Metabo-Endotypes of Asthma Reveal Clinically Important Differences in Lung Function: Discovery and validation in two TOPMed Cohorts

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Abstract

Current guidelines do not sufficiently capture the heterogeneous nature of asthma; a detailed molecular classification is needed. Metabolomics represents a novel and compelling approach to derive asthma endotypes, *i.e.,* subtypes defined by functional/pathobiological mechanisms. In two cohorts of asthmatics, untargeted metabolomic profiling and Similarity Network Fusion was used to derive and validate five "metabo-endotypes" of asthma, which displayed significant differences in asthma-relevant phenotypes including pre-bronchodilator and post-bronchodilator forced expiratory volume/forced vital capacity (FEV_1/FVC). The "most-severe" asthma metabo-endotype was defined by the lowest FEV_1/FVC and characterized by altered levels of phospholipids and polyunsaturated fatty acids, suggesting dysregulation of pulmonary surfactant homeostasis. This was supported by genetic analyses as members of this endotype were more likely to carry variants in key pulmonary surfactant regulation genes including *BMPR1B* (meta-analyzed $p=2.8x10^{-4}$) and *BMP3* (meta-analyzed $p=5.23x10^{-4}$). These findings suggest clinically meaningful endotypes can be derived and validated using metabolomic data. Interrogating the drivers of these metabo-endotypes can help understand their pathophysiology.

Introduction

Asthma affects 26 million children and adults in the U.S. and remains a leading cause of morbidity [1,2]. Asthma is characterized by variable reversible airflow obstruction, nonspecific airway hyperresponsiveness and airway inflammation; however, there is substantial heterogeneity in its etiology, pathology and manifestation[3]. Current guidelines for defining asthma, which categorize cases from mild to severe, do not sufficiently capture this heterogeneity, leading to suboptimal management strategies in certain subgroups [4]. A more detailed molecular classification of asthma is needed.

It is hypothesized there are multiple asthma endotypes, (*i.e.*, subtypes defined by their functional or pathobiological mechanisms) that confer clinically meaningful differences in patient outcomes [5]. Treatments and management strategies based on these underlying pathobiological mechanisms, rather than a 'one-size-fits-all' approach, may be more effective in terms of improved outcomes and optimized use of health-care resources. The relative contribution of genetics and environment to the formation of these mechanistically driven endotypes is likely to vary between endotypes. Metabolomics reflects genetics, environmental factors, and their interactions [6], and as the 'ome closest to phenotype provides real-time insight into the physiological state of an individual. As such it represents a novel and compelling approach to identifying asthma endotypes with the potential for biological insight and clinical translation.

Interrogating high-dimensional omic datasets to infer biological meaning can be challenging. Clustering methods have proven to be powerful in the identification of molecular subtypes of asthma that differ by atopic status, eosinophil count, and cytokine levels [5,7-11] but to date, none have taken an untargeted approach leveraging the global metabolome. In this study, we aim to derive and validate clinically meaningful "metabo-endotypes" of asthma.

Methods

The study schematic is described in **eFigure1.**

Study Populations

The study populations have previously been described. The Genetics of Asthma in Costa Rica Study (GACRS) [12] recruited 1,165 children aged 6–14 years with asthma (physician's diagnosis and ≥ 2 respiratory symptoms or asthma attacks in the prior year). At enrollment, all children completed a protocol including questionnaires, blood collection, and spirometry conducted with a Survey Tach Spirometer (Warren E. Collins; Braintree, MA) in accordance with American Thoracic Society recommendations [12]. Written parental and participating child consent/assent was obtained. The study was approved by the Mass General Brigham Human Research Committee at Brigham and Women's Hospital (Boston, USA); Protocol#: 2000-P-001130/55, and the Hospital Nacional de Niños (San José, Costa Rica).

The Childhood Asthma Management Program (CAMP) [13] (Clinicaltrials.gov: NCT00000575) is a completed randomized clinical trial of inhaled treatments for mild-to-moderate asthma (symptoms for > 6 months in the year prior to interview and PC_{20} < 12.5mg/mL) in children aged 5–12 at baseline. All children completed a similar protocol to GACRS. The study was approved by the institutional review board of Mass General Brigham Healthcare (Protocol#: 1999-P-001549/29), by all participating clinical centers and the Data Coordinating Center. Child assent and parental written consent was obtained. Participants who had available plasma samples with sufficient volume from the end of the trial visit (4 years post-baseline) were selected for this current study. (**eMethods**)

Metabolomic Profiling

Metabolomic profiling was conducted using a combination of four complimentary liquid chromatography tandem mass spectrometry (LC-MS) platforms as part of the Trans Omic Precision Medicine (TOPMed) initiative[14]. Three nontargeted LC-MS methods using high resolution, accurate mass (HRAM) profiling measured: i) polar and nonpolar lipids (C8-pos); ii) free fatty acids, bile acids, and metabolites of intermediate polarity (C18-neg); and iii) polar metabolites including amino acids, acylcarnitines, and amines (HILIC-pos). An additional targeted LC-MS profiling method measured intermediary metabolites including TCA cycle intermediates, purines and pyrimidines, and acyl CoAs (Amide-neg) [15,16]. Metabolite exclusions and QC are described in **eMethods (eTable1)**.

