Simultaneous quantitative profiling of clinically relevant immune markers in neonatal stool swabs to reveal inflammation

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An aberrant immune response developed early in life may trigger inflammatory bowel disease (IBD) and food allergies (e.g., celiac disease). Fecal levels of immune markers categorize an inflammatory response (e.g., food allergy, autoimmune) paralleled with the initial microbial colonization. The immunoaffinity assays are routinely applied to quantify circulating immune protein markers in blood/serum. However, a reliable, multiplex assay to quantify fecal levels of immune proteins is unavailable. We developed mass spectrometry assays to simultaneously quantify fecal calprotectin, myeloperoxidase, eosinophil-derived neurotoxin, eosinophil cationic protein, alpha-1-antitrypsin 1, and adaptive immunity effectors in 134 neonatal stool swabs. We optimized extraction and proteolytic protocol and validated the multiplex assay in terms of linearity of response (> 100; typically 0.04 to 14.77 µg/mg of total protein), coefficient of determination (R2; > 0.99), the limit of detection (LOD; 0.003 to 0.04 µg/mg of total protein), the limit of quantification (LOQ; 0.009 to 0.122 µg/mg of total protein) and robustness. The median CV of intra- and interday precision was 9.8% and 14.1%, respectively. We quantified breast milk-derived IGHA2 to differentiate meconium from feces samples and to detect the first food intake. An early life profiling of immune markers reflects disrupted intestinal homeostasis, and it is perhaps suitable for pre-symptomatic interception of IBD and food allergies.

Inflammatory bowel disease (IBD) and food allergies are a highly prevalent and diverse group of intestinal disorders, potentially resulting in chronic inflammation of the gastrointestinal tract (e.g., Crohn's disease, ulcerative colitis, and celiac diseases). IBD is an umbrella term for disorders with unknown etiology affecting 6.8 million people globally4. In Western countries, as high as 10% of the population reacts abnormally to a food allergen, with the highest prevalence among younger children2. The global prevalence of celiac disease was 1.4%, significantly greater in children than adults4. However, specific causes of food allergies are challenging to explore, and the prevalence is likely underreported4,5.

The early life or even prenatal exposure of the naïve immune system to human gut microbiota plays a prominent role in immunomodulation, potentially resulting in an inappropriate immune response and disorders6,7. The mode of delivery (i.e., cesarean section or vaginal birth), nutritional and lifestyle factors (e.g., breastfeeding, hygiene, probiotics), or medication (e.g., antibiotics) shapes the composition of the human gut microbiota, influencing the response of the immature immune system and homeostasis8–10. Naturally-born neonates benefit from early colonization by the vaginal microbiome11,12. On the contrary, adverse influence is attributed to the early colonization by nosocomial or skin microbiota after cesarean section13,14. Breastfeeding reportedly prevents allergies, asthma, and infections15. Mucosal immunoglobulins, prebiotics, and other breast milk components protect against disease and allergies via modulation of developing immune system and intestinal barrier function. Mucosal epithelial cells are arranged into a tight junctional complex during intestinal maturation and later form the permeable barrier between the intestinal lumen and the lamina propria16. In autoimmune diseases, the presence of allergens and pathogens causes intestinal inflammation and disruption of epithelial cells. Neutrophils, monocytes, eosinophils, white blood cells, and blood protein occur in the stool due to the intestinal barrier's lower integrity, causing inflammation.

The naïve immune system's inflammatory response traced via specific immunological markers in meconium or first feces can reveal a disease condition. Fecal calprotectin (CAL1, CAL2) and myeloperoxidase (MPO)

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The authors confirm that the data supporting this study’s findings are available within the article and supplementary material. The methods used in the study and described below were carried out following the relevant guidelines and regulations. The authors confirm that the data supporting this study’s findings are available within the article and supplementary material.

Table 1. Immune protein markers were assayed in meconium and feces. The proteotypic surrogate peptides for absolute quantification. The position of stable isotope-labeled arginine (R*; 1\(^{15}\)N\(_2\); + 10 Da mass shift) or lysine (K*; 1\(^{15}\)N\(_4\); + 8 Da mass shift) in the proteotypic sequence of internal standard synthetic peptides marked in bold.

