FECAL BIOMARKERS ACROSS GASTROINTESTINAL FOOD ALLERGY PHENOTYPES IN YOUNG CHILDREN

Charos G. Yakubova – Ph.D. student
Inobat M. Akhmedova – D.M.Sc.
Center for the Development of Professional Qualifications of Medical Workers under the Ministry of Health of the Republic of Uzbekistan (Tashkent, Uzbekistan)
inobatahm@gmail.com

Abstract. *The study compared fecal eosinophil-derived neurotoxin (EDN), calprotectin and zonulin in 130 children aged 3 months to 3 years with allergic enterocolitis or allergic enteropathy. EDN and calprotectin were significantly higher in enterocolitis, whereas zonulin did not differ. The findings indicate phenotype-related heterogeneity of intestinal inflammation.*

Keywords: *gastrointestinal food allergy, allergic enterocolitis, allergic enteropathy, eosinophil-derived neurotoxin, calprotectin, zonulin, fecal biomarkers, young children.*

INTRODUCTION.

Non-IgE-mediated gastrointestinal food allergy is not a single clinical entity but a group of immune-mediated disorders that present mainly in infancy and early childhood. The recognized spectrum includes food protein-induced allergic proctocolitis (FPIAP), food protein-induced enteropathy (FPE), and food protein-induced enterocolitis syndrome (FPIES) [2, 3, 5, 9, 13, 15, 24, 26, 40]. Because gastrointestinal symptoms are often nonspecific and may resemble other pediatric gastrointestinal conditions, diagnosis remains primarily clinical. Current guidance therefore emphasizes a compatible history, improvement after removal of the suspected food, recurrence or controlled reintroduction when appropriate, oral food challenge in selected cases, and exclusion of competing diagnoses [6, 11, 12, 17, 19, 21, 23, 25, 30, 36]. The subsequent allergic course is also not uniform; FPIAP, for example, has been linked to a higher frequency of later IgE-mediated food allergy [18].

This clinical variability is likely to reflect more than one mucosal inflammatory pathway. Studies of non-IgE-mediated gastrointestinal food allergy implicate eosinophil activation, innate immune signaling, mucosal inflammation, and epithelial-barrier disturbances, with the relative contribution of these processes differing across disorders and patients [20, 32, 35]. A phenotype-oriented biomarker approach may therefore be more informative than assuming a single inflammatory pattern for all gastrointestinal presentations.

Stool-based measurements offer a practical way to examine intestinal inflammatory activity without invasive sampling. EDN reflects products released during eosinophil activation and has been evaluated in several pediatric non-IgE-mediated food-allergy settings [16, 28, 29, 33]. Calprotectin provides a broader signal of intestinal inflammation, but values in infants vary considerably with age and between individuals, which limits interpretation of an isolated result [7, 27, 28, 33, 39]. Zonulin-related assays have also been used experimentally in gastrointestinal disorders, including allergic proctocolitis and FPIES, as putative indicators related to barrier biology [7, 8, 10, 34]. Importantly, assay identity is a methodological concern: commonly used commercial zonulin ELISAs may detect proteins other than pre-haptoglobin-2 [31].

The available biomarker literature does not show a consistent pattern. Higher EDN, calprotectin, or zonulin-related values have been reported in some pediatric cohorts [7, 27, 29], whereas other studies found broad overlap between affected and unaffected children or weak discrimination by individual markers [16, 28, 33]. A meta-analysis of fecal calprotectin in cow's milk protein allergy likewise identified substantial heterogeneity across studies [39]. Such inconsistency may arise from differences in age, phenotype definitions, dietary exposure at sampling, timing relative to symptoms or challenge, and laboratory methods. Moreover, much of the evidence concerns a single diagnosis, such as FPIAP, FPIES, or cow's milk protein allergy, rather than direct comparison of biomarker patterns across clinically defined gastrointestinal phenotypes [1, 7, 16, 27–29, 33].

The aim of this study was to compare fecal EDN, calprotectin, and zonulin concentrations between clinically defined AEC and AEP phenotypes in infants and young children, to assess associations among these biomarkers, and to explore their discriminatory performance individually and in combination.

METHODS.

Study design and participants

This prospective observational case-control study was conducted from July 2024 to July 2025 in the Department of Gastroenterology and the Consultative Diagnostic Polyclinic of the Republican Specialized Scientific and Practical Medical Center of Pediatrics, Tashkent, Republic of Uzbekistan. The study is reported in accordance with the STROBE recommendations.

