SUPPLEMENTARY MATERIAL

Measurement of the total serum immunoglobulin E (IgE), specific IgE and eosinophil levels

The total and specific IgE levels (IU/mL) were measured using the ImmunoCAP-CAP 1000 system (Phadia AB, Uppsala, Sweden) at 1 year of age. Concentrations of at least 0.35 kU/L (corresponding to CAP class 1) were considered positive. Atopy was defined as a ≥1 positive response. Eosinophils were counted using an automatic blood-cell counter (XE-100; Sysmex Co., Kobe, Japan) at 1 year of age.

Study subjects for target lipidome analysis

Gut sphingolipid metabolites were prospectively analyzed from 46 healthy infants, 30 infants with only AD and 82 FA with AD in infants at 6 months of age in available fecal samples. The study population consisted of 158 six-month-old infants involved in the Cohort for Childhood Origin of Asthma and Allergic Diseases (COCOA) [1], which was a previously established general population-based birth cohort. The Institutional Review Boards (IRBs) of Asan Medical Center approved this study (IRB No. 2021-1596). A diagnosis of AD was based on the Hanifin and Rajka's criteria by pediatric allergists [2]. The severity of AD was assessed using the Scoring Atopic Dermatitis (SCORAD). FA was diagnosed by a physician based on confirmed allergic sensitization, as evident by IgE levels higher than 0.35 kU/L corresponding to food antigen and history of IgE-mediated symptoms after food ingestion during the first year of life. Human feces were freeze-dried for 24 h using a benchtop manifold freeze drier and stored at -80°C until analysis.

J Investig Allergol Clin Immunol 2024; Vol. 34(4): 263-265
doi: 10.18176/jiaci.0979

Sample preparation for target lipidome analysis

Laboratory analysis was conducted using LC–tandem MS (LC-MS/MS). Internal standards were added to all calibration solutions, sample feces and quality control samples (QCs) prior to sample preparation, and they were prepared the same way as stated in the method. 12 batches were used for this study, and the same number of samples from each group were allocated across the batches. Unfortunately, sample feces were not enough to be used as pooled QCs. Instead pooled human plasma samples (Sigma-Aldrich) were used QC, and two QC runs were included in each batch. Internal standard solutions were added first to all samples during sample preparation. The analysis order was randomized among the samples in a batch. Several runs with blank samples, standard solutions, and QC samples were performed to check the robustness of the analytical method before study sample analysis. Principal component analysis (PCA) score plot of sample feces with QC samples was shown in supplementary Figure 3. QC samples were well clustered in PCA score plot, which represents that their analytical variability was low (Figure S3). The result showed that the overall performance of the targeted metabolomics platform used in this study was thought to be acceptable throughout the run.

For Sphingosine 1-phosphate (S1P), ~10 mg of the freeze-dried feces was homogenized well with an internal standard solution (200 µl of 1 µM C17 S1P solution). Then, 300 µl of chloroform, 200 µl of methanol, 50 µl of H_2O, and 10 µl of 10N NaOH were added to each solution, and vortex well. The aqueous layer was collected after centrifugation, where S1P was separated from other lipids under alkaline condition [3]. 50 uL of H_2O was added to the remaining solution and the aqueous layer was collected again after centrifugation. Then, S1P was re-extracted into the chloroform phase under acidic conditions using 400 µl of chloroform and 20 µl of 10M HCl. This step was repeated three times. The chloroform was evaporated, and the dried samples were reconstituted with methanol prior to LC-MS/MS analysis.

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Extraction efficiencies of S1P can be compared by the peak areas of internal standard (C17-S1P) in standard solution, sample feces, and QC sample, where standard solution did not follow sample preparation, while sample feces and QC sample were prepared as stated above. Extraction efficiencies was approximately 50-60%. For other sphingolipids and diacylglycerols, ~40 mg of feces was used and lipids were extracted by Bligh and Dyer method [4] after adding internal standard solutions (50 µl of 100 nM C18 ceramide-d7 and C18:1 Sphingomyelin-d_9 and 200 µl of 1 µM 1,3-19:0-d_5). Organic solutions containing the lipids were dried using a vacuum centrifuge and stored at –20°C until LC-MS/MS analysis. The dried matter was reconstituted with 50 uL of methanol and injected into the LC-MS/MS system. All lipid standards including internal standards were purchased from Avanti-Polar Lipids and Sigma-Aldrich.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS).