Statistical Methods

Derivation of "metabo-endotypes" in GACRS

We grouped 1151 subjects from the GACRS based on their metabolite residuals (adjusting for age, sex, and BMI (and race in CAMP) to account for their potential influence on the metabolome[17]) into distinct metabolomic-driven endotypes, using Similarity Network Fusion *(SNF)* [R package: SNFtool version 2.2][18,19] and spectral clustering[20] (**eMethods**). We examined whether the omic-derived alterations in biological pathway between the clusters (endotypes), resulted in measurable clinical or epidemiological differences using one-way analysis of variance (ANOVA) for continuous variables and chi-squared tests for categorical variables. We determined that we had very good-excellent power to detect differences across the endotypes (**eTable2**).

Validation of endotypes

In the CAMP cohort, we utilized the label propagation classifier approach as a machine learning method to predict which metabo-endotype the new subjects belonged to [18]. We then assessed the clinical and phenotypic characteristics of CAMP subjects within these GACRS-defined endotypes.

Identification of metabolomic drivers of meta-endotypes

We utilized independent logistic regression models and a one-endotype-versus-the-rest approach to identify the metabolites that contributed the most to the formation of each endotype. We meta-analyzed the GACRS and CAMP results using a random effects model.

All analyses were conducted in R version 4.0.0.

Results

Study Population

In GACRS, 1151 subjects with asthma had plasma samples available for metabolomic profiling, and in CAMP, 911 subjects with asthma had suitable plasma samples extracted at the end of trial **(**Table 1**)**. In the original CAMP trial, no significant difference in lung function outcomes between the study arms was found [21].

Table 1
Characteristics of the Discovery and Validation Populations

	DICOVERY GACRS (n = 1151)	**VALIDATION CAMP (n = 911)**
Age [yrs]; Mean (SD)	9.22 (1.88)	12.94 (2.14)
Sex; Male (%)	682 (59.3%)	549 (60.3%)
Female (%)	469 (40.7%)	362 (39.7%)
Height [cm]; Mean (SD)	132.66 (11.85)	155.89 (13.35)
Weight [kg]; Mean (SD)	33.02 (11.49)	53.23 (17.32)
BMI [kg/m2]; Mean (SD)	18.28 (3.77)	21.42 (4.70)
Race; White (%)	-	630 (69.2%)
Black (%)	-	117 (12.8%)
Hispanic (%)[a]	1151 (100%)	84 (9.2%)
Other (%)	-	80 (8.8%)
Treatment Arm[b]; Budesonide n (%)	-	270 (29.6%)
Nedocromil n (%)	-	269 (29.5%)
Placebo n (%)	-	372 (40.8%)

[a] *GACRS represents a unique population isolate where participants were selected on the basis of having 6 or more great-grandparents born within the central valley of Costa Rica*

[b] *The CAMP population were from a completed clinical trial*

GACRS Metabo-Endotypes

A total of 2, 2, 2, and 3 clusters of GACRS asthmatics were identified based on metabolite residuals from the C8-pos, C18-neg, HILIC-pos and Amide-neg platforms respectively. We applied SNF to fuse the networks from the four platforms, with convergence after 10 iterations, and spectral clustering resulting in five clusters designated as the asthma metabo-endotypes containing 213, 270, 222, 232, and 214, asthma cases respectively (**eFigure2**). Based on the Adjusted Rand Index (ARI), the fused network clusters were most similar to those of the Amide-neg platform (ARI = 0.297) (**eTable3**).

There was no difference between the clusters in terms of sex, age, BMI, vitamin D level, or current smoking status ($p > 0.5$) (Table 2). However, there was a significant difference across the endotypes in measures of lung function: pre-bronchodilator FEV_1/FVC ratio ($p = 8.25x10^{-5}$) for which endotype2 had the lowest ratio (mean = 83.1%, range = 50.6%-98.8%) and endotype3 the highest (mean = 86.5%, range = 64.2%-99.9%) and post-bronchodilator FEV_1/FVC ratio ($p = 1.82x10^{-5}$). Again, endotype2 (mean = 85.9%, range = 52.2%-100%) was the lowest and endotype3 (mean = 89.1%, range = 69.0%-100%) the highest. The same pattern was observed when considering percent predicted FEV_1/FVC ratio (pre-bronchodilator $p = 4.46x10^{-5}$, post-bronchodilator $p = 1.00x10^{-5}$) **(**Fig. 1**)**. There was also a significant difference in percent predicted FVC across the endotypes **(eFigure3).** The endotypes differed in the use of oral ($p = 0.007$) and inhaled ($p = 4.97x10^{-13}$) corticosteroids and the use of beta2-agonists ($p = 3.25x10^{-10}$). Asthma cases in endotype2 were the most likely to have taken oral steroids (57.8%) or beta2-agonists (30.4%) in the previous year, but the least likely to have taken inhaled steroids (33.7%). Endotype3 had the lowest number who reported use the use of beta2-agonists in the previous year (**eFigure4**). Consequently, endotype2 was designated the “most-severe” asthma endotype, and endotype3 the “least-severe”.

