

REVIEW PAPER

Biomarkers of intestinal epithelial damage in children with food allergies

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ABSTRACT

Food allergy has a significant negative impact on patients and their families, and it has become an important public health problem. Currently used diagnostic methods, such as skin prick tests (SPT) and determination of specific immunoglobulins E (sIgE), present limited sensitivity and specificity. Therefore, new tools are expected to improve the accuracy of the diagnostic work-up. A recent hypothesis of allergic reactions indicates a primary role of dysregulation of the epithelial barrier, which pioneers the role of biomarkers of intestinal damage in food allergy. The objective of this paper is to present the currently most promising biomarkers for the diagnosis, prognosis, and management of food allergy in children.

KEY WORDS:

children, food allergy, biomarkers.

INTRODUCTION

The diagnosis of food allergy (FA) in children is difficult because until now there has been no single, universal diagnostic method. The diagnostic difficulties result from the variety of offending foods, the variability of their sensitising properties, and the various mechanisms of allergic reactions. For these reasons, clinicians are still looking for more accurate diagnostic tools.

A recent hypothesis on the pathogenesis of allergic reactions indicates a crucial role of epithelial barrier impairment. According to Zissler *et al.*, the exploration of biomarkers for allergic rhinitis and allergic asthma has revealed 161 different, potential biomarkers; about 20 of them are exclusively derived from epithelium of the respiratory tract [1]. In terms of gastrointestinal (GI) diseases, until now biomarkers of the epithelial damage have been included in practice guidelines in the management of inflammatory

bowel disease (IBD). Furthermore, there is the potential for more epithelial-derived markers to be incorporated into diagnostic standards of such clinical entities as IBD, irritable bowel syndrome (IBS), short bowel syndrome, colorectal cancer, malabsorption syndrome, or FA [2].

This paper summarises the current state of knowledge on selected, most promising biomarkers of intestinal epithelial damage that might be useful in FA diagnostics.

GASTROINTESTINAL FOOD ALLERGY

FA can be defined as an abnormal immune response on exposure to consumed food, appearing reproducibly, and causing adverse reactions [3, 4].

According to current nomenclature, immunological mechanisms involved in allergy are classified as either immunoglobulin E (IgE)-mediated, non-IgE-mediated, or a mixture of IgE- and non-IgE-mediated [3]. In the ma-

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majority of cases, allergic diseases with GI manifestation are considered to be non-IgE-mediated; however, they might be presented with different immunological mechanisms as well [3].

The highest prevalence of FA is reported among the youngest children (up to 6–8% of the infant population), decreasing over the years, to around 3–4% of adolescents and 1–3% of adults [4].

GI allergies are classified by differences in symptomatology into various clinical entities. Food protein-induced enterocolitis syndrome (FPIES) is a severe non-immunoglobulin, IgE-mediated, gastrointestinal food hypersensitivity, which in an acute form manifests as intensive, repetitive vomiting, often accompanied with diarrhoea, leading to dehydration and lethargy. Together with food-protein-induced enteropathy (FPIE) and food protein-induced allergic proctocolitis (FPIAP) FPIES belongs to a group of non-IgE-mediated gastrointestinal food allergic disorders (non-IgE-GI-FA). These diseases primarily affect infants and are most commonly caused by cow's milk, soy, and rice proteins, although other food allergens can be triggers as well [5].

The diagnosis is based upon the presence of consistent clinical features with improvement following withdrawal of the suspected causal allergens. The results of skin prick tests or allergen-specific IgE antibodies are usually negative. In endoscopy, non-characteristic changes are manifested in the form of diffuse and/or haemorrhagic inflammation, without or with the involvement of the ileum and atrophy of the intestinal villi. Due to the lack of specific symptoms and laboratory tests, diagnosis of non-IgE-GI-FA is often challenging and time-consuming [4, 5].

THE FUNCTION OF THE INTESTINAL EPITHELIAL BARRIER

The epithelial layer is a structure forming a barrier that separates the human host from the external environment. Together with gut-associated lymphatic tissue, it is the part of the largest immunological organ of the human body [6].

The intestinal barrier is established by a single layer of epithelial cells organised into crypts and villi. It is continuously renewed by multipotential intestinal epithelial stem cells. The integrity of the epithelial barrier rests upon the structure of cells and their junctional complexes: desmosomes, zonula adherens, and tight junctions. In response to a variety of stimuli, tight junctions are continuously opened and closed creating the dynamic functional state of the intestinal barrier.

