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Unconjugated bilirubin is associated with protection from early-life wheeze and childhood asthma

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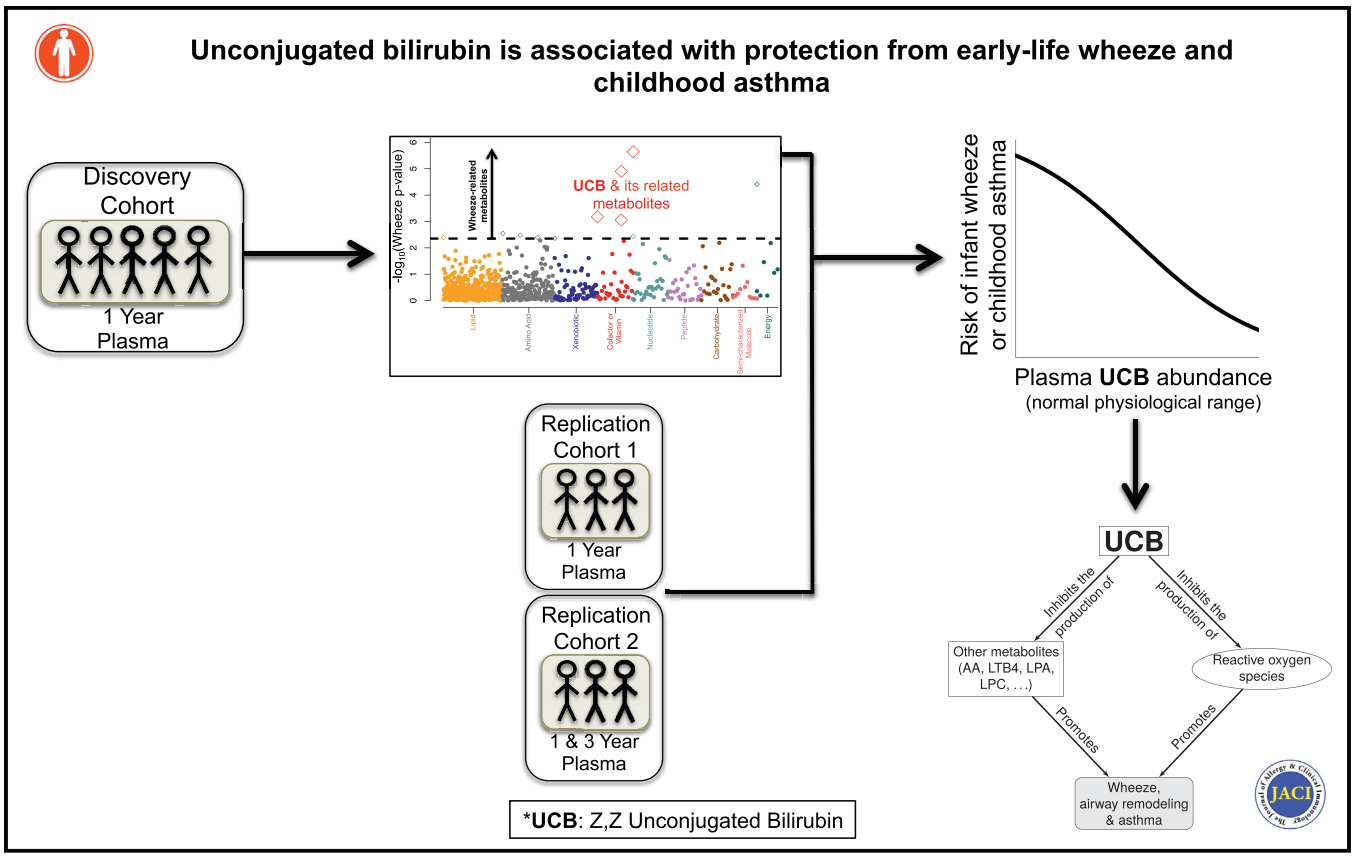
GRAPHICAL ABSTRACT



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Background: Wheeze and allergic sensitization are the strongest early-life predictors of childhood asthma development; the molecular origins of these early-life phenotypes are poorly understood.
Objectives: We sought to identify metabolites associated with early-life wheeze, allergic sensitization, and childhood asthma.
Methods: We conducted a nested case-control study using Environmental influences on Child Health Outcomes Program cohorts for discovery and independent replication. Wheeze and allergic sensitization were defined by number of wheeze episodes and positive specific IgE at age 1 year, respectively. Asthma was defined as physician diagnosis of asthma at age 5 or 6 years. We used untargeted metabolomics, controlling for observed and latent confounding factors, to assess associations between the plasma metabolome and early-life wheeze, allergy, and childhood asthma.
Results: Eighteen plasma metabolites were associated with first-year wheeze in the discovery cohort (n = 338). Z,Z unconjugated bilirubin (UCB) and its related metabolites exhibited a dose-response relationship with wheeze frequency; UCB levels were 13% (β = 0.87; 95% CI, 0.74-1.02) and 22% (β = 0.78; 95% CI, 0.68-0.91) lower in children with 1 to 3 and 4+ wheeze episodes compared with those who never wheezed, respectively. UCB levels were also associated with childhood asthma (β = 0.82; 95% CI, 0.68-0.98). Similar trends were observed in 2 independent cohorts. UCB was significantly negatively correlated with eicosanoid- and oxidative stress–related metabolites. There were no significant associations between metabolites and allergic sensitization.
Conclusions: We identified a novel inverse, dose-dependent association between UCB and recurrent wheeze and childhood asthma. Inflammatory lipid mediators and oxidative stress byproducts inversely correlated with UCB, suggesting that UCB modulates pathways critical to the development of early-life recurrent wheeze and childhood asthma. (J Allergy Clin Immunol 2021;■■■:■■■-■■■.)

Key words: *Wheeze, asthma, metabolomics, bilirubin, lipid mediators*

Abbreviations used
AA: Arachidonic acid
COAST: Childhood Origins of Asthma study
CREW: Children's Respiratory and Environmental Workgroup
FDR: False-discovery rate
INSPIRE: Infant Susceptibility to Pulmonary Infections and Asthma following RSV Exposure
LTB4: Leukotriene B4
sPLA2: Secretory phospholipase 2
UCB: Z,Z unconjugated bilirubin
UGT1A1: Uridine-diphosphoglucuronate glucuronosyltransferase 1A1
WISC: Wisconsin Infant Study Cohort

Asthma is among the most common chronic syndromes in children and results in substantial morbidity and annual health care expenditure.[1,2] The critical window for development of childhood asthma can be traced back to vulnerable fetal and early-life periods when the immune system and respiratory organs are still developing.[3] Because wheeze and allergic sensitization are the strongest early-life predictors of childhood asthma development,[4] understanding the molecular origins of these early-life and childhood phenotypes is critical for understanding asthma pathogenesis.

