. On the other hand, monocytes are able to synthetize ECP, except for when macrophage differentiation is ongoing [115].

Activated eosinophil tissue granulocytes can excrete ECP, in response to two kinds of stimuli: antibody dependent (IgG, IgA) and antibody independent (C3 and C5 complement components) [123, 124]. IL like IL-5 and IL-3, and the granulocyte–monocyte colony-stimulating factor (GM-CSF) have positive impact on this secretion. During the excretion of the ECP molecule from eosinophil granules, enzymatic deglycosylation occurs and converts the inactive ECP form with high molecular mass into a cytotoxic variant with low molecular mass [125].

Inhibitory effect on ECP secretion has been confirmed for cyclosporine A, dexamethasone, rapamycin, formoterol and pemirolast [126–128].

ECP exhibits numerous biological activities that may be classified into cytotoxic and non-toxic reactions.

Cytotoxic activity of ECP is effective against a wide range of microorganisms: parasites, Gram-negative and Gram-positive bacteria, viruses [129–131].

- *Antibacterial activity*: for antibacterial ECP activity, electrostatic interactions are important between negatively charged cellular membrane or cellular bacterial wall and positively charged ECP, followed by destabilization of bacterial membrane. Other mechanisms involve the formation of transmembrane pores that allow the transition of water and osmotic cell lysis. Specific interaction of ECP with lipopolysaccharides and peptidoglycans on the bacterial cell wall may result in bacterial cell aggregation and cell death [132–136].

- *Antihelminthic activity*: eosinophilia is present in the peripheral blood of patients with parasitary diseases and granulocyte and eosinophil infiltrates have been detected in tissue biopsy. However, the actual role of ECP and eosinophil granulocytes in parasitary infection has not been clarified. ECP seems to play a role in the isolation of pathogenic agent and the entire infected region in the form of granuloma. Blood cells, particularly eosinophil granulocytes, are part of the structure of such granulomas. The chemotactic action of ECP on fibroblasts is the first step towards the remodelling of the extracellular matrix. Moreover, ECP promotes the secretion of tumor growth factor-β (TGF-β), that is a pro-fibrotic mediator synthetized by fibroblasts [121, 137].

- *Antiviral activity*: antiviral action of ECP is mediated by ribonuclease activity [121]. ECP belongs to the family of ribonucleases A (RNase A) which cleave the single-strand RNA molecules [131, 138]. Ribonuclease activity is, however, the same in all genetic and post-translational ECP variants and is entirely independent of cytotoxic activity. Most of the studies have been performed on respiratory syncytial virus (RSV) [139, 140], showing a decrease of viral infectivity due to ECP, yet ribonuclease activity does not seem sufficient to explain the whole antiviral effect attributed to eosinophil granulocytes [121, 138].

- *Antihost activity*: in addition to its important role in host immune defence, ECP may also cause undesired side-effects on the host's own tissues via its cytotoxic activity. Neuronal damage has been described, as well as the damage of muscular cells and respiratory tract epithelial cells. One of the possible mechanisms of eosinophil-induced tissue destruction is based on the apoptotic action of ECP via activation of the caspase cycle [132, 141–143]. In

contrast, in several dermatoses, intracellular accumulation of ECP is crucial in damaging dermal cell, whilst RNase activity and cation-dependent cytotoxicity of the ECP molecule seem to cause skin lesions [121, 144].

- *Non-toxic activity*: several immunomodulatory properties (e.g. inhibition of T-cell proliferation, up-regulation of receptors and adhesion molecules on epithelial cells, or basophil histamine release) are involved in ECP non-toxic action [132, 137, 145]. Chemotactic action on fibroblasts is the first ECP action in tissue repair processes. Moreover, ECP stimulates the secretion of TGF-β, whose pro-fibrotic action alters the intracellular metabolism of fibroblasts. ECP mediates both the increase of proteoglycan synthesis and the inhibition of proteoglycan degradation, with consequent intracellular proteoglycan accumulation [121, 137, 146]. Moreover, ECP seems to play a role in atherogenesis as it enables adhesion of monocytes on endothelial cells, is involved in coagulation cascade, and has a stabilizing impact on the plaque [137, 145, 146].

Only activated eosinophil granulocytes release the granule content, and therefore, the determination of ECP concentration is a considerably more specific indicator of eosinophil inflammation than eosinophil granulocyte count in peripheral blood. ECP has been associated with several pathologic conditions, especially atopic diseases: allergic asthma, allergic rhinitis and perennial rhinitis, atopic eczema/dermatitis syndrome (AEDS) [116, 147–149]. Elevated serum values are proportional to the intensity of allergic inflammation and indicate acute allergen exposure. ECP levels are also augmented in several gastrointestinal disorders, some of which are IgE-associated: eosinophil diseases (esophagitis, gastro-enteritis and colitis), gastrointestinal food allergy and intestinal parasitoses. Also, ECP values are enhanced in non-IgE-dependent disorders such as non allergic asthma with aspirin intolerance, respiratory infections, sinonasal polyposis, Churg-Strauss disease and idiopathic hypereosinophilia (HES) syndrome [150].

Differential diagnostics of hypereosinophilia could be easier thanks to the evaluation of plasma ECP concentration and the ECP/eosinophil count ratio, as ECP levels and the above ratio are increased in patients with HES. Moreover, these values are higher in patients with reactive eosinophilia associated with malignancy than in those patients with reactive eosinophilia associated with inflammation. However, on the basis of ECP concentration and ECP/eosinophil count ratio, discriminating clonal from reactive eosinophilia is not possible [121, 151].