Table 2
Differences in characteristics between the five GACRS asthma metabo-endotypes

GACRS Variable		Endotype1		Endotype2		Endotype3		Endotype4		Endotype5		*p-value*
		n = 213		n = 270		n = 222		n = 232		n = 214		
Demographic Characteristics	Sex [Male, n (%)]	125	(58.7%)	160	(59.3%)	136	(61.3%)	133	(57.3%)	128	(59.8%)	*0.941*
	Age [mean (SD)]	9.21	(1.82)	9.23	(1.83)	9.27	(1.84)	9.14	(2.02)	9.28	(1.87)	*0.932*
	BMI [mean (SD)]	18.3	(3.84)	18.4	(3.66)	18.4	(3.83)	18.3	(3.81)	17.9	(3.73)	*0.577*
	Serum Vitamin D (ng/ml) [mean (%)]	37.4	(11.6)	37.6	(10.8)	35.2	(9.0)	37.5	(12.6)	38.0	(14.3)	*0.870*
	Current smoking exposure [Yes, n (%)]	53	(24.9%)	68	(25.2%)	53	(23.9%)	58	(25.0%)	52	(24.3%)	*0.995*
Lung Function	Pre-bronchodilator FEV1 [mean (SD)]	1.76	(0.48)	1.78	(0.46)	1.78	(0.48)	1.79	(0.56)	1.77	(0.53)	*0.980*
	Pre-bronchodilator FVC [mean (SD)]	2.09	(0.57)	2.16	(0.56)	2.08	(0.58)	2.13	(0.64)	2.11	(0.63)	*0.588*
	Pre-bronchodilator FEV1 / FVC [mean (SD)]	84.34	(8.60)	83.14	(6.64)	86.16	(7.03)	83.81	(8.52)	84.43	(8.36)	*8.25E-05**
	Post-bronchodilator FEV1 [mean (SD)]	1.85	(0.52)	1.87	(0.48)	1.86	(0.48)	1.88	(0.57)	1.85	(0.53)	*0.942*
	Post-bronchodilator FVC [mean (SD)]	2.13	(0.59)	2.19	(0.56)	2.1	(0.56)	2.17	(0.64)	2.14	(0.63)	*0.595*
	Post-bronchodilator FEV1 / FVC ([mean (SD)]	87.10	(7.55)	85.87	(6.06)	89.10	(5.82)	87.15	(7.22)	86.97	(7.45)	*1.82E-05**
	% predicted Pre-bronchodilator FEV1 [mean (SD)]	97.40	(16.60)	100.00	(16.40)	98.40	(16.90)	99.00	(18.30)	99.00	(17.60)	*0.453*
	% predicted Pre-bronchodilator FVC [mean (SD)]	103.00	(16.40)	108.00	(15.50)	101.00	(16.20)	105.00	(16.20)	105.00	(17.80)	*2.11E-4**
	% predicted Pre-bronchodilator FEV1 / FVC [mean (SD)]	94.80	(9.55)	93.00	(7.50)	96.90	(7.78)	94.20	(9.52)	95.00	(9.22)	*4.46E-05**
	% predicted Post-bronchodilator FEV1 [mean (SD)]	102.00	(16.10)	105.00	(15.20)	103.00	(16.20)	104.00	(16.80)	103.00	(16.20)	*0.192*
	% predicted Post-bronchodilator FVC [mean (SD)]	104.00	(15.80)	109.00	(15.10)	103.00	(15.60)	106.00	(16.00)	106.00	(17.20)	*9.95E-05**

GACRS Variable		Endotype1		Endotype2		Endotype3		Endotype4		Endotype5		*p-value*
		n = 213		n = 270		n = 222		n = 232		n = 214		
	% predicted Post-bronchodilator FEV1 / FVC [mean (SD)]	98.00	(8.46)	96.60	(6.81)	100.00	(6.37)	97.90	(8.04)	97.80	(8.24)	*1.00E-05**
Indices of Asthma Severity	Use of oral steroids in previous year [Yes, n (%)]	96	(45.1%)	156	(57.8%)	118	(53.2%)	126	(54.3%)	93	(43.5%)	*0.007**
	Use of inhaled steroids in previous year [Yes, n (%)]	138	(64.8%)	91	(33.7%)	139	(62.6%)	123	(53.0%)	97	(45.3%)	*4.97E-13**
	Use of short acting beta2-agonists in previous year [Yes, n (%)]	25	(11.7%)	82	(30.4%)	17	(7.7%)	45	(19.4%)	47	(22.0%)	*3.25E-10**
	Any asthma medication in previous year [Yes, n (%)]	209	(98.1%)	256	(94.8%)	217	(97.7%)	229	(98.7%)	204	(95.3%)	*0.046**
	Ever Hospitalized for asthma [Yes, n (%)]	80	(37.6%)	117	(43.3%)	95	(42.8%)	106	(45.7%)	90	(42.1%)	*0.522*
	Ever visited ER for asthma [Yes, n (%)]	208	(97.7%)	259	(95.9%)	214	(96.4%)	224	(96.6%)	206	(96.3%)	*0.886*
Allergic Phenotypes	Log10 blood eosinophils [mean (SD)]	2.56	(0.41)	2.67	(0.35)	2.55	(0.46)	2.58	(0.41)	2.60	(0.43)	*0.009**
	Eosinophilic asthma (count > 300) [Yes, n (%)]	127	(59.6%)	202	(74.8%)	142	(64.0%)	136	(58.6%)	143	(66.8%)	*0.006*
	Log10 IgE [mean (SD)]	2.52	(0.70)	2.48	(0.69)	2.52	(0.65)	2.45	(0.69)	2.57	(0.61)	*0.344*
	Number of positive skin prick tests [mean (SD)]	3.12	(1.77)	3.02	(1.91)	2.94	(1.89)	3.14	(1.83)	3.02	(1.77)	*0.784*
	Prevalent Hay Fever [Yes, n (%)]	79	(37.1%)	74	(27.4%)	80	(36.0%)	66	(28.4%)	67	(31.3%)	*0.076*
	Prevalent Atopic Dermatitis [Yes, n (%)]	10	(4.7%)	12	(4.4%)	7	(3.2%)	8	(3.4%)	17	(7.9%)	*0.142*

