# Metabotypes are linked to uncontrolled childhood asthma, gut microbiota, and systemic inflammation

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PII: S0091-6749(25)00457-9

DOI: https://doi.org/10.1016/j.jaci.2025.04.017

Reference: YMAI 16771

To appear in: *Journal of Allergy and Clinical Immunology*

Received Date: 20 December 2024

Revised Date: 14 April 2025

Accepted Date: 18 April 2025

Please cite this article as: Abdel-Aziz MI, Hashimoto S, Neerincx AH, Haarman EG, Cecil A, Lintelmann J, Witting M, Hauck SM, Kerssemakers N, Verster JC, Bang C, Franke A, Dierdorp BS, Dekker T, Metwally NKA, Duitman JW, Lutter R, Gorenjak M, Toncheva AA, Kheiroddin P, Harner S, Brandstetter S, Wolff C, Corcuera-Elosegui P, López-Fernández L, Perez-Garcia J, Almeida MM, Sardón-Prado O, Pino-Yanes M, Potočnik U, Kabesch M, Vijverberg SJH, Kraneveld AD, Maitland-van der Zee AH, SysPharmPediA consortium, Metabotypes are linked to uncontrolled childhood asthma, gut microbiota, and systemic inflammation, *Journal of Allergy and Clinical Immunology* (2025), doi: https://doi.org/10.1016/j.jaci.2025.04.017.

# Metabotypes are linked to uncontrolled childhood asthma, gut microbiota, and systemic inflammation

SysPharmPediA

Children (6-17 years old) with moderate-to-severe asthma

Serum metabolomics

Faecal metabolomics

Serum inflammatory proteins

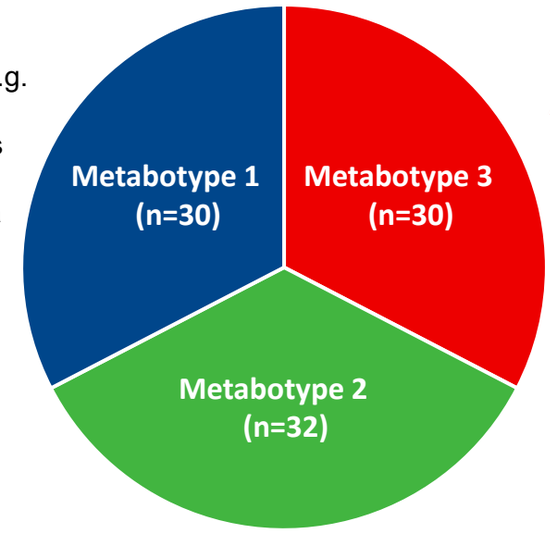
Faecal microbiome

24-h food diaries

Integrative clustering and phenotype discovery

Check association with the clusters

Three metabotypes




Metabotype 1 (n=30)

↑ Serum/fecal lipids, e.g. triglycerides, phosphatidylcholines
C-section deliveries
Uncontrolled asthma

↓ HDM sensitization, allergic rhinitis

Metabotype 3 (n=30)

↑ Fecal amino acids
Arginase activity
Brondodilator response
Exacerbations
Uncontrolled asthma
Medication prescription

↓ Serum sphingomyelins
Fecal bacterial diversity

Metabotype 2 (n=32)

↑ Serum carnitines, ω−3 fatty acids
Breast feeding ≥4 months
Protein intake

↓ Serum/fecal triglycerides
Uncontrolled asthma
Blood neutrophils, monocytes
Non-T2 inflammatory markers, e.g. IL-6
Sugar intake

Abbreviations: ω−3: omega-3, HDM: house dust mite, IL-6: Interleukin-6

**Metabotypes are linked to uncontrolled childhood asthma, gut microbiota, and systemic inflammation**

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**Authorship contribution:**

MIA-A has performed the analysis and drafted the initial version of the manuscript. MIA-A, AHM, ADK, and MK have contributed to the design of the analysis plan. All co-authors have contributed to the acquisition of data, interpretation of the analysis, revision, drafting, critical appraisal and ensuring accuracy and integrity of the analysis. All co-authors have provided final approval of the version to be published.

**Funding:**

The SysPharmPediA consortium has been supported by ZonMW [project number: 9003035001], Ministry of Higher Education, Science and Innovation of the Republic of Slovenia (MVZI) [contract number C330-16-500106]; the German Ministry of Education and Research (BMBF) [project number FKZ 031 L0088]; Instituto de Salud Carlos III (ISCIII) through Strategic Action for Health Research (AES) and European Community (EC) within the Active and Assisted Living (AAL) Program framework [award numbers AC15/00015 and AC15/00058] under the frame of the ERACoSysMed JTC-1 Call. M. I. Abdel-Aziz is supported by the Longfonds, Stichting Astma Bestrijding, and Amsterdam Public Health Institute in relation to this work.

**Conflict of Interest statement**

**JCV** reports receiving consulting fees from Eisai, KNMP, Med Solutions, Red Bull, and Toast, receiving travel support from Sen-Jam Pharmaceutical and personal stock options from Sen-Jam Pharmaceutical, outside the submitted work. **JWD** reports receiving grants paid to institution from Abbvie and Boehringer Ingelheim, outside of this submitted work. **RL** reports receiving grants paid to institution from Amsterdam UMC Foundation, Foresee and Eurostar, receiving fees for webinar contribution from Pfizer, receiving support for attending meeting/travel from the European Society for Clinical Investigation (ESCI), receiving fees from Sanofi for Participation on a Data Safety Monitoring/Advisory Board, and unpaid secretary/treasurer Trust of ESCI, outside of this submitted work. **SH (Susanne)** reports receiving payment or honoraria from Nutricia and Allergopharma, outside of this submitted work. **OS-P** reports receiving support from Faes Farma for travelling to Spanish Society of Pediatric Pulmonology congress June 2024, outside of the submitted work. **MP-Y** was funded by grants PID2020-116274RB-I00, SAF2017-83417R and the Ramon y Cajal program RYC-2015-17205 from the Spanish Ministry of Science, Innovation, and Universities (MICIU/AEI/10.13039/501100011033) and the European Regional Development Fund "ERDF A way of making Europe" by the European Union, was funded by Instituto de Salud Carlos III (ISCIII) through AES and EC within AAL framework, and was funded GlaxoSmithKline, Spain and CSL Behring, outside the submitted work. **MK** reports grants from European Union, German Ministry of Education and Research, Bavarian Ministry of Health, receiving consulting fees from AstaZeneca and receiving honoria from EAACI, Novartis, Nutricia, and Pari, outside the submitted work. **SJHV** reports receiving support for attending meeting/travel from the European Respiratory Society (ERS), being ERS officer Pediatric Assembly, receiving payment paid to institution for involvement in 3TR ABC (IMI project), which includes funding from AstraZeneca, GSK and Sanofi, outside the submitted work. **ADK** reports leadership role by being dean of the faculty of Science, Vrije Universiteit Amsterdam. **AHM** is the PI of a public private consortium (P4O2 (Precision Medicine for More Oxygen)) sponsored by

Health~Holland involving many private partners that contribute in cash and/or in kind (AbbVie, Boehringer Ingelheim, Breathomix, Clear, Fluidda, Ortec Logiqcare, Olive, Philips, Quantib-U, Smartfish, Clear, SODAQ, Thirona, Roche, TopMD, Novartis, RespiQ), received unrestricted research grant from GSK and Boehringer Ingelheim, received Vertex Innovation Award Grant, received Honoraria paid to Institution from Boehringer Ingelheim, Astra Zeneca and GSK, and reports that she is the Chair of DSMB of a study on BPD in neonates, outside of the submitted work. **MIA-A**, **SH (Simone)**, **AHN**, **EJH**, **AC**, **JL**, **MW**, **SMH**, **NK**, **CB**, **AF**, **BSD**, **TD**, **NKAM**, **MG**, **AAT**, **PK**, **SB**, **CW**, **PC-E**, **LL-F**, **JP-C**, **MM-A**, and **UP** report no conflicts of interest to disclose.

**Short running head: "Metabotyping in childhood asthma"**

**Article type:** Original article

**Word count:** 4,000 without abstract, references, tables, figures, and disclosures

**References number:** 46

**Tables:** 4 in the main manuscript + 8 supplementary

**Figures:** 3 in the main manuscript + 8 supplementary

**Online Supplementary Material:** This article has an online data supplement.

**Keywords:** Moderate-to-Severe Childhood Asthma; Metabotyping; Gut Microbiota; Inflammatory Markers; Personalized Medicine



**Clinical Implications**

Metabotyping reveals distinct childhood asthma subtypes with unique inflammatory profiles and gut microbiota composition, offering novel insights for targeted therapies and personalized management of moderate-to-severe childhood asthma.

**Capsule Summary**

Serum and fecal-based metabotypig identified three childhood asthma subtypes lined with asthma burden, and gut microbiota, and non-T2 inflammation supporting the role of certain , e.g. lipid, pathways in the development of severe childhood asthma.

**Background**

Childhood asthma has been linked to distinct metabolomic profiles.

**Objective**

To identify phenotypes (metabotypes) in children with moderate-to-severe asthma through integrative fecal and serum metabolome analysis.

