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

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Abstract: Background: The natural history of immunoglobulin (Ig) E-mediated diseases in preterm infants is still elusive. We aimed at developing a non-invasive tool for detecting specific IgE (sIgE) and eosinophil-derived neurotoxin (EDN) in neonatal fecal samples and evaluating its predictive value for the development of IgE-mediated diseases during the first year of life. Methods: We developed a stool extraction protocol, followed by freeze-drying and solubilization. The sIgEs were investigated in neonatal fecal samples from 21 preterm infants with a 300-allergen multiplex and confirmed by a capillary Western blot with a nano-immunoassay. EDN concentration was used to investigate the local eosinophilic component. Results: The multiplexed allergen assay detected sIgE in all of the samples. A Western blot was used to confirm the results. The frequency and levels of sIgE in the neonatal fecal samples differed between the infants who developed IgE-mediated diseases and the controls. Allergen specificity was associated with the development of cow's milk allergy (CMA) and asthma. The development of CMA was predicted by the sIgE response to milk proteins (sensitivity was 88%; specificity was 78%). The EDN levels predicted the development of IgE-mediated diseases (sensitivity was 100%; specificity was 75%). Conclusion: The non-invasive investigation of neonatal fecal sIgE is a promising tool for predicting the subsequent development of IgE-mediated diseases. Clinical Implications: The non-invasive sIgE and EDN profiling of neonatal fecal samples from preterm infants can predict the development of IgE-mediated diseases.

Keywords: fecal IgE; EDN; preterm birth; neonate; asthma; atopic dermatitis; cow's milk allergy; Western blot

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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 immunoglobulin E (sIgE) [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-sections but not preterm birth were related to food allergies [9] or

atopic dermatitis (AD) [10]. Identifying preterm infants at risk of developing asthma, food allergies, 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 predict later asthma, wheezing, or AD [11]. However, because such assays require blood samples, they are unlikely to be performed on a routine basis in pediatric clinics, and even less likely 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 1984 [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, the detection of fecal sIgE might support the pathophysiological, diagnostic, and prognostic evaluation of atopic and allergic conditions. Here, we provide a 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

2.1. Patients and Ethics

Neonatal fecal samples from 21 preterm infants aged between 0 and 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).

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

2.2. Fecal Sample Preparation

The EliA Stool Extraction Kit (Thermo Fisher Scientific, Uppsala, Sweden) was used for the stool preparation, 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 min at room temperature before centrifugation at 2000 rpm for 15 min at 4 °C. The supernatant was filtered using the 0.22 µm Millipak® Express 40 filter (Merck Millipore, Molsheim, France), freeze-dried for 24 h, and then resolubilized in one milliliter of extraction buffer to be used for further studies[4].

2.3. Allergen Multiplex Detection of Fecal Specific IgE

The detection of sIgE was first performed with the ALEX™ allergen multiplex (MacroArray DX, Vienna, Austria), which contained 300 allergen extracts ("whole allergens") and molecular allergens [19]. A modified protocol adapted to the stool extracts was developed. Briefly, the multiplex was incubated with a 1:5 dilution of the stool extract under delicate

stirring for two hours. After washing, the multiplex was incubated for 30 min with a dilution of anti-human IgE. After a second wash cycle, the enzyme substrate was added for 8 min, and the reaction was stopped by the addition of a stop solution. After drying the multiplex, the colorimetric intensity for each allergen point was measured by a camera. The images were digitized using proprietary software (Raptor™, MacroArray DX) and converted to kUA/L measurements of sIgE to the whole and molecular allergens. The measuring range was 0.30–50 kUA/L, and the lower limit was 0.1 kUA/L.

2.4. Western Blot Detection of sIgE

The detection of fecal sIgE was further performed with a Western blot as a control step. The JessTM Simple Western system (ProteinSimple, San Jose, CA, USA, a Bio-Techne Brand), which is an automated capillary-based size separation and nano-immunoassay system, and the manufacturer's standard method for the 12–230 kDa JessTM separation module (SM-W004) were used to investigate fecal sIgE to cow's milk proteins. First, commercially purified casein from cow's milk (Abcam, France) (1 μg/μL) was mixed with 1× sample buffer and fluorescent 5× 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 min. The ladder (12–230 kDa PS-ST01EZ) and the proteins were separated in capillaries as they migrated through a separation matrix at 375 volts. ProteinSimple proprietary photoactivated capture chemistry was used to immobilize the separated milk proteins on the capillaries. Stool extracts at a 1:2 dilution were added and incubated for 60 min. After a wash step, a goat HRP-conjugated anti-human IgE antibody (Abcam, France) diluted at 1:500 was added for 30 min. Finally, the chemiluminescent revelations of the ladder and the samples were established with peroxide/luminol-S. The digital images were analyzed with Compass for SW software (version 4.1.0, ProteinSimple), and the quantified data of the detected proteins were reported as molecular weight, chemiluminescence intensity, and signal/noise ratio.

