Article

Multiplex Specific IgE Profiling in Neonatal Stool of Preterms Predicts IgE-mediated Disease

Youssouf Sereme[1,2], Moïse Michel[1,2,3], Soraya Mezouar[1,2], Nicolas Orain[1,2], Renaud Cezar[3,4,5], Tu Anh Tran[4,5,6], Pierre Corbeau[3,5,7], Anne Filleron[4,5,6†] and Joana Vitte [1,2,8,†,*]

1 Aix Marseille Univ, IRD, APHM, MEPHI, Marseille, France
2 IHU Méditerranée Infection, Marseille, France
3 Immunology Department, University Hospital, Nîmes, France
4 IRMB, INSERM U1183, Montpellier, France
5 Montpellier University, Montpellier, France
6 Paediatrics Department, University Hospital Nîmes, Nîmes, France
7 Institute of Human Genetics, UMR9002, CNRS-Montpellier University, Montpellier, France
8 IDESP, INSERM UMR UA 11, University of Montpellier, Montpellier, France
†These authors share senior authorship of the manuscript
*Corresponding author: joana.vitte@inserm.fr; TEL.: (+33) 4 13 73 20 51; Fax: (+33) 4 13 73 20 52

Abstract: Background: Little is known about the natural history of immunoglobulin (Ig) E-mediated diseases in preterm infants, further hampered by the lack of noninvasive investigations. We aimed at developing a non-invasive tool for the study of IgE and eosinophil-derived neurotoxin (EDN) in neonatal fecal samples and evaluating its predictive value for the development of IgE-mediated diseases (cow's milk allergy, asthma, or atopic dermatitis) during the first year of life. **Methods:** We developed a stool extraction protocol, followed by freeze-drying and solubilization. sIgE responses were investigated in neonatal fecal samples from 21 preterm infants with a 300-allergen multiplex containing whole and molecular allergens and confirmed by capillary Western blot with nano-immunoassay. The local eosinophilic component was investigated by measuring the concentration of EDN. **Results:** The multiplexed allergen assay detected sIgE in all samples. Confirmation was obtained with Western blot. Frequency and levels of sIgE in neonatal fecal samples differed between infants who developed IgE-mediated diseases and controls. Neonatal fecal sIgE directed to milk proteins predicted later development of cow's milk allergy (specificity 88%, sensitivity 78%). Allergen specificity of neonatal fecal sIgE was associated with later development of cow's milk allergy and asthma. Neonatal fecal EDN levels predicted the development of IgE-mediated diseases (sensitivity 100%, specificity 75%). **Conclusion:** Non-invasive investigation of neonatal fecal sIgE is a promising tool for the prediction of subsequent development of IgE-mediated diseases.

Clinical Implications:
Non-invasive specific IgE profiling of neonatal fecal samples from preterm infants predicts later development of IgE-mediated diseases.

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Keywords: Fecal IgE; preterm birth; neonate; asthma; atopic dermatitis; cow's milk allergy; Western Blot

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Key Message

Neonatal fecal samples of preterm infants contain specific IgE directed to food and environmental allergens and eosinophilic activity predicting later development of cow's milk allergy and providing clues for the pathophysiology of atopic diseases.

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1. Introduction

The prevalence of allergic diseases has dramatically increased over the past century, especially in the pediatric population [1]. Allergic diseases in children are potential targets for preventive, diagnostic and therapeutic interventions[2]. The first three months after birth host a window of opportunity for allergic priming[3,4], building upon prenatal events such as transplacental allergen priming[5], transplacental transport of specific IgE[6], and early-life microbial colonization and immune response[7] These data suggest that shifting time frames, as seen in preterm infants, may lead to changes in later susceptibility to allergic diseases. Accordingly, data from cohort studies showed that children born prematurely were at increased risk for preschool wheezing and school-age asthma[8], while C-section but not preterm birth was related to food allergy[9] or atopic dermatitis (AD)[10]. Identifying preterm infants at risk for developing asthma, food allergy, or AD later in life would be particularly useful in this population.

Multiplexed allergen assays have proven their ability to identify distinct sensitization profiles in 3-year-old children, and to predict later asthma, wheeze, or AD[11]. However, such assays are performed with blood samples, making them unlikely to be performed on a routine basis in pediatric clinics, and even less in preterm infants[12]. Clinically useful IgE determination in local samples such as nasal, lacrimal, or salivary fluids has been reported[13-16]. The presence of IgE in children's fecal samples has been shown since 1985[17]. The pathophysiological significance of fecal IgE is supported by the demonstration of a gastrointestinal IgE reservoir of cells of the B lineage, involved in food allergy[18]. Hence, detection of fecal specific IgE (sIgE) might support the pathophysiological, diagnostic and prognostic evaluation of atopic and allergic conditions. Here, we provide the proof of concept for multiplex sIgE determination in meconial and neonatal stool samples from preterm infants and its predictive value for the subsequent development of cow's milk allergy (CMA), asthma, and AD.

2. Materials and Methods

Patients and ethics

Neonatal fecal samples from 21 preterm infants aged 0 to 14 weeks were investigated in this study. The infants were part of the birth cohort "Influence of intestinal microbiota implantation in preterm infants on microbiota and immune orientation at 3 years" (NCT02738411, Principal Investigator AF). The ancillary study presented here was approved by a joint committee of the Clinical Research Departments of the University Hospitals of Nîmes, France and the University Hospitals of Marseille, France (Research collaboration agreement 2018.1238).