**Methods**

Children from the Systems Pharmacology Approach to Uncontrolled Pediatric Asthma cohort with Global Initiative for Asthma treatment step ≥3 were recruited. Asthma control was defined by the Asthma Control Test and annual exacerbation history. Targeted metabolomics profiling of feces and serum was performed using liquid chromatography and flow injection electrospray ionization-triple quadrupole mass spectrometry. Similarity Network Fusion integrated fecal and serum metabolome profiles, followed by spectral clustering. Clusters were analyzed for differences in asthma characteristics, food diaries, fecal microbiota composition, and levels of serum inflammatory markers and blood cells.

**Results**

Integrative fecal and serum metabolome analysis of 92 children with moderate-to-severe asthma (median age: 11.5 years, 34% female) revealed three metabotypes. Metabotype1 had the lowest percentage of allergic rhinitis, with elevated serum ceramides and triglycerides. Metabotype2 had higher odds of asthma control, the highest percentage of children with ≥4 months of breastfeeding, reduced sugar intake, lowest levels of blood neutrophils and serum inflammatory markers, and with elevated serum acylcarnitines and ω-3 fatty acids. Metabotype3 included the highest percentage of uncontrolled asthma patients, with decreased serum cholesteryl esters, phosphatidylcholines, and sphingomyelins, elevated fecal amino acids, and reduced fecal microbiota diversity.

**Conclusions**

Metabotypes in children with moderate-to-severe asthma are linked to asthma control, distinct fecal microbiota and systemic inflammatory patterns. The findings suggest that metabotyping can be valuable in precision medicine approaches for asthma.

**Introduction**

Asthma is the most common chronic disease in children, characterized by airway inflammation with heterogeneity in clinical manifestations and treatment responses (1). The burden of childhood asthma increases with its increasing severity and lack of control due to the increased health-related complications and the lack of suitable treatment options (2).

Currently, asthma diagnosis and management are based on the evaluation of clinical symptoms, lung function tests, fractional exhaled nitric oxide, inflammatory biomarkers, and allergy testing (3). However, these methods have limitations in accurately reflecting the heterogeneity of asthma, particularly in childhood (4). Pinpointing asthma heterogeneity into clinically identifiable phenotypes (phenotyping) and understanding their underlying mechanisms (endotyping) will help to improve the care and treatment of asthma (4). Omics-guided classification of subjects with asthma has been emerging as a systems medicine strategy for phenotyping and endotyping asthma (4). It relies on using omics to help subtyping subjects with asthma while uncovering molecular pathways underlying the different subtypes. This may help to target specific molecular pathways for diagnosing or treating the different asthma subtypes.

Metabolites are small molecules involved in various biological processes, such as energy metabolism, immunity, inflammation, and oxidative stress (5). In addition, metabolites are influenced by the genetic makeup and environmental factors (6, 7), making them potential biomarkers reflecting the cellar state of asthma and its heterogeneity. Metabotyping is the process of subtyping individuals based on their metabolome profiles and is thought to help in subtyping subjects with different diseases and in guiding personalized therapeutic or nutritional decisions for each individual subject (8). In adults with asthma, metabotyping has been successfully performed using the metabolome profiles in different sampling compartments, such as exhaled breath and serum, showing strong associations to asthma severity and

inflammatory biomarkers (9, 10). Yet, limited research has been performed on childhood asthma. We hypothesize that metabolome profiles in serum and feces can reveal distinct clusters (metabotypes) in children with moderate-to-severe asthma.

The aims of this study are to: 1) perform unsupervised integrative clustering of serum and fecal metabolome profiles of children with moderate to severe asthma and assess whether they are linked to distinct disease characteristics; 2) elucidate the possible underlying nutritional and biological connections of the revealed phenotypic clusters by investigating 24-hr food diaries, the fecal microbiome and inflammatory markers.

## Methods

### *Study design*

Systems Pharmacology Approach to Uncontrolled Pediatric Asthma (SysPharmPediA) is a European multi-center observational study involving children aged 6-17 with physician-diagnosed asthma from the Netherlands, Germany, Spain, and Slovenia (11). Ethics approval was obtained, informed consent was given by parents/caregivers, and assent by children where appropriate. The study is registered at ClinicalTrials.gov (NCT04865575).

### *Participants*

Children (n=145) with moderate to severe asthma (GINA treatment step ≥3) were included and n=92 subjects providing paired samples for the analysis (Figure S1). Uncontrolled asthma was defined by (childhood) Asthma Control Test ((c)ACT) score ≤19 and/or severe exacerbations requiring hospitalization or emergency room (ER) visits or oral corticosteroid (OCS) use in the past year (12, 13). Subjects were evaluated for allergy, atopy, spirometry, fractional exhaled nitric oxide ($FE_{NO}$), blood inflammatory biomarkers, and medical history.



### Sample collection and omics analysis

Sample collection and detailed omics analyses are described in the online supplement. Subjects with available paired fecal and serum samples were compared to subjects not included in the study across different characteristics (Table S1).

### *Fecal and serum metabolomics analysis*

Metabolomic profiling was performed using the Biocrates MxP® Quant 500 kit (BIOCRATES Life Sciences AG, Innsbruck, Austria), which resulted in hundreds of metabolites belonging to various classes (Table

S2) and their relevant ratios. Metabolites with >40% missing values were excluded. Remaining values below the limit of detection (LOD) were imputed (14), and those with >25% coefficient of variation were excluded. Spearman correlation analysis was conducted between shared metabolites in serum and feces, and *P*-values were adjusted by Benjamini-Hochberg false discovery rate (FDR).

***Fecal 16S V3-V4 rRNA microbiome sequencing***

Amplicon sequencing was performed (15). Quality control was performed by DADA2 pipeline (16) with Silva database version 138 (17). The microbiome data are deposited on BioProject (PRJNA867125).

***Serum cytokines and chemokines***

Differential blood count was performed using fluorescence flow cytometry and a panel of serum cytokines and chemokines was measured using a Luminex multiplex assay as described (18). Protein data were imputed similarly to the metabolites.

***Food diaries***

Participants completed a 24-hour dietary recall diary one day before the study visits to estimate food intake (19). Nutrient calculations were performed using the 'eetmeter' tool (20).

**Data and statistical analysis**

The general data analysis workflow is shown in Figure S2 and described in detail in the online supplement. Briefly, clustering was based on serum and fecal metabolomics using similarity network fusion (SNF) (21). Both omics layers were normalized, scaled, and converted into Euclidean distances, then into patient affinity matrices. The matrices were fused into a single similarity matrix (parameters, K=10, α=0.5, and T=20) as recommended (21). The optimum number of clusters was determined using eigengap, rotation cost, silhouette's width, within cluster sum of squares and gap statistics (21). A

heatmap and kernel principal component analysis (PCA) were utilized to visualize the similarity network. The metabotypes were checked for differences in asthma characteristics using chi-square tests with Monte-Carlo simulation (10,000 permutations) or Kruskal-Wallis H tests as appropriate, followed by post-hoc analyses for nominally significant associations (22).

Covariates identified using directed acyclic graph (DAG, Figure S3), included age, sex, ethnicity, country of inclusion, body mass index-z-score, recent antibiotic intake, season of inclusion, corticosteroids (nasal/inhaled/oral) intake, and Medication Adherence Report Scale-5 scores, were adjusted for in a multivariate logistic regression model (asthma control was the dependent outcome and metabotypes and covariates were included as independent parameters). An additional model with a reduced number of covariates (age, sex, and country of inclusion) was then fitted to minimize the risk of overfitting. As a sensitivity analysis, atopic sensitization was included as an additional covariate to assess whether the association between metabotypes and asthma control was explained by atopy.

To benchmark whether the integrative clustering based on dual metabolomics blocks offers more clinical relevance than each individual metabolomics block, independent clustering was performed on separate omics layers and compared in relation to asthma burden using chi-square tests with Monte-Carlo simulation.

**Differences in omics layers between the clusters**

Fecal and serum metabolites, serum cytokines, and nutrient intake values were compared between metabotypes using the Kruskal Wallis H test, followed by FDR correction. Post-hoc analysis identified specific enriched/depleted metabolites per metabotype, and then grouped by metabolic classes. Differences in microbial richness and Shannon α-diversity were assessed between the metabotypes, and weighted UniFrac β-diversity was analyzed using PERMANOVA after adjusting for confounders. Microbiome Multivariable Associations with Linear Models 2 (MaAsLin 2 (23)) checked differences in

bacterial genera, with adjustments for most significant confounders with FDR correction. Additionally, we assessed the direct correlation between fecal microbiome diversity (α and β) and metabolic classes by calculating mean z-scores for metabolic classes and applying Spearman correlation with FDR correction, and PERMANOVA while adjusting for multiple confounders.

***Results***

***Combining fecal and serum metabolome revealed three different metabotypes***

Ninety-two subjects provided dual serum and fecal samples that successfully underwent metabolomics profiling. The correlation between paired fecal and serum metabolites was generally low, with a mean ± SD of 0.06 ± 0.14. Only five metabolites showed statistically significant correlations between paired sample types (correlation coefficients $r_s$>0.3 and q-value <0.05), specifically: 1,2-Diacylglycerol (C16:0/C18:1), Lyso-phosphatidylcholine (C16:0), Triacylglycerol (C16:0/C34:1), Triacylglycerol (C18:0/C36:2), and Triacylglycerol (dC18:1/C36:0). Similarity Network Fusion based on serum and fecal metabolome profiles yielded three distinct clusters (i.e. metabotypes) by spectral clustering (Figure 1), as evaluated by the majority vote of multiple indices (Table S3, and Figure S4A-E). The combination of serum and fecal metabolomics yielded more coherent clusters than each single omics block at all clustering K solutions (K 2:10, Figure S4F). In addition, each omics block separately divided the subjects into an inconsistent number of clusters (Table S3), in contrast to the combined omics integration, which divided the subjects consistently into 3 clusters using different statistical indices.

