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PhD Thesis

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Type 2 Inflammation and Symptom-Based Phenotypes in Childhood Asthma

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PAPERS

This thesis is written as a synopsis and is built around three scientific papers which are listed below. These papers will be referred to by their Roman numerals. The full versions of these scientific papers and their corresponding supplementary materials are included in the Appendix.

I. Levels of Total IgE versus Specific IgE during Childhood for Defining and Predicting T2-high Asthma

Tamo Sultan, Frederikke Skov, Nicklas Brustad, Nilo Vahman, Jakob Stokholm, Klaus Bønnelykke, Ann-Marie Malby Schoos*, Bo Chawes*.

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II. Dominance of IL-5 and CCL17 in Nasal Cytokine Profiles of Children with Type 2 Inflammation

Tamo Sultan, Nicklas Brustad, Julie Nyholm Kyvsgaard, Frederikke Skov, Laura Marie Hesselberg, Anton Kjellberg, Jonathan Thorsen, Nilo Vahman, Ann-Marie Malby Schoos, Susanne Brix, Klaus Bønnelykke*, Bo Chawes*.

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III. Coughing and Wheezing in Early Life Define Distinct Asthma and Lung Function Trajectories

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**Senior authors contributed equally*

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1. Zhu L, Munch IC, Lee SS, Mackey DA, **Sultan T**, Bønnelykke K, Chawes B, Larsen M, Brustad N. Prenatal and childhood exposure to smoking and retinal nerve fibre layer thickness: A meta-analysis of three independent birth cohorts. Acta Ophthalmol. 2026 Mar 7. doi: 10.1111/aos.70109. Epub ahead of print. PMID: 41794403. (**Impact Factor 2.8**)

2. **Sultan T**, Brustad N, Kyvsgaard JN, Skov F, Hesselberg LM, Kjellberg A, Thorsen J, Vahman N, Malby Schoos AM, Brix S, Bønnelykke K, Chawes B. Dominance of IL-5 and CCL17 in nasal cytokine profiles of children with type 2 inflammation. J Allergy Clin Immunol. 2025 Dec 4:S0091-6749(25)01181-9. doi: 10.1016/j.jaci.2025.11.009. Epub ahead of print. PMID: 41352441. (**Impact Factor 11.2**)

3. Skov FR, **Sultan T**, Fischer-Rasmussen K, Chawes BL, Stokholm J, Vahman N, Bønnelykke K, Schoos AM. Type 2-high airway inflammation in childhood asthma distinguishes a more severe phenotype. Pediatr Allergy Immunol. 2025 Feb;36(2):e70032. doi: 10.1111/pai.70032. PMID: 39905635; PMCID: PMC11794961. (**Impact Factor 4.5**)

4. **Sultan T**, Skov F, Brustad N, Vahman N, Stokholm J, Bønnelykke K, Schoos AM, Chawes B. Levels of total IgE versus specific IgE during childhood for defining and predicting T2-high asthma. World Allergy Organ J. 2024 Nov 21;17(12):100994. doi: 10.1016/j.waojou.2024.100994. PMID: 39650194; PMCID: PMC11621935. (**Impact Factor 4.3**)

5. **Sultan T**, Moser C, Smerup MH, Idorn L. Salmonella endocarditis in an immunocompetent 8-year-old with a mechanical aortic valve: a case report and literature review. Prog Pediatr Cardiol. 2024 Sep;74:101739. doi:10.1016/j.ppedcard.2024.101739. (**Impact Factor 0.8**)

6. **Sultan T**, Wong C. Presence and grade of undertreatment of pain in children with cerebral palsy. Scand J Pain. 2023 Jun 2;23(3):546-552. doi: 10.1515/sjpain-2022-0124. PMID: 37267482. (**Impact Factor 1.9**)

7. **Sultan T**, Marcinski PA. Gonoroisk konjunktivitis hos nyfødt i Grønland. Ugeskr Laeger. 2023 Jan 30;185(5):V71157. Danish. PMID: 36760191. (**Impact Factor 0.1**)

8. Rahimi H, **Sultan T**, Idorn L, Jensen LH. [Acute neonatal myocarditis with cardiogenic shock caused by enterovirus infection]. Ugeskr Laeger. 2021 Aug 30;183(35):V04210345. Danish. PMID: 34477088. (**Impact Factor 0.1**)

9. **Sultan T**, Nielsen JEK. [A teenager with a rare cause of migraine-like headaches and neurological deficits - syndrome of transient headache and neurologic deficits with cerebrospinal fluid lymphocytosis]. Ugeskr Laeger. 2020 Jan 27;182(5):V09190515. Danish. PMID: 32052739. (**Impact Factor 0.1**)

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1. Bundgaard MS, **Sultan T**, Kjellberg A, Meldgaard M, Brix S, Vahman N, Schoos AMM, Stokholm J, Chawes B, Bønnelykke K, Krogfelt KA, Thorsen J*, Kyvsgaard JN*. Pathogenic Airway Bacteria and Local Immune Responses During Early-Life Asthma-Like Episodes. Revision in Allergy. (**Impact Factor 14.3**)

2. Hauerslev M, Wang T, Kjellberg A, Luo Y, **Sultan T**, Thorsen J, Brix S, Sørensen S, Ernst M, Stokholm J, Bønnelykke K, Chawes B, Brustad N. Maternal antibiotic exposure alters the newborn metabolomic profile and increases the risk of respiratory infections in offspring: a 13-year longitudinal birth cohort study. Revision in Journal of Allergy and Clinical Immunology. (**Impact Factor 11.2**)

3. Luo Y, Eliasen AU, Fischer-Rasmussen K, Thorsen J, Brustad N, Chen L, Jensen SK, **Sultan T**, Thysen AH, Linneberg A, Werge T, Bybjerg-Grauholm J, Bækvad-Hansen M, Brix S, Stokholm J, Chawes B, Tingskov Pedersen CE*, Bønnelykke K*. Blood neutrophil genetics highlight the role of neutrophils in early childhood asthma and viral respiratory illnesses. Revision in Journal of Allergy and Clinical Immunology. (**Impact Factor 11.2**)

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5. **Sultan T,** Brustad N, Kyvsgaard JN, Jensen SK, Chen L, Wang T, Tingskov CE, Eliasen AU, Luo Y, Krogfelt KA, Smith BM, Schoos AMM, Vahman N, Thorsen J, Rasmussen MA, Stokholm J, Prince N, Lasky-Su J, Chawes B, Bønnelykke K. Coughing and Wheezing in Early Life Define Distinct Asthma and Lung Function Trajectories. Under review in European Respiratory Journal (**Impact Factor 21.2**)

6. Brustad N, **Sultan T**, Vahman N, Jensen SK, Aagaard K, Gørtz PM, Nielsen CH, Bønnelykke K, Chawes B. Prenatal high-dose vitamin D supplementation, offspring bone mineralization and fracture risk until age 13 years: a randomized clinical trial. Under review in The New England Journal of Medicine (**Impact Factor 78.5**)

7. Brustad N, Wang T, **Sultan T**, Jensen SK, Gomes B, Klein A, Schoos AMM, Kaiser H, Prince N, Lasky-Su J, Bønnelykke K, Chawes B. Early respiratory infection burden associates with risk of atopic dermatitis in childhood: a 10-year longitudinal study of two birth cohorts. Under review in JAMA Dermatology (**Impact Factor 11.5**)

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AG, Bønnelykke K. Genome-wide association study of childhood croup identifies susceptibility loci and highlights genetically shared mechanisms with related phenotypes. Under review in The American Journal of Human Genetics. (**Impact Factor 8.1**)

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For me, this section is akin to the closing credits of a movie. As I write this, I am awaiting the final formatting check. An acknowledgment is supposed to be long. Well, mine is, because this work has only been possible through the sacrifice and help of numerous people in my life.

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ABBREVIATIONS

AD = Atopic dermatitis

AC = Allergic conjunctivitis

AR = Allergic rhinitis

AUC = Area under the curve

BAL = Bronchoalveolar lavage

b-eos = Blood eosinophils

β2 = Beta-2

CCL = CC motif chemokine ligand

CDHR3 = Cadherin-related family member 3

CI = Confidence interval

CLR = Centered log-ratio

COPD = Chronic obstructive pulmonary disease

COPSAC = Copenhagen Prospective Studies on Asthma in Childhood

CVA = Cough-variant asthma

CXCL8 = C-X-C motif chemokine ligand 8

FDR = False discovery rate

FeNO = Fractional exhaled nitric oxide

FEV1 = Forced expiratory volume in 1 second

FVC = Forced vital capacity

GCP = Good Clinical Practice

GEE = Generalized estimating equations

GINA = Global Initiative for Asthma

GSDMB = Gasdermin B

GWAS = Genome-wide association study

ICS = Inhaled corticosteroids

IFN-γ = Interferon gamma

IgE = Immunoglobulin E

IL = Interleukin

IL1RL1 = Interleukin 1 receptor-like 1

ILC2 = Type 2 innate lymphoid cell

IQR = Interquartile range

IRRs = Incidence rate ratios

LABA = Long-acting beta-agonist

LRTIs = Lower respiratory tract infections

MAZ = Microbiota-by-age z-score

MMEF = Maximal mid-expiratory flow

MSD = Meso Scale Discovery

N = Number

NAR = Non-allergic rhinitis

ORs = Odds ratios

P = P-value

PBB = Protracted bacterial bronchitis

PCA = Principal component analysis

PC = Principal component

PD20 = Provocative dose causing a 20% fall in FEV1

ppb = Parts per billion

PRS = Polygenic risk score

QoL = Quality of life

RAST = Radioallergosorbent test

ROC = Receiver operating characteristic

RSV = Respiratory syncytial virus

RV = Rhinovirus

SAM = Synthetic absorptive matrix

SD = Standard deviation

sIgE = Specific immunoglobulin E

SNPs = Single nucleotide polymorphisms

SOP = Standard operating procedure

SPT = Skin prick test

sPLS = Sparse partial least squares

sRaw = Specific airway resistance

T2 = Type 2

TARC = Thymus and activation-regulated chemokine

Th2 = T helper 2 cell

tIgE = Total immunoglobulin E

TNF-α = Tumor necrosis factor alpha

TSLP = Thymic stromal lymphopoietin

Z-score = Standard score

SUMMARY

Childhood asthma is a chronic, heterogeneous disease that affects many children worldwide. It presents through observable traits and biological mechanisms, i.e. phenotypes and endotypes. Subgrouping by these features informs disease severity and treatment and is clinically valuable. Type 2 inflammation is central in atopic asthma, characterized by elevated blood eosinophils, fractional exhaled nitric oxide (FeNO) and plasma immunoglobulin E (IgE). These biomarkers reflect the underlying immune response via release of key cytokines. Beyond biomarkers, symptoms of cough and wheeze are common in early childhood and represent distinct clinical patterns related to asthma. However, the roles of IgE and cytokines in childhood type 2 inflammation remain insufficiently defined, especially in clinically applicable studies. Likewise, the implications of early-life cough and wheeze for later asthma, lung development, and type 2 inflammation are not well understood. **In this thesis**, we examine type 2 inflammation and symptom-based phenotypes to clarify their mechanistic and clinical roles in childhood asthma.

The roles of total and specific IgE as biomarkers of type 2 inflammation are less well defined than other used markers. **In Paper I**, longitudinal measurements by seven timepoints from the $COPSAC_{2000}$ and $COPSAC_{2010}$ birth cohorts were used to assess the predictive and endotyping value of total and specific IgE for type 2 (T2)-high and T2-low asthma, compared with blood eosinophils and FeNO. Total and specific IgE showed similar predictive and endotyping performance for discriminating T2-high asthma and T2-low asthma in early and later childhood and overlapped substantially with other type 2 biomarkers. These findings support total and specific IgE as useful biomarkers for type 2-high asthma.

It remains unknown whether airway cytokines can function as biomarkers of type 2 inflammation to the same extent as blood eosinophils, FeNO and IgE, or whether they provide airway-specific or superior performance relative to these other markers. **In Paper II**, 24 cytokines were measured non-invasively in nasal samples of epithelial lining fluid from six-year-old children in the $COPSAC_{2010}$ cohort. Using diseases linked to type 2 inflammation, which includes T2-high asthmatics, both allergic rhinitis and conjunctivitis, and atopic dermatitis, together with the established type 2 biomarkers from *Paper I*, we assessed their associations with airway cytokines. A consistent pattern emerged, with cytokines interleukin-5 (IL-5) and also C-C motif chemokine ligand 17 (CCL17) significantly elevated among type 2 inflammatory diseases and in children with elevation of type 2 relevant biomarkers. To compare predictive and endotyping performance, a supervised type 2 airway

cytokine score was compared with blood eosinophils, showing no difference. Effects were strongest for airway-specific type 2 diseases of T2-high asthma and allergic rhinitis. These findings indicate that nasal cytokine testing is a useful, non-invasive approach for airway-specific assessment of type 2 inflammation in children, with IL-5 and CCL17 particularly informative.

It remains uncertain whether early-life asthma-like symptom patterns hold predictive clinical value akin to biomarker profiling. **In Paper III**, over 5 million symptom daily diary entries quantified early-life cough and wheeze burden together with infection during the first three years of life in the $COPSAC_{2010}$ cohort. These symptoms were then used to define cough- and wheeze-dominant phenotypes in children with recurrent asthma-like symptoms. In mutually adjusted models in the full cohort, only greater cough burden independently predicted later asthma, whereas only greater wheeze burden independently predicted lower lung function. Phenotype-specific analyses of cough-only and cough+wheeze phenotypes showed higher asthma risk for both compared to healthy, but persistent lung function deficits only in the cough+wheeze phenotype, which also showed greater infection burden. Type 2 biomarker profiles did not differ between the phenotypes. Asthmatic signatures from early-life gut and airway microbiome, airway cytokines and genetics suggested overlapping type 2 and non-type 2 pathways in both phenotypes with a clear distinction from healthy children. These findings support symptom-based phenotyping as a complementary approach that may provide prognostic information beyond biomarker-based profiling.

In conclusion, total and specific IgE, alongside nasal cytokine profiles dominated by IL-5 and CCL17, were useful for identifying T2-high asthma in childhood. Early-life cough and wheeze burden captured distinct trajectories, with cough linked to later asthma and wheeze to persistent lung function deficits. Together, these results support integrating biomarker-based endotyping and symptom-based phenotyping to improve understanding and clinical management of childhood asthma.

DANSK RESUME

Børneastma er en kronisk, heterogen sygdom, som påvirker mange børn på verdensplan. Astma kommer til udtryk gennem observerbare træk og biologiske mekanismer, dvs. fænotyper og endotyper. Inddeling i grupper ud fra disse karakteristika kan bidrage til forståelsen af sygdommens sværhedsgrad og behandling og er klinisk relevant. Type 2-inflammation er central ved atopisk astma og er karakteriseret ved forhøjede niveauer af blodeosinofiler, fraktioneret ekshaleret nitrogenoxid (FeNO) og plasma-immunoglobulin E (IgE). Disse biomarkører afspejler den underliggende immunrespons via frigivelse af centrale cytokiner. Ud over biomarkører er hoste og hvæsen almindelige symptomer i den tidlige barndom og repræsenterer forskellige kliniske mønstre relateret til astma. Der er fortsat begrænset viden om betydningen af IgE og cytokiner ved type 2-inflammation hos børn, særligt i klinisk relevante studier. Tilsvarende er betydningen af tidlig hoste og hvæsen i relation til senere astmaudvikling, lungeudvikling og type 2-inflammation ikke velafklaret. **I denne afhandling** undersøges type 2-inflammation og symptombaserede fænotyper for at belyse deres mekanistiske og kliniske betydning for børneastma.

Funktionen af total og specifik IgE som biomarkører for type 2-inflammation er mindre veldefineret end for andre markører. I **Artikel I** blev gentagne målinger fra syv tidspunkter i fødselskohorterne $COPSAC_{2000}$ og $COPSAC_{2010}$ anvendt til at vurdere den prædiktive værdi af total og specifik IgE samt deres evne til at klassificere endotyper af type (T2)-høj og T2-lav astma sammenlignet med blodeosinofiler og FeNO. Total og specifik IgE viste tilsvarende prædiktiv evne og tilsvarende evne til endotypeklassifikation ved identifikation af T2-høj astma i både tidlig og senere barndom og var i betydelig grad overlappende med andre type 2-biomarkører. Disse fund understøtter total og specifik IgE som nyttige biomarkører for klassificering af T2-høj astma sammenlignet med T2-lav astma.

Det er fortsat uklart, om luftvejscytokiner kan fungere som biomarkører for type 2-inflammation i samme grad som blodeosinofiler, FeNO og IgE, eller om de giver luftvejsspecifik eller bedre ydeevne end disse øvrige markører. I **Artikel II** blev 24 cytokiner målt non-invasivt i nasal epitelvæske fra 6-årige børn i $COPSAC_{2010}$-kohorten. Med udgangspunkt i sygdomme relateret til type 2-inflammation, herunder T2-høj astma, allergisk rhinitis og konjunktivitis samt atopisk dermatitis, sammen med de etablerede biomarkører fra **Artikel I**, blev deres associationer med luftvejscytokiner undersøgt. Vi så et gennemgående mønster, hvor cytokinerne interleukin-5 (IL-5) og C-C motif chemokine ligand 17 (CCL17) var betydeligt forhøjede ved type 2-sygdomme og hos børn med forhøjede type 2-biomarkører. For at sammenligne den prædiktive værdi og evnen til at klassificere endotyper blev en

superviseret type 2-luftvejscytokinscore sammenlignet med blodeosinofiler, hvor ingen forskel blev påvist. Effekterne var stærkest for de mere luftvejsspecifikke type 2-sygdomme, T2-høj astma og allergisk rhinitis. Disse fund viser, at nasal cytokinprøvetagning er en nyttig, non-invasiv og luftvejsspecifik metode til at vurdere type 2-inflammation hos børn, hvor IL-5 og CCL17 var særligt informative.

Det er fortsat usikkert, om astmalignende symptommønstre tidligt i livet har en prædiktiv klinisk værdi på niveau med biomarkørprofilering. I **Artikel III** blev mere end 5 millioner daglige symptomregistreringer anvendt til at kvantificere tidlig hoste- og hvæsebyrde sammen med infektioner i de første tre leveår i $COPSAC_{2010}$-kohorten. Disse symptomer blev derefter anvendt til at definere hoste- og hvæsedominerede fænotyper hos børn med gentagne astmalignende symptomer. I fælles justerede modeller i hele kohorten var det kun større hostebyrde, der uafhængigt prædikterede senere astma ved 6-10 år, mens det kun var større hvæsebyrde, der uafhængigt prædikterede lavere lungefunktion ved 6-10 år. Fænotypespecifikke analyser af hostefænotypen uden hvæsen og hostefænotypen med hvæsen viste øget astmarisiko for begge sammenlignet med raske børn, men vedvarende lungefunktionsnedsættelse kun i hostefænotypen med hvæsen, som også havde en større infektionsbyrde. Type 2-biomarkørprofilerne adskilte sig ikke mellem fænotyperne. Astmatiske signaturer fra tidligt tarm- og luftvejsmikrobiom, luftvejscytokiner og genetik tydede på overlappende type 2- og non-type 2-biologi i begge fænotyper med en tydelig forskel fra raske børn. Disse fund understøtter symptombaseret fænotypeinddeling som en komplementær tilgang, der kan give prognostisk information ud over biomarkørbaseret profilering.

Sammenfattende var total og specifik IgE samt nasale cytokinprofiler domineret af IL-5 og CCL17 nyttige til at identificere T2-høj astma i barndommen. Tidlig hoste- og hvæsebyrde afspejlede forskellige forløb, hvor hoste var knyttet til senere astma og hvæsen til vedvarende lungefunktionsnedsættelse. Samlet set understøtter resultaterne en integreret anvendelse af biomarkørbaseret endotypeklassifikation og symptombaseret fænotypeinddeling for at forbedre forståelsen og den kliniske håndtering af børneastma.

PREFACE

Clinical work

During my PhD at the COPSAC research unit, which began in December 2022, I have spent half of my time contributing to clinical data collection in several projects and the other half on my own research, much of which has involved processing and preparing already collected clinical data to make them usable for analysis.

$COPSAC_{ACUTE}$ cohorts

In addition to my PhD research, I co-lead two multicenter randomized controlled trials in preschool asthma, currently conducted at 7 hospitals. One trial investigates whether high-dose vitamin D supplementation can reduce the risk of acute asthma-like exacerbations in children admitted with acute asthma-like symptoms. The other evaluates whether azithromycin can shorten hospital stays in preschool children admitted with acute asthma-like episodes. Both trials aim to improve the understanding of this phenotype and to optimize treatment strategies for preschool children at risk of severe exacerbations. The studies also include collection of multi-omics and symptom-based data that closely reflect the themes of my PhD, including airway cytokines, type 2 biomarkers, and diary-based symptom recordings.

For the past three years, I have co-led the day-to-day logistics of these large multicenter studies and supported their expansion to two additional sites. My responsibilities have included operational oversight and daily coordination of a multidisciplinary trial team of 7 research nurses, 10 physicians, and 2 medical students, staff supervision and training, database management and optimization, coordination with the GCP monitoring unit, active recruitment of hospitalized children during acute asthma-like episodes, development of recruitment procedures, scripts and standard operating procedures, data quality control, and funding-related administrative work.

$COPSAC_{2010}$ 13-year visit

During my PhD, I worked together with other MD PhD students on the extensive 13-year follow-up visit for the 700 children in the COPSAC2010 cohort, from initiation in December 2022 to completion in the summer of 2025. During these six-hour visits at the COPSAC research unit, I was part of the clinical team responsible for collecting continuous blood samples, urine samples, lung function measures, and other clinical assessments, alongside multiple omics data layers including airway

cytokines, nasal transcriptomics, skin lipidomics from tape strips, and airway pathogen data, as well as detailed medical history information. The overarching aim of this specific visit was deep phenotyping, with a focus on metabolism, individual responses to food intake, lifestyle exposures, and biological mechanisms of relevance for future disease development.

Naestved research unit within COPSAC

At the auxiliary COPSAC research clinic in Naestved, one of two COPSAC research units and the smaller of the two, which follows approximately one-third of the 700 children in the $COPSAC_{2010}$ cohort, I have secured funding for nursing staff and clinic operations. I have also undertaken clinical physician responsibilities related to asthma, allergy, atopic dermatitis, and other relevant pediatric concerns through acute visits, routine follow-up appointments, and telephone consultations, at times as the clinic's only employed physician, working together with the sole nurse, *Hanne Nissen*.

$COPSAC_{SEVERE}$ cohort

I have also contributed to data collection and data quality control for the $COPSAC_{SEVERE}$ cohort, the first nationwide pediatric study of severe asthma phenotypes in Denmark. As part of this project, a nurse-physician study team travelled to hospitals throughout Denmark to perform three-hour baseline visits and one-year follow-up visits, including detailed medical history interviews, lung function testing, omics data layers including, airway cytokines and proteomics, and other clinical assessments.

Research work

Harvard Medical School (research exchange)

From June to September 2025, I completed a short-term research fellowship at the Channing Division of Network Medicine, Harvard Medical School and Brigham and Women's Hospital, Boston, USA, as part of my PhD exchange. There, I worked under the close supervision of Associate Professor *Jessica Lasky-Su*, the current principal investigator of the VDAART cohort, together with senior researcher *Nicole Prince*, both experts in metabolomics and multi-omics. VDAART is a cohort similar to COPSAC2010, investigating the effects of high-dose prenatal vitamin D supplementation. The purpose of this stay was to deepen my understanding of omics integration in relation to **Paper III** and to support the development of future projects focusing more specifically on metabolomics

approaches to symptom-based phenotypes. During the fellowship, I also presented my work and took part in the local academic research environment.

National and International collaborations

In my PhD, I have also had the opportunity to represent COPSAC in collaborative projects within the pan-European ERS lung function trajectory consortium called Chronic Airway DiSeases Early sTratification (CADSET). Here I contributed to joint analyses of obstructive lung function trajectories and their associated early-life risk factors using available data from both $COPSAC_{2000}$ and $COPSAC_{2010}$ cohorts. My work with CADSET has allowed me to engage in international collaboration and to see my research as part of a broader European effort to understand the early origins of chronic airway disease.

For more than two years, I have also been an active board member of HYF/GNUF, the network for young researchers at Herlev and Gentofte Hospital. Here, I have contributed to developing a stronger learning and research environment for PhD students and other early-career researchers by organizing larger academic events designed to inspire young researchers and encourage collaboration. My responsibilities have included planning and coordinating events, securing funding, communication with speakers and participants, and supporting activities that strengthen the academic community at these two hospitals.

Omics environment

The COPSAC research unit facilitates a strong multidisciplinary research environment, and in my PhD I have been fortunate to be part of both the in-house Genetics Group and the Metabolomics Group. This has strengthened my understanding of omics data integration and helped me develop a broader perspective on how complex molecular data can be translated into clinically meaningful research for the patient.

Supervision

During my PhD, I contributed as an informal research supervisor for a completed bachelor's thesis by the medical student *Camille Ramm-Larsen*. The project involved an initial systematic review intended to serve as the foundation for a future meta-analysis. The idea for the project arose from my own PhD work, where it became clear that reference levels for total IgE, particularly in children, are

inadequate and poorly standardized. As part of the project, *Camille* screened 4,665 studies published over the past 10 years.

I am also currently involved in informal research supervision, including close statistical guidance, for ongoing projects involving one medical student (master thesis), one master's student in health sciences, and two PhD students, one MD and one MSc.

Funding

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Generative artificial intelligence statement

For this thesis, generative artificial intelligence was used only for limited language refinement and restructuring of author-written text. All scientific content, interpretations, and conclusions were developed independently by me.

INTRODUCTION

Childhood asthma: burden, heterogeneity, and clinical severity

One of the most frequent chronic diseases in childhood is asthma[3], with still increasing prevalences in recent decades[4]. It often debuts in early life[5], shaping these vulnerable beginnings of children. The core presentation of asthma is by symptoms of cough, wheeze, and breathlessness[6], with acute episodes of exacerbations typically triggered by respiratory infections or by exposure to allergens[7]. The burden of disease from asthma is high at multiple levels, from a patient-specific perspective, including both child and parent, and from a societal perspective, with considerable associated costs. Quality of life (QoL) in children with asthma is affected in domains of physical activity (e.g. absence of sport activities)[8], by school attendance[9] and performance[10], and by mental health[11], with higher asthma severity being associated with reduced QoL[12,13]. The disease burden also extends to the parents of the affected children, who similarly experience reduced QoL[14,15]. On a societal level, economic burdens of asthma is substantial, with high expenses from hospitalization and treatment and indirect costs from sick days[16,17]. These combined factors highlight why there is a continued need for research in childhood asthma.

A broad spectrum exists for asthma. This heterogeneity encompasses temporal patterns, persistence trajectories, symptom presentations, as well as asthma subtypes including endotypes and phenotypes. Progress towards precision medicine, where treatment is tailored to the individual, involves a greater understanding of underlying mechanisms of these different spectrums of asthma. Firstly, age at onset of symptoms matter as asthmatic children under five years are difficult to diagnose[18,19]. More precisely, it is difficult to differentiate between those with recurrent transient asthma-like symptoms of cough and wheeze, and those with symptoms that persist into school-age[20]. Here, the predominance of acute viral-induced infections in pre-school wheeze further complicates diagnosis, as episodes are typically self-limiting without progressing to later asthma[21]. From school-age, diagnosis of asthma becomes easier to support due to objective measurements of lung function and bronchodilator reversibility tests being more reliable to perform, which allows for a better clinical categorization[22,23]. Still, childhood asthma is defined with considerable variability, with a review of childhood asthma cohorts identifying 60 different definitions in 122 studies[24].

Another key component of this heterogeneity is severity, which guides the management of asthma. The Global Initiative for Asthma (GINA) currently defines severe asthma as a condition that is uncontrolled despite optimized guideline-based high dose treatment (inhaled corticosteroid [ICS]

combined with long-acting beta agonist), after addressing modifiable factors, or that worsens when this treatment is reduced[25]. These stringent definitions means that only between 2-10% of children and adolescents can be classified as having severe asthma[26,27]. A disproportionately large focus is, rightfully, placed on identifying and treating this group as children with severe asthma are more in need of disease control due to increased risk of adverse outcomes such as exacerbations[28]. More loosely, when discussing severity of asthma in clinical settings, a day-to-day disease burden is more relevant, including symptom frequency, medication use, exacerbation proneness, and impact on daily functioning. This broader heterogeneity can be addressed by stratifying children into phenotypes based on observable characteristics (traits) and endotypes based on underlying mechanisms (biology), supporting more targeted management. In asthma, type 2 inflammation represents one of the most important mechanistic axes for such stratification[29].

Type 2 inflammation

The inflammatory cascades that define asthma begin in the immune system. A central pathway in this process is type 2 inflammation. This immune pathway reflects an evolutionarily conserved host response to helminths (metazoan parasites), and in susceptible individuals it can become dysregulated due to environmental triggers such as allergens, leading to inflammation[30]. Type 2 inflammation spans both innate and adaptive immune responses in the affected tissue[31]. In airway epithelium, important immune cells are group 2 innate lymphoid cells (ILC2), mast cells, eosinophils, basophils and, importantly, T helper 2 (Th2) cells, with our understanding of the immune system still improving[32,33]. Upon relevant exposure, the release of these immune cells then leads to the production of key cytokines.

The most widely recognized type 2 cytokines are interleukin (IL)-4, IL-5, and IL-13, partly due to their long-established roles in the classical understanding of type 2 inflammation by th2 cell activation[31]. These three cytokines, together with others, act through complementary signalling pathways that are commonly grouped as the IL-4/IL-13 axis and the IL-5 axis. The IL-4/IL-13 axis, comprising the functionally similar cytokines IL-4 and IL-13, is characterized by Th2 cell-mediated responses and is closely linked to allergic sensitization and immunoglobulin E (IgE) production[34,35]. The IL-5 axis is driven by eosinophilic activity where IL-5 supports to eosinophil differentiation, maturation, and survival[36,37]. Both the IL-4/IL-13 and the IL-5 axis are central pathways in type 2 inflammatory diseases (i.e. type 2 [T2]-high asthma, allergic rhinitis, atopic dermatitis) and function as major targets for current biologic therapies[31]. As such, approved biologic therapies in children

and/or adults targeting these pathways include dupilumab (IL-4Rα, blocks IL-4/IL-13 signalling), tralokinumab and lebrikizumab (IL-13), mepolizumab and reslizumab (IL-5), benralizumab (IL-5Rα), as well as other type 2 targets such as tezepelumab (Thymic stromal lymphopoietin [TSLP]) and omalizumab (IgE)[38,39]. Biologics are usually reserved for the most treatment-refractory cases of children with type 2 inflammatory diseases[39].

The classical Th1/Th2 paradigm, which framed atopic inflammation in terms of Th1 and Th2 responses only, has also been expanded, reflecting a more complex immune interplay that includes additional pathways of Th17-associated inflammation, regulatory pathways and mixed responses[40,41]. Still, a consistently important immune cell in type 2 inflammation is the eosinophil. In non-atopic, otherwise healthy children, eosinophil levels are typically low, whereas they are elevated in type 2-associated diseases[42]. Eosinophils serve several immunological purposes, primarily being effector cells, with newer evidence of immunoregulatory function as well[43]. When type 2 inflammation occurs, eosinophils are produced and mature in the bone marrow, are released into the bloodstream, and then recruited into inflamed tissues (for example the lungs), where they contribute locally to the immune response[44,45]. Besides eosinophilic inflammation, another end-stage feature of type 2 inflammation is IgE antibodies. IgE is produced during allergic sensitization, when allergen-specific B cells are activated by allergen binding and undergo class switching to IgE-producing plasma cells[46,47].

Both eosinophils and IgE are hallmark biomarkers of chronic type 2 inflammation, and measuring their levels, typically in blood, can be used to approximate type 2 inflammatory status in children with associated diseases.

Measuring type 2 inflammation: biomarkers in childhood asthma

As type 2 inflammation is an important aspect in asthma, alongside rhinitis, and atopic dermatitis, having ways of measuring type 2 inflammatory status by proxy is highly valuable. In line with a precision medicine approach, identifying children with asthma endotypes more closely linked to type 2 inflammation could help determine those at higher risk of treatment-refractory disease and greater severity by symptoms and exacerbations, and those who may benefit from biologic therapy as a late-stage treatment option[48]. Here, several biomarkers for type 2 inflammation currently exist in children and adults. The clinical usefulness of a biomarker is measured by many factors, as the ideal biomarker should be easy and safe to use, cost-effective, consistent, accurate, and generalizable[49,50]. Likewise, the use of it can be prognostic or predictive, for diagnosis, or therapeutic (e.g. intensifying

treatment)[49,50]. A prognostic biomarker that predicts a later outcome would be highly valuable in clinical practice, especially in asthma, if it could help identify children at risk of later T2-high asthma or greater disease severity[51,52]. Another notable aspect is whether biomarker measurement is local or systemic, as biomarkers measured closer to the affected tissue may better reflect local changes than systemic spillover or byproducts measured in blood. In 2015, Papaioannou and colleagues reviewed asthma management guided by biomarkers and asked provocatively whether this approach was still science fiction or is a glimpse of the future[53].

In the clinic, chronic type 2 airway inflammation is currently assessed using blood eosinophils (b-eos) or sputum eosinophils, fractional exhaled nitric oxide (FeNO), total IgE, and allergic sensitization assessed by specific IgE to aeroallergens or food allergens or skin prick testing, and less frequently blood periostin[54]. B-eos a well-established, minimally invasive proxy biomarker of type 2 airway inflammation in asthma and are frequently used, with higher levels associated with greater exacerbation risk and better response to biologic therapy[55]. A strength of b-eos is its availability, as leukocyte counts from routine blood sampling are widely accessible worldwide. Eosinophilia in asthma is heterogeneous, is seen in both allergic and non-allergic asthma, and varies over time in a life course, meaning that age and timing of measurement will influence interpretation[55,56]. Another notable type 2 airway inflammatory biomarker is FeNO, and is in theory a more ideal biomarker as it is measured in the airways instead of systemically as with b-eos. FeNO is measured non-invasively during a controlled exhalation at 50 mL/s after inhalation to total lung capacity, limiting contamination from surrounding nitric oxide[57]. What is measured is the airway nitric oxide production (driven by the inducible nitric oxide synthase) produced by epithelial- and smooth muscle cells in the airways, and induced by IL-4 and IL-13[58].

Finally, circulating IgE levels are a measurable surrogate marker of type 2 inflammation in the body. IgE-mediated immune responses represent type I hypersensitivity and are typically triggered by allergens such as aeroallergens and food allergens[59]. One way to assess this is by measuring the total concentration of circulating IgE in peripheral blood, reported as total IgE. The biomarker of total IgE has long been used as a diagnostic and predictive biomarker for type 2 inflammation and to inform treatment response (i.e. omalizumab), but it is often perceived as less specific and less sensitive than other available biomarkers[60,61]. In parasite-endemic areas it is even less sensitive since it can be influenced by different host factors, including parasitic infections[62]. A point of contention is the lack of universally accepted cut-off values for defining which total IgE levels are “elevated”, particularly in younger children. As such, much of evidence rationale is actually based on a smaller, decades older, adult-focused and less generalizable population-based study, which could make standardized

reference values difficult, especially in children[63,64]. To improve the precision of total IgE as a biomarker, well-characterized pediatric cohorts are needed to evaluate clinically meaningful cut-off values, and larger population-based childhood studies to validate these thresholds and strengthen generalizability. A different blood-based and readily available IgE biomarker of type 2 inflammation is specific IgE. As the name suggests, it measures serum concentrations of allergen-specific IgE antibodies to individual aeroallergens and food allergens which makes it an appropriate surrogate marker for allergic sensitization[65,66]. Compared with total IgE, specific IgE has standardized assay thresholds and can additionally be reported on a class scale (radioallergosorbent test [RAST] classes, typically 0-6)[67]. Specific IgE to aeroallergens has been linked to current and later recurrent/persistent wheeze[68,69], and later asthma and other atopic disease[70–72]. We have also shown that polysensitization, assessed by specific IgE, is associated with a higher odd of later asthma compared with monosensitization[73]. Closely related is skin prick test (SPT) which is another proxy biomarker of allergic sensitization resembling the function of specific IgE. SPT can non-invasively assess panels of aeroallergen and food allergen sensitization and may capture an allergic sensitization signal that is partly independent of specific IgE[74]. However, sensitization measured by SPT, and particularly by specific IgE, in pediatric T2-high asthma is still not well understood, underlining the need for more studies in children, particularly longitudinal studies in deeply phenotyped cohorts, as this design would support more robust associations. Likewise, studies spanning multiple decades would strengthen interpretation, as sensitization appears to shift over recent time, including declining population-level specific IgE[75]. It has also been shown that allergic sensitization is present in a notable proportion of asymptomatic individuals and in individuals without atopic disease, adding nuance to whether the measured signal reflects a pre-disease state or merely biological variability[76,77].

Clinical endotypes in children: T2-high and T2-low asthma

T2-high asthma is the most common inflammatory endotype, with approximately 50–70% of individuals with asthma demonstrating the presence of type 2 inflammation[78,79]. It is characterized by elevated type 2 biomarkers and characteristic atopy, treatment-related and severity-related clinical patterns. The type 2 endotype is seen throughout life, but the literature is dominated by adult population studies, which may bias current concepts toward an adult-oriented lens on the disease[80]. Nevertheless, evidence suggests that T2-high asthma has early-life origins and is more prevalent in childhood than it is in adulthood[80]. In addition, children with severe asthma show a higher burden of type 2 inflammation, measured by type 2 biomarker elevation, than those with milder disease[81], and a subset of these children can be eligible for biologic therapy depending on age and inflammatory

profile[82]. In adults with T2-high asthma, it is more difficult to treat than T2-low asthma[83] and airway type 2 inflammation is often not completely eliminated by ICS treatment[84]. These characteristics outlines why this endotype is of such great importance, and why more research is especially needed in children.

T2-low asthma represents the other side of the coin. It may be underappreciated and understudied compared with T2-high asthma,[85] yet distinguishing the two holds clinical value. T2-low asthma is thought to be more neutrophilic and pauci-granulocytic by type 1 and type 17 immune response, and more steroid-resistant[86–88]. Pediatric studies on T2-low asthma are similarly scarce, just as with the T2-high counterpart. Children with T2-low asthma, defined by lack of type 2 biomarker elevation, have been shown to be more obese and older[89]. The treatment alternatives for these children are lacking, as they respond less well to ICS, and currently available biologics target type 2 inflammation instead of non-type 2 pathways[90–92]. Another possibility is that existing biomarkers may lack precision for identifying a type 2 immune response in school-aged children and thus are misclassifying T2-high children as T2-low, underscoring the need for more airway-specific biomarkers[85].

Why systemic biomarkers are limited for airway endotyping

With this rising era of precision medicine, major focus has been placed on developing tailored measurement techniques, in other words methods that measure disease activity directly at its site of origin. The blood-based biomarkers b-eos and IgE are systemic surrogate markers of type 2 inflammation, but they may not capture local airway processes. Progress in airway-specific type 2 assessment will likely require newer biomarkers that measure airway inflammation directly. Here, FeNO is the closest non-invasive and airway-specific measure, but greater sensitivity is still needed as it can be confounded by several factors, including allergic rhinitis, making FeNO generally more useful for confirming asthma rather than for excluding it[93,94]. Another non-invasive option which holds promise is induced sputum eosinophil count, where the percentage of eosinophilic cells is measured directly in sputum collected after provocation with inhalation of nebulized saline[95]. In children, induced sputum can be time-consuming and problematic from a technical standpoint, and can therefore lack direct clinical implementation in asthma management[95–97]. Lastly, bronchoalveolar lavage (BAL) provides direct sampling of the lower airways via bronchoscopy by analysing lower airway lining fluid[98]. Although clinically relevant, BAL is generally not feasible for routine use in

children with asthma because it is invasive, time-consuming and typically requires sedation or general anaesthesia[99].

Direct cytokine measurement, as cytokines are the key immunological signals driving the type 2 inflammatory response, is increasingly needed in the current precision medicine era of expanding opportunities. The main concerns are however that cytokine levels are dynamic and can be altered by several factors. Relative abundance in relation to other cytokines can affect detectability, meaning clinically relevant cytokines may be missed unless the measurement technique is sufficiently sensitive and high-resolution[100,101]. Sampling site is also inherently important, as local environments can shape immune signalling in ways that may not reflect biology in other compartments[102]. Timing is also important, as active infections can alter measured cytokine levels[103], and cytokine profiles can differ depending on whether sampling is performed during stable disease (e.g., routine visit) or during a flare-up (e.g., an asthma exacerbation)[104]. Examining new, non-invasive, and airway-specific biomarkers of type 2 inflammation in children can produce the breakthrough needed for improving diagnosis and management of T2-high asthma and associated diseases.

From early-life symptoms of cough and wheeze to later asthma

Continuing on the spectrum of asthma and asthma-like symptoms, both cough and wheeze are incredibly common in early childhood[105]. They are thought of as part of early life asthma-like phenotypes, but much is still not understood as both symptoms carry different annotations and encompass different conditions and diseases. By examining what these early symptoms represent, especially in the critical period of early childhood, it can help shape our understanding of later outcomes of childhood asthma, type 2 inflammation and lung development.

Wheeze is an early-life respiratory symptom that occurs in approximately half of all children at least once during the first year of life[106,107]. Wheezing disorders have in early life been connected to a range of trajectories, from transient to persistent forms, and some associated with progressive airway obstruction and inflammation[108,109]. Here, a clinically important subgroup experiences recurrent wheeze, reflecting a higher burden of airway infection in early life[110]. Recurrent wheeze is associated with an increased risk of persistent symptoms[111], and later asthma in childhood[112]. In clinical practice, the presence and frequency of wheeze guides treatment decisions in children with recurrent asthma-like symptoms[113,114]. Preschool wheeze is conventionally considered an early asthma-related

condition, although the terminology is controversial, placing it on a spectrum of asthma and asthma-like conditions and making it perhaps the most recognized symptom[106,115].

Relative to wheeze, coughing in early life is even more of a "black box" in terms of its underlying mechanisms and prognostic meaning. Cough is more common in children than in adults and is among the most frequent reasons for healthcare contacts[116]. The impact of chronic cough in a child is often felt throughout the household and can be a major stressor affecting QoL in both the child and the parents [117–120]. Determining a specific diagnosis in cough disorders is often challenging, as they span a broad spectrum that includes upper airway cough syndrome, post-infectious cough and protracted bacterial bronchitis (PBB)[121,122]. Of note is PBB, which is defined by a chronic wet cough lasting more than four weeks, is often under-recognized and undertreated[123]. It is commonly associated with bacterial pathogens such as *Haemophilus influenzae*, *Moraxella catarrhalis, and Streptococcus pneumoniae,* and typically will improve with an appropriate antibiotic course, sometimes requiring longer treatment if symptoms persist[123–125]. This highlights airway bacteria as a plausible contributor to early-life cough and motivates evaluating airway bacterial colonisation and infection as a factor of cough burden.

When discussing school-age phenotypes of coughing in the asthma spectrum, the diagnosis of cough variant asthma (CVA), characterized by persistent cough and some bronchodilator responsiveness in the absence of wheeze, is relevant[126]. A recent bibliometric study of CVA found that research in this field is still at an early stage[127]. Some studies suggests that CVA could be a precursor to "classic asthma", while other studies propose that it reflects a distinct phenotype with different underlying mechanisms[127,128].These diagnostic speculations are further complicated by the overlapping features between CVA and these other chronic cough disorders, and the common perception among clinicians that isolated cough is less likely to represent true asthma[129–131].

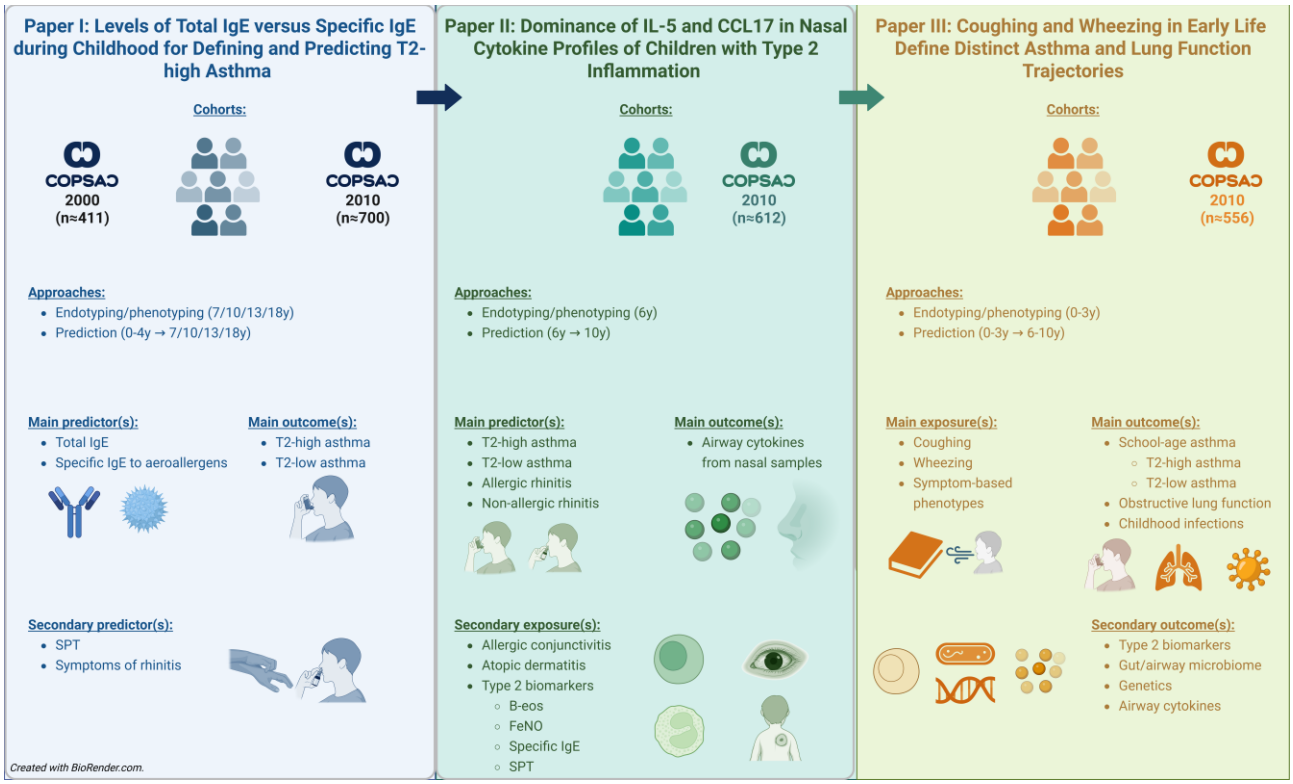
Connecting early symptoms to lifetime lung function

One of the longest-running prospective birth cohorts examining lung function development is the Tucson Children's Respiratory Study, initiated in the 1980s in America. This study demonstrated that trajectories of lung function are established early in life and track into later childhood and adulthood[132,133]. These observations highlight early-life lung development as a critical window for understanding the origins of obstructive airway disease and for identifying opportunities for early intervention. Longitudinal studies modelling wheeze phenotypes have further shown that lung function in early adulthood is lower across all wheeze-specific phenotypes compared with never-

wheeze children, with the lowest peak lung function observed among those with recurrent wheeze[134–136]. Since impaired peak lung function, reflecting suboptimal lung growth[137] and potentially early airway remodelling, is a key determinant of later chronic obstructive pulmonary disease (COPD) risk[138], these findings highlight the long-term consequences of early-life wheezing beyond just childhood. This is incredibly relevant as COPD is already the fourth leading cause of death worldwide and with case numbers projected to approach 600 million by 2050[139,140].

By contrast, it remains uncertain to what degree early-life cough affects lung function, particularly in cohorts with high-resolution symptom data that can disentangle cough from wheeze. The incremental effect of each wheeze episode on later lung function is also difficult to quantify, since most studies lack symptom data with enough temporal depth.

Unresolved questions and rationale for the present thesis



Thesis - Figure 1. *Conceptual overview of the three thesis papers, showing how predictors and outcomes are thematically connected between the papers. Created in BioRender. Bønnelykke, K. (2026) https://BioRender.com/j7abd33*

The GINA guidelines, used worldwide in asthma management, have over several iterations described type 2 inflammation in asthma as being supported by elevated b-eos and/or elevated FeNO (with suggested cut-offs for both) and/or the less clearly defined label of clinically "allergic/allergen-driven"[25,141]. This leaves several questions unanswered, particularly whether total IgE or specific IgE can meaningfully fulfil this label in children both predictively and for endotyping. Likewise, it remains unclear whether these IgE biomarkers are congruent with each other and with the better-characterized type 2 inflammatory biomarkers.