More and more experimental and clinical evidence suggests that the two major factors capable of altering intestinal permeability are high-energy diet and gut microbiota modifications [7].

Depending on the dose and strain, microbes of the human intestine are able to influence, via various mechanisms, the barrier strength and its functional integrity.

First of all, some probiotics, like *Lactobacillus plantarum* MB452, have been proven to directly alter tight junction protein expression [8]. Moreover, bacterial influence on gut permeability also results from releasing a variety of molecules: soluble peptides, cellular structural components, metabolites, or even toxins [9]. Those bacterial metabolites, called postbiotics, including short-chain fatty acids – acetate and butyrate, have been shown to directly enhance the intestinal epithelial barrier function and therefore protect against pathogens [10, 11]. What is more, microbes can influence the epithelial barrier also in an indirect manner – through their impact on host immune cells and the release of cytokines [12].

Manipulation of the gut microflora with probiotics, antibiotics, or microbial products results in both an attenuation of disease and a restoration of normal gut permeability [9].

According to pathophysiology of allergy, dysregulation of a T-helper type-2 response and hypersensitivity reactions upon antigen re-exposure are the key mechanisms that initiate IgE-sensitisation and recruitment of mast cells in the gastrointestinal mucosa. These pre-sensitised mast cells can be triggered directly by allergens in the lamina propria. During an effector phase of allergic inflammation, recruitment of mononuclear inflammatory cells results in the release of a number of proinflammatory cytokines as well as mast cell degranulation, which leads to increased intestinal permeability and transepithelial allergen transport, thereby inducing the inflammatory reaction [13].

BIOMARKERS OF GASTROINTESTINAL INFLAMMATION

The World Health Organisation (WHO) defines a “biological marker” as “any substance, structure, or process that can be measured in the body or its products whose detection indicates a particular disease state” [14]. The term “biomarker” contains multiple categories of disease indicators, including disease-specific molecules such as pathogen-specific proteins, host-response molecules such as immunoglobulins, physiological measurements such as blood pressure, or results from imaging technologies.

A useful biomarker should directly and uniquely identify a disease-causing agent, be easy to detect in multiple settings, and clearly identify whether a disease is currently active [15]. Nowadays, searching for proper biomarkers is one of the main research targets throughout medicine.

SUGAR ABSORPTION TEST

The non-invasive assessment of intestinal permeability in humans has been known for almost 30 years and served mainly as diagnostic screening for small bowel diseases. The basic tool for epithelial permeability assessment is the sugar absorption test (SAT). It is based on passive absorption of probes from the GI tract and com-

plete elimination by the urinary tract. After 5–6 hours of urine collection, samples are analysed using high-pressure liquid chromatography (HPLC) or liquid chromatography in combination with mass spectrometry (LC/MS).

The permeability of selected parts of the intestine is measured by applying specific probes: for assessment of stomach and proximal duodenum permeability – sucrose; for small intestine – lactulose; and for the whole gut – sucralose [16]. Using their combination, isolated colon permeability can be evaluated by 24-hour lactulose excretion subtracted from 24-hour sucrose excretion [17]. For this reason, a recent approach for assessing gut permeability is to use “multisugar tests”, like the one based on a combination of sucrose, lactulose, sucralose, erythritol, and rhamnose simultaneously [18].

However, until now, the lactulose-to-mannitol ratio (LMR) has been the most widely used measurement for routine diagnostics and scientific purposes [19]. Lactulose is a large molecule that consists of galactose and fructose. Due to the lack of specific enzymes converting lactulose in human intestine, this disaccharide is the most reliable measure for diagnostic purposes. Quantities of lactulose that are absorbed through intercellular spaces indicate small intestinal damage. In further parts of the GI tract lactulose is metabolised by residual bacteria or neutralised by other agents like mannitol. In contrast, mannitol is a small monosaccharide that is absorbed via trans/paracellular pathways of healthy small intestine and absorption of which is strictly dependent on the surface of the villi area. Determination of the probe ratio, rather than the rate of movement of any one probe, minimises confounding factors such as defects in collection, gastric retention, transit time, and renal clearance. LMR advantages include the following: safety, low cost, and satisfactory correlation with intestinal pathophysiology. As well as it being time consuming, Denno *et al.* revealed other limitations LMR such as: lack of standardisation of assay method and specimen collection, and no standard values for specific populations [20].