**The metabotypes show distinct clinical characteristics and are associated with asthma control, allergy, and blood neutrophils**

Subjects' baseline characteristics are shown in Table 1. The median age of the included participants was 11 years, 46% were female, 80% were White. The metabotypes showed statistically significant differences in mode of delivery, breastfeeding, country of inclusion, history of atopic sensitization (house dust mite, dog dander aeroallergens, and nuts food allergens), physician-diagnosed allergic diseases (allergic rhinitis and allergic conjunctivitis), blood inflammatory cells (neutrophils and monocytes), bronchodilator response and leukotriene antagonist add-on asthma medication. Particularly, Metabotype1 had the highest percentage of subjects with C-section deliveries (n=11/30 [37%]), and

lowest percentage of subjects with house dust mite (HDM) atopic sensitization (n=13/30 [43%]; allergic rhinitis (n=17/29 [59%]) and allergic conjunctivitis (n=15/28 [54%]). Furthermore, it had notable sensitization to pollens, mold, cat, and dog, but these findings were not statistically significant. Metabotype2 had the highest percentage of subjects with ≥4 months of breastfeeding (n=25/32 [78%]), lowest percentage of subjects with nut allergens (1/29 [3%]), the lowest blood monocytes (median=0.46, IQR:0.36-0.54) and neutrophils counts (median=2.60, IQR:1.92-3.54), and the lowest percentage of subjects prescribed leukotriene antagonists (n=0/32 [0%]). Metabotype3 had the highest percentage of subjects with dog dander aeroallergen sensitization (n=13/28 [46%]). Moreover, the metabotypes showed differences in characteristics associated with asthma burden; including asthma control, severe exacerbations in the past year, >10 missed school days, and non-infection related dry cough during the past year. In particular, Metabotype3 showed the largest percentage of subjects with uncontrolled asthma (unadjusted OR=4.41, 95% CI: 1.42, 15.7 and 4.37, 95% CI: 1.39, 15.8, relative to Metabotype2 and Metabotype1, respectively, all *P*-values <0.05). However, after adjusting for multiple confounders, both Metabotype3 and 1 showed statistically significantly higher odds of uncontrolled asthma in comparison to Metabotype2 (Table S4, adjusted OR = 11.7, 95% CI: 2.57, 88 for Metabotype3 and 7.34, 95% CI: 1.22, 68.9 for Metabotype1, both relative to Metabotype2, all *P*-values <0.05). There were no statistically significant associations between atopic sensitization or sensitization to HDM and asthma control, either alone (data not shown) or after adjusting for covariates, including metabotype classification (Table S5 and Table S6). Box 1 shows detailed summary of the phenotypic differences between the metabotypes.

**Integrative clustering exhibits stronger clinical relevance than single omics blocks**

Six different clustering solutions for the individual serum and fecal layers were obtained (based on K=2:4 highlighted in Table S3), none of which was significantly associated with asthma burden (including

asthma control status, severe asthma exacerbations in the past year, and missed school >10 days in the past year, all *P*-values>0.05, data not shown), with exception of the dry cough episodes in the past year merely showed associations to serum-based metabolome clustering solutions (*P*-values range: 0.012 to 0.087, with a lower effect size compared to clustering based on dual-omics integration), but not fecal-based metabolome clustering (data not shown). The significant association to different parameters of asthma burden is mainly seen when the clustering is performed on the integrative information of both omics blocks.

**The metabotypes reveal characteristic differences in metabolic classes**

***Serum metabolome***

Hundreds (n=377) of serum metabolites and associated ratios were differentially abundant between the metabotypes after FDR correction (excel file S1). Differences in serum metabolic classes are described in Table 2. Metabotype1 demonstrated enriched serum levels of ceramides, cholesteryl esters, phosphatidylcholines, triglycerides and diglycerides compared to the other metabotypes, but no characteristic depleted serum profiles. Metabotype2 showed enriched serum levels of acylcarnitines (particularly Tetradecadienylcarnitine), fatty acids (particularly Docosahexaenoic acid, an omega-3 fatty acid) and α-aminobutyric acid, and depleted serum levels of triglycerides, diglycerides and amino acids (particularly glutamate) compared to the other metabotypes. Metabotype3 showed elevated serum levels of triglycerides compared to Metabotype2, but less compared to Metabotype1 as well as an elevated ratio of ornithine-to-arginine (i.e., ornithine synthesis) compared to the other metabotypes. Moreover, it showed characteristic depleted serum levels of sphingomyelins, phosphatidylcholines, cholesteryl esters and ceramides as well as ratio of citrulline-to-ornithine (i.e., citrulline synthesis) compared to the other metabotypes. Notably, no differences in steroid hormones (cortisol and cortisone) among the 3 metabotypes were obesrved.

***Fecal metabolome***

Similarly, several fecal metabolites (n=136) and associated ratios were differentially abundant between the 3 metabotypes after FDR correction (excel file S2). Differences in fecal metabolic classes are shown in Table 3. Metabotype1 displayed enriched fecal levels of phosphatidylcholines, sphingomyelins and triglycerides, and depleted fecal levels of fecal amino acids compared to the other metabotypes. Metabotype2 did not show characteristic enriched levels of fecal metabolic classes, but showed depleted fecal levels of ceramides, phosphatidyl cholines, sphingomyelins, triglycerides, and diglycerides compared to the other metabotypes. Metabotype3 expressed elevated fecal levels of amino acids and ceramides compared to the other metabotypes, but did not show distinct depleted levels of fecal metabolic classes.

**The metabotypes were modestly associated with short-term dietary habits, but strongly associated with fecal microbiome**

Seventy-three (79.3%) out of the 92 subjects completed 24-hour food diaries before the baseline visit. The metabotypes showed nominally significant associations with total sugar and protein intake (Figure S5), however; the results were not significant after FDR corrections.

Metabotype3 showed significantly decreased richness and Shannon α-diversity in comparison to Metabotype2 and Metabotype1 in fecal microbiome (Figure S6). Moreover, PERMANOVA analysis revealed that the metabotypes were significantly different in the weighted UniFrac β-diversity measure (adjusted $R^2$=0.076, *P*-value=0.001). Figure S7 depicts the relative separation of the metabotypes based on the β-diversity. Figure 2 shows differentially abundant bacterial genera between the metabotypes. In particular, *Christensenellaceae R−7 group, Oscillospiraceae UCG−005, Oscillospiraceae NK4A214 group,*

*Coprococcus, Anaerovoracaceae Family XIII AD3011 group,* and *Victivallis* were more abundant, while *Lachnoclostridium* was less abundant in Metabotype2 in comparison to Metabotype3. No differentially abundant bacterial genera were found between Metabotype1 and Metabotype3 after FDR correction (Figure 2), and between Metabotype1 and Metabotype2 (data not shown for the latter). At the metabolic class level, the fecal microbiome showed more statistically significant associations with the fecal metabolome than with the serum metabolome at both α- and β-diversity levels (Figure S8 and Table S7).

**The metabotypes exhibits dysregulation of non-T2 asthma biomarkers**

In Metabotype2, decreased levels of serum IL-6 and IL-7 inflammatory markers were observed, while Metabotype1 showed elevated levels of TIMP-4 compared to the other metabotypes (all q-values<0.05, Figure 3).

**Discussion**

Using integrative analyses of fecal and serum metabolome profiles, we found that children with moderate-to-severe asthma could be stratified into three metabotypes that differed significantly by 1) asthma burden (asthma control, severe exacerbations, dry cough episodes, and missed school days), early life exposures (breastfeeding and mode of delivery) and inflammatory biomarkers (blood neutrophils and monocytes), 2) gut microbiota composition, and 3) serum inflammatory proteins. This study is regarded the first, to-date, to delineate multi-omics and clinical characteristics of metabotypes in moderate-to-severe childhood asthma.

Metabotype1 showed the lowest percentage of subjects with allergic rhinitis and allergic conjunctivitis, as well as the lowest percentage of subjects with atopic sensitization to house dust mite. However, it showed the highest percentage of atopic sensitization to food allergens, particularly nuts and showed notable sensitization to other aeroallergens such as pollens, mold, cat, and dog. These contrasting patterns of allergen sensitization warrant further investigation to determine the link between specific sensitizations and the metabolome. This metabotype showed higher odds for uncontrolled asthma compared to only Metabotype2 after adjusting for confounders, and showed the highest levels of blood neutrophils and monocytes, particularly in comparison to Metabotype2. Metabotype1 exhibited the highest levels of serum (ceramides, cholesteryl esters, phosphatidylcholines, triglycerides, and diglycerides), and fecal (sphingomyelins, phosphatidylcholines, and triglycerides) lipids compared to the other metabotypes. Furthermore, it demonstrated the lowest levels of fecal amino acids. Our results are partly in line with other findings described in the literature. It was reported that serum triglycerides were elevated in adults with asthma compared to healthy controls, even after adjusting for BMI, inflammatory markers, and medication intake (24). In another study, serum ceramides, particularly C16:0 and C24:0 , were elevated in uncontrolled asthma compared to controlled asthma, while high levels of CD66+

neutrophils were associated with high levels of sphingosine and C16:0 ceramide (25). Our findings suggest that elevation of specific lipids may be associated with uncontrolled asthma development. The high levels of both fecal and serum lipids may be partly associated with the high intake of a sugary diet in Metabotype1, particularly in comparison to Metabotype2. However, results were not statistically significant after multiple testing correction for the nutrients, which could be attributed to the small sample size of subjects/parents who filled in the food diaries. Interestingly, we did not observe differences between the metabotypes in fat intake or BMI, suggesting that we cannot fully capture the dietary habits of these children using the 24-hour food diaries. Therefore, further research is needed to investigate the consequence of long-term dietary intake over the metabolomics profiles. Moreover, Metabotype 1 is distinguished by elevated levels of TIMP-4 compared to the other metabotypes. TIMP-4 plays a crucial role in regulating the activity of matrix metalloproteinases (MMPs), which are involved in tissue remodelling and inflammation (26, 27). Limited research has been performed to investigate the role of TMP-4 in severe asthma pathophysiology, however, a previous study reported its link to COPD (28), suggesting its potential role in airway inflammation, which requires further investigation.

