

Intestinal permeability and fecal eosinophil-derived neurotoxin are the best diagnosis tools for digestive non-IgE-mediated cow's milk allergy in toddlers

Nicolas Kalach^{1,2}, Nathalie Kapel^{3,4,*}, Anne-Judith Waligora-Dupriet⁴, Marie-Christine Castelain¹, Marie Odile Cousin¹, Christine Sauvage¹, Fatimata Ba¹, Ioannis Nicolis⁵, Florence Campeotto^{2,4}, Marie José Butel⁴ and Christophe Dupont²

¹ Clinique Pédiatrique Saint Antoine, Hôpital Saint Vincent de Paul, Groupe Hospitalier de L'institut Catholique de Lille, Université Catholique, Lille, France

² Service d'Explorations Fonctionnelles Digestives Pédiatriques, AP-HP, Hôpital Necker Enfant Malade, Université Paris Descartes, Paris, France

³ Coprologie Fonctionnelle, AP-HP, Groupe Hospitalier Pitié-Salpêtrière, Paris, France

⁴ Ecosystème Intestinal, Probiotiques, Antibiotiques (EA 4065), Université Paris Descartes, Sorbonne Paris Cité, Paris, France

⁵ Biomathématiques (EA 2498), Université Paris Descartes, Sorbonne Paris Cité, Paris, France

Abstract

Background: Food allergy is a common problem in France involving 4%–6% of toddlers. As opposed to IgE-mediated cow's milk allergy (CMA), delayed-onset CMA, mostly, non-IgE-mediated, remains difficult to diagnose in toddlers. Our study assessed the diagnostic performances of intestinal permeability and of fecal markers, in comparison with the standard allergic work-up in children referred for CMA diagnosis.

Methods: Twenty-five consecutive children, mean age (standard deviation) 6.3 months (4.8) with digestive and/or extra-digestive manifestations suggesting CMA, were prospectively studied based on a standardized allergic work-up (specific cow's protein IgE and IgG, skin prick test, atopy patch test and oral open cow's milk challenge) and digestive work-up including fecal microbiota analysis, intestinal permeability determination (urinary lactitol/mannitol ratio) and fecal markers measurement, i.e., α_1 -antitrypsin, tumor necrosis factor- α , calprotectin, β -defensin2, secretory IgA and eosinophil-derived neurotoxin (EDN). Receiver operating

characteristic (ROC) curves were calculated for all markers in order to define cut-off levels.

Results: The cow's milk challenge was positive in 11 children and negative in 14. The global test performances, i.e., the number of true positive+negative cases/the total number of cases, were 76% for intestinal permeability; 72% for fecal EDN; contrasting with atopy patch test, 68%; IgE, 60%; skin prick test, 55% and IgG, 52%.

Conclusions: In this routine diagnosis allergy work-up for CMA in toddlers, the best efficacy was seen for intestinal permeability compared to IgE, IgG, skin prick test and atopy patch test. Moreover, fecal EDN in a single spot sample displayed a similar performance.

Keywords: cow's milk allergy; eosinophil-derived neurotoxin; fecal biomarkers; gut microbiota; intestinal permeability.

Introduction

The standard work-up for food allergy relies on the elimination of and challenge with the suspected food, either using a double-blind placebo controlled food challenge or according to simplified procedures exhibiting a good correlation with it (1–4). In addition to the food elimination/challenge procedure, the diagnostic work-up may rely on different tests.

For IgE-mediated disorders, skin prick tests (SPT), guided by the clinical history, provide a rapid method to screen patients for sensitivity to specific foods. Negative SPT responses essentially confirm the absence of IgE-mediated allergic reactivity, with a negative predictive value >95% (4, 5). Furthermore, the level of the IgE titers seems to be significantly related to the results of the oral food challenge (4). In the absence of immediate clinical reactions, delayed-onset allergies – most of the time related to a non-IgE-dependant mechanism – still present diagnostic difficulties (4, 5). Several authors have therefore investigated the use of the atopy patch test (APT) for the diagnosis of this condition showing that this APT could improve the diagnosis of conditions related to cow's milk allergy (CMA) (6–8).

Intestinal permeability (IP), which compares the urinary excretion of two orally absorbed non-metabolizable sugars, lactitol (L), a marker of larger molecule absorption and mannitol (M), a marker of small molecule absorption, is altered during small intestinal suffering (9–11). IP has been shown to be modified in adults and in children with food allergy: the offending food decreasing M urinary excretion and increasing both L excretion and L/M ratio, a phenomenon that occurs during both digestive and cutaneous manifestations (12, 13).

*Corresponding author: Pr. Nathalie Kapel, Laboratoire de Coprologie Fonctionnelle, Groupe Hospitalier Pitié-Salpêtrière, 47-83 Boulevard de l'Hôpital, 75013 Paris, France
Phone: +33 1 42162652, Fax: +33 1 42162654,
E-mail: nathalie.kapel@psl.aphp.fr

Received December 22, 2011; accepted April 19, 2012

Fecal markers evaluate non-invasively the intestinal inflammatory responses and have found a reliable place in gastroenterology, for diagnosing inflammatory processes (14). In contrast, data remain scarce and often controversial during food allergy. In addition, the composition of the microbiota has been incriminated in the development of food allergy (15) and seems to interact with intestinal permeability (16).

The aim of our study was 1) to confirm the performance of IP measurement for the diagnosis of CMA in toddlers, in comparison with a standard allergic work-up and 2) to assess the diagnostic performance of six non-invasive fecal markers [secretory IgA (SIgA) and β -defensin2 (HBD2), markers of intestinal immune stimulation; eosinophil-derived neurotoxin (EDN), marker of eosinophil activation and degranulation in the gut; α_1 -antitrypsin (AAT), marker of increased leakage of the gut, and tumor necrosis factor- α (TNF α) and calprotectin, markers of intestinal inflammation] in CMA diagnosis. Furthermore, in view of the importance of gaining more knowledge in this area, we investigated the changes of selected gut microbiota in toddlers with and without CMA.