Metabolomics is the analysis of low-molecular-weight compounds present in biological fluids and tissues, representing the end products of cellular activity.[5] Variation in the levels of these metabolites reflects variation in genetic, epigenetic, microbiota, and environmental exposures, and can provide insights into the biochemical processes involved in the development of complex childhood diseases, such as allergy and asthma.[6] Although there is some evidence that the early-life metabolome is associated with lung function trajectories and the development of childhood asthma and allergy,[7-9] these studies had limited sample sizes, lacked replication, and are therefore not generalizable to other populations. In addition, previous studies have not accounted for unmeasured confounders, including unmeasured dietary and environmental exposures, that can induce spurious associations, erroneously bolster predictive performance, and bias estimates.[10]

To fill this gap in knowledge, we used untargeted metabolomics to comprehensively profile the plasma metabolomes of more than 600 infants from 3 independent cohorts to identify and replicate associations between infant metabolites and metabolic pathways, and the phenotypes of wheeze and allergic sensitization at the first year of life, and diagnosis of childhood asthma. We used an approach robust to confounding, replicated results in independent cohorts, and identified the potential mechanisms through which these metabolites may protect against or potentiate these phenotypes.

METHODS

Study population and design

This study was conducted using National Institute of Health Environmental influences on Child Health Outcomes Program–funded Children's Respiratory and Environmental Workgroup (CREW) consortium cohorts. CREW consists of birth cohorts designed to understand the development of allergic disease and asthma in children.[11] Three CREW cohorts had available biospecimens and were included in this study: Infant Susceptibility to Pulmonary Infections and Asthma following RSV Exposure (INSPIRE), Wisconsin Infant Study Cohort (WISC), and Childhood Origins of Asthma study (COAST). These 3 cohorts include a general population cohort (INSPIRE),[12] a rural cohort stratified by residence on a dairy farm (WISC),[13] and a high-risk cohort on the basis of parental history of asthma or allergies (COAST).[14]

The study was designed as a nested case-control study (see this article's Methods section in the Online Repository and Fig E1 in this article's Online Repository at www.jacionline.org). Cases were defined as those with wheeze or allergic sensitization during the first year of life, and controls were defined as those who never wheezed and were not sensitized to aeroallergens or food during the first year of life. Cases and controls were matched on sex and age in months at the time of sampling, with priority given to those who had both plasma samples available for testing.

Outcome ascertainment

The diagnosis of wheeze at ages 1, 2, and 3 years was determined using elements from the International Study of Asthma and Allergies in Childhood questionnaire.[15] Children who did not wheeze in the previous 12 months were considered nonwheezers. Those who wheezed were categorized as follows on the basis of standard International Study of Asthma and Allergies in Childhood categorical responses: 1 to 3 wheezing episodes in the previous 12

months, and 4+ wheezing episodes in the previous 12 months.[16] Allergic sensitization (yes/no) to common aeroallergens (cat, dog, mold mix, and house dust mite mix) and food allergens (egg, milk, peanut) was defined on the basis of positive specific IgE testing of plasma (>0.1 kUA/L) at age 1 year. Asthma was defined as physician-diagnosed or parent-reported physician diagnosis of asthma and/or physician-prescribed asthma medications including systemic steroids for asthma exacerbations, at age 5 years (discovery cohort most recent time point available) or 6 years (replication cohorts prespecified outcome time point).

Untargeted metabolomics

Plasma samples were collected at age 1 year in all 3 cohorts. Additional plasma samples were collected at age 3 years in the COAST cohort. Plasma samples from INSPIRE were collected in EDTA anticoagulant, whereas plasma samples from WISC and COAST were collected in heparin anticoagulant. In total, we used 4 plasma metabolome data sets for this study. Plasma metabolome data from the largest cohort at age 1 year, INSPIRE, were used as the discovery data set, and the 3 data sets from the WISC (at age 1 year) and COAST (at age 1 and 3 years) cohorts were used for independent replication. Untargeted metabolic profiling and initial data processing was performed by Metabolon, Inc, Research Traingle Park, NC. We performed quality control (see Figs E2 and E3 in this article's Online Repository at www.jacionline.org) and used the Metabolomics Standard Initiative to classify metabolites as annotated (level 1 or 2; identified or putatively annotated compounds), semi-annotated (level 3; putatively characterized compound classes), and nonannotated (level 4; unknown compounds).[17] We defined a metabolite's abundance to be its LC-MS–determined peak area, which is proportional to concentration (see Fig E4 in this article's Online Repository at www.jacionline.org).[18]

Identifying and replicating wheeze- and sensitization-associated metabolites

We determined the relationship between a phenotype (wheeze, sensitization, or asthma) and metabolite levels using the method described in McKennan et al.[10] In brief, we excluded metabolites with more than 50% missing MS data, estimated latent factors that might confound the relationship between the phenotype and metabolite levels, and used linear regression to regress each metabolite's log-abundance onto the wheeze, sensitization, or asthma phenotypes, while accounting for age in months, sex (male/female), daycare attendance (yes/no), breast-feeding status (exclusively breast-fed or not in the first 6 months), and the latent factors. This uses inverse probability weighting, with weights estimated using McKennan et al,[10] to account for nonignorable missing MS data. Q values were used to control the false-discovery rate (FDR) at 20%. We further truncated the list of metabolites by considering a metabolite for downstream analysis only if it was annotated, its abundance was consistent with a wheeze-dose response, and if the direction of the association replicated in WISC, the replication cohort with the largest sample size. The wheeze-dose response, which is satisfied by metabolites whose abundance is strictly increasing or decreasing in the ordered wheeze variable, helped strengthen the biological underpinning of our findings. The analyses performed in each replication data set were identical to that in the discovery data set.

Determining a metabolite's correlation neighbors

We estimated the Pearson correlation between the log-abundances of a selected metabolite and all other metabolites in the discovery data set conditional on the phenotype of interest (cases and controls of sensitization or wheeze outcomes as defined in Outcome Ascertainment section), age, sex, daycare attendance, diet, and other latent confounding factors.[10] The selected metabolite's correlation neighbors were defined using a stringent FDR threshold of 10% to mitigate confounding-induced spurious associations that can arise in metabolomic correlation networks.[19]

Cross-phenotype meta-analysis

We used the replication data to pool information across the phenotypes of wheeze and asthma to explicitly test for replication. In brief, we tested the null hypothesis that the Z,Z unconjugated bilirubin (UCB) levels were the same in healthy controls (nonwheezers in previous year, nonwheezers in subsequent years, or not diagnosed with asthma) and cases (those who wheezed in the previous year, wheezed in subsequent years, or were diagnosed with childhood asthma, respectively). The alternative hypothesis was that the directions of the differences in UCB levels between healthy controls and any of the other 3 groups matched those directions of differences observed in the discovery data set. The test statistic, which corrects for correlation between phenotypes and estimates derived from data sets with overlapping participants, was designed to be standard normal under the null hypothesis, and reflect the directions of the aforementioned differences under the alternative hypothesis. Additional details are provided in this article's Online Repository at www.jacionline.org.

