

A kinetic study in adults with food hypersensitivity assessed as eosinophil activation in fecal samples

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Summary

Background Immune-mediated food hypersensitivity affecting the gut is difficult to evaluate, and objective tools to diagnose local gastrointestinal (GI) inflammatory reactions are lacking.

Objectives To determine whether allergic manifestations in adults with a history of food-related GI symptoms could be assessed in feces during symptomatic and non-symptomatic periods, using the surrogate markers, eosinophil cationic protein (ECP), eosinophil protein X (EPX) and myeloperoxidase (MPO).

Methods Thirteen subjects with food hypersensitivity-related GI symptoms, confirmed by a positive double-blind placebo-controlled food challenge (DBPCFC), were subjected to an open kinetic food challenge design for 6 weeks. Symptoms were recorded and scored during the 3-week study period and stool samples were obtained every day. The surrogate markers ECP, EPX and MPO were measured in the supernatants from feces samples.

Results A significant increase in abdominal pain, distension and flatulence was observed during challenge, with a gradual decrease during elimination diet. Both between days and subjects, EPX levels were more frequently increased compared to ECP and MPO. Individuals with a history of a short duration of symptoms had significantly higher mean levels of EPX and MPO than those with a longer duration of symptoms.

Conclusions An overall increase in levels of eosinophil markers, in particular EPX, was observed in feces from patients with food-related GI symptoms. However, rather than being a tool to differentiate symptomatic from non-symptomatic periods, EPX might be used for detecting an ongoing clinical or subclinical chronic inflammation, that may have an impact on the patient's clinical course of GI symptoms.

Keywords eosinophil cationic protein, eosinophil protein X, fecal samples, food-induced gastrointestinal hypersensitivity, kinetic study, neutrophil MPO

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Introduction

Various gastrointestinal (GI) symptoms occur in a large number of patients with food allergies and intolerance. Reactions to food may cause nausea, vomiting, abdominal pain and diarrhoea [1]. Adverse reactions to food is a major medical problem with up to 25% of the population reporting symptoms [2], but only 1–2% of the whole population has food allergy, as confirmed by double-blind, placebo-controlled food challenge (DBPCFC) [3, 4].

DBPCFC is considered the gold standard for the diagnosis of food hypersensitivity, although it does not discriminate between food allergy and non-immune-mediated food intolerance [5–8]. Owing to its time consumption and expense, it is rarely used in daily practice [2]. A weakness is that there is no international agreement on such essential issues as quantity of the test food used in trials, time between challenges, increasing of dose, etc [8, 9].

No single laboratory test or other procedures like skin prick test (SPT) or IgE-antibodies in blood can offer an effective diagnostic tool for food allergy. Especially, in many adult food hypersensitive patients negative in SPT and serum IgE-antibody, the mechanisms of intestinal hypersensitivity reactions to food are not well understood.

A number of studies have shown that the allergic reactions to food antigens are associated with infiltration of inflamma-

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tory cells, that upon activation release mediators contributing to the pathogenesis of the disorders [10–14]. One of the most notable inflammatory cells of allergic diseases is the eosinophil [15]. Upon activation, eosinophils release several cationic proteins from their granules e.g. major basic protein (MBP), eosinophil cationic protein (ECP), EPX/(EDN (eosinophil-derived neurotoxin)) and eosinophil peroxidases (EPO) [16]. These basic proteins exert a range of biologic effects contributing to local tissue damage and dysfunction [17].

Previous studies have shown that MBP and ECP are present in the sputum during severe asthma attacks [18], and are localized to sites of epithelial injury [19]. ECP is also present in bronchoalveolar lavage fluid [20] and in lavage fluids of the gut [21–23]. Increased levels of ECP and EPX in nasal lavage in atopic infants have also been demonstrated [24]. Studies by the present authors have also shown significantly increased numbers of MBP⁺ and IgE⁺ cells in duodenal biopsies in food-challenged DBPCFC-positive patients, with negative SPT and serum-IgE antibodies [12] and in IgE-mediated birch pollen-allergic patients in the pollen season (Magnusson J et al., in manuscript).