Moving beyond systemic biomarkers, there remains an unmet need for more precise markers of type 2 inflammation that better capture airway-specific activity. To be feasible in children, such biomarkers would need to be non-invasive. It remains uncertain whether nasal cytokines can serve this purpose, both for prediction and for endotyping.

Symptom-based phenotyping of recurrent troublesome lung symptoms in early life offers a scalable and clinically accessible approach to understanding asthma-like conditions. However, the biological basis of early-life symptom patterns, particularly isolated cough, remains poorly defined. It is unclear whether cough- and wheeze-dominant presentations reflect shared or distinct mechanisms, and whether they differ in inflammatory and biological profiles, including later asthma, type 2 inflammation, genetic risk, airway and gut microbiome composition and cytokine profiles. Clarifying these distinctions may support improved early risk assessment and management of childhood asthma.

AIM AND OBJECTIVES

The overall aim of this thesis was to characterize type 2 inflammation using biomarkers and phenotypes of childhood asthma and asthma-like diseases in early childhood.

For each paper the specific objective was:

PAPER I

To evaluate total and specific IgE as biomarkers of type 2 inflammation in childhood asthma.

PAPER II

To determine whether nasal airway cytokines can function as biomarkers of type 2 inflammation in children.

PAPER III

To assess the clinical and mechanistic implications of early-life cough and wheeze patterns for later school-age asthma, obstructive lung function, and type 2 inflammation.

METHODS

Mother-child cohorts

For this thesis, data were drawn from two Danish mother-child birth cohorts. For **Paper I**, the Copenhagen Prospective Study on Asthma in Childhood $(COPSAC)_{2000}$ cohort[142] and the $COPSAC_{2010}$ cohort[143] were used. For **Paper II** and **Paper III**, only the $COPSAC_{2010}$ cohort was used. Both $COPSAC_{2000}$ and $COPSAC_{2010}$ are ongoing prospective cohorts that follow children from birth to elucidate the mechanistic origins of childhood asthma and related type 2 inflammatory diseases (allergic rhinitis, atopic dermatitis, food allergy). Both cohorts are seen in two COPSAC clinics located in Gentofte, Copenhagen, Denmark and in Naestved, Denmark. In these cohorts, both acute and routinely scheduled clinical visits were conducted with cohort children, enabling deep phenotyping. Visits were performed by physicians at the COPSAC research unit together with clinical research assistants. Each scheduled visit followed a predefined protocol with specific research questions that changed over time. Multiple omics layers were collected longitudinally, including genetics, microbiome, exposome, metabolomics, proteomics, lipidomics and transcriptomics[144].

$COPSAC_{2000}$ was initiated in 2000 and recruited pregnant women with asthma. The cohort includes 411 children and has been followed prospectively with data available through 18 years of age, and a 26-year follow-up visit is currently being planned.[145–147]

$COPSAC_{2010}$ was initiated in 2010 as a population-based cohort with no maternal asthma prerequisite. The cohort includes 700 children and has been followed prospectively with data available through 10 years of age. The 13-year follow-up visit was recently completed, and data from that will be available soon. The cohort included a two-by-two factorial RCT of high-dose maternal vitamin D (2400 international units per day), fish oil (n-3 long-chain polyunsaturated fatty acids; 2.4 gram per day), the combination or placebo during pregnancy to test whether these prenatal interventions reduce asthma and other atopic outcomes in the offspring.[148–150]

Biomarkers of type 2 inflammation

Type 2 biomarkers

Longitudinal blood samples and sensitization assessments were collected for type 2 biomarkers at multiple timepoints in both cohorts and used in **Paper I-III**. These included b-eos, FeNO, total IgE, specific IgE, and SPT (*Additional details are provided in the Supplementary Materials for **Paper I***).

Peripheral b-eos count was measured at ages 7 years, 13 years, and 18 years in $COPSAC_{2000}$, and 6 years and 10 years in $COPSAC_{2010}$. Samples were analysed on *ADVIA 2120i* (Siemens Healthcare Diagnostics Inc) and *Sysmex XE* (Sysmex) and reported as $\times 10^9/L$[151]. For elevation of b-eos, the threshold of ≥0.30 was chosen in both cohorts. The primary cutoff was chosen to increase specificity, in line with thresholds used in GINA's guideline-based decision-making for type 2 targeted therapy[25,141]. Sensitivity analysis performed on both lower and higher values (≥0.15 and ≥0.50 $\times 10^9/L$).

FeNO was measured at the ages of 7, 13, and 18 years in the $COPSAC_{2000}$ cohort and at the ages of 6, 8, and 10 years in $COPSAC_{2010}$. Measurements were analysed on the *DENOX 88 module* (ECO MEDICS) and *NIOX Vero/Flex* (Aerocrine) and reported as parts per billion (ppb). To achieve best measurement quality, two assessments were performed, requiring agreement within approximately 5%, and with the mean of the two values then used. Elevated FeNO was defined as ≥20 ppb, based on a cutoff used in prior work and supported by guideline recommendations. [152–155]

Total IgE was measured in serum from peripheral venous blood at ages 6 months, 18 months, 4 years, 7 years, 13 years and 18 years in COPSAC2000, and at age 10 years in COPSAC2010. Measurements were analysed on *ImmunoCAP* (Thermo Fisher Scientific), and reported as kU/L, with a limit of detection of 2 kU/L. Elevated total IgE was defined using age-specific cut-off values 6 months: 7.3 kU/L, 18 months: 13 kU/L, 4 years: 40 kU/L, 7 years: 63 kU/L, 10-18 years: 85 kU/L. Cut-off values were based on assay provider reference values derived from a Scandinavian population, consistent with the present study. Sensitivity analyses were performed using a universal threshold of 50 kU/L.[156–158]

Specific IgE to aeroallergens was measured in blood at ages 6 months, 18 months, 4 years, 7 years, 13 years, and 18 years in $COPSAC_{2000}$ and at age 6 and 10 years in $COPSAC_{2010}$. Measurements were, as total IgE, analysed on *ImmunoCAP* (Thermo Fisher Scientific) and reported as kU/L. Aeroallergens

included were dog, cat, horse, birch, mugwort, grass, house dust (Dermatophagoides pteronyssinus/farinae), and molds (Alternaria alternata and Cladosporium herbarum), consistent with a Scandinavian screening panel. Elevated specific IgE was defined as ≥0.35 kU/L. Sensitivity analyses were performed using higher cutoffs corresponding to higher RAST classes.[74,159,160]

SPT to aeroallergens was measured at ages 7, 13, and 18 years in $COPSAC_{2000}$ and at age 6 and 10 years in $COPSAC_{2010}$. Tests were performed using extracts from *Soluprick* (ALK-Abelló) and interpreted as positive with a wheal diameter of ≥3 mm compared with the negative control. The aeroallergen panel matched the specific IgE panel.[74]

Airway cytokines

In **Paper II**, at 6 years of age in $COPSAC_{2010}$, 24 unique cytokines were measured in epithelial lining fluid samples collected from the nose using a synthetic absorptive matrix (SAM) filter paper. Filter papers (5×25 mm) were cut into "L" shapes by COPSAC research personnel to enable handling and reduce the risk of nasal displacement due to limited child cooperation. These upper airway samples were used as a proxy measure of lower airway inflammation. As nasal cytokine levels could be confounded by age, concurrent respiratory infection, season of year, intranasal and inhaled steroids, and batch effects, these variables were noted and accounted for later together with side-effects of crying, sneezing, nosebleed, and other technical difficulties. Sampling was performed during stable conditions and not during acute visits.

SAM filter paper was inserted with tweezers, with one filter paper placed in each nostril between the concha inferior and septum and positioned deep enough to still be visible in the vestibulum. Absorption time was 2 minutes, with intermittent gentle pressure on the nasal wings to keep the SAM filter paper in place. After 2 minutes, both filter papers were placed in a prelabelled NUNC tube, kept on ice, and transferred to a −80°C freezer as quickly as possible (up to 10 minutes on ice).

For analysis, the Meso Scale Discovery (MSD) assay platform was used due to high sensitivity[101]. The 24 selected cytokines were grouped into overall immune responses (type 1 response, type 2 response, type 17 response, regulatory response, and other immune responses), with some overlap between cytokines belonging to two groups. These groupings were defined a priori based on established immunologic roles and were not inherently specific to the nasal compartment.[103,161,162]

A similar sampling was also performed at 1 month of age in COPSAC2010 as a secondary outcome and used in a supervised model (*see section titled "Other" and supplementary materials for* ***Paper III***)[161,163].

Endotypes and phenotypes

Asthma and asthma-like endotypes and phenotypes

Asthma and asthma-like phenotypes were defined using predefined algorithm iterations aligned with cohort standard operating procedures (SOPs), selected to capture disease heterogeneity and match the purpose of each research hypothesis.

SOP-defined asthma was assigned based on recurrent troublesome lung symptoms of cough, wheeze and/or breathlessness lasting at least three days, with at least five episodes within six months. Alternatively, frequent symptoms occurring more than twice weekly within one month, including exercise-related symptoms, with a documented effect of inhaled beta-2 (β2)-agonist prompted a three-month treatment period with ICS. Relapse after stopping ICS was used to confirm asthma when applicable, defined as either two episodes lasting at least three days within a three month period or symptoms exceeding twice a week for two consecutive weeks. Severe acute symptoms requiring hospitalisation or physician assessment also qualified, and intermittent exercise- or allergy-triggered symptoms with documented β2-agonist response were included. If no symptoms or relapse occurred for one year, the diagnosis was terminated.

A stricter definition of objectively validated asthma was used when a high-specificity asthma outcome was required and feasible given the available statistical power[152]. This stricter definition was created based on European Respiratory Society (ERS) criteria[164]. Objectively validated asthma required fulfilment of at least two of the following criteria at any assessment during follow-up: (I) Airflow limitation, defined as sRaw above 1.6 kPa·s, FEV1 below 80% predicted, or FEV1/FVC below 80%, or; (II) bronchodilator reversibility, defined as a equal or above 12% increase in FEV1 or a equal or above 30% decrease in sRaw after inhaled short-acting β2-agonist; (III) airway inflammation, defined as FeNO equal or above 25 ppb; (IV) bronchial hyperresponsiveness, defined as more than 10% fall in FEV1 after exercise challenge or a equal or above 20% fall in FEV1 during methacholine challenge.

Type 2-high and type 2-low asthma were defined using a two-hit approach combining an asthma definition with type 2 biomarker elevation. In **Paper I**, objectively validated asthma together with type 2 status of using elevated blood eosinophils and/or FeNO. In **Paper II**, SOP-defined asthma defined at ICS initiation/relapse confirmation, type 2 status additionally incorporated sensitization to aeroallergens by either SPT and/or specific IgE (≥0.35), or total IgE (age-specific cut-offs, only available at 10 years of age).

In **Paper III**, symptom-based phenotypes were created from parent-reported daily symptom recordings during the first three years of life. Among children meeting the early-life SOP-defined asthma criteria based on recurrent troublesome lung symptoms, the cough-only phenotype was defined as zero days with wheeze during the first three years, while the cough+wheeze phenotype was defined as one or more days with wheeze during the same period. Remaining children were classified as the healthy control group. For school-age asthma, relapse-confirmed asthma and objectively validated asthma were used for later outcomes.[152,165,166]

Other type 2 & non type 2 diseases

For **Paper I**, symptomatic rhinitis was used as a symptom-based surrogate of type 2 airway involvement without overlapping with allergic sensitization measures, since specific IgE and SPT were used elsewhere as exposures and outcomes. Symptomatic rhinitis was assessed at the ages of 4, 7, 13, and 18 years in $COPSAC_{2000}$ and at age 6 and 10 years in $COPSAC_{2010}$. It was defined as at least two of four nasal symptoms (drippy nose, sneezing, itching of nose, blocked nose) without signs of common cold. Symptoms were required to occur within the relevant timeframe of suspected aeroallergen exposure.

For Paper II, allergic and non-allergic rhinitis at ages 6 and 10 years in $COPSAC_{2010}$ were defined using the same symptom definition (at least two of four symptoms without infection), but classified by concordance with allergic sensitization and seasonality. Allergic rhinitis required symptoms occurring during the relevant aeroallergen season together with sensitization by specific IgE and/or SPT to aeroallergens. If these criteria were not met, rhinitis was classified as non-allergic (*for additional details, see supplementary materials for **Paper II***).[167,168]

Atopic dermatitis diagnosis was defined at ages 6 and 10 years using the Hanifin and Rajka criteria in $COPSAC_{2010}$, with major and minor criteria assessed repeatedly at scheduled visits and when

presenting with relevant symptoms. Diagnosis of atopic dermatitis was used as secondary outcomes in **Paper II** (*for additional details, see supplementary materials for **Paper II***).[169]

Daily diary recordings

Daily symptom recordings were completed during the first three years of life in $COPSAC_{2010}$ and used in **Paper III**. Parents completed diary entries each day, indicating whether their child had cough, wheeze, or breathlessness together with common childhood infections. Parents were instructed in the meaning of these symptoms to help identify them. Using more than 5 million daily diary entries, episode variables were then constructed as ≥3 consecutive days with symptoms separated by ≥3 symptom-free days These continuous symptom variables, cough and wheeze episodes, were used as count variables. Likewise, to test dose-response relationships, clinically meaningful episode groups of 0–2, 3–5, 6–10, and ≥11 for cough and 0, 1–2, 3–5, and ≥6 for wheeze were selected based on the data-driven distribution. Lastly, based on days with any symptoms among those with recurrent troublesome lung symptoms, symptom-based asthma-like phenotypes of cough-only (zero days of wheeze) and cough+wheeze (both cough and wheeze) were created as described in previous section see (*Asthma and asthma-like endotypes and phenotypes)*. [170,171]

Common childhood infections and conditions, including cold, pneumonia, acute otitis media, tonsillitis, acute gastroenteritis, and fever, were likewise recorded daily during the first 3 years of life, and episode variables were created using the same algorithm, ≥3 days with symptoms of the given infection separated by ≥3 days without symptoms. During acute and scheduled visits (at least twice yearly), these infection recordings were validated by physicians at the COPSAC research unit. Children with unreliable data, rare disorders, or sufficient missingness were excluded from the analysis (*for additional details, see supplementary materials for **Paper III***).[172,173]

Lung function assessment

Lung function measurements were performed during scheduled visits at 6, 8, and 10 years of age in the $COPSAC_{2010}$ cohort and were used in **Paper III** as a primary outcome. Spirometry included forced expiratory volume in 1 second (FEV_1), forced vital capacity (FVC), FEV_1/FVC ratio, and maximal mid-expiratory flow (MMEF), and specific airway resistance (sRaw) was assessed by whole-body plethysmography, all using *MasterScope* systems (Erich Jäeger). Spirometry required multiple technically acceptable expiratory tests, from which the best FEV_1 and corresponding FVC

and MMEF were selected, whereas sRaw was derived as the mean of at least two technically acceptable plethysmography assessments. At 6 years of age only, bronchial hyperresponsiveness was evaluated by methacholine challenge (PD20), the cumulative dose causing a 20% drop in FEV_1, and at 10 years of age bronchodilator reversibility was assessed from pre- and post-bronchodilator spirometry 15 minutes after inhalation of a short-acting β2-agonist. All lung function measures were calibrated by regression for age, sex, and height using a cohort-internal reference population of children never diagnosed with asthma, using the relatively large lung healthy subgroup. Z-scores were then derived from this same reference group distribution. Low lung function was defined as values in the lowest decile of the reference distribution, below the 10^{th} percentile for spirometry ($z \leq -1.28$) and above the 90^{th} percentile for specific airway resistance (sRaw, $z \geq +1.28$) (*for additional details, see supplementary materials for* ***Paper III***).[172,174,175]

Other omics data layers

For **Paper III**, several omics data layers in $COPSAC_{2010}$ were used to comprehensively characterize these symptom-defined asthma-like phenotypes. This enabled a mechanistic understanding of how these phenotypes relate to asthma-relevant biological processes. All variables were selected *a priori* based on the underlying hypothesis and prior evidence of associations with asthma (*for additional details, see supplementary materials for* ***Paper III***).

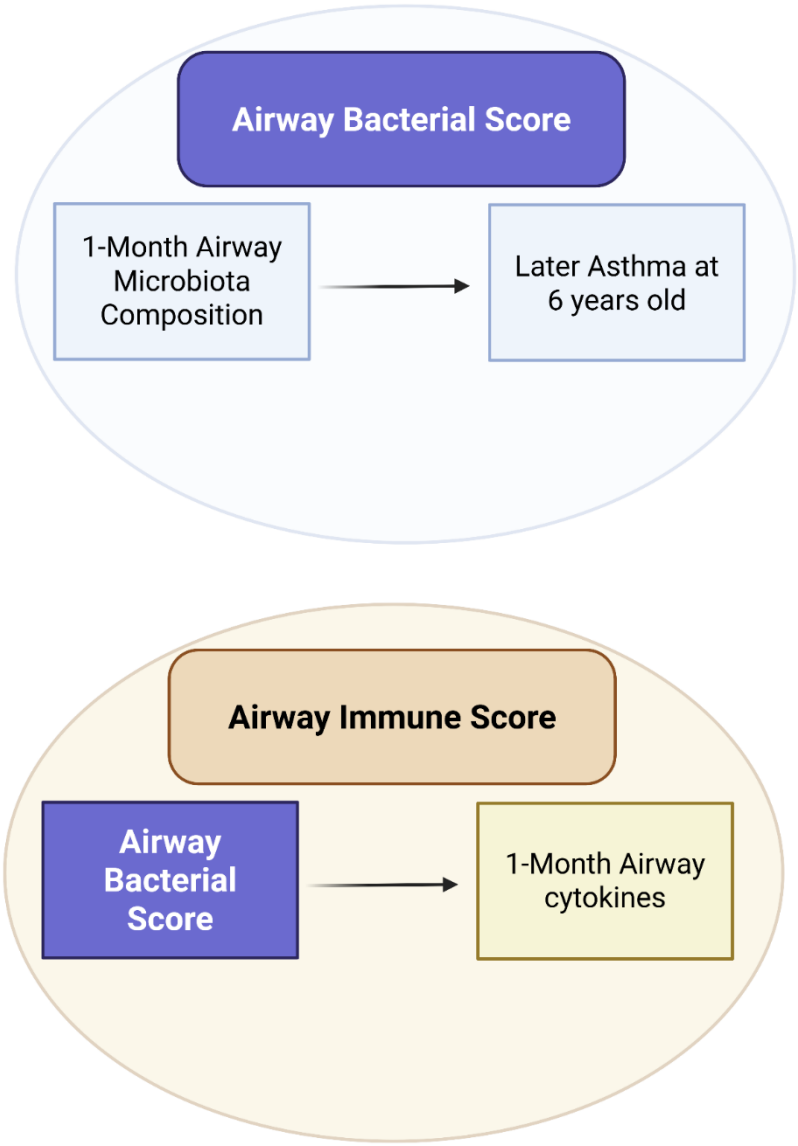
Gut microbiome

Gut microbiome maturity was assessed from stool samples collected at 1 week, 1 month, and 1 year of age using 16S rRNA gene sequencing. A microbiota by age z-score (MAZ) at 1 year was derived to quantify gut maturity relative to a non-asthma reference group, as has been previously published. For interpretability, the inverse value was used as a gut immaturity score, where higher values indicate greater immaturity and align directionally with other asthma-related scores.[176]

Airway scores

Two separate scores were derived using supervised modelling methods, as has been previously published (*see* ***Figure 2*** *and Statistical analysis,* ***Paper III***). First, an airway bacterial score was generated from 1-month airway microbiota composition based on hypopharyngeal sampling. This score was trained against asthma at 6 years of age and reflects an airway microbial signature of later asthma risk. Second, the airway bacterial score was used to derive a 1-month airway immune score

based on 18 nasal cytokines. The 1-month cytokines were measured using SAM filter paper, consistent with the 6-year cytokine measurements. Mediation analysis indicated that overlap between the airway immune score and bacterial score explained only approximately 10% of the airway immune score association with a six-year asthma diagnosis, supporting that the airway immune score reflects a largely distinct signal. This airway immune score therefore reflects a diverging asthma-related airway cytokine response.[163,177]



Thesis - Figure 2. *Overview of two airway asthma signature scores: airway bacterial score and airway immune score*

Genetics

Because genetic susceptibility is a key driver of asthma risk, five genetic scores were included, each reflecting distinct asthma or lung development related pathways, as has been previously published. A composite type 2 genetic score was constructed from risk single-nucleotide polymorphisms (SNPs) in interleukin 1 receptor-like 1 (*IL1RL1)*, *TSLP*, *IL13*, and *IL33* (two independent *IL33* variants), while a composite non-T2 genetic score was constructed from variants in *CDHR3* and *GSDMB*, incorporating their reported gene-to-gene interaction. These scores were coded as risk-allele dosages under an additive model (0, 1, 2) and weighted using published genome wide association studies (GWAS) effect estimates for childhood asthma, then summed to generate child-level scores. All composite genetic scores were standardized prior to analysis. In addition, polygenic risk scores (PRS) were computed for childhood asthma exacerbations (0 to 6 years), COPD, and dysanapsis using published GWAS summary statistics, and the resulting PRS were standardized before analysis. [178–181]

Airway pathogens during acute respiratory illnesses

During acute respiratory illness episodes lasting at least 3 days within the first 3 years of life, children were invited to the COPSAC research unit for physician assessment and biosampling, as previously described and published. Hypopharyngeal aspirates were obtained for bacterial culture with species-level identification, including *Haemophilus influenzae*, *Streptococcus pneumoniae*, and *Moraxella catarrhalis*. Nasopharyngeal aspirates were obtained for PCR-based viral testing, with primary analyses focused on known asthma-related respiratory viruses of respiratory syncytial virus (RSV) and rhinovirus (RV), including RV-C.[182]

Ethics

Ethical approvals were obtained in accordance with national guidelines. For $COPSAC_{2000}$, the study was approved by the local ethics committee (KF 01-289/96, H-16039498) and the Danish Data Protection Agency (2015-41-3696). For $COPSAC_{2010}$, the study was also approved by the local ethics committee (H-B-2008-093) and the Danish Data Protection Agency (2008-41-2599). For participation in both cohorts, parents provided oral and written informed consent. Both cohorts were monitored according to Good Clinical Practice (GCP) guidelines to ensure study quality and conducted in accordance with the Declaration of Helsinki.[143,146]

Statistical approach

Several statistical approaches were applied across the thesis. Categorical variables are presented as n (%) and continuous variables as mean (standard deviation [SD]) when approximately normally distributed, or median (interquartile range [IQR]) when otherwise. Associations were examined using regression and correlation methods, including linear regression, linear mixed models, generalized estimating equations (GEE), logistic regression, quasi-Poisson regression, multinomial models, and Spearman and Pearson correlations. Dimension-reduction and machine-learning approaches included unsupervised principal component analysis (PCA) and supervised sparse partial least squares (sPLS). Predictive performance was primarily evaluated using receiver operating characteristic (ROC) analyses with area under the curve (AUC) and accompanying predictive measures.

In **Papers I** and **III**, the primary aim was to quantify between-group differences as observed, similar to a clinical setting. Therefore, main analyses used no or minimal covariate adjustment to avoid attenuating differences that may be intrinsic to the phenotypes or lie on the causal pathway. In **Paper II**, covariate adjustment was applied to reduce bias from technical and contextual sources of variation in cytokine measurements, which can otherwise obscure biological differences (e.g., batch effects). Two-sided tests used $\alpha = 0.025$. P values were adjusted for multiple testing when applicable, with the applied adjustment stated where used. All analyses were performed in R (v4.2.1) using RStudio.
Paper-specific statistical approaches are described below.

Paper I

Overlap between elevated type 2 biomarkers was quantified in R by calculating proportions and then illustrated as Venn Diagrams using EulerAPE. Heatmaps were generated using Spearman correlation coefficients between type 2 biomarkers. For main analyses, logistic regression was used with T2-high vs T2-low at each time point.
For predictive analyses, early-life type 2 biomarkers (total IgE and specific IgE at 6 months, 18 months and 4 years) were modelled using logistic regression to discriminate T2-high versus T2-low asthma. Any elevation was coded as a binary yes, requiring all three time points to be available. These models were evaluated using ROC analysis, with sensitivity, specificity, and true and false positives reported. DeLong's test was used to compare AUCs. Vuong's likelihood ratio test was used to compare competing models based on total IgE alone, specific IgE alone, or their combination.

Sensitivity analyses included applying higher and lower cut-offs for type 2 biomarkers, comparing both T2-high and T2-low phenotypes with healthy controls, and adjusting models for sex, seasonality, paternal history of asthma, and atopic dermatitis ever

Paper II

Raw nasal cytokine concentrations (pg/mL) were transformed to enable robust between-child comparisons and to prevent analyses from being dominated by differences in cytokine scale, dispersion, and skewness. Cytokines were transformed using a predefined data processing method (log-transformation, scaling, and within-sample normalisation) to place markers on a comparable scale while accounting for variation in recovered sample material, with full details provided in the supplementary materials for **Paper II**. This overall approach has been applied in previous COPSAC nasal cytokine analyses at early-life visits and during acute respiratory episodes. Sensitivity analyses on the raw measurements confirmed consistent directionality and key associations before and after transformation. As an additional sensitivity analysis, a centered log-ratio (CLR) transformation was applied to assess robustness.

Supplementary materials describe this process in detail, including the transformation rationale and handling of values below the lower limit of detection and missingness (*see supplementary materials for **Paper II***).

Relationship between continuous levels of cytokines and type 2 inflammation biomarkers were summarised using Spearman's correlation heatmaps. Unsupervised cytokine structure was examined using principal component analysis (PCA), and linear models were used to test whether component 1 and 2 differed between T2-high asthmatics and asthma-free controls. Cytokine associations with type 2 inflammatory diseases and related biomarkers were analysed by adjusted linear regression, accounting for technical and contextual factors recorded at sampling and analysis (including batch-related variables, season, recent airway infection, and recent intranasal or inhaled steroid use). Multiple testing in the 24 cytokines was addressed using false discovery rate (FDR) adjustment.

For predictive modelling, a sPLS model was trained with log-transformed b-eos as the outcome and cytokines as predictors, using an 80/20 train-test split with 10-fold cross-validation repeated 10 times within the training set. A 15-feature model was selected based on root mean squared error and R^2. The resulting cytokine-derived type 2 score was compared with blood eosinophils using ROC analysis and DeLong's test for discrimination of type 2-associated diseases at six years, and for prediction at ten years in children.

Sensitivity analyses included alternative reference groups (biomarker-clean and type 2 disease-clean) to assess whether findings reflected disease-specific effects or a broader type 2 inflammatory profile.[163,177].

Paper III

Analyses of symptom diary recordings included only children with ≥90% completeness in total for the first three years of life. For count outcomes, quasi-Poisson models with a log link and exposure offsets (total valid diary days) were used to estimate incidence rate ratios (IRRs). Lung function was analysed using linear mixed-effects models with child ID as a random intercept and fixed-effect adjustment for visit age. GEE models estimated odds ratios (ORs) for asthma from 6 to 10 years, accounting for repeated asthma status within each child. Standardized continuous outcomes (biomarkers and omics-derived composite scores) were analysed using linear regression. Phenotype was analysed using multinomial regression, modelling the three-group outcome simultaneously to obtain more stable estimates than multiple pairwise models, with healthy as the reference category. Predictive performance was assessed using ROC analysis quantified by AUC, with DeLong's test for comparison and Youden's index to identify optimal cut-offs balancing sensitivity and specificity. Sensitivity analyses included adjustment for genetics using PRS, adjustment for maternal RCT interventions (vitamin D and fish oil), and use of stricter objectively validated asthma as a later outcome.

RESULTS

Paper I (main results)

Cohort-specific type 2 characteristics

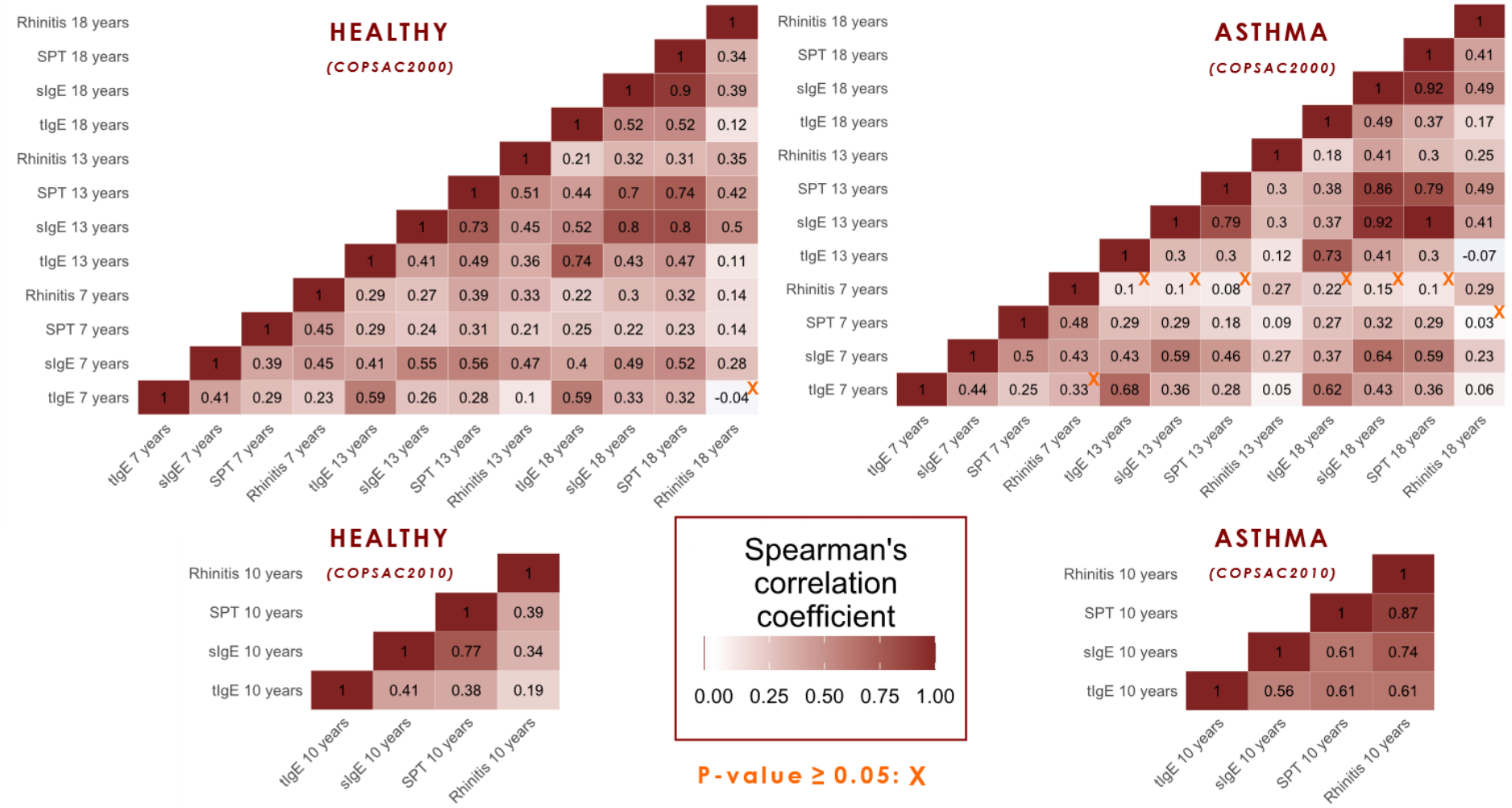
Cohort	COPSAC2000 (N = 411)			COPSAC2010 (N = 700)
Time point	7 years (%)	13 years (%)	18 years (%)	10 years (%)
Sex (Male)	169/336 (50.3)	180/362 (49.7)	183/371 (49.3)	330/640 (51.6)
Asthma (Yes)	47/336 (14.0)	62/362 (17.1)	94/371 (25.4)	42/640 (6.6)
T2-high asthma (Yes)	34/334 (10.2)	33/353 (9.3)	55/362 (15.2)	23/631 (3.6)
b-eos (Elevated)	204/311 (65.6)	106/308 (34.4)	92/337 (27.3)	208/511 (40.7)
FeNO (Elevated)	27/317 (8.5)	77/330 (23.3)	150/365 (41.1)	91/598 (15.2)
tIgE (Elevated)	122/296 (41.2)	129/315 (41.0)	173/355 (48.7)	248/532 (46.6)
sIgE (Elevated)	76/296 (25.7)	146/315 (46.3)	187/355 (52.7)	187/532 (35.2)
SPT (Elevated)	24/290 (8.3)	115/316 (36.4)	175/358 (48.9)	148/532 (27.8)
Rhinitis symptoms (Yes)	33/214 (15.4)	133/357 (37.3)	163/370 (44.1)	112/636 (17.7)

Paper I - Table 1: Cohort-specific characteristics of the study population. Type 2 biomarkers elevation was defined according to pre-specified thresholds. *Reproduced from Sultan T, et al. Levels of total IgE versus specific IgE during childhood for defining and predicting T2-high asthma. World Allergy Organ J. 2024;17(12):100994. © 2024. This manuscript version is made available under the CC-BY-NC-ND 4.0 license http://creativecommons.org/licenses/by-nc-nd/4.0/*

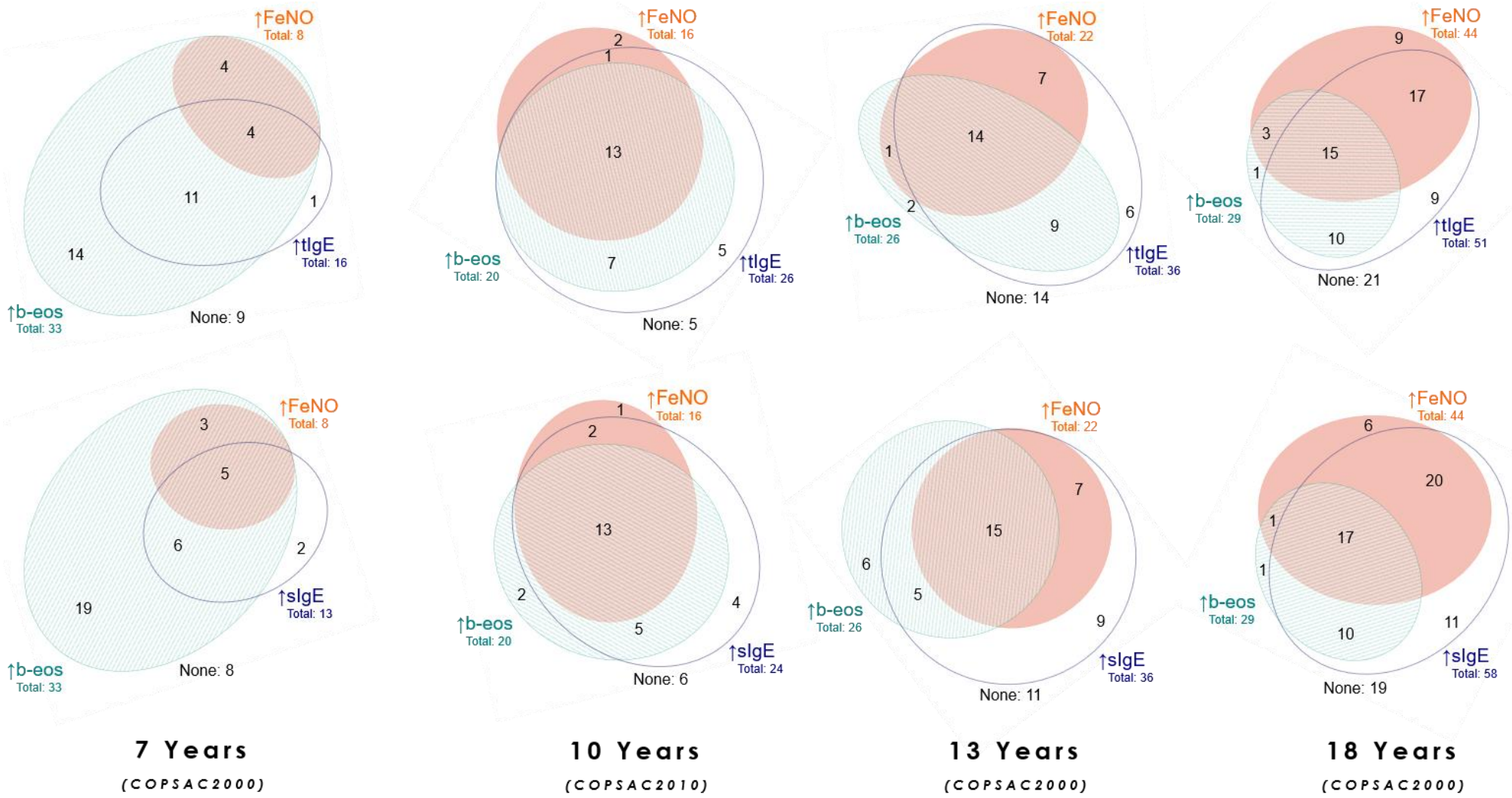
Paper I - Table 1 presents the characteristics of the study population in relation to asthma and type 2 biomarkers in both cohorts. Both asthma and T2-high asthma was more frequent in the high-risk $COPSAC_{2000}$ cohort. Overall, similar age-related trends were seen, with decreasing b-eos elevation, increasing FeNO, aeroallergen sensitization by specific IgE or SPT, and symptoms of rhinitis, and relatively stable total immunoglobulin E values. T2-low asthma was less frequent than T2-high asthma at all ages 7 (4.2%), 10 (1.6%), 13 (4.0%), and 18 years (8.3%).

Additionally, for early timepoints between ages 0-4 years we also saw increasing levels of total IgE at ages 0.5 (31.6%), 1.5 (37.5%), and 4 years (40.1%) and likewise for specific IgE at ages 0.5 (0.5%), 1.5 (3.4%), and 4 years (10%).

Type 2 biomarkers and asthma



Paper I - Figure 1: *Correlation in biomarkers of type 2 inflammation in asthmatic and non-asthmatic children. Reproduced from Sultan T, et al. Levels of total IgE versus specific IgE during childhood for defining and predicting T2-high asthma. World Allergy Organ J. 2024;17(12):100994. © 2024. This manuscript version is made available under the CC-BY-NC-ND 4.0 license http://creativecommons.org/licenses/by-nc-nd/4.0/*



Paper I - Figure 2: *Visualization by Venn diagram of asthmatic children with overlap of conventional type 2 biomarkers of b-eos and FeNO and either total IgE (top) or specific IgE (bottom). Reproduced from Sultan T, et al. Levels of total IgE versus specific IgE during childhood for defining and predicting T2-high asthma. World Allergy Organ J. 2024;17(12):100994. © 2024. This manuscript version is made available under the CC-BY-NC-ND 4.0 license http://creativecommons.org/licenses/by-nc-nd/4.0/*

Specific IgE values and RAST groupings at different ages were examined (**Paper I - Supplementary Table 2**). Likewise, details on monosensitization, polysensitization, and sensitization patterns to individual aeroallergens based on specific IgE were also examined (**Paper I - Supplementary Table 3**)

Correlation analyses showed modest correlations between the two IgE biomarkers at all time points in children with asthma (rho = 0.30 to 0.49, all $p<0.001$), with slightly stronger correlations in children without asthma (rho = 0.41 to 0.56, all $p<0.001$) (**Paper I - Figure 1**).

Descriptive Venn diagrams showed that total IgE and specific IgE identified a broadly similar proportion of children with asthma who also had elevated b-eos, FeNO, or both, consistent with conventional T2-high asthma definition. Both biomarkers also captured a small subgroup of children with asthma not identified by these conventional markers alone (**Paper I - Figure 2**).

Endotyping T2-high and T2-low asthma

In concurrent analyses at each time point, elevated total IgE was consistently associated with T2-high asthma compared with T2-low asthma from 7 to 18 years of age (all $p\leq0.03$). Elevated specific IgE showed a similar pattern, with a no association at age 7 years (Odds ratio: 2.00, p-value: 0.43) but stronger associations at older ages. Positive SPT and symptoms of rhinitis were also associated with T2-high asthma at several later time points, although findings were less consistent in early childhood (**Paper I - Table 2)**. By ROC discrimination analyses, total IgE showed higher specificity at age 7 years, with a shift toward higher sensitivity at later ages. Specific IgE showed a similar but less consistent pattern (**Paper I - Supplementary Table 5**).

Overall, when comparing biomarker endotyping capabilities using Vuong's likelihood ratio test of sole IgE models versus a combined IgE model, only age 13 years showed improved fit for the combined model compared with specific IgE alone, while no advantage was seen over total IgE alone (**Paper I - Supplementary Table 4**).

Predicting T2-high and T2-low asthma

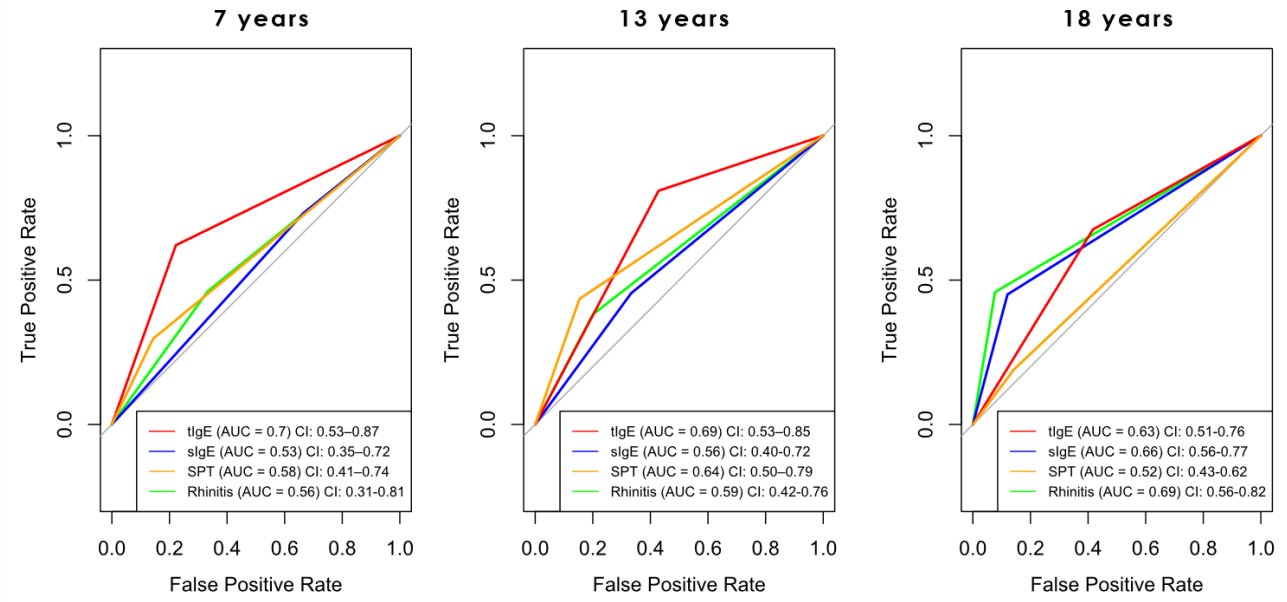
When early-life type 2 markers were related to later T2-high versus T2-low asthma, total IgE showed the most consistent signal, with positive associations at all ages. Specific IgE was associated with later T2-high asthma only at age 18 years. SPT positivity was not associated with later T2-high

asthma, while symptoms of rhinitis were associated with later T2-high asthma only at age 18 years (**Paper I - Table 2**).

	Elevated tIgE, sIgE, SPT or rhinitis symptoms and concurrent T2-high vs T2-low asthma				Elevated tIgE, sIgE, SPT or rhinitis symptoms at early age and later T2-high vs T2-low asthma			
	N (T2-high	Odds ratio	Confidence interval	P-value	N (T2-high)	Odds ratio	Confidence interval	P-value
7 Years								
tIgE	33	7.50	[1.20–146.49]	0.03	29	5.73	[1.14–43.37]	0.03
sIgE	33	2.00	[0.41–14.77]	0.43	30	0.73	[0.15–4.08]	0.70
SPT	31	3.27	[0.49–65.28]	0.24	27	2.53	[0.35–51.78]	0.42
Rhinitis	14	1.50	[0.19–14.25]	0.70	13	1.71	[0.24–15.75]	0.60
10 Years								
tIgE	23	10.50	[1.74–90.78]	0.03	-	-	-	-
sIgE	23	10.00	[1.88–67.27]	0.007	-	-	-	-
SPT	23	15.56	[2.83–115.51]	0.001	-	-	-	-
Rhinitis	23	7.13	[1.43–42.08]	0.02	23	3.67	[0.72–27.96]	0.12
13 Years								
tIgE	33	23.33	[5.70–127.87]	<0.001	21	5.67	[1.31–28.62]	0.02
sIgE	33	5.50	[1.64–20.39]	0.005	22	1.67	[0.44–6.88]	0.46
SPT	30	3.00	[0.94–10.08]	0.94	23	4.23	[0.87–31.52]	0.10
Rhinitis	33	3.26	[1.03–10.86]	0.04	21	2.46	[0.46–19.12]	0.32
18 Years								
tIgE	55	7.54	[2.87–21.40]	<0.001	40	2.91	[1.04–8.52]	0.04
sIgE	55	10.15	[3.67–30.75]	<0.001	40	6.00	[1.72–28.28]	0.004
SPT	54	10.00	[3.66–29.86]	<0.001	42	1.41	[0.36–7.05]	0.63
Rhinitis	55	4.85	[1.89–13.04]	<0.001	24	10.15	[1.60–200.12]	0.01

Paper I - Table 2: Cross-sectional endotyping and predictive logistic regression models to discriminate T2-high from T2-low asthma using either total or specific IgE. *Reproduced from Sultan T, et al. Levels of total IgE versus specific IgE during childhood for defining and predicting T2-high asthma. World Allergy Organ J. 2024;17(12):100994. © 2024. This manuscript version is made available under the CC-BY-NC-ND 4.0 license http://creativecommons.org/licenses/by-nc-nd/4.0/*

This pattern was supported by ROC-based discrimination analyses, with total IgE tending to perform slightly better than specific IgE, although the differences were not statistically significant (all p>0.20; **Paper I - Figure 3**). Total IgE also showed a shift from higher specificity at age 7 years to higher sensitivity at later ages, whereas this pattern was opposite for specific IgE (**Paper I - Supplementary Table 6**).



Paper I - Figure 3: *ROC curves for prediction of later T2-high versus T2-low asthma by elevated early-life type 2 markers. Reproduced from Sultan T, et al. Levels of total IgE versus specific IgE during childhood for defining and predicting T2-high asthma. World Allergy Organ J. 2024;17(12):100994. © 2024. This manuscript version is made available under the CC-BY-NC-ND 4.0 license http://creativecommons.org/licenses/by-nc-nd/4.0/*

Sensitivity analyses

Sensitivity analyses using alternative cut-offs for b-eos and total IgE, comparisons with healthy children instead of the T2-low asthma group, and covariate adjustment for sex, season, paternal symptoms, and ever atopic dermatitis showed similar results (**Paper I - Supplementary Tables 7-10**).