Samples were collected at the University Hospitals of Nîmes, France and stored at -80°C prior to investigation. For the present study, stool samples were selected retrospectively, considering the presence or absence of one of the following conditions at age 1 year: CMA, asthma (defined as at least three episodes of bronchiolitis), or AD. Twenty-one neonatal fecal samples from 21 neonates were analyzed. There were 4 meconium samples (from one child who later developed CMA, and 3 who were free of IgE-mediated disease at age 1), 5 samples taken at ages 2-5 weeks (comprising 1 child who later developed CMA, 1 asthma, 1 AD, 1 CMA and asthma, and 1 no IgE-mediated disease), 6 samples taken at ages 6-8 weeks (from 2 children who later developed asthma, 2 CMA and asthma, 1 AD, and 1 no IgE-mediated disease), and 6 samples taken at ages 9-14 weeks (from 1 child who later developed CMA, 1 CMA and AD, 2 CMA and asthma, and 2 no IgE-mediated diseases) **(Supplementary Table 1).**

Fecal sample preparation

The EliA Stool Extraction kit (Thermo Fisher Scientific, Uppsala, Sweden) for stool preparation was used following the manufacturer's instructions. Briefly, one gram of stool from each subject was solubilized in two milliliters of extraction buffer. The mixture was incubated for 20 minutes at room temperature before centrifugation at 2,000 rpm for 15

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minutes at 4°C. The supernatant was filtered using the 0.22 µm Millipak® Express 40 Filter (Merck Millipore, Molsheim, France), freeze-dried for 24 hours, and then resolubilized in one milliliter of extraction buffer and used for further studies[4].

Allergen multiplex detection of fecal specific IgE

Detection of sIgE was first performed with the ALEX™ allergen multiplex (MacroArray DX, Vienna, Austria), containing 300 allergen extracts ("whole allergens") and molecular allergens[19]. A modified protocol adapted to stool extracts was developed. Briefly, the multiplex was incubated with a 1:5 dilution of stool extract under delicate stirring for two hours. After washing, the multiplex was incubated for 30 minutes with a dilution of anti-human IgE. After a second wash cycle, the enzyme substrate was added for 8 minutes, and the reaction was stopped by the addition of the stop solution. After drying the multiplex, the colorimetric intensity for each allergen point was measured by a camera. The images were digitized by a proprietary software (Raptor™, MacroArray DX) and converted to kUA/L measurements of sIgE to whole and molecular allergens. The measuring range was 0.30 - 50 kUA/L, and the lower limit of was 0.1 kUA/L.

Western blot detection of sIgE

Detection of fecal sIgE was further performed with Western blot as a control step. JessTM Simple Western system (Protein Simple, San Jose CA, USA, a Bio-Techne Brand), an automated capillary-based size separation and nano-immunoassay system and the manufacturer's standard method for 12-230 kDa JessTM separation module (SM-W004) were used to investigate fecal sIgE to cow's milk proteins. First, commercial purified casein from cow's milk (Abcam France) (1µg/µL) was mixed with 0.1X Sample buffer and Fluorescent 5X Master mix to achieve a final concentration of 0.25 µg/µL in the presence of fluorescent molecular weight markers and 400 mM dithiothreitol. Then, this preparation was denatured at 95°C for 5 minutes. The ladder (12-230 kDa PS-ST01EZ) and the proteins were separated in capillaries as they migrated through a separation matrix at 375 volts. A ProteinSimple proprietary photoactivated capture chemistry was used to immobilize separated milk proteins on the capillaries. Stool extracts at a 1:2 dilution were added and incubated for 60 minutes. After a wash step, goat HRP-conjugated anti-human IgE antibody (Abcam, France) diluted 1:500 was added for 30 min. Finally, the chemiluminescent revelations of ladder and samples were established with peroxide/luminol-S. Digital images were analyzed with Compass for SW software (version 4.1.0, Protein Simple), and quantified data of the detected proteins were reported as the molecular weight, chemiluminescence intensity and signal/noise ratio.

Eosinophil activity assessment

Eosinophil activity was investigated by measuring eosinophil-derived neurotoxin (EDN). The concentration of EDN was measured in stool extracts using a fluoroimmunoenzymatic assay with the ImmunoCAP™ 250 platform (Thermo Fisher Scientific, Uppsala, Sweden). The measuring range was 2 - 200 µg/L. Samples yielding a result at 200 µg/L or greater were not assayed after dilution, owing to a lack of data on the linearity of measurements with diluted samples.

Data expression and analysis

Results were expressed as median and interquartile range (IQR), unless otherwise stated. The statistical calculations and analyses were conducted using the GraphPad Prism software (GraphPad Software, San Diego, USA). Comparisons between groups were made using the chi-square goodness-of-fit test and the binomial test for prevalence studies, and non-parametric Student, Kruskal-Wallis, ANOVA and Mann-Whitney tests as appropriate for quantitative analysis and comparison. A p value < 0.05 was considered statistically significant.

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3. Results

Demographic and clinical characterization of the study population

Infants were born at a median gestational age of 215 days (30.7 weeks), IQR 199-223, with a median birth weight of 1275 g, IQR 925-1490, and a median birth height of 39 cm, IQR 35-42. Regarding delivery, 17/21 (81%) were born by C-section and 4/21 (19%) by vaginal delivery. Antibiotic treatment during the peripartum period was received by 6/21 mothers (**Supplementary Table 1)**.

Association of fecal sIgE detection with age and the presence of doctor-diagnosed atopic conditions at the age of 1 year

All stool samples displayed allergen multiplex sIgE binding higher than the manufacturer's cut-off value of 0.30 kUA/L. A trend to increasing numbers of sIgE detection with age at sampling was observed, although not significant **(Figure 1a)**. However, a significant increase in sIgE detection with sampling age was found in infants who were diagnosed with CMA, asthma or AD at age 1 **(Figure 1b)**.