A total of 175 children aged 3 months to 3 years were included: 130 patients with gastrointestinal food allergy and 45 age-matched healthy controls. According to the predominant clinical phenotype, patients were classified as allergic enterocolitis (AEC; n=76) or allergic enteropathy (AEP; n=54).

Diagnostic criteria and clinical classification

The diagnosis of gastrointestinal food allergy was based on clinical history, gastrointestinal symptoms temporally associated with suspected food exposure, available laboratory findings, exclusion of alternative causes, and the response to dietary elimination followed by medically supervised reintroduction of the suspected food. Clinical assessment was informed by the symptom patterns described by Labrosse et al. [15] and relevant guidance on elimination and reintroduction. All 130 affected children were observed for 2–4 weeks during dietary elimination and showed improvement in gastrointestinal symptoms. Subsequent reintroduction of the suspected food was performed under medical supervision in all 130 children and was followed by recurrence of gastrointestinal symptoms in every case, supporting the clinical diagnosis. The reintroduction procedure is described here as medically supervised reintroduction rather than a standardized oral food challenge, because uniform challenge doses, observation schedules, and positivity criteria have not been documented in the available study description.

The AEC phenotype was characterized predominantly by enterocolitic/colonic manifestations, including diarrhea with blood and/or mucus in the stool, with or without vomiting. The AEP phenotype was characterized by persistent or recurrent enteropathy-type gastrointestinal manifestations, including chronic or recurrent diarrhea, with or without vomiting, together with clinical evidence of impaired intestinal absorption or nutritional consequences.

Phenotype classification was based on the predominant clinical symptom complex and associated clinical findings before analysis of fecal biomarker results. Endoscopy and intestinal biopsy were not performed. Accordingly, AEC and AEP were operational clinical phenotype categories rather than histologically confirmed or independently validated phenotype-specific diagnostic entities.

Eligibility criteria

Children aged 3 months to 3 years with a clinical diagnosis of gastrointestinal food allergy and a stool sample obtained before initiation of the elimination diet were eligible. Exclusion criteria were acute or chronic intestinal infection, inflammatory bowel disease, celiac disease, congenital gastrointestinal anomalies, primary immunodeficiency, severe systemic disease, and treatment with systemic antibiotics, glucocorticosteroids, or immunosuppressive therapy during the 4 weeks before enrollment. Insufficient clinical information to establish the diagnosis or phenotype was also an exclusion criterion.

Missing individual biomarker measurements did not result in exclusion from the overall cohort. Analyses of individual biomarkers were performed using available observations; models requiring simultaneous availability of multiple biomarkers were restricted to complete cases.

Clinical and laboratory assessment

At enrollment, standardized clinical assessment included documentation of gastrointestinal symptoms. The Cow's Milk-related Symptom Score (CoMiSS) was recorded, the SCORAD index was used when atopic dermatitis was present, and anthropometric assessment was performed according to WHO standards using WHO Anthro. Routine investigations included complete blood count and total IgE; allergen-specific IgE was measured when clinically indicated. Stool microscopy, fecal occult blood testing, and stool carbohydrate testing were also performed.

Fecal biomarker measurements

Fresh stool samples were collected before initiation of the elimination diet, transported under cooled conditions, and stored at −80 °C until analysis. EDN was measured using the IDK® EDN ELISA (Immundiagnostik AG, Bensheim, Germany; REF K6811), calprotectin using the IDK® Calprotectin ELISA (REF K6927), and zonulin using the IDK® Zonulin ELISA (REF K5600), according to the manufacturer's instructions. Biomarker results were not used to establish the diagnosis or assign the clinical phenotype.

Outcome measures

The primary outcomes were between-phenotype differences in fecal EDN, calprotectin, and zonulin concentrations. Secondary outcomes included associations among fecal biomarkers and available clinical and laboratory variables. The discriminatory performance of individual biomarkers and the combined model including EDN, calprotectin, and zonulin was evaluated as an exploratory outcome.

Statistical analysis

Statistical analyses were performed using jamovi version 2.7.37. Distributional normality was assessed using the Shapiro-Wilk test. Continuous variables are presented as mean ± standard deviation or median with interquartile range (IQR), as appropriate; categorical variables are presented as counts and percentages. EDN, calprotectin, and zonulin were compared between AEC and AEP using the Mann-Whitney U test. Other variables were analyzed using Student's t test, Mann-Whitney U test, chi-square test, or Fisher's exact test, as appropriate.