2.5. Eosinophil Activity Assessment

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

2.6. Data Expression and Analysis

The results were expressed as medians and interquartile ranges (IQRs), unless otherwise stated. The statistical calculations and analyses were conducted using the GraphPad Prism software, version is 9.5.1 (GraphPad Software, San Diego, CA, USA). The comparisons between the 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 were used as appropriate for the quantitative analysis and comparison. A p value of < 0.05 was considered statistically significant.

3. Results

3.1. Demographic and Clinical Characterization of the Study Population

The 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 their deliveries, 17/21 (81%) were born by C-sections, and 4/21 (19%) were born by vaginal delivery. Antibiotic treatments during the peripartum period were received by 6/21 mothers (Supplementary Table S1).

3.2. 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 that was higher than the manufacturer's cut-off value of 0.30 kUA/L. A trend of increasing numbers of sIgE detection with sampling age was observed, although it was 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 the age of 1 (Figure 1b).

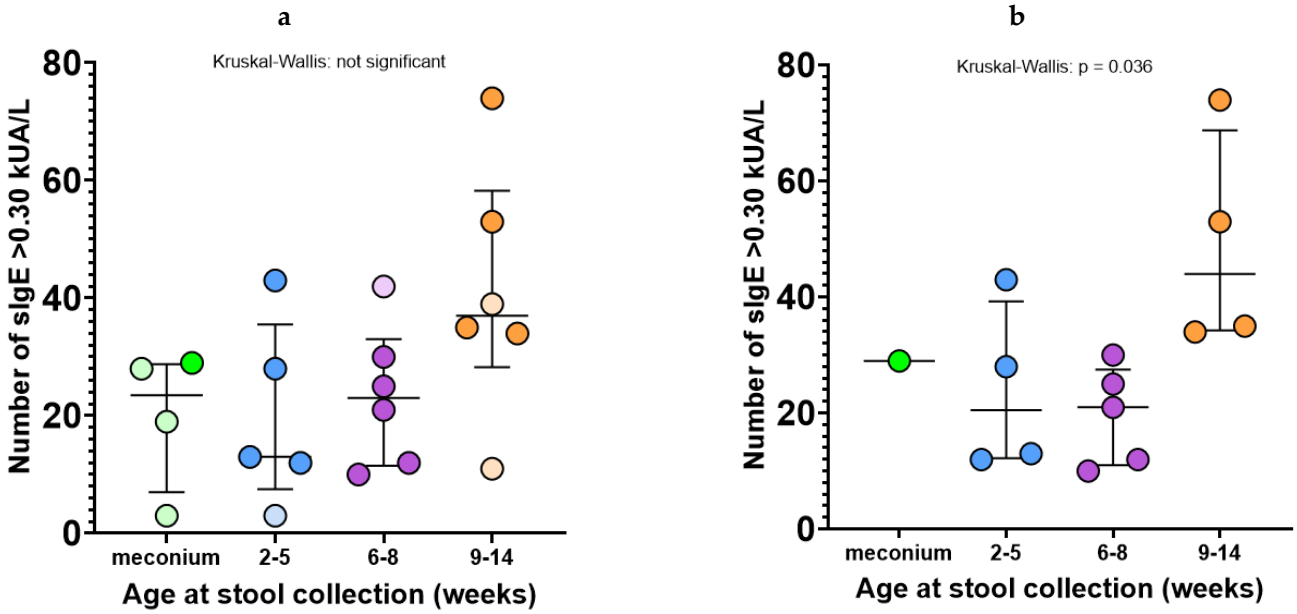

Figure 1. Number of multiplexed allergens displaying fecal sIgE binding as a function of age at stool collection. (**a**) All fecal samples; (**b**) fecal samples from patients diagnosed with CMA, asthma, or AD at the age of 1. The colors denote the age at which the samples were taken (green: meconial;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

A total of 186 of the 300 whole and molecular allergens showed sIgE binding with at least one stool sample (the complete list is provided in Supplementary Table S2). The sIgE binding was more frequent in samples from patients who developed IgE-mediated diseases, a finding that also held true for meconium (Table 1).

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 allergens yielding at least one positive response in the study population (3162 sIgE data, i.e., 21 samples × 186 allergens) was analyzed for each condition. The meconial samples, which were associated with one case of CMA but no asthma or AD, were analyzed separately from the later samples. The analysis was performed using Fisher's exact test. AD, atopic dermatitis; CMA, cow's milk allergy; OR, odds ratio; RR, relative risk. Asterisks indicate significant differences (* $p < 0.05$; **** $p < 0.0001$).

	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%)				

Table 1. *Cont.*

	Condition at Age 1 Year	n	sIgE+ Allergens (%)	sIgE− Allergens (%)	*p*	Level of Significance	RR (CI 95%)	OR (CI 95%)
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%)				

3.3. Fecal sIgE Detection as a Function of Allergen Families and Atopic Conditions at the Age of 1 Year

Next, the sIgE reactivity was addressed as a function of the target allergens. In order to improve the discrimination among the allergen family-related pathophysiological effects, the allergens were categorized following the IUIS classification [20] (Figure 2). Pollen, plant food whole allergens, and a mixture 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 (a mixture of allergen protein families precludes the analysis of individual protein family effects).