Adverse reactions to foods causing GI symptoms are sometimes accompanied by local inflammatory reactions, accordingly tools are needed to determine the presence and extent of such reactions locally. Data regarding the presence of increased levels of fecal ECP and EPX in food hypersensitive patients are scarce, particularly in relation to time [25–28]. We performed a kinetic study to investigate further the potential role of the eosinophil and the neutrophil in the GI tract by measuring fecal ECP, EPX and MPO over time during periods of variable symptoms. We also wanted to establish whether these markers could be used as indicators of inflammation, in SPT and serum IgE-antibody negative patients and to help explore the pathophysiological mechanisms in food hypersensitivity with GI symptoms.

Materials and methods

Study population

Thirteen consecutive patients with food hypersensitivity-related GI symptoms were included from the Department of Respiratory Medicine and Allergology, Sahlgrenska University Hospital, Gothenburg, Sweden. The median age and range of the three males and the 10 females was 52 years and 26–68 years. Of the patients, 10 were SPT (Soluprick, ALK, Hørsholm, Denmark) and serum-IgE antibody (Pharmacia CAP system[®], Uppsala, Sweden) negative to the tested foods.

The patients were verified positive to staple foods by means of DBPCFC (Table 1). The screening included diaries as well as elimination diets and open and blinded challenges. DBPCFC were performed with fresh food mixed in drinks, depending on whether the expected symptoms were objective or subjective with two or six challenges on separate days [29]. The criterion for positive challenge was the appearance of expected symptoms on active challenges and no symptoms with placebo. Some patients were challenged for several consecutive days, since they were expected from their clinical history to develop additional symptoms 3–5 days after exposure. After the challenges, the patients recorded their presence of symptoms in a symptom protocol during the following days, together with the time of onset and duration of the symptoms. When all the challenges were completed, the forms were assessed, and then the code was broken.

All six patients with milk hypersensitivity were SPT and serum IgE-antibody negative for milk and tolerated lactose. Furthermore, the presence of intestinal inflammatory diseases and coeliac disease was excluded in all patients according to international criteria [30]. Seven of the patients were positive in SPT for inhalant allergens and five patients had positive SPT for other foods. Clinical characteristics, including symptoms after DBPCFC and laboratory test results of the subjects, are shown in Table 1.

Table 1. Clinical features in the 13 DBPCFC positive patients at inclusion

No.	Age/sex	Food DBPCFC positive	SPT/serum IgE-antibody for DBPCFC food	Time of onset of symptom (h)	Duration of symptoms (h)	Symptoms after challenge	SPT inhalants/other foods than DBPCFC tested	IBS diagnosis
1.	68/F	Milk	–/–	1	12–24	AD, D, N	+/+	–
2.	68/F	Milk	–/–	5 min	12–24	AD, AP, A, D, N	–/–	+
3.	64/F	Milk	–/–	5 min	12–24	AD, D, OMB	–/–	+
4.	63/F	Wheat	–/–	5 min	24–48	AP, D, OI, RP	–/–	–
5.	56/F	Wheat	–/–	1	12–24	AP, D, OI, OMB	+/–	–
6.	55/M	Milk	–/–	1–24	24–48	AP, D	+/–	+
7.	52/F	Wheat	++/2	30 min	12–24	AD, AP, D, OI	+/+	+
8.	44/F	Milk	–/–	24	24–48	F, N, RP	–/–	+
9.	42/F	Wheat	++/4	24	24–48	AD, AP, D, F, N	+/+	+
10.	38/F	Meat	+/1	1–24	24–48	AP, N	+/+	+
11.	38/F	Soy	–/–	1	12–48	AD, D, E, F, N	+/+	+
		Milk	–/–					
		Egg	–/–					
12.	35/M	Wheat	–/–	24	24–48	AP, D	–/–	–
13.	26/M	Egg	–/–	1	12–24	AP, C/D	–/–	–

N = 13.

A = asthmatic symptoms, AD = abdominal distension, AP = abdominal pain, D = diarrhoea, E = eczema, F = flatulence, N = nausea, C = constipation, OI = oral itching, hoarseness, irritation at palate, swollen feeling in the throat, phlegm, pain and redness in the throat, OMB = oral mucosa blebs, pain and irritation in the tongue and palate, RP = rheumatic pain in wrists, shoulders and elbows.

– = negative, + = positive.