Materials and methods

Patients and study design

We designed a prospective descriptive study in the routine management of children with clinical manifestations, digestive and/or extra-digestive (eczema and/or wheezing), suggesting CMA. Children with gastroesophageal reflux disease and/or clinical gastritis symptoms underwent an upper gastrointestinal endoscopy with upper gastrointestinal biopsy specimens (at least two esophageal biopsy specimens looking for minor and major criteria of eosinophilic esophagitis, and four gastric biopsy specimen looking for *Helicobacter pylori* infection

according to the updated Sydney classification), whereas those with failure to thrive underwent a sweat test. Children were not enrolled if with anemia (hemoglobin <100 g/L), cystic fibrosis (pathological sweat chloride test >60 mEq/L), inflammatory bowel disease (typical endoscopic and pathologic lesions), celiac disease (positive blood anti-transglutaminase IgA and IgG antibodies and anti-endomysium IgA), digestive surgery, small-bowel bacterial overgrowth (positive hydrogen breath test), parasitic or infectious gastroenteritis (positive stool analysis or culture) and diabetes mellitus (hyperglycemia and abnormal glycosylated hemoglobin). Between December 2008 and June 2009, 25 consecutive children were enrolled, mean age (standard deviation) 6.3 months (4.8), range 1–18 months, 14 males and 11 females.

The allergic, digestive and fecal work-up were carried out on an outpatient basis: measurement of specific cow's milk protein (CMP)-IgE and IgG, CMP-SPT and CMP-APT; IP measurement by urine elimination of orally ingested L and M; fecal markers measurement, i.e., AAT, TNF α , calprotectin, HBD2, SIgA and EDN.

Following the initial standardized work-up, children were treated with a CMP elimination diet according to the recent ESPGHAN guidelines (1), based on an extensively hydrolyzed whey protein formula or an amino acid-derived formula when the use of the extensively hydrolyzed formula was not well tolerated or did not lead to clinical recovery within 4–8 weeks.

All children were subjected to a cow's milk challenge (CMC) after the elimination period (Figure 1).

A written parental consent was obtained from both parents and the study protocol was declared to the national research committee, i.e., La Commission Nationale de l'Information et des Libertés (CNIL) under the declaration number 1508869v0 and registered under the number of DC-2011-1393.

Specific cow's milk protein IgE and IgG

Sera cow's milk protein-IgE and IgG, whey-IgE and IgG, β -lactoglobulin-IgE and IgG, α -lactalbumin-IgE and IgG were analyzed using the ImmunoCap RAST tests (Phadia AB®, Uppsala, Sweden) calibrated with reference to the World Health Organization

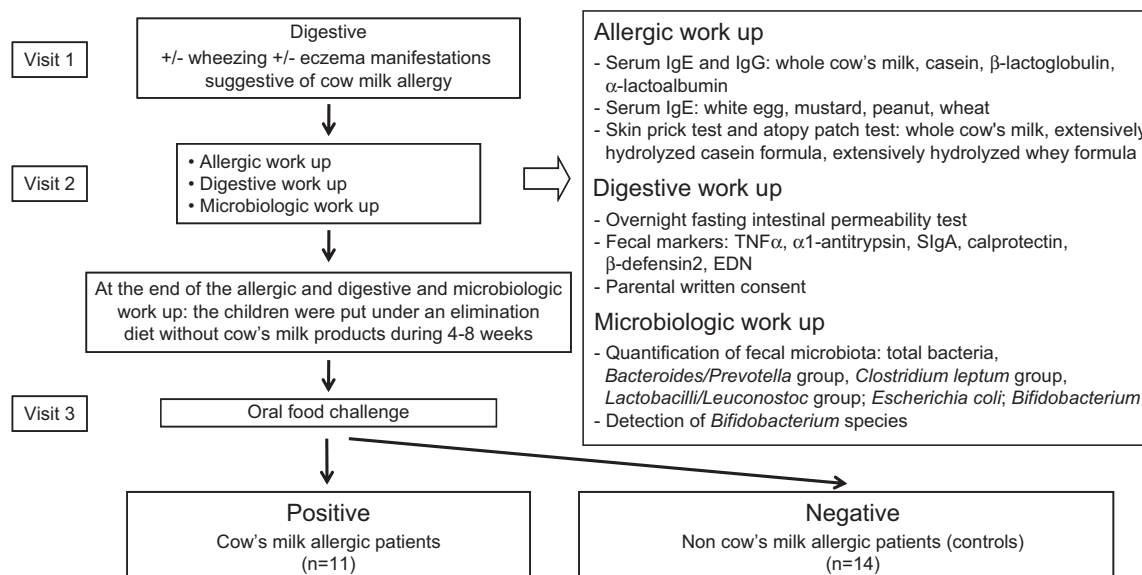


Figure 1 Study scheme.

standards for IgE. References data for those specific IgE and IgG titers were those of the manufacturer, considering values <0.10 KUA/L as non-detectable.

Cow's milk protein skin prick test

SPTs were performed with a drop of pasteurized half-skimmed cow's milk on the back of children. Histamine di-hydrochloride (ALK®, Copenhagen, Denmark), 10 mg/mL and glycerosaline were used as positive and negative controls. A positive reaction implied a wheal diameter 3 mm larger than the negative control after 15 min.

Cow's milk protein atopy patch test

The APT was applied using an aluminum cup (Finn Chamber®, Epitest Ltd, Hyrylä, Finland) as previously described (8).

Open oral cow's milk challenge

The open CMC was chosen since it is a reliable method in young children when performed under experienced professional observation (1, 2). The CMC was designed to be done in the hospital, according to (1–4), in order to file the conditions requiring such a medical setting, i.e., for children presenting a risk of anaphylaxis manifested by previous severe immediate clinical manifestations and/or positive cow's milk protein-IgE and/or SPT. The physician responsible for the CMC was blinded to the results of specific IgE, SPT and APT. The challenge used a standard cow's milk based infant formula. Increasing doses (1, 5, 10, 50, and 100 mL) were given at approximately 30-min intervals for 6 h, then the tolerance of increasing doses was monitored by a daily parental record of the child's symptoms. Clinical reactions and variation between parental opinions during the CMC were defined and analyzed according to the consortium of food allergy research, conducting an oral food challenge (3). CMA was defined as the disappearance of symptoms under cow's milk elimination and an unequivocal adverse reaction to challenge. A reaction within 2 h of challenge was regarded as immediate-onset type and others as delayed-onset type. The challenge was discontinued when a clinical reaction was noticed. To evaluate long-term tolerance and detect any false-negative result, all patients with a negative challenge at the end of the first week of surveillance went on consuming cow's milk and were seen 4–8 weeks later. Finally, children were considered controls if they had a negative CMC, and CMA children were those with a positive CMC according to traditional ESPGHAN criteria (1) as well as the newly defined criteria by the French Society of Allergology and Clinical Immunology (SFAIC) (2).