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

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

In contrast, a diagnosis of asthma at the age of 1 (n = 8) was associated with an increased frequency of sIgE reactivity to airborne allergens when compared to non-asthmatic controls (n = 9) (Table 2). The analysis performed after the exclusion of the patients with CMA as a possible confounder from the asthmatic and non-asthmatic groups did not alter this result (not shown).

		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

		faecal sIgE detected		no faecal sIgE detected	
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	

Figure 2. *Cont.*

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).

Figure 2. International Union of Immunological Societies (IUIS) classification of allergens. The allergen multiplex assay employed in this study comprised 300 allergen extracts and molecules representative of a wide array of plant and animal allergenic sources, of which 186 were bound by fecal sIgE. These 186 allergen extracts and molecules were grouped as a function of their state-of-the-art clinically relevant specificity and cross-reactivity [20]. The allergen extracts and molecules with defined specificity, e.g., fish extracts, allergens, or seed storage proteins, were assigned to corresponding groups. The cross-reactive families of allergen molecules were grouped together, e.g., profilins. The allergen extracts with extended cross-reactivity and limited informative values on genuine sensitization, e.g., grass pollen extracts, were excluded from the analysis.

Table 2. 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. Excluding the meconial samples, the results 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). Asterisks indicate significant differences (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

	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				

3.4. Quantitative Analysis of Neonatal Fecal sIgE Reactivity

First, the 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. The sIgE levels displayed considerable interindividual variation ($p < 0.0001$) but did not differ significantly between the controls and the patients who developed IgE-mediated diseases (Figure 3).

Second, we addressed the levels of sIgE for the relevant allergen families, as described in Figure 2. The sIgE reactivity was expressed as the sum of the sIgE levels directed to individual allergens in the family. The presence of CMA at the age of 1 was associated with the neonatal fecal sIgE recognition of milk allergens ($p = 0.003$, Figure 4a). An 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 4b). The levels of neonatal fecal sIgE to kiwifruit marker allergens ($p = 0.02$, Figure 4c), wheat allergens ($p = 0.03$, Figure 4d), and oleosin, a nut marker allergen family ($p = 0.02$, Figure 4e), were higher in the CMA patients than in the controls.

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

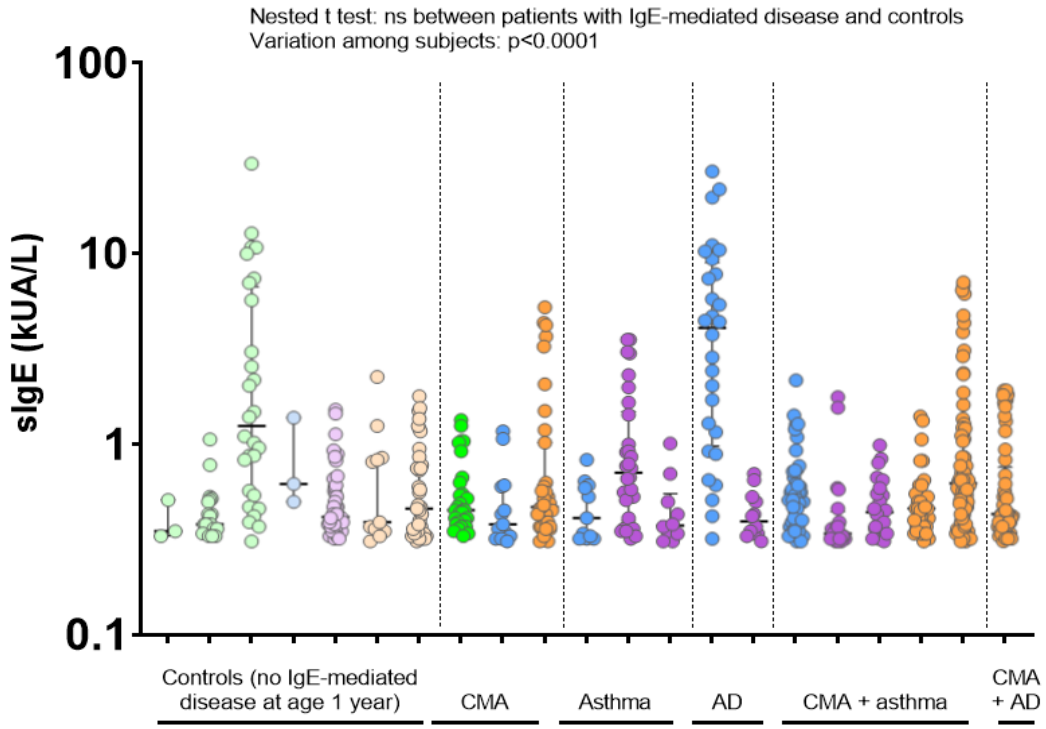

Figure 3. Levels of fecal sIgE reactivity in preterm infants according to the presence or absence of IgE-mediated disease at the age of 1 year. Values of sIgE greater than 0.30 kUA/L are represented. The colors denote the age at which the samples were taken (green: meconial; blue: 2–5 weeks; purple: 6–8 weeks; orange: 9–14 weeks), with the light shades denoting the control subjects and the dark shades denoting the patients who developed IgE-mediated diseases by the age of 1. AD, atopic dermatitis; CMA, cow's milk allergy; ns, not significant.