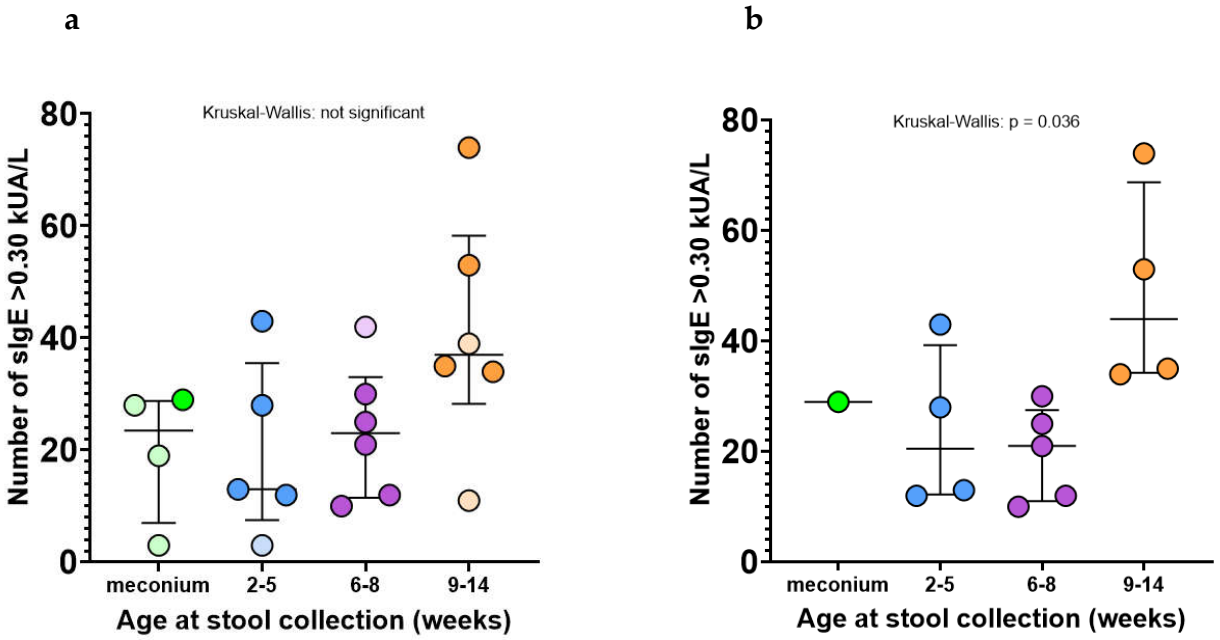

Figure 1. Number of multiplexed allergens displaying fecal sIgE binding as a function of age at stool collection. a, all fecal samples; **b**, fecal samples from patients diagnosed with CMA, asthma or AD at age 1. The colors denote the age at which samples were taken (green: meconial; blue: 2-5 weeks; purple: 6-8 weeks; orange: 9-14 weeks), with light shades denoting control subjects and dark shades denoting patients who developed IgE-mediated diseases by age 1. AD, atopic dermatitis; CMA, cow's milk allergy.

One hundred and eighty-six out of 300 whole and molecular allergens exhibited sIgE binding with at least one stool sample (**complete list provided in Supplementary Table 2**).

sIgE binding was more frequent in samples from patients who developed IgE-mediated diseases, a finding also holding true for meconium **(Table 1)**.

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Table 1. The prevalence of sIgE responses in neonatal fecal samples is predictive of doctor-diagnosed IgE-mediated conditions at the age of 1 year. The presence (sIgE+) or absence (sIgE-) of sIgE to each allergen among the 186 yielding at least one positive response in the study population was analyzed for each condition. Meconial samples, which were associated with one case of CMA but no asthma or AD, were analyzed separately from later samples. Analysis was done with Fisher's exact test. AD, atopic dermatitis; CMA, cow's milk allergy; OR, odds ratio; RR, relative risk.

	Condition at age 1 year	n	sIgE+ allergens (%)	sIgE- allergens (%)	P	Level of significance	RR (CI 95%)	OR (CI 95%)
CMA/meconium samples	CMA+	1	29 (16%)	157 (84%)	**0.014**	*	1.56 (1.11-2.10)	1.88 (1.13-3.05)
	CMA-	3	50 (9%)	508 (91%)				
Any IgE-mediated disease	AA+	13	390 (16%)	2028 (84%)	**0.027**	*	1.06 (1.01-1.11)	1.3 (1.03-1.67)
	AA-	4	95 (13%)	649 (87%)				
CMA	CMA+	8	298 (20%)	1190 (80%)	**<0.0001**	****	1.38 (1.27-1.50)	1.99 (1.64-2.43)
	CMA-	9	187 (11%)	1487 (89%)				
Asthma	Asthma+	8	249 (17%)	1239 (83%)	**0.043**	*	1.11 (1.01-1.22)	1.23 (1.01-1.49)
	Asthma-	9	236 (14%)	1438 (86%)				
AD	AD+	3	93 (17%)	465 (83%)	ns	0	1.10 (0.90-1.34)	1.13 (0.88-1.45)
	AD-	14	392 (15%)	2212 (85%)				

Fecal sIgE detection as a function of allergen families and atopic conditions at the age of 1 year

Next, sIgE reactivity was addressed as a function of the target allergens. In order to improve discrimination among allergen family-related pathophysiological effects, allergens were categorized following the IUIS classification[20] **(Table 2)**. Pollen and plant food whole allergens, as well as a mix of two molecular allergens belonging to distinct allergen protein families, Fra a 1+3 (strawberry PR-10 and nsLTP) were excluded from the analyses because of their complex allergenic contents at the molecular level (mixture of allergen protein families, precluding the analysis of individual protein family effects).

Meconial samples were analyzed separately from later samples because of (1) their exclusively prenatal build-up, (2) the small sample size (n = 4), and (3) the presence of IgE-mediated disease (CMA) at age 1 in only one child. The frequency of sIgE to allergen families (milk, storage proteins, cross-reactive pollen and food allergens, outdoor and indoor airborne allergens) did not differ as a function of CMA at age 1 (not shown).

Outside meconium, neonatal fecal samples from patients who were diagnosed as CMA at the age of 1 year (n = 8), as compared with controls who did not develop CMA (n = 9), displayed an increased frequency of sIgE reactivity to milk proteins, and to other allergen families involved in pediatric food allergy and asthma: kiwifruit marker allergens, storage proteins, and indoor airborne allergens (cockroach, mite, mold allergens and tropomyosin) **(Table 2)**.

In contrast, a diagnosis of asthma at age 1 (n = 8) was associated with an increased frequency of sIgE reactivity to airborne allergens when compared to non-asthmatic controls (n = 9) (**Table 3**). Analysis performed after exclusion of patients with CMA as a possible confounder from asthmatic and non-asthmatic groups did not alter this result (not shown).