Correlations were assessed using Spearman's rank coefficient (ρ). Missing biomarker values were not imputed. Exploratory ROC analyses generated AUC values with 95% confidence intervals, and cutoffs were selected using the Youden index. Exploratory multivariable binary logistic regression including EDN, calprotectin, and zonulin was performed in participants with complete measurements of all three biomarkers. All tests were two-sided, and $p<0.05$ was considered statistically significant. ROC, correlation, and multivariable analyses were considered hypothesis-generating and require independent validation.

RESULTS.

Study population

The study included 175 children: 130 with gastrointestinal food allergy and 45 healthy controls. Among affected children, 76 (58.5%) were classified as AEC and 54 (41.5%) as AEP. Of the 130 patients, 49 (37.7%) were aged 3–6 months, 30 (23.1%) were aged 6–12 months, and 51 (39.2%) were aged 1–3 years; 79 (60.8%) were boys and 51 (39.2%) were girls (Table 1).

Table 1.

Baseline demographic characteristics of the study population

Characteristic	GIFA (n=130)	AEC (n=76)	AEP (n=54)	Healthy controls (n=45)
Sex, n (%)				
Male	79 (60.8)	53 (69.7)	26 (48.1)	21 (46.7)
Female	51 (39.2)	23 (30.3)	28 (51.9)	24 (53.3)

Characteristic	GIFA (n=130)	AEC (n=76)	AEP (n=54)	Healthy controls (n=45)
Age group, n (%)				
3–6 months	49 (37.7)	20 (26.3)	29 (53.7)	17 (37.8)
6–12 months	30 (23.1)	19 (25.0)	11 (20.4)	13 (28.9)
1–3 years	51 (39.2)	37 (48.7)	14 (25.9)	15 (33.3)

Data are presented as n (%). GIFA, gastrointestinal food allergy; AEC, allergic enterocolitis; AEP, allergic enteropathy.

Fecal biomarkers according to clinical phenotype

Children with AEC had higher fecal EDN concentrations than those with AEP: 1200 (840–1600) vs 820 (540–1100) ng/mL (U=665; p=0.002). Fecal calprotectin was also higher in AEC: 690 (480–900) vs 450 (300–620) mg/kg (U=172; p=0.003). Zonulin was numerically higher in AEC, 185 (150–230) vs 140 (110–175) ng/mL, but the difference did not reach statistical significance (U=256; p=0.065) (Table 2).

Table 2.

Comparison of fecal inflammatory biomarkers between allergic enterocolitis and allergic enteropathy

Biomarker	AEC, median (IQR)	AEP, median (IQR)	Mann-Whitney U	P value
EDN (ng/mL)	1200 (840–1600)	820 (540–1100)	665	0.002
Calprotectin (mg/kg)	690 (480–900)	450 (300–620)	172	0.003
Zonulin (ng/mL)	185 (150–230)	140 (110–175)	256	0.065

Data are presented as median (IQR). EDN, eosinophil-derived neurotoxin; IQR, interquartile range; AEC, allergic enterocolitis; AEP, allergic enteropathy.

Correlation analysis

In the overall cohort, EDN showed a moderate positive correlation with calprotectin (ρ=0.454; p=0.004). A weaker positive correlation was observed between zonulin and calprotectin (ρ=0.294; p=0.048). Total IgE correlated negatively with EDN (ρ=−0.464; p=0.026); no statistically significant associations were found between total IgE and calprotectin or zonulin (Table 3).

Table 3.

Spearman correlation analysis of inflammatory biomarkers

Variables	Spearman's ρ	P value
Overall cohort		
EDN and calprotectin	0.454	0.004
Zonulin and calprotectin	0.294	0.048
Total IgE and EDN	−0.464	0.026
Total IgE and calprotectin	NS	>0.05
Total IgE and zonulin	NS	>0.05
AEC subgroup		
EDN and calprotectin	0.501	0.007

Variables	Spearman's ρ	P value
*AEP subgroup**		
EDN and zonulin	0.900	0.002

**Interpret with caution because of the limited number of observations. NS, not significant.*

Within the AEC subgroup, the positive correlation between EDN and calprotectin persisted (ρ=0.501; p=0.007). Within the AEP subgroup, EDN showed a strong positive correlation with zonulin (ρ=0.900; p=0.002). Because of the limited number of paired observations, subgroup correlations should be interpreted cautiously (Table 3).

Exploratory discriminatory performance

In ROC analysis, calprotectin showed the highest AUC among individual biomarkers for distinguishing AEC from AEP: 0.742 (95% CI 0.599–0.885; p<0.001), followed by EDN: 0.686 (95% CI 0.576–0.795; p<0.001). Zonulin yielded an AUC of 0.659 (95% CI 0.498–0.819; p=0.052).