Lipid levels except S1P were determined using a LC-MS/MS system equipped with an 1290 HPLC (Agilent, Waldbronn, Germany) and QTRAP 5500 (AB Sciex, Toronto, Canada). A reverse-phase column (Pursuit5 C18, 150 × 2.1 mm) was used with mobile phase A (5 mM ammonium formate/MeOH/tetrahydrofuran (500/200/300, v/v/v)) and mobile phase B (5 mM ammonium formate/MeOH/ tetrahydrofuran (100/200/700, v/v/v)). The LC was run at 200 µl/min and 35°C. 4500 V of ion spray voltage for electrospray ionization, and positive ion mode were used. For ceramide and sphingomyelin, the LC gradient was as follows: 50 % of B for 0 min, 50 % of B for 5 min, 50 to 70 % of B for 3 min, 70 % of B for 7 min, 70 to 90 % of B for 7 min, 90 % of B for 3 min, 90 to 50 % of B for 0.1 min, and 50 % of B for 4.9 min. For diacylglycerol, the LC gradient was as follows: 90 % of B for 0 min, 90 % of B for 6 min, 90

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to 95 % of B for 0.6 min, 95 % of B for 3.4 min, 95 to 90 % of B for 0.1 min, and 90 % of B for 1.9 min. Multiple reaction monitoring (MRM) was performed in the positive ion mode and the extracted ion chromatogram corresponding to the specific transition for each lipid was used for quantification. Details for MRM and instrumental conditions can be found in Table S2. The calibration range for each lipid was 0.1-1000 nM ($r^2 \geq 0.99$). Data analysis was performed by using Analyst 1.5.2 software.

S1P was measured using an LC-MS/MS system equipped with Ultimate3000 (Dionex) and LTQ-Orbitrap XL (Thermo Fischer Scientific). 3300 V of ion spray voltage for electrospray ionization, and negative ion mode were used. A reverse-phase column (Jupiter 5uM C4 column (50 x 1 mm)) was used with mobile phase A (0.1 % formic acid in H_2O) and mobile phase B (0.1 % formic acid in methanol). The LC was run at 300 µl/min and 35°C. The LC gradient was as follows: 10 % of B for 0 min, 10 to 90 % of B for 5 min, 90 % of B for 10 min, 90 to 10 % of B for 0.1 min, and 10 % of B for 4.9 min). Selected ion monitoring (SRM) was uesd in the negative ion mode and the extracted ion chromatogram corresponding to the specific transition (phosphate ion, PO_3^-, *m/z* 78.9585) for S1P was used for quantification. Details for SRM can be found in Table S2. Calibration range was 0.01 – 10 µM with $R^2 > 0.99$. Data analysis was performed by using Xcalibur 2.2 software.

Statistical analysis

Comparisons of the gut sphingolipid metabolites was tested using Mann-Whitney tests. *P*-values of Figure S1 were corrected for multiple comparisons using the Benjamini and Hochberg's false discovery rate (FDR). Kruskal–Wallis test and Mann-Whitney *U* test were used for continuous variables, and Chi-square test and Fisher's test were used for categorical variables. For multiple comparisons, the

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Bonferroni correction was used. Correlations between the total IgE, specific IgE to egg white, specific IgE to milk and eosinophils (%) at 1 year of age were analysed using the Spearman correlation test. All statistical analyses were conducted using SPSS statistical software, version 24.0 (SPSS Inc, Chicago, IL, USA), and prism, version 8.0.1 with $P < 0.05$ considered to indicate statistical significance.

Data availability

The Metabolite data are available at NIH Metabolomics Workbench, https://www.metabolomicsworkbench.org/ (accessed on 01 November 2023), with DataTrack ID numbers 4379 (study ID : ST002923) and 4380 (study ID : ST002924) for data of S1P and other sphingolipid metabolites.
The data can be accessed directly via its Project DOI: http://dx.doi.org/10.21228/M8ZX5S.

Reference

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doi: 10.18176/jiaci.0979