1
2

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		faecal sIgE detected		no faecal sIgE detected	
ANIMAL FOOD ALLERGEN FAMILIES	MILK ALLERGENS	Bos d 4 Bos d 8 Bos d 5	Ovi a_milk Cam d	Bos d_milk Cap h_milk Equ c_milk	
	SERUMALBUMIN	Equ c 3 Can f 3	Fel d 2 Sus d 1	Bos d 6	
	MEAT ALLERGENS	Bos d_meat Equ c_meat Gal d_meat	Ory_meat Sus d_meat	Mel g Ovi a_meat	
	FISH ALLERGENS	Clu h Clu h 1 Raj c Sal s 1	Sco s Sco s 1 Thu a Thu a 1 Xip g 1	Cyp c 1 Gad m Gad m 1 Gad m 2+3 Raj c Parvalbumin Sal s	
	EGG ALLERGENS	Gal d 2 Gal d 3 Gal d 4	Gal d 5 Gal d_white Gal d_yolk	Gal d 1	
PLANT FOOD MARKER ALLERGENS	KIWI MARKER ALLERGENS	Act d 1 Act d 5			
	WHEAT ALLERGENS	Tri a aA_TI Tri a 14 Tri a 19			
	STORAGE PROTEINS	Ara h 1 Ara h 6 Cor a 14 Gly m 5 Gly m 6 Jug r 6	Mac i 2S Albumin Pap s 2S Albumin Pis v 1 Pis v 2 Ses i Ses i 1 Sin a 1	Ana o 2 Ana o 3 Ara h 2 Ara h 3 Ber e 1 Cor a 11 Cor a 9	Fag e 2 Gly m 8 Jug r 1 Jug r 2 Jug r 4 Pis v 3
	OLEOSIN	Ara h 15		Cor a 12_RUO	
POLLEN AND PLANT FOOD CROSS-REACTIVE ALLERGEN PROTEINS	LIPID TRANSFER PROTEINS	Act d 10 Api g 2 Ara h 9 Can s 3 Jug r 3	Mal d 3 Ole e 7_RUO Pru p 3 Sola l 6 Vit v 1 Zea m 14	Api g 6 Art v 3 Cor a 8 Pla a 3	
	THAUMATIN-LIKE PROTEINS	Act d 2 Mal d 2			
	PR-10	Aln g 1 Api g 1 Bet v 1	Cor a 1.0401 Fag s 1 Fra e 1 Mal d 1	Ara h 8 Cor a 1.0103 Dau c 1 Gly m 4	
	PROFILIN	Bet v 2 Cuc m 2 Hev b 8	Mer a 1 Phl p 12	Pho d 2	
	OTHERS	Pru p 7_RUO Hev b 11 Hev b 6.02	Ole e 9 Pis v 4_RUO	Aln g 4 Bet v 6	Pla a 2 Phl p 7
AIRBORNE MARKER ALLERGENS	POLLEN MARKERS	Amb a 1 Amb a 4 Art v 1 Che a 1 Cup a 1	Cyn d 1 Phl p 1 Pla a 1 Pla l 1 Sal k 1	Cry j 1 Lol p 1 Par j 2	Ole e 1 Phl p 2 Phl p 5.0101 Phl p 6
	LIPOCALIN	Bos d 2 Can f 2 Can f 4 Can f 6 Cav p 1	Equ c 1 Fel d 4 Mes a 1_RUO Mus m 1 Ory c 1	Can f 1 Fel d 7 Phod s 1	
	OTHER EPITHELIAL ALLERGENS	Equ c 4 Ory c 2 Ory c 3		Can f_Fd1 Fel d 1	
	FUNGAL ALLERGENS	Asp f 1 Asp f 3 Asp f 4 Asp f 6	Cla h 8 Mala s 11 Mala s 6	Alt a 1 Alt a 6 Mala s 5	
	MITES	Der p 1 Der p 11 Der p 20	Der p 21 Der p 7 Tyr p 2	Blo t 10 Blo t 21 Blo t 5 Der f 1 Der f 2	Der p 2 Der p 23 Der p 5 Gly d 2 Lep d 2
	COCKROACH	Bla g 4 Bla g 9 Bla g 5		Bla g 1 Bla g 2	
	TROPOMYOSIN AND INVERTEBRATE ALLERGENS	Ani s 1 Cra c 6 Pen m 2	Per a 7 Der p 10	Ani s 3 Arg r 1	Pen m 1 Pen m 3 Pen m 4
	INSECT VENOM ALLERGENS	Ves v 5 Pol d 5	Ves v 1 Api m 10	Api m 1	
	LATEX MARKER ALLERGENS	Hev b 3		Hev b 1 Hev b 5	

faecal sIgE detected but excluded from allergen group analysis

Aca m	Cyn d	Par j	Sal k
Ail a	Dol spp	Pec spp.	Sec c_flour
All c	Fic b	Per a	Sec c_pollen
All s	Fra a 1+3	Pers a	Ses i
Amb a	Hel a	Pet c	Sin
Ana o	Jug r_pollen	Pha v	Sol spp.
Api m	Loc m	Phr c	Sol t
Ber e	Lol spp.	Pim a	Sus d_epithelia
Car i	Mac inte	Pis s	Ten m
Car p	Man i	Pol d	Tri fo
Che q	Mus a	Pop n	Tyr p
Chi spp.	Myt e	Pru av	Ulm c
Cic a	Ost e	Pyr c	Urt d
Cla h	Ovi a_epithelia	Rat n	Vac m
Cuc p	Pap s	Sac c	Ves v
			Zea m

This group comprises whole allergens and the combined Fra a 1+3 (association of LTP and PR-10).

Table 2. Working definition of allergen families (sIgE>0.30kUA/L).

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Table 3. Allergen families associated with increased frequency of neonatal fecal sIgE in patients diagnosed as allergic to cow's milk or asthmatic at the age of 1 year. Results excluding meconial samples were analyzed in cow's milk allergic patients (n = 8) compared to cow's milk tolerant controls (n = 9), and in asthmatic patients (n = 8) compared with non-asthmatic patients (n = 9).