Increased permeability detected by LMR has served as a screening method for enteropathy and FA as well as response to treatment, and for assessing the prognosis in coeliac disease and Crohn’s disease [21]. The role of increased intestinal permeability in developing FA was confirmed in a group of patients on a calcineurin inhibitor therapy after solid organ transplantation [22]. Kalach *et al.* concluded that LMR with a cut-off level of 3.95% reached a performance value of 76%, which exceeded the levels of IgE, IgG, skin prick test, or atopy patch test [23]. The authors underlined that the highest sensitivity was observed in patients with non-IgE-mediated allergy.

CITRULLINE

A growing number of data support the implementation of citrulline as a non-invasive, simple, and accurate tool for estimating intestinal permeability.

Citrulline is an amino acid produced from glutamine by intact small bowel enterocytes. Recent studies revealed that citrulline plasma concentration presents direct correlation with the mass of intestinal epithelial cells and thereby can be used as a marker of gut status in short bowel syndrome [24].

A study conducted on patients with coeliac and non-coeliac villous atrophy revealed that citrulline levels were significantly lower than in controls and increased during a year of successful treatment [25]. Other possible clinical indications for citrulline assessment include detection of intestinal damage during chemotherapy and necrotising enterocolitis (NEC) [26, 27].

In comparison to LMR, citrulline has been proven to be a more sensitive and specific method for bowel leakage diagnostics [28]. In a recently published systematic review and meta-analysis, it was estimated that its sensitivity and specificity on the level ~80% indicates intestinal insufficiency [29]. In a study of non-invasive biomarkers for assessment of villous abnormalities citrulline was proven to be the most reliable [30].

In terms of allergic diagnostics, citrulline may replace LMR in assessing intestinal permeability and seems to be a particularly promising marker for food-induced enteropathy. However, until now no sufficient data has been collected to establish citrulline’s role in FA diagnostics.

INTESTINAL-FATTY ACID BINDING PROTEIN

Another diagnostic tool for assessing epithelial cell integrity is intestinal-fatty acid binding protein (I-FABP). It is a member of larger protein family of fatty acid binding proteins (FABPs) that until now have been identified in such organs as the heart, intestine, liver, epidermal layer of skin, muscle, and fatty tissue. FABPs are small, water-soluble molecules that transport fatty acids from apical membrane of enterocyte to the endoplasmic reticulum for further biochemical reactions. Depending on the localisation in the GI system, three types of FABP were identified: intestinal FABP (I-FABP), found predominantly in the jejunum; liver FABP (L-FABP), derived mostly from liver; and ileal bile acid binding protein (I-BABP), exclusively bound to the ileum. The concentration of all FABPs can be obtained from plasma, serum, or urine using an enzyme-linked immunosorbent assay (ELISA) [31].

Urine measurements present a valuable alternative for circulating I-FABPs with the advantage of relatively non-invasive (and blood sparing) sample collection and detection over a longer period of time (half the time of circulating I-FABP – 11 minutes) [7].

I-FABP basal levels reflect the physiological turnover rate of enterocytes, which was confirmed by Derikx *et al.* [32]. This study involved a group of 34 patients with intestinal mucosal barrier injury resulting from myeloab-

lative therapy, presenting significant decrease in serum levels of I-FABP and I-BABP from the day of transplantation until day 14.

Elevated levels of both circulating and urine I-FABP are believed to indicate intestinal epithelial cell damage, which has been observed in patients with intestinal ischaemia, systemic inflammatory response syndrome (SIRS), and NEC [31, 33]. Schurink *et al.* showed that in terms of NEC, urine I-FABP could serve as a reliable biomarker for complicated course of disease; even in the earliest stages of the disease [7]. On the other hand, serum concentration of I-FABP has also been applied for the assessment of disease activity in celiac disease [34]. Therefore, I-FABP has emerged as a possible non-invasive marker for evaluating gut wall integrity loss and inflammation in FA. However, further studies are required to confirm such predictions.

REGENERATING GENE 1 α

Studies on the pathophysiology of autoimmune diseases led to the discovery of the regenerating protein (Reg) family. They are involved in the process of cell proliferation, differentiation, inflammation, and carcinogenesis [35]. In humans, until now, five functional Reg genes have been isolated: *REG1 α* , *REG1 β* , *REG3*, *HIP/PAP*, and *REG4* [36]. Reg family proteins were identified as acute phase reactants, anti-apoptotic factors, and growth agents [36].