Paper II (main results)

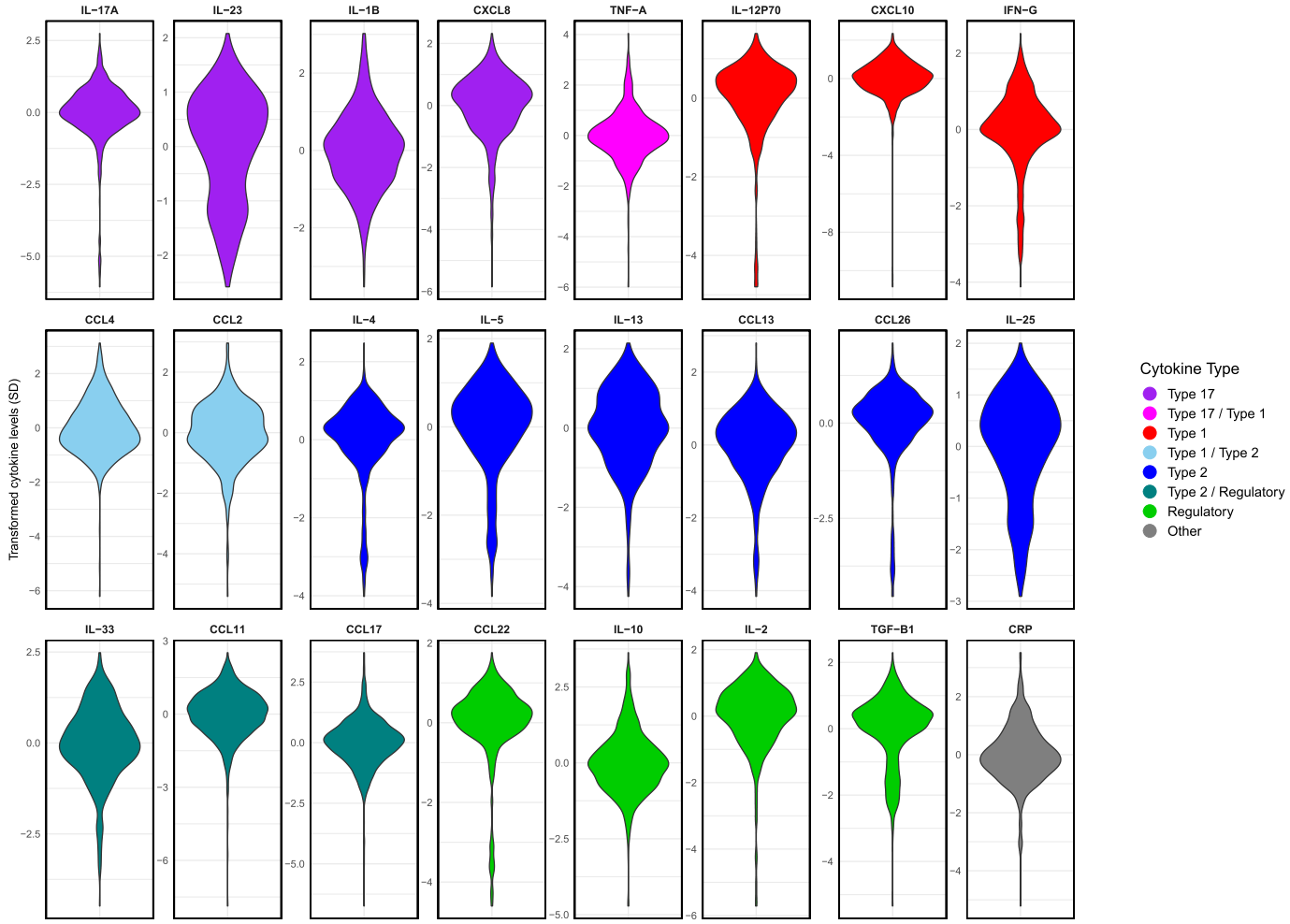
Cohort and airway cytokine sampling characteristics

Characteristic	Total (N = 612)		Asthma (n = 75)		Control group (n = 537)	
	Value	Missing	Value	Missing	Value	Missing
Demographics						
Sex (male)	310 (50.7)	0	46 (61.3)	0	264 (49.2)	0
Age (years)	6.0 [5.9-6.1]	0	6.0 [5.9-6.0]	0	6.0 [5.9-6.1]	0
T2 and non-T2 diseases						
T2-high asthma diagnosis (yes)	35 (5.7)	21 (3.4)	35 (46.7)	21 (28.0)	0	0
T2-low asthma diagnosis (yes)	19 (3.1)	21 (3.4)	19 (25.3)	21 (28.0)	0	0
AR (yes)	43 (7.0)	5 (0.8)	12 (16.0)	2 (2.7)	31 (5.8)	3 (0.6)
NAR (yes)	33 (5.4)	5 (0.8)	12 (16.0)	2 (2.7)	21 (3.9)	3 (0.6)
Allergic conjunctivitis (yes)	45 (7.4)	3 (0.5)	16 (21.3)	0	29 (5.4)	3 (0.6)
AD (yes)	75 (12.3)	7 (1.1)	18 (24.0)	0	57 (10.6)	7 (1.3)
T2 biomarkers						
Blood eosinophils (elevated)	217 (35.5)	129 (21.1)	30 (40.0)	14 (18.7)	187 (34.8)	115 (21.4)
FENO (elevated)	18 (2.9)	244 (39.9)	4 (5.3)	32 (42.7)	14 (2.6)	212 (39.5)
Blood specific IgE (elevated)	108 (17.7)	86 (14.1)	18 (24.0)	10 (13.3)	90 (16.8)	76 (14.2)
SPT (elevated)	42 (6.9)	81 (13.2)	9 (12.0)	11 (14.7)	33 (6.1)	70 (13.0)
Cytokine sampling						
Infection within 7 days of sampling (yes)	77 (12.6)	0	12 (16.0)	0	65 (12.1)	0
Nasal steroid within 5 days of sampling (yes)	6 (1.0)	0	2 (2.7)	0	4 (0.8)	0
Inhalation steroid within 2 weeks of sampling (yes)	23 (3.8)	0	22 (29.3)	0	1 (0.2)	0
Season of sampling: winter	106 (17.3)	0	11 (14.7)	0	95 (17.7)	0
Season of sampling: spring	131 (21.4)	0	17 (22.7)	0	114 (21.2)	0
Season of sampling: summer	171 (27.9)	0	24 (32.0)	0	147 (27.4)	0
Season of sampling: fall	204 (33.3)	0	23 (30.7)	0	181 (33.7)	0

Paper II - Table 1: *Study characteristics of children with/without asthma at six years of age. Elevation of type 2 biomarkers were set according to prespecified thresholds. Data are presented as median (IQR) or mean (SD). AR = allergic rhinitis; NAR = non-allergic rhinitis.*

At the scheduled 6-year visit in the COPSAC2010 cohort, nasal sample data were unavailable for 88 of 700 children (12.6%). The analysis therefore included 612 children, of whom 75 (12.3%) had an asthma diagnosis. In the full study population, 35 children (5.7%) were T2-high asthmatics, 19 (3.1%) were T2-low asthmatics, and 21 (3.4%) were unclassified because data on type 2 biomarkers were missing. Potential cytokine sampling-related factors were as shown, and models for regression were additionally adjusted for batch effect and age (**Paper II - Table 1**).

Standardized cytokine distributions by immune response type are presented in **Paper II - Figure 1**, with the corresponding pre-transformed raw cytokine distribution shown in **Paper II - Supplementary Figure 1**.



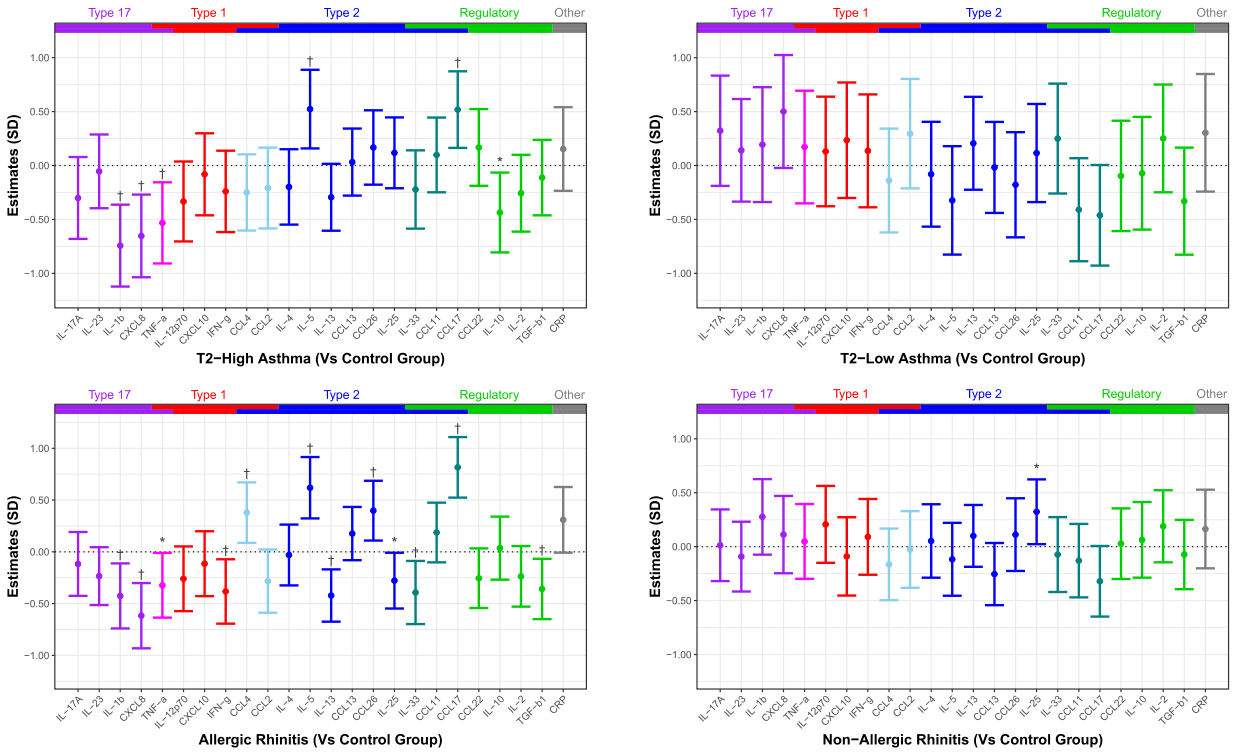
Paper II - Figure 1: *Airway cytokine distribution by immune response type. Reproduced from Sultan T, et al. Dominance of IL-5 and CCL17 in nasal cytokine profiles of children with type 2 inflammation. J Allergy Clin Immunol. 2025 Dec 4:S0091-6749(25)01181-9. doi:10.1016/j.jaci.2025.11.009. © 2025. This manuscript version is made available under the CC-BY-NC-ND 4.0 license http://creativecommons.org/licenses/by-nc-nd/4.0/*

Relationship between type 2 and non-type 2 diseases and airway cytokine levels

Compared with the non-asthmatic control group, T2-high asthmatics had higher IL-5 and CCL17 levels, corresponding to a 0.52-SD increase in IL-5 (p=0.03 after FDR correction) and a 0.52-SD increase in CCL17 (p=0.03 after FDR correction), while lower IL-1β, C-X-C motif chemokine ligand 8 (CXCL8), and tumor necrosis factor alpha (TNF-α) levels were also observed (**Paper II - Figure 2**).

Compared with the non-disease control group, those with allergic rhinitis had higher IL-5, CCL17, CCL26, and CCL4 levels, corresponding to a 0.62-SD increase in IL-5 (p=0.0006 after FDR

correction), a 0.82-SD increase in CCL17 (p<0.0001 after FDR correction), a 0.40-SD increase in CCL26 (p=0.03 after FDR correction), and a 0.38-SD increase in CCL4 (p=0.04 after FDR correction), together with a 0.31-SD decrease in IL-13 (p=0.001 after FDR correction), while lower IL-1β, CXCL8, and Interferon gamma (IFN-γ) levels were also observed (**Paper II - Figure 2**).

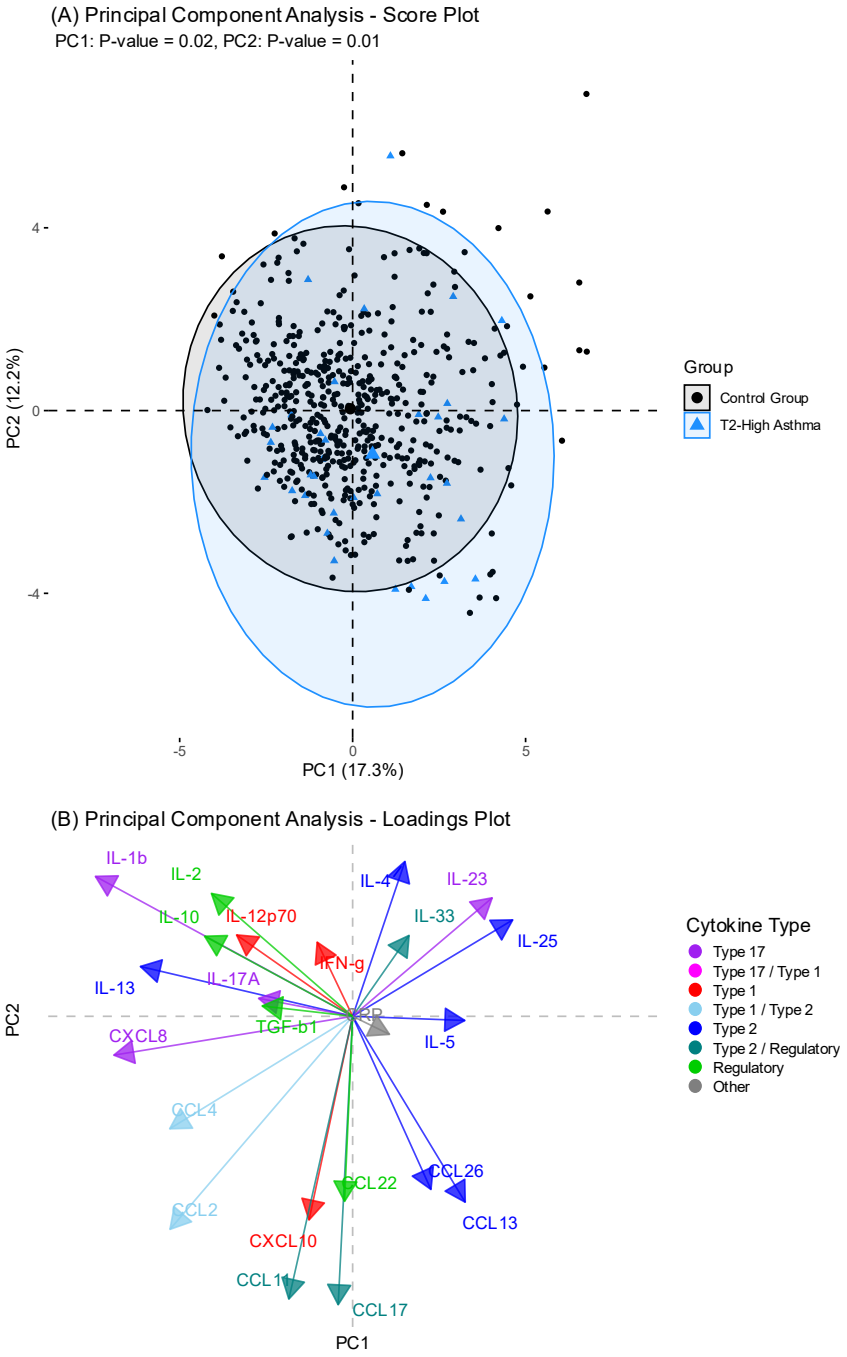


Paper II - Figure 2: *Airway cytokine levels by type 2 inflammation phenotypes and endotypes. Analyses were adjusted for cytokine sampling covariates using linear regression models. * denotes nominal statistical significance at p<0.05; † denotes significance after FDR adjustment at p<0.05. Reproduced from Sultan T, et al. Dominance of IL-5 and CCL17 in nasal cytokine profiles of children with type 2 inflammation. J Allergy Clin Immunol. 2025 Dec 4:S0091-6749(25)01181-9. doi:10.1016/j.jaci.2025.11.009. © 2025. This manuscript version is made available under the CC-BY-NC-ND 4.0 license http://creativecommons.org/licenses/by-nc-nd/4.0/*

Neither T2-low asthma nor non-allergic rhinitis showed significant associations compared with the non-disease control group after FDR correction (**Paper II - Figure 2**).

Head-to-head comparisons between type 2 and non-type 2 endotypes and phenotypes showed weaker signals than the disease versus control analyses. Although nominal associations followed a similar pattern, no airway cytokines differed between T2-high asthma (n=35) and T2-low asthma (n=19) after correcting for multiple testing. In contrast, children with allergic rhinitis (n=43) had higher CCL17 levels than those with non-allergic rhinitis (n=33) (**Paper II - Supplementary Figure 4**).

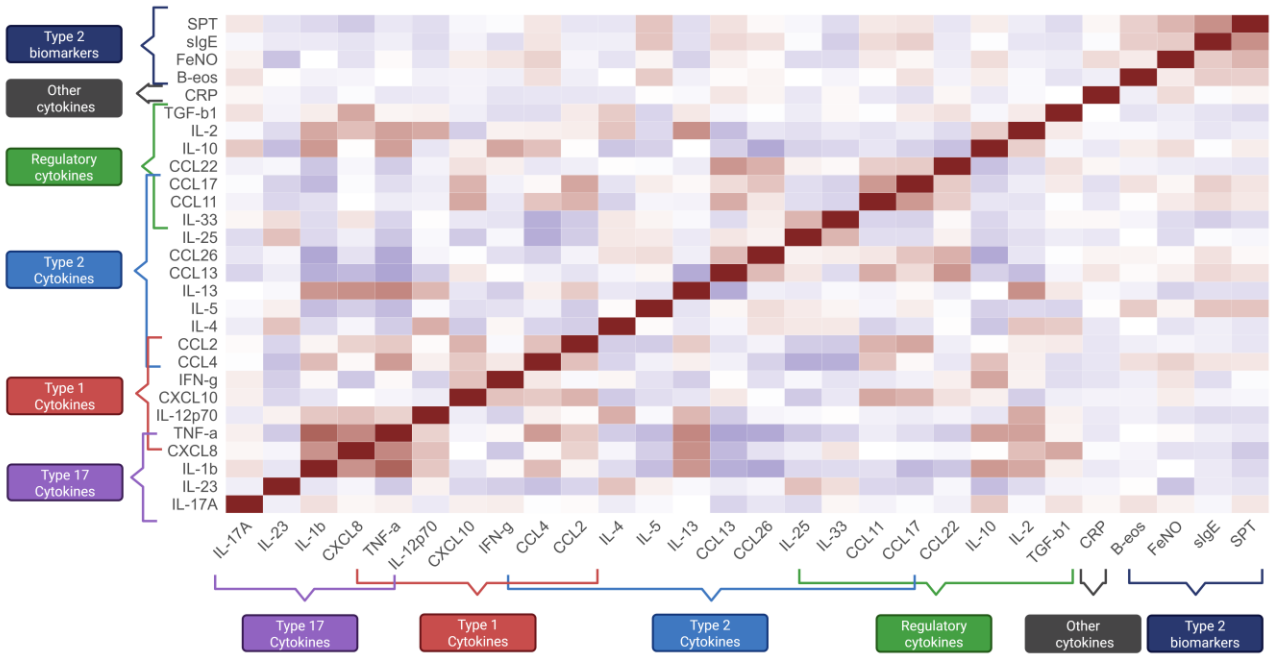
Other non-airway type 2 inflammatory diseases of allergic conjunctivitis and atopic dermatitis also showed weaker signals than the airway-specific diseases. Allergic conjunctivitis was associated with higher CCL17 levels, whereas no differences in airway cytokine levels were observed for atopic dermatitis after FDR adjustment (**Paper II - Supplementary Figure 3**).



Paper II - Figure 3: *Unsupervised principal component analysis shows airway cytokine patterns for T2-high asthma and non-asthmatic control group. Reproduced from Sultan T, et al. Dominance of IL-5 and CCL17 in nasal cytokine profiles of children with type 2 inflammation. J Allergy Clin Immunol. 2025 Dec 4:S0091-6749(25)01181-9. doi:10.1016/j.jaci.2025.11.009. © 2025. This manuscript version is made available under the CC-BY-NC-ND 4.0 license* http://creativecommons.org/licenses/by-nc-nd/4.0/

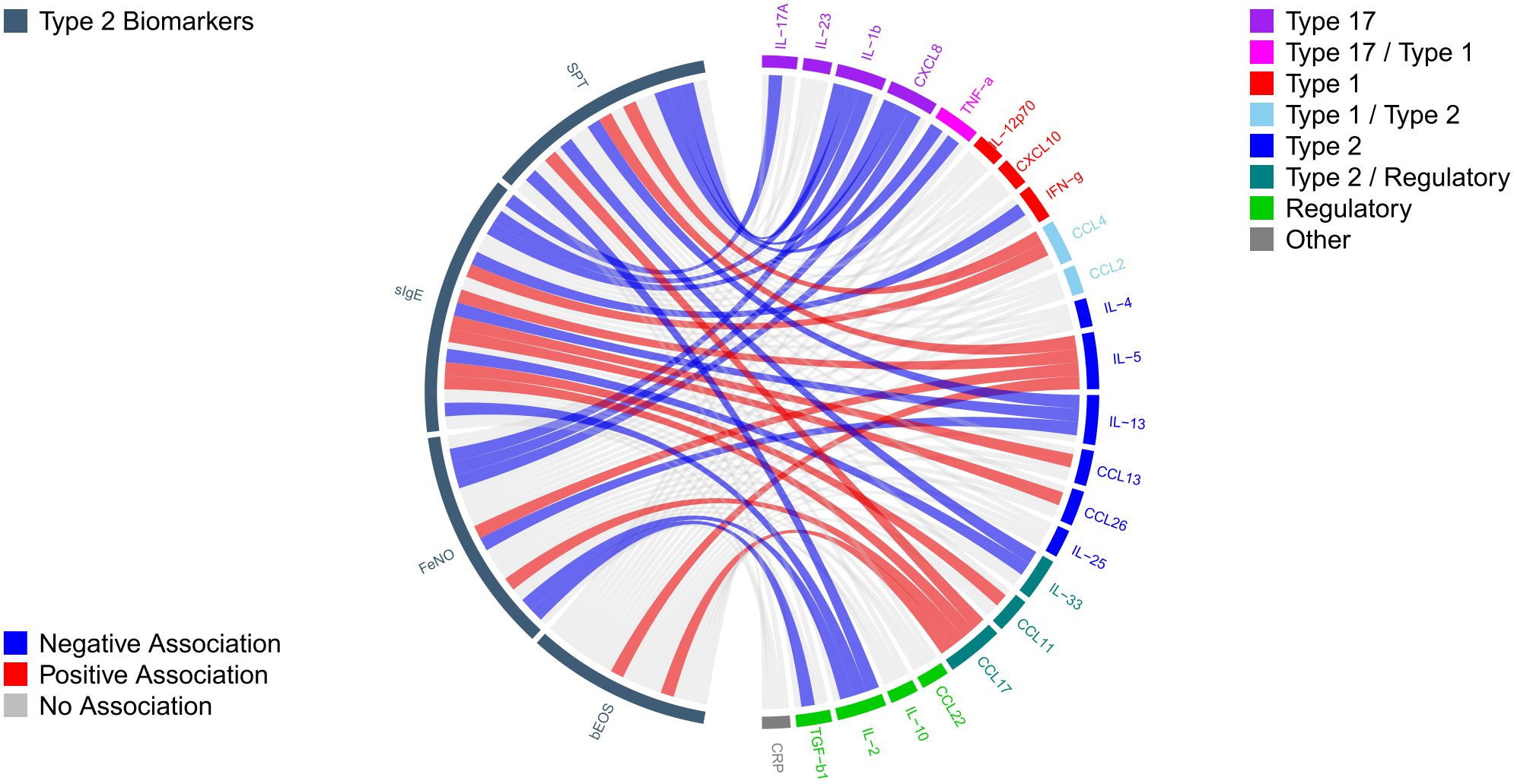
Using an unsupervised PCA approach, distinct cytokine patterns emerged in those with T2-high asthma and the non-asthmatic control group, consistent with the previous regression models. T2-high asthmatics clustered toward a positive PC1 and negative PC2 in the score plot, in line with IL-5, CCL17, CCL26, and CCL13. Both PC scores differed significantly between those with T2-high asthma and the non-asthmatic control group (**Paper II - Figure 3**).

Relationship between type 2 biomarkers and airway cytokine levels



Paper II - Figure 4: *Heatmap showing correlations between type 2 biomarkers and airway cytokines from nasal samples. Reproduced from Sultan T, et al. Dominance of IL-5 and CCL17 in nasal cytokine profiles of children with type 2 inflammation. J Allergy Clin Immunol. 2025 Dec 4:S0091-6749(25)01181-9. doi:10.1016/j.jaci.2025.11.009. © 2025. This manuscript version is made available under the CC-BY-NC-ND 4.0 license http://creativecommons.org/licenses/by-nc-nd/4.0/*

Correlations between continuous type 2 biomarkers and airway cytokines were examined, with sensitization variables analysed as binary outcomes using the predefined thresholds. IL-5, CCL17, CCL26, CCL13, and CCL4 were positively correlated with all measured biomarkers of type 2 inflammation, although correlations were low to modest (rho=0.02 to 0.26). IL-13 was inversely correlated with all type 2 biomarkers. Type 1, type 17, and regulatory cytokines were otherwise mostly uncorrelated or inversely correlated with type 2 biomarkers (**Paper II - Figure 4**).



Paper II - Figure 5: *Airway cytokine levels by type 2 biomarker elevation in all children. Analyses were adjusted for cytokine sampling covariates in linear regression models, and only results with FDR-adjusted p<0.05 are presented. Reproduced from Sultan T, et al. Dominance of IL-5 and CCL17 in nasal cytokine profiles of children with type 2 inflammation. J Allergy Clin Immunol. 2025 Dec 4:S0091-6749(25)01181-9. doi:10.1016/j.jaci.2025.11.009. © 2025. This manuscript version is made available under the CC-BY-NC-ND 4.0 license http://creativecommons.org/licenses/by-nc-nd/4.0/*

In the full study population, analyses adjusted for sampling covariates showed that, among FDR-significant findings, that the four measures of elevated type 2 biomarkers were most consistently positively associated with IL-5 and CCL17, with additional positive associations for CCL4 in two of the four measures. The most consistent negative associations were seen for IL-2, IL-1β, CXCL8, and IL-13, each in three of the four measures, followed by TNF-α and IL-33, each in two (**Paper II - Figure 5)**.

Airway cytokine profiles compared to conventional systemic b-eos

The discriminatory ability of b-eos and an sPLS-derived airway type 2 cytokine composite score was assessed in concurrent (endotyping) and future disease analyses (predictive). At age 6 years, b-eos showed a numerically higher AUC than the cytokine score for T2-high asthma versus the non-asthmatic control group (0.77 vs 0.65), whereas the cytokine score showed a numerically higher AUC than b-eos for allergic rhinitis versus the control group (0.75 vs 0.68), but neither difference was statistically significant (p=0.10 and p=0.07, respectively). For the predictive analyses at age 10 years, in those without type 2 disease or sensitization at age six years, b-eos and the airway type 2 score performed similarly for both T2-high asthma (AUC 0.61 vs 0.60, p=0.97) and allergic rhinitis (AUC 0.54 vs 0.52, p=0.84) (**Paper II - Supplementary Table 3**).

Sensitivity analyses

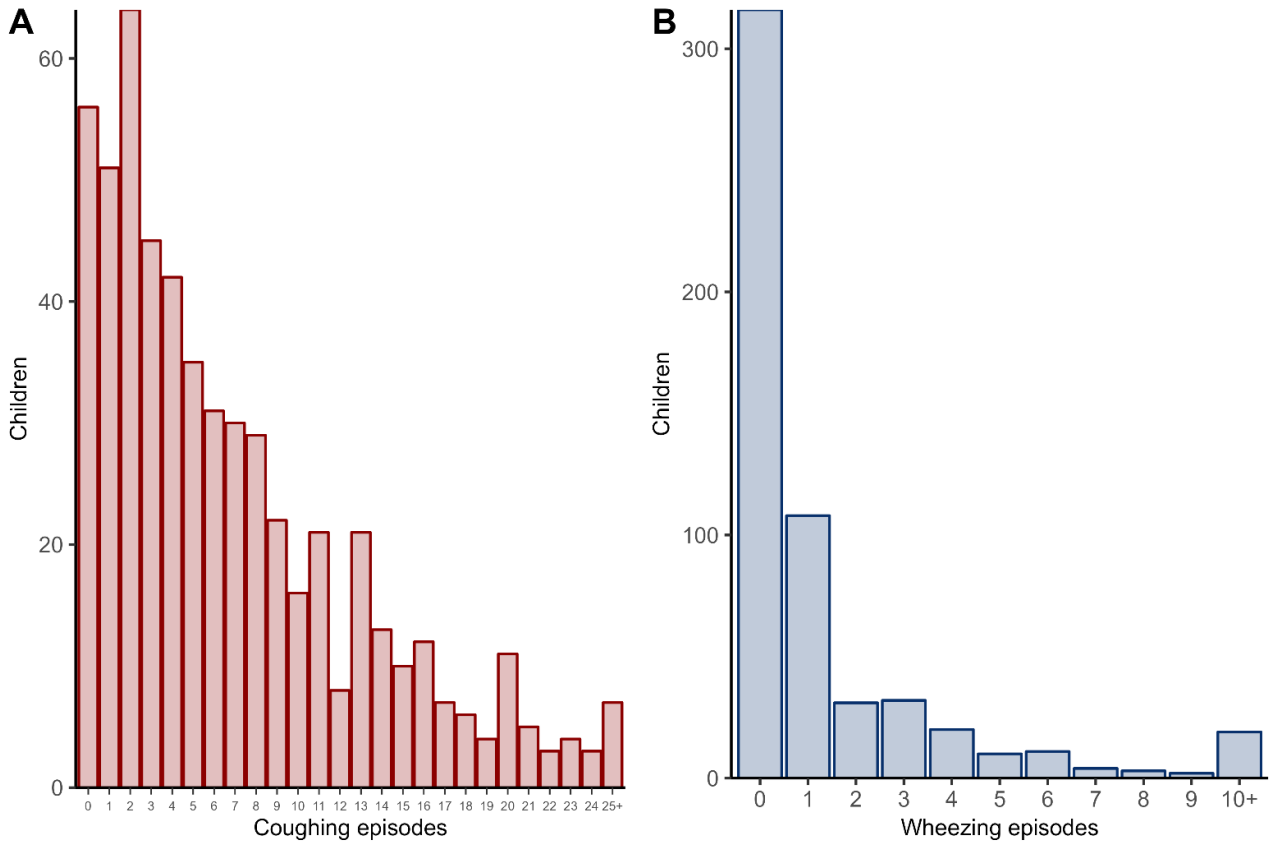
Sensitivity analyses with additional asthma adjustment, raw and alternative cytokine data transformation testing, a higher threshold for sensitization of aeroallergen-specific IgE, and different control group definitions supported the main findings, as the overall airway cytokine patterns remained largely unchanged (**Paper II – Supplementary Figures 5-11).**

Paper III (main results)

Characteristics and selection of the study population

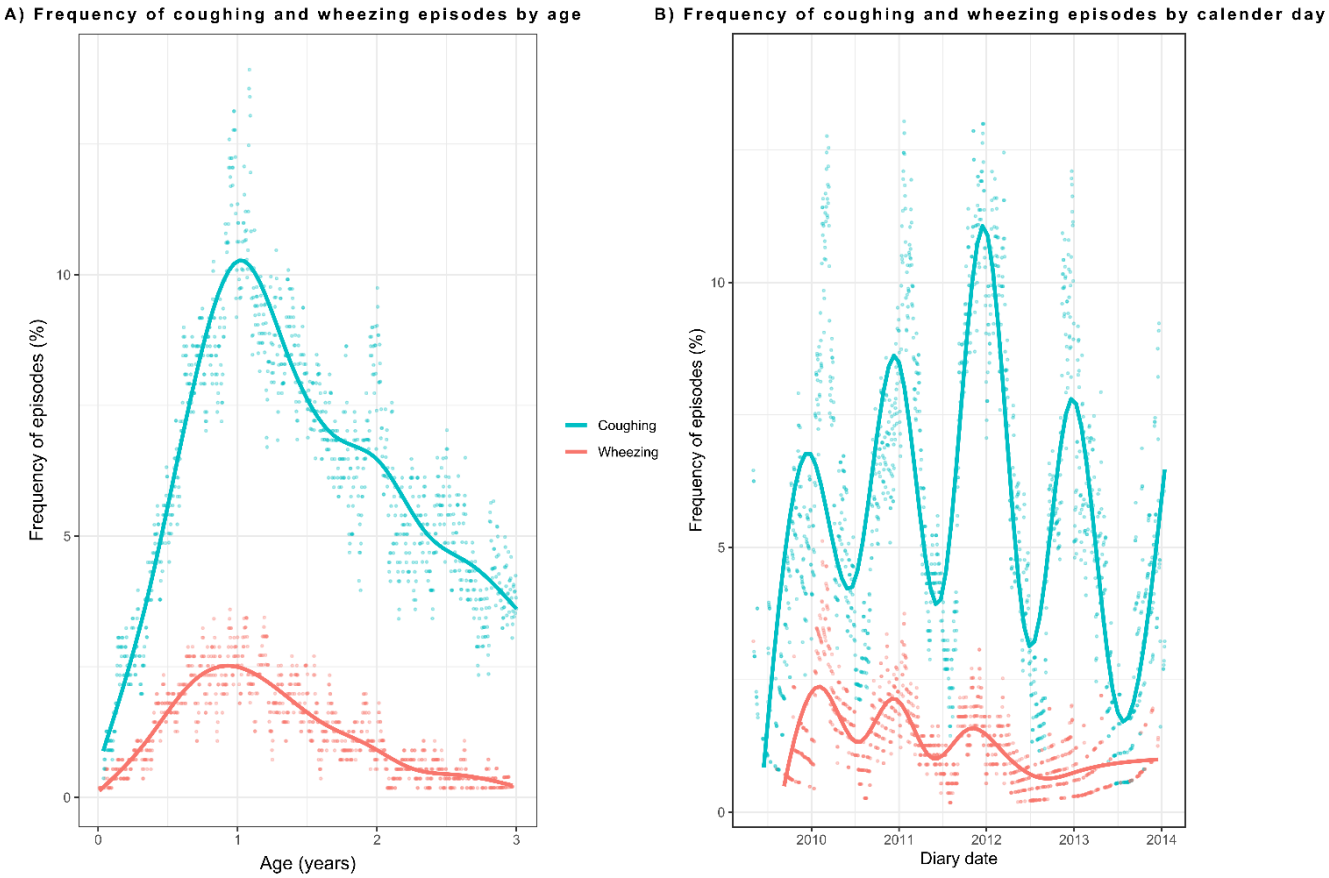
in $COPSAC_{2010}$, 144 (20.6%) out of 700 children were excluded because daily symptom diary completion was below 90% in the first three years of life. The analysis therefore included 556 children (79.4%). Comparison of baseline characteristics between included and excluded children showed that excluded children more often came from low-income households in the first year of life (p=0.03), were more often born by cesarean delivery (p=0.04), and were more often exposed to maternal smoking during pregnancy (p=0.04) (**Paper III - Supplementary Table 1**).

Early-life cough and wheeze as continuous traits (characteristics)



Paper III - Figure 1: *Distribution of coughing and wheezing episodes during the first 3 years of life in the full study population (n=556), with coughing shown in panel A and wheezing in panel B.*

Using daily symptom recordings during the first three years life, coughing episodes were more common and more widely distributed than wheezing episodes (**Paper III - Figure 1**). When investigating age-related and seasonal patterns of coughing and wheezing episodes, peaks around 1 year of age (12 months), and winter were seen (**Paper III - Figure 2**).



Paper III - Figure 2: *Age-related and seasonal patterns of coughing and wheezing episodes during the first 3 years of life in the full study population. Frequency of episodes by age (A) and temporal distribution from 2009 to 2014 (B), expressed as the proportion of children with an ongoing coughing or wheezing episode among those with valid diary entries on the corresponding day.*

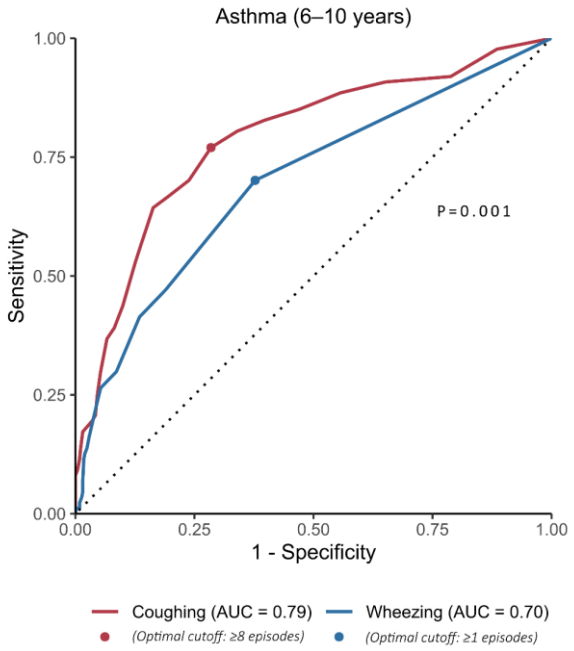
For daily symptom recordings, both symptom days and symptom episodes, defined by at least 3 consecutive days of symptoms separated by at least 3 symptom-free days, were available. For both symptoms, symptom days and symptom episodes were strongly correlated (rho=0.94), indicating that either metric captured symptom burden similarly. Episodes were therefore used as the primary metric because of greater clinical applicability. The number of coughing and wheezing episodes was moderately correlated (rho=0.51), whereas the number of coughing episodes was almost identical to the total number of episodes with any troublesome lung symptoms (rho=0.99) (**Paper III - Supplementary Figure 3**).

Early-life cough and wheeze as continuous traits (later asthma)

	Single-symptom model vs later asthma		Mutually adjusted model vs later asthma	
Exposure	OR (95% CI)	P-value	OR (95% CI)	P-value
Coughing episodes	1.18 (1.14; 1.23)	**<0.0001**	1.17 (1.12; 1.22)	**<0.0001**
Coughing episodes (no-wheezing subset)	1.14 (1.06; 1.22)	**0.0004**	—	—
Wheezing episodes	1.20 (1.12; 1.29)	**<0.0001**	1.04 (0.96; 1.13)	0.32

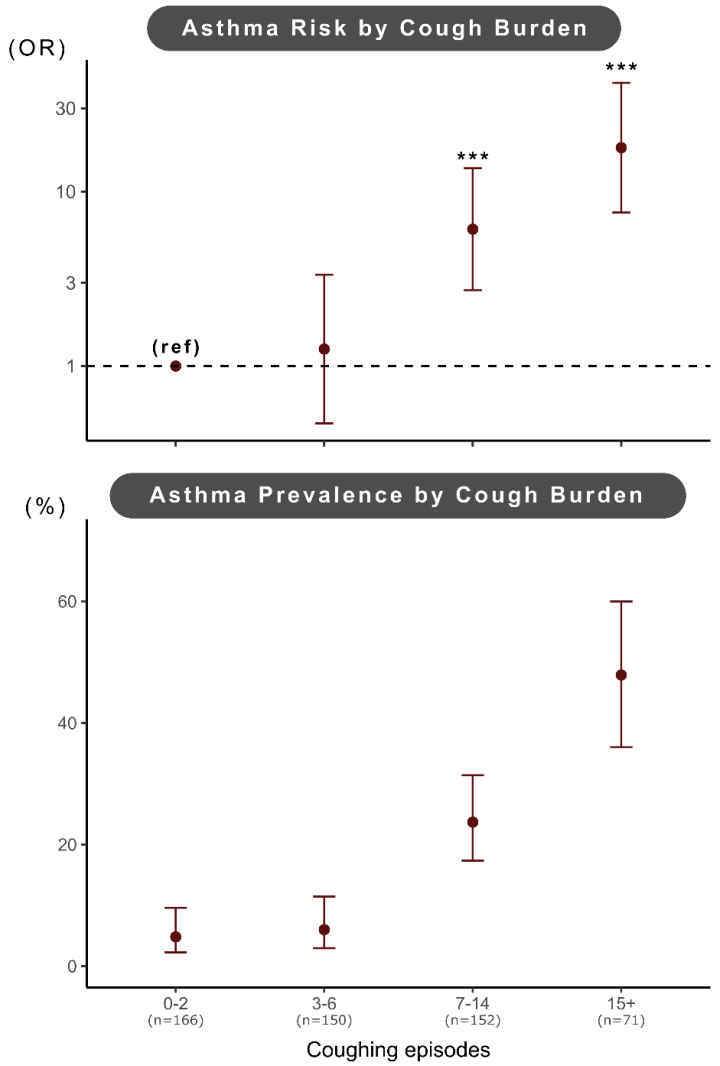
Paper III - Table 1: *Association between early-life symptom burden and later asthma. Odds ratios (ORs) with 95% confidence intervals (CIs) and p-values from logistic regression analyses of asthma between ages 6 and 10 years. Predictors were counts of cough and wheeze episodes from 0 to 3 years, modelled per 1 additional episode. All models were adjusted for sex, and mutually adjusted models included both coughing and wheezing episodes in the same model.*

Among 539 children with follow-up data, 87 (16.1%) had asthma between ages 6 and 10 years. Independent association between coughing and wheezing respectively, and later asthma 6-10 years were tested. In mutually adjusted models, the association between coughing episodes and later asthma remained largely unchanged after adjustment for wheezing (OR 1.17, 95% CI 1.12 to 1.22, p<0.0001). In contrast, wheezing episodes were not associated with later asthma after adjustment for coughing (p=0.32). Coughing episodes were also associated with later asthma in children without any wheezing (OR 1.14, p=0.0004) (**Paper III - Table 1**).



Paper III - Figure 3: *Predictive performance of early-life symptom burden for later asthma. RROC curves for asthma between ages 6 and 10 years based on cumulative coughing episodes (red) and wheezing episodes (blue) during the first 3 years of life. AUCs were compared using the DeLong test. The cutoff with the best sensitivity-specificity balance, defined by the maximum Youden's index, is shown as a dot on each curve.*

Predictive analyses showed that cough burden had a higher AUC than wheeze burden for predicting asthma between ages 6 and 10 years (0.79 vs 0.70, p=0.001) (**Paper III - Figure 3**). For both symptoms, discrimination was stronger in years 2 and 3 than in year 1 (**Paper III – Supplementary Figure 4A-C**).



Paper III - Figure 4: *Early-life cough burden and later asthma risk. Asthma risk and prevalence by cumulative number of coughing episodes during the first 3 years of life. The upper panel shows odds ratios (ORs) with 95% confidence intervals (CIs) for asthma between ages 6 and 10 years from sex-adjusted logistic regression, using the lowest category (0 to 2 episodes) as the reference group. The lower panel shows the corresponding asthma prevalence within each ordered cough category, with 95% Wilson confidence intervals shown as error bars. Children were categorized as 0 to 2 episodes, 3 to 6 episodes, 7 to 14 episodes, and at least 15 episodes. * p<0.05, ** p<0.01, *** p<0.001.*

Next, the incremental effect of symptom episodes was examined using symptom burden categories defined to reflect increasing episode burden in the data. Because only cough, and not wheeze, remained independently associated with later asthma in mutually adjusted models, the subsequent burden-group analyses focused on cough. Analyses of cough categories showed a dose-response relationship between early-life cough burden and later asthma. Compared with children in the lowest group with 0 to 2 coughing episodes, children in the highest group with at least 15 episodes had markedly higher odds of later asthma (OR=17.84, p<0.0001). Asthma prevalence between ages 6 and 10 years increased from 4.8% in children with 0 to 2 episodes to 48.0% in those with at least 15 episodes (**Paper III - Figure 4**).

Early-life cough and wheeze as continuous traits (later lung function)

Cough burden 0-3 years and later lung function						
	Unadjusted		**Wheezing-adjusted**		**No-asthma subset (Wheezing-adjusted)**	
Outcome	β (95% CI)	P-value	β (95% CI)	P-value	β (95% CI)	P-value
FEV1	-0.02 (-0.03; 0.00)	**0.026**	0.00 (-0.02; 0.01)	0.91	-0.01 (-0.03; 0.01)	0.47
FVC	0.00 (-0.01; 0.01)	0.67	0.00 (-0.01; 0.01)	0.96	-0.01 (-0.03; 0.01)	0.36
FEV1/FVC	-0.02 (-0.03; -0.01)	**0.0003**	0.00 (-0.01; 0.01)	0.86	0.00 (-0.01; 0.02)	0.71
MMEF	-0.02 (-0.04; -0.01)	**0.0001**	0.00 (-0.02; 0.01)	0.64	0.00 (-0.02; 0.02)	0.88
sRAW	0.01 (0.00; 0.02)	*0.052*	0.00 (-0.02; 0.01)	0.52	0.00 (-0.02; 0.02)	0.93
Wheeze burden 0-3 years and later lung function						
	Unadjusted		**Coughing-adjusted**		**No-asthma subset (Coughing-adjusted)**	
Outcome	β (95% CI)	P-value	β (95% CI)	P-value	β (95% CI)	P-value
FEV1	-0.06 (-0.09; -0.03)	**<0.0001**	-0.06 (-0.09; -0.03)	**0.0004**	-0.07 (-0.11; -0.03)	**0.0007**
FVC	-0.01 (-0.04; 0.01)	0.36	-0.01 (-0.04; 0.02)	0.41	-0.02 (-0.06; 0.01)	0.21
FEV1/FVC	-0.08 (-0.11; -0.06)	**<0.0001**	-0.08 (-0.11; -0.06)	**<0.0001**	-0.08 (-0.11; -0.05)	**<0.0001**
MMEF	-0.09 (-0.12; -0.07)	**<0.0001**	-0.09 (-0.12; -0.06)	**<0.0001**	-0.10 (-0.14; -0.06)	**<0.0001**
sRAW	0.06 (0.04; 0.08)	**<0.0001**	0.06 (0.04; 0.09)	**<0.0001**	0.06 (0.02; 0.09)	**0.001**

Paper III - ***Table 2****. Early-life coughing and wheezing episodes in relation to later lung function (z-score). Associations with lung function at ages 6, 8, and 10 years were examined using linear mixed models with child-specific random intercepts and fixed effects for time. Exposures were the number of coughing and wheezing episodes. Three models are*

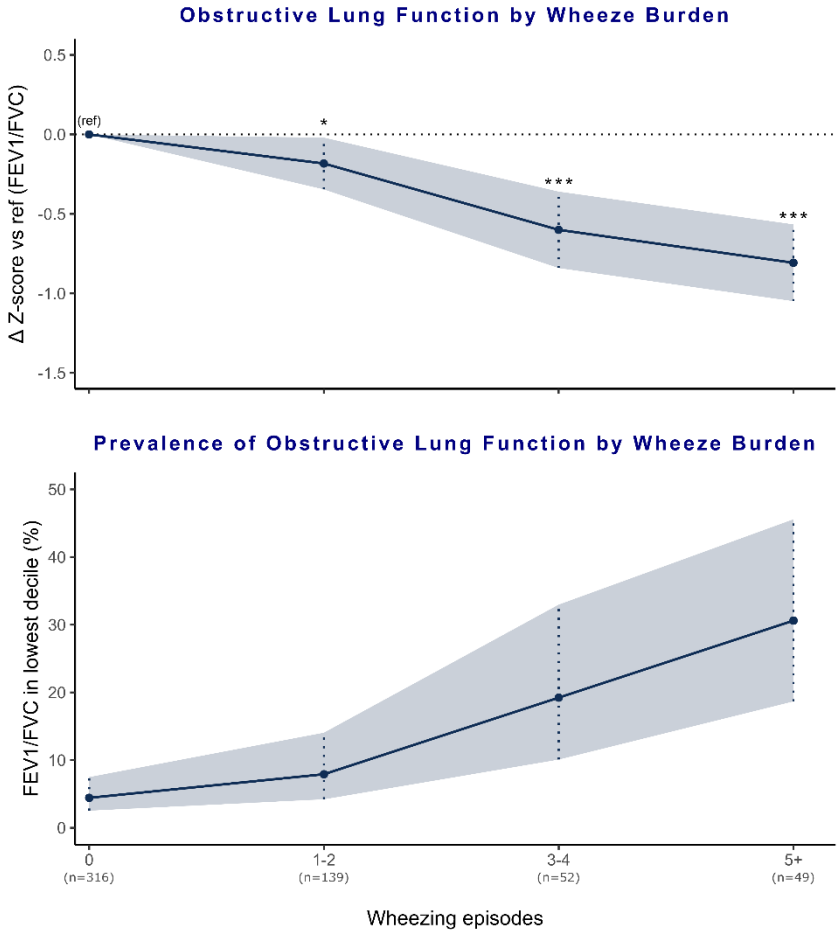
shown: unadjusted, mutually adjusted for both symptom episode types, and mutually adjusted in the subset without asthma between ages 6 and 10 years.

In mutually adjusted analyses, a higher burden of wheezing episodes in the first 3 years of life was associated with all measures of obstructive lung function, including lower FEV1, FEV1/FVC, and MMEF, and higher sRaw. These findings were largely unchanged in analyses restricted to children without asthma between ages 6 and 10 years. In contrast, cough burden showed no independent association with lung function parameters (**Paper III - Table 2**).