	Condition at age 1 year	sIgE+ allergens (%)	Total group allergen count	P	Level of significance	RR (CI 95%)	OR (CI 95%)
Milk allergens (Bos d 4, Bos d 5, Bos d 8, Ovi a _milk, Cam d)	CMA+	10 (25%)	40	**0.002**	**	2.2 (1.4-3.1)	14.7 (2.2-162)
	CMA-	1 (2%)	45				
Kiwifruit marker allergens (Act d 1, Act d 5)	CMA+	6 (38%)	16	**0.03**	*	2.4 (1.2-4.3)	10.8 (1.2-129)
	CMA-	1 (5.3%)	18				
Storage proteins	CMA+	29 (28%)	104	**<0.0001**	***	1.9 (1.5-2.4)	5.3 (2.2-11.4)
	CMA-	8 (7%)	117				
Indoor airborne allergens	CMA+	32 (19%)	168	**0.0007**	***	1.6 (1.3-2.0)	3.2 (1.6-6.5)
	CMA-	13 (7%)	189				
Indoor and outdoor airborne allergens	CMA+	52 (19%)	280	**0.01**	*	1.3 (1.1-1.6)	1.8 (1.1-2.9)
	CMA-	35 (11%)	315				
Indoor and outdoor airborne allergens	Asthma+	51 (18%)	280	**0.003**	**	1.4 (1.1-1.7)	2.1 (1.3-3.4)
	Asthma-	30 (10%)	315				

Quantitative analysis of neonatal fecal sIgE reactivity

First, sIgE levels measured against the 186 whole and molecular allergens yielding at least one positive result were analyzed without considering the specificity of their allergen targets. Levels of sIgE displayed considerable interindividual variation ($p<0.0001$) but did not differ significantly between controls and patients who developed IgE-mediated diseases (**Figure 2**).

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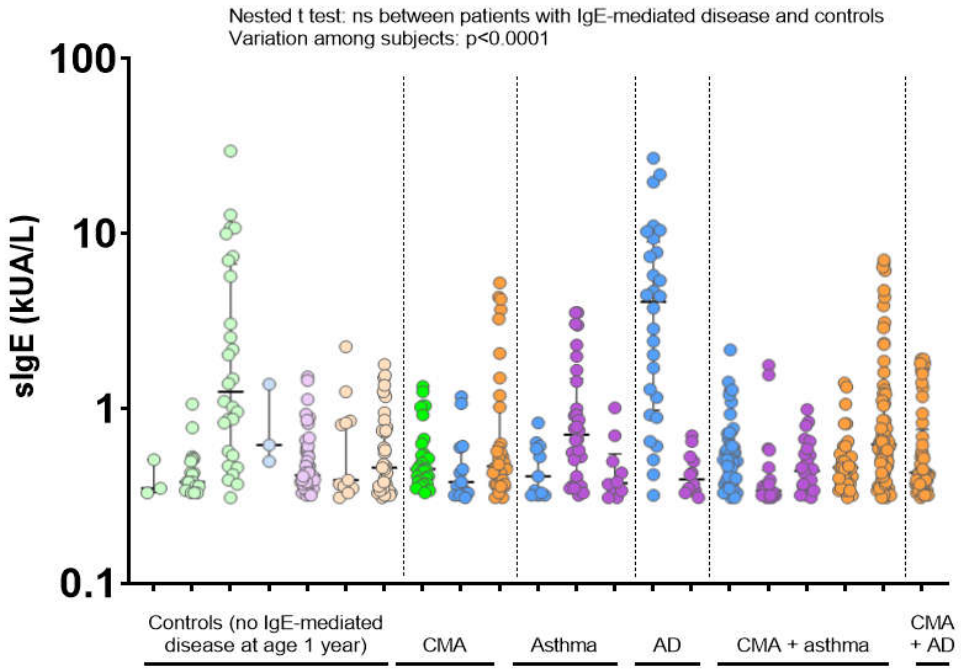

Figure 2. Levels of fecal sIgE reactivity in preterm infants according to the presence or absence of IgE-mediated disease at age 1 year. Values of sIgE greater than 0.30 kUA/L are represented. The colors denote the age at which samples were taken (green: meconial; blue: 2-5 weeks; purple: 6-8 weeks; orange: 9-14 weeks), with light shades denoting control subjects and dark shades denoting patients who developed IgE-mediated diseases by age 1. AD, atopic dermatitis, CMA, cow's milk allergy, ns, not significant.

Second, we addressed levels of sIgE to relevant allergen families, as described in **Table 2**. sIgE reactivity was expressed as the sum of sIgE levels directed to individual allergens comprised in the family. The presence of CMA at age 1 was associated with neonatal fecal sIgE recognition of milk allergens (p = 0.003, **Figure 3a**). ROC analysis using the level of neonatal fecal sIgE reactivity to milk allergens as a predictor of CMA occurrence during the first year of life yielded 88% (CI95%: 53-99%) sensitivity, 78% (CI95: 45-96%) specificity, and a likelihood ratio of 3.9 for a cut-off value of 0.41 kUA/L, p = 0.0045 (**Figure 3b**). Levels of neonatal fecal sIgE to kiwifruit marker allergens (p = 0.02, **Figure 3c**), wheat allergens (p = 0.03, **Figure 3d**), and oleosin, a nut marker allergen family (p = 0.02, **Figure 3e**) were higher in CMA patients than in controls.