Table 2. Clinical history and atopy-related features in the 13 DBPCFC-positive patients with histories of food-related gastrointestinal symptoms

Case	Life-long food hyper-sensitivity	Foods reacted at as a child	Asthma	Rhinitis and/or conjunctivitis	Eczema	Atopic family
1.	+	Chocolate, pear, egg: AP, D until teenaged, then milk products	—	+se	+se	+
2.	—	—	+se	+se	+se	—
3.	+	Milk products: AP, D, F, V 5 ya	—	—	—	—
4.	—	—	—	—	+5–9 ya	+
5.	—	—	—	—	—	+
6.	—	—	—	—	—	+
7.	—	—	—	—	+7–10 ya	+
8.	+	Milk products: AP, F, N, teenaged	—	—	+10 ya	—
9.	—	—	—	—	—	—
10.	—	—	—	—	—	—
11.	—	—	+1 ya	+1 ya	+se	+
12.	+	Egg, chocolate: E, 5 ya	—	—	+se	—
13.	+	Egg; AP, D 1 ya	+3 ya	+se	—	—

N = 13.

ya = years of age at initial onset of symptoms.

se = since early childhood, <1 ya.

Other abbreviations same as in Table 1.

The clinical history showed that five of the 13 patients had life-long food hypersensitivity with GI symptoms. Seven of the patients had eczema, four had rhinitis and/or conjunctivitis and three had asthma as children, with onset in early age and remission of the symptoms in the teenage years. An atopic family history was found in six of the 13 patients, Table 2.

The patients were asked to answer a questionnaire if they had severe GI symptoms in birch pollen season, and if they fulfilled the irritable bowel syndrome (IBS) criteria (Rome II criteria, described elsewhere) [31]. The study was performed out of birch pollen season from January to March 2001 to ensure minimum of exposure to birch pollen and limited exposure of cross-reaction to alder and hazel.

Experimental design

The patients were subjected to a 6-week long study period, (Fig. 1), starting with a 'wash-out' week (7 days), when all foods that could cause symptoms were avoided. In the following week, the patients were still on the elimination diet and fecal samples were obtained at every time of defecation. In the third week, the patients still collected feces samples in the same manner as in week 2, but they were now challenged for 7 days with the type of food that they were positive for in DBPCFC. The patients were instructed to consume that particular food in doses that would give rise to significant symptoms. In the fourth week, the patients were encouraged to an elimination diet, and fecal samples were obtained at every time of defecation. In weeks 5 and 6, the patients still followed the elimination diet and fecal samples were taken only once a week (day 7). Symptoms of abdominal pain, distension, flatulence and nausea were scored 1–3 by the subjects and registered in their symptom diaries. The subjects were also requested to record the amount of challenged foods, motion frequency and stool consistency. During the whole study period antihistamines, corticosteroids, leucotriene antagonists and sodium cromoglycates, and in case of birch

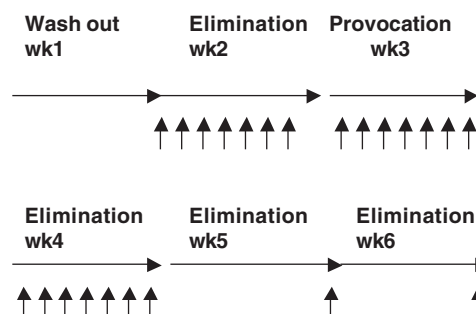


Fig. 1. Study design. Vertical arrows indicate days with collection of stool samples.

pollen allergy, birch-related foods (hazelnut, kiwi fruit, peanut, apple, pear), which could influence the results were avoided.

Informed consent was obtained from the participants. The Ethics Committee of the Medical Faculty, University of Gothenburg approved the study protocol.

Feces preparation

Stool samples were collected in plastic tubes using a spatula in the patients' home and kept in the freezer until delivery to the clinic of Asthma and Allergy. Samples were then kept at -30°C , until analysis. Processing of stool samples was carried out according to Peterson et al. [32]. Briefly, approximately 0.1–1.5 g of thawed feces were weighted and diluted five times by adding four volumes (v/w) of an extraction buffer consisting of PBS, pH 7.4, supplemented with 10-mM EDTA, 0.2% CTAB (*N*-cetyl-*N,N,N*-trimethylammonium bromide), 20% glycerol, 0.05% Tween20 and 1% BSA. The mixture was homogenized using a Polytron PT1200 CL mixer (Kinematica AG, Luzern, Switzerland) for 5–90 s until a homogenous solution was obtained. An aliquot of 0.5 mL of the homogenate was thereafter further diluted 20

times in the extraction buffer and centrifuged at 20 800 *g* for 30 min at 5 °C. The supernatant without particles was frozen at -27 °C for later analysis.