Mean and standard errors for the specified metric in each endotype are shown

There was also evidence of a significant difference in allergic phenotypes across the endotypes. Endotype2 has the highest levels of blood eosinophils (log10 eosinophil count = 2.67, range: 1.0 to 3.41) and the highest percentage of individuals with eosinophilic asthma (74.8%) defined as > 300 cells/μL [22] (**eFigure5**).

Validating Metabo-endotypes in CAMP

The recapitulated endotypes contained 99, 375, 45, 207 and 185 CAMP asthma cases. The significant difference across endotypes for FEV_1/FVC ratio pre- and post- bronchodilator validated in CAMP with an almost identical pattern (Table 3 **and** Fig. 1). Given that prior to sample collection CAMP subjects had been randomized to differing treatment regimens, we could not directly compare medication use and no significant differences were observed (**eFigure6**).

Table 3
Differences in characteristics between the five CAMP asthma metabo-endotypes

CAMP Variable		Endotype1		Endotype2		Endotype3		Endotype4		Endotype5		p-value
		n = 99		n = 375		n = 45		n = 207		n = 185		
Demographic Characteristics	Sex [Male, n (%)]	58	(58.6%)	229	(61.1%)	32	(71.1%)	125	(60.4%)	105	(56.8%)	0.496
	Age [mean (SD)]	13.06	(2.38)	12.91	(2.10)	12.32	(1.61)	12.95	(2.19)	13.08	(2.12)	0.288
	BMI [mean (SD)]	21.18	(4.60)	21.43	(4.66)	20.74	(4.93)	21.31	(4.19)	21.84	(5.31)	0.598
	Serum Vitamin D (ng/ml) [mean (%)]	33.94	(14.61)	31.55	(14.15)	30.49	(13.59)	28.3	(14.31)	26.75	(11.86)	4.83E-05*
	Current smoking exposure [Yes, n (%)]	9	(9.1%)	55	(14.7%)	6	(13.3%)	26	(12.6%)	32	(17.3%)	0.399
	Race [White, n (%)]	72	(72.7%)	262	(69.9%)	32	(71.1%)	140	(67.6%)	124	(67.0%)	0.644
Lung Phenotypes	Pre-bronchodilator FEV1 [mean (SD)]	2.55	(0.70)	2.54	(0.74)	2.51	(0.65)	2.62	(0.80)	2.54	(0.71)	0.771
	Pre-bronchodilator FVC [mean (SD)]	3.29	(0.99)	3.29	(0.94)	3.08	(0.85)	3.36	(1.05)	3.30	(0.91)	0.518
	Pre-bronchodilator FEV1 / FVC [mean (SD)]	78.31	(7.39)	77.56	(8.89)	82.38	(8.51)	78.47	(9.03)	77.21	(9.46)	0.008*
	Post-bronchodilator FEV1 [mean (SD)]	2.78	(0.78)	2.76	(0.77)	2.68	(0.67)	2.85	(0.85)	2.79	(0.75)	0.614
	Post-bronchodilator FVC [mean (SD)]	3.32	(1.00)	3.34	(0.94)	3.12	(0.83)	3.4	(1.07)	3.36	(0.92)	0.506
	Post-bronchodilator FEV1 / FVC ([mean (SD)]	84.36	(6.11)	83.07	(7.42)	86.56	(6.83)	84.51	(7.38)	83.42	(7.05)	0.009*
	% predicted Pre-bronchodilator FEV1 [mean (SD)]	94.4	(13.6)	93.7	(14.3)	96.0	(12.2)	94.6	(13.4)	94.4	(14.3)	0.831
	% predicted Pre-bronchodilator FVC [mean (SD)]	106.0	(13.8)	105.0	(12.5)	102.0	(11.5)	105.0	(12.1)	107.0	(12.1)	0.264
	% predicted Pre-bronchodilator FEV1 / FVC [mean (SD)]	89.6	(8.38)	89.1	(10.2)	94.6	(9.34)	90.0	(10.4)	88.8	(10.8)	0.011*
	% predicted Post-bronchodilator FEV1 [mean (SD)]	103.0	(12.6)	102.0	(12.6)	102.0	(11.2)	103.0	(11.9)	103.0	(12.7)	0.640
	% predicted Post-bronchodilator FVC [mean (SD)]	107.0	(14)	107.0	(12.1)	103.0	(10.9)	107.0	(12.2)	108.0	(12.3)	0.210
	% predicted Post-bronchodilator FEV1 / FVC [mean (SD)]	96.6	(6.79)	95.4	(8.43)	99.4	(7.03)	96.9	(8.59)	95.8	(8.05)	0.016*
Indices of Asthma Severity	Use of prednisone since last visit [Yes, n (%)]	21	(21.2%)	68	(18.1%)	8	(17.8%)	34	(16.4%)	29	(15.7%)	0.787
	Use of albuterol since last visit [Yes, n (%)]	80	(80.8%)	313	(83.5%)	33	(73.3%)	170	(82.1%)	151	(81.6%)	0.530
	Ever hospitalized for asthma [Yes, n (%)]	10	(10.1%)	36	(9.6%)	2	(4.4%)	25	(12.1%)	19	(10.3%)	0.659
	Ever visited ER for asthma [Yes, n (%)]	33	(33.3%)	129	(34.4%)	10	(22.2%)	77	(37.2%)	54	(29.2%)	0.238