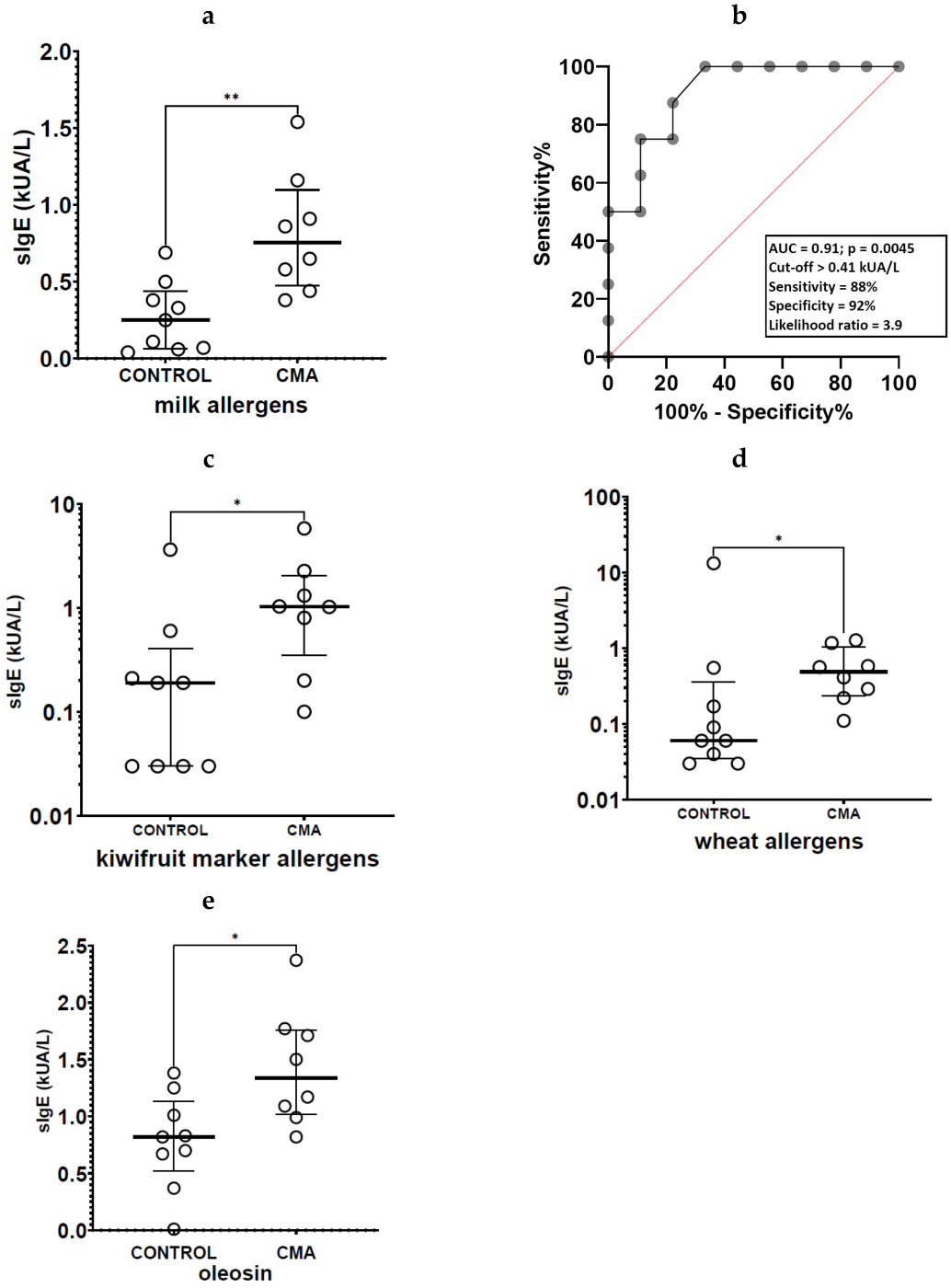

Figure 4. Levels of neonatal fecal sIgE directed to milk allergens and other common food allergens. (**a**) The levels of neonatal fecal sIgE to milk allergens are predictive of CMA during the 1st year of life (p = 0.003); (**b**) An ROC analysis shows that a cut-off value of 0.41 kUA/L for neonatal 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**–**e**) The 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 compared to the controls who did not. Asterisks indicate significant differences * $p < 0.05$; ** $p < 0.01$.

3.5. 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. The WB confirmed the presence of fecal sIgE binding to bovine casein, with ten samples that were strongly positive and four others that showed low binding (Figure 5). An intermethod comparison using clear positive and negative WB results showed agreement for nine samples and disagreement for eight samples (six 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 twelve without CMA displayed sIgE reactivity in the WB assay (Figure 5).