Metabotype2 showed the most favorable clinical profile (highest odds for asthma control and lowest percentage of dry cough episodes in the past year) compared to the other metabotypes. Metabotype2 had the lowest levels of blood neutrophils and monocytes compared to the other metabotypes. Furthermore, it showed the most favorable metabolic profile characterized by depletion of triglycerides in feces and serum and elevation of omega 3 fatty acids, acylcarnitines and α-aminobutyric acid in serum, which partly corresponds to reported evidence in the literature. For instance, a study demonstrated that elevated levels of omega-3 fatty acids in erythrocyte membranes positively correlate with improved asthma control in children (29). Another study revealed that maternal intake of fish oil-derived omega-3 fatty acids during pregnancy was linked to a reduction in asthma risk later in life (30). Moreover, children with moderate persistent asthma exhibited reduced levels of serum carnitines

compared to healthy controls; meanwhile, supplementation of L-carnitine to these asthmatic children showed improvement in asthma control relative to those receiving a placebo (31). Red meats, followed by poultry, fish, and dairy products are the main dietary sources of carnitines. Therefore, the elevated levels of carnitines in Metabotype2 may be partly explained by the high reported amounts of protein intake assessed by the 24h-food diaries within this metabotype. Metabotype2 also showed the most favorable microbial profile, characterized by enrichment of some beneficial and/or butyrate-producing bacteria (32-34), such as *Christensenellaceae R−7* group, *Coprococcus*, and members of *Oscillospiraceae* family particularly in comparison to Metabotype3. Moreover, Metabotype2 showed decreased levels non-T2 pro-inflammatory proteins in comparison to the other metabotypes, which is in line with their favorable clinical, metabolomics, and microbial profiles. The decreased levels of non-T2 pro-inflammatory markers, such as IL-6, MMP3 and TNF-α, in this metabotype may be partly explained by the elevated serum levels of α-aminobutyric acid within this metabotype. α-aminobutyric acid is reported to inhibit polarization and activity of M1 macrophages that can secrete these cytokines (35). Moreover, these markers play a significant role in asthma pathophysiology and exacerbations (27, 36), suggesting that Metabotype 2 has a lower inflammatory status compared to the other metabotypes.

Metabotype3 exhibited the highest burden of asthma in comparison to the other metabotypes, as characterized by the highest percentage of patients with uncontrolled asthma and the highest percentage of at least one severe asthma exacerbation requiring a burst of OCS intake ≥3 days in the past 12 months. Subjects within this group were more likely to be prescribed add-on asthma medications, particularly LTRAs, suggesting that these medications were prescribed to control their severe symptoms. This metabotype exhibited elevated ornithine-to-arginine ratio in serum compared to the other metabotypes. Ornithine-to-arginine ratio is a marker of arginase activity, where arginase is an enzyme that catalyzes the conversion of arginine to ornithine in the final step of the urea cycle (37). This finding is in line with a previous study also showing an increased arginase activity in serum of children

with asthma or wheezing (n=21) admitted to ER with acute exacerbations compared to controls (n=15) (38). Increased arginase activity and decreased citrulline synthesis can reflect iNOS inhibition (i.e. decreased endogenous nitric oxide production) in this metabotype (39), while inhibition of iNOS is thought to promote bronchoconstriction, airway remodeling and worsen disease control (40, 41). In addition, this metabotype showed dysregulated lipid profiles in serum characterized by the depletion of sphingomyelins, as well as other lipid classes, including phosphatidylcholines, cholesteryl esters, and ceramides. Sphingomyelins and phosphatidylcholines are classes of phospholipids, therefore, their depletion may suggest an imbalance in phospholipid metabolism. Phospholipids and ceramides are components of the lipid membranes as well as the airway surfactants (42), and decreased levels could reflect abnormalities in normal structure and function of the airways. Thus, the direct relationship between serum levels of these metabolites and airway function requires further investigation. Notably, this metabotype showed elevation of some triglycerides in comparison to Metabotype2, however, the overall number of elevated triglycerides was significantly less than Metabotype1. Again, this highlights that elevated triglycerides are associated with childhood asthma severity. Moreover, this metabotype showed characteristic elevated fecal levels of amino acids and ceramides compared to the other metabotypes. Increased levels of fecal amino acids have been observed in other inflammatory diseases, such as inflammatory bowel disease (43), suggesting that this may underline an ongoing inflammatory state in Metabotype3.

Distribution of subjects within the metabotypes was significantly different according to the country of inclusion. This is expected, considering that subjects recruited from different countries have different dietary habits and are exposed to different environments. Yet, we could still detect significant differences in asthma characteristics after adjusting for countries and other potential confounders, including medication intake. This suggests that the metabolic processes are strongly linked to childhood asthma control and may play a role in disease development. It has been reported that factors such as

age, sex, ethnicity, and corticosteroids intake can influence the metabolome profiles of individuals (44, 45). In contrast, we did not observe significant differences in these factors, and neither the dose of inhaled corticosteroids nor oral corticosteroids intake among the 3 metabotypes. Interestingly, a larger percent of subjects within Metabotype2 were breastfed for equal to or more than 4 months compared to the other clusters, while the mode of delivery for a larger percent of subjects within Metabotype1 was C-section. These findings suggest that early life exposures are associated with the metabolic makeup of children with asthma. Further research is needed evaluate whether these early life exposures have causal effects on the metabolic profiles later in life.

The association of the metabolic profiles with disease burden in children with moderate-to-severe asthma can have clinical implications. Asthma endotyping is crucial for the appropriate choice of the medications and the personalized disease management. In contrast to adulthood asthma, endotyping in childhood asthma is challenging due to the difficulty of obtaining sputum and/or invasive samples. Therefore, metabolomics profiling from minimally invasive/non-invasive specimens could be a solution. Moreover, our findings highlight potential metabolic pathways that can be targeted to improve disease control in children with moderate-to-severe asthma. In Metabotype2, favorable metabolic (e.g., increased acylcarnitines and ω-3 fatty acids and decreased triglycerides in serum) and gut microbiome (e.g., increased diversity and butyrate producing bacteria) profiles were associated with improved asthma control in those children. Therefore, further investigations required to assess if targeting microbiome (e.g., probiotics) or metabolome (e.g., ω-3 or carnitines) or diet-based supplement/healthy diet can play a role in the therapeutic management of severe childhood asthma.

This study has several strengths. First, we comprehensively assessed the metabolome profiles using a large panel of targeted metabolic profiling that encompass several metabolic classes across two sampling compartments (serum and feces). This allowed us to adequately characterize children with moderate-to-

severe asthma with respect to their metabolic phenotyping, while liking it to other omics features. Second, the analysis recruited children from 4 different European countries, making the findings more generalizable than single center studies. Third, the analysis approach utilized is unsupervised and its clinical relevance is driven mainly by the metabolome profiles. Finally, this study is regarded as one of the first attempts to uncover integrative metabolome-driven endotypes and elucidate targets for precision medicine within children with moderate-to-severe asthma.

However, this study has some limitations. First, although we recruited children from 4 European countries, the overall cohort sample size with both fecal and serum metabolome was limited, mainly due to difficulties in recruiting children with moderate-to-severe disease and their reluctance to provide some sample types, such as feces (11). This raises the question of whether there are more metabotypes in children with asthma that our sample size could not adequately cover. However, this highlights that multi-center collaboration is essential in the multi-omics investigating of moderate-to-severe childhood asthma. Second, we investigated the metabolome at one time point. Longitudinal shifts in the metabolomics profiles have been previously reported (46) and further research should examine the over-time stability of the metabotypes and their relationship to the asthma pathophysiology. Third, we have only collected the 24-hour food diaries in a subset of the cohort which limits statistical power. In addition, the 24-hour history may not necessarily reflect the long-term dietary habits which can have a stronger influence on the metabolome profiles. Fourth, we were not able to validate these findings in an external cohort. This is attributed to the scarceness of moderate-to-severe childhood asthma cohorts where comprehensive metabolic profiling has been performed. Finally, given the study's observational nature, bias due to selection and unmeasured confounders cannot be ruled out.

In conclusion, this study has delineated three distinct metabotypes among children with moderate-to-severe asthma, revealing significant differences in asthma burden, and underlying molecular and

inflammatory profiles. These findings underscore the importance of metabolic profiling in understanding childhood asthma and highlight the potential for metabolomics to aid in asthma endotyping and personalized management. Further research to investigate whether targeting specific metabolic pathways could offer safe and effective therapeutic options in children with uncontrolled disease.