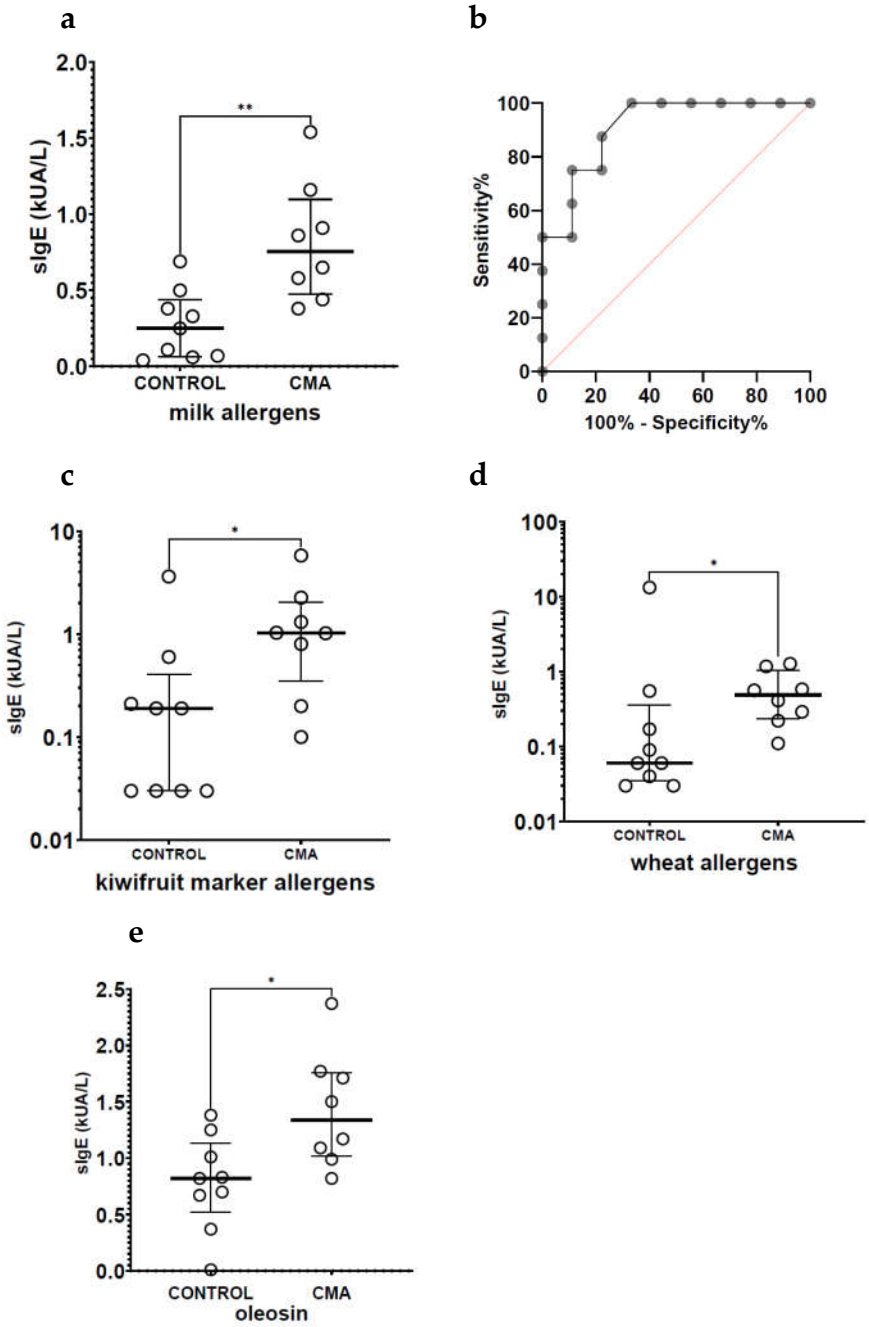

Figure 3. Levels of neonatal fecal sIgE directed to milk allergens and other common food allergens. a, Levels of neonatal fecal sIgE to milk allergens are predictive of CMA during the 1st year of life (p 0.003)**; b,** ROC analysis shows that a cut-off value of 0.41 kUA/L for neonatal fecal sIgE to milk allergens predicts CMA during the 1st year of life with a sensitivity of 88% (CI95%: 53-99%) and a specificity of 78% (CI95: 45-96%), p = 0.0045; **c, d, e:** Levels of neonatal fecal sIgE to kiwifruit marker allergens (p 0.02), wheat allergens (p 0.03), and oleosin (p 0.02)are higher in patients who developed CMA during the 1st year of life as compared to controls who did not.

Levels of sIgE reactivity to allergen families did not show preferential increase to allergens from specific families in asthmatic or AD patients (not shown). Similarly, meconial samples did not exhibit allergen-specific differences between the CMA patient and the three cow's milk tolerant subjects according to allergen families (not shown).

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Identification of cow's milk protein sIgE by Western blot in stool samples

As a validation method for the multiplex detection of fecal sIgE, we investigated the presence of IgE binding to full-length bovine casein containing α, β, and κ-casein isoforms (95% purity) using the capillary Western blot (WB) JessTM. According to the manufacturer's instructions, binding at 28-35 kDa denoted the presence of sIgE directed to casein. WB confirmed the presence of fecal sIgE binding to bovine casein, with 10 strongly positive samples and 4 others showing low binding (**Figure 4**). Intermethod comparison using clear positive and negative WB results showed agreement for 9 samples and disagreement for 8 samples (6 with positive WB and negative multiplex). From a clinical predictive perspective, six out of nine neonatal fecal samples from patients later diagnosed with CMA, and eight out of 12 without CMA, displayed sIgE reactivity in the WB assay (**Figure 4**).