CAMP Variable		Endotype1		Endotype2		Endotype3		Endotype4		Endotype5		p-value
		n = 99		n = 375		n = 45		n = 207		n = 185		
Allergic Phenotypes	Log10 blood eosinophils [mean (SD)]	2.47	(0.40)	2.43	(0.48)	2.45	(0.36)	2.40	(0.48)	2.31	(0.58)	0.050*
	Eosinophilic asthma (count > 300) [Yes, n (%)]	54	(54.5%)	196	(52.3%)	24	(53.3%)	104	(50.2%)	79	(42.7%)	0.343
	Log10 IgE [mean (SD)]	2.77	(0.62)	2.64	(0.62)	2.50	(0.70)	2.64	(0.68)	2.56	(0.69)	0.086
	Number of positive skin prick tests [mean (SD)]	6.09	(3.69)	6.14	(4.42)	4.70	(3.53)	6.20	(4.37)	5.99	(4.25)	0.312
	Prevalent Hay Fever [Yes, n (%)]	47	(47.5%)	165	(44.0%)	29	(64.4%)	88	(42.5%)	93	(50.3%)	0.061
	Prevalent Atopic Dermatitis [Yes, n (%)]	23	(23.2%)	98	(26.1%)	15	(33.3%)	59	(28.5%)	60	(32.4%)	0.371

The differences in log10 eosinophil count and hay fever prevalence seen in GACRS were borderline significant across the endotypes in CAMP (p = 0.050 and p = 0.061, respectively) although the patterns differed (**eFigure5**). Additionally, there was evidence that vitamin D levels differed significantly across the endotypes in CAMP ($4.83x10^{-5}$).

Key metabolite endotype drivers

In a meta-analysis of GACRS and CAMP, 147, 256, 161, 332 and 269 metabolites were significantly associated with membership of endotypes1, 2, 3, 4, 5 respectively after Bonferroni correction and restriction to those metabolites with concordant directions of effect (**eTables4-8, eFigure7**). There was some crossover in the metabolites associated with each endotype (**eTable9, eFigure8**), although the direction of effect often differed. For example, 9,10-diHOME, which has been shown to correlate with lung function[23], was at higher levels in the "most-severe", endotype2 (ß=0.595, $p = 1.78x10^{-28}$) relative to all other endotypes, but lower in the "least-severe" endotype3 (ß=-0.4000, $p = 2.23x10^{-15}$). Similarly, linoleic acid (ß=0.595, $p = 1.78x10^{-28}$) and arachidonic acid (ß=-0.564, $p = 1.04x10^{-5}$), which are also thought to play key roles in lung function [23,24], were lower in endotype2 relative to all other endotypes, but higher in the less serve endotype1. We also identified a number of metabolites unique to each endotype (**eTable10**). However, in general lipid, and in particular phospholipid, levels were among the greatest drivers of membership. Endotype2 was characterized by increased levels of lysophospholipids, phosphatidylcholines (PC), phosphatidylethanolamines (PE), bile acid metabolites, sphingomyelins, triacylglycerols and decreased levels of PUFAs (Fig. 2).

Pathways/classes are named if ≥ 5 metabolites belonging to that class are associated with membership in a given direction (or if they are the most abundant pathway/class for the endotype in a given direction)

Short Chain Carnitines - carbon chain length ≤ 8; Medium Chain Carnitines – carbon chain length 9 to ≤ 16; Long Chain Carnitine – carbon chain length > 16

Genetic drivers of the "most-severe" endotype

Finally, we sought to identify genetic variants that may be associated with membership of the "most-severe" low PUFA, high phospholipid endotype (Endotype2) compared to all other endotypes based on available whole genome sequencing data [14,25]. We explored 1,115,764 SNPs after employing a minor allele frequency < 0.05 and $r^2 > 0.2$. Using an additive genetic model adjusting for the first four principal components, age and sex, and meta-analyzing the results across GACRS and CAMP, no SNPs were significant in the meta-analysis at a Bonferroni threshold of $4.48x10^{-8}$. Therefore, we employed a nominal p-value threshold of 0.01 to account for five endotypes and filtered on a concordant direction of effect and a nominal p-value < 0.05 in both cohorts, resulting in 1382 SNPs associated with membership (**eFigure9**). SNPs were annotated to genes using the biomaRt package [26] and gene set enrichment analysis was conducted using gProfileR [27]. This identified an enrichment of "anatomical structure morphogenesis" ($p = 1.8x10^{-5}$) and other processes that may be involved in lung development, as well as the microRNA hsa-miR-4517, which has been shown to be altered between asthmatic and normal bronchial epithelial cells [28]. Among the top SNPs associated with membership **(eTable11)** were those mapping to genes associated with pulmonary function and disease including rs7751017 in *SMOC2* ($p = 6.31x10^{-5}$) and rs11099459 in *BMP3*, $p = 5.23x10^{-4}$); the regulation of pulmonary surfactant homeostasis, rs2120834 in *BMPR1B* ($p = 2.8x10^{-4}$), biosynthesis of glycoproteins including, rs7125946 in *GALNT18* ($p = 1.01x10^{-4}$), rs1648282 in *DUOX1* ($p = 2.12x10^{-4}$) and the immune inflammatory response including rs1294053 in *SPSB1* ($2.57x10^{-4}$) (**eTable12)**.