An exploratory EDN cutoff of ≥1119.65 ng/mL corresponded to a sensitivity of 46.7% and specificity of 89.4%; for calprotectin, a cutoff of ≥441 mg/kg yielded a sensitivity of 59.5% and specificity of 83.3%; for zonulin, a cutoff of ≥101.8 ng/mL yielded a sensitivity of 90.0% and specificity of 46.7% (Table 4).

Table 4.

Exploratory discriminatory performance of individual fecal biomarkers for differentiating allergic enterocolitis from allergic enteropathy

Biomarker	Cut-off	AUC (95% CI)	Sensitivity (%)	Specificity (%)	P value
EDN (ng/mL)	≥1119.65	0.686 (0.576–0.795)	46.7	89.4	<0.001
Calprotectin (mg/kg)	≥441	0.742 (0.599–0.885)	59.5	83.3	<0.001
Zonulin (ng/mL)	≥101.8	0.659 (0.498–0.819)	90.0	46.7	0.052

AUC, area under the receiver operating characteristic curve; CI, confidence interval; EDN, eosinophil-derived neurotoxin. Cut-offs were selected using the Youden index. These analyses are exploratory and require independent validation.

Exploratory multivariable analysis

The multivariable binary logistic regression model including EDN, calprotectin, and zonulin in participants with complete data was statistically significant (χ^2=13.3; df=3; p=0.004). Calprotectin was independently associated with clinical phenotype (OR=1.009; 95% CI 1.002–1.020; p=0.009). EDN did not reach statistical significance by the Wald test (OR=0.997; 95% CI 0.994–1.000; p=0.053), and zonulin was not independently associated with phenotype (OR=1.009; 95% CI 0.988–1.030; p=0.387) (Table 5). The combined model yielded an AUC of 0.875 (95% CI 0.734–1.000; p<0.001), sensitivity of 77.8%, specificity of 91.7%, and overall accuracy of 87.9% (Table 5). Because the model was developed in complete cases and evaluated in the same cohort without independent validation, these estimates are considered exploratory.

Table 5.

Exploratory multivariable logistic regression and performance of the combined biomarker model

A. Multivariable logistic regression analysis

Predictor	β	OR (95% CI)	Wald P value
EDN	−0.00326	0.997 (0.994–1.000)	0.053
Calprotectin	0.00885	1.009 (1.002–1.020)	0.009
Zonulin	0.00919	1.009 (0.988–1.030)	0.387

B. Combined model performance

Parameter	Value	Parameter	Value
Model χ^2	13.3	Sensitivity, %	77.8
df	3	Specificity, %	91.7
P value	0.004	Positive predictive value, %	77.8
McFadden pseudo-R^2	0.345	Negative predictive value, %	91.7
Cox-Snell R^2	0.333	Accuracy, %	87.9
Nagelkerke R^2	0.482	Positive likelihood ratio	9.333
AUC (95% CI)	0.875 (0.734–1.000)	Negative likelihood ratio	0.242
P value (AUC)	<0.001		

DISCUSSION.

In this prospective cohort, the three fecal markers did not behave uniformly across the two clinically defined gastrointestinal food-allergy phenotypes. AEC was associated with higher EDN and calprotectin concentrations than AEP, while the difference in zonulin was not statistically significant. The positive correlation between EDN and calprotectin, observed overall and within AEC, further indicates that the inflammatory burden captured by these markers varies across clinical presentations. The data therefore favor a model of inflammatory heterogeneity rather than a single biomarker signature shared by all gastrointestinal food allergy.

EDN has been studied as a stool marker of eosinophil-associated activity in non-IgE-mediated food allergy [16, 28, 29, 33]. The higher EDN values observed in AEC are compatible with greater eosinophil-associated inflammatory activity, but our observational design cannot identify the responsible cellular mechanism. Calprotectin showed a similar between-phenotype pattern and the largest AUC among the individual markers. Its clinical specificity is limited, however, and fecal concentrations are particularly variable in infancy [7, 27, 28, 33, 39]. We therefore interpret both findings as evidence of differing intestinal inflammatory activity, not as validated diagnostic signatures for AEC or AEP.

EDN and calprotectin were positively correlated, suggesting that eosinophil-associated activity and broader intestinal inflammation may increase together in at least a subset of children. This statistical association should not be interpreted as evidence that one pathway causes the other; mechanistic confirmation would require tissue-level or longitudinal experimental data.