Findings were unchanged after adjustment for genetic predisposition by COPD and dysanapsis genetic scores (**Paper III - Supplementary Table 3**). A similar but attenuated pattern was seen for post-bronchodilator lung function at 10 years for FEV1, FEV1/FVC, and MMEF suggesting partly fixed obstruction, with similar results in children without later asthma. (**Paper III - Supplementary Table 4).** By year, wheeze burden showed the largest effect estimates in year 3 (**Paper III - Supplementary Table 5**).

Next, the incremental effect of wheezing episodes was examined using symptom burden categories reflecting increasing episode burden. Because only wheeze, and not cough, showed an independent association with later lung function in mutually adjusted models, these analyses focused on wheeze. Analyses of wheeze categories showed a dose-response relationship between early-life wheeze burden and later obstructive lung function. Compared with children with no wheezing episodes (lowest group), children with at least 5 episodes (highest group) had a lower FEV1/FVC z score (β=-0.81, $p<0.0001$). The proportion with FEV1/FVC below the lowest population decile increased from 4.4% in children with no wheezing episodes to 30.6% in those with at least 5 episodes, corresponding to an OR of 9.52 ($p<0.0001$) (**Paper III - Figure 5 & Supplementary Tables 6-7**).

Similar dose-response relationships were seen for FEV1, MMEF, and sRaw (**Paper III - Supplementary Figure 5).**



Paper III - Figure 5: *Early-life wheeze burden and later obstructive lung function. Lung function and prevalence of obstructive lung function by cumulative number of wheezing episodes during the first 3 years of life. The upper panel shows estimated mean differences in FEV_1/FVC z scores with 95% confidence intervals (CIs) from linear mixed models using the lowest category (0 episodes) as the reference group. The lower panel shows the corresponding prevalence of obstructive lung function within each ordered wheeze category, with 95% Wilson confidence intervals shown as error bars. Obstructive lung function was defined as FEV_1/FVC below the 10^{th} percentile based on the healthy reference population. Children were categorized as 0 episodes, 1 to 2 episodes, 3 to 4 episodes, and at least 5 episodes. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.*

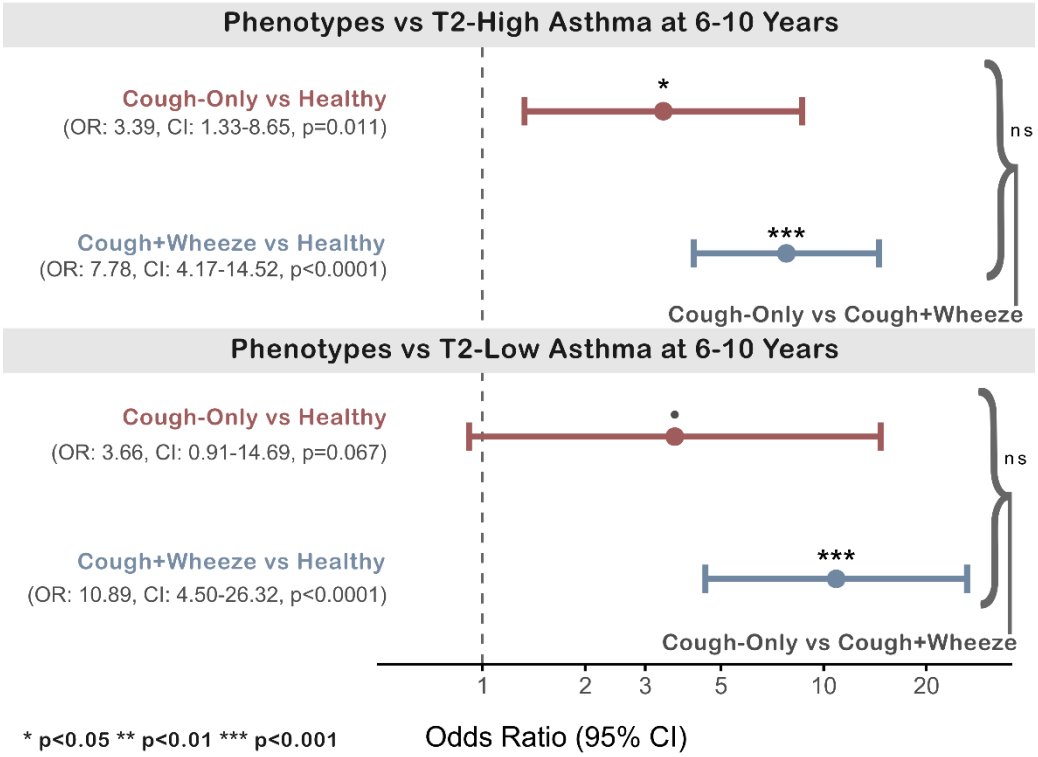
Cough-only and cough+wheeze phenotypes

Next, mechanistically relevant phenotypes of recurrent asthma-like symptoms were evaluated. Among included children, 175 (31.5%) met the definition of recurrent troublesome lung symptoms during the first 3 years of life. Of these, 46 (26.3%) were classified as the cough-only phenotype, defined by no recorded wheeze days, and 129 (73.7%) as the cough+wheeze phenotype.

Compared with healthy children, the cough-only phenotype was characterized mainly by male sex and fewer older siblings, whereas the cough+wheeze phenotype showed broader associations with

parental asthma, smoke exposure, and adverse birth characteristics (**Paper III - Supplementary Table 11**).

The symptom and cough burden during the first 3 years generally was higher in the cough+wheeze phenotype than in the cough-only phenotype. The median (IQR) number of coughing episodes in the first three years of life was 3 (1 to 6) in healthy children, 12 (9 to 15) in the cough-only phenotype, and 14 (9 to 18) in the cough+wheeze phenotype (**Paper III - Supplementary Table 8**).

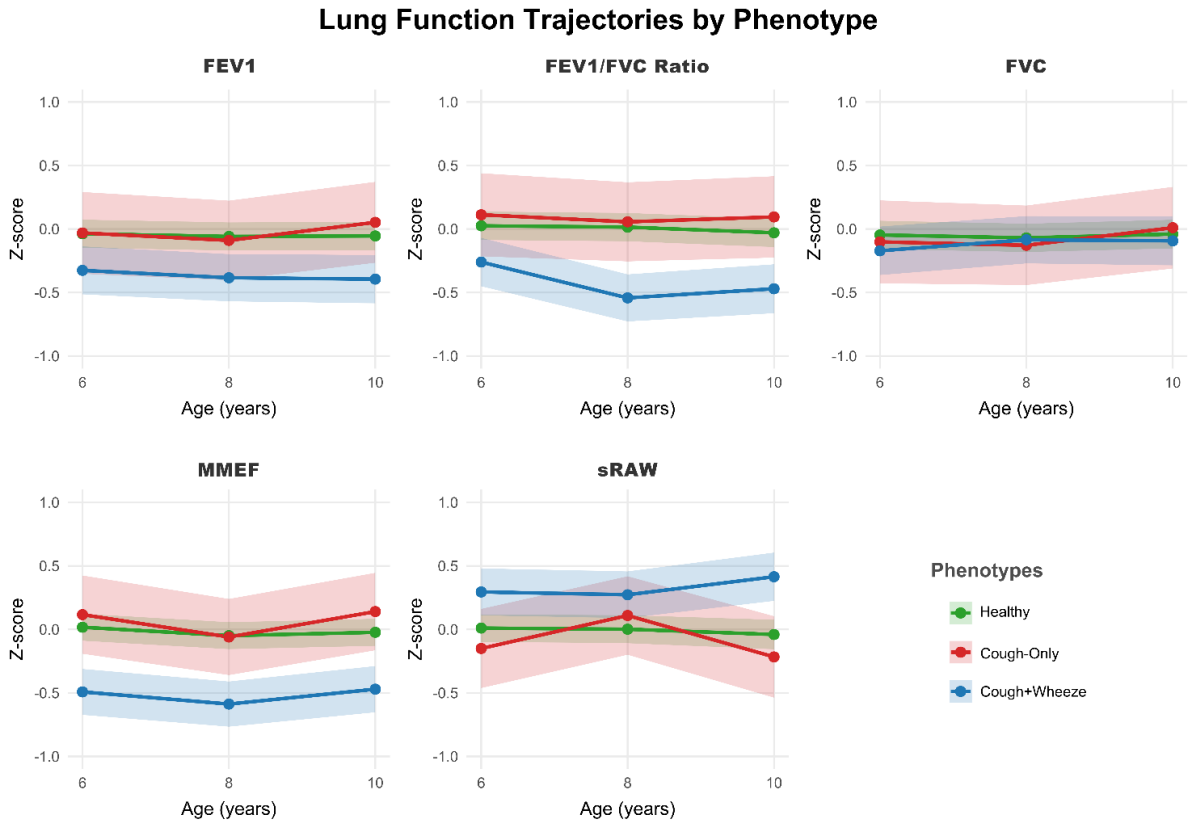


Paper III - Figure 6: *E-Figure 7. Phenotypes and risk of T2-high and T2-low asthma. Associations of the early-life cough-only phenotype and cough+wheeze phenotype, relative to healthy children, with subsequent T2-high asthma and T2-low asthma between ages 6 and 10 years. T2-high asthma was defined by elevation of established type 2 biomarkers, otherwise asthma was classified as T2-low.*

For later asthma, both the cough-only phenotype (GEE OR 6.26, p<0.001) and the cough+wheeze phenotype (GEE OR 10.47, p<0.001) were associated with higher odds of asthma between ages 6 and 10 years compared with healthy children, with no difference between the two phenotypes (**Paper III - Supplementary Figure 6**).

Findings were similar when analyzing by endotypes of T2-high and T2-low asthma (**Paper III - Supplementary Figure 7**).

For later lung function, the cough-only phenotype had lung function similar to that of healthy children at ages 6 to 10 years. In contrast, the cough+wheeze phenotype had lower FEV1, FEV1/FVC, and MMEF, higher sRaw, lower post-bronchodilator FEV1/FVC, and greater bronchial responsiveness than healthy children. The cough+wheeze phenotype also had lower lung function measurements than the cough-only phenotype (**Paper III - Supplementary Table 9**). These differences were stable over time, with no phenotype-by-age interaction (all p≥0.17) (**Paper III - Figure 6**).



Paper III - Figure 7: *Longitudinal lung function trajectories by phenotype. Estimated marginal mean z-scores of FEV1, FEV1/FVC, FVC, MMEF, and sRaw at ages 6, 8, and 10 years in healthy children and in children with early-life cough-only and cough+wheeze phenotypes. Trajectories were derived from linear mixed models. Shaded areas indicate CIs.*

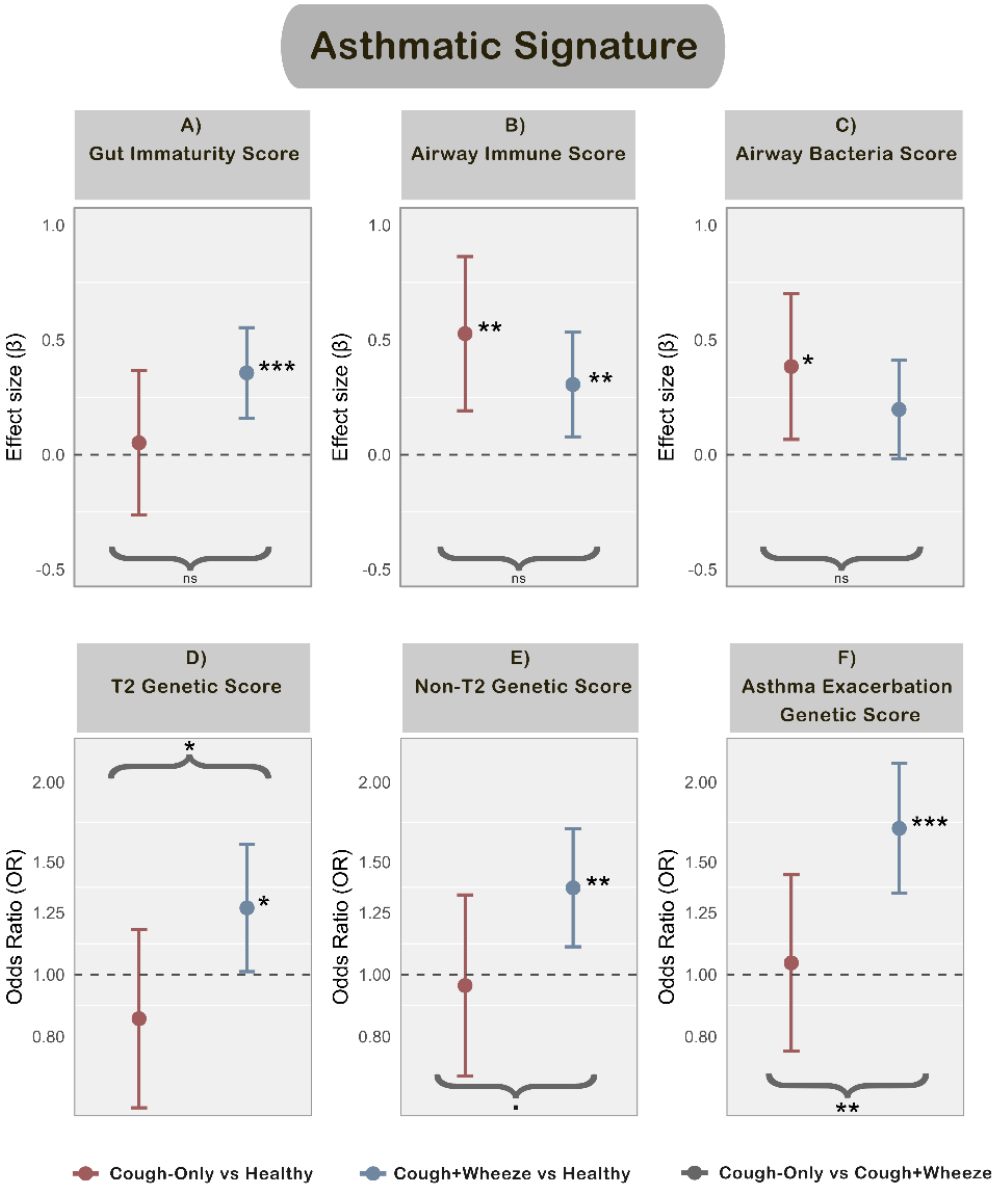
For later type 2 biomarkers, there were no significant differences in b-eos, FeNO, total IgE, or specific IgE between phenotypes at ages 6, 8, or 10 years (**Paper III - Supplementary Table 10**).

For burden of common childhood infections, both the cough-only and cough+wheeze phenotypes had more episodes of cold and fever than healthy children. Additionally, the cough+wheeze phenotype had more episodes of pneumonia, acute otitis media and gastroenteritis. Between phenotypes, the cough-only phenotype had fewer pneumonia episodes than the cough+wheeze phenotype (IRR 0.47, p=0.007) (**Paper III - Table 3**).

Daily Diary Outcomes	Healthy (n=381)	Cough-Only (n=46)	Cough-Only vs Healthy		Wheeze (n=129)	Cough+Wheeze vs Healthy		Cough-Only vs Cough+Wheeze	
(Episodes)	(Median, IQR)	(Median, IQR)	(IRR, 95% CI)	P-value	(Median, IQR)	(IRR, 95% CI)	P-value	(IRR, 95% CI)	P-value
Cold	9 (6; 14)	17 (10; 20)	1.58 (1.36; 1.83)	**<0.0001**	15 (11; 20)	1.54 (1.39; 1.70)	**<0.0001**	1.03 (0.87; 1.20)	0.76
Fever	5 (3; 7)	6 (4; 9)	1.24 (1.04; 1.48)	**0.019**	6 (4; 9)	1.25 (1.11; 1.41)	**0.0002**	0.99 (0.81; 1.20)	0.91
Pneumonia	0 (0; 1)	0 (0; 1)	1.38 (0.79; 2.40)	0.25	1 (0; 2)	2.95 (2.22; 3.92)	**<0.0001**	0.47 (0.27; 0.81)	**0.007**
Acute Otitis Media	1 (0; 2)	1 (0; 3)	1.30 (0.91; 1.87)	0.15	1 (0; 3)	1.41 (1.12; 1.78)	**0.0004**	0.92 (0.63; 1.36)	0.69
Tonsillitis	0 (0; 0)	0 (0; 1)	0.75 (0.32; 1.78)	0.52	0 (0; 1)	1.31 (0.84; 2.04)	0.24	0.58 (0.23; 1.42)	0.23
Acute Gastroenteritis	1 (1; 2)	1 (1; 3)	1.19 (0.90; 1.58)	*0.22*	2 (1; 3)	1.35 (1.13; 1.60)	**0.0007**	0.88 (0.66, 1.19)	0.42

Paper III - Table 3: *Early-life infection burden by phenotype. Values are median (IQR) based on daily diary recordings of infections during the first 3 years of life. Infection episodes were defined as at least 3 consecutive symptom days separated by at least 3 symptom-free days and verified by a physician. Incidence rate ratios (IRRs) with 95% confidence intervals (CIs) were estimated using quasi-Poisson regression with log completed diary days as an offset and adjustment for sex, older sibling at birth, and age at daycare start.*

By microbial triggers, different patterns were also observed. Bacterial and viral triggers were evaluated per sampled acute respiratory episode, meaning that the IRRs reflect pathogen detection per sampled acute episode rather than the total number of illnesses. After adjustment for sampling intensity, rhinovirus was more common in the cough+wheeze phenotype than in healthy children (IRR 1.33, p=0.044) and showed a similar tendency compared with the cough-only phenotype (p=0.066) (**Paper III - Supplementary Table 12**).



Paper III - Figure 8: *Asthma-related signatures of the phenotypes. Gut microbiome was assessed from stool 16S data at 1 week, 1 month, and 1 year and summarized as inverse Microbiota-by-Age Z-scores (MAZ). Airway immune and bacterial scores were derived at 1 month from nasal cytokines and hypopharyngeal microbiota using supervised models trained on later asthma. Genetic scores reflected T2 risk, non-T2 risk, and severe early-life asthma exacerbation risk. Panels A-C show β estimates from linear models, and panels D-F show odds ratios (ORs). All models were adjusted for sex.*

For gut immaturity, the gut immaturity score was higher in the cough+wheeze phenotype than in healthy children, with no difference between the 2 phenotypes (**Paper III - Figure 8A**).
For airway scores, both phenotypes showed similar associations with the asthma-associated airway immune score and airway bacterial score compared with healthy children (**Paper III - Figure 8B-C**).

For genetics, the cough+wheeze phenotype had higher values for all 3 genetic scores than both healthy children and the cough-only phenotype, whereas the cough-only phenotype did not differ from healthy children (**Paper III - Figure 8D-F**).
When examined individually, the COPD genetic score showed a borderline increase in cough+wheeze versus healthy children (OR=1.23, CI: 0.99 to 1.54, p=0.065), whereas the dysanapsis score was not associated (OR 1.16, CI: 0.94 to 1.44, p=0.17). No differences were observed for the cough-only phenotype in comparisons with either healthy children or the cough+wheeze phenotype.

Sensitivity analyses

Sensitivity analyses supported the main findings, as results for coughing episodes were consistent when using a stricter, objectively validated asthma definition and when analysing T2-high and T2-low asthma separately (**Paper III - Supplementary Table 2**). Findings were also unchanged after adjustment for vitamin D and fish oil supplementation during pregnancy.

DISCUSSION

Main findings

In **Paper I**, elevated total IgE and aeroallergen-specific IgE were evaluated as biomarkers of the allergic component of T2-high asthma, endotyped by elevated b-eos or FeNO, in two Danish birth cohorts comprising ~1,111 children. Cross-sectionally, both biomarkers were associated with T2-high asthma during school age, with total IgE showing associations at all examined time points and specific IgE showing similar associations from age 10 years onward. Both biomarkers also identified a subset of children with asthma who were not captured by blood eosinophils and FeNO alone. In predictive analyses, elevated early-life total IgE was associated with later T2-high versus T2-low asthma at several time points, whereas specific IgE was significantly associated only at 18 years. Total IgE showed a shift from higher specificity at 7 years to higher sensitivity at later ages, particularly in cross-sectional analyses. This pattern was also seen for specific IgE cross-sectionally but was less consistent in predictive analyses. However, direct comparisons did not show significant overall differences in prediction between the two IgE biomarkers. In general, total IgE and specific IgE appeared to function well as stand-alone biomarkers, with no clear overall benefit from combining them. SPT and parent-reported symptoms of rhinitis also showed usefulness, particularly for cross-sectional non-invasive endotyping and phenotyping and, for symptoms of rhinitis, later risk assessment within these well-characterized cohorts.

In **Paper II**, airway cytokine patterns were examined in 612 six-year-olds from the $COPSAC_{2010}$ population based cohort during stable conditions, both across type 2 inflammatory diseases and across elevated type 2 biomarkers in the full study population. A novel nasal epithelial lining fluid sampling method enabled measurement of 24 cytokines. IL-5 and CCL17 were the most consistent signals and were elevated in relation to type 2 inflammatory diseases and biomarker elevation, including b-eos, FeNO, specific IgE, and SPT. The signal was most pronounced in the airway-related endotypes of T2-high asthma and allergic rhinitis, while allergic conjunctivitis mainly showed increased CCL17. In contrast, non-allergic rhinitis, T2-low asthma, and atopic dermatitis did not show clear airway cytokine patterns. Direct endotype comparisons did not reveal a clear difference between T2-high and T2-low asthma, whereas allergic rhinitis showed higher CCL17 than non-allergic rhinitis. Known type 2 cytokines of IL-4 and IL-13 were not elevated as expected. Finally, an sPLS-derived type 2 airway cytokine score was compared with b-eos as a conventional blood-based and systemic

biomarker and showed similar performance for both endotyping at age 6 years and prediction to age 10 years.

In **Paper III**, 556 children with prospective daily symptom recordings during the first 3 years of life were studied to determine how early cough and wheeze relate to later asthma, obstructive lung function and selected mechanistic asthma signatures at ages 6 to 10 years. First, cough and wheeze were examined as continuous episode counts in relation to later outcomes. In mutually adjusted models, only cough remained independently associated with later asthma, while wheeze did not. Early-life cough also predicted later asthma better than wheeze, with an AUC of approximately 0.79 versus 0.70. A clear dose-response relationship was observed, with asthma prevalence increasing ten-fold from ~5% in children with the lowest cough burden to ~50% in those with the highest burden, corresponding to an OR of ~18-fold higher odds of asthma. In contrast, only wheeze, not cough, remained independently associated with later obstructive lung function. Higher wheeze burden was associated with a dose-dependent increase in the risk of obstructive lung function, and children with the highest wheeze burden had 9.5-fold higher odds of FEV1/FVC in the lowest decile. The corresponding reduction in FEV1/FVC reached -0.81 z-scores. These deficits persisted after bronchodilation, although the effect estimates were attenuated. To better understand the clinical and biological patterns, symptom-based phenotypes of cough-only (zero days of wheeze) and cough+wheeze were defined among children with recurrent troublesome lung symptoms, alongside a healthy control group. Both phenotypes were associated with increased risk of later asthma, whereas obstructive lung function was specific to the cough+wheeze phenotype. The cough+wheeze phenotype also had a higher burden of childhood infections, including pneumonia, more frequent rhinovirus detection during acute episodes and higher genetic susceptibility scores related to both type 2 and non-type 2 pathways. Both phenotypes shared asthma-associated airway immune and bacterial signatures, whereas gut immaturity appeared more pronounced in the cough+wheeze phenotype.

Strengths and limitations

Strengths

Overall, this thesis had multiple strengths. Using two deeply phenotyped mother-child cohorts, $COPSAC2_{000}$ and $COPSAC_{2010}$, enabled both longitudinal analyses and mechanistic analyses using repeated measures, infection and pathogen data, and omics data layers. Both cohorts were prospective

and followed SOPs throughout, supporting high data quality and consistent, methodologically robust diagnoses. From **Papers I** to **III**, multiple type 2 biomarkers were included, creating a rare opportunity for studying type 2 inflammation throughout childhood and adolescence. To our knowledge, few studies have access to such a broad biomarker panel spanning childhood and adolescence, enabling direct comparisons across biomarkers and timepoints.

Specifically for **Paper I**, a major strength was the longitudinal assessment of type 2 biomarkers, particularly total and specific IgE measured at 6 months, 18 months, and 4 years of age. This early-life repeated IgE sampling is uncommon, particularly when combined with later IgE assessments. In addition, the use of robust and objectively validated asthma diagnoses supported reliable observational analyses.

Specifically for **Paper II**, a major strength was the nasal filter paper sampling performed in more than 600 children, which differs from most airway cytokine studies in scale. This approach has been implemented within the COPSAC research unit, which has applied similar sampling at other timepoints and has the technical expertise required for this biosampling. Furthermore, the MSD platform provides a robust assay for cytokine quantification[101].

Specifically for **Paper III**, a major strength was the high-resolution symptom recordings during the first three years of life. Few cohorts have symptom data at this level of detail, providing day-by-day information on cough and wheeze across the first ~1095 days of life. Another major strength was the extensive lung function data, spanning spirometry (FEV1, FVC, FEV1/FVC, MMEF) and airway resistance (sRaw), with additional testing of bronchial hyperresponsiveness and bronchodilator response, allowing assessment of different aspects of airflow limitation. In addition, high-resolution infection data from symptom recordings, combined with bacterial and viral pathogen testing during acute respiratory illness, enabled mechanistic evaluation of infections as drivers of early-life cough and wheeze. Finally, integrating gut and airway microbiome, cytokines and genomics added a broader biological context and improved understanding of underlying mechanisms for symptom-based phenotypes.

Limitations

Overall, this thesis had several limitations that should be acknowledged. First, when discussing type 2 inflammation, disease severity is important, as type 2 inflammation is more commonly seen in children with difficult-to-treat asthma[83]. T2-high asthma is a clinically relevant endotype, particularly in relation to severe disease and potential biologic treatment. However, no children in the COPSAC

cohorts received biologic treatment, and due to the stringent treatment algorithms, asthma cases were generally well treated and not typically classified as severe or difficult-to-treat. Thus, the findings may be less generalizable to children with more severe, treatment-refractory T2-high asthma. Similarly, endotyping into T2-high and T2-low asthma required biomarker availability, and handling of missing data meant that some children could not be endotyped. This particularly affected the T2-low group, where absence of type 2 biomarker elevation required complete biomarker data. Missing biomarker data may not be random, and this could have introduced selection bias. This leads to the next overarching limitation, namely the limited number of asthma cases. As the number of asthma cases was low in some cross-sectional analyses, especially in the general population-based $COPSAC_{2010}$ cohort, statistical power may have been affected. This also necessitated the use of broader asthma definitions rather than relying solely on the objectively validated asthma diagnosis based on ERS criteria. At the same time, including more symptom history-based asthma cases may partly have reduced the risk of restricting analyses to more moderate-to-severe asthma, as milder cases could therefore also be captured Finally, and most importantly, the thesis is limited by the inherent risk of bias in observational cohort studies. Associations can therefore only be interpreted with caution, without firm conclusions regarding directionality or causality. Larger and independent cohorts would strengthen precision and robustness of our findings, including through external validation in independent cohorts, while clarification of causality would require randomized controlled or other interventional study designs rather than observational data alone.

Specifically for **Paper I**, an important limitation was the small number of cases which increased the standard errors, resulting in wider confidence intervals around the estimated associations, leading to greater uncertainty in their interpretation. Also, the predictive analyses were conducted only in the $COPSAC_{2000}$ cohort, which consists of children born to mothers with asthma. As atopy and sensitization are closely linked to asthma, this maternal high-risk asthma background may have influenced the distribution of type 2 inflammatory biomarkers in that cohort[183]. This may also have affected classification into T2-high and T2-low endotypes, particularly where cut-offs were applied. However, the cross-sectional analyses included both the high-risk $COPSAC_{2000}$ cohort, at three time points, and the population-based $COPSAC_{2010}$ cohort at one time point, which strengthens the robustness and generalizability of the observed cross-sectional associations. Likewise, as universally accepted cut-offs for type 2 biomarkers are not available in children and adolescents, the applied thresholds may have been influenced by the study population and the measurement methods used. To address these limitations, sensitivity analyses using both lower and higher cut-off values were performed and showed similar results. Also, aeroallergen sensitization during the first 4 years of life

was less common than elevated total IgE, which likely reduced the ability to detect significant predictive associations for specific IgE.

Specifically for **Paper II**, an important limitation was the limited statistical power, which reduced the ability to detect meaningful within-group differences, particularly between T2-high and T2-low asthma and between allergic and non-allergic rhinitis. In addition, the primary analyses used healthy controls rather than comparisons within disease groups, although in a clinical setting endotyping is more relevant among children who already have the disease. This may limit the clinical interpretability of the findings. As an additional limitation, these healthy control groups were defined in relation to the disease under study, meaning that children classified as healthy for one analysis could still have other type 2 inflammatory diseases, such as rhinitis or atopic dermatitis. Another limitation is that airway immune profiles were assessed using upper airway samples, and therefore reflect nasal site immunology in particular, which may not fully represent inflammatory processes in the lower airways due to anatomical compartment differences. In addition, the assay used may have had limited sensitivity for low-abundance cytokines such as IL-4 and IL-13, which are more difficult to quantify reliably without measurement in separate optimized assays[101]. Lastly, the supervised sPLS-derived type 2 airway cytokine score was trained using blood eosinophils, and blood eosinophil elevation was later also included in the definition of T2-high asthma. This introduces some degree of circularity, which should be considered when interpreting the observed associations.

Specifically for **Paper III**, an important limitation was that daily cough and wheeze symptoms were recorded in parent-reported diaries and were therefore dependent on proxy reporting. This could have led to misinterpretation of symptoms, particularly wheeze. However, this was partly mitigated by repeated parental instruction in diary completion before registration and during the biannual visits at the COPSAC research unit, as well as during acute respiratory illness episodes. It is also a limitation that we did not measure infant lung function at birth and therefore could not investigate to which extent the wheeze-lung function associations represent pre-existing lung function deficits as a risk factor for wheezing episodes. As phenotypes at ages 0-3 years were based on an algorithm including coughing symptoms, associations with school-age asthma may be partly circular. This concern is reduced by the 3 to 7-year interval between phenotype definition and asthma assessments and supported by sensitivity analyses using objectively verified asthma diagnoses less dependent on symptoms. Finally, the finding of an independent association between cough and later asthma is limited by the lack of a validation cohort, as replication would strengthen the robustness of this result.

Although few studies have similarly high-resolution diary data, replication in other cohorts with granular symptom recordings could still help verify the finding.

Interpretation

We examined and compared different endotypes and phenotypes of asthma and asthma-like diseases in childhood and adolescence. A major focus of this thesis was type 2 inflammation in childhood asthma, including a deeper exploration of both established and potential biomarkers. **Papers I** and **II** addressed this aim by investigating total and specific IgE, and airway cytokines measured in nasal samples, respectively.

In **Paper I**, we re-examined the known biomarker landscape of type 2 inflammation. As no sole biomarker can capture the whole type 2 inflammatory pathway, we wanted to test the independent and additive contribution of IgE biomarkers[184]. For total IgE, it interestingly showed promise in biomarker capabilities as it has perhaps been regarded as a broader marker of type 2 activity, reflecting overall circulating IgE burden rather than a targeted allergen immune response[185,186]. It is widely available worldwide, but it can be difficult to interpret because of the lack of uniform reference values in both children and adults[185,187]. We observed that, compared with specific IgE, total IgE showed more consistent associations with T2-high than T2-low asthma, both in early-life analyses linking measurements from the first 4 years of life to later diagnosis, and cross-sectionally at each time point between 7 and 18 years. This may suggest that total IgE could act as an early marker of processes partly independent of sensitization, which is known to develop later in life, and may also have value for endotyping[186,188]. This aligns with a pediatric component-resolved study suggesting that allergen-specific IgE patterns become more complex with age, particularly in children with asthma, supporting that the informative value of specific IgE may increase as sensitization becomes more prevalent[189].

Conversely, no overall improved predictive or endotyping value was observed when total IgE and aeroallergen-specific IgE were combined, compared with either biomarker alone. This likely reflects overlap in the biological information captured by the two markers, despite total IgE representing a broader IgE signal and specific IgE a more allergen-directed response. We also observed overlap between elevated total and specific IgE and the established type 2 biomarkers of b-eos and FeNO in children with asthma, while each biomarker also identified partly distinct groups. This supports the view that no single biomarker captures the full spectrum that is type 2 inflammation, and that a

multimarker approach may provide a more complete, although still indirect, characterization of the underlying biology[190]. Similarly, Frøssing et al assessed a type 2 biomarker panel consisting of b-eos, FeNO, and total IgE in 166 adults with severe asthma [190,191]. Here, they found that 70% had at least one biomarker elevated, 39% had two or more elevated, and only 15% had elevation of all three, underscoring substantial heterogeneity in biomarker overlap which we can speculate is similar in a pediatric population. They further showed discordance between systemic and airway eosinophilia, with eosinophilia present in both blood and sputum in only ~50% of patients who showed eosinophilia at either site. Together, these findings support the notion that systemic biomarkers do not fully reflect airway inflammation. **In Paper I**, skin prick testing and parent-reported allergic rhinitis symptoms, two non-invasive modalities, also showed some endotyping utility without requiring blood sampling. Building on both the need for airway-relevant assessment and the potential of non-invasive approaches, **Paper II** examined nasal cytokines as a novel, non-invasive, and potentially airway-specific method for type 2 endotyping.

In **Paper II**, elevated nasal sample IL-5 and CCL17 levels were observed in six-year-old children with several type 2 inflammatory diseases, including T2-high asthmatics, and in those with elevation of type 2 biomarkers. IL-5 is an established type 2 cytokine with a central role in eosinophilic inflammation making its consistent association in the present study biologically plausible and providing proof of concept for nasal cytokine sampling as a biomarker approach[192]. This is particularly relevant because IL-5 is generally considered a low-abundance cytokine, yet elevated levels were still detected using the MSD assay under stable conditions[193]. Moreover, as IL-5 has been connected to airway remodelling, the process of structural airway wall change linked to chronic inflammation in asthma, it may represent an important pathway that can be captured by these nasal samples[194]. Biologic therapies targeting the IL-5 pathway, including mepolizumab and benralizumab, are already used in severe eosinophilic asthma in children as young as this study population of 6 years [195,196]. This raises the question whether this way of non-invasive cytokine profiling could eventually have value in identifying airway inflammatory patterns relevant to IL-5 treatment response, although this was not examined here. Conversely, a small-scale pilot study of 12 adults with severe eosinophilic asthma found no difference in nasal secretion cytokine levels, including IL-5, before and after 24 weeks of mepolizumab treatment[197]. Larger longitudinal studies are therefore needed, particularly to determine whether nasal cytokine profiles can predict or track response to IL-5-targeted therapy.

We also found elevated nasal CCL17 levels, with stronger associations than IL-5 with airway-specific type 2 inflammatory diseases and biomarkers. CCL17, also known as thymus and activation-regulated

chemokine (TARC), is a type 2-associated cytokine involved in Th2 cell recruitment and allergic asthma[198]. CCL17 has been proposed as a potential biomarker of T2-high asthma, although current evidence still remains limited[199,200]. A 2002 case-control study (n=56) reported elevated plasma CCL17 levels in childhood asthma compared with healthy controls, with positive associations with total IgE and cat allergen-specific IgE [201]. More broadly, CCL17-related signatures are likewise being investigated as biomarkers of treatment response in severe asthma[202]. Also, in pediatric atopic dermatitis, dupilumab significantly reduced circulating CCL17 levels to values similar to those of healthy controls[203]. Together, these findings suggest that CCL17 may be an underrecognized cytokine/chemokine with potential as an airway-specific biomarker of type 2 inflammation.

By comparison, studies investigating nasal cytokine levels in asthma remain scarce, and comparability between studies is further complicated by differences in sampling methods and assay platforms. In a smaller pediatric study, Doulatpanah et al. measured nine cytokines in children with asthma, both with (n=10) and without (n=10) elevated total IgE, compared with healthy controls (n=20), using a similar, although not identical, sampling and assay approach. In line with our study, they found increased IL-5 levels, but also reported elevated IL-13, which was not observed here[204]. Using a similar sampling method, Hansel et al. found that adults with allergic asthma (n=28) had elevated nasal IL-5 levels at baseline compared to healthy controls (n=11), despite the study being conducted in the context of experimental rhinovirus infection, which supports the present findings under stable conditions[205]. Finally, Melo et al. reported that adults with allergic asthma (n=29), assessed using a similar sampling method, had increased nasal IL-5, but not IL-13, compared with healthy controls (n=17)[206]. Our study extends these findings by supporting IL-5 as a relevant type 2 biomarker in asthma in a larger and deeply phenotyped cohort, while also identifying additional signals through a broader cytokine panel.

In our study, nasal sample IL-5 and CCL17 levels were strongest in allergic rhinitis, thereafter T2-high asthma, allergic conjunctivitis, and finally atopic dermatitis, suggesting either weaker signals in conditions more distant from the nasal site or in those less directly related to the airway. These findings add complexity to the measurement and interpretation of nasal sampling, as local immune activity at the sampling site is also likely to influence the results. We have previously in our other cohort of $COPSAC_{2000}$ found a similar increase in both nasal IL-5 and CCL17 in 8-year-olds with allergic rhinitis (n=15) compared to healthy controls (n=10)[207]. Furthermore, a mucosal tissue analysis in allergic rhinitis showed higher epithelial CCL17 expression in the nasal mucosa than in

healthy controls [208]. This suggests that our findings should be interpreted cautiously, as they may reflect local nasal epithelial immune activity rather than airway type 2 inflammation.

Building on the complexity of *in vivo* studies, no elevations in IL-4 or IL-13 were observed in relation to any type 2 inflammatory diseases or biomarkers in the present study, and IL-13 levels were instead decreased. This contrasts with what might have been expected and highlights the importance of real-world studies, such as ours, examining whether cytokine signals align with current biological assumptions, particularly when evaluating their biomarker potential. IL-4 and IL-13 appear to be low-abundance cytokines in nasal samples and may be difficult to quantify reliably, even with sensitive multiplex assays such as MSD used in this study[209]. Similarly, Bossley et al. reported no elevation in BAL IL-5, IL-4, or IL-13 in 53 adolescents with severe treatment-refractory asthma compared with healthy controls, using the Luminex assay, which is comparable to the MSD assay used in our study[210].

For this sampling method, clear elevations of key type 2 cytokines were observed outside acute disease exacerbations, supporting the biological sensitivity of the approach. Unlike more stable clinical biomarkers, cytokine measurements are particularly sensitive to preanalytical and analytical conditions, which may affect their reliability[211,212]. Nevertheless, we still identified robust disease-related signals in this study. This is notable because many pediatric nasal cytokine studies have instead been performed during either natural viral exacerbations[213,214], or acute wheeze[215], both settings where cytokine responses are amplified and therefore easier to detect. In $COPSAC_{2010}$, children were also sampled during acute viral respiratory illnesses in the first 3 years of life, where a cytokine pattern of increased type 1 and regulatory cytokines, together with decreased type 2 and type 17 cytokines, was observed[103]. Together, these findings support that nasal epithelial lining fluid sampling can capture a biologically meaningful immune activity not only during acute inflammatory episodes but also in more stable type 2 disease states.

For **Papers I** and **II**, minimally invasive and non-invasive biomarkers of type 2 inflammation were examined, including the established but less commonly used blood-based markers total IgE and specific IgE, as well as the more investigational nasal SAM filter paper method. There remains an unmet need for improved biomarkers of type 2 inflammation, particularly in therapeutic contexts[216]. In general, novel pediatric biomarkers are still at an early stage of development[217]. In 2019, European Academy of Allergy & Clinical Immunology (EAACI) convened a task force on non-invasive diagnostic approaches in allergic disease, culminating in a position paper that

emphasized the need for further development and validation of minimally invasive, site-specific biomarker-based methods[218]. This thesis answers that call by evaluating the utility of total and specific IgE in relation to T2-high asthma and by directly comparing blood eosinophils with an sPLS-derived airway type 2 cytokine score. The finding that this airway cytokine score performed comparably to blood eosinophils supports the potential of non-invasive diagnostic approaches for assessing type 2 inflammation.

In **paper III,** we demonstrate that early-life asthma-like symptoms of cough and wheeze are linked to distinct school-age trajectories of asthma and obstructive lung function, respectively, with incremental dose-response relationships observed for both. Preschool asthma research and clinical practice have traditionally centered on wheeze, while early-life coughing has remained a clinical and mechanistic black box. What is proposed here may represent a paradigm shift in the understanding of early-life asthma-like symptoms. More specifically, the findings suggest that cough symptoms alone, rather than wheeze, may provide an independent predictive signal for later school-age asthma, and that cough predicts later asthma more accurately than wheeze, with an approximately 0.10 higher AUC. This challenges the wheeze-centric view that has long shaped clinical thinking around early-life asthma-like symptoms.

Some studies have examined the role of early-life cough in the development of later asthma, including its interplay with wheeze symptoms. Rabe et al. found that a composite symptom-based model based on 11 early-life respiratory and non-respiratory symptoms, assessed by two parent-reported questionnaires before 1 year of age, predicted school-age asthma, and that predictive ability remained even when wheeze was excluded from the model[219]. In our study, cough burden showed stronger predictive performance at 2 to 3 years of age than at 1 year, suggesting that the associations reported by Rabe et al. might have been stronger had symptom assessment extended beyond infancy. Boudewijn et al. found, in more than 3,000 children, that parent-reported nocturnal dry cough, assessed yearly by questionnaire from ages 2 to 7 years, was associated with later asthma at 8 years of age, even in the absence of wheeze[220]. This is consistent with our findings, although cough in our study was assessed more broadly rather than being limited to only nocturnal symptoms. Using prospectively recorded daily diaries, our study highlights early-life cough as a dose-dependent predictor of later asthma and suggests that focusing solely on wheeze may be insufficient.

Symptoms of wheeze were the only symptom domain that showed an independent, incremental dose-response association with obstructive lung function patterns at ages 6 to 10 years, reflected by lower FEV1, FEV1/FVC ratio, and MMEF, and higher sRaw. Consistent with our findings, several birth

cohorts have shown that early-life wheeze phenotypes linked to persistent impairments in lung function during childhood and adolescence[135,221,222]. The present study adds to this literature through high-resolution prospective daily recordings, which showed that each additional wheeze episode was associated with a progressively more obstructive lung function pattern. We also found similar associations in children who never developed asthma, suggesting that the relationship between wheeze and obstructive lung function may reflect a broader physiological or developmental airway process rather than asthma alone. Likewise, postbronchodilator spirometry showed only partial improvement, indicating that these obstructive changes were only partly reversible. In light of evidence suggesting that reduced early-life lung function may contribute to later COPD[223], early-life wheeze may warrant greater attention as a marker of long-term airway dysfunction, regardless of asthma status. Here, it would be important to include lung function measurements as early as possible, preferably from infancy, to better understand causality and the temporal sequence of these associations[224].

The underlying mechanisms responsible for the distinct cough and wheeze associations are not clear from our study. To better approach these mechanistic layers while retaining clinical relevance, two phenotypes were defined: a cough-only phenotype, consisting of children with recurrent asthma-like symptoms but no wheeze days, and a cough+wheeze phenotype, consisting of children with recurrent asthma-like symptoms and wheeze days. Cough and wheeze episodes are likely triggered by infections, primarily viral infections[225,226], and this is supported by the seasonal pattern observed for both symptom episodes in our study. Still, we saw a distinct infectious pattern that separated both the symptom-based phenotypes. Compared with healthy controls, the cough+wheeze phenotype had a higher burden of most common childhood infections, whereas the cough-only phenotype was only associated with more episodes of cold, suggesting that upper respiratory tract infections may be a main driver of this phenotype. By contrast, pneumonia episodes were the only infection type distinguishing the two phenotypes, suggesting that lower respiratory tract infections (LRTIs) may be more closely linked to the cough+wheeze phenotype. However, causality cannot be inferred, as the association may be bidirectional as airway mechanisms predisposing to wheeze may increase susceptibility to LRTIs, while recurrent LRTIs may also contribute to wheeze[227]. Again, consistent with the life-course perspective of lung function, a meta-analysis found that early-life LRTI leads to an increased risk of COPD later in life[228].

Both symptom-based phenotypes also remained distinct in relation to later obstructive lung function, with the cough+wheeze phenotype showing a more obstructive pattern than both the cough-only phenotype and healthy controls. To explore potential mechanisms, polygenic risk scores for

dysanapsis and COPD were examined. The aim was to assess whether the combination of wheeze and obstructive lung function could reflect dysanapsis, where airway growth is disproportionate to lung volume[229], or whether it might indicate early genetic susceptibility to later COPD. No clear evidence supported either hypothesis. The COPD PRS showed only a borderline association, while the dysanapsis PRS was not associated yet was still directionally as expected. Importantly, the absence of clear associations does not exclude these mechanisms, as PRS-based inference is complex and can be limited by phenotypic heterogeneity[230]. Another possible interpretation is that the airway-related processes linking early wheeze to later obstructive lung function may reflect early airway remodelling, consistent with previous studies in preschool children with recurrent wheeze showing structural airway changes compatible with remodelling[231,232].

Next, bacterial and viral pathogens were examined to assess whether a pattern emerged that differentiated the phenotypes. We found that, even in a conservative analysis restricted to acute respiratory illnesses lasting more than three days and accounting for episode burden, rhinovirus remained more frequent in the cough+wheeze phenotype, showing its potential role in this phenotype. This potential role of rhinovirus in the cough+wheeze phenotype is particularly notable, as this phenotype was characterized by both increased later asthma risk and more obstructive lung function, two outcomes that have also been linked to early-life rhinovirus infections[233–235]. The cough-only phenotype was, however, not associated with an increased rate of any specific bacterial or viral pathogens. It could have been hypothesized that this cough-related phenotype would show increased colonization with *Haemophilus influenzae*, *Moraxella catarrhalis*, or *Streptococcus pneumoniae* during acute respiratory episodes, given its similarities to protracted bacterial bronchitis (PBB). These three bacteria are usually found in PBB[236], which is characterized by persistent wet cough, and has been linked to increased risk of later asthma[237]. However, in our study we did not find any increase in those three bacteria, and we could not subclassify our cough data to fit diagnostic criteria for PBB and instead focused on a broader view and implications of parent-reported cough. We did, however, observe similar airway bacterial scores in both phenotypes, with the greatest separation between the cough-only phenotype and healthy controls, suggesting that the airway microbial signature associated with later asthma risk may be more pronounced in this phenotype. Together, these results suggest that similarities and differences between the two symptom-based phenotypes may reflect partly distinct underlying mechanisms.

Although defined during the first 3 years of life, before any asthma diagnosis, our cough-only phenotype showed some conceptual and clinical similarities to cough-variant asthma (CVA) described at later ages. However, these entities are not equivalent. The present phenotype reflects an

early-life asthma-like symptom pattern, whereas CVA is typically defined at school age or later in children with established asthma. Previous pediatric studies vary in interpretation, with some regarding CVA as a precursor condition[238] and others as a true asthma phenotype[239,240]. We observed that later lung function in the cough-only phenotype was preserved and comparable to healthy controls. Few pediatric studies have examined similar early-life cough phenotypes, but in studies of CVA, where asthma is already established rather than predicted, spirometry has generally been normal or near normal[241–243].

In contrast to **Papers I** and **II**, **Paper III** examined type 2 inflammation using T2-high and T2-low asthma, as well as biomarker elevation, as later outcomes of the symptom-based phenotypes. No differences in type 2 biomarkers were observed later in childhood, at ages 6 to 10 years, between the phenotypes and healthy controls. Since both phenotypes represent heterogeneous groups defined by symptom patterns, a clear later type 2 biomarker signal was not expected. Similarly, the associations with later asthma did not distinguish between T2-high and T2-low asthma, suggesting that the phenotypes capture both ends of the spectrum. The airway immune score, derived from an sPLS model combining 18 infancy cytokines to capture an asthma-related immune pattern rather than a specifically type 2 profile, was also similar between groups. Finally, the cough+wheeze phenotype appeared more genetically driven, as it showed higher type 2 and non-type 2 genetic scores than healthy controls, whereas the cough-only phenotype did not differ from healthy controls. Taken together, these findings suggest that the early-life symptom-based phenotypes do not map onto later type 2 inflammatory profiles. Rather, they appear to reflect broader and more clinically relevant heterogeneity, particularly in relation to later asthma risk, lung function, and genetic susceptibility.

CONCLUSIONS AND PERSPECTIVES

This thesis examined different phenotypes and endotypes of childhood and adolescent asthma and asthma-like conditions, with a particular focus on the role of type 2 inflammation in these characterizations.