Determination of ECP, EPX and MPO in feces

ECP, EPX and MPO were analysed using commercial radioimmunoassays according to the manufacturer's instructions (Pharmacia Diagnostics AB).

Levels of inflammation markers were adjusted to feces semidry weight as described [32]. Using the feces preparation method and assays described above, we have previously shown the upper normal limit (95th percentile) of ECP, EPX and MPO to be 6.4, 1.7 and 8.8 µg/g, respectively, when assessed in feces obtained from 44 healthy individuals with a median age of 43.5 years (range 18–73) [32].

Determination of IgE-antibodies in serum

The food-specific IgE-antibodies were measured with Pharmacia CAP system according to the manufacturer's instructions (Pharmacia Diagnostics AB).

Skin prick test

SPT was performed and the reactions were graded according to the recommendations of the Standardization Committee of the Northern Society of Allergology [33]. Weal reactions corresponding to the 10 mg/mL histamine reference were recorded as +++; double this size +++, half the histamine reference size was recorded as ++ and half of that size as +.

Statistical analysis

Averaged values of symptom scores were calculated as mean values. The statistical analyses were carried out using Statistica® on a PC. The Wilcoxon matched pairs test and

Mann–Whitney *U*-test for unpaired data were applied. Correlation was calculated using Spearman's correlation. The level for significance was set at $P < 0.05$.

Results

Symptoms

Two of six birch pollen allergic patients, as well two patients not birch pollen allergic, reported severe GI symptoms (abdominal distension, nausea, diarrhoea, flatulence and abdominal pain) in the birch pollen season. Eight patients fulfilled the IBS criteria, Table 1.

Recording of symptoms at home by the study participants revealed that two subjects, nos. 1 and 5, had increased baseline symptoms of abdominal pain and distension. The other 11 patients had minor symptoms during the wash out and the first elimination diet period or decreased further during the first elimination period. In all subjects a significant, but variable increase in abdominal pain, distension and flatulence was observed during the challenge week (Fig. 2). A gradual decrease in these symptoms was then observed during elimination diet the following 3 weeks. The symptoms were generally mild to moderate during food challenge, except in three individuals (nos. 1, 5 and 8). These three individuals showed a fast and considerable increase of symptoms, both severe abdominal pain and distension. Two other individuals (nos. 11, 12) experienced severe abdominal pain and severe distension, respectively. The individual mean scores of distension and abdominal pain during the study period correlated well ($R_s = 0.7159$, $P < 0.0001$). No change was observed regarding number of motions during the 6 study weeks. Symptoms of headache, joint pain and uncomfortable abdominal sensation were experienced in three individuals and nausea was experienced in five individuals.

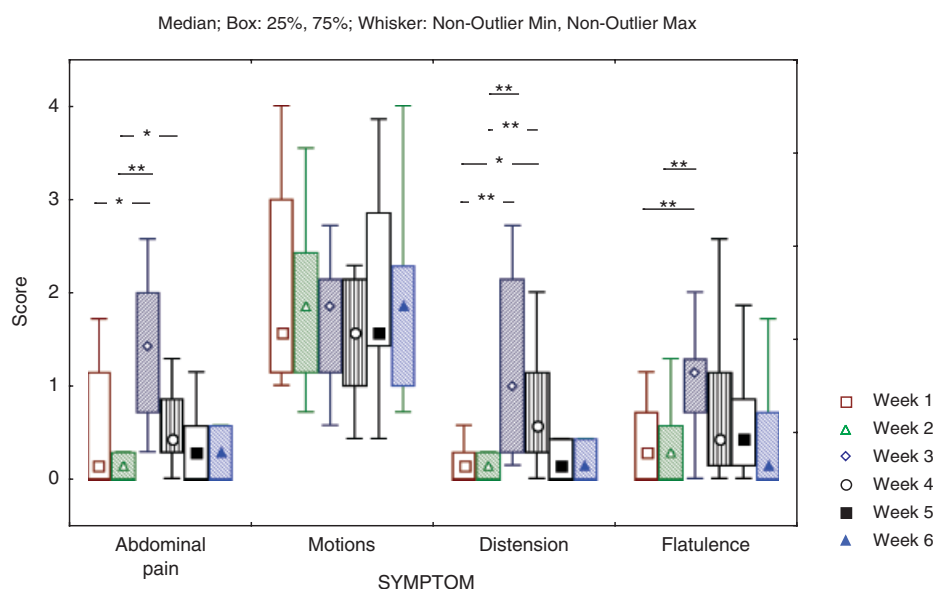


Fig. 2. Major symptoms experienced during the study. Median scores of the whole study group are based on individual mean scores of the respective study weeks. * $P < 0.05$, ** $P < 0.01$ (Wilcoxon's matched pairs test).