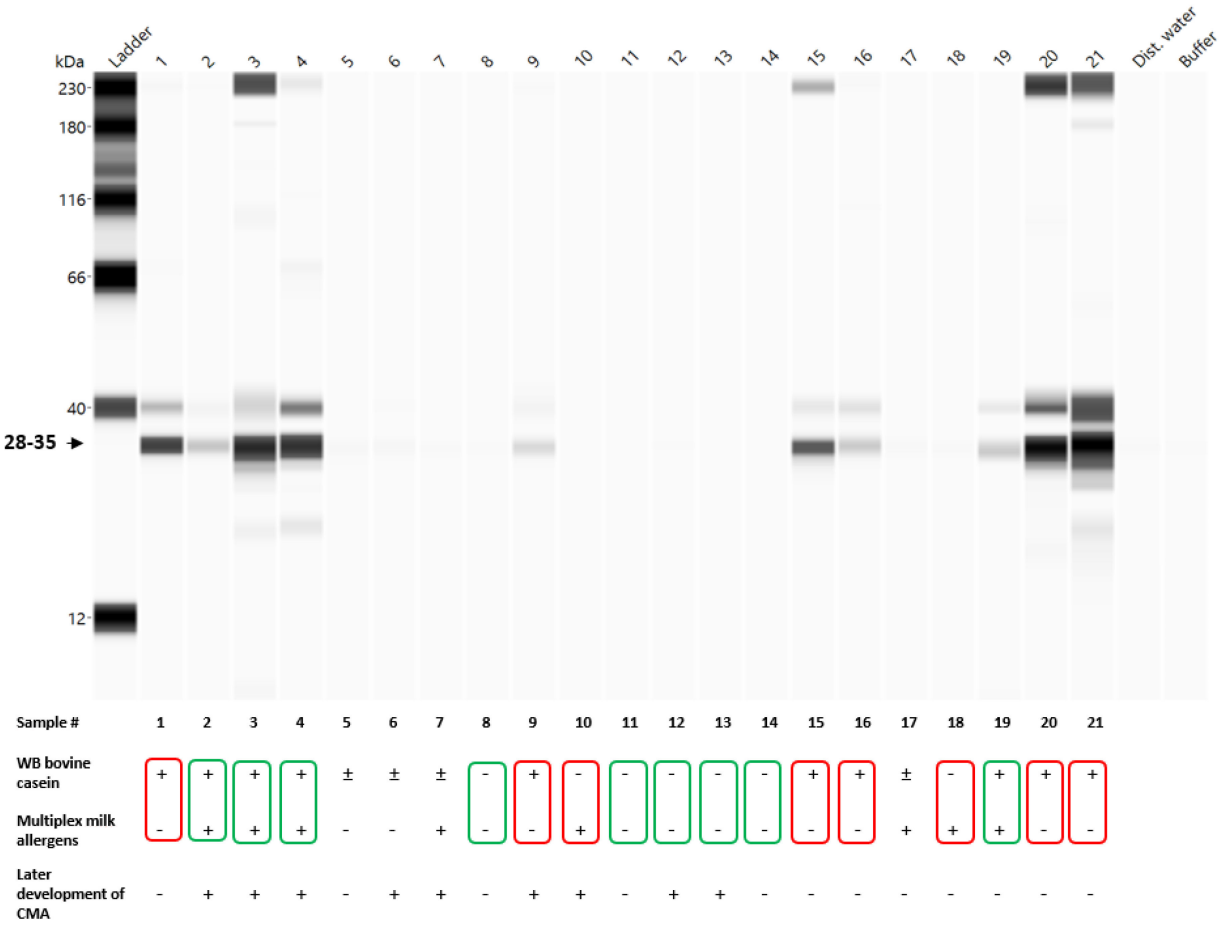

Figure 5. Western blot JessTM using full-length bovine casein. Western blot with bovine casein containing α, β, and κ-casein isoforms (95% purity) was used as a validation method for the detection of sIgE in neonatal fecal samples. The CMA status at the age of 1 and the results of the sIgE detection with multiplexed milk allergens are shown. Intermethod agreement (green frame) was found in nine samples; disagreement (red frames) was found in eight samples. Four samples were not considered for the intermethod agreement assessment due to low WB IgE binding.

3.6. Fecal EDN Detection and Quantification

EDN was detected in all of the samples, from meconium to the age of 14 weeks (Figure 6a). There was a non-significant trend of increasing EDN concentrations in neonatal samples from infants who developed IgE-mediated diseases by the age of 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 an EDN concentration below the median of the CMA group (Figure 6a). Excluding the meconial samples from the analysis, the 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 the controls (median 31 µg/L, IQR 21–159); $p = 0.016$ (not shown). An ROC analysis using fecal EDN concentrations in the 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 6b).