Leukotriene B4 mediation analysis

Because leukotriene B4 (LTB4) was observed in only 14% of the samples in the discovery data set, we coded it as binary (present or absent) when assessing its role in mediating UCB's association with wheeze to circumvent statistical issues that arise when treating nonignorable missing MS data as continuous.[10] We assessed the effect of UCB on LTB4 levels and of LTB4 levels on wheeze risk using standard and ordinal logistic regression, respectively. Additional details are provided in this article's Online Repository at www.jacionline.org.

RESULTS

Population characteristics and metabolite identification

Characteristics of the INSPIRE population and the 2 replication cohorts (WISC and COAST) have been previously published.[12-14] Characteristics of the nested case-control sample used in this study are summarized in Table I, and a detailed description is provided in this article's Online Repository at www.jacionline.org. First-year wheeze was not associated with aeroallergen or food sensitization ($P = .50$ and .72) in the discovery data set, indicating insignificant overlap between the 2 phenotypes.

We identified 938 annotated and 265 nonannotated metabolites in the INSPIRE 1-year plasma discovery data set, and between 1039 and 1321 total metabolites in each of the 4 additional data sets. There was large (824 of 1512) overlap in metabolites identified from INSPIRE, WISC, and COAST (first- and third-year) plasma samples (see Table E1 and Fig E5 in this article's Online Repository at www.jacionline.org). The metabolites M-1 ($C_{17}H_{20}N_2O_5$), M-2 ($C_{17}H_{20}N_2O_5$), and M-3 ($C_{17}H_{18}N_2O_4$) were retrospectively semi-annotated (Metabolon, Inc) to be bilirubin degradation products. (See Table E2 and Fig E6 in this article's Online Repository at www.jacionline.org. Fig E6 was modified from Jasprova et al.[20])

Identifying plasma metabolites associated with allergic sensitization

No metabolites in the INSPIRE plasma data set were significantly correlated with aeroallergen sensitization at a 20% FDR. However, INSPIRE children with sensitization to at least 1 food in the first year of life had significantly increased plasma alpha-D-glucuronic acid than those without a food sensitization, and the direction of the association was replicated in the COAST 1-year plasma data set ($P < .05$; see Fig E7 in this article's Online Repository at www.jacionline.org). Correlation analysis showed that alpha-D-glucuronic acid had 3 correlation neighbors (see Table E3 in this article's Online Repository at www.jacionline.org).

TABLE I. Nested case-control study sample characteristics in discovery and replication cohorts

Characteristic	Discovery cohort (INSPIRE)	Replication cohort (WISC)	Replication cohort (COAST)	
	EDTA plasma (1 y)*	Heparin plasma (1 y)	Heparin plasma (1 y)	Heparin plasma (3 y)
Birth weight (g), median (IQR) {$P = .74$}	3433 (596)	3458 (621)	3402 (667)	3572 (702)
Gestational age (wk), median (IQR) {$P = .15$}	39 (1)	39 (2)	39 (3)	40 (2)
Maternal years of education (school), median (IQR)	14 (2)	See below	See below	See below
Maternal education level {$P = 1.4 \times 10^{-17}$}				
Did not graduate high school (HS)	31 (9)	0 (0)	0 (0)	3 (2)
Graduated HS, but no college	76 (22)	13 (9)	4 (6)	7 (6)
Attended college, but did not graduate	87 (26)	13 (9)	12 (18)	21 (17)
Graduated college	144 (43)	113 (78)	49 (73)	95 (75)
Missing	0 (0)	6 (4)	2 (3)	1 (1)
Sex {$P = .60$}				
Male	186 (55)	74 (51)	37 (55)	72 (57)
Female	152 (45)	71 (49)	30 (45)	55 (43)
Race {$P = 5.3 \times 10^{-14}$}				
White	228 (67)	137 (94)	63 (94)	115 (91)
Black	77 (23)	4 (3)	0 (0)	5 (4)
Other	14 (4)	4 (3)	1 (1)	1 (1)
Multirace	19 (6)	0 (0)	3 (4)	6 (5)
Maternal marital status {$P = 8.6 \times 10^{-11}$}				
Single	135 (40)	8 (6)	NA	NA
Married	196 (58)	128 (88)	NA	NA
Divorced/separated	7 (2)	4 (3)	NA	NA
Missing	0 (0)	5 (3)	NA	NA
Child exclusively breast-fed for the first 6 mo {$P = 9.8 \times 10^{-13}$}				
Yes	134 (40)	48 (33)	6 (9)	6 (5)
No	204 (60)	97 (67)	61 (81)	121 (95)
First-year daycare attendance {$P = 3.0 \times 10^{-4}$}				
Yes	122 (36)	65 (45)	36 (54)	69 (54)
No	216 (64)	80 (55)	31 (46)	58 (46)
Maternal smoking during pregnancy {$P = .0019$}				
Yes	46 (14)	6 (4)	2 (3)	9 (7)
No	292 (86)	134 (92)	17 (25)	23 (18)
Missing	0 (0)	5 (3)	48 (72)	95 (75)
Paternal asthma {$P = 6.0 \times 10^{-5}$}				
Yes	61 (19)	16 (12)	19 (29)	42 (34)
No	259 (81)	120 (88)	47 (71)	82 (66)
Maternal asthma {$P = 8.1 \times 10^{-7}$}				
Yes	75 (22)	32 (22)	21 (31)	59 (46)
No	263 (78)	107 (74)	45 (67)	67 (53)
Unknown	0 (0)	1 (1)	0 (0)	0 (0)
Missing	0 (0)	5 (3)	1 (1)	1 (1)
Maternal allergic rhinitis {$P = 8.8 \times 10^{-20}$}				
Yes	86 (25)	23 (16)	29 (43)	74 (58)
No	252 (75)	112 (77)	24 (36)	39 (27)
Unknown	0 (0)	4 (3)	7 (10)	13 (10)
Missing	0 (0)	6 (4)	7 (10)	6 (5)
Maternal eczema {$P = 4.9 \times 10^{-8}$}				
Yes	67 (20)	29 (20)	28 (42)	43 (34)
No	271 (80)	110 (76)	23 (34)	62 (49)
Missing	0 (0)	6 (4)	16 (24)	22 (17)
First-year wheeze {$P = .035$}				
Never wheeze	226 (67)	112 (77)	52 (78)	94 (74)
1-3 wheezing episodes	46 (14)	32 (22)	11 (16)	30 (24)
4+ wheezing episodes	66 (20)	1 (1)	4 (6)	3 (2)
Second-year wheeze {$P = .49$}				
Never wheeze	245 (72)	89 (61)	53 (79)	93 (73)
1-3 wheezing episodes	58 (17)	28 (19)	11 (16)	27 (21)
4+ wheezing episodes	35 (10)	1 (1)	3 (4)	7 (6)
Missing	0 (0)	27 (19)	0 (0)	0 (0)
Third-year wheeze {$P = .81$}				
Never wheeze	269 (80)	78 (54)	52 (78)	100 (79)

(Continued)

TABLE I. *(Continued)*

Characteristic	Discovery cohort (INSPIRE)	Replication cohort (WISC)	Replication cohort (COAST)	
	EDTA plasma (1 y)*	Heparin plasma (1 y)	Heparin plasma (1 y)	Heparin plasma (3 y)
1-3 wheezing episodes	48 (14)	15 (10)	11 (16)	18 (14)
4+ wheezing episodes	21 (6)	1 (1)	4 (6)	9 (7)
Missing	0 (0)	51 (35)	0 (0)	0 (0)
Asthma diagnosis {$P = .87$}				
Yes	72 (21)		34 (27)	16 (24)
No	201 (59)		85 (67)	47 (70)
Missing	65 (19)		8 (6)	4 (6)

Values are n (%) unless otherwise indicated. When appropriate, the P value for the feature's heterogeneity across the 3 cohorts is given in curly parentheses.
*Some columns may add up to 99% or 101% because of rounding.