**Acknowledgement:**

The SysPharmPediA is a consortium effort and we would like to acknowledge the help and expertise of the individuals and groups whose names are mentioned in the attached SysPharmPediA consortium list. In addition, we would like to thank all the children and parents/caregivers who participated in the SysPharmPediA study.

Some of the drawn objects in the Graphical Abstract were adopted from Servier, Somersault18:24 and National Institutes of Health BioArt corporations, which are used under creative commons license.

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**Tables:**

**Table 1:** Demographic and Clinical Characteristics of the metabotypes.

| Characteristics | All subjects (N=92) | Metabotype 1 (n=30) | Metabotype 2 (n=32) | Metabotype 3 (n=30) | *P*-value |
|---|---|---|---|---|---|
| Age in years, median (IQR) | 11.54 (9.65, 13.44) | 11.74 (9.65, 14.11) | 11.58 (9.59, 12.84) | 11.16 (9.65, 13.20) | 0.880 |
| Female, n (%) | 31/92 (34%) | 10/30 (33%) | 14/32 (44%) | 7/30 (23%) | 0.249 |
| White Caucasian, n (%) | 73/92 (79%) | 26/30 (87%) | 27/32 (84%) | 20/30 (67%) | 0.120 |
| BMI z-score, median (IQR) | 0.38 (-0.37, 1.34) (n=91) | 0.25 (-0.60, 1.41) (n=29) | 0.33 (-0.32, 1.11) (n=32) | 0.44 (-0.33, 1.26) (n=30) | 0.895 |
| **Mode of delivery (C-section), n (%)** | **20/91 (22%)** | **11/30 (37%)** | **6/32 (19%)** | **3/29 (10%)** | **0.048** |
| **Breastfeeding (≥4 months), n (%)** | **51/91 (56%)** | **12/30 (40%)** | **25/32 (78%)** | **14/29 (48%)** | **0.006** |
| Residential location, n (%) | | | | | 0.193 |
| • City | 30/90 (33%) | 5/30 (17%) | 13/32 (41%) | 12/28 (43%) | |
| • City center | 8/90 (9%) | 2/30 (7%) | 2/32 (6%) | 4/28 (14%) | |
| • Rural area | 15/90 (17%) | 6/30 (20%) | 6/32 (19%) | 3/28 (11%) | |
| • Village | 37/90 (41%) | 17/30 (57%) | 11/32 (34%) | 9/28 (32%) | |
| Smoking exposure, n (%) | 25/90 (28%) | 8/30 (27%) | 10/32 (31%) | 7/28 (25%) | 0.874 |
| **Uncontrolled asthma*, n (%)** | **58/92 (63%)** | **16/30 (53%)** | **17/32 (53%)** | **25/30 (83%)** | **0.019** |
| **Severe asthma exacerbations in the past 12 months, n (%)** | **50/92 (54%)** | **12/30 (40%)** | **15/32 (47%)** | **23/30 (77%)** | **0.010** |
| **Dry cough apart from infection in the past 12 months, n (%)** | **70/90 (78%)** | **26/29 (90%)** | **18/32 (56%)** | **26/29 (90%)** | **0.001** |
| **Dry cough at night apart from infection in the past 12 months, n (%)** | **63/90 (70%)** | **25/29 (86%)** | **17/32 (53%)** | **21/29 (72%)** | **0.013** |
| **>10 days of missed school due to asthma in the past 12 months, n (%)** | **14/90 (16%)** | **6/29 (21%)** | **1/32 (3%)** | **7/29 (24%)** | **0.049** |
| **Country of inclusion, n (%)** | | | | | **$1\times10^{-04}$** |
| • **Spain** | **49/92 (53%)** | **4/30 (13%)** | **30/32 (94%)** | **15/30 (50%)** | |
| • **Germany** | **22/92 (24%)** | **19/30 (63%)** | **0/32 (0%)** | **3/30 (10%)** | |
| • **The Netherlands** | **12/92 (13%)** | **5/30 (17%)** | **0/32 (0%)** | **7/30 (23%)** | |

| Characteristics | All subjects (N=92) | Metabotype 1 (n=30) | Metabotype 2 (n=32) | Metabotype 3 (n=30) | *P*-value |
|---|---|---|---|---|---|
| • **Slovenia** | **9/92 (10%)** | **2/30 (7%)** | **2/32 (6%)** | **5/30 (17%)** | |
| Atopy[#], n (%) | 79/90 (88%) | 23/30 (77%) | 29/32 (91%) | 27/28 (96%) | 0.061 |
| • Aeroallergen combined | 78/91 (86%) | 22/30 (73%) | 29/32 (91%) | 27/29 (93%) | 0.067 |
| ○ **HDM** | **68/91 (75%)** | **13/30 (43%)** | **29/32 (91%)** | **26/29 (90%)** | **$1\times10^{-04}$** |
| ○ Grass pollens | 43/88 (49%) | 18/30 (60%) | 11/30 (37%) | 14/28 (50%) | 0.213 |
| ○ Mold | 7/70 (10%) | 3/23 (13%) | 0/22 (0%) | 4/25 (16%) | 0.199 |
| ○ Cat | 28/82 (34%) | 11/30 (37%) | 4/23 (17%) | 13/29 (45%) | 0.109 |
| ○ **Dog** | **24/80 (30%)** | **8/28 (29%)** | **3/24 (12%)** | **13/28 (46%)** | **0.029** |
| • Food allergen combined | 19/66 (29%) | 6/14 (43%) | 4/29 (14%) | 9/23 (39%) | 0.057 |
| ○ **Nuts** | **15/64 (23%)** | **6/12 (50%)** | **1/29 (3%)** | **8/23 (35%)** | **0.001** |
| ○ Egg | 6/57 (11%) | 1/9 (11%) | 1/29 (3%) | 4/19 (21%) | 0.124 |
| ○ Milk | 8/60 (13%) | 3/11 (27%) | 1/29 (3%) | 4/20 (20%) | 0.078 |
| ○ Fish | 1/55 (2%) | 0/9 (0%) | 1/29 (3%) | 0/17 (0%) | 1 |
| Comorbid allergy diagnosed (ever), n (%) | | | | | |
| • **Allergic rhinitis** | **68/87 (78%)** | **17/29 (59%)** | **27/30 (90%)** | **24/28 (86%)** | **0.006** |
| • **Allergic conjunctivitis** | **63/85 (74%)** | **15/28 (54%)** | **27/30 (90%)** | **21/27 (78%)** | **0.005** |
| • Atopic dermatitis | 39/85 (46%) | 8/23 (35%) | 19/32 (59%) | 12/30 (40%) | 0.145 |
| (childhood) Asthma Control Test ((c)ACT) z-score, median (IQR) | 0.95 (0.33, 1.55) (n=91) | 1.03 (0.35, 1.55) (n=30) | 1.19 (0.72, 1.69) (n=31) | 0.72 (-0.17, 1.28) (n=30) | 0.132 |
| Spirometry: | | | | | |
| • $FEV_1$ pre-salbutamol z-score, median (IQR) | -0.45 (-1.45, 0.24) (n=91) | -0.62 (-1.55, 0.02) (n=29) | -0.33 (-1.18, 0.13) (n=32) | -0.42 (-1.48, 0.26) (n=30) | 0.781 |
| • $FEV_1$ post-salbutamol z-score, median (IQR) | -0.12 (-0.93, 0.65) (n=90) | -0.63 (-1.28, 0.66) (n=28) | -0.09 (-0.74, 0.48) (n=32) | -0.10 (-0.70, 0.65) (n=30) | 0.595 |
| • **Positive bronchodilator response ($FEV_1$ ≥200 mL and ≥12%), n (%)** | **19/91 (21%)** | **4/29 (14%)** | **4/32 (12%)** | **11/30 (37%)** | **0.016** |
| $FE_{NO}$ (ppb), n (%) | 15.00 (8.60, 36.30) (n=85) | 15.00 (7.35, 31.00) (n=27) | 12.70 (8.70, 32.85) (n=31) | 17.00 (10.35, 47.15) (n=27) | 0.631 |