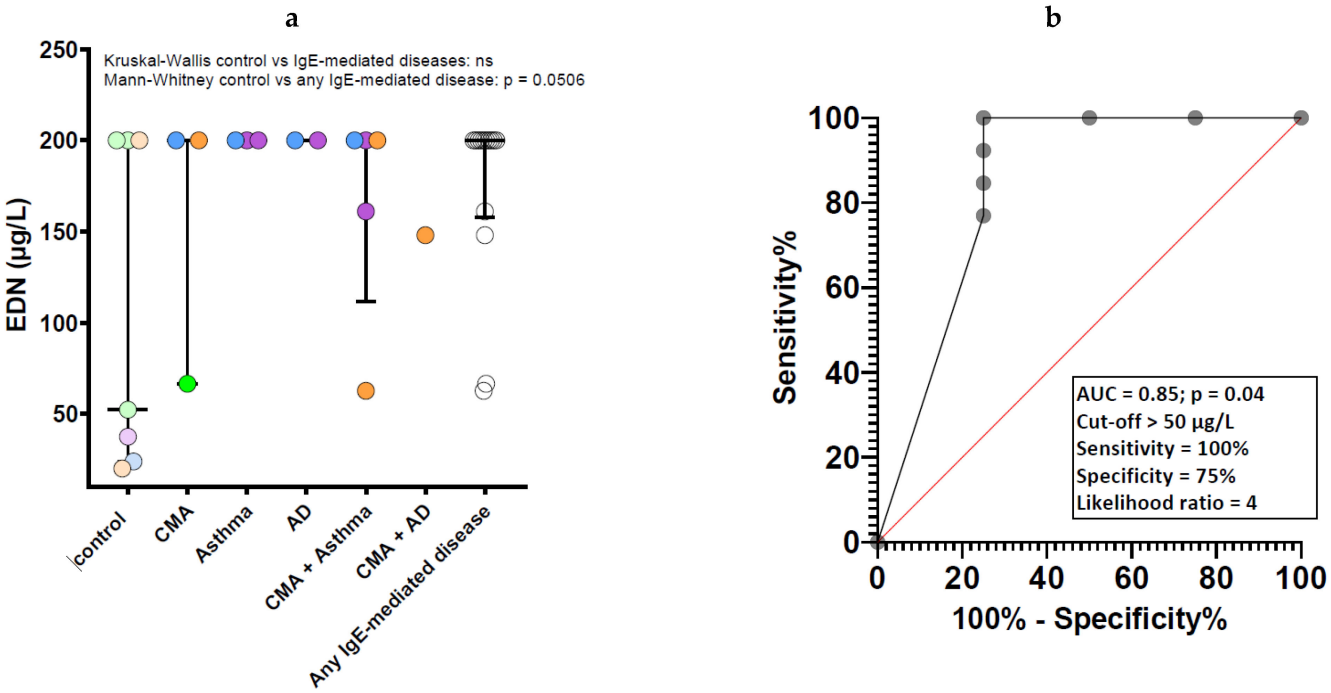

Figure 6. Fecal EDN concentration in neonatal fecal samples. (**a**) EDN was detectable in all samples, with a non-significant increasing trend in patients who developed IgE-mediated diseases during the 1st year of life; (**b**) An ROC analysis shows that a cut-off value of 50 µg/L EDN in fecal samples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

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 to conduct an extensive investigation of IgE sensitization.

First, we demonstrated that sIgE was present in all fecal samples, including meconium samples, and directed against a variety of food and environmental allergens. The fecal sIgE detection with multiplexed allergens was validated using WB as a distinct method and commercial bovine casein as a different source of allergens. Neonatal fecal sIgE was demonstrated with both methods. The 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, the allergen specificity of neonatal fecal sIgE was associated with the 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 first manifestation of the atopic march, in line with our finding of increased frequency and levels of neonatal fecal sIgE to food allergens, which are frequently involved in childhood food allergies, such as the kiwifruit marker allergens Act d 1 (cysteine-esterase) and Act d 5 (kiwellin), peanut and nut marker allergens (storage proteins, oleosin Ara h 15), and the wheat allergens Tri a 19 (omega-5 gliadin), anti-trypsin inhibitor, and Tri a 14 (wheat nonspecific lipid protein), as well as indoor and outdoor airborne allergens. In our study, the development of asthma during the first year of life was associated with an increased detection 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).

A puzzling finding was the detection of neonatal fecal sIgE directed to an array of environmental allergens, such as cockroaches (Bla g 1, Bla g 2, Bla g 4, Bla g 5, Bla g 9, and Per a 7), the soft 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, and 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].

Eosinophilic inflammation is often associated with IgE-related diseases. Here, we measured the eosinophilic granule protein EDN in the neonatal fecal samples and found increased levels in patients who later developed CMA, asthma, or AD and a significant predictive value for the subsequent development of CMA, supporting the pathophysiological relevance of neonatal fecal EDN in IgE-mediated diseases in preterm infants. In at-term infants, non-IgE-mediated CMA was not associated with variations in fecal EDN [25]. Fecal EDN concentrations in our preterm population were higher in meconium samples than in samples taken between 2 and 14 weeks after birth, in contrast with findings from a study of at-term infants [26].