Intestinal permeability test

IP test was performed on the morning following an overnight fast and after voiding the overnight urine. Children were given a mixture of a 10% M and L in aqueous solution at a dosage of 0.1 g/kg of body weight for each sugar. All urine was collected for the 5 following hours with gentamicine as a preservative. The urine volume was measured, and aliquots stored frozen at –80°C until analysis. Urine samples were analyzed using gas chromatography performed with a Perkin Elmer AutoSystem KL (Perkin Elmer, Waltham, MA, USA) equipped with a flame ionization detector. Prior to measurement, 3 mL of urine was deionized using 0.5 g of Amberlite IRN-150 resin then centrifuged for 10 min at 1500 g. Nine hundred and fifty µL of

deionized urine added with 50 µL of internal standard [a mixture of inositol and turanose (v/v) at a final concentration of 5 mg/mL] was then lyophilized and submitted to derivatization by addition of 400 µL of a mixture composed of hexamethyldisilazane and trimethylchlorosilane in pyridine (3:1:9) (Supelco analytical, Bellefonte, PA, USA) for 1 h at 60°C. Samples were dried under nitrogen, and then extracted with 200 µL hexane. One µL of hexane solution was injected via the autosampler in a capillary column (30 m×0.25 mm ID Elite N 9316076, Perkin Elmer) with helium as gas carrier at a 1.5 bar head pressure. Temperature program was performed from 150 to 250°C with 20°C per min. Peak areas were automatically integrated and, M and L areas reported to inositol and turanose areas, respectively. Results were expressed as mg/L and IP (%) as L/M ratio (L/M, %).

Fecal markers measurement

For each child, a spot sample of feces was collected from the diapers immediately after defecation and stored at –80°C until assayed. Frozen stool samples were thawed at room temperature immediately before analysis.

Fecal AAT was measured using an immunonephelometric method adapted on the BN ProSpec® system (Siemens, Deerfield, IL, USA). Weighed stool samples were homogenized 1:5 (w/v) with NaCl 0.15 M for 1 h at 4°C, then centrifuged at 10,000 g for 15 min at 4°C and the supernatants were used for analysis which was performed at two different dilutions (1:5 and 1:500 final dilutions) to avoid any prozone phenomena. Fecal TNFα was determined using an ELISA (TNF-α EASIA, Biosource, Belgium) adapted for fecal samples as previously described (17). Fecal calprotectin, HBD2, SigA and EDN were analyzed using ELISA methods (Calprest®, Eurospital, Trieste, Italy or Immundiagnostik, Bensheim, Germany) according to the manufacturers' instructions. All samples were analyzed in duplicate for ELISA methods. The detection limits for each parameter were the following: AAT=10 µg/g of stool, TNFα=30 pg/g of stool, calprotectin=15 µg/g of stool, HBD2=7 ng/g of stool, SigA=170 µg/g of stool and EDN=120 ng/g of stool.

Fecal microbiota analysis

Fecal microbiota was assessed using real-time qPCR. For DNA extraction, stools were thawed, homogenized and 125 mg aliquot was used to extract total DNA using the chemical guanidium isothiocyanate and the mechanical bead beating method as previously described (18). Real-time qPCR was performed using an ABI 9700 Sequence Detection System apparatus with system software version 2.3 (Applied-Biosystems). TaqMan® qPCR was used to quantify total bacteria populations and the dominant (>1% of fecal bacteria population) bacterial groups, and genera: *Clostridium leptum* group, *Bacteroides/Prevotella* group and *Bifidobacterium*. qPCR using SYBR-Green® was performed for the sub-dominant bacterial species *Escherichia coli* and for the *Lactobacillus/Leuconostoc/Pediococcus* group. Primers and probes used were described by Furet et al. (19). Standard curves were obtained from serial dilutions of a known concentration of plasmid DNA containing a 16S rRNA gene insert from each species or group. The coefficients of correlation between log₁₀ colony forming units (CFU) and rRNA gene copy numbers for each species and group were obtained from rrnDB (20), enabling calculation of the number of CFU/g of feces. When a species or bacterial group was not detected, the values of 3 log₁₀ CFU/g of feces, i.e., corresponding to about half of the detection limit, was used. *Bifidobacterium* species identification was performed directly on fecal DNA using simplex PCR with 16S

rDNA-gene targeted species specific primers adapted from Mullié et al. (21).

Statistical analysis

Statistical tests were performed with the Statview program (Abacus®, CA, USA) and R software environment for statistical computing and graphics. Calculation of mean, SD, 95% confidence interval (CI), median and extremes were done for all quantitative parameters. Differences between qualitative parameters were analyzed by the χ^2 -test. Differences between quantitative parameters were analyzed using the non-parametric Mann-Whitney test. A principal component analysis was performed for a global analysis of the microbiota. Correlations between age and laboratory parameters were analyzed using the non-parametric Spearman test. A p-value of <0.05 was accepted as statistically significant. Receiver operating characteristic (ROC) curves were carried out to evaluate the ability of the IP test and fecal markers to predict CMA allergy and to accurately define cut-off levels. The performances of both the allergic work-up, IP test and laboratory markers were compared with CMC with calculation of sensitivity, specificity, positive predictive value, negative predictive value and overall accuracy of the test (the sum of true-positive and true-negative results divided by the total number of test).

Results

Population and allergic work-up

Patients' characteristics and diagnostic procedure are presented in Tables 1 and 2. The CMC was positive in 11 patients, referred to as CMA patients, and negative in 14, referred to as controls. All CMA patients had the delayed-onset type.

CMA was IgE-mediated, i.e., with positive IgE-CMP titers, in three patients and non-IgE-mediated, i.e., with negative IgE-CMP titers, in eight, one control exhibiting a positive CMP-IgE titer. A positive CMP-IgG titer was seen in one CMA patient. The CMP-SPT was positive in one IgE-mediated CMA patient. APTs were positive in four CMA patients (two IgE and two non-IgE-mediated) and in one control.