Inflammation markers

Inflammation markers were measured in the fecal samples collected every day during weeks 2–4. EPX levels during this 3-week period, were increased ($> 1.7 \mu\text{g/g}$ feces) compared to normal levels during the entire study period in four individuals (1, 2, 5 and 7) [32]. Five subjects had increased levels during 1–10 days, whereas one individual had increased levels during 19 days (Figs 3a and b). Three individuals had normal levels of EPX. In contrast, no individual had continuously increased levels of ECP ($> 6.4 \mu\text{g/g}$ feces) during weeks 2–4. The ECP levels were increased during 1–10 days in six individuals, whereas one individual had increased levels during 12 days. Six individuals had normal levels of ECP. MPO levels were not increased ($> 8.8 \mu\text{g/g}$ feces) during the

entire study period in any subject, but were increased 1–10 days in five individuals and 11–20 days in three subjects (Figs 4a and b). Five individuals had normal levels of MPO.

Relation between clinical symptoms and inflammation markers

Individuals with a history of symptoms, of short duration (12–24 h, $n = 6$, Table 1) had significantly higher mean levels of EPX and MPO than those with a longer duration of symptoms ($n = 7$) ($P < 0.01$ and 0.05) (Figs 3a and b and 4a and b). However, the marker response to the 7 days of challenge was generally unclear, except for one patient, no. 8. The relation between the clinical parameters and the