Primarily, regenerating gene 1 α (*REG1 α*) expression was found in various types of tissues in the course of autoimmune diseases, like in salivary glands in Sjögren's syndrome or in pancreatic islets in type 1 diabetes. Further studies revealed the highest expression of *REG1 α* within the small intestine [35]. Until now *REG1 α* involvement was confirmed in such diseases as gastritis, pancreatitis, GI cancer, IBD, and cystic fibrosis [35]. However, at this point the most promising field for *REG1 α* practise application is celiac disease. In a preliminary study by Planas *et al.* increased serum levels of *REG1 α* reflected the destructive process of the villi, followed by significant reduction after a gluten-free diet [37]. Singh *et al.* compared serum concentration of *REG1 α* and plasma levels of I-FABP with their tissue expression in duodenal biopsies in 131 patients with CD, before and after a gluten-free diet. The results showed that levels of both, I-FABP and *REG1 α* were significantly higher before treatment, while their tissue expression was lower before treatment. After introduction of a gluten-free diet, a decrease in plasma I-FABP and serum *REG1 α* levels was observed, parallel to a significant increase of tissue expression [30].

Because celiac disease and FA share a similar pathophysiological process of immunological inflammation, biomarkers like *REG1 α* might have diagnostic value for both of them.

FAECAL CALPROTECTIN

The allergic inflammation process developing within intestinal wall involves multicellular infiltration including neutrophils. Activated neutrophils infiltrate the gut mucosa and products of their activation, like faecal calprotectin (FC), can be later detected in faeces [38].

FC is a zinc- and calcium-binding protein complex composed of one light and two heavy chains of the S100 family. It is a main neutrophil cytosol protein of a chemotactic, antibacterial, and proliferative properties. Although mostly associated with neutrophils, in smaller amounts, calprotectin is also present in monocytes, macrophages, bone marrow cells, squamous epithelial cells, mucosal epithelial cells, microvascular endothelial cells, and activated fibroblasts [39]. FC is known to be resistant to proteolytic enzymes, which makes it an easily accessible and reliable diagnostic tool [38].

Increased levels of FC indicate and closely correlate with the severity of inflammatory infiltration within the intestinal wall [40]. Furthermore, elevated FC may even precede typical inflammatory findings in endoscopic examination, which has been observed in IBD [41]. However, it should be remembered that the concentration of FC in stool varies with age. The cut-off point for adults and children over four years of age was established at the level of 50 $\mu\text{g/g}$ [42]. In terms of IBD diagnostics, higher sensitivity was observed for concentrations over 100 $\mu\text{g/g}$ [43]. Cut-off points in younger age groups are more difficult to present because the level of FC depends on a number of factors, such as gestational and/or postnatal age or feeding patterns with either breastmilk or milk formula. In a study by Ezri *et al.* the cut-off level was found to be < 350 $\mu\text{g/g}$ in the first year of life and < 275 $\mu\text{g/g}$ during childhood [44].

Based on literature overview, FC measurement in children was valuable in the diagnostics of IBD, infectious diarrhoea, GI complications in juvenile idiopathic arthritis, NEC, colonic polyps, non-steroidal anti-inflammatory drug-induced enteropathy, cystic fibrosis, and follow-up in coeliac disease [45, 46]. Until now its most significant role is focused on diagnosis, the monitoring of remission and mucosal healing, and in the prediction of relapse in paediatric IBD.

Nevertheless, a growing body of clinical data suggest that FC is a valuable test for FA diagnostics in children [23, 45–48]. Waligora-Dupriet *et al.* found that the level of FC in infants with FA was twice that of those without FA [45]. Beser *et al.* indicated that in non-IgE-mediated phenotypes of FA, FC tends to present significantly higher levels than in IgE-mediated ones (889 \pm 278 $\mu\text{g/g}$ vs. 392 \pm 209 $\mu\text{g/g}$), leading to the conclusion that it is also more useful to determine relapses and the follow-up of patients in the non-IgE-mediated group [47]. Finally, Kukkonen *et al.*, in a prospective study upon over 230 children participating in a randomised double-blind placebo-

controlled allergy-prevention trial with probiotic strains, noted that increased calprotectin levels at six months of age were associated with lower risk of later atopic sensitisation [48].