The eight cases of non-IgE-mediated CMA, symptoms were categorized, based on the description by Sicherer and Sampson (4) as food protein-induced enterocolitis syndrome (FPIES) in three cases, eosinophilic gastroenteropathy (EGEP) in four cases, and finally, gastrointestinal dysmotility (GID) in one case (Table 1). Upper gastrointestinal endoscopy revealed peptic esophagitis in seven children with CMA and in seven controls. None of the explored children showed *H. pylori* infection or >15 eosinophilic infiltration by high power field or eosinophilic oesophagitis in their upper gastrointestinal biopsy specimens (Tables 1 and 2). Finally, none of the explored children showed a positive sweat test.

Fecal microbiota

The microbiota did not differ significantly between CMA patients and controls using principal component analysis. *E. coli* was detected in all infants, the *Lactobacillus*/

Leuconostoc group in nearly all infants (over 80%) and the *C. leptum* group in about half of them, with no differences between allergic and controls. The *Bacteroides/Prevotella* group was detected in 64% of CMA infants and 79% of controls (NS), but the mean level of colonization tended to be lower in CMA patients (median 4.6 vs. 8.1 log₁₀CFU/g of feces, p=0.08) (Figure 2). *Bifidobacterium* were detected in 82% CMA and all control patients (NS). This genus was dominant, i.e., above 9 log₁₀CFU/g of feces, in 63% of CMA patients and in all controls with no difference between groups. The main *Bifidobacterium* species detected were *B. breve* and *B. longum*, with no *B. adolescentis*.

Intestinal permeability

Individual results of IP test are presented in Tables 3 and 4. No correlation with age was seen (r=−0.12, p=0.56). The median L/M (%) was significantly higher in CMA patients (4.77, range: 1.50–7.89), than in controls (3.06, 1.16–10.62, p=0.05). The ROC analysis showed an area under the curve (AUC) of 0.73 with an optimal cut-off value of 3.95% (Figure 3A). Using this cut-off, the sensitivity (Se) and the specificity (Sp) of the test for the diagnosis of CMA were 82% and 71%, respectively. The positive (PPV) and negative predictive values (NPV) for CMA allergy were 69% and 83%, respectively. Only 2/11 infants with CMA had L/M ratio lower than 3.95%. Finally, the accuracy performance of this IP test was 76% (Table 5).

Fecal markers

Fecal TNF α was under the limit of detection in all infants. Calprotectin, AAT, HBD2, SIgA and EDN did not differ significantly between CMA and control patients (Tables 3 and 4). All markers, except AAT (r=−0.34, p=0.18), were negatively correlated with age: calprotectin, r=−0.43, p=0.03; HBD2, r=−0.53, p=0.01; SIgA r=−0.66, p=0.001 and EDN, r=−0.34, p=0.06. ROC curves showed that EDN was related with CMA condition, allowing the determination of an optimal cut-off value for CMA risk of 2818 ng/g (Figure 3B). This cut-off value gave a sensitivity of only 55% but a specificity of 86%. The PPV and NPV for CMA allergy were 75% and 71%, respectively and only 2/14 control infants had fecal EDN level over the cut-off value (Table 5). The association of IP test and/or EDN gave a PPV of 73% and a NPV of 79%.

Discussion

This pilot descriptive prospective study suggests a cut-off level for IP in the diagnosis of delayed-onset reactions (digestive or eczematous) during CMA in toddlers and the role of fecal EDN as a potential non-invasive tool in this setting. The diagnostic performances of IP and EDN are especially interesting in children with non-IgE-mediated CMA, for whom the performance of the current allergic work up is poor.

Table 1 Clinical manifestations, atopic past history, allergic work-up and final diagnosis of patients with CMA.

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7	Patient 8	Patient 9	Patient 10	Patient 11
Age, months	4	4	1	4	11	9	13	2	6	18	1
Sex	Male	Female	Male	Male	Male	Female	Female	Female	Female	Male	Male
Clinical manifestations	GERD, eczema and wheezing	GERD, FTT and eczema	GERD, eczema and wheezing	Colic, constipation, eczema	GERD and FTT	Constipation, FTT and eczema	FTT and eczema	GERD, vomiting and RAP	Colic, GERD and eczema	Colic, vomiting and chronic diarrhea	GERD, FTT and chronic diarrhea
Personal atopic past history	Yes	Yes	No	No	No	Yes	Yes	No	No	No	No
Familial atopic past history	Yes	No	Yes	Yes	Yes	No	No	No	Yes	Yes	Yes
Skin prick test											
Whole cow's milk	ND	Negative	Positive	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
Peptijunior	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Pregestimil	ND	Negative	ND	ND	ND	ND	ND	ND	ND	ND	ND
Atopy patch test											
Whole cow's milk	Negative	Positive	Positive	Negative	Negative	Negative	Positive	Positive	Negative	Negative	Negative
Peptijunior	ND	Negative	ND	ND	ND	ND	Positive	ND	Negative	ND	ND
Pregestimil	ND	Positive	ND	ND	ND	ND	Negative	ND	Negative	ND	ND
IgE											
Whole cow's milk, KUA/L	0.24	Negative	18.6	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
Casein, KUA/L	Negative	0.69	0.64	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
β-Lactoglobulin, KUA/L	Negative	0.24	21	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
α-Lactalbumin, KUA/L	Negative	Negative	0.99	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
IgG											
Whole cow's milk	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
Casein	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
β-Lactoglobulin	Negative	Negative	Positive	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
α-Lactalbumin	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
CMC	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
Final diagnosis	CMA-IgE mediated, EGEP	CMA-IgE mediated, EGEP	CMA-IgE mediated, EGEP	CMA-Non IgE mediated, EGEP	CMA-Non IgE mediated, EGEP	CMA-Non IgE mediated, FPIES	CMA-Non IgE mediated, EGEP	CMA-Non IgE mediated, GID	CMA-Non IgE mediated, EGEP	CMA-Non IgE mediated, FPIES	CMA-Non IgE mediated, FPIES
Elimination diet	Peptijunior®	Pregestimil®	Peptijunior® and breastfeeding	Peptijunior®	Pregestimil®	Neocate®	Pregestimil®	Nutrigen AA®	Nutrigen AA®	Neocate®	Nutrigen AA® and breastfeeding

CMA, cow's milk allergy; CMC, open oral cow's milk challenge; EGEP, eosinophilic gastroenteropathies; FPIES, food protein-induced enterocolitis syndrome; FTT, failure to thrive; GERD, gastro-oesophageal reflux disease; GID, gastrointestinal dysmotility; Peregestimil®, extensively hydrolyzed casein formula (eHF-Casein); Peptijunior®, extensively hydrolyzed whey formula (eHF-Whey); ND, not done; Neocate® and Nutrigen AA®, Amino acids formula; RAP, recurrent abdominal pain.