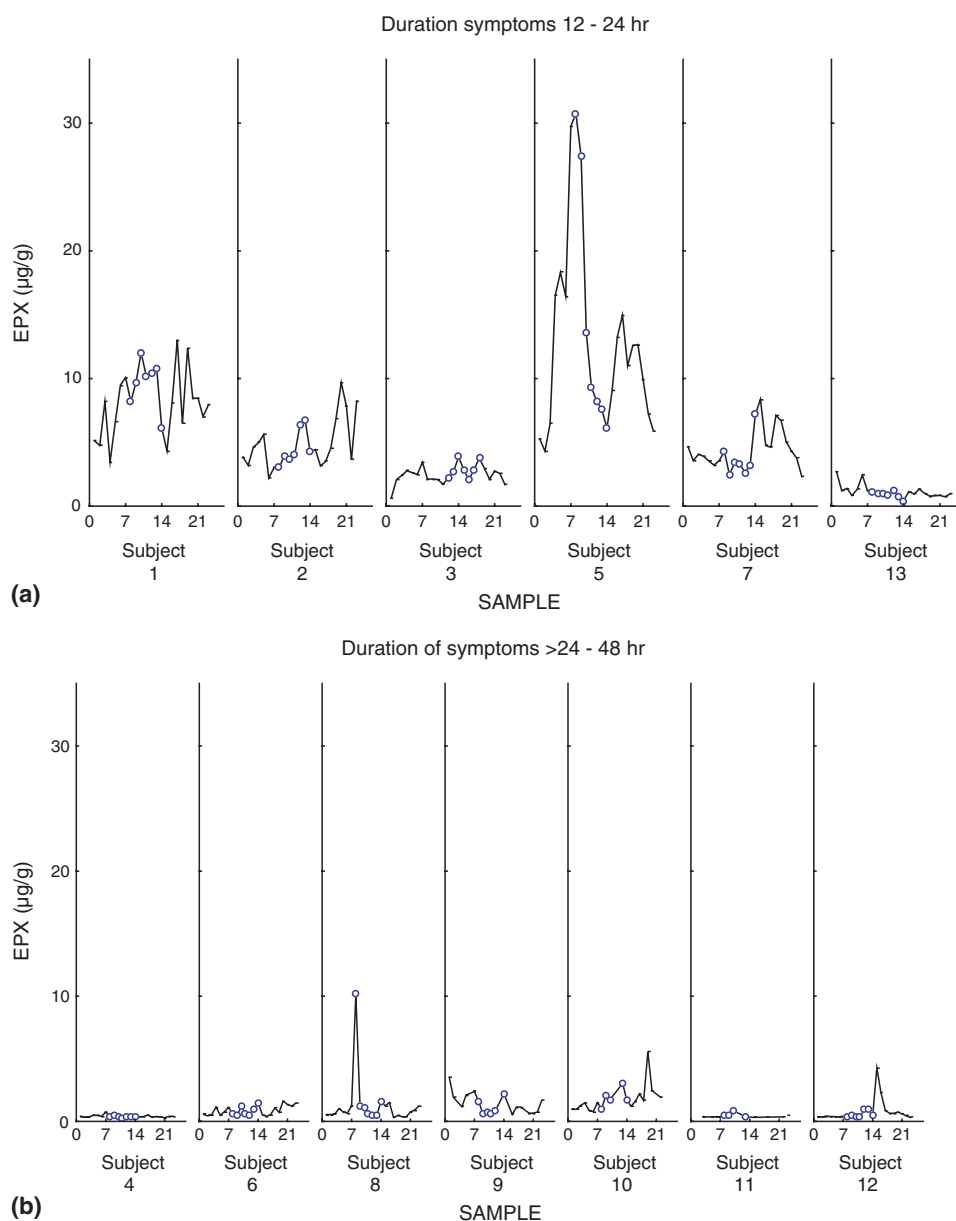


Fig. 3. Daily feces levels of EPX in subjects with different history of symptom duration. (a) Symptom duration between 12 and 24 h, (b) symptom duration between 24 and 48 h. Open circles denote days with food challenge. EPX levels $< 1.7 \mu\text{g/g}$ are considered as normal.