Discussion

In this study, we identified five asthma metabo-endotypes with differing lung function and clinical characteristics driven by distinct metabolomic pathways. We further determined a potential genetic component to the most severe endotype. Crucially, we were able to validate these findings in an independent cohort of childhood asthmatics.

Although several studies have attempted to identify asthma endotypes [29,30], these have been somewhat limited and have tended to use one of two general approaches: *a priori* definitions of a phenotype based on characteristics of subjects; or pathobiologic differences in sputum or bronchoscopy specimens [5,31–34]. The resulting endotypes have often demonstrated high overlap in important clinical features rendering them challenging for clinical use. More importantly, they provide little information on underlying mechanisms. Among the few studies utilizing omic data to derive clusters [29,30,35–37] sample sizes have been small; however, results support the existence of multiple heterogeneous asthma subtypes with differing molecular profiles and pathophysiological pathways. Although several studies have incorporated metabolomics into their exploration of endotypes [7,9,10,38,39], to date none have leveraged unsupervised clustering of the global blood metabolome to sub-phenotype asthmatics.

There were significant differences in asthma-relevant phenotypic characteristics across our endotypes, specifically with regard to lung function. Based on these metrics, endotype2 was characterized as the "most-severe", with individuals in this group demonstrating the greatest degree of airflow obstruction and reporting the highest usage of oral corticosteroids and beta2-agonists. Endotype3 was designated the "least-severe" endotype using these same metrics. We recapitulated these endotypes in an independent population based on their metabolome and observed almost identical differences in lung function across the endotypes. Although differences in medication usage were not validated, we hypothesize that this is because CAMP blood was collected at the end of a clinical trial, which would have dictated use of steroids in the previous year, as well as differences in the prescribing and therapeutic approaches applied by the respective health systems of the Costa Rican-based discovery cohort, and USA-based validation cohort. In contrast, FEV_1/FVC, which replicated between the two populations, provides a more objective measure.

We further observed differences in allergic characteristics between the discovery endotypes. In GACRS, the "most-severe" endotype displayed the highest blood eosinophil counts and proportion of eosinophilic asthmatics. This was not replicated in CAMP which may be explained by the underlying differences in immune phenotypes between the two populations, with CAMP displaying significantly higher log(IgE) levels (mean [SD] 2.63 [0.65] versus 2.50 [0.67] in GACRS, $p = 1.57x10^{-5}$)), prevalence of hay fever (53.2% versus 31.8%, $p < 2.2x10^{-16}$) and eczema (28.0% versus 4.7%, $p < 2.2x10^{-16}$), and mean number of total skin prick tests (6.05 [2.38]) versus 3.05 [1.84], $p < 2.2x10^{-16}$).

The "most-severe" endotype2 was characterized by increased levels phosphocholines, bile acid metabolites and sphingomyelins and decreased levels of both n3 and n6 long chain PUFAs. While individuals in endotype1 who showed better metrics of lung function demonstrated higher levels of the same PUFAs. This may be explained by the fact that PUFAs have been shown to play a role in pulmonary function and disease through their role in maintenance of the pro-inflammatory – pro-resolvin pathways[24,40].

Intriguingly, PUFAs have also been demonstrated to be important in the homeostasis of pulmonary surfactant [41], which lines the inner surface of the lung and works to lower surface tension and prevent alveolar collapse as well as playing a role in innate immune defense[42]. Consequently, dysregulation of surfactant homeostasis has been implicated in pulmonary diseases and reduced lung function in both adults and children. Pulmonary surfactant has multiple integrated and highly regulated lipid metabolite components including phospholipids (phosphatidylcholines, phosphatidylglycerols, phosphatidylethanolamines), triglycerides, cholesterols, fatty acids and sphingomyelins[43], which were found to be among the greatest drivers of endotype membership and therefore lung function. A large number were associated with membership of the "most-severe endotype". Endotype4 which was associated with lower levels of many of these same metabolites also displayed less severe phenotypes. This leads to the hypothesis that differences in pulmonary surfactant homeostasis reflected in the blood may underlie the severity metrics observed in this endotype, which is further supported by the genetic analysis. Several SNPs mapping to genes involved in the regulation of pulmonary surfactant homeostasis were associated with membership, including *BMPR1B* and *BMP3*, members of the bone morphogenic protein family that signal through transmembrane serine-threonine kinase receptors to influence lung morphogenesis and support neonatal respiratory function via enhanced expression of surfactant glycoproteins [44,45]. These pulmonary surfactant glycoproteins play critical roles in the innate immune response and anti-inflammatory effects [46], and among genes mapping to the significant SNPs for the "most-severe" endotype were also a number involved in the biosynthesis of glycoproteins including *GALNT18* and *DUOX1*. Several SNPs also mapped to genes involved in the immune inflammatory response such as *SPSB1*, which may explain the high eosinophil count in this endotype.