Table 2 Clinical manifestations, atopic past history, allergic work-up and final diagnosis of patients without CMA (control patients).

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7	Patient 8	Patient 9	Patient 10	Patient 11	Patient 12	Patient 13	Patient 14
Age, months	9	16	9	1	12	4	2	4	3	2	7	5	2	12
Sex	Female	Male	Female	Male	Male	Female	Female	Female	Male	Male	Female	Male	Male	Male
Clinical manifestations	Colic, GERD and wheezing	Colic, FTT, GERD, chronic constipation and eczema	Chronic diarrhea	GERD	FTT	Colic, vomiting and chronic diarrhea	Colic, vomiting and chronic diarrhea	GERD	GERD, vomiting, constipation and eczema	Vomiting	FTT, GERD, wheezing and chronic diarrhea	GERD, vomiting, constipation and eczema	GERD, colic, vomiting	FTT
Familial atopic past history	Yes	Yes	No	Yes	Yes	Yes	No	No	No	Yes	Yes	Yes	Yes	No
Skin prick test														
Whole cow's milk	Negative	Negative	Negative	ND	Negative	Negative	ND	Negative	Negative	ND	Negative	Negative	ND	Negative
Peptijunior	ND	ND	ND	ND	Negative	ND	ND	Negative	ND	ND	ND	Negative	ND	Negative
Pregestimil	ND	ND	ND	ND	Negative	ND	ND	Negative	ND	ND	ND	Negative	ND	Negative
Atopy patch test														
Whole cow's milk	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Positive	Negative	Negative
Peptijunior	ND	ND	ND	ND	Negative	ND	Negative	Negative	ND	ND	Negative	Negative	ND	Negative
Pregestimil	ND	ND	ND	ND	Negative	ND	Negative	Negative	ND	ND	ND	Positive	ND	Negative
IgE														
Whole cow's milk, KUA/L	Negative	Negative	Negative	Negative	0.2	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
Casain, KUA/L	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	ND	Negative
β -Lactoglobulin, KUA/L	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	ND	Negative
α -Lactalbumin, KUA/L	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	ND	Negative
IgG														
Whole cow's milk	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	ND	Negative	Negative	ND	Negative
Casain	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	ND	Negative	Negative	ND	Negative
β -Lactoglobulin	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	ND	Negative	Negative	ND	Negative
α -Lactalbumin	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	ND	Negative	Negative	ND	Negative
IgE														
White egg, KUA/L	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	0.9 (Positive)	Negative	Negative
Peanut, KUA/L	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
Mustard, KUA/L	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
Wheat, KUA/L	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
CMC	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
Final diagnosis	Peptic oesophagitis	Peptic oesophagitis and alimentary habits troubles	Intestinal bacterial overgrowth	Peptic oesophagitis	Neuro-metabolic disease	Peptic oesophagitis	Irritable bowel syndrome	Denutrition by food intake insufficiency	Peptic oesophagitis	Peptic oesophagitis	Intestinal bacterial overgrowth	Sensitization against white egg	Peptic oesophagitis	Denutrition by food intake insufficiency
Elimination diet	Pregestimil®	Pregestimil®	Pregestimil®	Breastfeeding and Neocate®	Pregestimil®	Pregestimil®	Pregestimil®	Nutramigen AA®	Pregestimil®	Pregestimil®	Pregestimil®	Pregestimil®	Breastfeeding/ Nutramigen AA®	Nutramigen AA®

CMA, cow's milk allergy; CMC, open oral cow's milk challenge; FTT, failure to thrive; GERD, gastro-oesophageal reflux disease; Peregestimil®, extensively hydrolyzed casein formula (eHF-Casein); Peptijunior®, extensively hydrolyzed whey formula (eHF-Whey); ND, not done; Neocate® and Nutramigen AA®, Amino acids formula; RAP, recurrent abdominal pain.

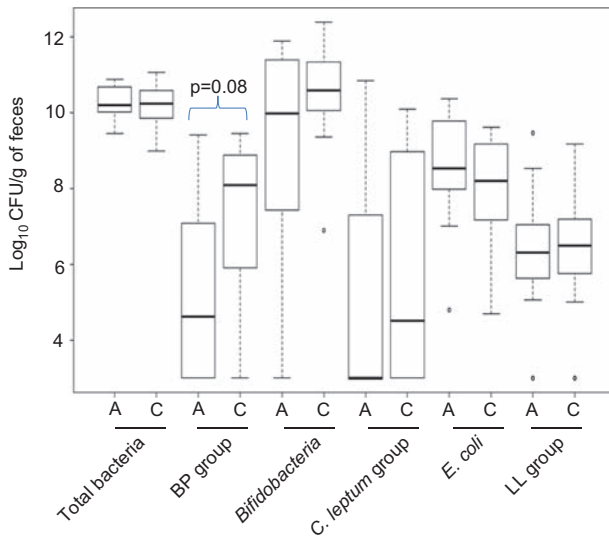


Figure 2 Level of colonization with main facultative anaerobic and strict anaerobic groups represented as a box plot. The box plot shows median (central horizontal line), the 25th centile (lower box border), and the 75th centile (upper box border). The lower and upper horizontal lines refer to the 10th and the 90th centile, respectively. A, allergic patients; BP group, *Bacteroides/Prevotella* group; C, control patients; LL group, *Lactobacillus/Leuconostoc* group.