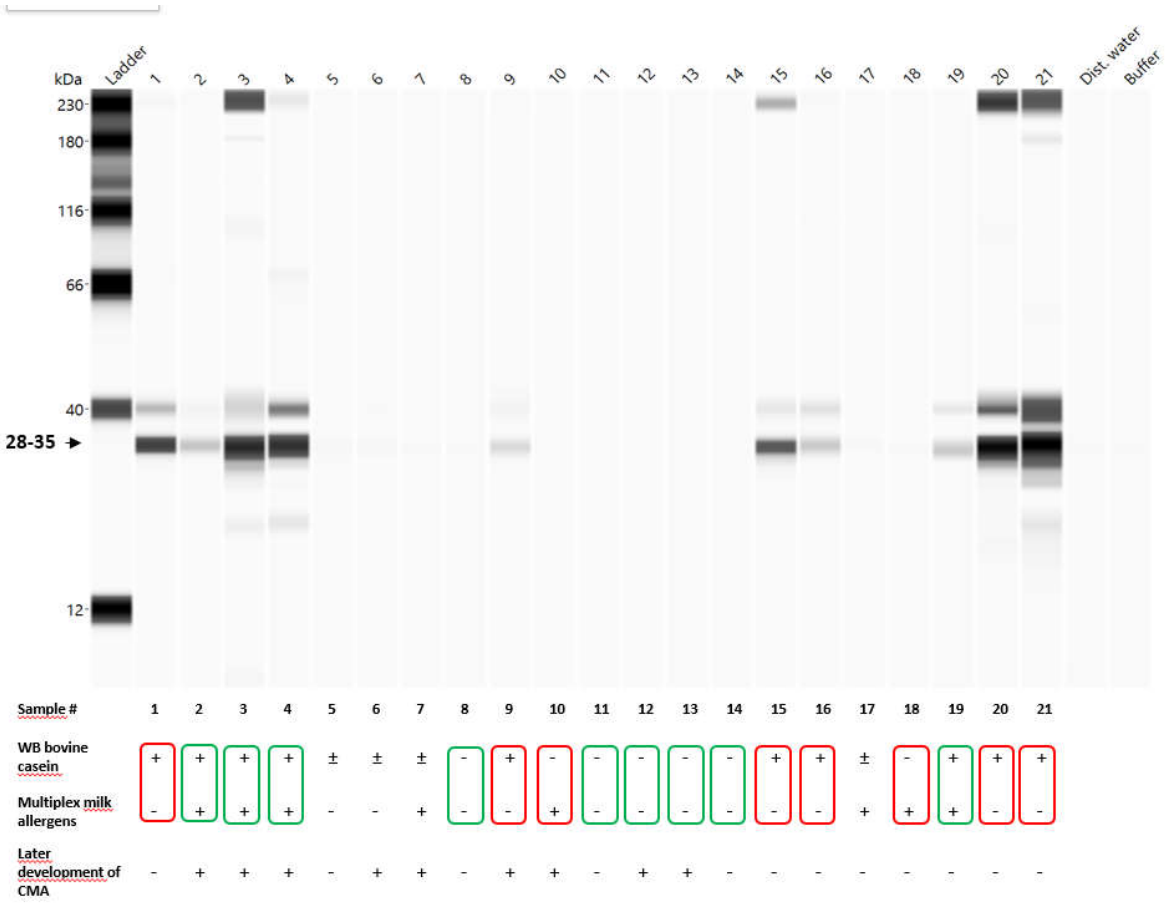

Figure 4. Western blot JessTM using full-length bovine casein. Western blot with bovine casein containing α, β, and κ-casein isoforms (95% purity) was used as a validation method for the detection of sIgE in neonatal fecal samples. The CMA status at age 1 and results of sIgE detection with multiplexed milk allergens are shown. Intermethod agreement (green frame) was found in 9 samples; disagreement (red frames) was found in 8 samples. Four samples with low WB IgE binding were not considered for intermethod agreement assessment.

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Fecal EDN detection and quantification

EDN was detected in all samples, from meconium to age 14 weeks (**Figure 5a**). There was a non-significant trend to increasing EDN concentrations in neonatal samples from infants who developed IgE-mediated diseases by age 1 year (median 200 μg/L, IQR 158/200), as compared to those who did not (median 52 μg/L, IQR 24-200). However, two out of three control meconial samples displayed EDN concentrations at the upper limit of the measuring range, while the meconial CMA sample demonstrated EDN concentration below the median of the CMA group (Figure 5a). Excluding meconial samples from the analysis, samples taken between 2 and 14 weeks displayed higher EDN concentrations in patients who later developed IgE-mediated diseases (median 157 μg/L, IQR 157-200), as compared with controls (median 31 μg/L, IQR 21-159), p = 0.016 (not shown). ROC analysis using fecal EDN concentrations in samples taken between 2 and 14 weeks as a predictor of IgE-mediated diseases during the first year of life yielded 100% (CI95%: 77-100%) sensitivity, 75% (CI95: 30-99%) specificity, and a likelihood ratio of 4.0 for a cut-off value of 50 μg/L, p = 0.04 (**Figure 5b**).

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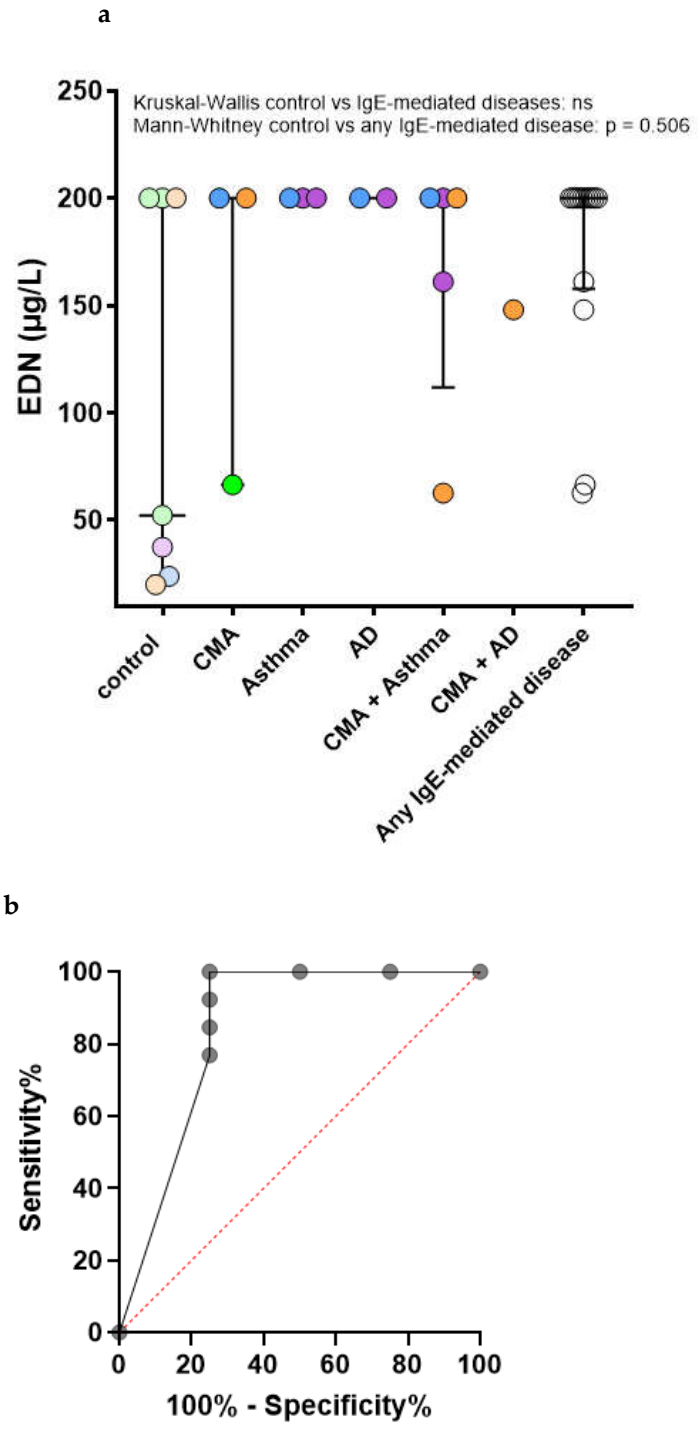