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., 6300 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.

5. Conclusions

Further studies are warranted for the validation of sIgE profiling of fecal samples as a non-invasive diagnostic and predictive tool for the early recognition of allergic sensitization in clinical practices. This approach is expected to improve the early diagnosis of allergies while limiting the risk and discomfort associated with current medical procedures. From a pathophysiological viewpoint, a deeper insight will be gained into the role of sIgE and EDN in the fetal and neonatal gut, breast versus bottle feeding, and the introduction of solid foods.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/allergies3010005/s1, Table S1: Age at fecal sampling and distribution of IgE-mediated conditions diagnosed during 1st year of life. Table S2. Complete list of multiplexed allergens (whole allergens and molecules) assessed in the study.

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Informed Consent Statement: Informed consent was obtained from both parents at infants' birth.

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

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

References

1. Platts-Mills, T.A.E. The allergy epidemics: 1870–2010. *J. Allergy Clin. Immunol.* **2015**, *136*, 3–13. [CrossRef] [PubMed]
2. Halken, S.; Muraro, A.; de Silva, D.; Khaleva, E.; Angier, E.; Arasi, S.; Arshad, H.; Bahnson, H.T.; Beyer, K.; Boyle, R.; et al. EAACI guideline: Preventing the development of food allergy in infants and young children (2020 update). *Pediatr. Allergy Immunol.* **2021**, *32*, 843–858. [CrossRef] [PubMed]
3. Hornef, M.W.; Torow, N. 'Layered immunity' and the 'neonatal window of opportunity'—Timed succession of non-redundant phases to establish mucosal host-microbial homeostasis after birth. *Immunology* **2020**, *159*, 15–25. [CrossRef] [PubMed]
4. Sereme, Y.; Michel, M.; Mezouar, S.; Guindo, C.O.; Kaba, L.; Grine, G.; Mura, T.; Mege, J.L.; Tran, T.A.; Corbeau, P.; et al. A Non-Invasive Neonatal Signature Predicts Later Development of Atopic Diseases. *J. Clin. Med.* **2022**, *11*, 2749. [CrossRef] [PubMed]
5. Szépfalusi, Z.; Pichler, J.; Elsässer, S.; van Duren, K.; Ebner, C.; Bernaschek, G.; Urbanek, R. Transplacental priming of the human immune system with environmental allergens can occur early in gestation. *J. Allergy Clin. Immunol.* **2000**, *106*, 530–536. [CrossRef]
6. Msallam, R.; Balla, J.; Rathore, A.P.S.; Kared, H.; Malleret, B.; Saron, W.A.A.; Liu, Z.; Hang, J.W.; Dutertre, C.A.; Larbi, A.; et al. Fetal mast cells mediate postnatal allergic responses dependent on maternal IgE. *Science* **2020**, *370*, 941–950. [CrossRef]
7. Sereme, Y.; Guindo, C.O.; Filleron, A.; Corbeau, P.; Tran, T.A.; Drancourt, M.; Vitte, J.; Grine, G. Meconial Methanobrevibacter smithii suggests intrauterine methanogen colonization in preterm neonates. *Curr. Res. Microb. Sci.* **2021**, *2*, 100034.
8. Sonnenschein-van der Voort, A.M.; Arends, L.R.; de Jongste, J.C.; Annesi-Maesano, I.; Arshad, S.H.; Barros, H.; Basterrechea, M.; Bisgaard, H.; Chatzi, L.; Corpeleijn, E.; et al. Preterm birth, infant weight gain, and childhood asthma risk: A meta-analysis of 147,000 European children. *J. Allergy Clin. Immunol.* **2014**, *133*, 1317–1329. [CrossRef]
9. Mitselou, N.; Hallberg, J.; Stephansson, O.; Almqvist, C.; Melén, E.; Ludvigsson, J.F. Cesarean delivery, preterm birth, and risk of food allergy: Nationwide Swedish cohort study of more than 1 million children. *J. Allergy Clin. Immunol.* **2018**, *142*, 1510–1514. [CrossRef]
10. Haataja, P.; Korhonen, P.; Ojala, R.; Hirvonen, M.; Paassilta, M.; Gissler, M.; Luukkaala, T.; Tammela, O. Asthma and atopic dermatitis in children born moderately and late preterm. *Eur. J. Pediatr.* **2016**, *175*, 799–808. [CrossRef]
11. Wickman, M.; Lupinek, C.; Andersson, N.; Belgrave, D.; Asarnoj, A.; Benet, M.; Pinart, M.; Wieser, S.; Garcia-Aymerich, J.; Baar, A.; et al. Detection of IgE Reactivity to a Handful of Allergen Molecules in Early Childhood Predicts Respiratory Allergy in Adolescence. *EBioMedicine* **2017**, *26*, 91–99. [CrossRef]