Several studies highlighted differences in the composition of bacterial communities in the feces of children with or without allergic diseases; however, available epidemiological studies remain controversial. In our study, no significant differences were seen between groups as observed by Adlerberth et al. (22), with noticeably no quantitative or qualitative differences in *Bifidobacterium* colonization, as previously described (23). However, a trend towards a decrease occurred in the *Bacteroides* group in allergic infants as previously described in 12-month-old infants (24). *Bacteroides* are part of the dominant gut microbiota and members of *Bacteroides* genus acts on the Treg lineage differentiation pathway in the gut, to actively induce mucosal tolerance (25). Moreover, infants colonized with *B. fragilis* at 1 month of age had more IgA-secreting and IgM-secreting cells/10⁶ mononuclear cells at 2 months than infants not colonized (26).

In patients with atopic dermatitis suspect of CMA, Isolauri and Turjanmaa (27) reported positive SPTs in 67% of cases with acute-onset reactions to challenge and positive APTs in 89% of those with delayed-onset-reactions. Combining SPT and APTs significantly enhanced the diagnosis accuracy of specific food allergies (86% sensitivity and 72% specificity). However, Majamaa et al. (28) reported results similar to ours in children under the age of 2 years suspected of CMA with sensitivities of only 26, 14 and 44% for cow's milk-specific IgE, SPT and APT, respectively, in children with challenge-proven CMA.

IP is known to vary according to age and to be altered in children with CMA (13). For Jalonen (29), IP changes

Table 3 Intestinal permeability test and fecal markers in patients with CMA.

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7	Patient 8	Patient 9	Patient 10	Patient 11
Age, months	4	4	1	4	11	9	13	2	6	18	1
Sex	Male	Female	Male	Male	Male	Female	Female	Female	Female	Male	Male
CMA	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
Intestinal permeability test, %	4.76	5.09	3.98	3.95	5.25	2.86	0.45	4.46	7.89	7.59	6.4
Fecal markers											
TNFα, pg/g	<30	<30	<30	<30	<30	<30	<30	<30	<30	<30	<30
Calprotectine, μg/g	94	512	120	15	<15	21	100	46	361	<15	107
AAT, mg/g	0.45	0.29	0.26	0.19	0.03	0.19	0.74	0.14	1.08	<0.01	0.31
HBD2, ng/g	210	111	94	47	7	11	130	75	64	43	127
IgAs, ng/g	1030	2538	10,590	2775	585	1758	<170	698	3111	190	5783
EDN, ng/g	2818	2926	3654	3168	146	2362	2534	1840	3074	<50	2868

AAT, α₁-antitrypsin; CMA, cow's milk allergy; CMC, open oral cow's milk challenge; EDN, eosinophil-derived neurotoxin; HBD2, β-defensin2; TNFα, tumor necrosis factor-α.

Table 4 Intestinal permeability test and fecal markers in patients without CMA (control patients).

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7	Patient 8	Patient 9	Patient 10	Patient 11	Patient 12	Patient 13	Patient 14
Age, months	9	16	9	12	4	4	2	4	3	2	7	5	2	12
Sex	Female	Male	Female	Male	Female	Female	Female	Female	Male	Male	Female	Male	Male	Male
CMA	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
Intestinal permeability test, %	4.32	1.16	0.03	10.62	2.21	3.16	3.11	4.59	2.38	2.24	3.26	3.01	1.87	5.96
Fecal markers														
TNF α , pg/g	<30	<30	<30	<30	<30	<30	<30	<30	<30	<30	<30	<30	<30	<30
Calprotectine, μ g/g	172	23	96	189	36	267	29	305	455	157	28	50	31	<15
AAT, mg/g	0.41	0.08	0.22	0.31	0.04	0.51	0.22	0.16	1.49	0.25	0.08	0.13	0.07	0.07
HBD2, ng/g	62	15	50	199	15	35	39	128	791	279	<7	133	121	7
IgAs, ng/g	951	576	1065	11,750	2211	6058	2988	3039	1800	1068	665	512	2520	1545
EDN, ng/g	1326	664	2534	1562	548	1650	994	2414	3214	3066	2100	2802	692	2240

AAT, α -antitrypsin; CMA, cow's milk allergy; CMC, open oral cow's milk challenge; EDN, eosinophil-derived neurotoxin; HBD2, β -defensin2; TNF α , tumor necrosis factor- α .

AAT, α_1 -antitrypsin; CMA, cow's milk allergy; CMC, open oral cow's milk challenge; EDN, eosinophil-derived neurotoxin; HBD2, β -defensin2; TNF α , tumor necrosis factor- α .

were similar whether children exhibited gastrointestinal or skin manifestations with a significant rise in L/M ratio following cow milk protein ingestion by day 3 in both situations. In a previous study, the sensitivity of the test for CMA was 70% in infants aged 6–12 months and only 58% in children over 3 years. In the present work, no correlation was seen between L/M ratio and age. This could be related to the young age of the enrolled children since all of them were <18 months old. We confirm the diagnosis value of IP in this population of toddlers, the performance of the test reaching 76% after optimization of the cut-off level to 3.95%. Noteworthy, the best test performance was observed in delayed-onset digestive non-IgE-mediated CMA patients as compared to other allergic diagnosis tools.

Fecal eosinophil-derived proteins (ECP, EPX) have been previously investigated in infants with atopic dermatitis and food allergy even in the absence of overt gastrointestinal symptoms (30). Saarinen et al. (31) showed that infants with intestinal symptoms during the challenge had higher fecal ECP levels than those with other symptoms or those tolerating cow milk, suggesting an eosinophilic degranulation in the intestine. Our protocol was designed to evaluate the diagnostic performance of the fecal tests: we measured fecal EDN using a method easier and more reliable than for fecal ECP. Moreover, EDN appeared to be more sensitive and more closely correlated with the severity of the illness as demonstrated for serum and urinary measurements in atopic asthma and dermatitis (32, 33). Although non-significant, fecal EDN levels were higher in CMA patients compared with controls leading to a PPV better than for the IP test. These high levels in children with symptoms suggesting eosinophilic conditions are in agreement with the current knowledge (34). The lack of significance in our study could be related to the small population and to the absence of exclusion regimen at enrollment. Such as for the IP test, false negative results were reduced compared with current allergic work-up tests, suggesting the value of both tests in the diagnosis procedure: the diagnosis accuracy was 76% for IP and 72% for EDN.