A potential difference in blood eosinophil count between asthma endotypes is in agreement with existing sub-phenotyping studies of asthmatics. Clustering of 9 clinical variables identified and validated in the ADEPT and U-BIOPRED cohorts identified four groups with distinct clinical and biomarker profiles, one of which had a "moderate, hyper-responsive, eosinophilic" phenotype, with moderate asthma control, mild airflow obstruction and predominant Type-2 inflammation [47]. Similarly, three separate studies of exhaled breath samples showed that models based on volatile organic compounds (VOCs) could classify asthma as eosinophilic or neutrophilic with high accuracy [9,38,48]. Eosinophil count also forms a component of the commonly cited T-helper type 2 (Th2)-high and Th2-low endotypes [49]. However, such subgroups have not yet demonstrated clear clinical utility [29]. More promisingly, endotypes based on gene expression profiles in participants from U-BIOPRED were shown to differ in their responses to oral corticosteroids [37], while another study on exhaled breath demonstrated that levels of VOCs could help to predict steroid responsiveness [39]. An important facet of omic-driven endotypes is their ability to inform therapeutic and management approaches. Results in our discovery cohort suggested differences in medication usage between the metabo-driven endotypes, but validation was complicated by differences between the cohorts, and further investigation is required.

There were a number of limitations to these analyses. All participants were under 18 years of age at blood collection. Additional work in older populations is required to determine the generalizability of the findings to adult-onset asthma, although, we note that early-onset asthma may represent the larger public health burden due to its higher prevalence [50]. Cluster analysis is a descriptive method, and groups can be defined even when there is no underlying structure in the data; however, we addressed this by assessing the clinical characteristics of the clusters in two different populations. It should be noted these clusters are based on a single timepoint, and repeated sampling is necessary to assess their temporal stability, although encouragingly, previous clustering studies in asthmatic populations have demonstrated good longitudinal cluster stability [47]. Endotypes were derived via metabolomic profiling of blood. The utility of blood for asthma studies is supported by the literature [51] and has the benefits of being readily accessible, vital for the development of clinically translatable biomarkers. However, future studies should address the replicability of these endotypes in different biosamples, particularly those closest to the lung such as sputum.

There are also several strengths to this study. It employs a unique design, utilizing a bottom-up approach from molecular signatures to clinical endotypes, unlike the majority of studies that have clustered based on phenotype, potentially missing mechanistic information. The patient similarity networks were generated using state-of-the-art profiling platforms, providing broad coverage and highly reproducible data. We leveraged machine learning approaches to derive endotypes and most crucially we were able to validate our findings in an independent population with comparable metabolomic, phenotypic and clinical data, despite underlying differences between the cohorts.

Conclusion

In conclusion, asthma represents a spectrum of disorders with heterogenous etiologies and clinical presentations, yet its clinical definition has remained unchanged for more than 50 years [29]. A significant proportion of asthmatics do not respond to the “one-size-fits-all” management approach, and it is these patients who are responsible for the majority of asthma related health care costs and economic burden [52]. This study, which is by far the largest to leverage metabolomics for asthma endotyping and the first to use an unsupervised metabolome-wide clustering approach, proposes five novel validated asthma

metabo-endotypes. These endotypes provide strong candidates for more precise asthma management strategies while informing on underlying mechanisms, paving the way for more personalized approaches to asthma management and a new era of precision medicine.

Declarations

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Author Contributions

Conceptualization, R.S.K., J.L-S., S.T.W.; methodology, R.S.K., K.M., B.D.H., S. C. H; validation, M.H.; formal analysis, R.S.K., K.M.; investigation, R.S.K., J.L-S.; resources, R.S.K, J.L-S., S.T.W., C.C., R.G., M.H.C., J.C.C.; data curation, R.S.K, K.M., M.H.; writing—original draft preparation, R.S.K., J.L-S.; writing—review and editing, K.M., M.H., B.D.H., C.C., R.G., M.H.C., C.E.W., P.K., M.J.M., S.H. C, J.C.C., S.T.W.; supervision, J.L-S., S.T.W., M.H.C., C.E.W., P.K.; funding acquisition, R.S.K, J.L-S., S.T.W., J.C.C.

Competing Interests

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Data Availability

RSK has full access to all the data in the study and takes responsibility for the integrity of the data analysis. The metabolomic data were generated as part of the NHLBI Trans-Omics for Precision Medicine Initiative (TOPMed). These data will be released to the scientific community in their entirety via NIH-designated repositories according to the TOPMed data release timeline. Full details can be found at https://www.nhlbiwgs.org/topmed-data-access-scientific-community.

Code Availability

All statistical analyses were conducted using freely available packages in R version 4.0.0; all such packages are stated and referenced in the methods and supplementary methods.