In **Paper I**, the predictive and endotyping potential of total IgE and specific IgE as type 2 biomarkers was evaluated in two Danish birth cohorts comprising ~1,111 children with follow-up through childhood and adolescence. Both biomarkers were cross-sectionally associated with T2-high asthma and performed similarly as individual markers, with no significant overall difference in predictive performance and no clear added value from combining them. Total IgE showed more consistent associations at all time points, whereas predictive associations for specific IgE were less consistent, which may partly reflect limited power at early ages when aeroallergen sensitization was less frequent. These findings support both total IgE and specific IgE as beneficial biomarkers for endotyping T2-high asthma in childhood, though validation in broader, more heterogeneous populations is still needed.

In **Paper II**, 24 airway cytokines were measured in epithelial lining fluid by nasal sampling from 612 six-year-old children to examine their relation to type 2 inflammation. Cytokines of IL-5 and CCL17 emerged as the most consistent cytokine signals, with the clearest elevations observed in the more airway-related conditions, particularly T2-high asthma and allergic rhinitis. An sPLS-derived type 2 airway cytokine score was then compared with b-eos and showed similar performance for T2 immune endotyping at age 6 years and prediction to age 10 years. Together, these findings support this type of cytokine sampling as a non-invasive and airway-specific approach to immune endotyping. However, substantial work remains before such methods could be translated into clinical practice, and further studies in larger and more diverse pediatric populations are needed, ideally also clarifying how disease activity and sampling timing influence cytokine profiles.

In Paper III, daily symptom recordings from the first 3 years of life in 556 children, comprising more than five million diary entries, showed that cough and wheeze carry different prognostic implications. Only cough was independently associated with later asthma risk, whereas only wheeze was independently associated with later obstructive lung function. Among children with recurrent troublesome lung symptoms, symptom-based phenotyping showed that both the cough-only phenotype, defined by no recorded wheeze days, and the cough+wheeze phenotype were associated

with later asthma risk, whereas reduced lung function was specific to the cough+wheeze phenotype. Both phenotypes shared asthma-related airway immune and bacterial signatures. The cough+wheeze phenotype also showed higher genetic susceptibility in both type 2 and non-type 2 pathways. No clear differences in later type 2 biomarker levels were observed between phenotypes. The two phenotypes had a similar cold burden, but differed in relation to pneumonia and rhinovirus infections, which were more evident in the cough+wheeze phenotype.

Overall, this thesis highlights the heterogeneous nature of asthma and asthma-like conditions in childhood and adolescence. The findings do not support a one-size-fits-all approach. Instead, they suggest that both established and novel biomarkers, together with symptom-based phenotyping, may contribute to more precise characterization of disease and may support future progress toward precision medicine and improved diagnostics. Taken together, the results show that type 2 inflammation is an important but incomplete immune response for understanding pediatric asthma. While Papers **I-II** support biomarker-based immune endotyping of T2-high disease, **Paper III** shows that early-life symptom patterns capture additional clinically relevant heterogeneity, particularly in relation to later asthma risk and lung function development. A more integrated approach combining biomarkers, immune profiling and longitudinal symptom data may therefore provide a stronger basis for earlier risk stratification and more tailored management of childhood asthma in the future.

Future research

This thesis highlights several areas for further study.

In **Paper I**, total and specific IgE were compared for their value in endotyping within two cohorts of children with relatively well-controlled asthma. Since type 2 inflammation is closely linked to more severe asthma, future studies should examine these biomarkers in children with severe disease, particularly those considered for biologic therapy. A relevant next step would be to evaluate these biomarkers in the nationwide Danish severe pediatric asthma cohort that I worked with during the PhD (*see Preface*), where IgE biomarkers were also available. This would allow assessment of whether the findings from the COPSAC cohorts also generalize to children with severe asthma.

In **Paper II**, we assessed stable type 2 disease-associated patterns of airway cytokines measured using nasal SAM filter papers. Certain cytokines showed promising and consistent signals, supporting their potential for endotyping. An important next step would be to investigate these cytokine patterns

longitudinally before and after biologic treatment in children with T2-high asthma, to assess whether nasal airway cytokine levels reflect treatment response. Such a study would likely require a prospective design and inclusion of both children and adults, as biologic treatment remains more restricted and less well studied in children than in adults[82].

Bridging **Papers II** and **III**, an important next step would be to investigate differences in airway cytokine patterns in symptom-based phenotypes during both acute asthma-like episodes and stable disease periods. This may be examined further in the two early-life RCT studies that I co-led during my PhD (*see Preface*). In these studies, prospective diary recordings would allow phenotypic classification based on cough and wheeze burden, while paired airway cytokine measurements obtained during hospitalization and again at a scheduled follow-up approximately one year later would enable within-child comparisons between acute and stable disease states.

In **Paper III**, the causal direction of the association between wheeze and obstructive lung function was questioned. As infant lung function measurements preceding the onset of wheeze were not available in COPSAC2010, this could not be studied within that cohort. In $COPSAC_{2000}$, wheeze episodes were objectively validated during acute visits to the COPSAC research unit and recorded together with novel infant lung function measurements, providing an opportunity to investigate this question further.

Likewise, in **Paper III**, we attempted to explore potential mechanisms underlying the wheeze-related obstructive lung function findings. However, the molecular mechanisms linking early-life wheeze burden to later obstructive lung function development remain insufficiently understood. An omics-driven approach offers a direct route from symptom patterns to mechanism. In pulmonary research, unbiased approaches, including proteomics, have uncovered disease endotypes[244]. However, omics analyses have not yet been used to distinguish and understand differences between symptom-based phenotypes of childhood asthma. This gap is important in a life-course perspective. Lange et al. reported that individuals who develop COPD may follow different lung function trajectories, including persistently low lung function from early adulthood and accelerated decline from a higher starting level[245]. Building on this perspective, a 2022 commentary in *The Lancet Respiratory Medicine* discussed how to identify and treat young "low flyers", those with persistently low lung function trajectories who may be at increased risk of later COPD[246]. One possible next step would therefore be to examine early-life recurrent wheezers using proteomic profiling at multiple timepoints. Given that such data are available in $COPSAC_{2010}$ at birth, 6 months, 18 months, and 6

years, this would be a natural opportunity to identify proteins involved in these underlying processes, including potential markers of airway remodelling and candidate molecular targets.

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Appendix: Paper I, II, III

I. Levels of Total IgE versus Specific IgE during Childhood for Defining and Predicting T2-high Asthma

Tamo Sultan, Frederikke Skov, Nicklas Brustad, Nilo Vahman, Jakob Stokholm, Klaus Bønnelykke, Ann-Marie Malby Schoos*, Bo Chawes*.

Published in World Allergy Organization Journal, November 2024[1]

II. Dominance of IL-5 and CCL17 in Nasal Cytokine Profiles of Children with Type 2 Inflammation

Tamo Sultan, Nicklas Brustad, Julie Nyholm Kyvsgaard, Frederikke Skov, Laura Marie Hesselberg, Anton Kjellberg, Jonathan Thorsen, Nilo Vahman, Ann-Marie Malby Schoos, Susanne Brix, Klaus Bønnelykke*, Bo Chawes*.

Published in The Journal of Allergy and Clinical Immunology, December 2025[2]

III. Coughing and Wheezing in Early Life Define Distinct Asthma and Lung Function Trajectories

Tamo Sultan, Nicklas Brustad, Julie Nyholm Kyvsgaard, Signe Kjeldgaard Jensen, Liang Chen, Tingting Wang, Casper Emil Tingskov, Anders Ulrik Eliasen, Yang Luo, Karen Angeliki Krogfelt, Benjamin McDonald Smith, Ann-Marie Malby Schoos, Nilo Vahman, Jonathan Thorsen, Morten Arendt Rasmussen, Jakob Stokholm, Nicole Prince, Jessica Lasky-Su, Bo Chawes, Klaus Bønnelykke.

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PAPER I

Levels of Total IgE versus Specific IgE during Childhood for Defining and Predicting T2-high Asthma

Tamo Sultan, Frederikke Skov, Nicklas Brustad, Nilo Vahman, Jakob Stokholm, Klaus Bønnelykke, Ann-Marie Malby Schoos*, Bo Chawes*.

**Senior authors contributed equally*

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Levels of total IgE vs. specific IgE during childhood for defining and predicting T2-high asthma

6 months | 18 months | 4 years | 7 years | 10 years | 13 years | 18 years

Early childhood

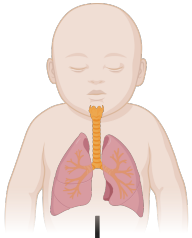
Later childhood and adolescence

Study aim:

1) Can elevated total or specific IgE define T2-high asthma in later childhood and adolescence?

2) Can elevated total or specific IgE in early childhood predict later risk of T2-high asthma?

T2-high asthma[1]



[1]Defined as asthma with:

1) Blood eosinophil count ≥ 0.30 10^9/L
or
2) Fractional exhaled nitric oxide ≥ 20 ppb

Mother-child cohorts

COPSAC2000 (n=411)

and

COPSAC2010 (n=700)

Versus

Total IgE

- Great overlap with other T2-high markers
- Significant association with T2-high asthma at ages 7/10/13/18 years
- Elevated total IgE at early childhood has a significant association with T2-high asthma at ages 7/13/18 years

Specific IgE

- Great overlap with other T2-high markers
- Significant association with T2-high asthma at ages 10/13/18 years
- Elevated specific IgE at early childhood has a significant association with T2-high asthma at age 18 years

Abbreviations:
IgE: Immunoglobulin E

In conclusion

Both total IgE and specific IgE are equally **useful stand-alone biomarkers for defining and predicting risk of T2-high asthma in childhood and adolescence**

WORLD ALLERGY ORGANIZATION

COPSAC
COPENHAGEN PROSPECTIVE STUDIES ON ASTHMA IN CHILDHOOD

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WORLD ALLERGY ORGANIZATION JOURNAL

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Levels of total IgE versus specific IgE during childhood for defining and predicting T2-high asthma

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ABSTRACT

Background: T2-high asthma is characterized by elevated blood eosinophils (b-eos), and/or fractional exhaled nitric oxide (FeNO), and/or being "allergy-driven", which is not well-defined.

Objective: To investigate the role of total and specific immunoglobulin E (tIgE/sIgE) for defining and predicting T2-high asthma in childhood as biomarkers of "allergy-driven".

Methods: We utilized data from the COPSAC2000 (n = 411) and COPSAC2010 (n = 700) mother-child cohorts with repeated measurements of tIgE, sIgE, b-eos and FeNO through childhood. We defined T2-high asthma by elevated b-eos ($\geq 0.3 \times 10^9$/L) and/or FeNO ($\geq$20 ppb) and analyzed association with elevated tIgE (age-specific cut-offs) and sIgE ($\geq$0.35 kU/L) using logistic regression at ages 7/10/13/18 years. Further, we analyzed the association between elevated tIgE and sIgE at age 0–4 years and later risk of T2-high asthma using logistic regression and ROC models.

Results: Elevated tIgE was associated with risk of T2-high asthma at all time points, whereas elevated sIgE showed similar results at ages 10/13/18 years. There was no overall model fit preference for a combination of tIgE and sIgE instead of tIgE or sIgE alone using Vuong's Likelihood-Ratio-Test, Akaike or Bayesian Information Criterion. Further, elevated tIgE at age 0–4 years was associated with later risk of T2-high asthma at all time points (AUC = 0.63–0.70, sensitivity = 0.62–0.81, specificity = 0.57–0.78), whereas elevated sIgE at 0–4 years was only associated with T2-high asthma at 18 years (AUC = 0.66, sensitivity = 0.45, specificity = 0.88). There were no significant differences in AUC values between tIgE and sIgE (DeLong's test).

Conclusion: Elevated tIgE and sIgE are equally useful stand-alone biomarkers for defining and predicting risk of T2-high asthma in childhood.

Keywords: Asthma, Pulmonary eosinophilia, Paediatric asthma

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INTRODUCTION

Childhood asthma affects 10–15% of all European school-aged children with an increasing prevalence globally over the past decades.[1-3] A common asthma inflammatory phenotype is T2-high asthma, which is defined by either 1) being "allergy-driven"; 2) elevated sputum eosinophil count ≥2% and/or elevated blood eosinophil count (b-eos) ≥0.15 10^9/L; and/or 3) elevated fractional exhaled nitric oxide (FeNO) ≥20 parts per billion (ppb).[4] The latter 2 have clearly defined international cut-off values used by The Global Initiative for Asthma (GINA), whereas "allergy-driven" is not well-defined and could be characterized by either age-specific elevated total immunoglobulin E (tIgE) and/or elevated specific immunoglobulin E (sIgE) ≥0.35 kU/L towards aeroallergens and/or relevant symptoms on exposure.[4]

Most difficult-to-treat and severe asthma in childhood and adolescence can be classified as T2-high, which usually debuts in early childhood.[5,6] T2-high asthma in children is often well-controlled with inhaled corticosteroids in low to moderate daily doses, possibly with the addition of a second controller. In refractory severe cases biological treatment targeting key inflammatory mediators are needed.[7-9] Therefore, early identification, prediction and correct diagnosis of T2-high asthma in childhood is important in order to develop personalized care and precision medicine.[10]

The objective of this study was to assess and compare the value of tIgE and sIgE from infancy until adulthood for defining and predicting the risk of T2-high asthma in childhood using existing data from the extensively characterized Copenhagen Prospective Studies on Asthma in Childhood 2000 (COPSAC2000) and 2010 (COPSAC2010) mother-child cohorts.[11,12] Additionally, we investigated whether aeroallergen skin prick test (SPT) positivity or parent-reported rhinitis symptoms could serve the same purpose for defining and predicting risk of T2-high asthma in childhood.

METHODS

The COPSAC2000 cohort

COPSAC2000 is a prospective study conducted at a single center in Denmark, comprising 411 children born between 1998 and 2001 who were at-risk by virtue of maternal physician-diagnosed asthma. Enrollment of the children occurred at 1 month of age with scheduled clinical visits every 6 months until 7 years of age and thereafter at 13 and 18 years along with acute visits as needed. Longitudinal data was collected, including detailed clinical and environmental measures, alongside multi-omics profiling, to understand early-life asthma and allergy development.[11]

The COPSAC2010 cohort

COPSAC2010 is a prospective study of 736 pregnant women and their 700 children born between 2008 and 2011 with similar endpoints as the COPSAC2000 cohort but using an unselected population. Scheduled clinical visits were done at 1 week, 1, 3, 6, 12, 18, 24, 30, and 36 months, and regularly afterwards until 6 years of age and thereafter at ages 8 and 10 years along with acute visit as needed. In addition to similar endpoints, COPSAC2010 incorporates assessments of body composition, metabolism, neurodevelopment and multi-omics profiling to provide a broader understanding of how early-life exposures and comorbidity influence asthma and allergy, and offering validation of findings in a larger, more diverse population.[12,13]

Type-2 biomarkers

Blood eosinophil count was measured at ages 6 and 18 months and 4, 7, 13, and 18 years for COPSAC2000, and at ages 18 months and 6 and 10 years for COPSAC2010.

FeNO was measured at scheduled visits at ages 5, 6, 7, 13 and 18 years and at acute visits for COPSAC2000 and at ages 6 and 10 years for COPSAC2010 using a NIOX Vero (Aerocrine AB, Solna, Sweden) and a Denox 88 (ECO Medics AG, Switzerland) in accordance with international guidelines.[14]

Total IgE was determined at the same ages as b-eos. We used age-specific cut-off values of 7.3, 13, 40, 63, 85, and 85 kU/L for ages 6 and 18 months and 4, 7, 13 and 18 years respectively as recommended by the manufacturer.[15,16]

Specific IgE levels for aeroallergens were determined at the same ages as b-eos for

COPSAC2000 and at 10 years of age for COPSAC2010. sIgE was measured at additional ages for COPSAC2010 without simultaneous tIgE measures. A screening cut-off value of ≥0.35 kU/L was used (ImmunoCAP, Phadiatop Infant™ and Phadiatop™, Thermo Fisher Scientific, Uppsala, Sweden) followed by analysis of individual allergen sIgE levels by ImmunoCAP in screening positive samples for dog, cat, horse, birch, house dust mites, timothy grass, mugwort, and molds.

Skin prick test for aeroallergens were determined at the same ages as b-eos for COPSAC2000 and at 10 years of age for COPSAC2010 using standard allergen extracts for the same 8 aeroallergens as sIgE. A positive SPT reaction was defined as an average wheal diameter that exceeded the negative control by ≥ 2 mm up to 18 months of age and ≥3 mm for ages beyond 18 months.

Diagnosis of asthma and rhinitis

Asthma: In both cohorts, troublesome lung symptoms defined as clinically significant cough, wheeze, or dyspnea explained to the parents as wheeze or whistling sounds, breathlessness, or recurrent troublesome cough severely affecting the well-being of the child were recorded in a daily diary from birth as a dichotomous score (yes/no). Asthma before age 7 years was prospectively determined by physicians employed at the COPSAC research unit using strict predefined standard operating procedure as described in **Supplementary methods**.[17,18] Asthma after age 7 years was objectively validated in accordance with the European Respiratory Society guidelines (Supplementary Table 1).[19]

T2-high asthma was defined by elevated b-eos ≥0.3 10^9/L and/or FeNO≥20 ppb.[4] This cut-off value for b-eos is the recommended upper limit for initiation of biologic treatment targeting type-2 inflammation in refractory asthma cases ensuring higher specificity for this study.[4]

Rhinitis was defined as parent-reported symptoms of at least 2 symptoms of runny nose, sneezing, itchy nose, or blocked nose in the absence of infection. This assessment was conducted during acute visits for both cohorts, and additionally at ages 7, 13 and 18 years for COPSAC2000, and at ages 6 and 10 years for COPSAC2010.

Statistical analysis

Descriptive statistics are presented as frequencies and proportions. Counts used for Venn diagram visualization were estimated in R and digitally drawn with ellipses using the software EulerAPE version 3 to achieve area-proportional dimensions.[20] Spearman's rank correlation coefficient was calculated for all type-2 biomarkers. Cross-sectional association analyses were done using logistic regression models calculating odds ratio (OR) with 95% confidence interval (CI) and associated p-values (P) using an α value of 0.05. Comparison of models for tIgE and sIgE was done using Vuong's Likelihood-Ratio-Test (LRT) for model selection and the Akaike Information Criterion (AIC) and Bayesian Information Criterion (BIC).[21] Predictive analysis with logistic regression models were performed with corresponding receiver operating characteristic (ROC) analysis estimating area under the curve (AUC) values with 95% CI, sensitivity, specificity, positive and negative predictive values to compare the predictive performance of elevated tIgE, sIgE, and SPT ever at age 0–4 years in relation to development of T2-high asthma compared to T2-low asthma as well as cross-sectionally. This analysis was repeated for rhinitis symptoms ever at age 0–6 years. Comparison of AUC values were done using DeLong's test. Statistical analyses were performed using R version 4.2.1 (R Core Team, 2022).

Ethics

The studies were approved by the Copenhagen Ethics Committee (COPSAC2000: KF 01–289/96, COPSAC2010: H–B-2008-093) and the Danish Data Protection Agency (COPSAC2000 & COPSAC2010: 2015-41-3696). Parental and participant consent was obtained.

RESULTS

In COPSAC2000, a total of 155 (55.8%) children had elevated tIgE, 48 (17.0%) had elevated sIgE, and 26 (10.6%) had a positive SPT during their first 4 years of life. Levels of sIgE and radioallergosorbent test (RAST) class distributions at various time points are detailed in Supplementary Table 2. Information on mono- and polysensitization, along with sIgE sensitization to specific aeroallergens, is provided in Supplementary Table 3. In COPSAC2000, 31

(14.9%) children experienced rhinitis symptoms during their first 6 years of life, which was 44 (6.7%) children in COPSAC2010. In COPSAC2000, the percentages of asthma cases at ages 7, 13, and 18 years were 14.0%, 17.1%, and 25.4%, respectively, while in COPSAC2010, it was 6.6% at age 10 years. In COPSAC2000, the percentages of T2-high asthma cases at ages 7, 13 and 18 years were 10.2%, 9.3%, and 15.2%, respectively, while in COPSAC2010, it was 3.6% at age 10 years. The distribution of children with asthma and elevated type-2 biomarkers in COPSAC2000 and COPSAC2010 is outlined in Table 1. No children included from either cohort had diagnoses of primary immune deficiency, including hyper IgE syndrome, recurrent severe infections, or parasitic infections at any point during the study.

The correlation between elevated tIgE, elevated sIgE, positive SPT and reported rhinitis symptoms is visualized in Fig. 1. In COPSAC2000, moderate Spearman's rank correlation coefficients were identified between tIgE and sIgE at ages 7, 13, and 18 years, for both healthy children (0.41, 0.41, 0.52) and children with asthma (0.44, 0.30, 0.49). In COPSAC2010, a moderate correlation between tIgE and sIgE was observed at age 10 years for both healthy children (0.41) and children with asthma (0.56). All p-values were less than 0.001 for the correlations between tIgE and sIgE. In COPSAC2000, symptoms of allergic rhinitis were more strongly correlated with sIgE than tIgE at all time points in both healthy children (0.39 vs 0.12) and children with asthma ($\geq$0.30 vs $\geq$ 0.12). Similarly, in COPSAC2010 at age 10 years, symptoms of allergic rhinitis were more strongly correlated with sIgE than tIgE in both healthy children (0.34 vs 0.19) and children with asthma (0.74 vs 0.61).

Visualizations of the overlap between elevated b-eos, elevated FeNO and either elevated tIgE or sIgE among children with a confirmed asthma diagnosis are shown for both cohorts in Fig. 2 at ages 7–18 years. We observed a similar overlap of asthma with elevated b-eos and/or FeNO and either elevated tIgE or sIgE at ages 10 (63.6% vs 60.6%), 13 (56.6% vs 50.9%) and 18 years (49.4% vs 55.3%), and a slightly diverging overlap at age 7 years (34.9% vs 25.6%). Additionally, there was a similar number of children with asthma, who had isolated elevation of either tIgE or sIgE at 7 years (2.3% vs 4.7%), 10 years (15.2% vs 12.1%), 13 years (11.3% vs 17.0%), and 18 years (10.6% vs 12.9%).

Cross-sectional analyses of tIgE and sIgE vs T2-high/low asthma

Logistic regression was performed on the cross-sectional association between elevated tIgE and

Cohort	COPSAC2000 (N = 411)			COPSAC2010 (N = 700)
Time point	7 years (%)	13 years (%)	18 years (%)	10 years (%)
Sex (Male)	169/336 (50.3)	180/362 (49.7)	183/371 (49.3)	330/640 (51.6)
Asthma (Yes)	47/336 (14.0)	62/362 (17.1)	94/371 (25.4)	42/640 (6.6)
T2-high asthma (Yes)[a]	34/334 (10.2)	33/353 (9.3)	55/362 (15.2)	23/631 (3.6)
b-eos (Elevated)	204/311 (65.6)	106/308 (34.4)	92/337 (27.3)	208/511 (40.7)
FeNO (Elevated)	27/317 (8.5)	77/330 (23.3)	150/365 (41.1)	91/598 (15.2)
tIgE (Elevated)	122/296 (41.2)	129/315 (41.0)	173/355 (48.7)	248/532 (46.6)
sIgE (Elevated)	76/296 (25.7)	146/315 (46.3)	187/355 (52.7)	187/532 (35.2)
SPT (Elevated)	24/290 (8.3)	115/316 (36.4)	175/358 (48.9)	148/532 (27.8)
Rhinitis symptoms (Yes)	33/214 (15.4)	133/357 (37.3)	163/370 (44.1)	112/636 (17.7)

Table 1. Distribution of children with asthma and elevated type-2 biomarkers for each clinical visit during school-age. Elevated blood eosinophil count (b-eos) was defined as $\geq$0.30 10^9/L, fractional exhaled nitric oxide (FeNO) as $\geq$20 ppb, total immunoglobulin E (tIgE) as $\geq$ age-specific cutoff values, specific immunoglobulin E (sIgE) as $\geq$0.35 kU/L and skin prick test (SPT) as positive for tested aeroallergens.
[a]*Missing data for T2-high asthma was 2/9/9/9 children at 7/10/13/18 years with either b-eos or FeNO missing.*

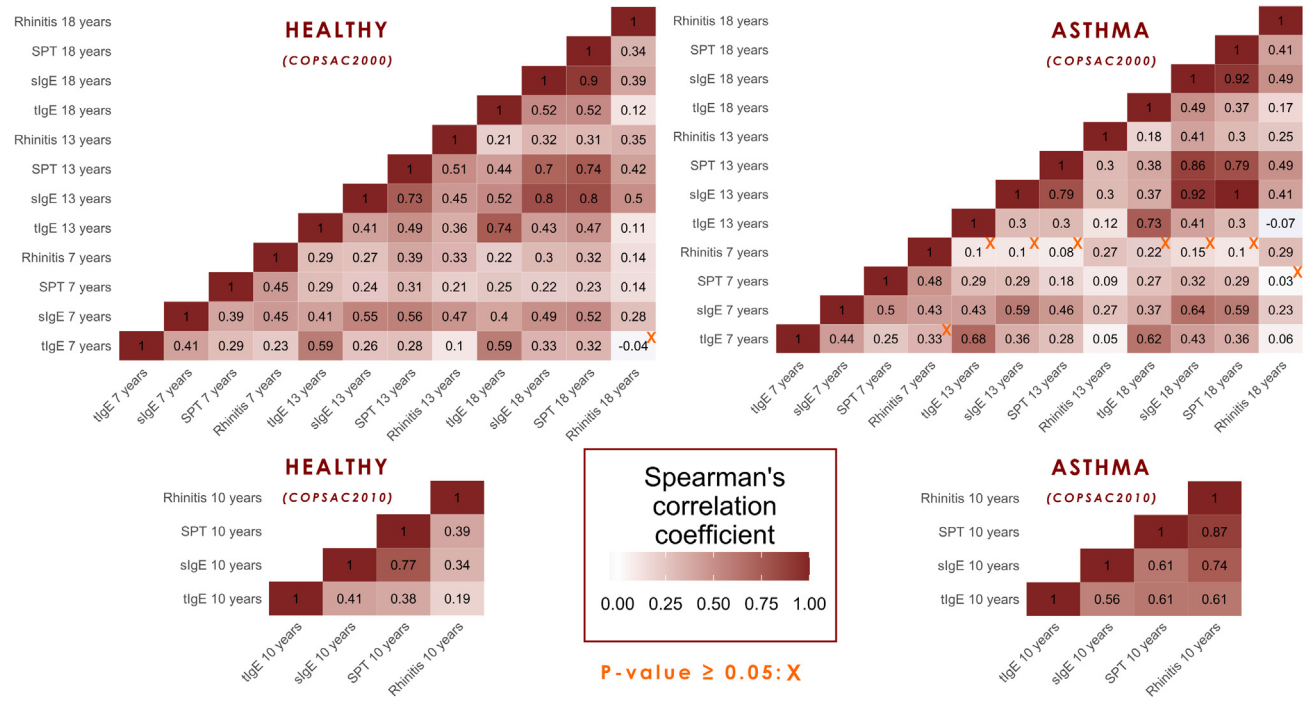

Fig. 1 Heatmap of correlation between type-2 biomarkers in COPSAC2000 and COPSAC2010 at ages 7, 10, 13 and 18 years. Elevated total immunoglobulin E (tIgE ≥ age-specific cutoff values), specific immunoglobulin E (sIgE ≥0.35 kU/L), positive aeroallergen skin prick test (SPT) and rhinitis symptoms (Rhinitis) were compared. P-values are ≤0.05 unless stated otherwise

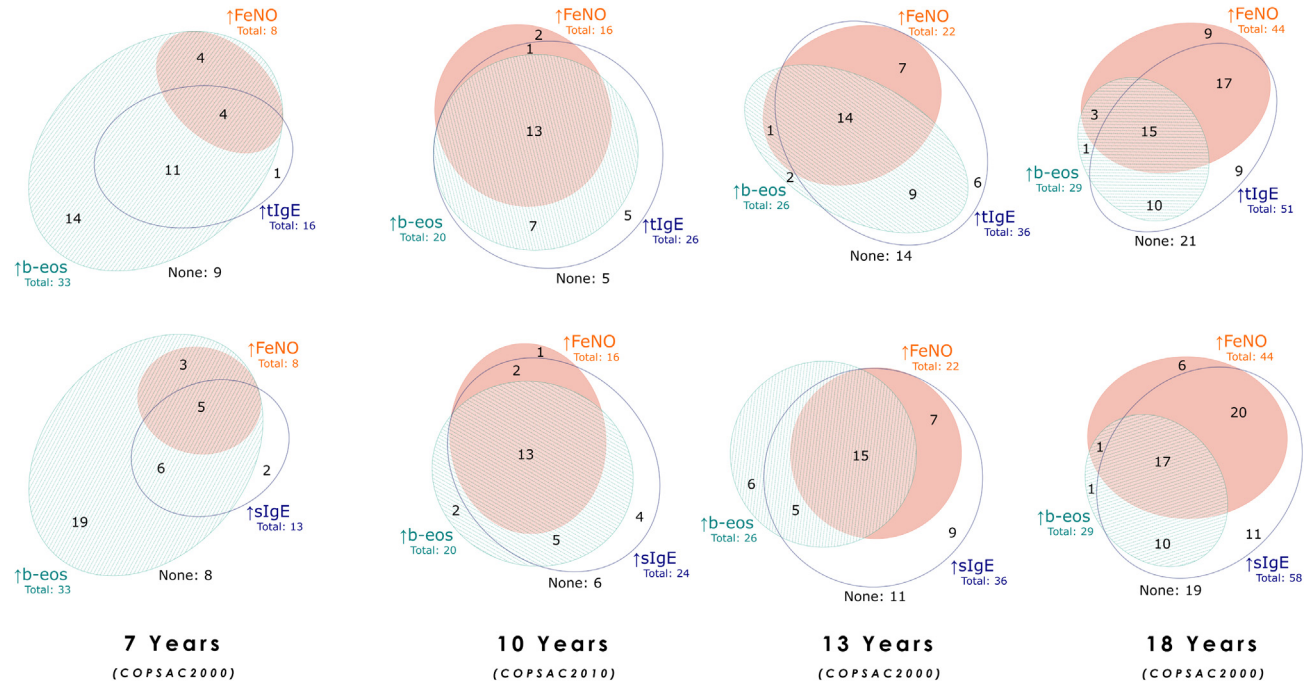

Fig. 2 Venn diagram of children with asthma diagnosis at school-age time points in relation to elevated blood eosinophil count (b-eos) ≥0.30 10^9/L, fractional exhaled nitric oxide (FeNO) ≥20 ppb and either total immunoglobulin E (tIgE) ≥63 kU/L at 7 years, and ≥85 kU/L at 10, 13, and 18 years or specific immunoglobulin E (sIgE) ≥0.35 kU/L

-	Elevated tIgE, sIgE, SPT or rhinitis symptoms and concurrent T2-high vs T2-low asthma				Elevated tIgE, sIgE, SPT or rhinitis symptoms at early age and later T2-high vs T2-low asthma			
-	N (T2-high	Odds ratio	Confidence interval	P-value	N (T2-high)	Odds ratio	Confidence interval	P-value
7 Years								
tIgE	33	7.50	[1.20–146.49]	0.03	29	5.73	[1.14–43.37]	0.03
sIgE	33	2.00	[0.41–14.77]	0.43	30	0.73	[0.15–4.08]	0.70
SPT	31	3.27	[0.49–65.28]	0.24	27	2.53	[0.35–51.78]	0.42
Rhinitis	14	1.50	[0.19–14.25]	0.70	13	1.71	[0.24–15.75]	0.60
10 Years								
tIgE	23	10.50	[1.74–90.78]	0.03	–	–	–	–
sIgE	23	10.00	[1.88–67.27]	0.007	–	–	–	–
SPT	23	15.56	[2.83–115.51]	0.001	–	–	–	–
Rhinitis	23	7.13	[1.43–42.08]	0.02	23	3.67	[0.72–27.96]	0.12
13 Years								
tIgE	33	23.33	[5.70–127.87]	<0.001	21	5.67	[1.31–28.62]	0.02
sIgE	33	5.50	[1.64–20.39]	0.005	22	1.67	[0.44–6.88]	0.46
SPT	30	3.00	[0.94–10.08]	0.94	23	4.23	[0.87–31.52]	0.10
Rhinitis	33	3.26	[1.03–10.86]	0.04	21	2.46	[0.46–19.12]	0.32
18 Years								
tIgE	55	7.54	[2.87–21.40]	<0.001	40	2.91	[1.04–8.52]	0.04
sIgE	55	10.15	[3.67–30.75]	<0.001	40	6.00	[1.72–28.28]	0.004
SPT	54	10.00	[3.66–29.86]	<0.001	42	1.41	[0.36–7.05]	0.63
Rhinitis	55	4.85	[1.89–13.04]	<0.001	24	10.15	[1.60–200.12]	0.01

Table 2. Logistic regression models assessing association between T2-high asthma and elevated total immunoglobulin E (tIgE ≥ age-specific cutoff values), specific immunoglobulin E (sIgE ≥0.35 kU/L), aeroallergen skin prick test (SPT) or rhinitis symptoms (Rhinitis). Model 1 examines cross-sectional associations at ages 7–18 years. Model 2 explores early-life risk factors (0–4 years for IgE/SPT, 0–6 years for rhinitis symptoms) for later T2-high asthma

sIgE and T2-high asthma defined by either elevated b-eos and/or FeNO at ages 7, 10, 13, and 18 years (Table 2). Using age-specific cut-off values, elevated tIgE was significantly associated with an increased risk of T2-high asthma at ages 7 years (OR = 7.5, 95% CI = 1.2–146.5, P = 0.03), 10 years (10.5, 1.7–90.8, P = 0.03), 13 years (23.3, 5.7–127.9, P < 0.01) and 18 years (7.5, 2.9–21.4, P < 0.01). Elevated sIgE was also significantly associated with an increased risk of T2-high asthma at ages 10 years (10.0, 1.9–67.3, P < 0.01), 13 years (5.5, 1.6–20.4, P < 0.01), and 18 years (10.2, 3.7–30.8, P < 0.01), but not at age 7 years (2.0, 0.4–14.8, P = 0.43).

Similarly, a positive SPT was significantly associated with an increased risk of T2-high asthma at ages 10 years (15.6, 2.8–115.5, P < 0.01) and 18 years (10.0, 3.7–29.9, P < 0.01), but not at ages 7 years (3.3, 0.5–65.3, P = 0.24) or 13 years (3.0, 0.9–

10.1, P = 0.94). Rhinitis symptoms were also significantly associated with an increased risk of T2-high asthma at ages 10 years (7.1, 1.4–42.1, P = 0.02), 13 years (3.3, 1.0–10.9, P = 0.04), and 18 years (4.9, 1.9–13.0, P < 0.01), but not at age 7 years (1.5, 0.2–14.3, P = 0.70) (Table 2).

To compare whether associations with T2-high asthma were stronger for tIgE, sIgE, or a combination of both, Vuong's LRT and AIC/BIC calculations were performed for model selection. This was done for either elevated tIgE or sIgE in relation to concurrent T2-high asthma in comparison to using a combination model of both tIgE and sIgE. These analyses showed that at age 13 years there was a significant fit preference for using a combination model of both tIgE and sIgE instead of sIgE alone (P < 0.01), but there were no significant preferences at ages 7, 10, or 18 years (p-values>0.10). Further, there was no fit preference for using a combination model of both tIgE and sIgE instead of tIgE alone (p = 0.61) (Supplementary Table 4).

ROC analyses for the cross-sectional performance of elevated tIgE and sIgE for determining risk of T2-high asthma are shown in Supplementary Table 5. Using DeLong's test there was a significant difference between the AUC values for tIgE and sIgE at age 13 years (P = 0.04) but not at ages 7, 10, or 18 years (p-values>0.09).

Early life tIgE and sIgE vs later T2-high/low asthma

Logistic regression was performed to investigate the association between elevated tIgE and sIgE in early childhood and the later risk of developing T2-high asthma (Table 2). Elevated tIgE at ages 0–4 years was significantly associated with an increased risk of having T2-high asthma at ages 7 years (5.7, 1.1–43.4, P = 0.03), 13 years (5.7, 1.3–28.6, P = 0.02), and 18 years (2.9, 1.0–8.5, P = 0.04). Elevated sIgE at ages 0–4 years was also significantly associated with an increased risk of T2-high asthma at age 18 years (6.0, 1.7–28.3, P < 0.01), but not at ages 7 years (0.7, 0.2–4.1, P = 0.70) or 13 years (1.7, 0.4–6.9, P = 0.46).

Conversely, an early positive SPT was not significantly associated with the risk of T2-high asthma at ages 7 years (2.5, 0.4–51.8, P = 0.42), 13 years (4.2, 0.9–31.5, P = 0.10) or 18 years (1.4, 0.4–7.1, P = 0.63). Parentally reported rhinitis symptoms at ages 0–6 years was significantly associated with the risk of having T2-high asthma at age 18 years (10.2, 1.6–200.1, P = 0.01) but not at ages 7 years (1.7, 0.2–15.8, P = 0.60), 10 years

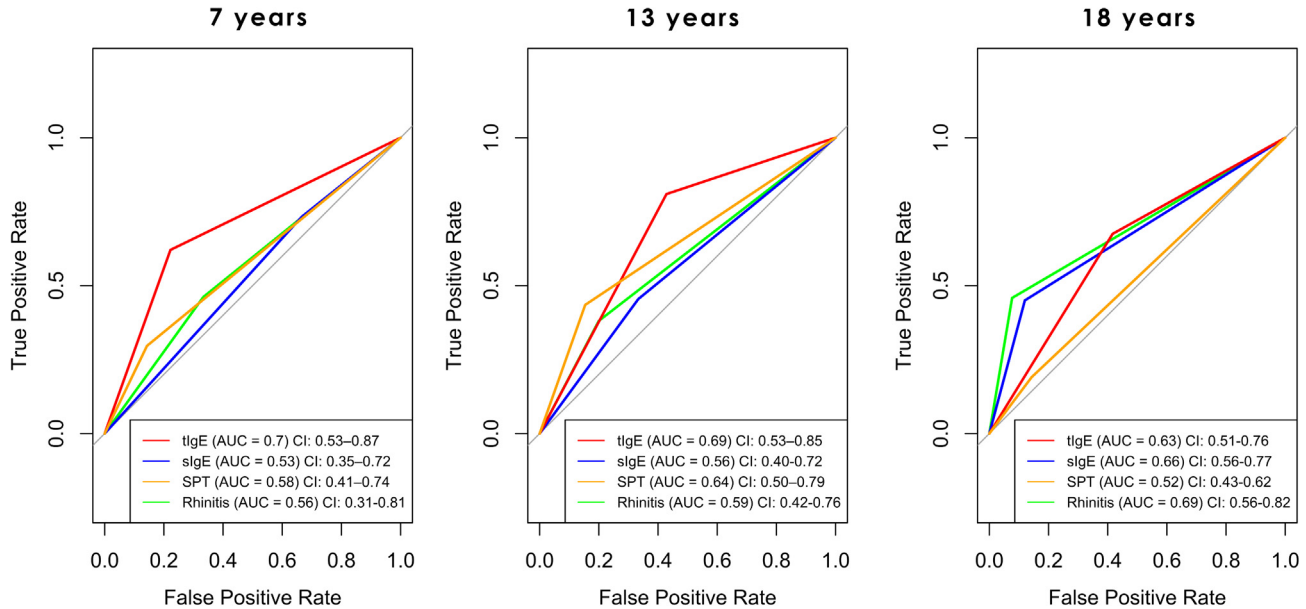

Fig. 3 Coupled Receiver operating characteristic (ROC) curves of logistic models for elevated total immunoglobulin E (tIgE), specific immunoglobulin E (sIgE), positive aeroallergen skin prick test (SPT) and rhinitis symptoms (yes/no) between age 0–6 years in relation to T2-high asthma at later time points. Corresponding area under curve (AUC) and confidence interval (CI) values are shown

(3.7, 0.7–28.0, P = 0.12) or 13 years (2.5, 0.5–19.1, P = 0.32) (Table 2).

ROC analyses and corresponding ROC curves for the performance of early childhood elevated tIgE and sIgE for predicting risk of developing T2-high asthma after age 7 years is shown in Fig. 3 and Supplementary Table 6. At ages 7, 13, and 18 years, the AUC values [95% CI] for tIgE were 0.70 [0.53–0.87], 0.69 [0.53–0.85], and 0.63 [0.51–0.76], respectively, while those for sIgE were 0.53 [0.35–0.72], 0.56 [0.40–0.72], and 0.66 [0.56–0.77]. Likewise, the positive predictive value (PPV) and negative predictive value (NPV) were calculated for tIgE at 7 years (PPV/NPV: 90%/39%), 13 years (74%/67%), and 18 years (73%/52%), and for sIgE at 7 years (79%/27%), 13 years (67%/45%), and 18 years (86%/50%). Using DeLong's test there were no significant differences between the AUC values for tIgE and sIgE at ages 7, 13, or 18 years (p-values>0.20). Sensitivity and specificity for tIgE were 0.62/0.78 at 7 years, 0.81/0.57 at 13 years, and 0.68/0.58 at 18 years, while for sIgE, they were 0.73/0.33 at 7 years, 0.45/0.67 at 13 years, and 0.45/0.88 at 18 years.

For SPT, the AUC values were 0.58 [0.41–0.74], 0.64 [0.50–0.79], and 0.52 [0.43–0.62] for ages 7, 13, and 18 years, with corresponding PPV/NPV values of 89%/24%, 83%/46%, and 73%/35%. Likewise, for rhinitis symptoms, the AUC values were 0.56 [0.31–0.81], 0.64 [0.47–0.81], 0.59 [0.42–0.76], and 0.69 [0.56–0.82] for ages 7, 10, 13, and 18 years, with corresponding PPV/NPV values of 75%/36%, 85%/40%, 80%/38%, and 92%/48% (Fig. 3 and Supplementary Table 6).

Sensitivity analyses

Exploration of a higher cut-off value for b-eos ($\geq 0.5 \times 10^9$/L) and a uniform not age-specific cut-off value for elevated tIgE (≥50 kU/L) yielded similar results for both cross-sectional analyses and for early life elevated tIgE and sIgE in relation to risk of developing T2-high asthma (Supplementary Tables 7–8). Likewise, comparing T2-high asthma to healthy children instead of T2-low yielded similar associations (Supplementary Table 9). Additionally, sensitivity analyses adjusting for confounders such as sex, atopic dermatitis, seasonal allergies, and parental predisposition to asthma yielded no significant changes from the unadjusted model (Supplementary Table 10).

DISCUSSION

We found that elevated tIgE and sIgE had similar cross-sectional associations with T2-high asthma from age 10 years and above, whereas elevated tIgE was also associated with T2-high asthma at age 7 years unlike sIgE. There were almost interchangeable overlapping areas of elevated tIgE and sIgE with T2-high asthma from age 7. Importantly, either tIgE or sIgE were solely elevated for a considerable number of children with asthma during adolescence that otherwise would not be defined as having T2-high asthma using only elevated b-eos and/or FeNO. Further, elevated levels of both tIgE and sIgE in early childhood was associated with an increased risk of developing T2-high asthma though this was only significant at 18 years for sIgE, whereas it was significant across all time points in childhood for tIgE. However, there were no significant differences in AUC values for tIgE vs sIgE. These findings suggest that elevated tIgE and sIgE are equally useful stand-alone biomarkers for defining and predicting risk of T2-high asthma in childhood.

We found that up to 17% of the children with asthma had elevated tIgE and/or sIgE without accompanying elevated b-eos and/or FeNO. These children could be defined as having T2-high asthma, but it is uncertain whether T2-high asthma should be defined solely by elevated tIgE or sIgE levels or whether combining both would be preferential.[22,23] A previous study has shown that the risk of developing asthma in childhood is greatest when multiple type-2 biomarkers are elevated in combination as opposed to when only 1 biomarker is elevated.[24] In contrast, we found no benefit using a combination model of both elevated tIgE and sIgE in relation to the risk of T2-high asthma compared to using either elevated tIgE or sIgE alone. However, children with asthma with an isolated elevated IgE without other elevated type-2 biomarkers could also be children, who do not have the conventional T2-low asthma phenotype but instead a similar phenotype of T2-low with elevated IgE as reported in adults.[25]

Other studies have shown that both elevated tIgE and sIgE are associated with increased

severity of asthma.[26,27] Being able to correctly identify and predict risk of T2-high asthma could lead to improved personalized treatment, better asthma control, and lessen the burden of asthma exacerbations in refractory cases, where there is a need for specific treatments with biologics and ultimately lead the way for precision medicine.[7,10,28,29] As such, the possibility of categorizing asthma early in life into T2-high and T2-low groups and predicting the inflammatory phenotype later in childhood with a higher accuracy has a clinical perspective for clinicians treating childhood asthma. Our findings suggested that both tIgE and sIgE are equally useful biomarkers during childhood and adolescence to define risk of T2-high asthma. However, elevated tIgE exhibited a more consistent association with T2-high asthma at every time point from age 7 years, whereas elevated sIgE was associated with risk of T2-high asthma from age 10 years.

Our findings suggest that both tIgE and sIgE could serve as valuable biomarkers for predicting risk of T2-high asthma later in life. Elevated tIgE performed slightly better than elevated sIgE with a significant association to T2-high asthma at all time points and overall showed higher AUC values, but these were not significantly different than the sIgE AUC values. These findings are consistent with previous studies, which have shown that elevated tIgE and sIgE levels during childhood were both significant predictors for the development of wheeze and asthma,[24,30,31] with our study notably contextualizing these associations in children with T2-high asthma. Generally, the overall predictive performance of both tIgE and sIgE, as indicated by their AUC, were relatively low ($\leq$0.70), with sIgE only being significantly better than random at 18 years of age. In our study, we observed age-related variations in the predictive power of tIgE and sIgE. Notably, tIgE demonstrated superior accuracy to sIgE in identifying T2-high children and adolescents, with the best PPV observed at 13 years (tIgE vs sIgE, 90% vs 79%). In contrast, sIgE surpassed tIgE in terms of PPV at 18 years (86% vs 73%). However, for NPV, tIgE appeared to outperform sIgE at all time points (39–67% vs 27–50%).

There is an ongoing debate whether sIgE or SPT is the most informative tool for identifying clinically significant sensitization,[32–34] particularly as type-2 biomarkers, with limited consensus in the existing literature. Though SPT is associated with pain and anxiety in children,[35,36] it is seen as minimally invasive in comparison to venipuncture for sIgE measurements.[37] We observed similar cross-sectional risks of T2-high asthma for sIgE vs SPT, whereas early childhood sIgE was a stronger predictor than SPT for development of T2-high asthma.

Allergic rhinitis frequently coexists with asthma in children.[38] Our analysis of parentally reported rhinitis symptoms independent of allergic sensitization testing showed that this was non-inferior to elevated tIgE and sIgE for defining and predicting T2-high asthma. This could prove useful in a clinical setting, as evaluating symptoms of rhinitis is straightforward and does not require any form of blood testing, making it a completely non-invasive approach. However, reported rhinitis symptoms may encompass several other causes than allergy in a general practice or asthma clinic unlike our closely monitored cohorts, where the parents and children where prospectively trained in recognizing asthma and allergy symptoms.

One important limitation of this study is the small number of children with asthma in the cohorts, which hampers the ability to study T2-high asthma subtypes and may have caused lack of association specifically for elevated sIgE, positive SPT, and reported rhinitis symptoms at early ages, where sensitization to aeroallergens is not very prevalent compared to elevated tIgE. We also observed a difference in the prevalence of T2-high asthma between the 2 cohorts. The prevalence was lower in the COPSAC2010 cohort at 10 years compared to the COPSAC2000 cohort at 13 years (3.6% vs 9.3%), likely due to cohort differences, such as the asthma at-risk status in COPSAC2000. Also, children from an asthma at-risk cohort are more likely to have a genetic predisposition to asthma and atopy, which may influence the association between tIgE and sIgE levels and the development of T2-high asthma. Specifically, an asthma at-risk cohort may have higher baseline levels of tIgE and sIgE, making it difficult to determine if elevated IgE is related to the development of T2-high asthma or simply a reflection of the genetic background.[27] However, we found similar results at age 10 years in the population-based COPSAC2010 cohort, suggesting that our findings are representative of the general

population. Additionally, exploring a higher cut-off value for b-eos and a uniform not age-specific cut-off for tIgE did not alter our findings. Previous studies have examined age-related changes in tIgE levels, and while several reference intervals have been suggested, there is still variability in the definition of normal range for different age groups.[39,40] Comparison between children with T2-high asthma and healthy children was similar to that between children with T2-high and T2-low asthma children. Finally, it is a limitation that we did not include regular assessment of asthma severity or control using, e.g., the Asthma Control Test (ACT) or Asthma Control Questionnaire (ACQ) tools.