EOSINOPHIL-DERIVED NEUROTOXIN

As mentioned above, allergic inflammation is associated with recruitment of leukocytes, particularly eosinophils. They are mobilised in response to Th2 stimuli characteristic for allergic inflammation and parasitic helminth infection. Parallel to neutrophils, their activation leads to the release four major cytosolic proteins, including eosinophil-derived neurotoxin (EDN) [49]. From previous studies, it is also known that eosinophil protein X (the terms EPX and EDN refer to the same molecule) together with eosinophil cationic protein (ECP) belong to the so-called RNase A superfamily [49]. Since its first purification in 1981, scientists have uncovered neurotoxic, chemotactic, transductive (via Toll-like receptors), and antiviral properties – particularly against respiratory syncytial virus (RSV) [50].

EDN concentration was studied in plasma, urine, and stools in a number of inflammatory diseases, including asthma and atopic dermatitis [51–53]. In terms of FA, Ada *et al.* studied a group of children diagnosed with FPIES, in whom the level of EDN in stools was tested before and after oral food challenge. It revealed strong positive correlation of the biomarker, reaching its maximum concentration after 24 hours of exposure [53]. Further studies confirmed the findings for FPIP and FPIE, together with an observation of particularly high EDN levels in bloody stools [54]. Kalach *et al.* estimated the diagnostic accuracy for EDN to be 72% and concluded that faecal EDN appeared to be more sensitive and closely correlated with the severity of illness than blood and urinary measurements in atopic dermatitis and atopic asthma [23].

TUMOUR NECROSIS FACTOR α

The last but not least promising biomarker of FA is tumour necrosis factor α (TNF- α). Since the discovery of

TNF in 1962, research has revealed a further 19 agents, creating the TNF superfamily and demonstrating their proinflammatory properties. The role of TNF- α in inflammation, apoptosis, proliferation, angiogenesis, metastasis, and morphogenesis has been well-documented and used in diagnostics in neurology (depression, bipolar disorder, epilepsy, Alzheimer's disease, Parkinson's disease, multiple sclerosis), pulmonary diseases (asthma, chronic bronchitis, chronic obstructive pulmonary disease, acute lung injury, acute respiratory distress syndrome), and autoimmune diseases (uveitis, multiple sclerosis, systemic lupus, arthritis, psoriasis, and Crohn's disease) [55].

Currently it is believed that TNF- α also plays a crucial part in the pathophysiology of allergy. Two separate studies by Hayman *et al.* and Rodriguez *et al.* have shown that TNF- α initiates a process of increased intestinal permeability [56, 57]. This role of TNF- α was confirmed by further studies indicating high molecule expression in epithelial cells and mononuclear blood cells derived from the intestines of patients with FPIES [58]. It is interesting that TNF- α was also detected in the stools of patients undergoing oral food challenge with confirmed cow's milk protein allergy [59]. Upon antigen-specific cytokine secretion profiles of 89 blood samples from Japanese children with FA Morita *et al.* concluded that particularly high levels of TNF- α indicate a critical role of that cytokine in intestinal epithelial cell damage and eosinophil infiltration [60]. The authors suggest that utility of TNF- α testing expands beyond FPIES and can be useful for both IgE-mediated and non-IgE-mediated subtypes of allergy. Further studies are warranted to confirm the diagnostic value of TNF- α .

CONCLUSIONS

The presented paper indicates that there are a number of promising markers for non-IgE-mediated FA diagnostics (Table 1). Some of them, such as LMR and FC, seem to play a well-established role in the process of diagnosing and monitoring the disease's activity. Others, like citrulline, EDN, and TNF- α , need further prospective confirmatory studies on a larger scale. However, it is unlikely that any of the selected methods will become a single,

TABLE 1. Potential biomarkers according to their origin (epithelium, infiltrate derived and matrix)

Origin		LMR	Citrulline	FC	I-FABP	REG1 α	EDN	TNF- α
Epithelium derived	Enterocyte integrity	X	X					
	Enterocyte injury			X	X			
	Enterocyte regeneration					X		
Infiltrate derived							X	X
Matrix	Blood	X	X		X	X	X	X
	Urine	X			X		X	
	Faeces			X				x
	Biopsies		X		X	X		

LMR – lactulose-to-mannitol ratio, FC – faecal calprotectin, I-FABP – intestinal fatty acid binding protein, REG1 α – regenerating gene 1 α , EDN – eosinophil-derived neurotoxin, TNF- α – tumour necrosis factor α

stand-alone test, particularly in terms of the delayed onset, non-IgE-mediated mechanism, which is most common for FA. It is more probable that the combination of those will meet satisfactory levels of sensitivity and specificity for simple, accurate, and non-invasive FA diagnostics.

DISCLOSURE

The authors declare no conflict of interest.

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