Conclusions

The present study confirms the efficiency of IP for the diagnosis of CMA in toddlers compared with the routine allergy work-up, i.e., CMP-specific IgE, IgG, SPT and APT. In addition, we show that measurement of fecal EDN on a single spot sample is promising in this population. This suggests the usefulness of these markers in the diagnosis of delayed-onset digestive non-IgE-mediated CMA in toddlers. Further prospective confirmatory studies on large-scale population are now needed.

Besides, we confirm some variation in intestinal microbiota in this population with a trend to decrease in the *Bacteroides* group which belongs to the dominant microbiota and is known to participate in Treg modulation.

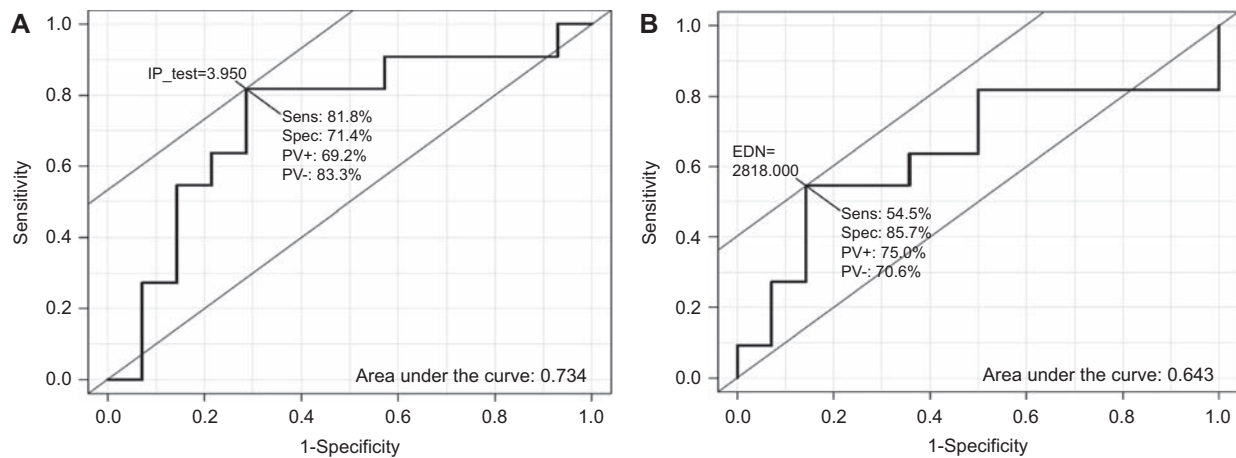


Figure 3 ROC curve of IP test (A) and fecal EDN (B) for prediction of CMA.

Table 5 Allergic work-up and fecal markers tests performances in the 25 studied children, i.e., 11 CMA children and 14 controls.

	Se, %	Sp, %	PPV, %	NPV, %	False positive, n	False negative, n	Performance ^a
Allergic work up							
SPT	9.1	100	100	58.3	0	10	1+10/20 (55)
APT	36.4	92.9	80	65	1	7	4+13/25 (68)
IgE	27.3	92.9	75	61.9	1	9	2+13/25 (60)
IgG	9.1	100	100	58.3	0	11	0+12/23 (52)
Intestinal permeability	82	71	69	83	4	2	9+10/25 (76)
Fecal markers ^b							
EDN	55	86	75	71	2	5	6+12/25 (72)
Calprotectine	18	93	67	59	1	10	1+13/25 (56)
AAT	55	71	60	67	4	7	4+10/25 (56)
HBD2	82	36	50	71	9	2	9+15/25 (56)
SigA	46	71	56	63	4	6	5+11/25 (60)

^aTest performance, Calculated as the number of true positive+negative cases/the total number of cases; ^bCut-off values yielded using ROC curves, IP, %: 3.96; EDN, ng/g: 2818; Calprotectine, μ g/g: 361; α 1-AT, mg/g: 0.26; HBD2, ng/g: 39; SIgA, μ g/g: 2538.

AAT, α -antitrypsin; APT, atopy patch test; CMA, cow's milk allergy; EDN, eosinophil-derived neurotoxin; HBD2, β -defensin2; NPV, negative predictive values; PPV, positive predictive values; Se, sensitivity; Sp, specificity; SPT, skin prick test.

Acknowledgments

This scientific work was supported by a scientific grant from Bayer Schering[®] laboratory allocated to the Faculté Libre de Médecine de Lille, Catholic University-Lille, France. This grant was mainly used to cover laboratory analysis expenses for urinary and fecal markers.

Conflict of interest statement

Authors' conflict of interest disclosure: The authors stated that there are no conflicts of interest regarding the publication of this article. Research support played no role in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; or in the decision to submit the report for publication.

Research funding: None declared.

Employment or leadership: None declared.

Honorarium: None declared.

References

- Vandenplas Y, Koletzko S, Isolaurie E, Hill D, Orange AP, Brueton M, et al. Guidelines for the diagnosis and management of cow's milk protein allergy in infants. Arch Dis Child 2007; 10:902–8.
- Rancé F, Deschildre A, Villard-Truc F, Gomez SA, Paty E, Santos C, et al. Oral food challenge in children: an expert review. Position paper of the section of Pediatrics French Society of Allergy and Clinical Immunology (SFAIC) and the Pediatric Society of Pulmonology and Allergy (SP2A). Eur Ann Allergy Clin Immunol 2009;41:35–49.
- Nowak-Węgrzyn A, Assa'ad AH, Bahna SL, Bock SA, Sicherer SH, Teuber SS, et al. On behalf of the adverse reactions to food committee of the American Academy of Allergy, Asthma and Immunology. Work group report: oral food challenge testing. J Allergy Clin Immunol 2009;S365–8.
- Sicherer SH, Sampson HA. Food allergy. J Clin Allergy Immunol 2010;125(2 Suppl 2):S116–25.