ECP is present in numerous body fluids such as plasma, serum, sputum, bronchoalveolar lavage (BAL), saliva, nasal lavage, tears, jejunal fluid, faeces, synovial fluid [145].

Faecal ECP is intensively investigated as a novel potential marker of IBD and eosinophil gastroenteritis [152, 153]. Faecal ECP levels are not dependent on the number of eosinophil granulocytes or serum ECP values, which makes this type of measurement a potential intestinal marker. Moreover, faecal ECP levels do not differ in dependence to age or gender. Serum and faecal ECP mean and median values have been evaluated in a population of healthy children. Serum values are, respectively, 13.50 and 9.54 mcg/L, whereas faecal values are 1.93 and 1.20 mcg/g [154].

11. ECP and CMPA

ECP values are a valid index to evaluate eosinophil granulocytes activity, even more than eosinophil peripheral blood count. In particular, serum ECP values have been demonstrated to strictly correlate with disease activity in CMPA: ECP levels were determined during a diet with and without cow's milk in a patient with eosinophilic enteritis. ECP levels were considerably elevated during the diet with milk, although they returned to normal values several months after milk was withdrawn [155].

To demonstrate inflammation and increased protein leakage from the gut during a cow's milk elimination-challenge test in faecal samples of infants presenting with different symptoms suggestive of cow's milk allergy, ECP levels were measured in faecal samples of 208 infants with a mean age of 7 months. Pre-challenge samples were obtained after a mean 3-weeks elimination period, while post-challenge samples were collected 4 days after starting the challenge. Among these infants, pre- and post-challenge ECP levels were increased in those reacting after 24 h than in those reacting within 1 h. Moreover, pre-challenge levels of ECP were higher in those showing intestinal symptoms. Therefore, in infants with slowly Developing gastrointestinal symptoms, enhanced faecal ECP values could be helpful in discriminating patients from those who tolerate cow's milk. Serial follow-up of faecal ECP can be useful to evaluate the degree of intestinal inflammation and to determine an appropriate time for a challenge test. Nevertheless, faecal ECP values are not diagnostic tools for cow's milk allergy [156].

Kapel et al. [107] also evaluated faecal ECP values before and after the oral challenge in 13 patients with GI-CMPA, not showing any significant change. No data are available about timing of measurement.

Kristjánsson et al., using rectal protein challenge, investigated the local inflammatory reaction to gluten and CM protein in adult patients with CD in remission, but still complaining gastrointestinal symptoms. In 20 celiac patients and 15 healthy controls, rectal challenges with wheat gluten and dried cow's milk powder were conducted. Fifteen hours after challenge, the reaction of intestinal mucosa was recorded evaluating local secretion of neutrophil and eosinophil granule molecules such as myeloperoxidase (MPO) and ECP. At the same time, mucosal release of nitric oxide (NO) was measured. Compared to healthy controls, patients with CD showed significant increases in rectal NO and MPO concentrations measured 15 h after challenge with both CM and gluten, while ECP was increased to a similar extent in the two groups. Therefore, a rectal challenge with CM protein seems to induce a local inflammatory mucosal reaction in patients with CD but not in healthy controls. Ten out of 20 patients showed abnormal increases in both MPO and NO as a reaction to CM challenge, but no increase in ECP, indicating the absence of eosinophil activation at least 15 h after challenge [157].

More studies are needed to evaluate the correct time of faecal ECP measurement.

Therefore, we can assume that ECP is not a reliable faecal marker, as its values are not constantly increased in CMPA. This is possibly due to the lack of agreement on the correct time to perform ECP measurement in stool samples, as this faecal marker does not seem to

increase immediately after CM exposure. Moreover, as previously said, faecal concentration of ECP seems to be enhanced particularly in patients with immediate-type reactions to the cow's milk challenge (IgE mediated). This can help understanding the different results reached in the cited studies.

12. Conclusions

In the light of this, calprotectin and TNF-α seem to be the most useful faecal marker in the management of non-IgE-mediated GI-CMPA. They can be helpful in achieving a diagnosis, as they can easily differentiate constitutive intestinal epithelial disorders, such as MVA or ED, and immune-inflammatory disorders, such as AIE or IC. Moreover, faecal calprotectin and TNF-α are useful for treatment follow-up, as their values in the stools markedly increase after intake of cow's milk protein, due to the reactivation of mucosal inflammation.

The main difference between TNF-α and calprotectin is that the first is highly susceptible to degradation. Therefore, calprotectin measurement might be preferable and more appropriate to the clinical setting.

Beta-defensins seem to provide a first-line defence against infection, promoting interactions between the innate and adaptive immunity in newborn infants. Their values are mainly influenced by microbiota and are up-regulated during NEC. No significant correlations have been reported with GI-CMPA, so far. Therefore, β-defensins are not considered a useful faecal marker in the management of CMPA.

ECP is one of the most important proteins released by activated eosinophil granulocytes, and the determination of its concentration is a considerably more specific indicator of eosinophil inflammation than eosinophil granulocyte count in peripheral blood.

ECP can be measured in several biological samples, serum and stools among them. In individuals with CMPA, serum ECP values increase considerably during the diet with milk, then returning to normal values several months after milk withdrawal. Also faecal ECP values tend to increase after exposure to cow's milk protein, but this does not happen immediately. ECP values usually increase for several hours, or even days, after CM exposure. Unfortunately, the lack of agreement on when to measure ECP in stool samples after CM exposure, leads ECP not to be considered a reliable faecal marker in CMPA nowadays, as further researches are still needed in order to understand the correct time to perform faecal ECP measurement.

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