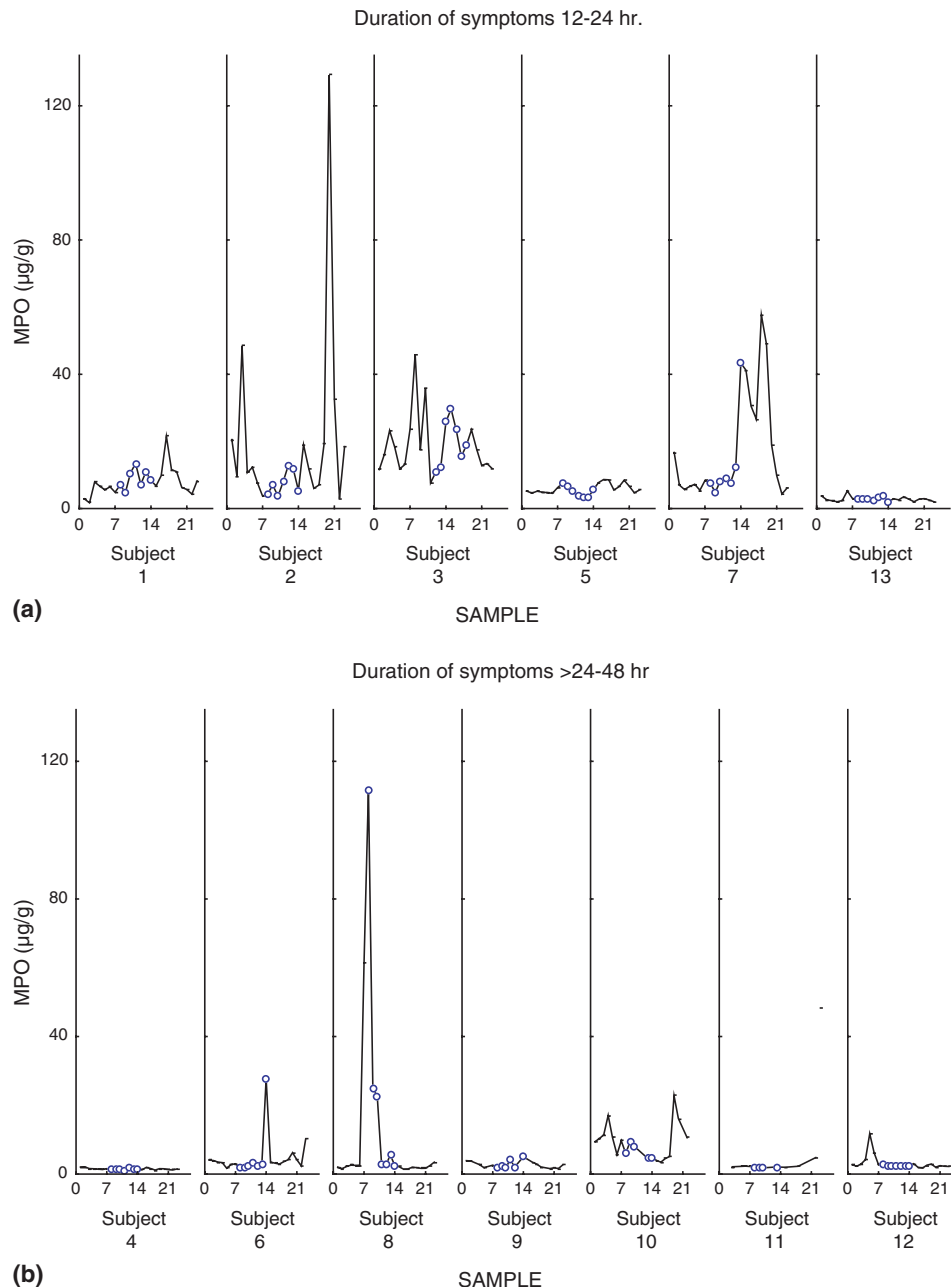


Fig. 4. Daily feces levels of MPO in subjects with different history of symptom duration. (a) Symptom duration between 12 and 24 h, (b) symptom duration between 24 and 48 h. Open circles denote days with food challenge. MPO levels $< 8.8 \mu\text{g/g}$ are considered as normal.

inflammation markers was evaluated. A poor agreement was observed on a daily basis between clinical parameters such as abdominal pain, distension, frequency of flatulence, frequency of motions and nausea, and the inflammation markers. However, considering the possibility that inflammation and clinical symptoms were related, but not necessary coincided, a comparison was made between the average symptoms and the average levels of ECP, EPX and MPO during weeks 2, 3 and 4. During week 2, a clear relation was found between the mean levels of EPX and mean scores of distension ($R_s = 0.7345$, $P = 0.0042$) and abdominal pain ($R_s = 0.6632$, $P = 0.0135$) (Fig. 5). In contrast, the relation

between ECP and symptom scores was less clear and not significant. Further, a weak, but significant, correlation was found between MPO and mean motion frequency during week 2 ($R_s = 0.3843$, $P = 0.048$). During week 3, the relation between mean EPX and mean scores of abdominal distension was still significant ($R_s = 0.6455$, $P = 0.0172$), but the relation to abdominal pain failed to reach significance ($R_s = 0.4862$, $P = 0.092$). However, a significant correlation was observed between mean ECP and mean scores of abdominal distension during week 3 ($R_s = 0.6235$, $P = 0.0228$). In contrast, during week 4, no significant correlation was observed between feces markers and symptoms.

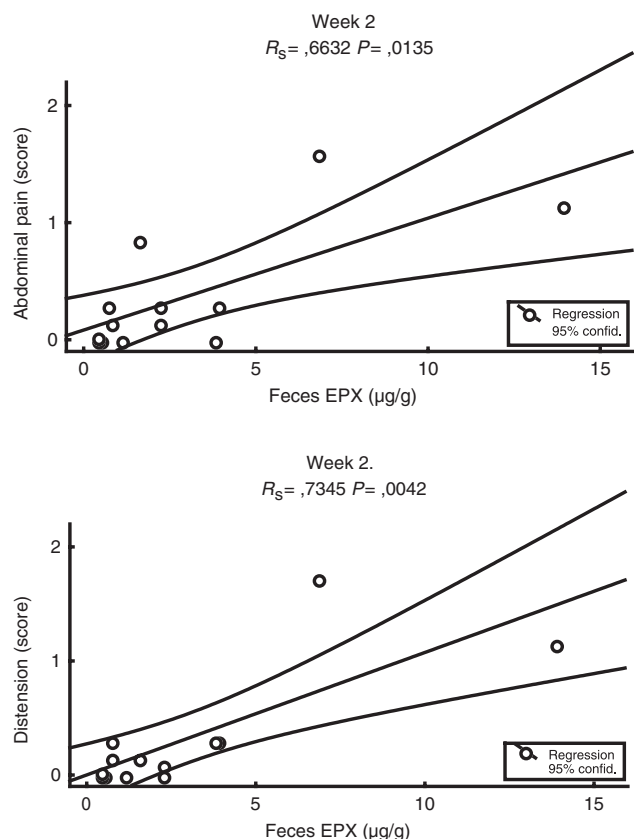


Fig. 5. The relation between symptoms and feces levels of EPX during week 2. Correlation between mean levels of feces EPX and (a) mean scores of abdominal pain and (b) mean scores of abdominal distension during week 2. Spearman's correlation coefficient, R_s , is given in the graph.