5. Sampson HA. Update on food allergy. *J Allergy Clin Immunol* 2004;113:805–19.
6. Niggemann B, Reibel S, Wahn U. The atopic patch test (APT) – a useful tool for the diagnosis of food allergy in children with atopic dermatitis. *Allergy* 2000;55:281–5.
7. de Boissieu D, Wagué JC, Dupont C. The atopy patch tests for detection of cow's milk allergy with digestive symptoms. *J Pediatr* 2003;142:203–5.
8. Kalach N, Soulaines P, de Boissieu D, Dupont C. A pilot study of the usefulness and safety of a ready-to-use atopy patch test (Diallertest) versus a comparator (Finn Chamber) during cow's milk allergy in children. *J Allergy Clin Immunol* 2005;116:1321–6.
9. Dupont C, Goutail-Flaud MF. Alterations of intestinal permeability to sugars in infants following neonatal surgery. *J Pediatr Gastroenterol Nutr* 1990;11:66–71.
10. Isolauri E, Juntunen M, Wiren S, Vuorinen P, Koivula T. Intestinal permeability changes in acute gastroenteritis: effects of clinical factors and nutritional management. *J Pediatr Gastroenterol Nutr* 1989;8:466–73.
11. Carratù R, Secondulfo M, de Magistris L, Iafusco D, Urio A, Carbone MG, et al. Altered intestinal permeability to mannitol in diabetes mellitus type I. *J Pediatr Gastroenterol Nutr* 1999;28:264–9.
12. Dupont C, Barau E, Molkhou P, Raynaud F, Barbet JP, Dehenin L. Food-induced alterations of intestinal permeability in children with cow's milk-sensitive enteropathy and atopic dermatitis. *J Pediatr Gastroenterol Nutr* 1989;8:459–65.
13. Kalach N, Rocchiccioli F, de Boissieu D, Benhamou PH, Dupont C. Intestinal permeability in children: variation with age and reliability in the diagnosis of cow's milk allergy. *Acta Paediatr* 2001;90:499–504.
14. Tibble J, Teahon K, Thjodleifsson B, Roseth A, Sigthorsson G, Bridger S, et al. A simple method for assessing intestinal inflammation in Crohn's disease. *Gut* 2000;47:506–13.
15. McLoughlin RM, Mills KH. Influence of gastrointestinal commensal bacteria on the immune responses that mediate allergy and asthma. *J Allergy Clin Immunol* 2011;127:1097–107.
16. Round JL, Mazmanian SK. The gut microbiota shapes intestinal immune responses during health and disease. *Nat Rev Immunol* 2009;9:313–23.
17. Kapel N, Roman C, Caldari D, Sieprath F, Canioni D, Khalfoun Y, et al. Fecal tumor necrosis factor- α and calprotectin as differential diagnostic markers for severe diarrhea of small infants. *J Pediatr Gastroenterol Nutr* 2005;41:396–400.
18. Mangin I, Suau A, Magne F, Garrido D, Gotteland M, Neut C, et al. Characterization of human intestinal bifidobacteria using competitive PCR and PCR-TTGE. *FEMS Microbiol Ecol* 2006;55:28–37.
19. Furet JP, Firmesse O, Gourmelon M, Bridonneau C, Tap J, Mondot S, et al. Comparative assessment of human and farm animal faecal microbiota using real-time quantitative PCR. *FEMS Microbiol Ecol* 2009;68:351–62.
20. Klappenbach JA, Saxman PR, Cole JR, Schmidt TM. rrndb: the ribosomal RNA operon copy number database. *Nucleic Acids Res* 2001;29:181–4.
21. Mullié C, Odou MF, Singer E, Romond MB, Izard D. Multiplex PCR using 16S rRNA gene-targeted primers for the identification of bifidobacteria from human origin. *FEMS Microbiol Lett* 2003;222:129–36.
22. Adlerberth I, Strachan DP, Matricardi PM, Ahmé S, Orfei L, Aberg N, et al. Gut microbiota and development of atopic eczema in 3 European birth cohorts. *J Allergy Clin Immunol* 2007;120:343–50.
23. Waligora-Dupriet AJ, Campeotto F, Romero K, Mangin I, Rouzaud G, Ménard O, et al. Diversity of gut Bifidobacterium species is not altered between allergic and non-allergic French infants. *Anaerobe* 2011;17:91–6.
24. Björkstén B, Sepp E, Julge K, Voor T, Mikelsaar M. Allergy development and the intestinal microflora during the first year of life. *J Allergy Clin Immunol* 2001;108:516–20.
25. Round JL, Mazmanian SK. Inducible Foxp3+ regulatory T-cell development by a commensal bacterium of the intestinal microbiota. *Proc Natl Acad Sci USA* 2010;107:12204–9.
26. Grönlund MM, Arvilommi H, Kero P, Lehtonen OP, Isolauri E. Importance of intestinal colonisation in the maturation of humoral immunity in early infancy: a prospective follow up study of healthy infants aged 0–6 months. *Arch Dis Child Fetal Neonatal Ed* 2000;83:F186–92.
27. Isolauri E, Turjanmaa K. Combined skin prick and patch testing enhances identification of food allergy in infants with atopic dermatitis. *J Allergy Clin Immunol* 1996;97:9–15.
28. Majamaa H, Moiso P, Holm K, Kautanen H, Turjanmaa K. Cow's milk allergy: diagnostic accuracy of skin prick and patch tests and specific IgE. *Allergy* 1999;54:346–51.
29. Jalonen T. Identical intestinal permeability changes in children with different clinical manifestations of cow's milk allergy. *J Allergy Clin Immunol* 1991;88:737–42.
30. Majamaa H, Laine S, Miettinen A. Eosinophil protein X and eosinophil cationic protein as indicators of intestinal inflammation in infants with atopic eczema and food allergy. *Clin Exp Allergy* 1999;29:1502–6.
31. Saarinen KM, Sarnesto A, Savilahti E. Markers of inflammation in the feces of infants with cow's milk allergy. *Pediatr Allergy Immunol* 2002;13:188–94.
32. Kim KW, Lee KE, Kim ES, Song TW, Sohn MH, Kim KE. Serum eosinophil-derived neurotoxin (EDN) in diagnosis and evaluation of severity and bronchial hyperresponsiveness in childhood asthma. *Lung* 2007;185:97–103.
33. Goto T, Morioka J, Inamura H, Yano M, Kodaira K, Igarashi Y, et al. Urinary eosinophil derived neurotoxin concentrations in patients with atopic dermatitis: a useful clinical marker for disease activity. *Allergol Int* 2007;56:433–8.
34. Morita H, Nomura I, Shoda T, Saito H, Matsumoto K. Fecal eosinophil-derived neurotoxin is significantly elevated in non-IgE dependent gastrointestinal allergies, especially in subtypes showing bloody stool [abstract]. *J Allergy Clin Immunol* 2012;129:A353.