CONCLUSION

Elevated tIgE and sIgE are equally relevant stand-alone biomarkers for defining and predicting risk of T2-high asthma in childhood, particularly during adolescence, with SPT performing similarly. The consistent utility of both tIgE and sIgE as T2-high biomarkers across age groups underscores their role for improving diagnostic accuracy of T2-high asthma, providing clinicians with valuable tools for asthma phenotyping. Parent-reported rhinitis symptoms were also useful in our closely monitored birth cohorts for defining and predicting risk of T2-high asthma without any need for blood samples or skin prick tests. These findings are useful for clinicians working with childhood asthma to develop personalized care and precision medicine.

Abbreviations

ACQ: Asthma Control Questionnaire; **ACT**: Asthma Control Test; **AIC**: Akaike information criterion; **AUC**: Area under the curve; **BIC**: Bayesian Information Criterion; **b-eos**: Blood eosinophil count; **CI**: Confidence Interval; **COPSAC2000**: Copenhagen Prospective Studies on Asthma in Childhood 2000; **COPSAC2010**: Copenhagen Prospective Studies on Asthma in Childhood 2010; **FeNO**: Fractional exhaled nitric oxide; **GINA**: The Global Initiative for Asthma; **IL**: Interleukin; **IgE**: Immunoglobulin E; **LRT**: Likelihood-Ratio-Test; **OR**: Odds ratio; **P**: P-value; **Ppb**: Parts per billion; **RAST**: Radioallergosorbent test; **ROC**: Receiver operating characteristic; **sIgE**: Specific immunoglobulin E; **tIgE**: Total immunoglobulin E

Availability of data and materials

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials.

Author's contributions

The guarantor of the study is BC, from conception and design to conduct of the study and acquisition of data, data analysis, and interpretation of data. TS has written the first draft of the manuscript. All co-authors have provided important intellectual input and contributed considerably to the analyses and interpretation of the data. All authors guarantee that the accuracy and integrity of any part of the work have been appropriately investigated and resolved and all have approved the final version of the manuscript. The corresponding author had full access to the data and had final responsibility for the decision to submit for publication. No honorarium, grant, or other form of payment was given to any of the authors to produce this manuscript.

Ethics approval

The studies were approved by the Copenhagen Ethics Committee (COPSAC2000: KF 01–289/96, COPSAC2010: H–B-2008-093) and the Danish Data Protection Agency (COPSAC2000 & COPSAC2010: 2015-41-3696). Parental and participant consent was obtained.

Author's consent for publication

All the authors reviewed the final draft and provided consent for publication.

Governance

We are aware of and comply with recognized codes of good research practice, including the Danish Code of Conduct for Research Integrity. We comply with national and international rules on the safety and rights of patients and healthy subjects, including Good Clinical Practice (GCP) as defined in the EU's Directive on Good Clinical Practice, the International Conference on Harmonisation's (ICH) good clinical practice guidelines and the Helsinki Declaration. Privacy is important to us which is why we follow national and international legislation on General Data Protection Regulation (GDPR), the Danish Act on Processing of Personal Data and the practice of the Danish Data Inspectorate.

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Declaration of competing interest

All authors declare no potential, perceived, or real conflict of interest regarding the content of this manuscript. The funding agencies did not have any role in design and conduct of the study; collection, management, and interpretation of the data; or preparation, review, or approval of the manuscript. No pharmaceutical company was involved in the study.

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Appendix A. Supplementary data
Supplementary data to this article can be found online at https://doi.org/10.1016/j.waojou.2024.100994.

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Title:

Levels of total IgE vs. specific IgE during childhood for defining and predicting T2-high asthma

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Document: Online Supplementary Materials

ONLINE REPOSITORY MATERIALS

Supplementary methods:

Asthma diagnosis: Asthma before 7 years of age was prospectively determined by physicians employed at the COPSAC research unit using a predefined standard operating procedure with 4 mandatory criteria: 1) Diary-verified persistent wheeze defined as 5 episodes of troublesome lung symptoms lasting at least 3 continuous days within 6 months or continuous symptoms lasting at least 4 weeks; 2) Symptoms judged by the COPSAC physicians to be typical of asthma (e.g. exercise induced symptoms, prolonged nocturnal cough, recurrent cough outside common cold, symptoms causing wakening at night); 3) Intermittent need of rescue inhaled β2-agonist; and 4) Response to a 3-month trial of inhaled corticosteroids initiated when the criteria for persistent wheeze were met and relapse after cessation.

OBJECTIVE MEASUREMENTS OF ASTHMA

1.	**Obstructive lung function:**	FEV1<80% and/or FEV1/FVC<80% and/or specific airway resistance >1.6 kPa/s
2.	**Bronchial reversibility:**	Increase in FEV1≥12 % and/or decrease in specific airway resistance ≥ 30% after administration of inhaled short-acting beta2-agonist
3.	**Bronchial hyperreactivity:**	Decrease in FEV1 of >10% during exercise challenge test and/or decrease in FEV1≥ 20% during methacholine challenge test with a provocation dose ≤ 8mg/ml
4.	**Airway inflammation:**	Elevated FeNO≥25ppb

Supplementary Table 1: Asthma after age 7 years was objectively validated assuring that there were at least two accompanying objective measures in accordance with European Respiratory Society guidelines (18).

Time points	RAST Class 0 (<0.35)	RAST Class 1 (0.35-0.69)	RAST Class 2 (0.70-3-49)	RAST Class 3 (3.50-17.49)	RAST Class 4 (17.50-49.99)	RAST Class 5 (50.00-100.00)	RAST Class 6 (>=100.00)	Missing (NA)
6 months	367/369 (99.5%)	0/369 (0.0%)	1/369 (0.3%)	0/369 (0.0%)	1/369 (0.3%)	0/369 (0.0%)	0/369 (0.0%)	42
18 months	344/358 (96.1%)	1/358 (0.3%)	9/358 (2.5%)	3/358 (0.8%)	1/358 (0.3%)	0/358 (0.0%)	0/358 (0.0%)	53
4 years	262/319 (82.1%)	12/319 (3.8%)	18/319 (5.6%)	14/319 (4.4%)	7/319 (2.2%)	3/319 (0.9%)	3/319 (0.9%)	92
7 years	220/296 (74.3%)	9/296 (3.0%)	22/296 (7.4%)	17/296 (5.7%)	15/296 (5.1%)	7/296 (2.4%)	6/296 (2.0%)	115
10 years*	345/532 (64.9%)	24/532 (4.5%)	40/532 (7.5%)	35/532 (6.6%)	39/532 (7.3%)	18/532 (3.4%)	31/532 (5.8%)	168
13 years	169/315 (63.8%)	10/315 (3.8%)	27/315 (10.2%)	28/315 (10.6%)	32/315 (12.1%)	26/315 (9.8%)	23/315 (8.7%)	96
18 years	168/355 (75.0%)	17/355 (4.8%)	30/355 (13.4%)	48/355 (21.4%)	42/355 (18.8%)	29/355 (12.9%)	21/355 (9.4%)	56

*COPSAC2010

Supplementary Table 2:

This table presents the distribution of specific IgE levels across various time points for COPSAC2000 (411 children) and COPSAC2010 (700 children). Elevated specific IgE levels to aeroallergens were classified into seven radioallergosorbent test (RAST) classes based on kU/L ranges.

Time points	Mono-sensitization	Poly-sensitization	Dog	Cat	Horse	Birch	House Dust Mites	Timothy Grass	Mugwort	Molds
6 months	2/369 (0.5%)	0/369 (0.0%)	0/369 (0.0%)	1/369 (0.3%)	0/369 (0.0%)	0/369 (0.0%)	0/369 (0.0%)	1/369 (0.3%)	0/369 (0.0%)	0/369 (0.0%)
18 months	12/356 (3.4%)	1/356 (0.3%)	4/357 (1.1%)	3/358 (0.8%)	1/358 (0.3%)	0/358 (0.0%)	2/358 (0.6%)	5/358 (1.4%)	0/357 (0.0%)	0/358 (0.0%)
4 years	32/319 (10.0%)	25/319 (7.8%)	13/319 (4.1%)	9/319 (2.8%)	9/319 (2.8%)	19/319 (6.0%)	19/319 (6.0%)	27/319 (8.5%)	6/319 (1.9%)	8/319 (2.5%)
7 years	20/296 (6.8%)	56/296 (18.9%)	20/296 (6.8%)	15/296 (5.1%)	10/296 (3.4%)	26/296 (8.8%)	35/296 (11.8%)	39/296 (13.2%)	11/296 (3.7%)	10/296 (3.4%)
10 years*	71/532 (13.3%)	116/532 (21.8%)	50/532 (9.4%)	53/532 (10.0%)	22/532 (4.1%)	77/532 (14.5%)	109/532 (20.5%)	115/532 (21.6%)	50/532 (9.4%)	28/532 (5.3%)
13 years	25/315 (7.9%)	121/315 (38.4%)	41/315 (13.0%)	52/315 (16.5%)	28/315 (8.9%)	54/315 (17.1%)	92/315 (29.2%)	100/315 (31.7%)	43/315 (13.7%)	19/315 (6.0%)
18 years	63/355 (17.7%)	124/355 (34.9%)	67/355 (18.9%)	64/355 (18.0%)	-	75/355 (21.1%)	121/355 (34.1%)	132/355 (37.2%)	54/355 (15.2%)	21/355 (5.9%)

*COPSAC2010

Supplementary Table 3: This table provides detailed information on mono- and polysensitization to aeroallergens and the distribution of specific IgE sensitizations (≥0.35 $\mathrm{kU/L}$) to common environmental triggers of asthma, such as dog, cat, horse, birch, house dust mites, timothy grass, mugwort, and molds, across different time points for COPSAC2000 (411 children) and COPSAC2010 (700 children). If data for one aeroallergen component is missing (NA), sensitization was recorded as missing

Models at time point	Variance test (p-value)	Non-nested LRT Model 1 > 2 in fit (P-value)	Non-nested LRT Model 2 > 1 in fit (P-value)	AIC/BIC differences: (Which model fit to prefer?)
7 years				
tIgE (1) vs. Combination IgE (2)	0.19	-	-	-
sIgE (1) vs. Combination IgE (2)	0.02	0.90	0.10	Neither
10 years				
tIgE (1) vs. Combination IgE (2)	0.07	-	-	-
sIgE (1) vs. Combination IgE (2)	0.04	0.41	0.59	Neither
13 years				
tIgE (1) vs. Combination IgE (2)	<0.001	0.40	0.61	Neither
sIgE (1) vs. Combination IgE (2)	0.02	1.00	0.005	Combination IgE
18 years				
tIgE (1) vs. Combination IgE (2)	<0.001	0.66	0.34	Neither
sIgE (1) vs. Combination IgE (2)	<0.001	0.42	0.58	Neither

Supplementary Table 4: Cross-sectional logistic regression model comparison to determine a superior fit regarding risk of T2-high asthma. The models use either total immunoglobulin E (tIgE) or specific immunoglobulin E (sIgE) alone or a combination of both (combination IgE). If the variance test was significant (H0=P<0.05) it indicated a potential distinguishable model difference and a Likelihood-Ratio-Test for model selection would then be performed.

	AUC	CI 95%	Sensitivity	Specificity	PPV	NPV
7 years						
tIgE	0.68	0.55-0.81	0.45	0.90	0.94	0.33
sIgE	0.57	0.41-0.72	0.33	0.80	0.85	0.27
SPT	0.59	0.45-0.73	0.29	0.89	0.90	0.27
Rhinitis	0.55	0.27-0.83	0.50	0.60	0.78	0.30
10 years						
tIgE	0.71	0.53-0.88	0.91	0.50	0.81	0.71
sIgE	0.74	0.56-0.91	0.87	0.60	0.83	0.67
SPT	0.79	0.62-0.95	0.87	0.70	0.87	0.70
Rhinitis	0.71	0.53-0.89	0.83	0.60	0.83	0.60
13 years						
tIgE	0.81	0.69-0.92	0.91	0.70	0.83	0.82
sIgE	0.68	0.55-0.81	0.82	0.55	0.75	0.65
SPT	0.63	0.49-0.77	0.67	0.60	0.71	0.55
Rhinitis	0.64	0.50-0.78	0.73	0.55	0.73	0.55
18 years						
tIgE	0.73	0.63-0.83	0.76	0.70	0.82	0.62
sIgE	0.74	0.64-0.84	0.85	0.63	0.81	0.70
SPT	0.75	0.65-0.85	0.83	0.67	0.82	0.69
Rhinitis	0.68	0.58-0.79	0.76	0.60	0.78	0.58

Supplementary Table 5: Coupled Receiver operating characteristic (ROC) performance analysis of cross-sectional logistic models for elevated total immunoglobulin E (tIgE), specific immunoglobulin E (sIgE), aeroallergen skin prick test (SPT) or rhinitis symptoms (Rhinitis) in relation to T2-high asthma. Area under the curve (AUC), confidence interval (CI), sensitivity, specificity, true positive value (PPV), and negative positive value (NPV) are shown.

-	AUC	CI 95%	Sensitivity	Specificity	PPV	NPV
7 years						
tIgE	0.70	0.53–0.87	0.62	0.78	0.90	0.39
sIgE	0.53	0.35–0.72	0.73	0.33	0.79	0.27
SPT	0.58	0.41–0.74	0.30	0.86	0.89	0.24
Rhinitis	0.56	0.31-0.81	0.46	0.67	0.75	0.36
10 years						
Rhinitis	0.64	0.47-0.81	0.48	0.80	0.85	0.40
13 years						
tIgE	0.69	0.53-0.85	0.81	0.57	0.74	0.67
sIgE	0.56	0.40-0.72	0.45	0.67	0.67	0.45
SPT	0.64	0.50-0.79	0.43	0.85	0.83	0.46
Rhinitis	0.59	0.42-0.76	0.38	0.80	0.80	0.38
18 years						
tIgE	0.63	0.51-0.76	0.68	0.58	0.73	0.52
sIgE	0.66	0.56-0.77	0.45	0.88	0.86	0.50
SPT	0.52	0.43-0.62	0.19	0.86	0.73	0.35
Rhinitis	0.69	0.56-0.82	0.46	0.92	0.92	0.48

Supplementary Table 6: Coupled Receiver operating characteristic (ROC) predictive performance analysis of logistic models for elevated total immunoglobulin E (tIgE), specific immunoglobulin E (sIgE), aeroallergen skin prick test (SPT) or rhinitis symptoms (Rhinitis) between ages 0-6 years in relation to T2-high asthma at later time points. Area under the curve (AUC), confidence interval (CI), sensitivity, specificity, true positive value (PPV), and negative positive value (NPV) are shown.

-	Elevated tIgE, sIgE, SPT or rhinitis symptoms and concurrent T2-high vs T2-low asthma				Elevated tIgE, sIgE, SPT or rhinitis symptoms at early age and later T2-high vs T2-low asthma			
-	N (T2-high)	Odds ratio	Confidence interval	P-value	N (T2-high)	Odds ratio	Confidence interval	P-value
7 years								
tIgE	20	7.13	[1.88 – 32.29]	0.003	19	8.17	[2.03 – 39.66]	0.003
sIgE	20	6.67	[1.63 – 35.07]	0.01	19	4.12	[0.96 – 22.12]	0.07
SPT	20	15.55	[2.45 – 307.01]	0.002	18	12.00	[1.80 – 241.03]	0.008
Rhinitis	14	1.50	[0.19 – 14.25]	0.70	13	1.71	[0.24 – 15.75]	0.60
10 years								
tIgE	21	6.79	[1.17 – 55.80]	0.03	-	-	-	-
sIgE	21	13.30	[2.39 – 111.17]	0.003	-	-	-	-
SPT	21	60.0	[7.69 – >999.99]	<0.001	-	-	-	-
Rhinitis	23	7.13	[1.43 – 42.08]	0.02	23	3.67	[0.72 – 27.96]	0.12
13 years								
tIgE	27	17.05	[3.95 – 120.74]	<0.001	16	2.18	[0.53 – 10.15]	0.29
sIgE	27	9.33	[2.49 – 46.50]	<0.001	17	1.65	[0.44 – 6.38]	0.46
SPT	25	3.86	[1.22 – 13.26]	0.02	19	8.33	[1.72 – 62.86]	0.007
Rhinitis	33	3.26	[1.03 – 10.86]	0.04	21	2.46	[0.46 – 19.12]	0.32
18 years								
tIgE	48	3.53	[1.44 – 9.00]	0.006	34	3.14	[1.14 – 9.09]	0.03
sIgE	48	6.89	[2.56 – 20.47]	<0.001	34	10.50	[3.00 – 49.97]	<0.001
SPT	47	6.39	[2.43 – 18.28]	<0.001	36	2.29	[0.59 – 11.33]	0.24
Rhinitis	55	4.85	[1.89 – 13.04]	<0.001	24	10.15	[1.60 – 200.12]	0.01

Supplementary Table 7: Exploration of using a higher cut-off value for blood eosinophil count (b-eos). Logistic regression of the association between elevated total immunoglobulin E (tIgE ≥ age-specific cutoff values), specific immunoglobulin E (sIgE ≥0.35 kU/L), aeroallergen skin prick test (SPT) or rhinitis symptoms (Rhinitis) and T2-high asthma defined by elevated b-eos (≥0.50 10^9/L) and/or fractional exhaled nitric oxide (≥20 ppb).

-	Elevated tIgE and concurrent T2-high vs T2-low asthma				Elevated tIgE at early age and later T2-high vs T2-low asthma			
tIgE	**N (T2-high)**	**Odds ratio**	**Confidence interval**	**P-value**	**N (T2-high)**	**Odds ratio**	**Confidence interval**	**P-value**
7 years	33	8.47	[1.36 – 165.40]	0.02	29	>99.99	[NA – NA]	0.003
10 years	23	22.00	[2.79 – 475.50]	0.002	-	-	-	-
13 years	33	59.43	[9.58 – >999.99]	<0.001	21	5.96	[1.38 – 32.86]	0.02
18 years	55	5.11	[1.90 – 14.57]	0.001	40	4.64	[1.53 – 16.29]	0.006

Supplementary Table 8: Exploration of using a universal cut-off value for total immunoglobulin E. Logistic regression of the association between elevated tIgE (≥50 kU/L) and T2-high asthma defined by elevated blood eosinophil count (≥0.30 10^9/L) and/or fractional exhaled nitric oxide (≥20 ppb).

-	Elevated tIgE, sIgE, SPT or rhinitis symptoms and concurrent T2-high asthma vs healthy				Elevated tIgE, sIgE, SPT or rhinitis symptoms at early age and later T2-high asthma vs healthy			
-	N (T2-high)	Odds ratio	Confidence interval	P-value	N (T2-high)	Odds ratio	Confidence interval	P-value
7 years								
tIgE	33	1.20	[0.57 – 2.49]	0.62	29	1.37	[0.63 – 3.14]	0.43
sIgE	33	1.43	[0.63 – 3.06]	0.37	30	1.85	[0.72 – 4.37]	0.17
SPT	31	5.82	[2.20 – 14.90]	<0.001	27	4.09	[1.50 – 10.57]	0.004
Rhinitis	14	5.88	[1.81 – 19.35]	0.003	13	5.95	[1.75 – 19.97]	0.004
10 years								
tIgE	23	13.18	[3.81 – 82.97]	<0.001	-	-	-	-
sIgE	23	13.90	[4.70 – 59.66]	<0.001	-	-	-	-
SPT	23	19.27	[6.45 – 82.91]	<0.001	-	-	-	-
Rhinitis	23	26.56	[9.65 – 93.68]	<0.001	23	17.62	[6.97 – 44.62]	<0.001
13 years								
tIgE	33	19.39	[6.66 – 82.57]	<0.001	21	3.53	[1.25 – 12.62]	0.02
sIgE	33	6.24	[2.65 – 17.21]	<0.001	22	6.14	[2.34 – 15.96]	<0.001
SPT	30	4.49	[2.04 – 10.49]	<0.001	23	11.23	[3.97 – 32.64]	<0.001
Rhinitis	33	5.27	[2.41 – 12.47]	<0.001	21	5.49	[1.86 – 15.97]	0.002
18 years								
tIgE	55	3.80	[1.99 – 7.70]	<0.001	40	1.62	[0.80 – 3.43]	0.19
sIgE	55	6.27	[2.99 – 14.83]	<0.001	40	5.07	[2.39 – 10.83]	<0.001
SPT	54	6.70	[3.27 – 15.20]	<0.001	42	3.06	[1.11 – 8.15]	0.03
Rhinitis	55	5.22	[2.73 – 10.60]	<0.001	24	6.88	[2.61 – 18.31]	<0.001

Supplementary Table 9: Comparison of T2-high vs healthy children. Logistic regression of the association between elevated total immunoglobulin E (tIgE ≥age-specific cutoff values), specific immunoglobulin E (sIgE ≥0.35 kU/L), aeroallergen skin prick test (SPT) or rhinitis symptoms (Rhinitis) and T2-high asthma defined by elevated b-eos (≥0.30 10^9/L) and/or fractional exhaled nitric oxide (≥20 ppb).

-	Elevated tIgE and concurrent T2-high vs T2-low asthma: Analysis adjusted for covariates				Elevated sIgE and concurrent T2-high vs T2-low asthma: Analysis adjusted for covariates			
-	N (T2-high)	Odds ratio	Confidence interval	P-value	N (T2-high)	Odds ratio	Confidence interval	P-value
7 YEARS								
Model **1**	33	7.27	[1.16 – 142.28]	0.03	33	1.87	[0.37 – 14.01]	0.46
Model **2**	33	9.31	[1.16 – 187.30]	0.02	33	2.09	[0.42 – 15.67]	0.40
Model **3**	32	7.30	[1.12 – 145.40]	0.04	32	2.03	[0.39 – 15.71]	0.42
Model **4**	35	8.51	[1.34 – 168.35]	0.02	35	2.23	[0.43 – 17.22]	0.36
Model **5**	32	10.92	[1.51 – 233.87]	0.02	32	2.07	[0.36 – 17.69]	0.43
10 YEARS								
Model **1**	23	10.54	[1.73 – 91.94]	0.01	23	10.13	[1.89 – 69.46]	0.006
Model **2**	23	12.27	[1.81 – 132.87]	0.01	23	18.28	[2.36 – 393.77]	0.004
Model **3**	23	9.57	[1.53– 84.14]	0.02	23	9.43	[1.72 – 65.20]	0.009
Model **4**	23	12.00	[1.85 – 121.66]	0.008	23	11.92	[2.06 – 101.59]	0.005
Model **5**	23	8.32	[1.27 – 75.63]	0.03	23	8.28	[1.44 – 58.80]	0.02
Model **6**	23	12.02	[1.33 – 198.87]	0.03	23	25.59	[2.25 – 842.58]	0.007
13 YEARS								
Model **1**	33	31.87	[6.80 – 246.38]	<0.001	33	11.03	[2.48 – 78.98]	0.005
Model **2**	32	23.33	[5.59 – 133. 03]	<0.001	32	7.48	[2.05 – 32.56]	0.004
Model **3**	32	21.16	[5.09– 117.00]	<0.001	32	5.60	[1.61 – 22.02]	0.008
Model **4**	33	>99.99	[NA – NA]	<0.001	33	7.72	[2.21 – 32.69]	0.002
Model **5***	32	29.23	[6.24 – 230.82]	<0.001	32	32.098	[4.92 – 659.21]	0.003
18 YEARS								
Model **1**	55	7.03	[2.60 – 20.54]	<0.001	55	8.50	[2.87 – 27.70]	<0.001
Model **2**	55	7.41	[2.81 – 21.11]	<0.001	55	10.72	[3.81– 33.55]	<0.001
Model **3**	53	6.86	[2.59 – 19.65]	<0.001	53	9.29	[3.32 – 28.50]	<0.001
Model **4**	55	7.76	[3.06– 21.17]	<0.001	55	8.59	[3.27 – 24.43]	<0.001
Model **5**	53	6.47	[2.39– 19.02]	<0.001	53	5.82	[1.96– 18.69]	0.001

*Excluding atopic dermatitis ever between 0-18 years

COPSAC2000 (10 years): Model 1 (Sex), Model 2 (Seasonality), Model 3 (Paternal history of asthma symptoms), Model 4 (atopic dermatitis ever between 0-18 years), Model 5 (Sex, seasonality, paternal history of asthma symptoms and atopic dermatitis ever between 0-10 years).

COPSAC2010 (7, 13 and 18 years): Model 1 (Sex), Model 2 (Seasonality), Model 3 (Maternal history of asthma symptoms), Model 4 (Paternal history of asthma symptoms), Model 5 (atopic dermatitis ever between 0-10 years), and Model 6 (Sex, seasonality, maternal history of asthma symptoms, paternal history of asthma symptoms and atopic dermatitis ever between 0-10 years).

Supplementary Table 10: Adjusted analysis of elevated tIgE and sIgE in relation to concurrent T2-high vs T2-low asthma, accounting for covariates. Separate logistic regression models were conducted for each covariate (sex, atopic dermatitis, seasonality, parental history of asthma), followed by a combined model that includes all covariates. Seasonality was defined based on pollen allergy seasons, using a binary variable to indicate whether the test was conducted during the peak pollen months (May, June, July, August, September) in Denmark.

PAPER II

Dominance of IL-5 and CCL17 in Nasal Cytokine Profiles of Children with Type 2 Inflammation

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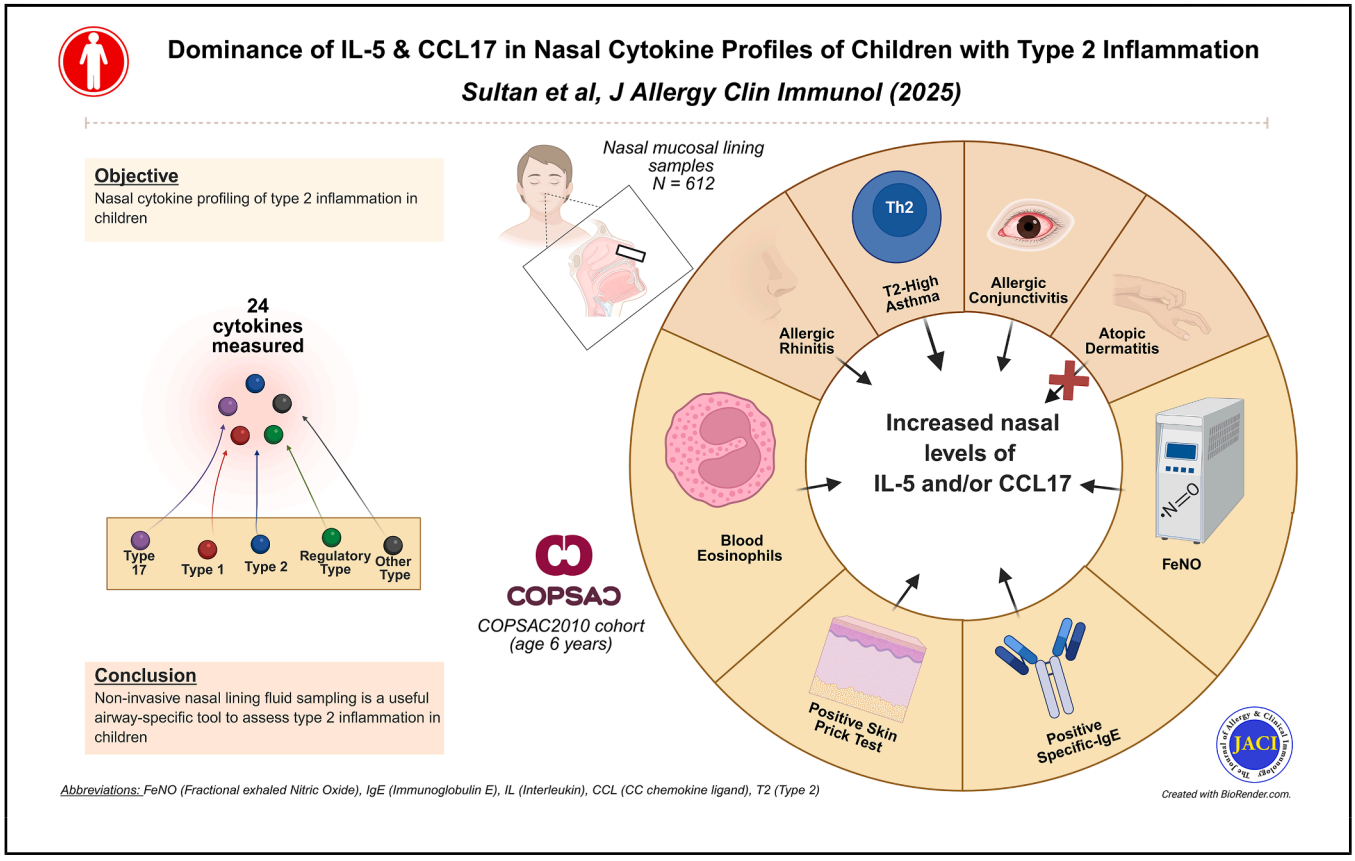
GRAPHICAL ABSTRACT



Capsule summary: Nasal IL-5 and chemokine C-C motif ligand 17 levels reflect T2 inflammation in children with T2 diseases and perform comparably to blood eosinophils for phenotyping asthma and allergic disease.

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Dominance of IL-5 and CCL17 in nasal cytokine profiles of children with type 2 inflammation

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Background: Type 2 (T2) inflammation, driven by T_H2 cells and associated cytokines, has been linked with asthma and allergic rhinitis. Early detection and immunophenotyping of T2-related diseases in childhood are crucial for effective targeted therapies.
Objective: We evaluated the usefulness of a noninvasive method to measure nasal cytokine profiles for airway-specific immunophenotyping of T2 inflammation in children.
Methods: Nasal epithelial lining fluid was analyzed for levels of 24 cytokines in 612 children at age 6 years from the Danish $COPSAC_{2010}$ mother–child cohort with prospective diagnoses of T2 diseases (T2-high asthma, allergic rhinitis, allergic conjunctivitis, and atopic dermatitis), non-T2 diseases (T2-low asthma, nonallergic rhinitis) and assessment of T2 biomarkers (blood eosinophils, fractional exhaled nitric oxide, specific IgE and skin prick test for aeroallergens). Associations between nasal cytokine levels, T2 diseases, and biomarkers were analyzed by Spearman correlation, linear regression models, and sparse partial least squares models.
Results: Children with T2-high asthma and allergic rhinitis had increased nasal IL-5/chemokine C-C motif ligand (CCL) 17 levels, while children with allergic conjunctivitis showed elevated CCL17. No associations were observed for atopic dermatitis, T2-low asthma, or nonallergic rhinitis. Positive correlations were also observed between nasal IL-5/CCL17 levels and all T2 biomarkers. A sparse partial least squares–derived nasal cytokine T2 inflammation score performed equally to blood eosinophils in cross-sectional and predictive analyses of T2 diseases.
Conclusion: Nasal epithelial lining fluid sampling is a valuable, noninvasive, and airway-specific tool for immunophenotyping T2 inflammation in 6-year-old children. Nasal IL-5 and CCL17 are consistent biomarkers of T2 inflammation and disease. (J Allergy Clin Immunol 2025;■■■:■■■-■■■.)

Key words: *Biomarkers, cytokines, nasal sampling, pediatric asthma, T2 inflammation*

Type 2 (T2) inflammatory reactions characterize a specific immune response pathway that primarily protects against environmental exposures and parasitic infections.[1] While traditionally linked to T_H2 cells,[2] it is now well established that multiple other immune cells contribute to T2 inflammation. These include T2 innate lymphoid cells, alternatively activated macrophages (M2), eosinophils, basophils, mast cells, and tuft cells.[3] Collectively, these cells drive the production of hallmark T2 cytokines such as IL-4, IL-5, and IL-13, along with other cytokines. As such, the regulation of cytokines plays an important role in T2 inflammation.

In addition to T2 responses, immune reactions are categorized into several other response phenotypes on the basis of the expression of cardinal cytokines involved in mediating the different effector functions. These include type 17 (T17)-inducing cytokines (C-X-C motif chemokine ligand 8 [aka CXCL8], IL-23, and IL-17A) important for activation of cells leading to clearance of extracellular pathogens, type 1 (T1) cytokines (IL-12p70, IFN-γ) mediating intracellular defense reactions, and regulatory type cytokines (eg, IL-10, TGF-β1) that dampen responses.[4,5]

Dysregulation in the T2 immune response is involved in T2 immune-associated diseases such as T2-high asthma and allergic rhinitis (AR), as opposed to non-T2 diseases such as T2-low asthma and nonallergic rhinitis (NAR).[6,7] Distinguishing between T2 and non-T2 diseases is clinically important because it has implications for treatment and prognosis.[8-10] Children with T2-high asthma, for instance, benefit more from inhaled corticosteroids and targeted biological therapies,[8] while T2-low asthma is less responsive to these treatments.[9,11] The prevalence of T2 diseases has increased in recent decades,[12] with both disease severity[13] and quality of life[14] being important factors in children with T2-high asthma.

Identifying useful biomarkers for T2-high asthma is essential for early detection, immunophenotyping, and personalized treatment plans.[15,16] Key biomarkers used to identify T2 inflammation include blood eosinophils, fractional exhaled nitric oxide (FENO), total and specific IgE, and skin prick test (SPT).[17] Cytokines, such as IL-4, IL-5, and IL-13, are well-known T2 cytokines, all of which can be targeted with available biologics in children with severe asthma.[18] Blood cytokines are considered unreliable as biomarkers of T2-high asthma as a result of their variability and limited stability,[19-21] whereas levels of nasal

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Abbreviations used
AD: Atopic dermatitis
AR: Allergic rhinitis
CCL: Chemokine C-C motif ligand
CI: Confidence interval
$COPSAC_{2010}$: Copenhagen Prospective Studies on Asthma in Childhood 2010
FDR: False discovery rate
FENO: Fractional exhaled nitric oxide
NAR: Nonallergic rhinitis
PC1/2: Principal component 1/2
PCA: Principal component analysis
P_{FDR}: FDR-adjusted *P* value
SD: Standard deviation
Simoa: Single-molecule array
SPT: Skin prick test
T1/2/17: Type 1/2/17

cytokines may offer a more specific reflection of local immune responses. Likewise, other blood-based biomarkers face similar limitations as systemic proxies, failing to accurately reflect local airway mechanisms.[22]

Nasal sampling offers a practical and child-friendly alternative to more invasive and cooperation-demanding procedures, such as bronchoalveolar lavage, bronchial biopsy, or induced sputum, which are not as well suited as nasal sampling for large-scale pediatric studies or routine clinical settings.[23] Moreover, the nasal mucosa shares key immunologic features with the lower airways, including epithelial cell composition and cytokine signaling patterns, making it a relevant surrogate for studying airway inflammation in children.[24,25] As such, there is a growing need for noninvasive biomarkers that can more accurately identify and immunophenotype children with T2 diseases, which could potentially be met by assessing airway immune profiles to optimize treatment strategies.[26]

The objective of this study was to evaluate whether nasal mucosal lining fluid sampling represents a useful and noninvasive method for characterizing airway inflammation and immunophenotyping children with T2 inflammation. We investigated the associations between nasal cytokine levels and T2-associated diseases, including T2-high asthma and AR, as well as established systemic biomarkers of T2 inflammation such as blood eosinophils, FENO, specific IgE, and SPT. This approach may offer insights into whether nasal cytokines reflect local immune activity relevant to T2 disease phenotypes.

METHODS

Study cohort

Data from the Copenhagen Prospective Studies on Asthma in Childhood 2010 ($COPSAC_{2010}$) mother–child cohort with 700 children enrolled at birth from Zealand, Denmark, were used for this study.[27] The children underwent regular monitoring for symptoms of asthma, allergy, and/or eczema through scheduled clinical assessments and acute care visits. Data from the 6-year visit were used for the cross-sectional analysis, while data from the 10-year visit were utilized for predictive analysis.

Disease definitions

Asthma was defined as current asthma at age 6 years, diagnosed by clinicians at $COPSAC_{2010}$, following a predefined and validated algorithm that included symptom assessment, bronchodilator reversibility, a trial of inhaled corticosteroids, or relapse at cessation of corticosteroids.[28,29]

T2-high asthma was defined as asthma accompanied by at least one elevated T2 biomarker: any of blood eosinophils $\geq 0.30 \times 10^9$/L, FENO ≥ 20 ppb, specific IgE ≥ 0.35 IU/mL, or positive SPT result to aeroallergens.[30]

T2-low asthma was defined as asthma without any elevated T2 biomarkers.

AR was defined by parent reports of at least two troublesome symptoms of runny nose, blocked nose, itchy nose, or sneezing, occurring in the absence of a common cold and matching the seasonal or perennial pattern of aeroallergen sensitization, confirmed by specific IgE and/or SPT. The full algorithm is provided in the Methods section of this article's Online Repository available at www.jacionline.org.

Other definitions. NAR was defined as the presence of troublesome rhinitis symptoms without sensitization to any aeroallergen, based on specific IgE and/or SPT. Allergic conjunctivitis, in contrast, was defined solely on the basis of at least two parent-reported symptoms of red, itchy, watery, or swollen eyes in the absence of an eye infection and without requiring sensitization. Atopic dermatitis (AD) was diagnosed according to Hanifin and Rajka's criteria, requiring a combination of major and minor clinical features, including chronic or relapsing dermatitis, typical morphology and distribution, personal or family history of atopy, and pruritus.[31,32] Control groups were defined as children without a diagnosis of the disease in focus.

T2 biomarkers

Blood eosinophils were measured from blood samples as previously detailed.[33] FENO levels were measured with a NIOX Flex device (Aerocrine, Solna, Sweden) or a Denox 88 device (Eco Medics, Dürnten, Switzerland), with the average of two measurements used.[34] Specific IgE for common aeroallergens (horse, dog, cat, birch, timothy grass, mugwort, house dust mite, mold) was measured by ImmunoCAP blood testing.[35] SPT for aeroallergens was identical to those in the specific IgE panel. A positive SPT result was defined as a mean wheal diameter that was at least 3 mm larger than the negative control.[35]

Nasal cytokine sampling

A filter paper based on a synthetic absorptive matrix (fibrous hydroxylated polyester sheets, Accuwik Ultra, Pall Life Sciences, Portsmouth, United Kingdom) was inserted into each nasal cavity for 2 minutes to sample the nasal mucosal lining fluid of the children at age 6 years. Thereafter, the two strips were removed and then immediately frozen at −80°C (for a maximum of 10 minutes) for later immunoassay. The sampling method has previously been used and validated in neonates and infants.[36-39]

Cytokine selection and preprocessing

In this study, 24 cytokines were selected *a priori* on the bases of their known roles in distinct immune response pathways: T17, T1, T2, regulatory, and other. The cytokines were measured in

duplicate samples per with the Meso Scale Discovery immunoassay kits. Before measurement, proteins were extracted using 400 μL of freshly prepared Milliplex Assay buffer (L-AB, EMD Millipore, Billerica, Mass) containing one EDTA-free protease inhibitor tablet (Protease Inhibitor Cocktail, catalog 11873580001, Roche, Basel, Switzerland) per 25 mL of buffer. TGF-β1 values were not measured for 30 samples because of limited sample material. The distribution of individual raw cytokine levels across immune response types before and after data transformation is shown in Figs E1 and E2 in the Online Repository available at www.jacionline.org. Details on minimum concentration values (see Table E1 in the Online Repository) for individual cytokines are provided in the Methods section in the Online Repository.

Cytokine data processing and covariate adjustment

Raw measured levels of *in vivo* nasal cytokines (pg/mL) were log transformed and *z* score transformed to ensure equal weight to all cytokines. Here, missing values for the 30 (0.20%) of 14,688 cytokine TGF-β1 samples were imputed using the *k* nearest neighbors method. Sensitivity analyses (data not shown) confirmed that the results were not significantly affected by this imputation approach. Subsequently, data were reexponentiated and total-sum normalized within samples (cytokine level divided by the sum of all cytokine levels in the sample). Finally, data were again log transformed and *z* score transformed to unit standard deviation (SD) to address within-sample collinearity, as previously published.[37,39,40] An alternative data transformation that used centered log-ratio transformation[41] was performed as a sensitivity analysis; details are provided in the Methods section in the Online Repository.

To ensure data reliability, several variables were registered during sampling that could influence cytokine expression: sampling extraction date, analysis date, analysis plate, season of sampling,[42] receipt of nasal steroids <5 days before sampling,[43] receipt inhalation steroids <2 weeks before sampling,[44] and airway infection <1 week before sampling.[39,45,46] To minimize confounding effects, these factors were adjusted for as covariates.

Statistical analysis

Demographic variables were presented as counts and percentages for categorical variables and as medians with interquartile ranges for continuous variables. The association between continuous levels of nasal cytokines and T2 biomarkers was visualized by a heat map, with correlation coefficients calculated by the Spearman method.

A principal component analysis (PCA) was performed to identify cytokines that contributed most to the variance in the data. Furthermore, linear models were used to test whether the first two principal components (PC1 and PC2) differed between children with T2-high asthma and the asthma-free control group.

A sparse partial least squares model was applied with log-transformed and scaled blood eosinophils as the outcome (Y matrix) and 15 nasal cytokines as predictors (X matrix) to obtain a T2 cytokine score. The predictive capacity of the derived T2 nasal cytokine score was evaluated against blood eosinophils by receiver operating characteristic analysis. This included binary T2 diseases at 6 years for all children and at 10 years for children without T2 disease or sensitization at 6 years. Finally, the DeLong test was used to statistically compare the predictive performance of the T2 nasal cytokine score and blood eosinophils.

The association between nasal cytokine levels and T2 diseases and T2 biomarkers was examined by covariate-adjusted linear regression models. T2 diseases were analyzed as binary variables compared to healthy controls, while T2 biomarkers were analyzed dichotomized (elevated, yes/no) in the entire sample population. Statistical significance was set at $P < .05$, with multiple testing corrected via the false discovery rate (FDR, 5%). Statistical analyses were performed by R v4.2.1 software (www.r-project.org).

Ethical approval

The $COPSAC_{2010}$ research project has been approved by the Danish Data Protection Agency (2015-41-3696) and the Danish Ethical Committee (H-B-2008-093).

RESULTS

T2 disease incidence and T2 biomarkers at 6 years of age in $COPSAC_{2010}$

Of the entire $COPSAC_{2010}$ mother–child cohort (n = 700), nasal samples were available for 612 children (87.4%), with 1 (0.2%) outlier being removed. A total of 75 (12.3%) of these children had an asthma diagnosis. Of the 75 with asthma, 35 (46.7%) had T2-high asthma, 19 (25.3%) had T2-low asthma, and 21 (28%) had disease that was not categorized because data were missing for one or more of the T2 biomarkers required for stratification. Among the 35 children classified as having T2-high asthma, 18 (51%) fulfilled one criterion, 10 (29%) fulfilled two, 5 (14%) fulfilled three, and 2 (6%) fulfilled all four criteria. Of the 107 (20.3%) of 526 children sensitized by specific IgE and the 42 (7.9%) of 531 children sensitized by SPT, 35 (32.7%) of 107, and 35 (83.3%) of 42 were positive by both sensitization methods, respectively. Baseline characteristics of the entire study population—that is, children both with and without asthma (control group)—are outlined in Table I.

Association between nasal cytokine levels and T2 diseases at 6 years of age

Children with T2-high asthma versus children without asthma had increased levels of IL-5 (SD, beta estimate [β] = 0.52; 95% confidence interval [CI], 0.16, 0.89; FDR-adjusted *P* value [P_{FDR}] = .03) and C-C motif chemokine ligand (CCL) 17 (β = 0.52; 95% CI, 0.16, 0.87; P_{FDR} = .03) (Fig 1).

Further, children with versus without AR had increased levels of IL-5 (β = 0.62; 95% CI, 0.32, 0.92; P_{FDR} = .0006), CCL17 (β = 0.82; 95% CI, 0.52, 1.11; P_{FDR} < .0001), CCL26 (β = 0.40; 95% CI, 0.11, 0.69; P_{FDR} = .03), and CCL4 (β = 0.38; 95% CI, 0.09, 0.67; P_{FDR} = .04), along with decreased levels of IL-13 (β = −0.31; 95% CI, −0.48, −0.15; P_{FDR} = .001) (Fig 1).

Similarly, children with versus without allergic conjunctivitis had increased levels of CCL17 (P_{FDR} = .001) (see Fig E3 in the Online Repository available at www.jacionline.org). There were no FDR-significant differences in nasal cytokines in children with versus without AD (Fig E3). Further, for non-T2 diseases, there were also no FDR-significant differences in levels of any

TABLE I. Demographics and T2 biomarker status in children with and without asthma at 6 years

Characteristic	Total (N = 612)		Asthma (n = 75)		Control group (n = 537)	
	Value	Missing	Value	Missing	Value	Missing
Demographics						
Sex (male)	310 (50.7)	0	46 (61.3)	0	264 (49.2)	0
Age (years)	6.0 [5.9-6.1]	0	6.0 [5.9-6.0]	0	6.0 [5.9-6.1]	0
T2 and non-T2 diseases						
T2-high asthma diagnosis (yes)	35 (5.7)	21 (3.4)	35 (46.7)	21 (28.0)	0	0
T2-low asthma diagnosis (yes)	19 (3.1)	21 (3.4)	19 (25.3)	21 (28.0)	0	0
AR (yes)	43 (7.0)	5 (0.8)	12 (16.0)	2 (2.7)	31 (5.8)	3 (0.6)
NAR (yes)	33 (5.4)	5 (0.8)	12 (16.0)	2 (2.7)	21 (3.9)	3 (0.6)
Allergic conjunctivitis (yes)	45 (7.4)	3 (0.5)	16 (21.3)	0	29 (5.4)	3 (0.6)
AD (yes)	75 (12.3)	7 (1.1)	18 (24.0)	0	57 (10.6)	7 (1.3)
T2 biomarkers						
Blood eosinophils (elevated)	217 (35.5)	129 (21.1)	30 (40.0)	14 (18.7)	187 (34.8)	115 (21.4)
FENO (elevated)	18 (2.9)	244 (39.9)	4 (5.3)	32 (42.7)	14 (2.6)	212 (39.5)
Blood specific IgE (elevated)	108 (17.7)	86 (14.1)	18 (24.0)	10 (13.3)	90 (16.8)	76 (14.2)
SPT (elevated)	42 (6.9)	81 (13.2)	9 (12.0)	11 (14.7)	33 (6.1)	70 (13.0)
Cytokine sampling						
Infection within 7 days of sampling (yes)	77 (12.6)	0	12 (16.0)	0	65 (12.1)	0
Nasal steroid within 5 days of sampling (yes)	6 (1.0)	0	2 (2.7)	0	4 (0.8)	0
Inhalation steroid within 2 weeks of sampling (yes)	23 (3.8)	0	22 (29.3)	0	1 (0.2)	0
Season of sampling: winter	106 (17.3)	0	11 (14.7)	0	95 (17.7)	0
Season of sampling: spring	131 (21.4)	0	17 (22.7)	0	114 (21.2)	0
Season of sampling: summer	171 (27.9)	0	24 (32.0)	0	147 (27.4)	0
Season of sampling: fall	204 (33.3)	0	23 (30.7)	0	181 (33.7)	0

Continuous variables are presented as medians [interquartile ranges] and categorical variables as nos. (%). Baseline characteristics are shown for entire study population, including children with asthma and children without asthma (control group), at age 6 years. T2 biomarkers are considered elevated when FENO is ≥20 ppb, blood eosinophils are $\geq 0.30 \times 10^9$/L, specific IgE to aeroallergens is ≥0.35 IU/mL, and SPT to aeroallergens is positive.

nasal cytokines among children with T2-low asthma compared to children without asthma or among children with NAR compared to children without rhinitis (Fig 1).

Direct phenotype comparisons were conducted to explore within-disease airway immune profile differences. Cytokine expression patterns in these comparisons were directionally consistent with those observed in disease versus healthy control analyses. No nasal cytokines differed significantly between children with T2-high and T2-low asthma after FDR correction (see Fig E4 in the Online Repository available at www.jacionline.org). In contrast, CCL17 was significantly increased in children with allergic compared to NAR (P_{FDR} = .009) (Fig E4). Raw cytokine concentrations by disease group are provided in Table E2 in the Online Repository.