| Protein number | Gene name | Name | Abbreviation | Proteotypic sequence |
|---------------|-----------|------|--------------|----------------------|
| P01009-1      | SERPINA1  | Alpha-1-antitrypsin isom 1 | A1AT-1 | AVLTIDEK |
| P01876        | IGHA1     | Immunoglobulin heavy constant alpha 1 | IGHA1 | TPLATLISK |
| P01876 + P01877 | IGHA1 + 2 | Immunoglobulin heavy constant alpha 1 and 2 | IGHA1 + 2 | SAVQGQPER |
| P01877        | IGHA2     | Immunoglobulin heavy constant alpha 2 | IGHA2 | DASGATFTWTPSGK |
| P12724        | RNASE3    | Eosinophil cationic protein | ECP | NQNTFLK |
| P10153        | RNASE2    | Eosinophil-derived neurotoxin | EDN | DPOQPVVPVHDLR |
| P05164        | MPO       | Myeloperoxidase | MPO | VVLEGIDPILR |
| P05109        | S100-A8   | Calprotectin 1 | CAL1 | ALENSIDYTHK |
| P06702        | S100-A9   | Calprotectin 2 | CAL2 | DLQNFLK |

Table 1 reflects the intestinal mucosal barrier homeostasis. The multiplex assay is suitable for classifying inflammatory response and potentially reveals a propensity of IBD and food allergies (Fig. 1).
Figure 1. Scheme of immune protein markers’ role in the intestinal inflammatory response, barrier function, and homeostasis.

Table 2. The validation parameters of assessed proteotypic peptides, i.e., the linearity of response, assay range, LOD, and LOQ. All concentrations in (nM) for peptide surrogates and in (µg/mg) for target protein in total protein (by BCA). The position of stable isotope-labeled arginine (R*: 13C6H14O2 15N4; + 10 Da mass shift) or lysine (K*: 13C6H14O2 15N2; + 8 Da mass shift) in the proteotypic sequence of internal standard synthetic peptides marked in bold.
Protein extraction protocol. We precipitated proteins by adding 1 ml of 80% IPA to the sample and orbital shaking (5 min, 1600 rpm). We centrifuged samples (2 min, 12,000 × g) and removed 50 µL of supernatant. The residual sample volume (950 µL), including the swab, was dried in speed vac overnight (minimum 6 h). Dried samples were reconstituted in 1500 µL of buffer (50 mM ABB with 5 g/l SDC) and homogenized (Benchmark Scientiﬁcs, Bead Blaster 24 homogenizer, 4 pulses × 30 s; 4 m/s; inter-time 10 s; ambient temperature). Next, we centrifuged samples (3000 × g; 10 min), transferred a supernatant (500 µL) into a clean vial, and centrifuged the vial again (12,000 × g; 5 min). After the second centrifugation, the supernatant (400 µL) was transferred into another clean vial for the trypsin digestion of extracted proteins. Total protein concentration in 134 neonatal meconium or feces swabs extracts (Table S2) were assessed in 10 µL of protein extract using the BCA assay (cat. #23225). We subjected the protein extract (50 µL) to the trypsin digestion protocol.

Sample fresh weight total protein content. We prepared quality control (QC) material pooling fresh meconium and feces samples from 12 randomly selected neonates. Various QC material amounts were accurately weighed (25, 50, 75, 100, 125, 150 mg) on a swab in triplicate. We extracted proteins from feces swabs as described above and determined the protein content in protein extracts using the BCA assay (Table S3). We established the correlation between the stool sample fresh weight and total protein content and used QC swabs for method validation and reproducibility assessment.

Trypsin proteolytic protocol. We reduced and alkylated protein extracts (50 µL) by adding 5 µL of 200 mM DTT (10 min at 95°C) and consequently adding 5 µL of 400 mM IAA (30 min at ambient temperature in the dark). We added a working solution (10 µL; 500–600 nM) of the SIL-TCT peptide internal standards into a sample. Next, we added trypsin (3 µL; 1 µg/µL) approximately in the ratio of 1:70 to the total protein content and incubated samples at 37°C (orbital shaking 200 rpm). We quenched the trypsin digestion after 5 h by adding 200 µL of 2% FA and peptides were purified using solid-phase extraction (SPE, Oasis HLB prime; 96-well plate format, 30 mg; Waters, Milford, MA). Samples were loaded on SPE, washed with 300 µL of 2% FA, and eluted with 200 µL 50% ACN with 2% FA. SPE eluates were dried in speed vac, reconstituted in 50 µL 5% ACN with 0.1% FA, resulting in 100–120 nM SIL-TCT internal standard concentrations in the sample (Table 2), and analyzed by UHPLC/SRM-MS.

Proteolysis time optimization. Protein extracts from individually extracted QC swabs (n = 3) were combined (1200 µL) and divided into 21 identical aliquots (50 µL) to prepare the time-lapse experiment in triplicate. We did not add trypsin to samples without incubation (time 0 h), spiked with proteotypic SIL peptides (added 10 µL of 1.5 µM mixed working solution, all proteotypic sequences listed in Table 1). Trypsin was added to all incubated samples, spiked with SIL-TCT internal standards (all proteotypic sequences listed in Table 1). We quenched the trypsin digestion after 1, 3, 5, 17, 20, and 24 h of incubation. The 2% FA (200 µL) was added to all samples, followed by SPE processing and UHPLC/SRM-MS analysis. The reproducibility of trypsin digestion was tested using SIL-TCT peptides SAVQGPPER, DASGATFTWTSSGK, TPLTATLSK, AVLTIDETK, DLQNFLK, LGHPDTLNQGEFK, ALNSIDVYHK, DPPQYPVVPVHLDR, NQNTFLR, and VVLEGGIDPILR.