Discovery analyses identifying plasma metabolites associated with wheeze

The abundances of 18 metabolites were significantly associated with first-year wheeze, which included 5 nonannotated, 3 semi-annotated, and 10 annotated metabolites (see Fig 1). The semi-annotated bilirubin degradation products M-1 and M-2 were the metabolites most associated with wheeze ($q = 3.1 \times 10^{-4}$, 8.8×10^{-4}). M-1 and M-2, along with the third semi-annotated bilirubin degradation product M-3, were highly correlated with the significantly annotated metabolite UCB (correlations = 0.62, 0.57, and 0.87, $P < 10^{-16}$). The abundances of all 3 of these bilirubin-related metabolites in children with 1 to 3 and 4+ wheezing episodes were approximately the same as, and substantially less than, their abundances in children without wheeze, respectively (see Fig 2). However, because these metabolites were only semi-annotated, we used them to support, rather than make standalone, biological conclusions.

The annotated metabolites that were significantly associated with first-year wheeze included succinate, iminodiacetate, *N*-(2-furoyl) glycine, 3-amino-2-piperidone, 5,6-dihydrouridine, UCB, linoleoyl-linolenoyl-glycerol (18:2/18:3), pyroglutamine, transurocanate, and cysteine-s-sulfate. Succinate, the most significant annotated metabolite, along with the marginally significant metabolite aconitate ($q = 0.248$), is involved in the citric acid cycle and has previously been observed in serum, urine, and exhaled breath condensate metabolomes to predict mild adult and childhood asthma.[21] Table E4 in this article's Online Repository at www.jacionline.org contains a complete list of all 18 metabolites and their association with first-year wheeze.

Of the above-mentioned 10 annotated wheeze-associated metabolites, 3 (3-amino-2-piperidone, 5,6-dihydrouridine, and UCB) exhibited wheeze-dose responses. The abundances of 3-amino-2-piperidone and 5,6-dihydrouridine were higher in children who wheezed than in those who did not wheeze (see Table E4). However, the direction of these 2 associations failed to replicate in the WISC cohort and were therefore excluded from further analysis.

In contrast, UCB abundance was 13% lower ($\beta_{1\text{-}3} = 0.87$; 95% CI, 0.74-1.02) and 22% lower ($\beta_{4+} = 0.78$; 95% CI, 0.68-0.91), on average, in children with 1 to 3 wheezing episodes and 4+ wheezing episodes compared with children with no wheeze, respectively (see Fig 3, *A*). These results were recapitulated by UCB's nearly significant photoisomer E,Z/Z,E unconjugated bilirubin ($q = 0.214$; see Fig E8 in this article's Online Repository at www.jacionline.org); we observed a similar relationship between plasma UCB abundance and childhood asthma ($\beta_{\text{asthma}} = 0.82$; 95% CI, 0.68-0.98; Fig 3, *B*), and the direction of this association replicated in WISC. Therefore, UCB was the only wheeze-associated metabolite that we considered for further analysis.

Replication of the association between UCB and wheeze

We used the replication data to assess the replicability of the cross-sectional association between UCB levels and wheeze and, given the relationship with future asthma in the discovery data, further evaluate the effect of UCB on childhood asthma. Fig 3, *A*, shows that the magnitude and direction of UCB's cross-sectional association with 4+ wheezing episodes, and with the exception of the data set with the smallest sample size, 1 to 3 wheezing episodes, were consistent in all replication data sets.

The results in Fig 3, *B*, show that the dependence of childhood asthma on UCB levels observed in the discovery data replicate. The strong associations with asthma observed in the year 1 and 3 COAST data sets (1-sided $P = .00376$ and .0252) suggest that UCB also protects against future asthma. Furthermore, the relationships between the semi-annotated bilirubin degradation products M-1, M-2, and M-3 and both wheeze and asthma mirrored all aforementioned associations (see Fig 2), suggesting that this UCB-related pathway might protect against wheeze and future asthma. We remark that the correlation coefficients between estimates derived from the year 1 and 3 COAST data sets, which contained overlapping individuals, are negligibly small, meaning it suffices to treat estimates from all 4 data sets in Figs 2 and 3 as independent (for details, see this article's Methods section in the Online Repository and Table E5 in this article's Online Repository at www.jacionline.org).

Lastly, we performed a cross-phenotype meta-analysis, which pools information across replication cohorts and the phenotypes wheeze and asthma, to explicitly test that the direction of the associations between UCB levels and respiratory phenotypes observed in the discovery data set replicate in the replication data sets. The significant replication P value of .00638 is congruent with the results in Fig 3, and suggests that the relationship between UCB levels and respiratory phenotypes is consistent across populations.

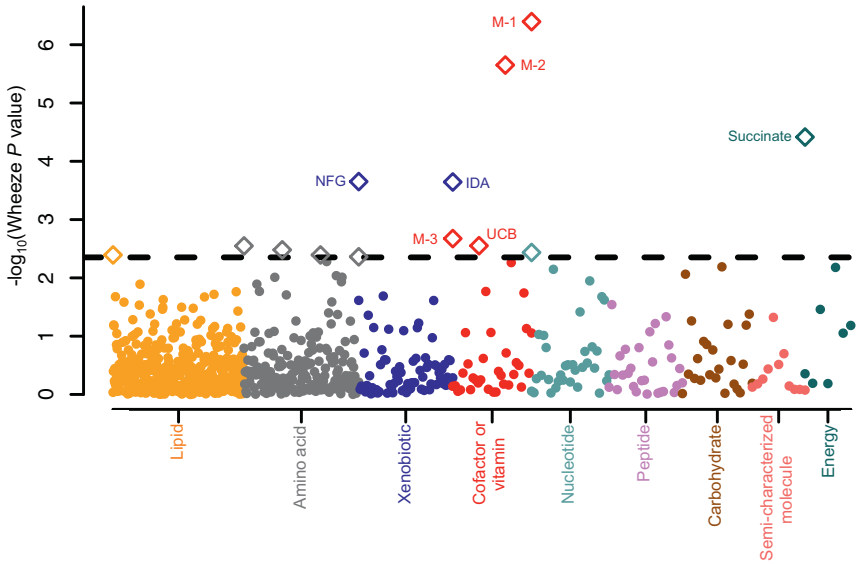


FIG 1. A Manhattan-like plot of *F* test *P* values from the discovery data set (INSPIRE) for the null hypothesis that metabolite abundance in plasma is not dependent on wheeze status, where metabolites are grouped by Metabolon, Inc–defined pathways. The dashed black line is the 20% FDR threshold, and points above and below that line are labeled with diamonds and solid circles, respectively. UCB, its 3 semi-annotated putative degradation products M-1, M-2 and M-3, as well as the 3 most significant annotated metabolites succinate, *N*-(2-furoyl)glycine (NFG), and iminodiacetate (IDA), are labeled.