Interrelationships between nasal cytokine levels and T2 biomarkers

Positive correlations were identified between all T2 biomarkers and IL-5 (Spearman correlation coefficient *[r]* = 0.05 to 0.26), CCL17 (r = 0.04 to 0.21), CCL26 (r = 0.02 to 0.13), CCL13 (r = 0.02 to 0.17), and CCL4 (r = 0.11 to 0.20) (Fig 2). Conversely, IL-13 was negatively correlated with all T2 biomarkers (r = −0.07 to 0.21). Overall, T17, T1, and regulatory type cytokines were either negatively correlated or not correlated with T2 biomarkers. IL-5 was not correlated with IL-4 (r = 0.02) but was negatively correlated with IL-13 (r = −0.13) and was positively correlated with CCL17 (r = 0.08). CCL13 and CCL26 were positively correlated (r = 0.22). Between sensitization methods, both specific IgE and SPT were positively correlated (r = 0.50).

The associations between the 24 nasal cytokines and elevated T2 biomarkers were thereafter analyzed in the entire study population in covariate-adjusted models (Fig 3). Elevated blood eosinophils were associated with increased levels of IL-5 (SD, β = 0.36; 95% CI, 0.19, 0.54; P_{FDR} = .001) and CCL17 (β = 0.29; 95% CI, 0.10, 0.47; P_{FDR} = .03). Elevated FENO was associated with increased levels of IL-5 (β = 1.07; 95% CI, 0.61, 1.52; P_{FDR} = .0001), CCL17 (β = 0.67; 95% CI, 0.21, 1.12; P_{FDR} = .01), and decreased levels of IL-13 (β = −0.12; 95% CI, −0.19, −0.06; P_{FDR} = .002).

Children with versus without sensitization to any aeroallergen by specific IgE ≥ 0.35 IU/mL had increased levels of IL-5 (β = 0.47; 95% CI, 0.26, 0.68; P_{FDR} = .0001), CCL17 (β = 0.61; 95% CI, 0.40, 0.81; P_{FDR} < .0001), CCL4 (β = 0.26; 95% CI, 0.05, 0.46; P_{FDR} = .03), CCL13 (β = 0.23; 95% CI, 0.05, 0.41; P_{FDR} = .03), CCL26 (β = 0.34; 95% CI, 0.14, 0.54; P_{FDR} = .005), CCL11 (β = 0.25; 95% CI, 0.05, 0.45; P_{FDR} = .03), and decreased levels of IL-13 (β = −0.27; 95% CI, −0.45, −0.10; P_{FDR} = .01).

Similarly, children with versus without a positive SPT result to any aeroallergen had increased levels of IL-5 (β = 0.65; 95% CI, 0.34, 0.95; P_{FDR} = .0003), CCL17 (β = 0.50; 95% CI, 0.21, 0.79; P_{FDR} = .003), CCL4 (β = 0.39; 95% CI, 0.09, 0.69; P_{FDR} = .03), and decreased levels of IL-13 (β = −0.58; 95% CI, −0.83, −0.34; P_{FDR} < .0001).

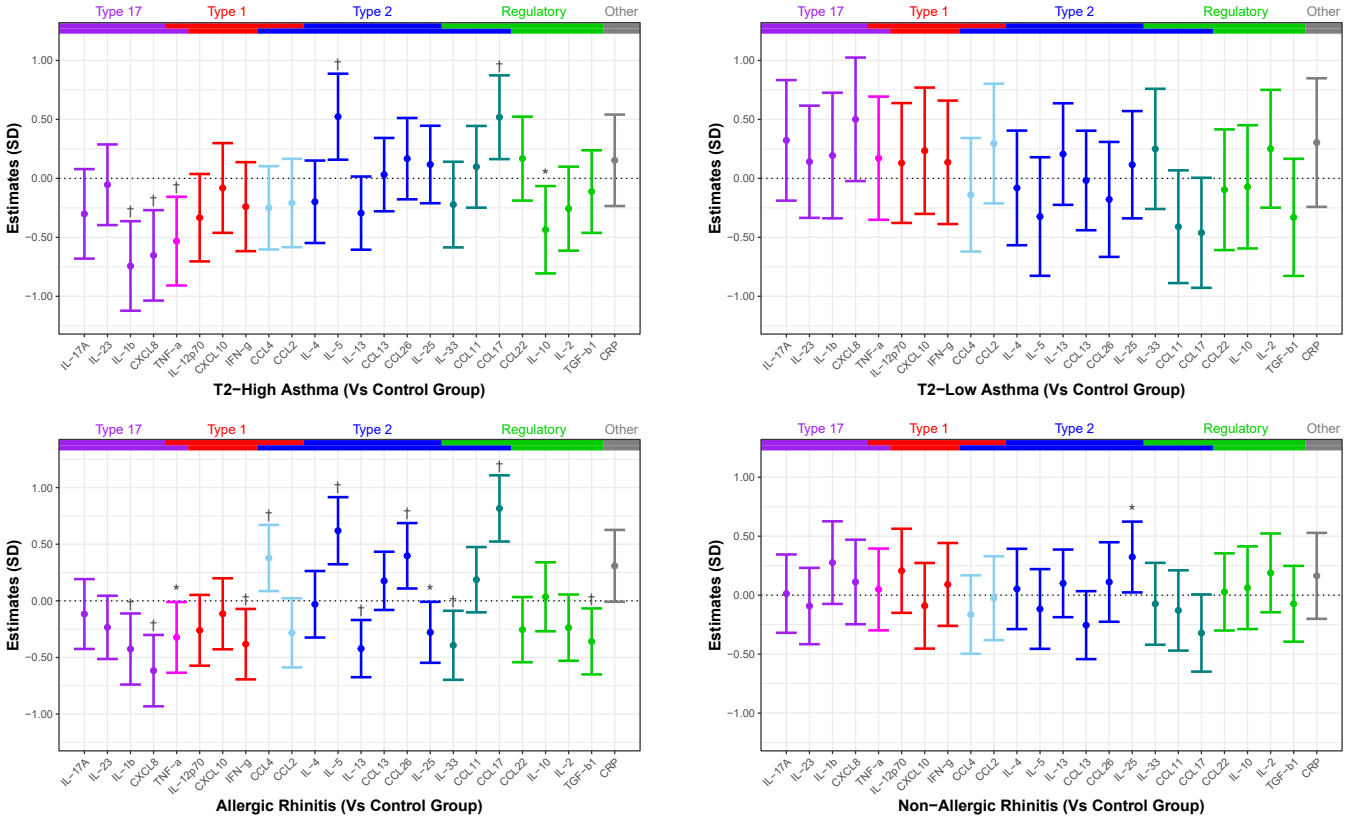

FIG 1. Associations between nasal cytokines and T2 and non-T2 diseases at age 6 years. Forest plots depicting association between nasal cytokines and T2 and non-T2 diseases vs control group (children without respective diseases) at age 6 years. Linear regression models were adjusted for age, sex, sampling season, sampling plate, analysis date, extraction date, airway infection less than 1 week before sampling, and prior receipt of nasal and inhalation steroids before sampling. *$P < .05$, †$P_{FDR} < .05$. Per disease panel, complete cases: T2-high, n = 569 (35/534); T2-low, n = 553 (19/534); AR, n = 571 (43/528); NAR, n = 561 (33/528).

Nasal cytokine patterns and their role in T2-high asthma

A PCA of the 24 nasal cytokines showed significantly higher PC1 values and lower PC2 values in children with T2-high asthma compared to healthy children ($P = .02$ and $P = .01$, respectively) (Fig 4, *A*). IL-5, CCL13, and CCL26 were positively associated with T2-high asthma along the positive PC1 axis (Fig 4, *B*), while CCL17, CCL13, and CCL26 were also positively associated with T2-high asthma along the negative PC2 axis. CCL13 and CCL26 showed similar directionality in both PC1 and PC2. Conversely, IL-13 exhibited an inverse relationship with IL-5, CCL13, and CCL26.

Evaluation of nasal cytokines as a biomarker of T2 disease

Model performance of blood eosinophils versus the sparse partial least squares–derived T2 nasal cytokine score was evaluated cross-sectionally at 6 years for T2 diseases and predictively at 10 years for children without T2 disease or sensitization at 6 years.

Cross-sectionally at 6 years, the area under the curve values for T2-high asthma (defined by FENO, specific IgE, and SPT), AR, allergic conjunctivitis, and AD were comparable between blood eosinophils and the T2 nasal cytokine score. The DeLong test indicated no significant differences ($P > .05$) (see Table E3 in the Online Repository available at www.jacionline.org).

Predictively at 10 years, the two models exhibited similar performance for T2-high asthma, AR, allergic conjunctivitis, and AD. The DeLong test again showed no significant differences in area under the curve values ($P > .05$) (Table E3).

Sensitivity analyses

The associations between nasal cytokine levels and both T2 biomarkers and nonasthma T2 diseases remained significant after further adjusting for asthma in a sensitivity analysis (see Fig E5 in the Online Repository available at www.jacionline.org). Sensitivity analysis using the alternative centered log-ratio transformation (see Fig E6 in the Online Repository) and PCA of raw cytokines (see Fig E7 in the Online Repository) yielded similar results, confirming the consistency of the findings regardless of transformation method. Sensitivity analysis using a higher sensitization cutoff for specific IgE (≥0.70 IU/mL) yielded similar cytokine patterns with even stronger signals (see Fig E8 in the Online Repository).

Sensitivity analyses comparing (1) each disease group to controls without any T2 diseases, (2) to controls without any T2 biomarker elevations, and (3) to children with biomarker

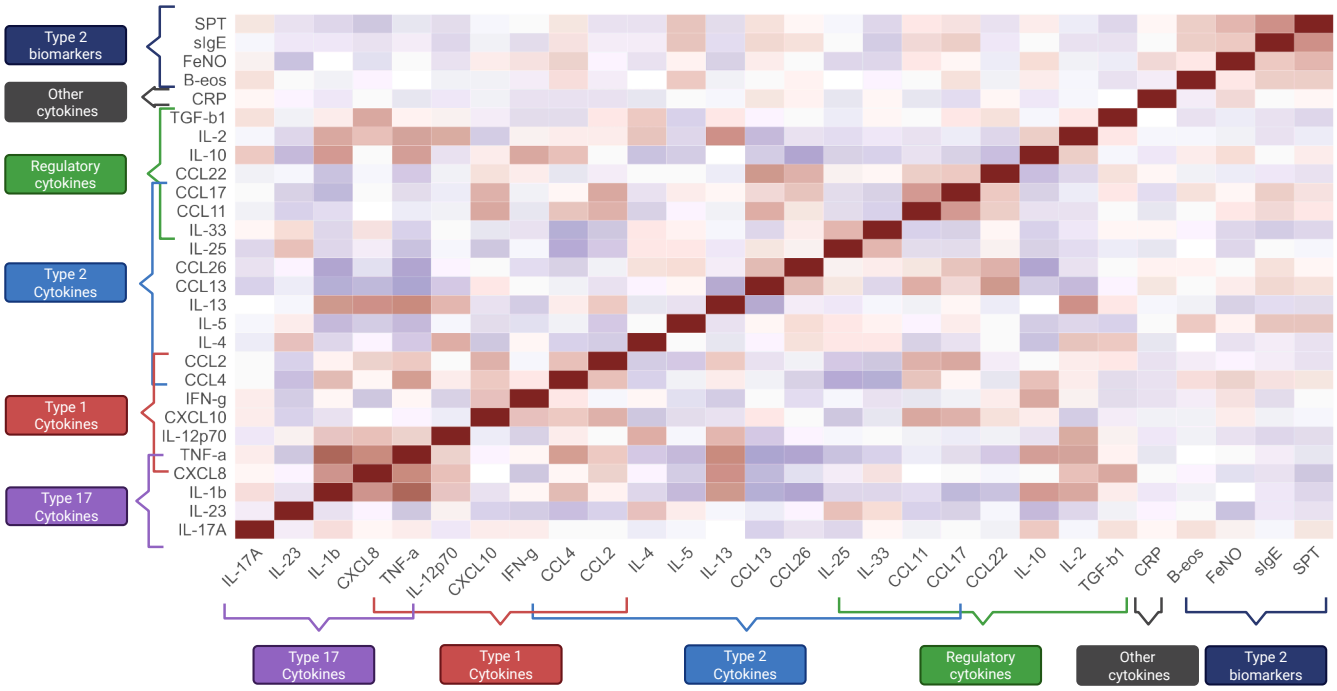

FIG 2. Correlation between nasal cytokines and conventional T2 biomarkers. Heat map showing correlations between nasal cytokines and T2 biomarkers by Spearman correlation coefficient. Positive correlations are shown in *red* and negative correlations in *blue*. Complete cases: N = 274.

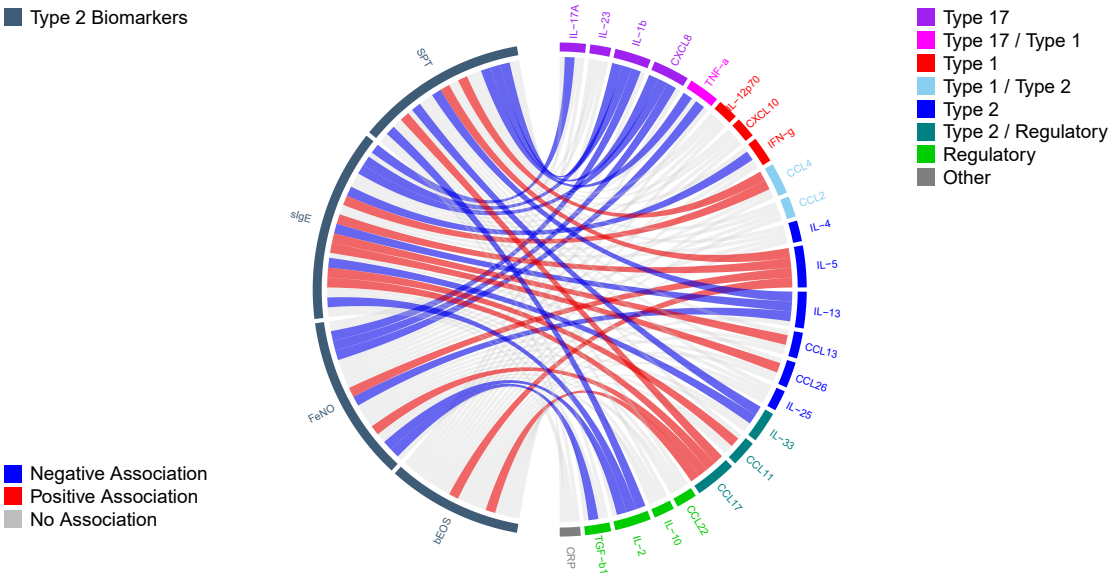

FIG 3. Nasal cytokine associations with elevated T2 biomarkers in children at age 6 years. Circos plot illustrating FDR-adjusted associations between 24 nasal cytokines and T2 biomarkers in whole study population, derived from linear models. Elevated T2 biomarkers were defined as FENO ≥ 20 ppb, blood eosinophils ≥ 0.30 × 10^9/L, specific IgE to aeroallergens ≥ 0.35 IU/mL, and positive SPT result to aeroallergens. Of note, increased levels of IL-5 and CCL17 were consistently observed across all elevated T2 biomarkers. In contrast, decreased levels of IL-13 were associated with elevated FENO, specific IgE, and positive SPT, while IL-4 levels showed no significant changes. Per biomarker panel, complete cases: blood eosinophils, n = 480 (216/264); FENO, n = 366 (18/348); specific IgE, n = 523 (106/417); SPT, n = 529 (41/488). *bEOS,* Blood eosinophils; *sIgE,* specific IgE.

elevations but no disease showed consistent cytokine directions across comparator definitions (see Figs E9–E11 in the Online Repository available at www.jacionline.org). Signals were strongest in controls without any T2 diseases or T2 biomarker elevations (Figs E9 and E10), while using biomarker-positive but asymptomatic controls (Fig E11) led to attenuation,

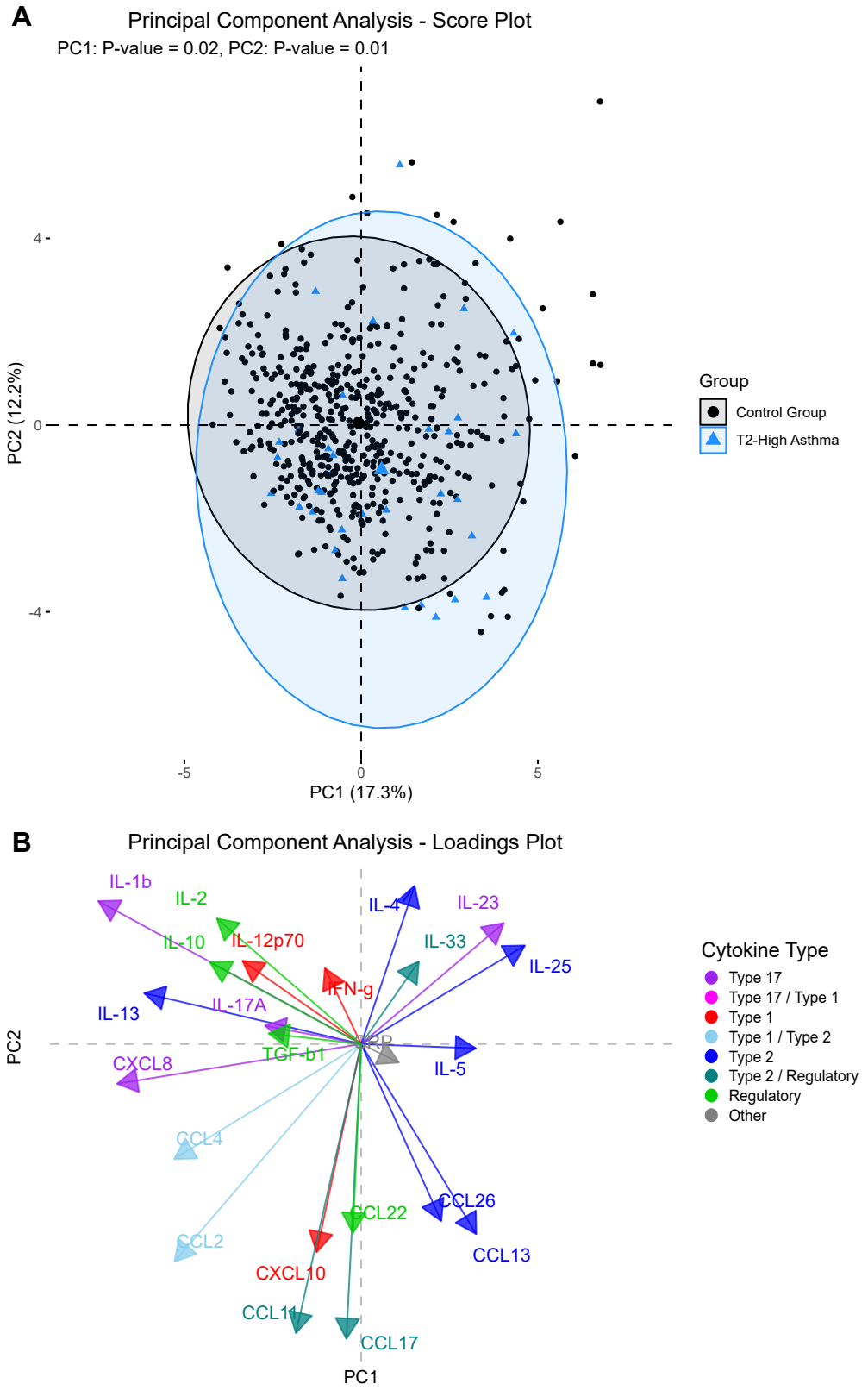

FIG 4. PCA reveals nasal cytokine patterns in T2-high asthma. PCA score plot **(A)** and loading plot **(B)** of nasal cytokine levels in children with T2-high asthma and children without asthma (control group) at 6 years of age. IL-5, CCL13, and CCL26 were positively associated with T2-high asthma along positive PC1 axis (P = .02), while CCL17, CCL13, and CCL26 were also positively associated with T2-high asthma along negative PC2 axis (P = .01). Conversely, IL-13 exhibited inverse relationship with IL-5, CCL13, and CCL26. N = 572 (35/537).

particularly for asthma and conjunctivitis, whereas AR retained strong associations.

DISCUSSION

Our study demonstrated specific *in vivo* airway immune profiles in T2 diseases, including T2-high asthma, AR, allergic conjunctivitis, and AD, and the T2 biomarkers of blood eosinophils, FENO, and aeroallergen sensitization assessed by specific IgE and SPT. The airway immune profiles were obtained by using a noninvasive technique to collect 612 nasal mucosal lining fluid samples to measure levels of 24 nasal cytokines in children at age 6 years. We found that increased upper airway levels of IL-5 and CCL17 were consistently associated with the majority of T2 diseases and biomarkers. In contrast, IL-13 was negatively associated, and IL-4 exhibited no associations. Moreover, children with T2-low asthma and NAR showed no distinct airway immune profiles compared to controls. This noninvasive, airway-specific method was useful and showed similar discriminative and predictive performance to conventional blood testing for identifying T2 inflammation.

There is limited research on this topic in children, and well-powered studies are especially needed. A study investigated nasal lavage cytokine profiles in a manner similar to our study but in a much smaller setting, examining 29 children with T2-high asthma compared to 28 healthy children (mean age, 17 years).[47] They measured 9 cytokines, IL-4, IL-5, IL-6, IL-10, IL-13, IL-17A, IL-33, IFN-γ, and TNF-α, and found no significant differences in nasal levels of IL-4, IL-5, and IL-13 between the two groups, but they found an inverse relationship between IL-5 and IL-13, which is consistent with our findings. Likewise, another similar small sample study investigated the nasal cytokine profiles of 20 children with T2-high asthma (defined by total IgE > 100 IU/mL) with disease in clinical remission and compared them to 20 healthy children (mean age, 9 years).[48] They analyzed a limited panel of 9 cytokines: IL-4, IL-5, IL-6, IL-12p70, IL-13, IL-33, granulocyte-macrophage colony-stimulating factor, thymic stromal lymphopoietin, and periostin. They found that levels of IL-5 and IL-13 were significantly elevated in children with T2-high asthma, while IL-4 did not show significant differences. Our findings for IL-5 and IL-4 are consistent with their results, but unlike them, we found no association between IL-13 and T2-high asthma. Additionally, in 2008, we conducted a case–control study using an asthma at-risk cohort (mean age, 8.2 years), comparing nasal cytokine profiles of 15 children with AR to 10 healthy controls. That study showed that children with AR had increased levels of IL-5, CCL17, and IL-13, but not IL-4.[49] In contrast, we now observe a negative association between IL-13 and AR, while IL-5 and CCL17 remain consistent with our prior findings.

We found a clear pattern of nasal cytokine levels across investigated T2 diseases and T2 biomarkers, with a significant increase in cytokines known to be associated with T2 inflammation, particularly IL-5. This increase in upper airway IL-5 level aligns with the limited studies on T2-high asthma and AR in adults, which have used various sampling techniques, including sinonasal tissue,[50] nasal lavage fluid,[51,52] and nasal epithelial lining fluid.[53,54] The consistency of our results is supported by evidence identifying IL-5 as a high-confidence therapeutic target across multiple allergic diseases, including asthma, eczema, and food allergy, because of its consistent upregulation and involvement in allergic responses.[55]

Surprisingly, we found that children with T2 diseases and elevated T2 biomarkers had equivalent levels of IL-4 and lower levels of IL-13 compared to healthy controls, which contradicts common expectations for these hallmark T2 cytokines. This may be attributed to the variable sensitivity and reliability of specifically IL-4 and IL-13 detection across different cytokine analysis platforms, as reported by others.[24,56] IL-4 and IL-13 have both shown poor correlation and high variability across multiple platforms,[57] suggesting that results for these cytokines may not be as accurate as, for example, IL-5. Other *in vivo* studies have also shown neutral or downregulated levels of IL-4 or IL-13 in nasal fluid from adults with allergic asthma[53] and with AR[54] compared to healthy controls. These findings, including both our results and those from prior studies, show the complexity and variability in *in situ* cytokine analysis, making it difficult to obtain results that accurately reflect *in vitro* stimulatory conditions. Importantly, our study sampled children during a clinically stable disease phase, which may reflect a resting mucosal immune state with neutral IL-4 and downregulated IL-13 activity, despite systemic sensitization. This may partly explain the unexpected IL-4 and IL-13 profile, a possibility also hypothesized in a nasal cytokine profiling study of 137 adults with AR, where individuals sampled under natural allergen exposure, without prior allergen provocation, showed reduced IL-4 and IL-13 levels compared to controls.[54] Furthermore, nasal cytokines primarily reflect local mucosal immune activity, which may differ from systemic immune responses due to anatomic compartmentalization. This phenomenon has been observed in both airway disease and mucosal immunology, where cytokine expression can diverge between nasal tissue and blood, underscoring the need for further studies of tissue-specific sampling to improve immune phenotyping in T2 inflammation.[58,59]

In addition to IL-5, we found that CCL17 was the most prominent cytokine associated with T2 diseases and T2 biomarkers, as shown by the correlation analysis and regression models. This is consistent with current literature, where blood CCL17 has previously been reported as a reliable biomarker in T2-high asthma,[60,61] AR,[62-65] and AD[66] in children and adults. Furthermore, CCL17 has been investigated as a possible therapeutic target in asthma and other T2 diseases.[67] Likewise, dupilumab, a monoclonal antibody blocking the IL-4 receptor, has been shown to reduce serum CCL17 levels across all T2 diseases.[68-70] Our findings suggest that upper airway CCL17 levels reflect T2 immune activity in children and may serve as a candidate marker for further exploration in nasal epithelial lining fluid, highlighting its potential as a biomarker in nasal epithelial lining fluid, which has not been demonstrated before.

Although specific IgE identified a broader group of sensitized children compared to SPT, the two methods were strongly correlated and linked to similar nasal cytokine profiles, particularly elevated IL-5 and CCL17. The airway immune patterns were consistent across sensitization methods, with SPT-positive children showing the same cytokine signature as those identified by specific IgE. These findings were robust to a higher specific IgE threshold (≥0.70 IU/mL), supporting the use of either method for capturing local T2 inflammation.

Interestingly, IL-5 and CCL17 were most strongly associated with AR, followed by T2-high asthma, allergic conjunctivitis, and

AD. This gradient likely reflects anatomic differences in immune activity captured by nasal sampling. Because nasal mucosa is directly involved in rhinitis, local cytokine expression may be more pronounced, while the lower signal in asthma likely reflects reduced spillover from the lower airways. These findings suggest that nasal cytokine profiling primarily reflects local mucosal inflammation and may be more sensitive to upper than lower airway disease.

While our findings support a distinct T2 nasal cytokine profile in children with T2 diseases, including T2-high asthma, recent studies have proposed broader immune endotypes within pediatric asthma specifically. One study analyzing nasal epithelial transcriptomes from more than 450 children with asthma aged 6 to 20 years identified T2-high, T17-high, and T2/T17-low profiles.[71] The children clustered into the non-T2 groups, even among those with allergic sensitization, suggesting that transcriptomic profiling may capture broader immune heterogeneity than clinically defined T2-high asthma. In contrast, our cytokine-based approach showed consistent upregulation of IL-5 and CCL17 in children with elevated T2 biomarkers and T2-high asthma, supporting a close link between nasal cytokine levels and clinical phenotyping. A second study applied integrated multiomics profiling of bronchial brushings and bronchoalveolar lavage from 73 children aged 1 to 18 years, combining transcriptomic, microbial, and metabolomic data.[72] It identified two distinct trajectories, one T2 associated, which was linked to progression toward T2-high asthma, and another neutrophilic and T1 associated, which was linked with preschool wheeze. These findings emphasize that transcriptomic and cytokine-based immune profiling may each capture unique aspects of airway inflammation and highlight the value of nasal cytokine profiling as a direct and accessible method to assess T2 inflammation in early childhood.

We also found that increased levels of the well-established T2 biomarkers CCL13[73] and CCL26[74] were both associated with T2 diseases and T2 biomarkers. These two cytokines were highly correlated in the PCA and correlation analysis, suggesting a shared function of CCL13 and CCL26. This indicates that these two cytokines play a coordinated role in the airway inflammatory processes characteristic of T2 diseases *in vivo*.

Non-T2 diseases—that is, T2-low asthma and NAR—exhibited no significant upregulation or downregulation in their airway immune profiles compared to their respective control groups, in contrast to the distinct cytokine patterns observed in T2 diseases.

A strength of this study is the large-scale approach, utilizing nasal samples from 612 children, which enhances the reliability and robustness of our findings. The use of a noninvasive filter paper method for sampling nasal lining fluid is a significant advantage, allowing for easy and repeatable collection of samples from children, minimizing discomfort, and ensuring better compliance. Nasal epithelial lining fluid absorption has been shown to be a superior sampling method for upper airway immune response determination compared to nasal washes in children and adults.[75] Similarly, among the commonly used cytokine detection methods, the Meso Scale Discovery platform, used in our study, exhibited the highest sensitivity.[57] Among the T2 diseases, AD showed the least distinct airway-specific results compared to T2-high asthma and AR, suggesting a location-dependent correlation with testing, which is expected.

There are limitations to our study, including the fragility of measuring cytokines in nasal lining fluid, which can be affected by various factors, including sample handling and processing. Despite the established relevance of upper airway sampling reflecting lower airway findings,[24] there remains some variability in cytokine detection across different platforms, particularly for IL-4 and IL-13. Single-molecule array (Simoa) assays show higher analytical sensitivity than conventional ELISA for IL-4 in serum, while for IL-13 a qualified Simoa assay showed reliable low-level detection with strong agreement to a high-sensitivity reference.[76,77] Thus, Simoa is a plausible platform to verify these cytokines in nasal epithelial lining fluid, provided the assay is qualified for synthetic absorptive matrix samples. Future studies should validate cytokine measurements, especially for IL-4 and IL-13, using additional detection methods beyond the Meso Scale Discovery platform to address potential platform-specific limitations and strengthen result reliability. Underlying T2 diseases or biomarker elevations in controls may have weakened group differences, which was underscored by sensitivity analyses using controls without any T2 diseases or biomarker elevations, showing the strongest signals. Comparisons with biomarker-positive but asymptomatic controls showed consistent cytokine patterns, but diminished signals, especially for asthma and conjunctivitis, reflecting reduced contrast and power, though effect directions remained concordant and rhinitis signals persisted. Furthermore, the study population was racially and ethnically homogeneous, as the $COPSAC_{2010}$ cohort primarily includes children of Danish descent, which may limit generalizability of our findings when applied to more diverse populations. Although we stratified asthma into T2-high and T2-low phenotypes, the number of children with T2-high asthma, and especially T2-low asthma, was limited. This may have reduced the power to detect subtle immunologic differences, both relative to healthy controls and between asthma subgroups. This is important given that T2-low asthma is often associated with neutrophilic inflammation- and neutrophil-associated cytokine responses, which may not have been captured by the airway immune profiles in this study.[78-80] Finally, although our study demonstrated consistent associations between nasal cytokines and T2 diseases and biomarkers, it was not designed to evaluate diagnostic accuracy or establish clinical utility. These findings should therefore be interpreted as initial steps toward understanding the potential role of nasal cytokines in airway immunophenotyping.

In conclusion, increased levels of IL-5 and CCL17 in nasal epithelial lining fluid in 6-year-old children from the $COPSAC_{2010}$ cohort were associated with T2 diseases and biomarkers. Our findings propose a noninvasive and airway-specific sampling method useful for immunophenotyping. Future studies are needed to further explore the clinical relevance and potential utility of nasal cytokine profiling in broader and more diverse pediatric populations.

DISCLOSURE STATEMENT

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Governance: We are aware of and comply with recognized codes of good research practice, including the Danish Code of Conduct for Research Integrity. We comply with national and international rules on the safety and rights of patients and healthy subjects, including Good Clinical Practice (GCP) as defined in the EU's Directive on Good Clinical Practice, the International Conference on Harmonisation's (ICH) good clinical practice guidelines and the Helsinki Declaration. Privacy is important to us, which is why we follow national and international legislation on General Data Protection Regulation (GDPR), the Danish Act on Processing of Personal Data, and the practice of the Danish Data Inspectorate.

Disclosure of potential conflict of interest: A.-M. M. Schoos has been a paid speaker for Thermo Fisher Scientific; and is on an advisory board for ALK. J. Thorsen has received speaking fees from AstraZeneca. The rest of the authors declare that they have no relevant conflicts of interest.

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Key messages

- **IL-5 and CCL17 were consistently elevated in nasal samples from children with T2-high asthma and AR, identifying them as dominant T2 cytokines in airway inflammation.**
- **Nasal cytokine profiles correlated with conventional T2 biomarkers and performed comparably to blood eosinophils in identifying T2 diseases, both cross-sectionally and predictively.**
- **Noninvasive nasal sampling may offer a feasible approach for investigating airway immune profiles in children with T2 inflammatory diseases.**

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Title:

Dominance of IL-5 and CCL17 in Nasal Cytokine Profiles of Children with Type 2 Inflammation

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SUPPLEMENTARY METHODS

1.a: Allergic and Non-Allergic Rhinitis Algorithm

Allergic rhinitis (AR) was defined as the presence of parent-reported rhinitis symptoms (runny nose, blocked nose, itchy nose, or sneezing) in combination with sensitization to relevant aeroallergens. Sensitization was determined by either a positive skin prick test (wheal diameter ≥3 mm) or elevated specific IgE (≥0.35 kU/L). To establish clinical relevance, the child was also required to have ≥2 days of rhinitis symptoms in at least one of the allergen's expected exposure months.

The allergen-season symptom windows were defined as:

- Birch: April or May
- Grass: May, June, July, or August
- Mugwort: July or August
- Horse, Cat, Dog: Year-round (no season-specific timing)
- House dust mite (D. pteronyssinus and D. farinae): September through March
- Mould (Cladosporium herbarum): September through March

A child was classified as having allergic rhinitis if sensitization was present and symptoms were reported on ≥2 days during the matching allergen season.

Non-allergic rhinitis was defined as having rhinitis symptoms (on ≥2 days in any month) but without sensitization to any aeroallergen by either test of skin prick test or specific-IgE to aeroallergens. Children without symptoms or with missing sensitization data were coded as missing.

1.b: Nasal Cytokines

The cytokines analyzed include type 17 (IL-17A, IL-23, IL-1β, CXCL8), mixed type 17 and type 1 (TNF-α), type 1 (IL-12p70, CXCL10, IFN-γ), mixed type 1 and type 2 (CCL2, CCL4), type 2 (IL-4, IL-5, IL-13, CCL13, CCL26, IL-25), mixed type 2 and regulatory type (IL-33, CCL11, CCL17), and regulatory type (CCL22, IL-10, IL-2, TGF-β1), and other (C-reactive protein (CRP)).

The cytokines were measured in duplicate samples per child using Meso Scale Discovery immunoassay kits. TGF-β1 values were not measured for 30 samples due to limited sample material. Undetectable samples (0 values) were replaced by half the minimum value detected for a given cytokine to allow for log-transformation.

1.c: Minimum Concentration Values of Individual Cytokines

Raw immune mediator level data is presented in pg/mL, determined from the mean of duplicate samples using Meso Scale Discovery immunoassays. The minimum concentration value represents the lowest detectable concentration of each cytokine. For samples with undetectable levels (zero values), these have been assigned a value equal to half of the minimum concentration value (MIN) to facilitate log transformation. **Supplementary Table E1** outlines the minimum concentration values for each cytokine.

1.d: Nasal Cytokines (Extraction)

Prior to measurement of cytokines, the proteins were extracted using 400 uL of freshly prepared Milliplex Assay buffer (L-AB, Millipore) containing one complete protease inhibitor tablet (EDTA-free Protease Inhibitor Cocktail, #11873580001, Roche) per 25 mL of buffer.

The following plate setup was used:

- IL-25, IL-33: U-plex (K15067L-2)
- IL-5, IL-17A: V-plex (K151A9H-2)
- CCL2, CCL4, CCL11, CCL13, CCL17, CCL22, CCL26, CXCL10: V-plex (K151A9H-2)
- CRP: V-plex (K151A9H-2)
- IL-23: V-plex (K151A9H-2)
- IFN-ɣ, IL-1b, IL-2, IL-4, CXCL8, IL-10, IL-12p70, IL-13, TNF-α: V-plex (K151A9H-2)
- TGF-β1: U-plex (K151XWK-2)

This setup allowed for identification of all major immune response phenotypes.

1.e: Data Transformation Rationale

Distributions of raw individual immune marker levels exhibit considerable variation in scale, relative dispersion, and skewness **(Supplementary Figure E1)**. Skewness hampers the application of parametric statistical methods, which are critical when accounting for covariates. To address these issues, we replaced 0-values with ½*minimum detectable value for a given cytokine and applied log-transformation to reduce skewness and z-scaling to standardize scale and relative dispersion.

Principal component analysis (PCA) of the raw data **(Supplementary Figure E7)** showed uniformly positive PC1 loadings across all immune markers, indicating a strong positive correlation among them. This suggests that variations in immune marker levels are primarily driven by a shared underlying factor. Raw marker levels are influenced by variability in the amount and concentration of the sampling material, which is inherently difficult to standardize. To account for this variability, we re-exponentiated the data to enable total cytokine-sum scaling, followed by re-application of log transformation and z-scaling.

1.f: Alternative Data Transformation method of CLR

One of the most widespread transformations in compositional data analysis is the CLR (Center Log-Ratio) transformation (Aitchison 1986). As a sensitivity analysis, raw immune marker levels were CLR-transformed:

$$clr(x) = \log \frac{x_i}{g(x)}$$

Where $x = [x_1, x_2, \dots, x_D]$ represents the parts of the composition (immune markers), where D is the total number of parts. And the geometric mean, $g(x)$, is:

$$g(x) = \left(\prod_{i=1}^{D} x_i \right)^{\frac{1}{D}}$$

Based on a product rather than a sum, the geometric mean preserves proportional relationships and allows comparisons independent of scale. In contrast to the transformation used in the main text, differences in relative dispersion among markers affect their influence on the transformation.

The raw data was transformed using the following steps:

1. Replacement of 0-values: Half of the smallest non-zero value for the respective cytokine.
2. Imputation of Missing Values for 30 TGF-β1 values (NAs): Using log-ratio expectation-maximization (zCompositions, lrEM), which accounts for compositionality.
3. CLR Transformation

The analysis yielded results similar to the transformation used in the main text (**Supplementary Figure E6**).

1.g: sPLS Model Cross-Validation

We employed a single-component (N=1) model with cross-validated predictions, using 10-fold cross-validation repeated 10 times, and an 80/20 train test split. This approach was designed to enhance interpretability and minimize the risk of overfitting. Model performance was evaluated using root-mean-square error (RMSE) and coefficient of determination (R^2).

1.h: Outlier Handling

Sixteen outliers were identified by their PC1 and PC2 scores, with one being removed from analysis due to an unusually high score (>18). None of the outliers exhibited biological or testing differences that could explain their deviation from the other samples, and sensitivity analysis yielded the same results without outliers (data not shown).

Table E1. Samples with replaced 0-values and minimum concentration (MIN) for all cytokines.

Cytokine	MIN (pg/mL)	0-values n (%)	Missing data n (%)
IL-12p70	0.002	15 (2.5%)	0 (0%)
IFN-γ	0.011	46 (7.5%)	0 (0%)
CXCL10	0.107	2 (0.3%)	0 (0%)

TNF-α	0.057	1 (0.2%)	0 (0%)
CCL2	0.346	1 (0.2%)	0 (0%)
CCL4	0.052	1 (0.2%)	0 (0%)
IL-33	0.003	19 (3.1%)	0 (0%)
IL-25	0.009	118 (19.3%)	0 (0%)
IL-4	0.001	47 (7.7%)	0 (0%)
IL-5	0.001	55 (9.0%)	0 (0%)
IL-13	0.256	1 (0.2%)	0 (0%)
CCL11	0.535	1 (0.2%)	0 (0%)
CCL13	0.966	24 (3.9%)	0 (0%)
CCL26	0.020	28 (4.6%)	0 (0%)
CCL17	0.119	1 (0.2%)	0 (0%)
CCL22	0.142	32 (5.2%)	0 (0%)
IL-23	0.003	179 (29.2%)	0 (0%)
IL-17A	0.002	12 (2.0%)	0 (0%)
CXCL8	0.055	1 (0.2%)	0 (0%)
IL-1b	0.191	1 (0.2%)	0 (0%)
IL-10	0.020	1 (0.2%)	0 (0%)
IL-2	0.016	5 (0.8%)	0 (0%)
TGF-β1	0.210	96 (15.7%)	30 (4.9%)
CRP	1.445	9 (1.5%)	0 (0%)

Cytokine	T2-high asthma	T2-low asthma	Allergic rhinitis	Non-allergic rhinitis	Allergic conjunctivitis	Atopic dermatitis	Healthy controls
IL-12p70	0.69 (0.26; 1.53)	0.55 (0.37; 1.40)	0.56 (0.25; 1.44)	0.69 (0.40; 1.62)	0.65 (0.23; 1.56)	0.63 (0.25; 1.32)	0.62 (0.30; 1.26)
IFN-γ	3.80 (1.46; 10.30)	5.30 (1.64; 18.86)	6.60 (1.65; 15.29)	3.32 (1.59; 9.37)	5.56 (1.73; 19.48)	4.27 (1.12; 12.19)	3.78 (1.29; 2.09)
CXCL10	4890 (1398; 9909)	4964 (1659; 9895)	5543 (1160; 10187)	2822 (1021; 5956)	5543 (1063; 8711)	3266.35 (941.4; 8182)	2958 (962.8; 7486)
TNF-α	7.20 (3.09; 12.99)	6.48 (5.08; 11.54)	4.91 (1.47; 19.68)	5.26 (3.09; 9.77)	4.93 (2.81; 12.99)	5.95 (2.34; 13.67)	5.27 (2.58; 11.50)
CCL2	60.74 (20.93; 103.8)	61.00 (26.60; 96.31)	40.50 (16.64; 86.38)	39.08 (16.50; 89.18)	40.50 (17.96; 92.83)	41.54 (14.92; 80.51)	40.49 (22.15; 76.85)
CCL4	69.65 (19.84; 417.4)	68.25 (24.86; 243.4)	99.99 (27.73; 390.6)	47.51 (30.15; 124.4)	85.70 (32.65; 413.3)	65.91 (18.99; 179.8)	49.51 (22.15; 118.2)
IL-33	0.98 (0.41; 2.78)	1.10 (0.46; 2.32)	0.73 (0.29; 3.04)	0.85 (0.51; 2.78)	0.83 (0.44; 2.44)	1.13 (0.35; 2.36)	1.16 (0.41; 2.97)
IL-25	0.41 (0.12; 0.68)	0.50 (0.13; 0.71)	0.39 (0.11; 0.67)	0.71 (0.22; 1.17)	0.47 (0.19; 0.87)	0.41 (0.08; 0.85)	0.39 (0.09; 0.93)
IL-4	0.34 (0.16; 0.78)	0.18 (0.04; 0.62)	0.43 (0.09; 0.93)	0.31 (0.18; 0.69)	0.37 (0.12; 0.78)	0.27 (0.09; 0.69)	0.30 (0.11; 0.69)
IL-5	0.46 (0.27; 3.39)	0.26 (0.16; 0.43)	2.93 (0.63; 8.29)	0.27 (0.04; 0.45)	1.00 (0.27; 6.06)	0.51 (0.17; 3.05)	0.37 (0.15; 0.77)
IL-13	12.09 (5.55; 18.56)	11.33 (7.48; 15.45)	7.91 (4.63; 14.69)	8.77 (5.26; 11.97)	7.91 (4.76; 14.09)	9.20 (4.86; 19.34)	9.62 (5.08; 17.10)
CCL11	139.9 (83.80; 319.3)	87.66 (60.42; 188.5)	142.9 (72.67; 259.0)	84.44 (49.63; 208.9)	136.1 (68.36; 299.7)	115.6 (56.75; 236.6)	102.5 (60.81; 185.3)
CCL13	12.66 (6.51; 25.66)	10.28 (4.91; 16.92)	14.91 (6.38; 34.56)	10.31 (5.40; 14.63)	13.29 (6.51; 25.65)	11.52 (5.94; 18.02)	9.80 (5.97; 15.67)
CCL26	38.49 (9.74; 83.43)	14.94 (10.66; 33.49)	38.21 (10.23; 89.51)	14.06 (6.50; 24.82)	24.82 (9.45; 52.69)	18.08 (7.51; 40.96)	16.39 (7.22; 30.30)
CCL17	29.67 (14.48; 51.18)	18.12 (11.54; 28.82)	30.17 (14.23; 50.52)	11.55 (6.48; 26.53)	28.83 (10.73; 40.47)	19.66 (8.65; 34.00)	16.60 (10.87; 24.85)
CCL22	79.93 (34.53; 148.7)	77.79 (39.97; 106.4)	52.44 (31.77; 138.1)	58.79 (19.85; 84.04)	63.77 (28.80; 116.6)	59.53 (23.36; 132.7)	54.83 (30.12; 100.3)
IL-23	0.32 (0.00; 1.07)	0.59 (0.01; 0.93)	0.53 (0.15; 1.23)	0.17 (0.00; 1.62)	0.32 (0.00; 1.12)	0.35 (0.00; 1.10)	0.53 (0.00; 1.32)
IL-17A	4.42 (1.77; 8.11)	4.28 (1.99; 7.64)	6.57 (1.17; 16.09)	3.52 (1.42; 7.76)	6.57 (1.40; 9.57)	4.77 (1.30; 11.59)	3.45 (1.32; 8.38)
CXCL8	3783 (1357; 5496)	4342 (2929; 6004)	2400 (870.8; 5579)	3250 (2409; 5335)	2719 (1283; 5403)	3551 (1252; 5655)	3764 (1570; 5664)

IL-1b	33.01 (15.03; 63.72)	56.29 (20.13; 126.8)	30.11 (9.91; 162.0)	36.46 (21.78; 132.3)	25.69 (11.43; 185.5)	42.76 (11.10; 120.1)	38.97 (13.96; 108.9)
IL-10	1.81 (0.58; 3.85)	1.84 (0.61; 3.47)	2.49 (0.60; 7.52)	1.79 (0.65; 2.57)	2.21 (0.65; 7.29)	2.23 (0.54; 5.10)	1.29 (0.62; 3.99)
IL-2	1.90 (0.91; 3.43)	1.86 (1.52; 3.72)	1.57 (0.83; 4.15)	1.64 (0.91; 2.91)	1.63 (0.87; 3.28)	1.61 (0.81; 3.58)	1.62 (0.90; 3.17)
TGF-β1	72.29 (25.32; 175.2)	38.79 (4.99; 84.33)	48.07 (0.10; 186.4)	40.65 (11.15; 89.54)	50.54 (8.40; 144.6)	42.26 (1.45; 97.63)	53.88 (9.86; 113.7)
CRP	1098 (329.0; 3920)	576.8 (335.52; 5945)	1166 (395.4; 5979)	638.5 (196.2; 2390)	1132 (392.5; 9084)	687.6 (180.6; 2454)	552.2 (184.9; 1788)

Table E2. Raw cytokine concentrations by group. *Raw nasal epithelial lining fluid cytokine concentrations reported as median and interquartile range (IQR) in pg/mL for T2-high asthma, T2-low asthma, allergic rhinitis, non-allergic rhinitis, allergic conjunctivitis or atopic dermatitis and healthy controls with no asthma, rhinitis, atopic dermatitis or allergic conjunctivitis. Values are untransformed and provided to illustrate absolute levels and variability. This table is descriptive; inferential analyses in the manuscript use normalized, variance-stabilized data.*

-	Age	Biomarker Model	AUC	CI 95%	Sensitivity	Specificity	DeLong's Test (P-value)
T2-high asthma*	6	B-eos	0.77	0.68–0.86	1.00	0.46	0.10
	6	Nasal Cytokine Score	0.65	0.48–0.81	0.55	0.83	
Allergic Rhinitis	6	B-eos	0.68	0.60 – 0.77	0.85	0.47	0.07
	6	Nasal Cytokine Score	0.75	0.69 – 0.86	0.74	0.76	
Allergic Conjunctivitis	6	B-eos	0.67	0.57 – 0.77	0.59	0.70	0.85
	6	Nasal Cytokine Score	0.66	0.55 – 0.77	0.56	0.78	
Atopic dermatitis	6	B-eos	0.56	0.48 – 0.64	0.45	0.68	0.62
	6	Nasal Cytokine Score	0.53	0.44 – 0.62	0.23	0.88	
T2-high asthma**	10	B-eos	0.61	0.40 – 0.81	0.80	0.56	0.97
	10	Nasal Cytokine Score	0.60	0.26 – 0.94	0.60	0.79	
Allergic Rhinitis	10	B-eos	0.54	0.39–0.68	1.00	0.22	0.84
	10	Nasal Cytokine Score	0.52	0.33–0.70	0.66	0.55	
Allergic Conjunctivitis	10	B-eos	0.50	0.37 – 0.63	0.63	0.45	0.68
	10	Nasal Cytokine Score	0.54	0.40–0.69	0.84	0.32	
Atopic dermatitis	10	B-eos	0.62	0.44–0.80	0.8	0.55	0.28
	10	Nasal Cytokine Score	0.52	0.56–0.81	0.80	0.48	

Table E3. ***Blood eosinophils versus sPLS-derived T2 Nasal Cytokine Score.*** Coupled Receiver Operating Characteristic (ROC) predictive performance analysis of logistic models for logarithm-transformed and scaled blood eosinophils (B-eos) versus sPLS-derived T2 Nasal Cytokine Score. The analysis first evaluates cross-sectional diagnostic performance at 6 years for type 2 diseases (T2-high asthma, allergic rhinitis, allergic conjunctivitis, atopic dermatitis) and then assesses predictive performance at 10 years for children classified as healthy at 6 years without any type 2 disease or sensitization to later type 2 diseases. Metrics include Area Under the Curve (AUC), Confidence Interval (CI), sensitivity, specificity, and DeLong's t-test for differences between models. For this model, 15 cytokines were chosen (IL-5, CCL17, CCL4, CCL11, CCL13, IL-13,

IL-25, CXCL8, IL-4, CCL2, IL-33, TGF-β1, CXCL10, IL-12p70, and IFN-γ) based on root-mean-square deviation (RMSE) and coefficient of determination (R^2).