Comparison with previous studies illustrates why a phenotype-specific interpretation may be necessary. Rycyk et al. reported higher fecal EDN and calprotectin in infants with FPIAP [29], whereas Roca et al. found marked variability and limited discrimination in children with non-IgE-

mediated cow's milk protein allergy [28]. In a prospective cohort, Sun et al. found no association of EDN, calprotectin, or zonulin with FPIAP [33]. Lemoine et al. reported similar baseline EDN and calprotectin values in children with FPIES on an elimination diet and controls, but both markers rose after a failed oral food challenge [16]. The broader calprotectin literature is also heterogeneous [39]. Differences in phenotype, age, diet at the time of sampling, proximity to allergen exposure, diagnostic criteria, and analytical methods could all contribute. In our cohort, stool was obtained before the elimination diet was started.

Zonulin did not significantly separate AEC from AEP. Although zonulin-related measurements have been used in pediatric gastrointestinal research as markers associated with barrier function [7, 8, 34], the biological meaning of a commercial assay result is not straightforward. Scheffler et al. demonstrated that a widely used commercial ELISA did not measure pre-haptoglobin-2 as originally presumed [31]. Accordingly, the zonulin values in this study are best regarded as assay-derived exploratory measurements and should not be equated directly with quantitative intestinal permeability.

The three-marker model produced an AUC of 0.875, but this estimate comes from the subset with complete EDN, calprotectin, and zonulin data and from evaluation in the same cohort used to fit the model. We also did not perform a formal comparison between the combined-model AUC and the AUCs of the individual biomarkers. The model should therefore be viewed as a hypothesis-generating signal rather than evidence that the combination is superior; external prospective validation is required.

Previous research provides additional context for this phenotype-oriented interpretation. Non-IgE-mediated gastrointestinal allergy has been described as clinically heterogeneous and linked with broader atopic trajectories [22]; symptom resolution after elimination also varies in timing among affected children [4]. Contemporary work emphasizes that dietary avoidance has nutritional consequences requiring individualized management [37], and feeding difficulties represent an additional, variably defined burden in food-allergic children [14]. A recent prospective multicenter FPIES study further documented acute, chronic, atypical, and switching phenotypes with different clinical courses [38]. Together, these observations support describing gastrointestinal food allergy in terms of clinically meaningful heterogeneity rather than treating it as a biologically uniform condition.

These fecal measurements are not substitutes for established diagnostic pathways. For non-IgE-mediated gastrointestinal food allergy, diagnosis continues to depend on clinical assessment, dietary elimination and reintroduction, and oral food challenge when clinically appropriate [6, 11, 12, 17, 19, 21, 23, 25, 30, 36]. EDN and calprotectin may instead serve as adjunctive indicators of intestinal inflammatory activity. Their reproducible clinical role will require prospective studies with standardized phenotype definitions, longitudinal sampling, age-aware interpretation, prespecified analyses, and validation in an independent population.

Strengths and limitations

Strengths of this study include its prospective design, standardized clinical assessment, collection of stool samples before initiation of the elimination diet, and simultaneous evaluation of biomarkers reflecting different aspects of intestinal inflammatory activity. In all 130 affected children, improvement during a 2–4-week elimination period and recurrence of gastrointestinal symptoms following medically supervised reintroduction provided additional clinical support for food allergy. Several limitations should be acknowledged. First, the single-center design limits generalizability. Second, AEC and AEP were classified according to predominant clinical symptom patterns rather than independently validated phenotype-specific criteria; endoscopy and intestinal biopsy were not performed, so histological confirmation was unavailable. The available description does not establish a uniform, formally standardized oral food challenge protocol. Third, individual biomarker measurements were missing for some participants; analyses were therefore performed using available cases, whereas the multivariable model was restricted to complete cases with all three biomarker

measurements. Finally, the combined model was developed and evaluated in the same cohort and lacked independent validation. These limitations require cautious interpretation of the discriminatory analyses.

CONCLUSION.

Fecal biomarker profiles differed between clinically defined gastrointestinal food allergy phenotypes in infants and young children. AEC was associated with higher fecal EDN and calprotectin concentrations than AEP, whereas zonulin did not differ significantly between phenotypes. These findings support heterogeneity in local intestinal inflammatory activity across clinical presentations of gastrointestinal food allergy. The potential value of combining fecal biomarkers for phenotypic discrimination remains exploratory and requires prospective validation in larger independent cohorts.

Acknowledgments

The authors sincerely acknowledge the leadership of the Republican Specialized Scientific and Practical Medical Center of Pediatrics for their continuous support and for providing the clinical setting and facilities necessary for this study.

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