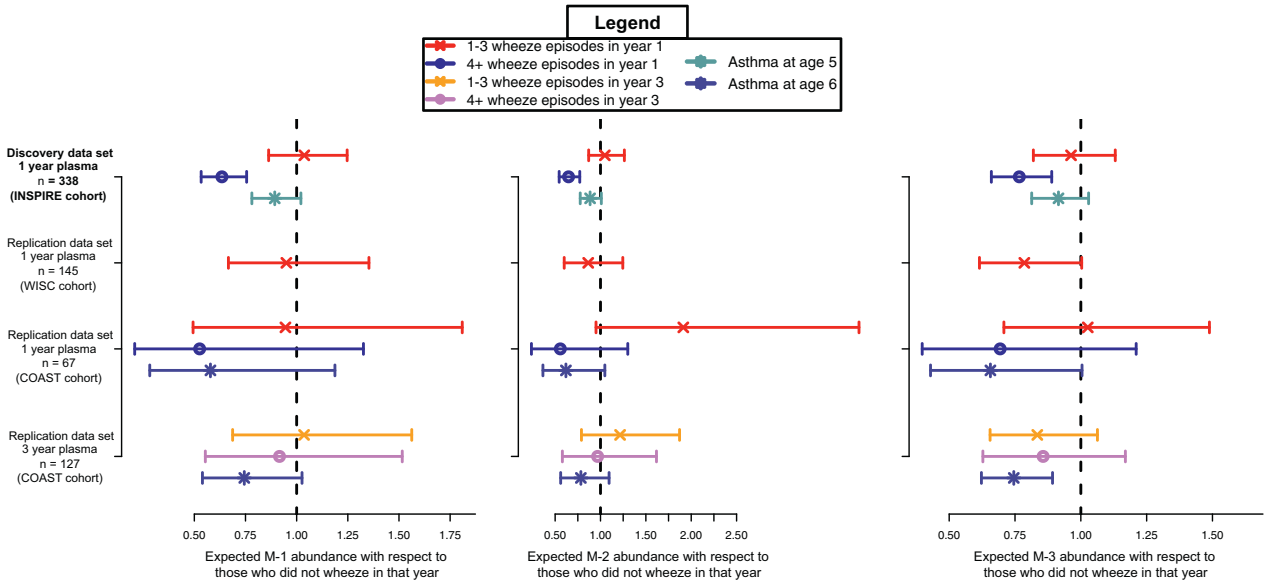


FIG 2. Estimates and 95% CIs for the expected abundance of the semi-annotated metabolites M-1, M-2, and M-3 (chemical formulas $C_{17}H_{20}N_2O_5$, $C_{17}H_{20}N_2O_5$, and $C_{17}H_{18}N_2O_4$, respectively) in the plasma of children who wheezed and have asthma with respect to those who did not wheeze and did not have asthma, respectively. The discovery cohort on the y-axis is in bold font, and the replication cohorts are in regular font; age is given in years and n is the sample size.

Metabolites correlated with plasma UCB identify potential mechanisms of action contributing to early-life wheeze

To identify potential mechanisms through which UCB protects against wheezing and asthma, we next sought to determine UCB's correlation neighbors. We identified 32 metabolites whose log-abundances were significantly correlated with those of UCB's (see Fig 4). Besides the 2 semi-annotated bilirubin degradation products, the metabolites most positively correlated with UCB were UCB's photoisomers (E,Z/Z,E unconjugated bilirubin and E,E unconjugated bilirubin) and biliverdin (an intermediate in the catabolism of heme to UCB).[22] Although UCB's photoisomers exhibited a wheeze-dose response, their associations with first-year wheeze were not significant at a 20% FDR (see Fig E8).

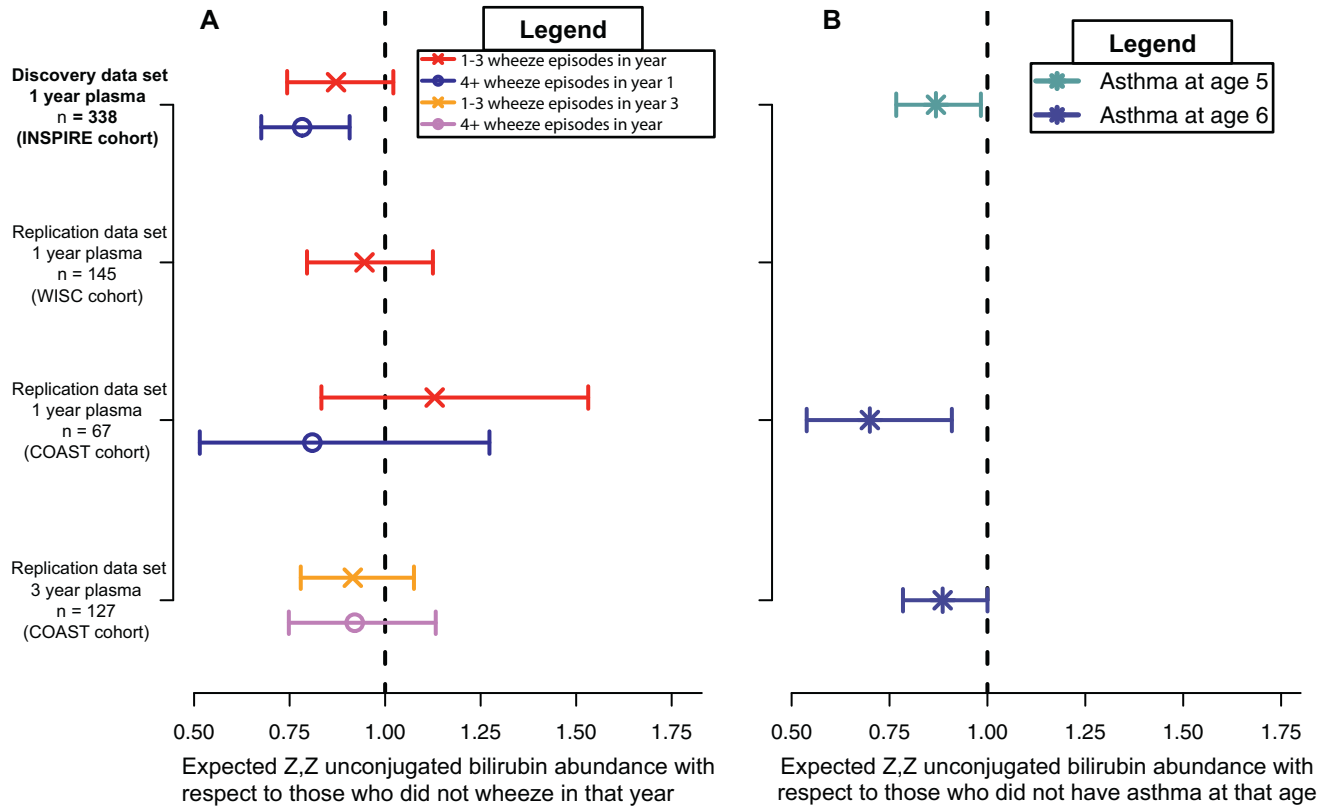


FIG 3. Estimates and 95% CIs for the expected abundance of UCB in the plasma of children who wheezed (**A**), and who were diagnosed with asthma (**B**). The discovery cohort on the y-axis is in bold font, age is given in years, and n is the sample size. Children in the WISC cohort were not yet old enough to define asthma.