12. Ansotegui, I.J.; Melioli, G.; Canonica, G.W.; Caraballo, L.; Villa, E.; Ebisawa, M.; Passalacqua, G.; Savi, E.; Ebo, D.; Gomez, R.M.; et al. IgE allergy diagnostics and other relevant tests in allergy, a World Allergy Organization position paper. *World Allergy Organ J.* **2020**, *13*, 100080. [CrossRef]
13. Nunes, M.P.O.; van Tilburg, M.F.; Tramontina Florean, E.O.P.; Guedes, M.I.F. Detection of serum and salivary IgE and IgG1 immunoglobulins specific for diagnosis of food allergy. *PLoS ONE* **2019**, *14*, e0214745. [CrossRef]
14. Leonardi, A.; Borghesan, F.; Faggian, D.; Plebani, M. Microarray-based IgE detection in tears of patients with vernal keratoconjunctivitis. *Pediatr. Allergy Immunol.* **2015**, *26*, 641–645. [CrossRef]
15. Aghayan-Ugurluoglu, R.; Ball, T.; Vrtala, S.; Schweiger, C.; Kraft, D.; Valenta, R. Dissociation of allergen-specific IgE and IgA responses in sera and tears of pollen-allergic patients: A study performed with purified recombinant pollen allergens. *J. Allergy Clin. Immunol.* **2000**, *105*, 803–813. [CrossRef]
16. Chawes, B.L.; Wolsk, H.M.; Carlsson, C.J.; Rasmussen, M.A.; Følsgaard, N.; Stokholm, J.; Bonnelykke, K.; Brix, S.; Schoos, M.A.M.; Bisgaard, H. Neonatal airway immune profiles and asthma and allergy endpoints in childhood. *Allergy* **2021**, *76*, 3713–3722. [CrossRef]
17. Kolmannskog, S.; Haneberg, B.; Marhaug, G.; Bolle, R. Immunoglobulin E in Extracts of Feces from Children. *Int. Arch. Allergy Immunol.* **1984**, *74*, 50–54. [CrossRef]
18. Hoh, R.A.; Joshi, S.A.; Lee, J.-Y.; Martin, B.A.; Varma, S.; Kwok, S.; Nielsen, S.C.A.; Nejad, P.; Haraguchi, E.; Dixit, P.S.; et al. Origins and clonal convergence of gastrointestinal IgE + B cells in human peanut allergy. *Sci. Immunol.* **2020**, *5*, eaay4209. [CrossRef]
19. Heffler, E.; Puggioni, F.; Peveri, S.; Montagni, M.; Canonica, G.W.; Melioli, G. Extended IgE profile based on an allergen macroarray: A novel tool for precision medicine in allergy diagnosis. *World Allergy Organ. J.* **2018**, *11*, 7. [CrossRef]
20. IUIS/WHO Allergen Nomenclature. Available online: http://allergen.org (accessed on 3 January 2023).
21. Daffos, F.; Grangeot-Keros, L.; Lebon, P.; Forestier, F.; Pavlovsky, M.C.; Chartier, M.; Pillot, J. Prenatal diagnosis of congenital rubella. *Lancet* **1984**, *324*, 1–3. [CrossRef]
22. Casas, R.; Björkstén, B. Detection of Fel d 1-immunoglobulin G immune complexes in cord blood and sera from allergic and non-allergic mothers: Fel d 1-IgG immune complexes in cord blood and sera. *Pediatr. Allergy Immunol.* **2001**, *12*, 59–64. [CrossRef] [PubMed]
23. Starkl, P.; Gaudenzio, N.; Marichal, T.; Reber, L.L.; Sibilano, R.; Watzenboeck, M.L.; Fontaine, F.; Mueller, A.C.; Tsai, M.; Knapp, S.; et al. IgE antibodies increase honeybee venom responsiveness and detoxification efficiency of mast cells. *Allergy* **2022**, *77*, 499–512. [CrossRef]
24. Starkl, P.; Watzenboeck, M.L.; Popov, L.M.; Zahalka, S.; Hladik, A.; Lakovits, K.; Radhouani, M.; Haschemi, A.; Marichal, T.; Reber, L.L.; et al. IgE Effector Mechanisms, in Concert with Mast Cells, Contribute to Acquired Host Defense against *Staphylococcus aureus*. *Immunity* **2020**, *53*, 793–804. [CrossRef] [PubMed]
25. Roca, M.; Donat, E.; Rodriguez Varela, A.; Carvajal, E.; Cano, F.; Armisen, A.; Ekoff, H.; Cañada-Martínez, A.J.; Rydell, N.; Ribes-Koninckx, C. Fecal Calprotectin and Eosinophil-Derived Neurotoxin in Children with Non-IgE-Mediated Cow's Milk Protein Allergy. *J. Clin. Med.* **2021**, *10*, 1595. [CrossRef] [PubMed]
26. Vidova, V.; Benesova, E.; Klanova, J.; Thon, V.; Spacil, Z. Simultaneous quantitative profiling of clinically relevant immune markers in neonatal stool swabs to reveal inflammation. *Sci. Rep.* **2021**, *11*, 10222. [CrossRef]