| Characteristics | All subjects (N=92) | Metabotype 1 (n=30) | Metabotype 2 (n=32) | Metabotype 3 (n=30) | *P*-value |
|---|---|---|---|---|---|
| White blood cell count (×$10^9$/L), median (IQR) | | | | | |
| • Eosinophils | 0.40 (0.27, 0.65) | 0.34 (0.21, 0.47) | 0.50 (0.29, 0.84) | 0.38 (0.28, 0.62) | 0.151 |
| • **Neutrophils** | **3.06 (2.30, 3.93)** | **3.40 (2.75, 4.12)** | **2.60 (1.92, 3.54)** | **3.14 (2.45, 4.30)** | **0.047** |
| • Lymphocytes | 2.58 (2.23, 3.09) | 2.88 (2.23, 3.10) | 2.50 (2.31, 3.10) | 2.58 (2.05, 3.04) | 0.864 |
| • Basophils | 0.05 (0.03, 0.07) | 0.05 (0.03, 0.06) | 0.05 (0.03, 0.08) | 0.04 (0.03, 0.06) | 0.175 |
| • **Monocytes** | **0.52 (0.40, 0.60) (n=83)** | **0.56 (0.46, 0.66) (n=21)** | **0.46 (0.36, 0.54) (n=32)** | **0.54 (0.42, 0.62) (n=30)** | **0.030** |
| Inclusion season, n (%) | | | | | 0.180 |
| • Winter | 20/92 (22%) | 4/30 (13%) | 8/32 (25%) | 8/30 (27%) | |
| • Spring | 29/92 (32%) | 10/30 (33%) | 10/32 (31%) | 9/30 (30%) | |
| • Summer | 29/92 (32%) | 14/30 (47%) | 6/32 (19%) | 9/30 (30%) | |
| • Autumn | 14/92 (15%) | 2/30 (7%) | 8/32 (25%) | 4/30 (13%) | |
| Asthma medications, n (%) | | | | | |
| • ICS dose: | | | | | 0.388 |
| ○ Low | 49/92 (53%) | 18/30 (60%) | 18/32 (56%) | 13/30 (43%) | |
| ○ Medium | 27/92 (29%) | 7/30 (23%) | 11/32 (34%) | 9/30 (30%) | |
| ○ High | 16/92 (17%) | 5/30 (17%) | 3/32 (9%) | 8/30 (27%) | |
| • SABA | 82/92 (89%) | 26/30 (87%) | 28/32 (88%) | 28/30 (93%) | 0.768 |
| • LABA | 89/92 (97%) | 30/30 (100%) | 32/32 (100%) | 27/30 (90%) | 0.070 |
| • OCS | 1/92 (1%) | 0/30 (0%) | 0/32 (0%) | 1/30 (3%) | 0.654 |
| • **LTRA** | **13/92 (14%)** | **5/30 (17%)** | **0/32 (0%)** | **8/30 (27%)** | **0.008** |
| • Anticholinergics | 9/92 (10%) | 5/30 (17%) | 1/32 (3%) | 3/30 (10%) | 0.176 |
| • Biologicals (Omalizumab or Mepolizumab) | 7/92 (8%) | 3/30 (10%) | 1/32 (3%) | 3/30 (10%) | 0.554 |
| • GINA step: | | | | | 0.528 |
| ○ Step 3 | 46/92 (50%) | 17/30 (57%) | 17/32 (53%) | 12/30 (40%) | |
| ○ Step 4 | 39/92 (42%) | 10/30 (33%) | 14/32 (44%) | 15/30 (50%) | |

| Characteristics | All subjects (N=92) | Metabotype 1 (n=30) | Metabotype 2 (n=32) | Metabotype 3 (n=30) | *P*-value |
|---|---|---|---|---|---|
| ○ Step 5 | 7/92 (8%) | 3/30 (10%) | 1/32 (3%) | 3/30 (10%) | |
| MARS-5 (≥21), n (%) | 81/86 (94%) | 26/28 (93%) | 30/31 (97%) | 25/27 (93%) | 0.727 |

For continuous measures (variables), the number of samples available for a specific measure is provided only when data were missing. *P*-values were calculated using Pearson's chi-square test with Monte Carlo simulation (10,000 permutations) or the Kruskal-Wallis H test as appropriate.

Entries with statistically significant P values (<0.05) are shown in boldface.

*Uncontrolled asthma is defined based on (childhood) Asthma Control Test ((c)ACT) score ≤19 and/or severe exacerbations requiring hospitalization or emergency room (ER) visits or oral corticosteroid (OCS) use in the past year

#Atopic sensitization refers to physician history (ever) of sensitization to aeroallergens or food allergens by a positive skin prick test result (wheal diameter ⩾3 mm) and/or positive allergen-specific IgE (⩾0.35 kU/L).

Definition of abbreviations: BMI = body mass index; $FE_{NO}$ = fractional exhaled nitric oxide; $FEV_1$ = forced expiratory volume in 1 second; GINA = Global Initiative for Asthma; HDM = house dust mite; ICS = inhaled corticosteroids; IQR = interquartile range; LABA = long-acting β-agonist; LTRA = leukotriene antagonist; MARS-5 = Medication Adherence Report Scale-5 ; OCS = oral corticosteroids; SABA = short-acting β-agonist;

**Table 2:** Number of statistically significant enriched or depleted serum metabolites grouped by metabolic classes across the 3 metabotypes.

| Metabolite class | Enriched serum metabolome | | | | Depleted serum metabolome | | | |
|---|---|---|---|---|---|---|---|---|
| | Metabotype 1 | Metabotype 2 | Metabotype 3 | *P*-value * | Metabotype 1 | Metabotype 2 | Metabotype 3 | *P*-value * |
| Amine oxides | 0 | 1 | 0 | <0.0001 | 0 | 0 | 0 | <0.0001 |
| Amino acids | 0 | 3 | 2 | | 0 | 4 | 2 | |
| Carnitines | 0 | 3 | 0 | | 1 | 0 | 1 | |
| Ceramides | 12 | 0 | 0 | | 0 | 0 | 4 | |
| Cholesteryl esters | 10 | 0 | 0 | | 0 | 0 | 8 | |
| Cresols | 0 | 0 | 0 | | 0 | 0 | 0 | |
| Fatty acids | 0 | 4 | 0 | | 0 | 0 | 0 | |
| Phosphatidylcholines | 46 | 5 | 0 | | 1 | 1 | 18 | |
| Sphingomyelins | 1 | 0 | 0 | | 0 | 0 | 7 | |
| Triglycerides | 73 | 0 | 26 | | 0 | 144 | 0 | |
| Diglycerides | 5 | 1 | 2 | | 0 | 5 | 0 | |
| Vitamins and cofactors | 1 | 0 | 0 | | 0 | 0 | 0 | |

**P*-values were calculated for overall metabolic classes.

**Table 3:** Number of statistically significant enriched or depleted fecal metabolites grouped by metabolic classes across the 3 metabotypes.

| Metabolite class | Enriched stool metabolome | | | | Depleted stool metabolome | | | |
|---|---|---|---|---|---|---|---|---|
| | Metabotype 1 | Metabotype 2 | Metabotype 3 | *P*-value * | Metabotype 1 | Metabotype 2 | Metabotype 3 | *P*-value* |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Amino acids | 0 | 2 | 12 | <0.0001 | 6 | 0 | 1 | 0.016 |
| Bile acids | 0 | 0 | 0 | | 0 | 0 | 0 | |
| Biogenic amines | 0 | 0 | 1 | | 0 | 0 | 0 | |
| Carboxylic acids | 0 | 0 | 1 | | 0 | 0 | 0 | |
| Carnitines | 0 | 1 | 0 | | 0 | 1 | 0 | |
| Ceramides | 0 | 0 | 3 | | 0 | 15 | 0 | |
| Fatty acids | 0 | 0 | 0 | | 0 | 0 | 0 | |
| Phosphatidylcholines | 16 | 0 | 0 | | 1 | 9 | 0 | |
| Sphingomyelins | 2 | 0 | 0 | | 0 | 7 | 0 | |
| Triglycerides | 2 | 0 | 0 | | 0 | 19 | 0 | |
| Diglycerides | 0 | 0 | 0 | | 0 | 6 | 0 | |
| Hormones and related | 0 | 0 | 1 | | 0 | 0 | 0 | |
| Monosaccharides | 0 | 0 | 0 | | 0 | 0 | 0 | |

**P*-values were calculated for overall metabolic classes.

**Box 1:** Summary overview for the differences in the study characteristics between the metabotype.

***Metabotype 1***

Metabotype1 included 30 (33%) subjects, with a large percentage of children recruited from Germany (n = 19 of 30 [63%]; overall $P = 1\times10^{-04}$, post hoc $q = <0.001$). Metabotype1 had the lowest percentage of subjects with history of ≥4 months of breastfeeding (n = 12 of 30 [40%]; overall $P = 0.006$, post hoc $q = 0.031$), highest percentage of subjects with a Caesarian-section mode of delivery, however; the latter was not statistically significant at post-hoc level (n = 11 of 30 [37%]; overall $P = 0.048$, post hoc $q = 0.106$), lowest percentage of subjects with history of house dust mite atopic sensitization (n = 13 of 30 [43%]; overall $P = 1\times10^{-04}$, post hoc $q = <0.001$), lowest percentage of subjects with physician-diagnosed allergic rhinitis (n = 17 of 29 [59%]; overall $P = 0.006$, post hoc $q = 0.011$), and allergic conjunctivitis (n = 15 of 28 [54%]; overall $P = 0.005$, post hoc $q = 0.015$). However, it had the highest percentage of subjects with nut allergens sensitization (n = 6 of 12 [50%]; overall $P = 0.001$, post hoc $q = 0.048$). This metabotype did not show association with asthma control at post-hoc level (n = 16 of 30 [53%]; overall $P = 0.019$, post hoc $q = 0.359$), however; it showed higher odds for uncontrolled asthma when compared to Metabotype2 after adjusting for multiple covariates. In addition, it showed the highest percentage of subjects with dry cough episodes at night, with a trend of statistically significant difference at post-hoc level (n = 25 of 29 [86%]; overall $P = 0.013$, post hoc $q = 0.062$).