Critical metabolites in the eicosanoid pathway, including arachidonic acid (AA), were among the 15 metabolites negatively correlated with UCB log-abundance. Given AA's role in the generation of eicosanoids, we next sought to determine whether the only leukotriene we identified, LTB4, which was identified in 14% of the INSPIRE plasma samples and was most prevalent in children who wheezed ($P = .0180$; see Fig 5, *A*), was related to UCB abundance. Plasma UCB levels were associated with an increase in the odds of observing LTB4 ($P = 6.19 \times 10^{-4}$; see Fig 5, *B*), which indicates that LTB4 may mediate the relationship between UCB and wheeze (see Fig 5, *C*). This statistically significant inverse relationship persisted even when we accounted for differences in AA abundance. The only other eicosanoid we identified was 12-hydroxyeicosatetraenoic acid, which was observed in 24% of the INSPIRE children. Although not statistically significant, there was an inverse relationship between UCB abundance and the presence of 12-hydroxyeicosatetraenoic acid (see Fig E9 in this article's Online Repository at www.jacionline.org).

The levels of plasma oxidative stress byproducts, such as glycerophosphorylcholine and glycerophosphoethanolamine (products of hypochlorous acid–induced plasmalogen degradation),[23] as well as 5-oxoproline (a glutamic acid derivative),[24] were inversely proportional to UCB abundance (see Table E6 in this article's Online Repository at www.jacionline.org).

DISCUSSION

To address the significant gaps in our understanding of the molecular mechanisms underlying asthma development, we studied the plasma metabolome profile of wheeze and allergic sensitization, the 2 most significant early-life risk phenotypes for asthma development, and childhood asthma. We applied a novel and rigorous statistical approach that corrects for nonrandom missing data and unobserved confounder factors,[10] and using independent replication cohorts, replicated the direction and magnitude of the effects estimated in the discovery cohort.

We identified 18 plasma metabolites that were significantly associated with first-year wheeze in discovery analysis. Among the 10 annotated wheeze-associated metabolites, only UCB, a byproduct of the normal catabolism of heme (see Fig E10 in this article's Online Repository at www.jacionline.org), exhibited a replicable dose-response relationship with first-year wheeze, in which higher plasma UCB levels were associated with fewer wheezing episodes. The magnitude and direction of the association, as well as the dose response, were consistent across both high-risk and non–high-risk cohorts. In addition, UCB levels were protectively associated with childhood asthma, suggesting that reduced UCB levels precede wheeze and asthma development. Lastly, and quite remarkably, the relationship between wheeze and the 3 semi-annotated putative bilirubin degradation products mirrored all of the aforementioned wheeze- and asthma-UCB associations in all 4 data sets, indicating that derivatives of UCB may also aid in protecting against infant wheeze.

Our finding of a strong protective association of UCB and its related metabolites with early-life wheeze and childhood asthma is consistent with recent findings demonstrating that mildly elevated bilirubin levels have a protective effect in respiratory conditions.[25,26] However, these observational studies were