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Figures

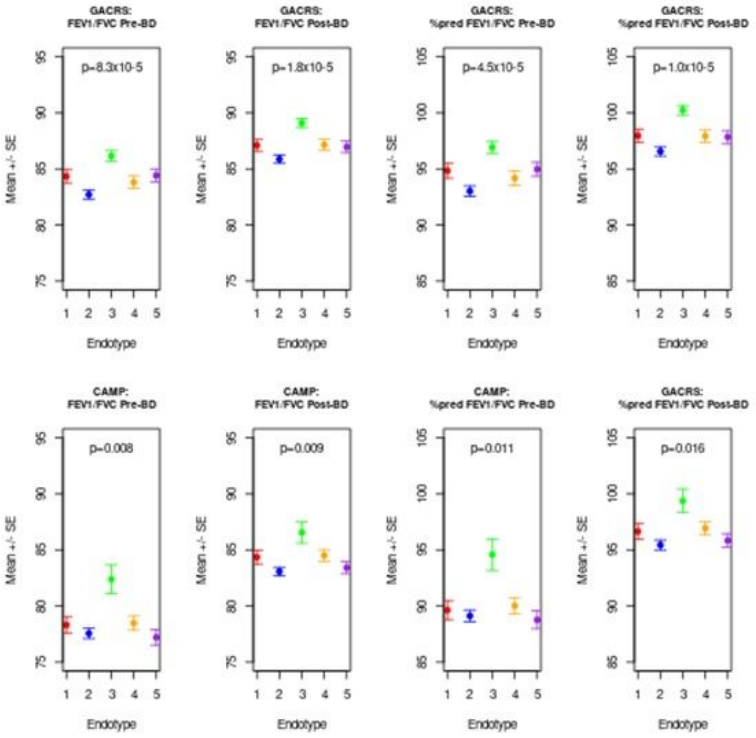

Figure 1

FEV1/FVC ratio Pre and Post-bronchodilator demonstrating Significant Difference Across Endotypes in Both the Genetic Epidemiology of Asthma in Costa Rica Study (GACRS) and The Childhood Asthma Management Program (CAMP). Mean and standard errors for the specified metric in each endotype are shown

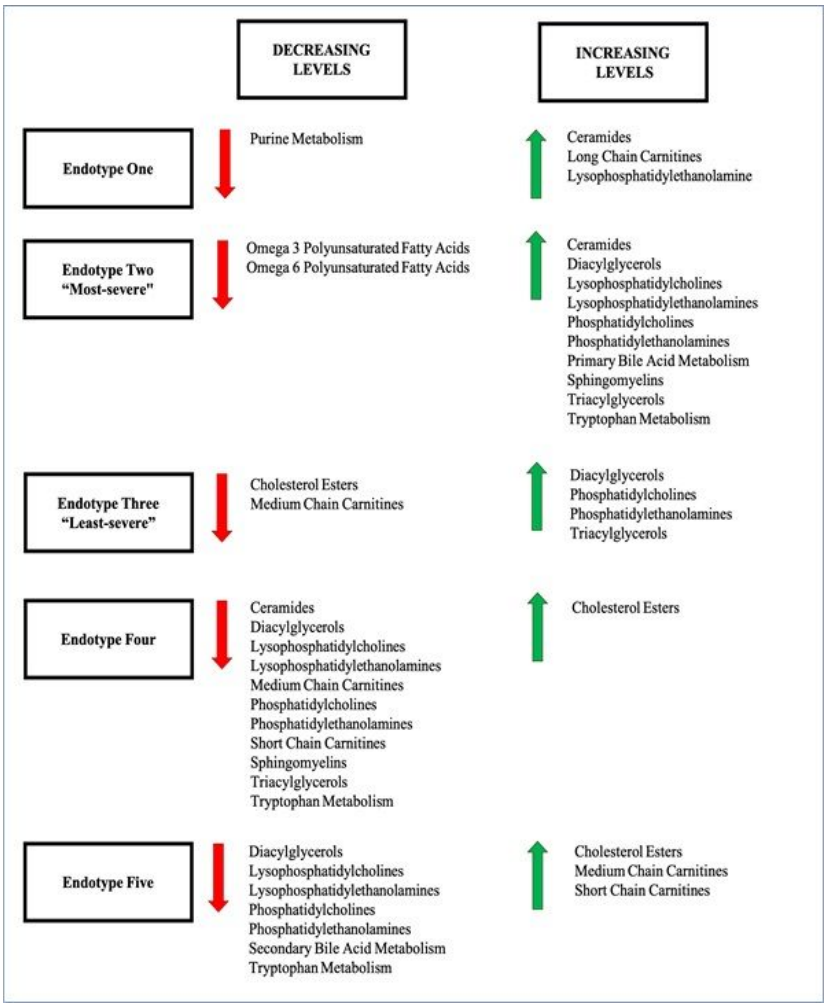

Figure 2

Pattern of Metabolomic Pathways/Classes driving Membership of each Endotype. Pathways/classes are named if ≥5 metabolites belonging to that class are associated with membership in a given direction (or if they are the most abundant pathway/class for the endotype in a given direction) Short Chain Carnitines - carbon chain length ≤ 8; Medium Chain Carnitines – carbon chain length 9 to ≤ 16; Long Chain Carnitine – carbon chain length >16

Supplementary Files

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