***Metabotype 2***

Metabotype2 included 32 (35%) subjects, and was dominated by children recruited from Spain (n = 30 of 32 [94%]; overall $P = 1\times10^{-04}$, post hoc $q = <0.001$). Metabotype2 had the highest percentage of subjects with history of ≥4 months of breastfeeding (n = 25 of 32 [78%]; overall $P = 0.006$, post hoc $q = 0.002$), a high percentage of subjects with history of house dust mite atopic sensitization (n = 29 of 32 [91%]; overall $P = 1\times10^{-04}$, post hoc $q = 0.030$), a trend for the lowest percentage of subject with dog dander aeroallergen sensitization at post-hoc level (n = 3 of 24 [12%]; overall $P = 0.029$, post hoc $q = 0.076$), lowest percentage of subjects with nut allergens (n = 1 of 29 [3%]; overall $P = 0.001$, post hoc $q = 0.004$) and the highest percentage of subjects with physician-diagnosed allergic conjunctivitis (n = 27 of 30 [90%]; overall $P = 0.005$, post hoc $q = 0.041$). This metabotype showed the most favorable clinical profile in terms of asthma burden and inflammatory cell profile amongst the three metabotypes. In particular, both Metabotype3 and Metabotype1 showed higher odds for uncontrolled asthma after adjusting for covariates (adjusted OR = 11.7, 95% CI: 2.57, 88 for Metabotype3 and 7.34, 95% CI: 1.22, 68.9 for Metabotype1, relative to Metabotype2, all *P*-values <0.05). In addition, it showed the lowest percentage of subjects with dry cough episodes (n = 18 of 32 [56%]; overall $P = 0.001$, post hoc $q = 0.002$), a trend for the lowest percentage of subjects with >10 days of missed school days at post-hoc level (n = 1 of 32 [3%]; overall $P = 0.049$, post hoc $q = 0.094$), the lowest blood monocytes counts ([median = 0.46, IQR: 0.36, 0.54], overall $P = 0.030$, post-hoc $q = 0.039$ in comparison to Metabotype1 [median = 0.56, IQR: 0.46, 0.66], and post-hoc $q = 0.084$ in comparison to Metabotype3 [median = 0.54, IQR: 0.42, 0.62]), and a trend for lowest blood neutrophils counts ([median = 2.60, IQR: 1.92, 3.54], overall $P = 0.047$, post-hoc $q = 0.059$ in comparison to Metabotype1 [median = 3.40, IQR: 2.75, 4.12], and post-hoc $q = 0.100$ in comparison to Metabotype3 [median = 3.14, IQR: 2.45, 4.30]). Moreover, this metabotype had the lowest percentage of subjects who were prescribed leukotriene antagonists add-on asthma medication (n = 0 of 32 [0%]; overall $P = 0.008$, post hoc $q = 0.027$).

***Metabotype 3***

Metabotype3 included 30 (33%) subjects. Metabotype3 had a high percentage of subjects with history of house dust mite atopic sensitization (n = 26 of 29 [90%]; overall $P = 1\times10^{-04}$, post hoc $q = 0.050$), and a trend for the highest percentage of subjects with dog dander aeroallergen sensitization at post-hoc level (n = 13 of 28 [46%]; overall $P = 0.029$, post hoc $q = 0.076$). This metabotype was the most associated with increased asthma burden. In particular, this metabotype showed the highest percentage of subjects with uncontrolled asthma (n = 25 of 30 [83%]; overall $P = 0.019$, post hoc $q = 0.030$) and had the highest odds for uncontrolled asthma in comparison to Metabotype2 after adjusting for covariates (adjusted OR = 11.7, 95% CI: 2.57, 88, all *P*-value <0.05). In addition, it showed the highest percentage of subjects with severe asthma exacerbation in the past year (n = 23 of 30 [77%]; overall $P = 0.010$, post hoc $q = 0.017$), a trend for the highest percentage of subjects with a positive bronchodilator response (n = 11 of 30 [37%]; overall $P = 0.016$, post hoc $q = 0.056$), and the highest percentage

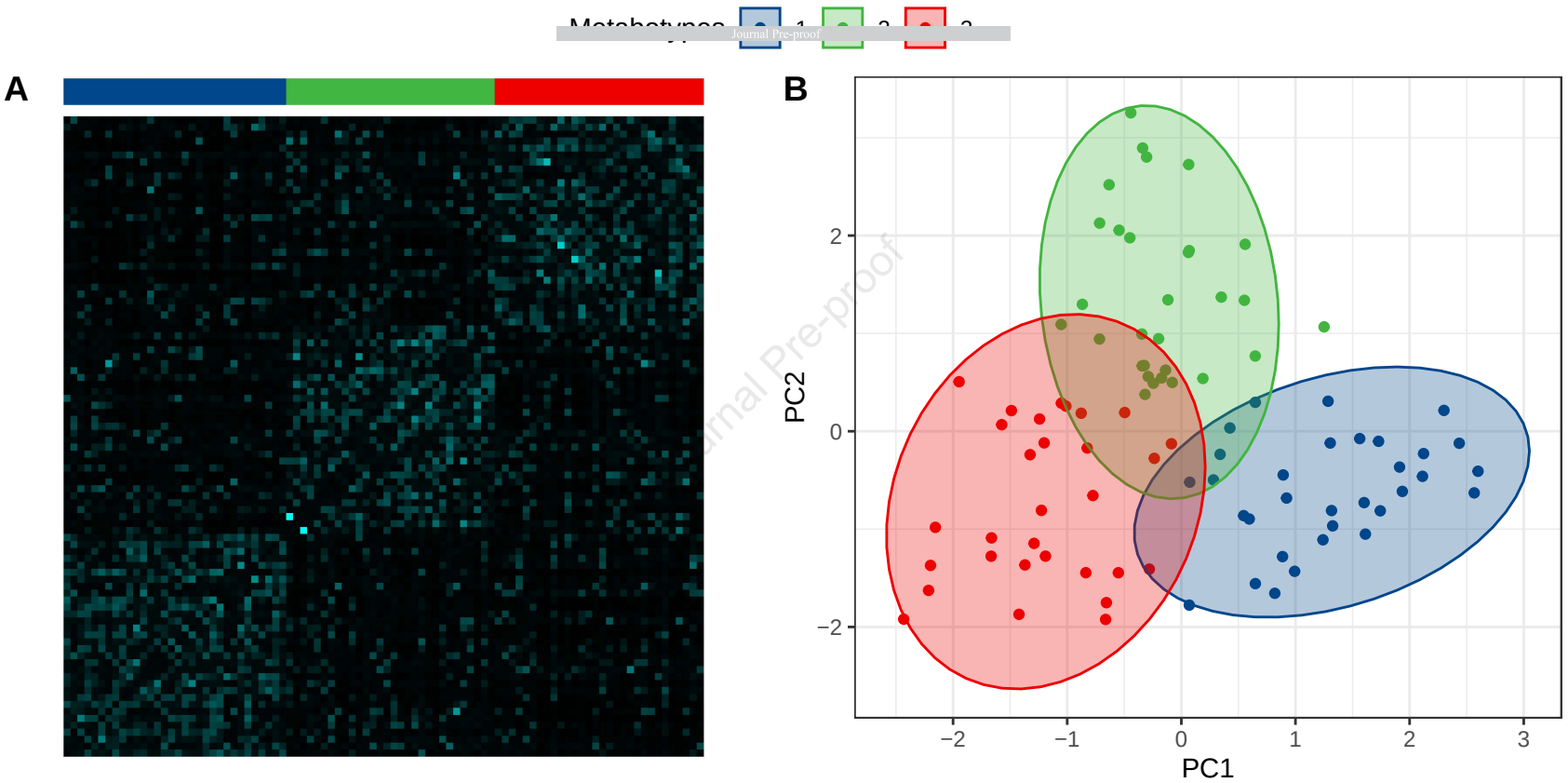
of subjects who were prescribed Leukotriene antagonists add-on asthma medication (n = 8 of 30 [27%]; overall *P* = 0.008, post hoc *q* = 0.049).