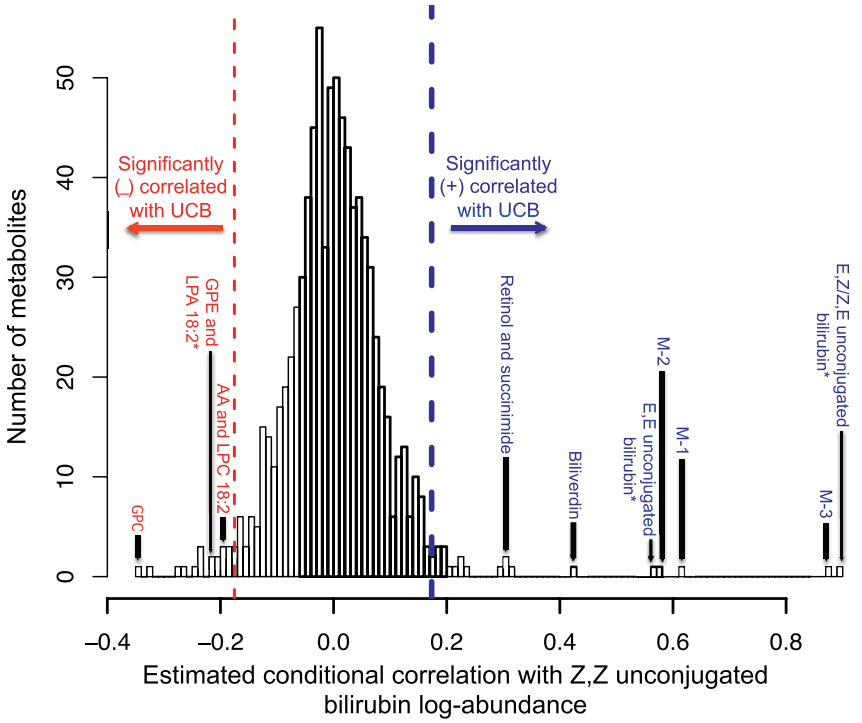


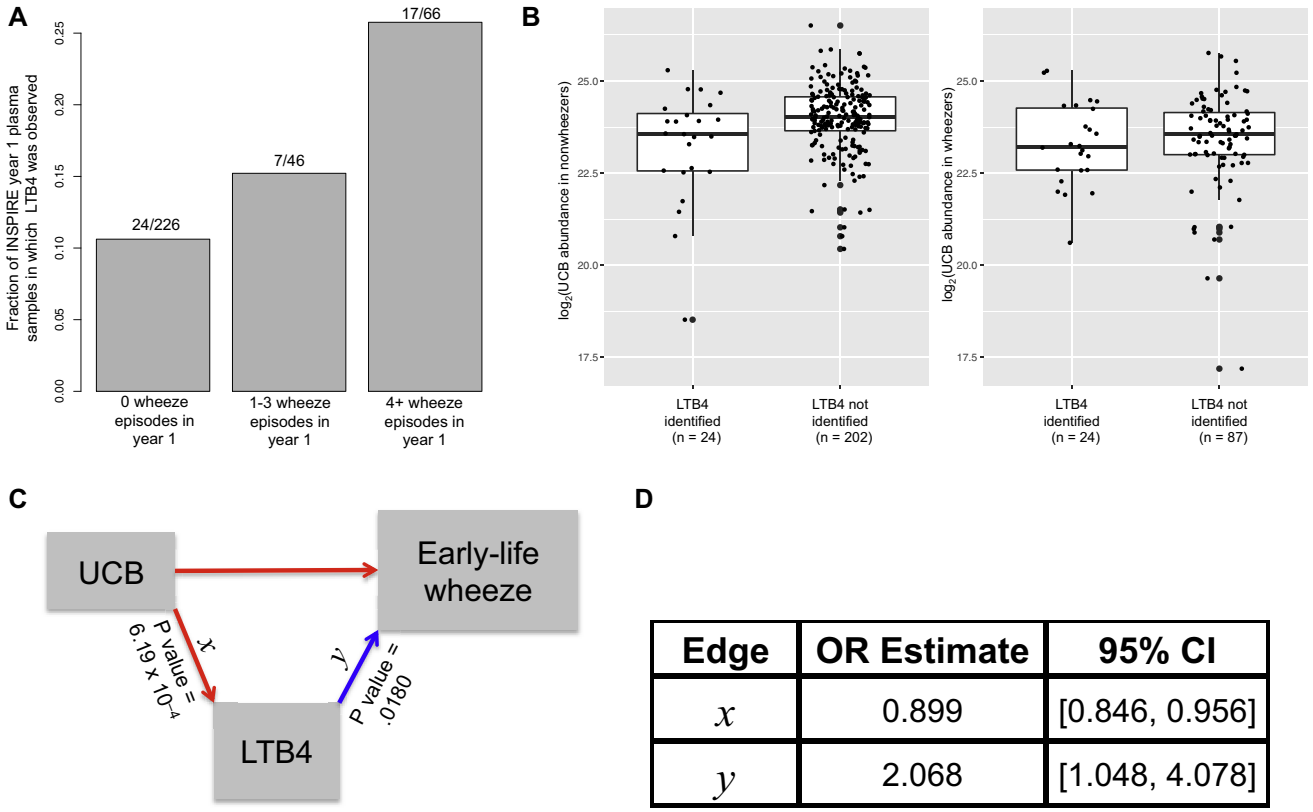
FIG 4. Histogram of the estimates for the conditional correlation between the log-abundances of UCB and all other annotated metabolites with less than or equal to 50% missing data in the discovery data set. The red and blue dashed lines are drawn at the correlation coefficients for metabolites corresponding to the 10% FDR threshold, where metabolites to the left and right of those lines are defined as UCB's correlation neighbors. Abbreviated metabolites are glycerophosphorylcholine (GPC), glycerophosphoethanolamine (GPE), lysophosphatidic acid 18:2* (LPA 18:2*), AA, and lysophos-phatidylcholine 18:2 (LPC 18:2). Metabolites with an appended "*" are annotated metabolites with a Metabolomics Standard Initiative level 2 (as opposed to level 1) identification.

conducted in adult populations and only considered total bilirubin, the sum of conjugated and unconjugated bilirubin concentration. Furthermore, no previous study had the resolution to assess the relative impact of bilirubin's isomers. Finally, this is the first prospectively followed case-control study to find an association between bilirubin and risk of wheeze, and childhood asthma, as well as the first to identify possible protective pathways through which bilirubin exerts its potential protective effect.

Contrary to our study, previous studies have demonstrated that hyperbilirubinemia is associated with an increased risk for developing asthma.[27-31] However, these studies did not measure bilirubin, and instead relied on a diagnosis of jaundice or a history of phototherapy. Moreover, a recent study in infants measured total serum bilirubin (TSB) and found that there was no difference between children with low to modest levels of TSB (≤5.9 mg/dL) and the highest levels of TSB (>18 mg/dL).[32] Kuzniewicz et al[33] was also only able to show that infants with TSB concentrations 2 to 3 times higher than those within the normal physiological range are at an increased risk of developing asthma. In contrast, our study measured unconjugated bilirubin and demonstrated that higher concentrations, within the normal physiological range, are associated with protection from asthma. This suggests that if bilirubin is causal for asthma development, our and those results from studies using upper extreme values suggest that the relationship between bilirubin levels and asthma risk follows a parabolic relationship, where moderately low and extremely high levels of bilirubin confer a greater risk for wheeze and asthma development.

Murine models of asthma indicate that UCB has many anti-inflammatory properties.[34] However, the mechanisms by which UCB is associated with reduced early-life wheeze and childhood asthma in humans are unknown. We therefore used a correlation analysis, along with a literature review, to posit pathways through which UCB protects infants against wheeze and asthma (see Fig 6). Notably, UCB levels were inversely proportional to those of AA and LTB4, which is consistent with experimental results that show that UCB inhibits many members of the secretory phospholipase A2 (sPLA2) family of enzymes (including sPLA2IIA) in a dose-dependent manner at physiologically relevant concentrations.[35,36] UCB's inhibition of sPLA2IIA *in vitro* is even irreversible and independent of substrate concentrations.[35] The observation that the significant inverse relationship between UCB abundance and LTB4 presence persisted even after adjusting for AA abundance is congruent with UCB's role as a 5-lipoxygenase inhibitor.[36] The dependence of UCB's inhibition of sPLA2IIA and 5-lipoxygenase on UCB's lipophilicity[36] may help explain why E,E unconjugated bilirubin levels are not as closely associated with wheeze, because Z to E isomerization of bilirubin reduces the intramolecular hydrogen bonding and makes the molecule less lipophilic.[37] These results suggest that UCB may curb LTB4 production during respiratory infections, which would decrease monocyte and leukocyte migration and chemotaxis, as well as the production of neutrophil-generated superoxide in the airways.[38,39]

We also observed a significant negative correlation between plasma UCB and lysophosphatidylcholine 18:2 and



Edge	OR Estimate	95% CI
x	0.899	[0.846, 0.956]
y	2.068	[1.048, 4.078]

FIG 5. A, The relationship between year 1 wheeze frequency and LTB4 presence in the discovery cohort (INSPIRE). **B,** UCB abundance in 1 year plasma as a function of whether or not LTB4 was identified in the discovery cohort. "Wheezers" are defined as children with at least 1 wheeze episode in year 1. **C,** The hypothesized relationship between UCB, LTB4, and wheeze, where red and blue edges indicate significant inverse and direct relationships, respectively. The *P* values for edges *y* and *x* are those associated with Fig 5, *A*, and *B*, respectively. **D,** The odds ratio (OR) estimates and corresponding 95% CIs for edges *x* and *y*, which quantify the associations plotted in Fig5, *B*, and *A*, respectively.

lysophosphatidic acid 18:2 levels. sPLA2s cleave membrane phospholipids to produce lysophosphatidylcholine 18:2, which is then hydrolyzed by autotaxin to produce lysophosphatidic acid 18:2.[40,41] This suggests that UCB may protect against early-life wheeze and childhood asthma in part by inhibiting the synthesis of lysophosphatidic acids, which are upregulated in human bronchial lavage fluid during allergic inflammation[42] and contribute to airway injury, fibrosis, and vascular permeability.[43]

In addition, UCB acts as a potent source antioxidant by inhibiting nicotinamide adenine dinucleotide phosphate (NADPH) oxidase,[22] which is the source of many reactive oxygen species. In addition to alleviating airway inflammation and hyperresponsiveness,[44,45] this activity could account for the negative correlations between plasma UCB levels and the hypochlorous acid–induced plasmalogen degradation products glycerophosphorylcholine and glycerophosphoethanolamine, as well as the oxidative stress indicator 5-oxoproline.