Discussion

Classical diagnostic tools, such as SPT and measurement of specific IgE in serum, are of limited value for confirming the diagnosis of food allergy. Elevated total IgE or high levels of specific IgE may suggest the presence of allergy. However, normal values of such parameters or negative SPT do not exclude intestinal food allergy, as shown by several studies [11, 34, 35].

In the present study, only 3/13 DBPCFC-positive subjects were either SPT positive or had serum IgE antibodies for tested food. This is in agreement with Bischoff [34], who found that most of the patients, who were positive in the colonoscopic allergen provocation (COLAP) test, had negative skin tests and low levels, if any, of specific IgE in serum against food antigens. Moreover, Bischoff [34] observed significantly higher serum levels of ECP and EPX in patients with positive COLAP than in those with negative COLAP. However, we failed to show any significant variation in the serum levels of ECP after positive DBPCFC in 11 of the patients included in the present study ([12], data not shown).

In the present study, we observe an overall local increase in levels of these eosinophil markers, in particular EPX, in feces from patients with a history of GI symptoms caused by hypersensitivity to food, irrespective of atopy status and SPT findings. Thus, elevated levels of EPX were found in 10 of the 13 patients on one or more occasions during the study.

Elevated fecal levels of the eosinophil markers ECP and EPX have previously been found in lower frequency, approximately 30%, in patients with intestinal food allergy [25, 34]. One explanation for this discrepancy in frequency could be the use of the improved sample treatment procedure in the present study [32]. Two weeks of elimination diet did not clear the GI symptoms or decrease the inflammation marker levels in feces of some patients. Our results indicate that some patients have an ongoing clinical or subclinical chronic inflammation in the GI tract characterized by an eosinophil infiltration, together with an increased cellular turnover or activation. Neutrophil activity (MPO) was, however, generally less pronounced in these patients, except for a few patients in whom the neutrophil activity dominated. Accordingly, 11 of the patients enrolled in the present study were previously shown to have significantly increased number of eosinophils in the duodenal mucosa after 7 days of food challenge [12]. Interestingly, the number of eosinophils was still significantly increased, compared to healthy individuals, after 2–3 months of elimination diet, even when the patients were asymptomatic [12].

The present study also included a 7-day period of open food challenges at home, which rendered a heterogeneous response with respect to inflammation markers and symptoms, with some patients seemingly responding very scantily. The reason for this could be that the study was designed as an open challenge study, which by itself may be associated with a risk of poor compliance of the recommended intake of the challenge foodstuff.

Eight of 13 patients fulfilled the ROME II criteria of IBS [31], and thus we cannot exclude that mechanisms other than a direct effect of the challenging foods are operating in some of the patients. Indeed, low-grade inflammation of the gut involving mast cells has been observed in subgroups of IBS patients [36, 37].

The eosinophil and neutrophil activities did not coincide with the daily symptoms, and this was especially noted during challenge. It is, however, noteworthy that the individual mean levels of the eosinophil marker EPX were closely related to average symptoms such as distension and abdominal pain before food challenge during week 2 and to distension during the challenge week. This implies that an ongoing inflammation could be of importance for the clinical course of food-related GI symptoms.

Interestingly, we observed that the majority of those patients who showed high levels of EPX during the study had a history of relatively short duration of symptoms (12–24 h), together with short time of onset of symptoms after challenge. This pattern of symptoms, together with eosinophil activity, may be a response similar to that described in atopic dermatitis as cutaneous late-phase response to allergen [38] with mast cell activation, synthesis and release of cytokines and chemokines like IL-5 and eotaxin and subsequent infiltration inflammatory cells such as eosinophils. In contrast, patients with a history of late onset (1–24 h) and long duration (>24 h) of symptoms did not show any increase in the inflammatory markers studied. An involvement of a T cell mediated (delayed-type) hypersensitivity cannot be excluded as the cause for symptoms in the latter group of patients. In summary, measurement of EPX in feces could be a valuable tool in the examination of the inflammatory status of the gut. However, the option of using

these markers, as an objective tool in the evaluation of a food challenge, should be addressed in separate studies with standardized blinded food challenges.

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