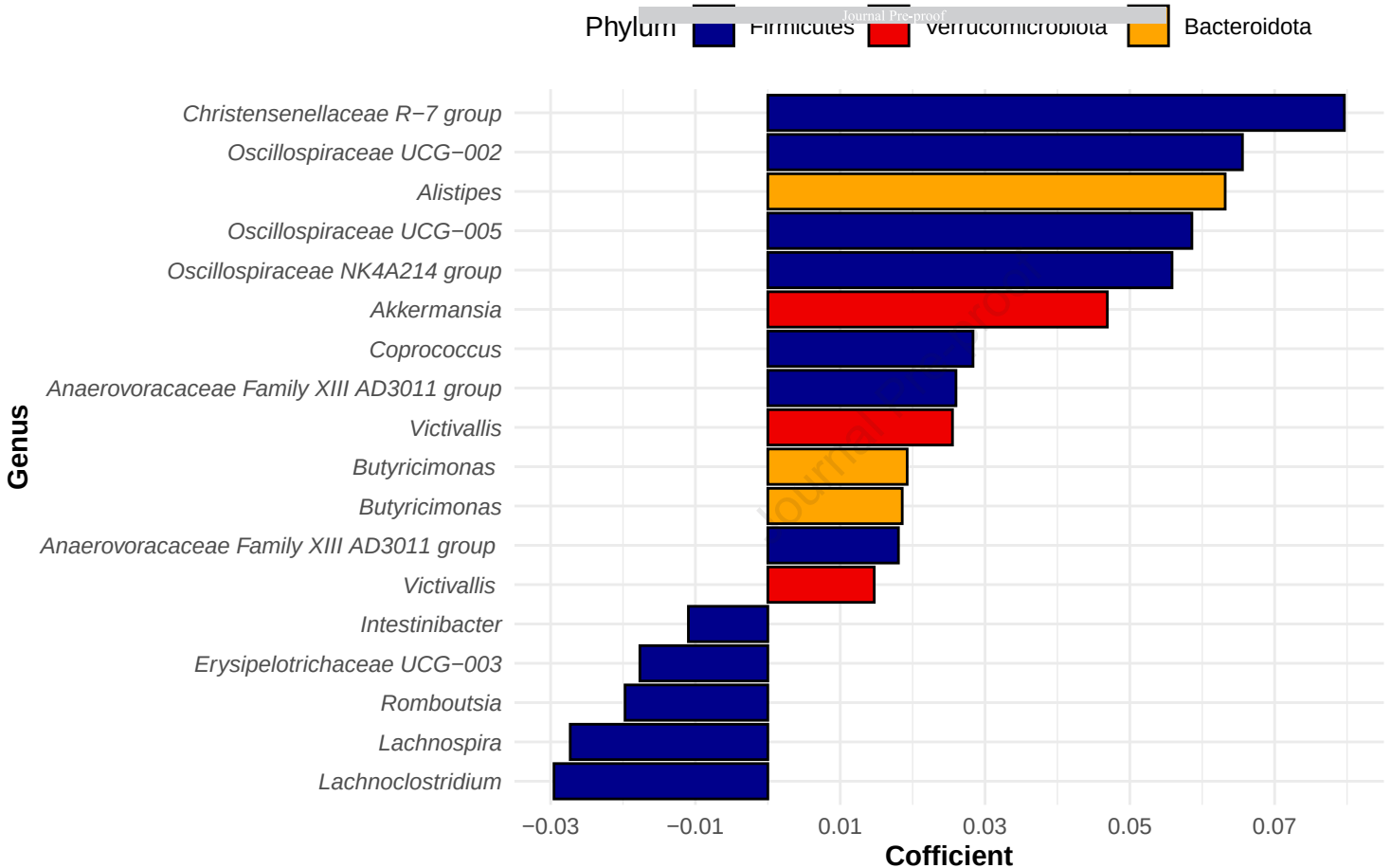
**Figure legends:**

**Figure 1: A;** Heatmap of Similarity Network Fusion (SNF) of serum and fecal metabolomics in the SysPharmPediA moderate-to-severe childhood asthma cohort, showing 3 identifiable clusters (metabotypes). **B;** Kernel principal component analysis (PCA) plot showing the separation of the metabotypes based on their similarity network of the two metabolomics blocks based on the 2D depiction of the first two principal components with the 95% confidence ellipses.

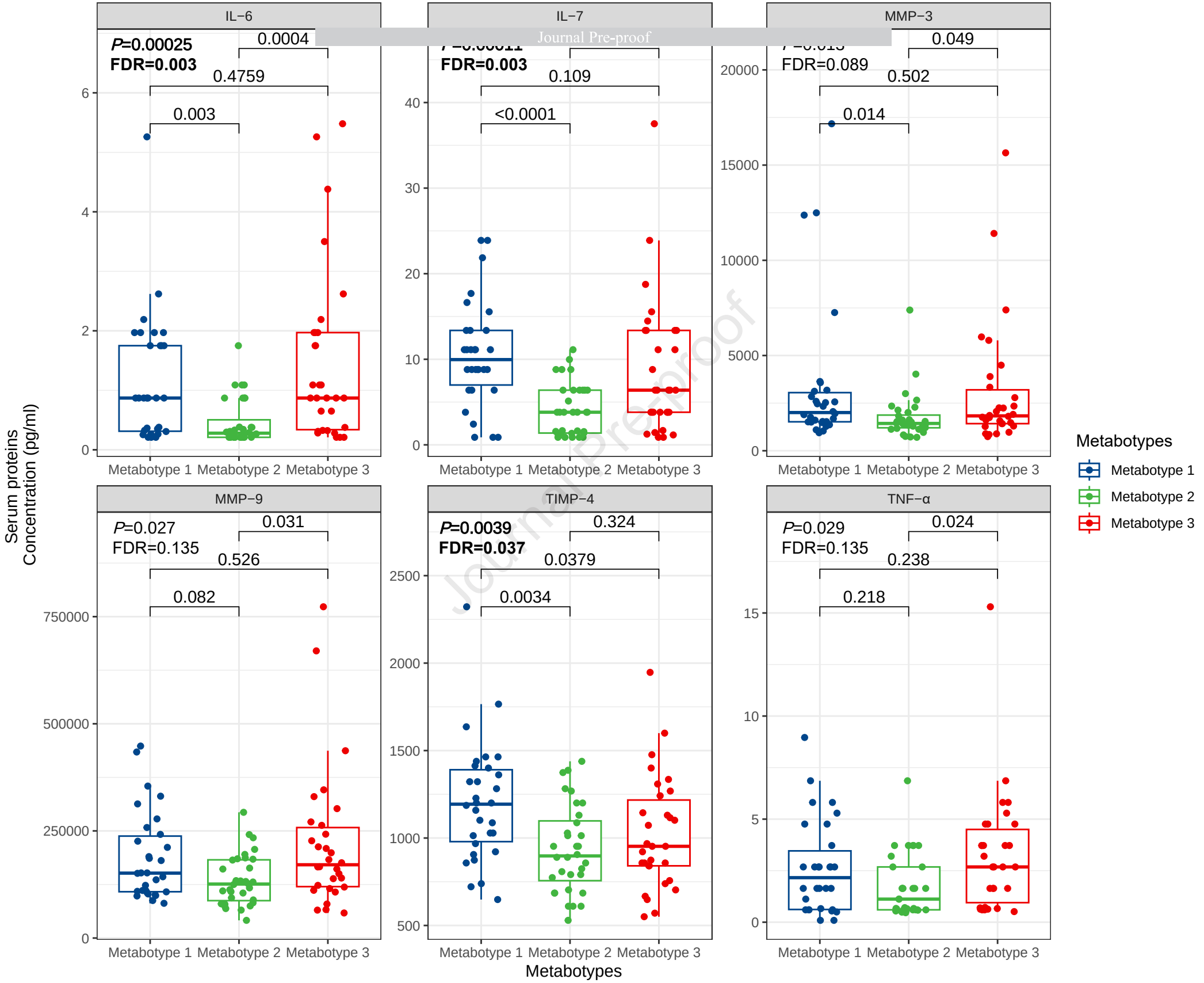
**Figure 2:** Microbiome Multivariable Associations with Linear Models 2 (MaAsLin 2) plot showing differentially abundant bacterial genera between the metabotypes (*P*-values<0.05), while those with statistically significant associations after multiple testing correction (*q*-values<0.05) are highlighted in boldface. The bars of bacterial genera were colored according to their respective phyla. Metabotype3 with the least diverse microbial profile was chosen as a reference group for comparison.

**Figure 3:** Box-and-Whisker plots of the statistically significant serum cytokines and chemokines in the three metabotypes, while the one with FDR corrected *P*-values <0.05 are shown in boldface.






| Metabotype | *P*-value | *q*-value |
|---|---|---|
| Cluster2 | **<0.01** | **0.037** |
| Cluster2 | 0.017 | 0.323 |
| Cluster2 | 0.032 | 0.351 |
| Cluster2 | **<0.01** | **0.017** |
| Cluster2 | **<0.01** | **0.017** |
| Cluster1 | 0.024 | 0.351 |
| Cluster2 | **<0.01** | **0.044** |
| Cluster2 | **<0.01** | **0.026** |
| Cluster2 | **<0.01** | **0.017** |
| Cluster1 | 0.032 | 0.351 |
| Cluster2 | 0.029 | 0.351 |
| Cluster1 | 0.019 | 0.323 |
| Cluster1 | 0.036 | 0.366 |
| Cluster2 | 0.05 | 0.477 |
| Cluster2 | 0.027 | 0.351 |
| Cluster1 | <0.01 | 0.155 |
| Cluster2 | 0.03 | 0.351 |
| Cluster2 | **<0.01** | **0.026** |



SysPharmPediA

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**Funding:**

The SysPharmPediA consortium is supported by ZonMW [project number: 9003035001], the Ministry of Education, Science, and Sport of the Republic of Slovenia [contract number C330-16-500106]; the German Ministry of Education and Research (BMBF) [project number FKZ 031L0088]; Instituto de Salud Carlos III (ISCIII) through Strategic Action for Health Research (AES) and European Community (EC) within the Active and Assisted Living (AAL) Program framework [award numbers AC15/00015 and AC15/00058] under the frame of the ERACoSysMed JTC-1 Call. M.P.-Y. was funded by the Ramón y Cajal Program (RYC-2015-17205) by the Spanish Ministry of Science and Innovation (MICINN), the State Research Agency, and the European Re-gional Development Fund from the European Union (MICINN/AEI/FEDER, UE, grant SAF2017-83417R). J.P.-G. was supported by a Ph.D. fellowship (FPU19/02175) granted by MICINN. U.P. and M.G. were funded by Slovenian Research Agency (research core funding No. P3-0067). M.I.A.-A. was funded by the Egyptian Government Ph.D. Scholarships. The STOPPA study was funded by the Swedish Research Council project grant 2018-02640 and the Swedish Asthma and Allergy Research Foundation.

**Input Spain:**

Human samples and related data used in this study were provided by the Basque Biobank/Biodonostia Node www.biobancovasco.org and were processed following standard operating procedures with appropriate approval of the relevant Ethics Committee and Scientific Advisory Board.