Selected reaction monitoring mass spectrometry protein assays. Samples were injected (2 µL) on the UHPLC system (1260 series Agilent, CA) equipped with an analytical column (C18 Peptide CSH; 1.7 µm, 2.1 mm i.d. × 100 mm; cat. #186006937; Waters, Milford, MA) thermostated at 40°C. The mobile phase consisted of solution A (0.1% FA in water) and solution B (0.1% FA in ACN). The flow rate was 300 µL/min, and the gradient elution program consisted of analytical (0–30.9 min) and re-equilibration part (31–35 min): 0.0 min 5% B; 25 min 30% B; 25.5 min 95% B; 30.9 min 95% B; 31 min 5% B; 35 min 5% B. A standard-flow electrospray was used to couple the UHPLC system with a triple quadrupole mass spectrometer (AJS 6455A, Agilent, CA). Electrospray source operated in positive ion mode (capillary voltage 3.5 kV; gas flow rate 11 L/min at 130°C; sheath gas pressure 25 PSI at 400°C; nozzle voltage 500 V). We monitored 98 transitions per the dynamic SRM mode analysis, with 2 min window scheduled around peptide experimental RT. SRM signature transitions were equivalent for proteotypic peptide and corresponding SIL internal standard, i.e., a single SRM quantifier transition and 2–4 additional qualifier SRM transitions were acquired (Table S4).

Protein assay validation and reproducibility. We extracted proteins from all 134 meconium and feces swabs using the protocol and pooled protein extracts (70 µL) from all individual samples. We prepared the dilution series adding SIL-TCT or SIL peptides (10 µL) into the pooled extract and applied trypsin digestion protocol and UHPLC-SRM. We determined the linearity range, the limit of detection (LOD), the limit of quan-
The median CV precision was 14.1%, all CV values < 16.2%, except for peptide TPLTATLSK (IGHA1) with the CV (29.9% CV). The peptide VVLEGGIDPILR (MPO) concentration in the QC sample was below LOQ.

Validation of multiplex protein assay. Protein assays for A1AT-1, IGHA1, IGHA2, IGHA1+2, ECP, EDN, MPO, CAL1, and CAL2 were validated using matrix-matched calibration curves for each SIL-TCT peptide standard in terms of linearity of response, R^2, LOD, and LOQ. Protein assays were linear within the range of two orders of magnitude at minimum, typically from 0.04 to 14.77 µg/mg of the total protein content. All R^2 were > 0.99. LODs and LOQs of target proteins in the sample matrix ranged from 0.003 to 0.04 µg/mg and from 0.009 to 0.122 µg/mg of total protein content, respectively (Table S5). Surrogate peptides were not detected in the control sample (0 h, no trypsin added). The surrogate peptides’ yields were optimal within 5 h of incubation; CV < 16.7% (Figure S2).

Robustness of multiplex protein assays. Meconium or feces FW collected on a swab ranged between 25 and 150 mg, corresponding to 0.97–10.34 µg/µL of extracted total protein concentration. The extraction efficiency or kinetics of trypsin digestion and overall protein assay accuracy may be affected by variable protein concentration in extracts. We tested the robustness of A1AT-1, IGHA1, IGHA2, IGHA1+2, ECP, EDN, MPO, CAL1, and CAL2 quantification in QC sample stool extracts varied in total protein concentration (i.e., 0.1; 0.5; 1; 2; 4; 6 and 8 µg/µL, range 5–400 µg/50 µL of extract). The assay was robust as all protein concentrations were determined within CV < 20.6% (Table S8 and Figure S3), except for low abundant EDN (CV 25.9%).

Immune markers in meconium and feces. Meconium initially excreted by neonates transitions into a first feces around the second day after birth due to breastfeeding. We classified 64 samples as meconium (IGHA < 10 µg/mg of total protein) and 70 samples as the first feces samples. We tested the robustness of A1AT-1, IGHA1, IGHA2, IGHA1+2, ECP, EDN, MPO, CAL1, and CAL2 quantification in QC sample stool extracts varied in total protein concentration (i.e., 0.1; 0.5; 1; 2; 4; 6 and 8 µg/µL, range 5–400 µg/50 µL of extract). The assay was robust as all protein concentrations were determined within CV < 20.6% (Table S8 and Figure S3), except for low abundant EDN (CV 25.9%).
potential contribution to meconium EDN (Fig. 3). If ECP and EDN in meconium and feces are derived only from eosinophils, the EDN/ECP ratio in meconium and feces is expected similar. The EDN/ECP ratio in meconium was substantially higher than in the first feces suggesting a contribution from other white blood cells to the total meconium EDN level. However, the different EDN/ECP ratios may also reflect changes in eosinophil protein expression after the first day of life.