Figure 5. Fecal EDN concentration in neonatal fecal samples.**a,** EDN was detectable in all samples, with a non-significant increasing trend in patients who developed IgE-mediated diseases during the 1st year of life; **b,** ROC analysis shows that a cut-off value of 50 µg/L EDN in fecal samples taken between 2 and 14 weeks predicts IgE-mediated diseases during the 1st year of life with a sensitivity of 100% (CI95%: 77-100%) and a specificity of 75% (CI95: 30-99%), p = 0.04. The colors denote the age at which samples were taken (green: meconial; blue: 2-5 weeks; purple: 6-8 weeks; orange: 9-14 weeks), with light shades denoting control subjects and dark shades denoting patients who developed IgE-mediated diseases by age 1. AD, atopic dermatitis; CI, confidence interval; CMA, cow's milk allergy.

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4. Discussion

We have recently shown that the fecal immune profile of preterm infants is associated with the development of atopic conditions during the first year of life[4]. Here, we present the proof of concept for neonatal fecal sIgE determination as a predictive tool for the development of atopic/allergic conditions in preterm infants. We took advantage of the non-invasive nature of fecal sampling and the miniaturization of allergen multiplex platforms allowing extensive investigation of IgE sensitization.

First, we demonstrated that sIgE was present in all fecal samples, including meconium, and directed against a variety of food and environmental allergens. Fecal sIgE detection with multiplexed allergens was validated using WB as a distinct method and commercial bovine casein as a different source of allergen. Neonatal fecal sIgE was demonstrated with both methods. Detection of meconial sIgE adds to the report of transplacental transport of IgE[6]. Alternatively, meconial sIgE might originate from the fetus, as specific immunological reactions during pregnancy have been demonstrated[21]. The ability of allergens to cross the human placental barrier has also been shown *in vitro*[22]. Indeed, fetal exposure to allergens occurs as early as the twentieth week of pregnancy[5]. In our hands, a higher prevalence of meconial sIgE of any specificity was associated with the development of CMA, but not asthma or AD, during the first year of life.

From a clinical viewpoint, allergen specificity of neonatal fecal sIgE was associated with later development of IgE-mediated conditions. CMA occurrence during the first year after birth was predicted by neonatal fecal sIgE to milk proteins with a sensitivity of 88% and a specificity of 78%, suggesting a possible application as a screening method. CMA often presents as the entry mode of the atopic march, which might relate to our finding of increased frequency and levels of neonatal fecal sIgE to food allergens frequently involved in childhood food allergies, such as kiwifruit marker allergens Act d 1 (cystein-esterase) and Act d 5 (kiwellin), peanut and nut marker allergens (storage proteins, oleosin Ara h 15), and wheat allergens Tri a 19 (omega-5 gliadin), anti-trypsin inhibitor, and Tri a 14 (wheat nonspecific lipid protein), and indoor and outdoor airborne allergens. In our hands, later occurrence of asthma was associated with a higher frequency of neonatal fecal sIgE to outdoor and indoor airborne allergens. We did not find associations between AD development and neonatal fecal sIgE, which might be related to the low prevalence of AD in our study population (3/21).

IgE-related diseases often associate with eosinophilic inflammation. Here, we measured the eosinophilic granule protein EDN in neonatal fecal samples and found increased levels in patients who later developed CMA, asthma or AD, supporting the pathophysiological relevance of neonatal fecal sIgE reactivity. Intriguingly, EDN concentrations were higher in meconium than in samples taken between 2 and 14 weeks after birth.

A puzzling finding was the detection of neonatal fecal sIgE directed to an array of environmental allergens such as cockroach (Bla g 1, Bla g 2, Bla g 4, Bla g 5, Bla g 9, Per a 7), the tick *Argas reflexus* (Arg r 1), the fish parasite *Anisakis simplex* (Ani s 1), and insect venoms (Api m 1, Api m 10, Ves v 1, Ves v 5, Pol d 5). We speculate that such sIgE responses might relate to a broader function of immune defense of the mast cell-IgE couple, as suggested by recent findings[23,24], harnessing the ability of maternal, and presumably fetal, sIgE to sensitize fetal mast cells [6].

The strengths of our study are the development of a standardized, non-invasive method for fecal sIgE determination and its validation as a proof of concept for extensive sIgE profiling with miniaturized allergen multiplex assays in a cohort of preterm infants, allowing the assessment of 300 molecular and whole allergens in each subject, i.e. 6,300 IgE results in this study's population sample of 21 infants. The major weaknesses are the retrospective design, the low sample size, and the lack of serial samples from the same patient.

Further studies are warranted for the validation of sIgE profiling of fecal samples as a non-invasive diagnostic and predictive tool for early recognition of allergic sensitization in clinical practice. This approach is expected to improve early diagnosis of allergy while

limiting the risk and discomfort associated with current medical procedures. From a pathophysiological viewpoint, deeper insight will be gained into the role of sIgE and EDN in the fetal and neonatal gut, of breast versus bottle feeding and introduction of solid foods.

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Data Availability Statement: Data are available upon request from Dr ANne Filleron, PI of the Primibiota cohort" (**NCT02738411).**

Conflicts of Interest: JV reports speaker and consultancy fees in the past 5 years from Meda Pharma (Mylan), Novartis, Sanofi, Thermo Fisher Scientific, outside the submitted work. The other authors declare no competing interests in relation to this study.

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