Given UCB's possible role in protecting against early-life wheeze and childhood asthma, it is therefore important to understand the underlying sources of variation in blood UCB levels, because this could be important for future therapeutic interventions. UCB is excreted by the liver, where the first and rate-limiting step is conjugation with glucuronic acid catalyzed by uridine-diphosphoglucuronate glucuronosyltransferase 1A1 (UGT1A1).[46,47] Individuals with slightly impaired UGT1A1 activity typically have elevated blood UCB levels, which is commonly observed in individuals with a genetic variant in the *UGT1A1* gene (Gilbert's syndrome).[22] The enzyme UGT1A1 can also be inhibited by other metabolites. For example, retinol (vitamin A) competitively inhibits UGT1A1 at physiologically relevant intensities *in vitro*.[48] The observation that plasma retinol and UCB levels were significantly positively correlated in the INSPIRE cohort may therefore be due to inhibition of the conjugation of UCB by retinol. Future studies addressing sources of UCB variation in healthy children, including genetic, epigenetic, and microbiome contributions, as well as diet and exposure histories, may aid in asthma prevention strategies.

Our study benefits from a large discovery cohort, as well as 2 independent replication cohorts. We were able to take advantage of the infrastructure created by Environmental influences on Child Health Outcomes and CREW birth cohorts, using harmonized data sets from birth cohorts that were specifically designed to study wheeze and asthma. Another important strength is that our analysis pipeline accounts for both latent confounding factors and nonrandom missing mass spectrometry data, which helps ensure our results are accurate and replicable.[10] Limitations of this observational study include uncertain causality in the association between metabolite levels and wheeze. However, the finding of an inverse dose-response relationship of UCB levels and number of wheeze episodes and the fact that UCB levels are inversely

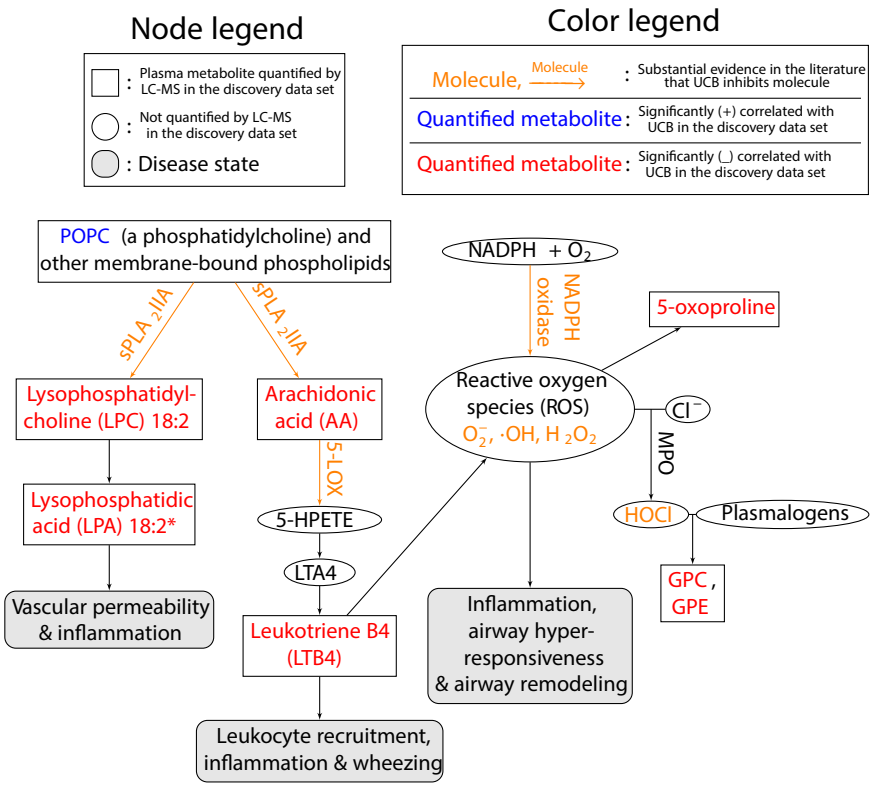


FIG 6. A proposed mechanistic diagram by which UCB present in blood is associated with reduced risk of infant wheezing. Abbreviated compounds are POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine), 5-LOX (5-lipoxygenase), LTA4 (leukotriene A4), MPO (myeloperoxidase), HOCl (hypochlorous acid), GPC (glycerophosphorylcholine), GPE (glycerophosphoethanolamine), and NADPH (nicotinamide adenine dinucleotide phosphate). All directed edges have been identified in previous literature. POPC intensity was positively associated with 4+ wheeze risk, and with the exception of GPE, the abundances of all metabolites depicted in red were negatively associated with 4+ wheeze risk in the discovery data set. Metabolites with an appended "*" are annotated metabolites with a Metabolomics Standard Initiative level 2 (as opposed to level 1) identification.

associated with future wheeze and childhood asthma support the hypothesis that UCB may, in fact, protect against wheeze and asthma development. Second, the plasma samples collected from the discovery and replication data sets were collected using different anticoagulants, the former with EDTA and the latter with heparin. Although this could cause differences in metabolome results, we were encouraged by the degree of metabolite overlap across cohorts and the robustness of the association between UCB and wheeze despite differences in anticoagulant.

Conclusions

We identified a novel inverse association of unconjugated bilirubin and its related metabolites with wheeze and childhood asthma and mapped its likely mechanisms of action *in silico*. These observations set the stage for mechanistic studies in other experimental systems to confirm these relationships, and may provide new therapeutic approaches to attenuate wheezing illnesses in infants, and potentially reduce the subsequent risk for childhood asthma.

We acknowledge the Environmental influences on Child Health Outcomes (ECHO) program and the Children's Respiratory and Environmental Workgroup (CREW) cohort members. Please see list of members in this article's Online Repository at www.jacionline.org. We also thank all the families who participated in this study.

Key messages

- **Protective association of unconjugated bilirubin and its related metabolites on early-life wheeze and asthma.**
- **Several inflammatory lipid mediators and oxidative stress byproducts were inversely correlated with unconjugated bilirubin, suggesting that unconjugated bilirubin modulates pathways critical to the development of early-life wheeze phenotypes and asthma.**
- **Results indicate that higher levels of bilirubin within the normal physiological range are associated with salutary effects on infant wheeze and asthma.**

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