A1AT-1, IGHA1, IGHA2, IGHA1 + 2, ECP, EDN, MPO, CAL1, and CAL2 levels (Table S2) in meconium and feces (n = 134), sorted by decreasing concentration of IGHA were cross-correlated using the Spearman correlation factor (Fig. 4). The excretory IGHA1 + 2 in feces and meconium negatively correlated with A1AT-1 (Spearman correlation factor of −0.6), as previously reported32. Li et al. demonstrated decreasing calprotectin levels in neonatal stool between 1 to 18 months45. Indeed, we detected a negative correlation of IGHA1 + 2 with both CAL1 and CAL2 (Spearman correlation factor of −0.4). MPO and ECP moderately correlate with IGHA1 + 2 (Spearman correlation factor of 0.67 and 0.59, respectively). MPO was associated with ECP (Spearman correlation factor of 0.72) and ECP with EDN (Spearman correlation factor of 0.63).

Birth mode influence on immune protein levels in meconium and feces. Meconium and feces samples were from neonates delivered vaginally (VD) and via cesarean section (CS). VD reportedly fosters the

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**Figure 2.** Levels of immune protein markers in meconium and feces: (A) A1AT-1, (B) EDN, (C) ECP, (D) MPO, (E) CAL1, (F) CAL2. Mann–Whitney U test **** *P* < 0.0001.
Eubiotic bacterial colonization of the intestinal lumen. Conversely, skin commensals, opportunistic pathogens, or nosocomial bacteria primarily colonize CS neonates' intestinal lumen. CAL1 and CAL2 levels indicate the neutrophil type of inflammation in response to a pathogen's presence in the intestinal lumen. The comparison of CAL1 and CAL2 levels in the feces of VD (n = 58) and CS (n = 12) neonates revealed significantly higher CAL1 levels (P value 0.0439) in CS neonates (Figure S4). CAL1 and CAL2 are components of the calprotectin dimer complex, but the CAL2 level was not significantly different between CS and VD neonates (P value 0.0622). However, fecal calprotectin levels were highly varied in neonates, and the sample size was limited. Meconium CAL1 and CAL2 in CS and VD neonates were not significantly different (Figure S4). Increased fecal calprotectin is a potential marker of dysbiosis and low-grade inflammation of the gastrointestinal tract in CS neonates.

Figure 3. ECP and EDN levels in meconium and feces. (A) meconium (n = 41) and (B) feces (n = 70), Mann–Whitney U test, *P < 0.05, ****P < 0.0001.

Figure 4. Correlation matrix plot for IGHA, A1AT-1, IGHA1, IGHA2, IGHA1 + 2, ECP, EDN, MPO, CAL1, and CAL2 levels in neonatal stool samples (n = 134).
Conclusions
In summary, we developed and validated a multiplex mass spectrometry-based protein assay for absolute quantification of adaptive immunity effectors (i.e., IGHV1, IGHV2) and immune protein markers (i.e., A1AT, ECP, EDN, MPO, CAL1, CAL2) in neonatal meconium and feces swabs. While circulating immune protein markers are routinely quantified in blood/serum, neonatal excretory levels are not established. We determined the stool (fresh weight) collected on a swab by measuring protein concentration in protein extracts. Information on the precise amount collected on a stool swab is required for absolute protein quantification. Breast milk-derived IGHV2 differentiates meconium from feces to study the influence of breastfeeding on intestinal barrier function maturation. Absolute quantification of multiple immune protein markers characterizes the type of inflammation in the intestinal lumen. We found significantly higher fecal calprotectin levels in neonates delivered via Cesarean section relative to vaginal delivery birth. The finding may indicate a low-grade inflammation in response to microbial dysbiosis in CS neonates.

Data availability
The mass spectrometry data were deposited to the PANORAMA Repository (https://panoramaweb.org/%20o%20Masaryk%20-%20RECETOX/Vidova_MultiMec_Assay/project-begin?view?pageId=Raw%20Data).

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**Author contributions**

V.V. performed laboratory experiments, acquired and processed mass spectrometry data, and drafted the manuscript; E.B. participated in the laboratory experiments and data analysis; J.K. participated in the study design; V.T. advised in study design and data interpretation; Z.S. designed the study, advised on the data interpretation, and wrote the manuscript. All authors reviewed and approved the final version of the manuscript.

**Competing interests**

The authors declare no competing interests.

**Additional information**

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