**Defined as asthma at 6 years with either elevated FeNO and/or specific-IgE and/or skin prick test without elevated blood eosinophils as a criteria*

***Defined as asthma at 10 years with either elevated blood eosinophils and/or FeNO and/or specific-IgE and/or skin prick test and/or total_IgE*

Figure E1. Distribution of raw nasal cytokine levels by immune response type. *Violin plot of 24 nasal epithelial lining cytokine levels expressed in pg/mL before multistep data transformation. Cytokines are colored depending on the primary inflammatory response type they induce/represent. N=612.*

(See next page)

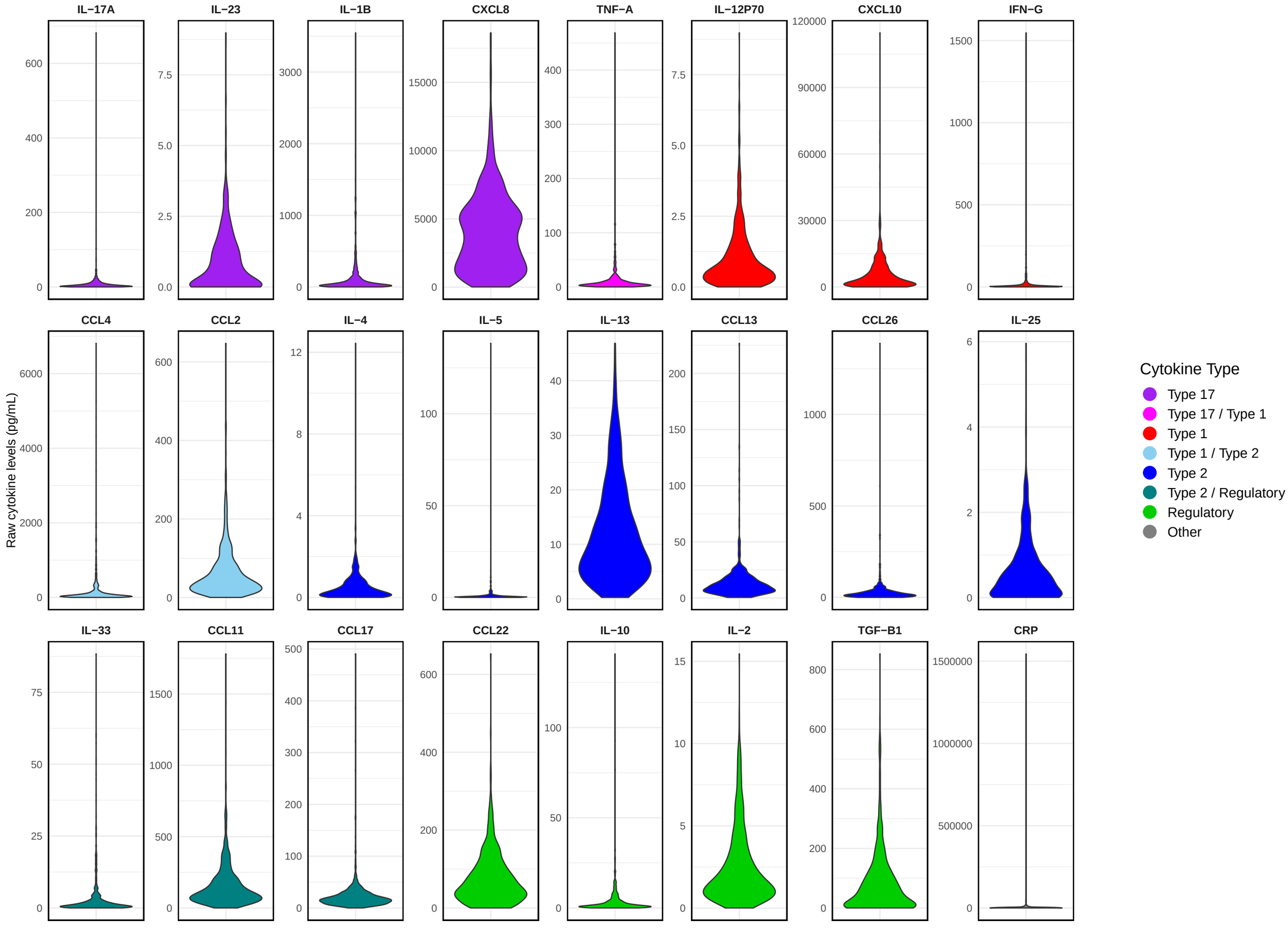

IL−17A
IL−23
IL−1B
CXCL8
TNF−A
IL−12P70
CXCL10
IFN−G
CCL4
CCL2
IL−4
IL−5
IL−13
CCL13
CCL26
IL−25
IL−33
CCL11
CCL17
CCL22
IL−10
IL−2
TGF−B1
CRP
Raw cytokine levels (pg/mL)
Cytokine Type
Type 17
Type 17 / Type 1
Type 1
Type 1 / Type 2
Type 2
Type 2 / Regulatory
Regulatory
Other

Figure E2. Distribution of transformed nasal cytokine levels by immune response type. *Violin plot of 24 nasal epithelial lining cytokine levels expressed in standard deviations (SD) after multistep data transformation. Cytokines are colored depending on the primary inflammatory response they induce/represent. N=612.*

(See next page)

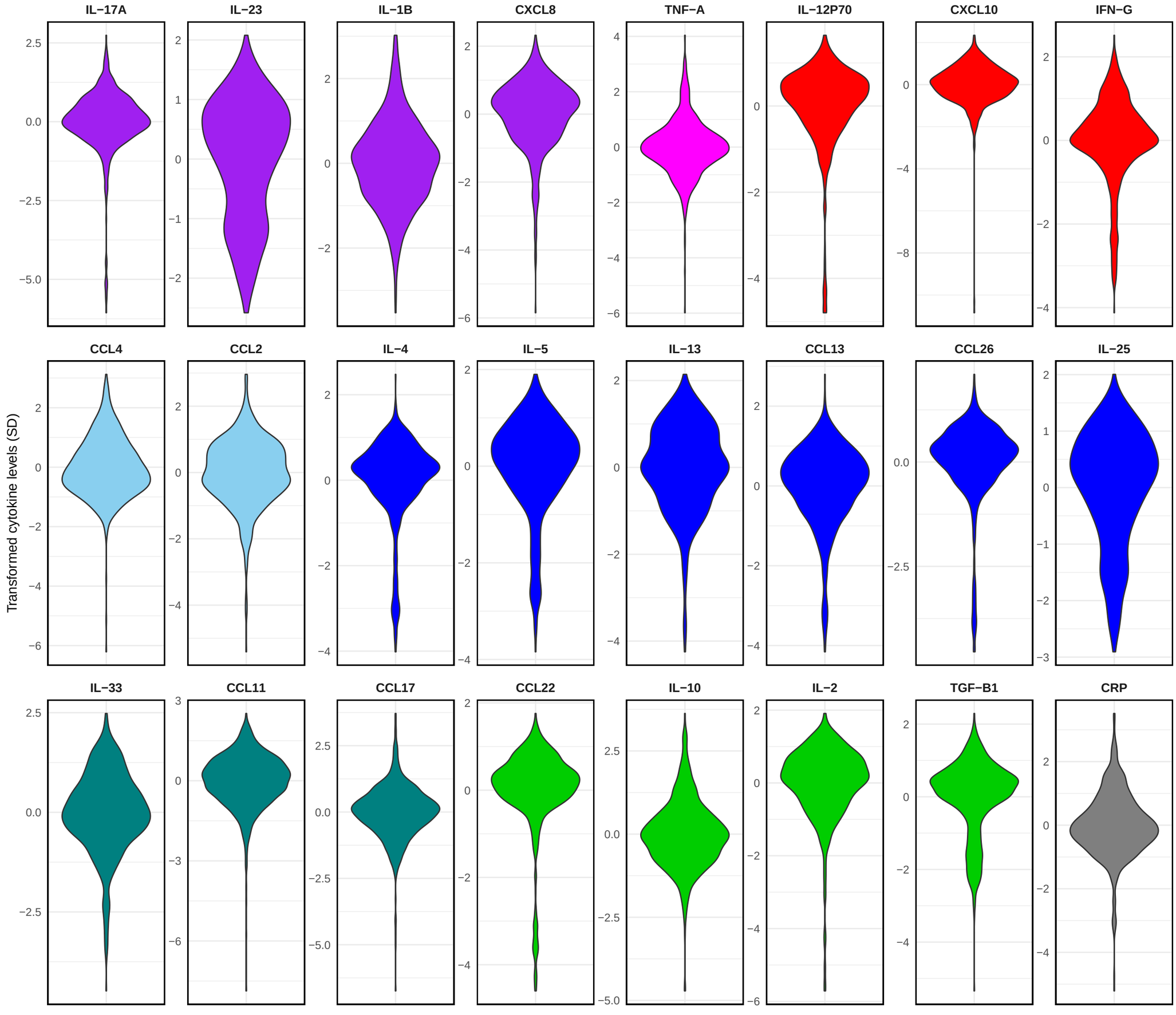
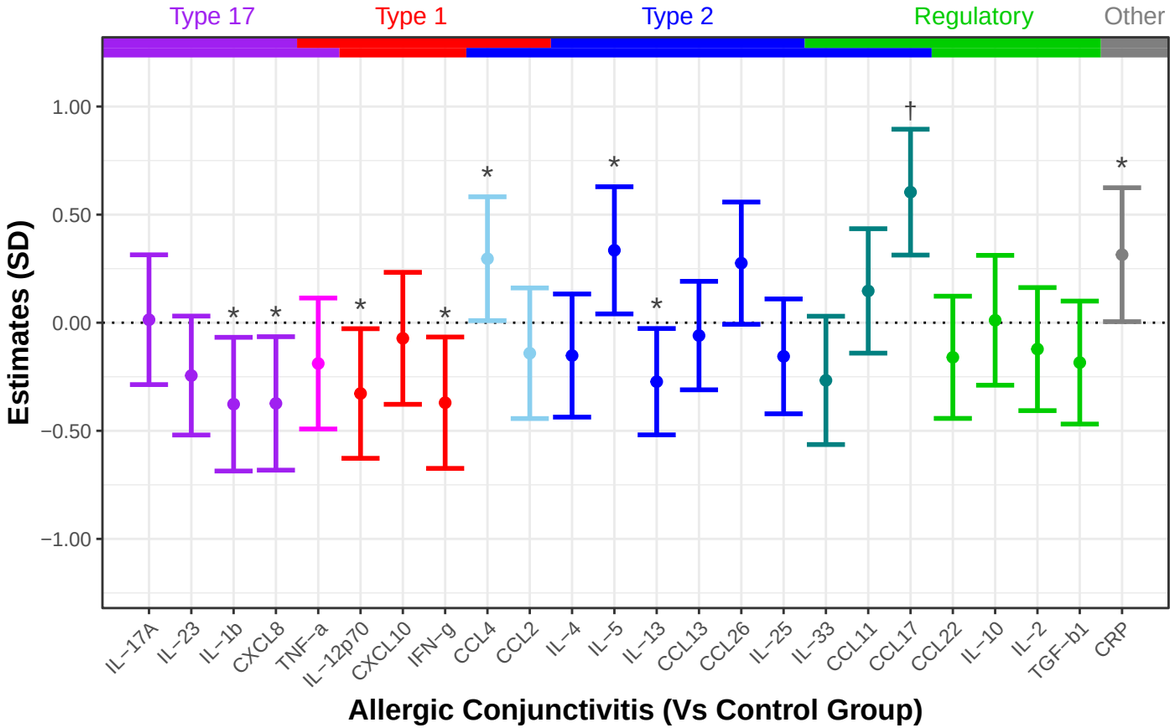

IL−17A
IL−23
IL−1B
CXCL8
TNF−A
IL−12P70
CXCL10
IFN−G
CCL4
CCL2
IL−4
IL−5
IL−13
CCL13
CCL26
IL−25
IL−33
CCL11
CCL17
CCL22
IL−10
IL−2
TGF−B1
CRP
Transformed cytokine levels (SD)
Cytokine Type
Type 17
Type 17 / Type 1
Type 1
Type 1 / Type 2
Type 2
Type 2 / Regulatory
Regulatory
Other

Figure E3. Association between nasal cytokines and allergic conjunctivitis and atopic dermatitis. *Forest plots depicting the association between airway cytokines and type 2 diseases of allergic conjunctivitis and atopic dermatitis versus control group (children without respective diseases) at age 6 years. Linear regression models were adjusted for age, sex, sampling season, sampling plate, analysis date, extraction date, airway infection less than one week prior to sampling, and prior use of nasal and inhalation steroids before sampling. P < 0.05 (*), FDR-adjusted p < 0.05 (†). Per outcome panel: allergic conjunctivitis N=606 (45/561), atopic dermatitis N=602 (75/527).*

(See next page)


Type 17
Type 1
Type 2
Regulatory
Other
1.00
0.50
0.00
−0.50
−1.00
Estimates (SD)
IL−17A
IL−23
IL−1b
CXCL8
TNF−a
IL−12p70
CXCL10
IFN−g
CCL4
CCL2
IL−4
IL−5
IL−13
CCL13
CCL26
IL−25
IL−33
CCL11
CCL17
CCL22
IL−10
IL−2
TGF−b1
CRP
Allergic Conjunctivitis (Vs Control Group)

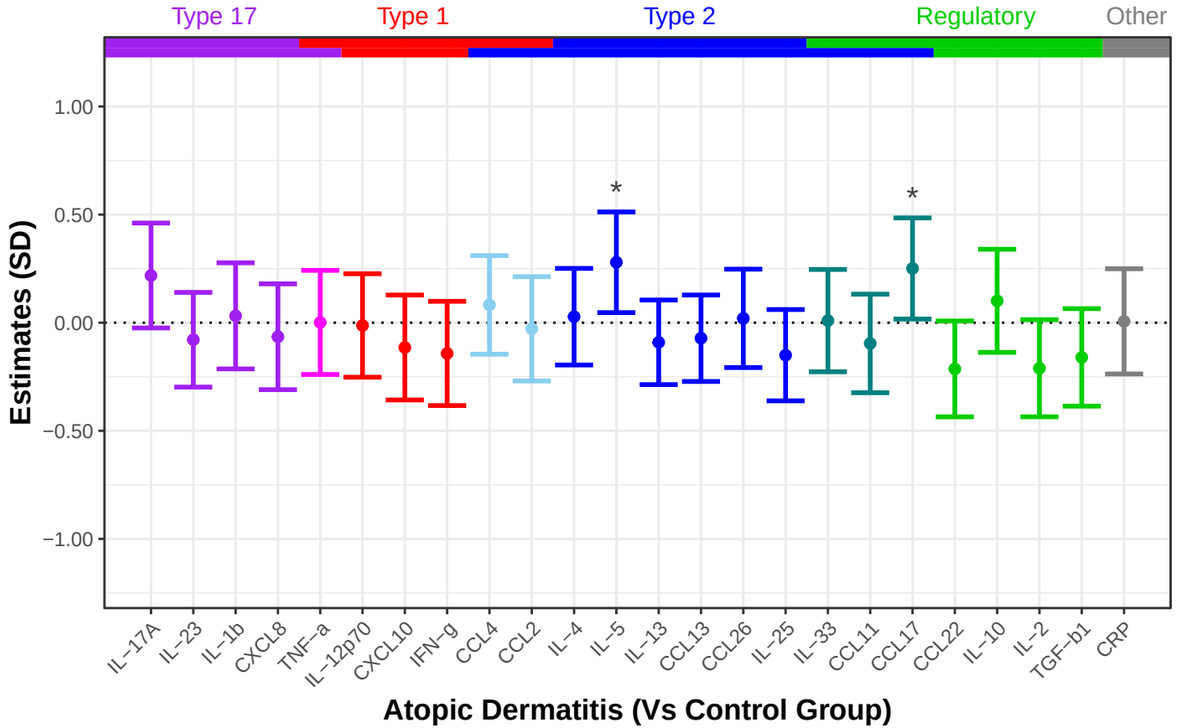

Type 17
Type 1
Type 2
Regulatory
Other
1.00
0.50
0.00
−0.50
−1.00
Estimates (SD)
IL−17A
IL−23
IL−1b
CXCL8
TNF−a
IL−12p70
CXCL10
IFN−g
CCL4
CCL2
IL−4
IL−5
IL−13
CCL13
CCL26
IL−25
IL−33
CCL11
CCL17
CCL22
IL−10
IL−2
TGF−b1
CRP
Atopic Dermatitis (Vs Control Group)

Figure E4. Direct comparisons of nasal cytokine levels. *The left plot shows T2-high asthma vs T2-low asthma. The right plot shows allergic rhinitis vs non-allergic rhinitis. Forest plots from covariate-adjusted linear regression. Models were adjusted for age, sex, sampling season, sampling plate, analysis date, extraction date, and use of nasal and inhalation steroids. P < 0.05 (*), FDR-adjusted p < 0.05 (†). Per disease panel, complete cases: T2-high vs T2-low N=54 (35/19); AR vs NAR N=76 (43/33).*

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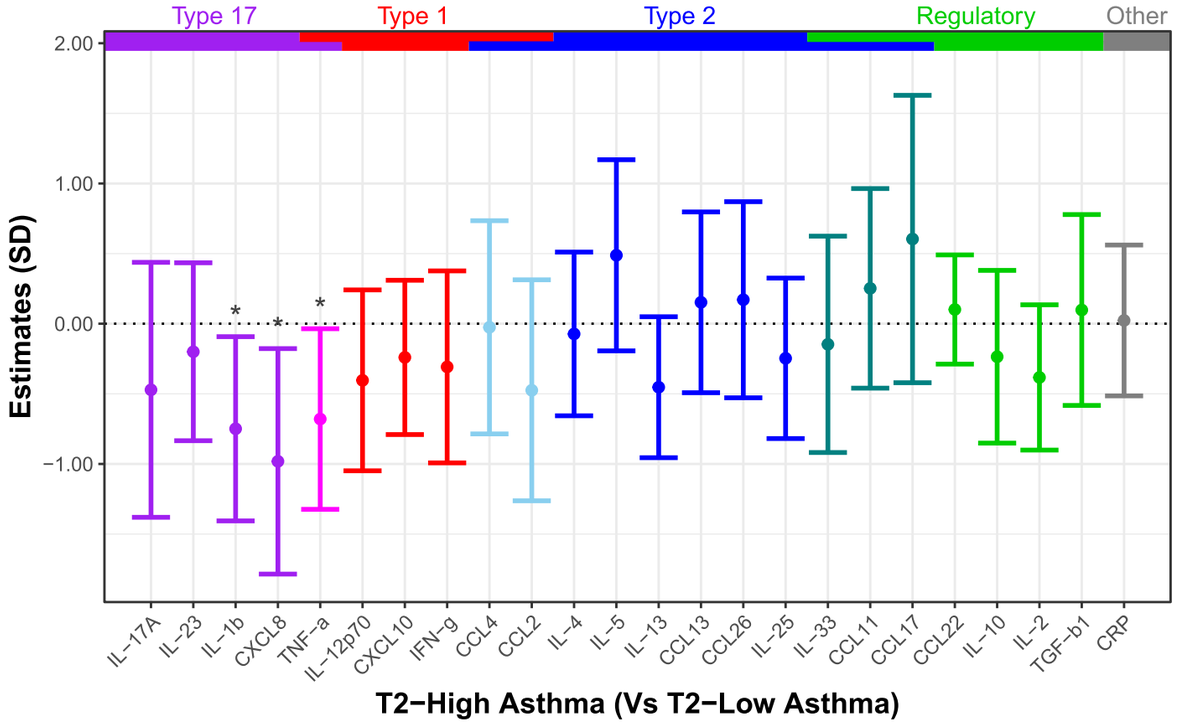
Type 17
Type 1
Type 2
Regulatory
Other
2.00
1.00
0.00
−1.00
Estimates (SD)
*
*
*
IL−17A
IL−23
IL−1b
CXCL8
TNF−a
IL−12p70
CXCL10
IFN−g
CCL4
CCL2
IL−4
IL−5
IL−13
CCL13
CCL26
IL−25
IL−33
CCL11
CCL17
CCL22
IL−10
IL−2
TGF−b1
CRP
T2−High Asthma (Vs T2−Low Asthma)

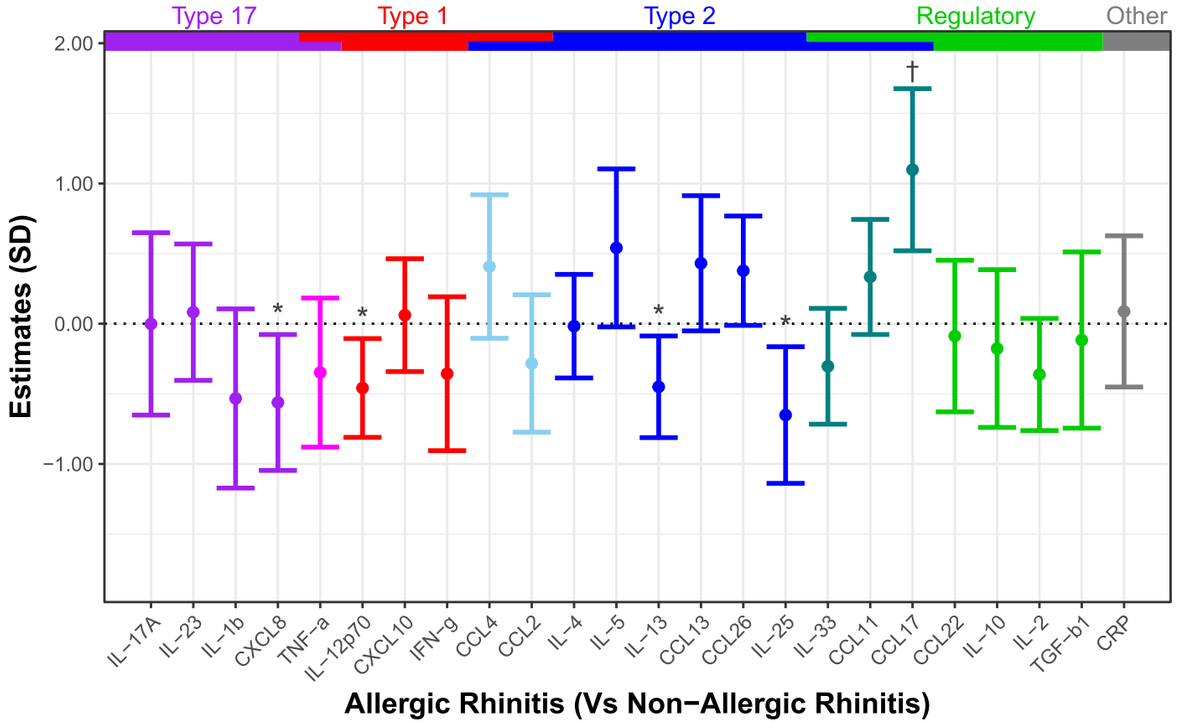
Type 17
Type 1
Type 2
Regulatory
Other
2.00
1.00
0.00
−1.00
Estimates (SD)
*
*
*
*
†
IL−17A
IL−23
IL−1b
CXCL8
TNF−a
IL−12p70
CXCL10
IFN−g
CCL4
CCL2
IL−4
IL−5
IL−13
CCL13
CCL26
IL−25
IL−33
CCL11
CCL17
CCL22
IL−10
IL−2
TGF−b1
CRP
Allergic Rhinitis (Vs Non−Allergic Rhinitis)

Figure E5. Sensitivity analysis: Type 2 outcomes adjusted for asthma. *Forest plots depicting the association between airway cytokines and non-asthma type 2 diseases and type 2 biomarkers at age 6 years. Linear regression models were adjusted for asthma, age, sex, sampling season, sampling plate, analysis date, extraction date, airway infection less than one week prior to sampling, and prior use of nasal and inhalation steroids before sampling. P < 0.05 (*), FDR-adjusted p < 0.05 (†). Per disease outcomes, complete cases: AR N=571 (43/528), NAR N=561 (33/528), allergic conjunctivitis N=606 (45/561), atopic dermatitis N=602 (75/527). Per biomarker panels, complete cases: blood eosinophils N=480 (216/264), FeNO N=366 (18/348), specific-IgE N=366 (18/348), SPT N=529 (41/488).*

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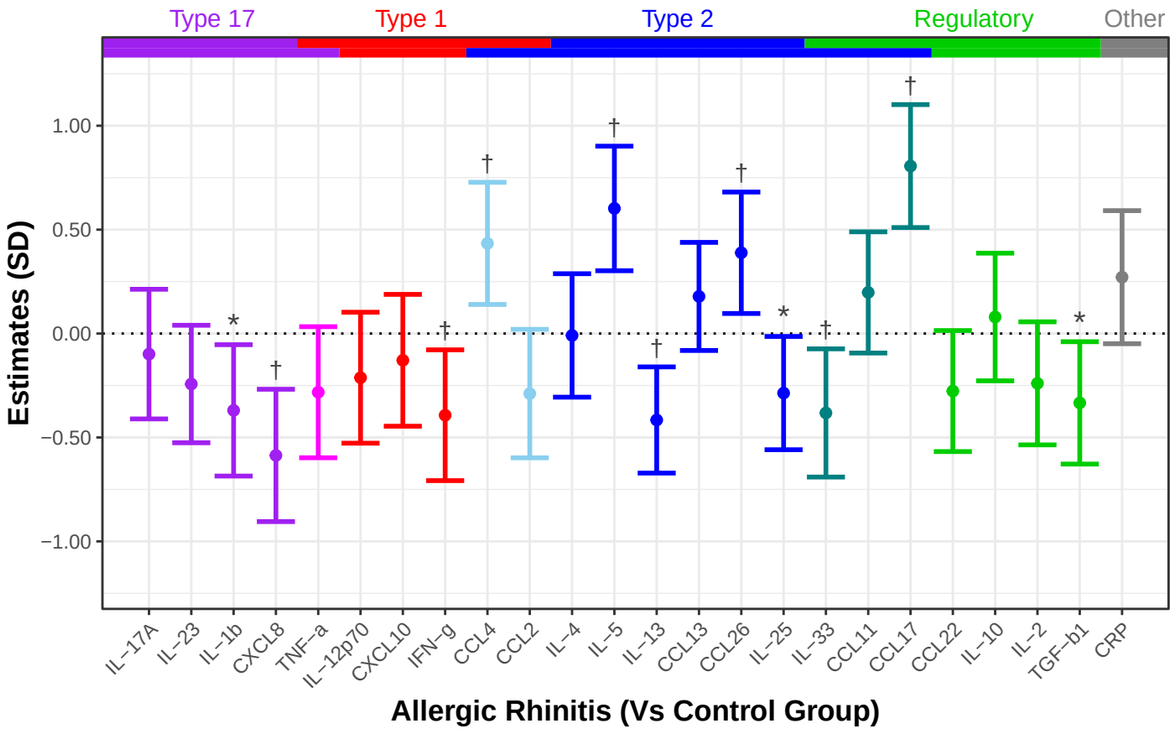
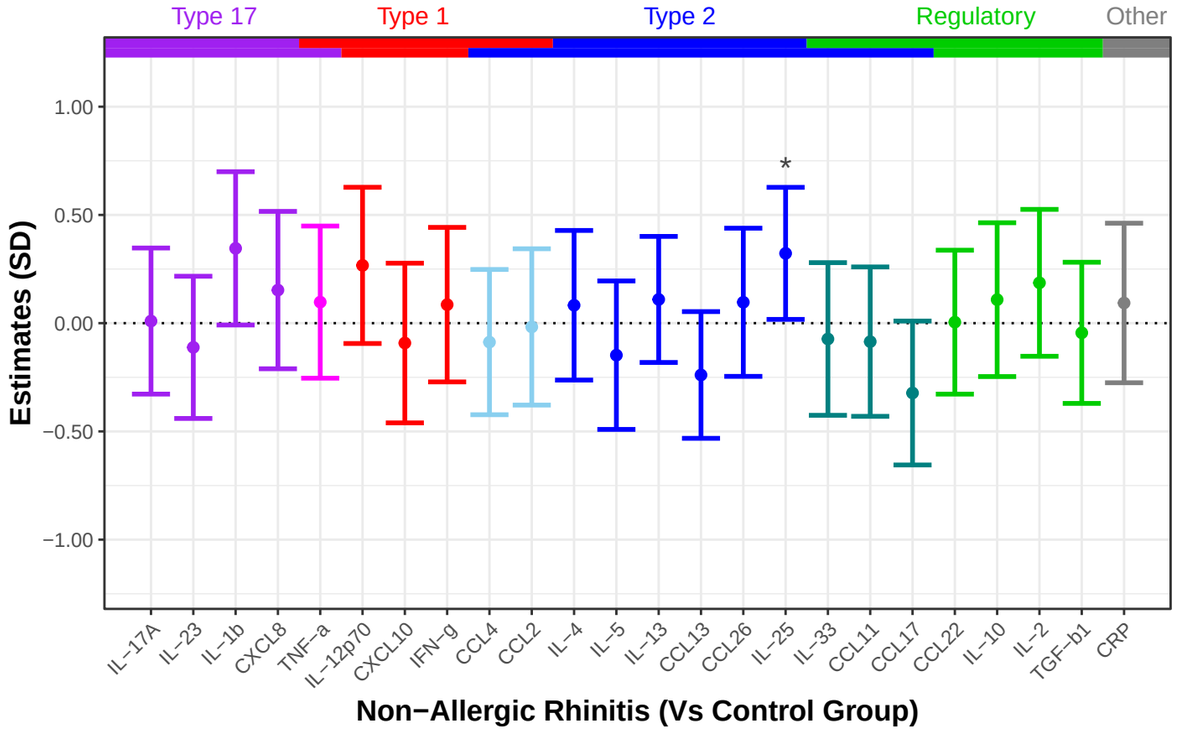
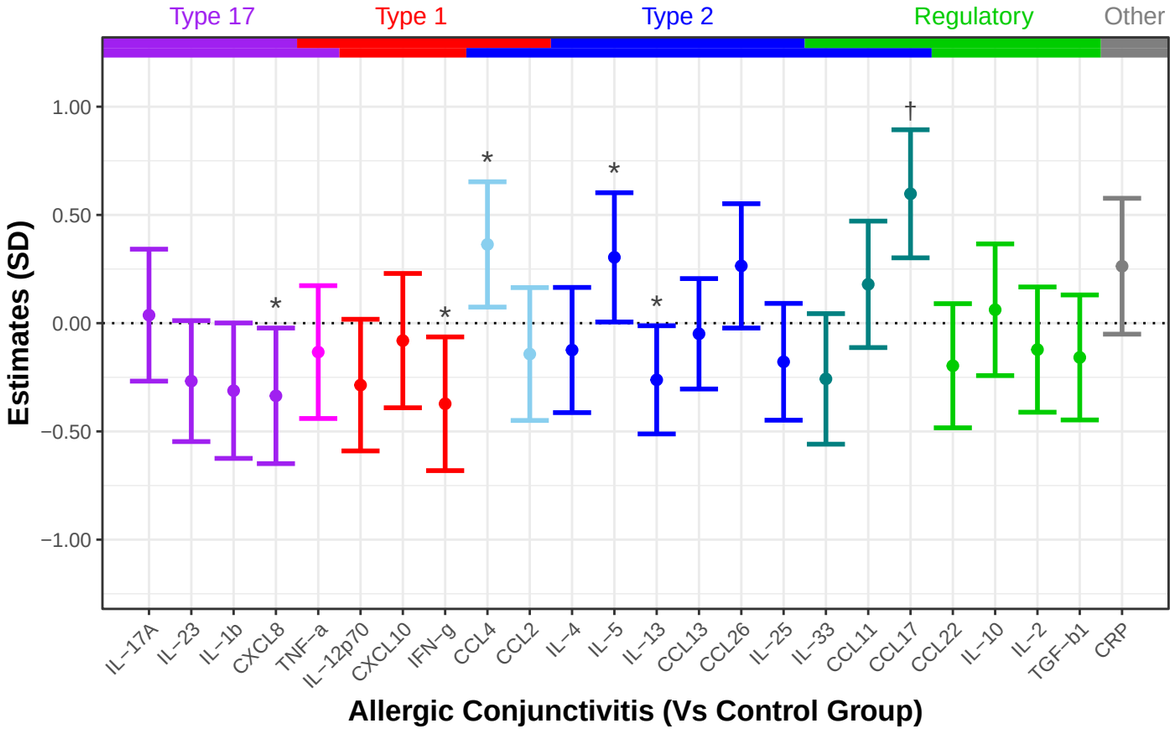
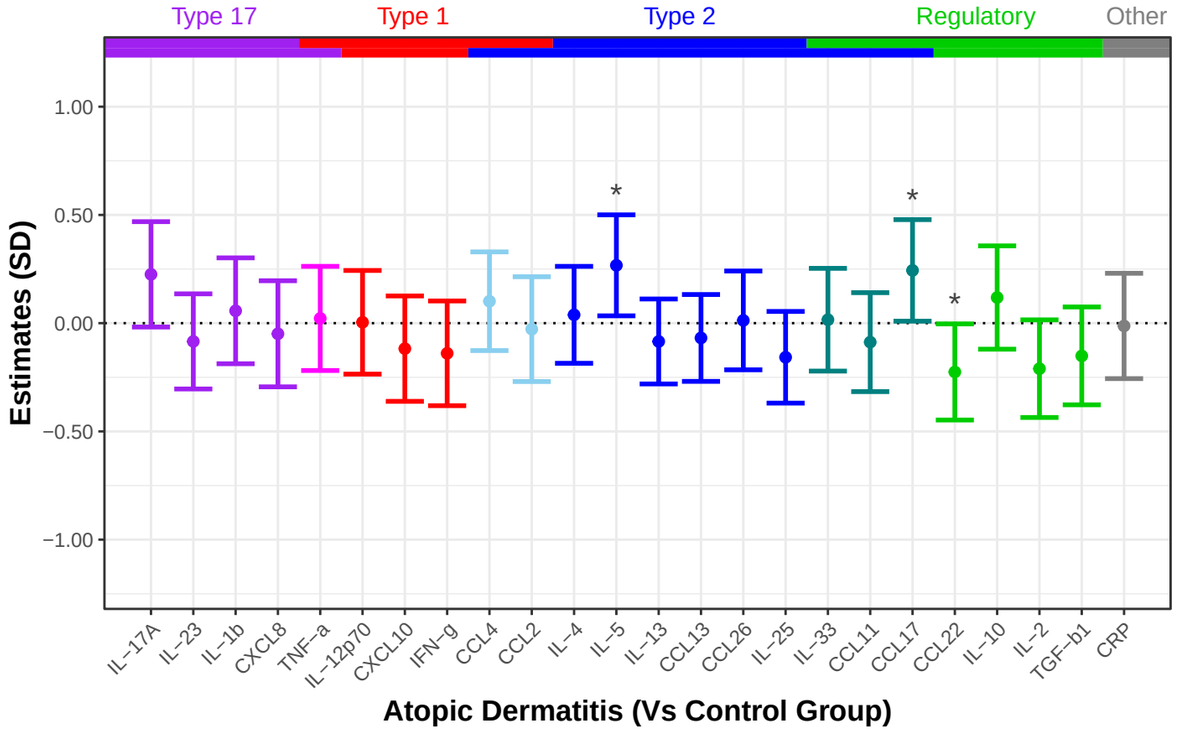

Type 17
Type 1
Type 2
Regulatory
Other
Estimates (SD)
1.00
0.50
0.00
−0.50
−1.00
IL−17A
IL−23
IL−1b
CXCL8
TNF−a
IL−12p70
CXCL10
IFN−g
CCL4
CCL2
IL−4
IL−5
IL−13
CCL13
CCL26
IL−25
IL−33
CCL11
CCL17
CCL22
IL−10
IL−2
TGF−b1
CRP
Allergic Rhinitis (Vs Control Group)


Type 17
Type 1
Type 2
Regulatory
Other
Estimates (SD)
1.00
0.50
0.00
−0.50
−1.00
IL−17A
IL−23
IL−1b
CXCL8
TNF−a
IL−12p70
CXCL10
IFN−g
CCL4
CCL2
IL−4
IL−5
IL−13
CCL13
CCL26
IL−25
IL−33
CCL11
CCL17
CCL22
IL−10
IL−2
TGF−b1
CRP
Non−Allergic Rhinitis (Vs Control Group)


Type 17
Type 1
Type 2
Regulatory
Other
Estimates (SD)
1.00
0.50
0.00
−0.50
−1.00
IL−17A
IL−23
IL−1b
CXCL8
TNF−a
IL−12p70
CXCL10
IFN−g
CCL4
CCL2
IL−4
IL−5
IL−13
CCL13
CCL26
IL−25
IL−33
CCL11
CCL17
CCL22
IL−10
IL−2
TGF−b1
CRP
Allergic Conjunctivitis (Vs Control Group)


Type 17
Type 1
Type 2
Regulatory
Other
Estimates (SD)
1.00
0.50
0.00
−0.50
−1.00
IL−17A
IL−23
IL−1b
CXCL8
TNF−a
IL−12p70
CXCL10
IFN−g
CCL4
CCL2
IL−4
IL−5
IL−13
CCL13
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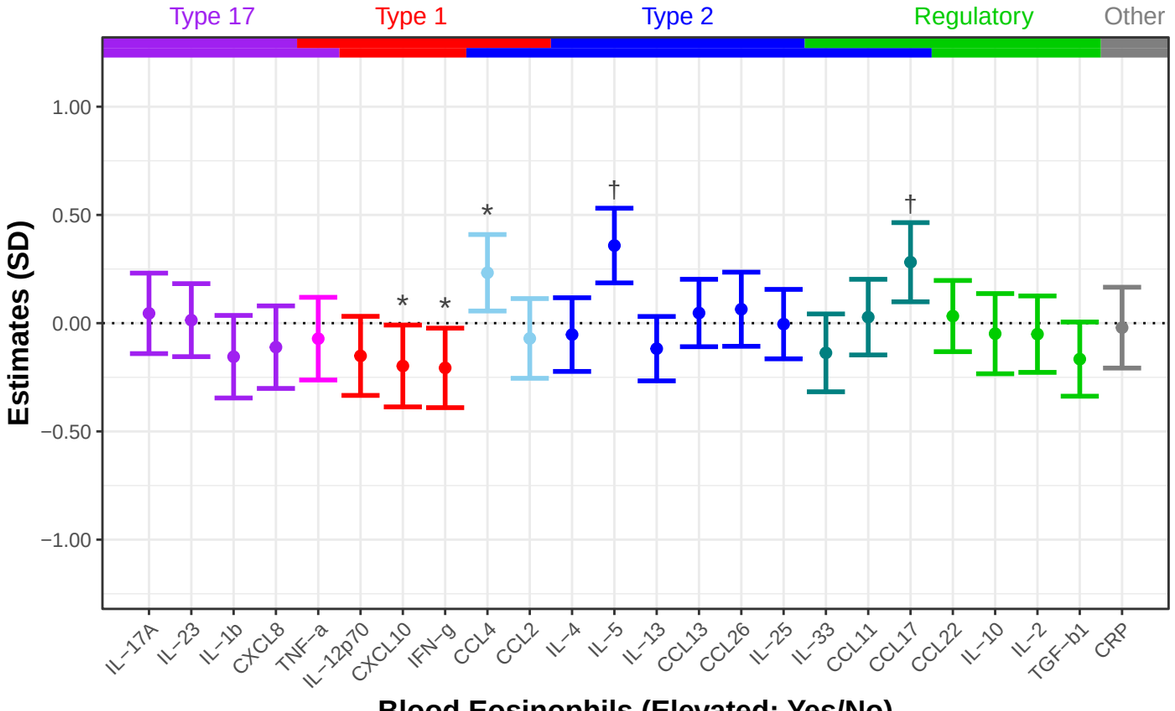
IL−10
IL−2
TGF−b1
CRP
Atopic Dermatitis (Vs Control Group)


Type 17
Type 1
Type 2
Regulatory
Other
Estimates (SD)
1.00
0.50
0.00
−0.50
−1.00
IL−17A
IL−23
IL−1b
CXCL8
TNF−a
IL−12p70
CXCL10
IFN−g
CCL4
CCL2
IL−4
IL−5
IL−13
CCL13
CCL26
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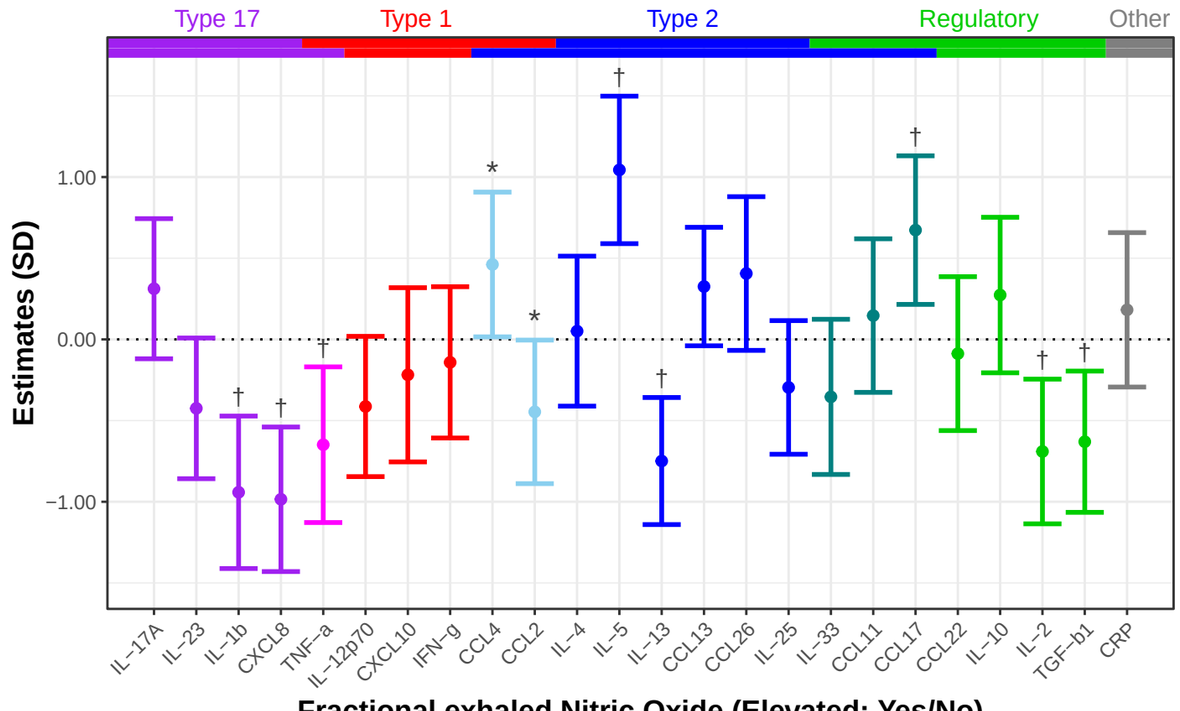
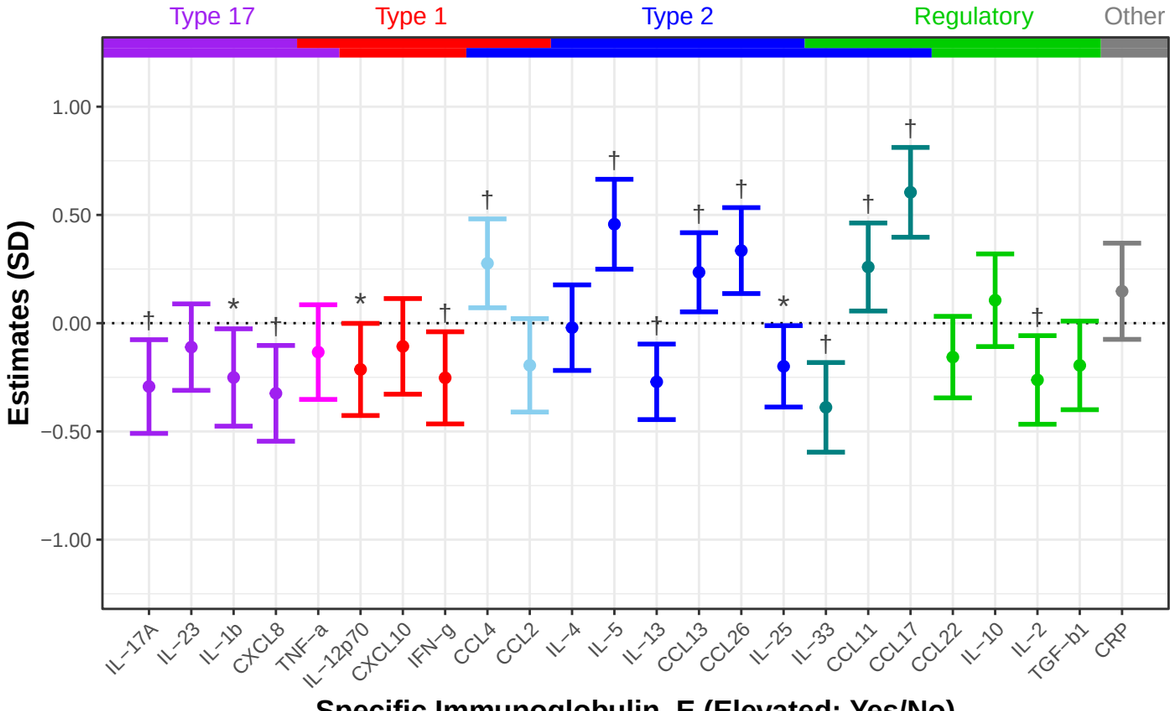
CCL17
CCL22
IL−10
IL−2
TGF−b1
CRP
Blood Eosinophils (Elevated: Yes/No)


Type 17
Type 1
Type 2
Regulatory
Other
Estimates (SD)
1.00
0.00
−1.00
IL−17A
IL−23
IL−1b
CXCL8
TNF−a
IL−12p70
CXCL10
IFN−g
CCL4
CCL2
IL−4
IL−5
IL−13
CCL13
CCL26
IL−25
IL−33
CCL11
CCL17
CCL22
IL−10
IL−2
TGF−b1
CRP
Fractional exhaled Nitric Oxide (Elevated: Yes/No)


Type 17
Type 1
Type 2
Regulatory
Other
Estimates (SD)
1.00
0.50
0.00
−0.50
−1.00
IL−17A
IL−23
IL−1b
CXCL8
TNF−a
IL−12p70
CXCL10
IFN−g
CCL4
CCL2
IL−4
IL−5
IL−13
CCL13
CCL26
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IL−2
TGF−b1
CRP
Specific Immunoglobulin E (Elevated: Yes/No)

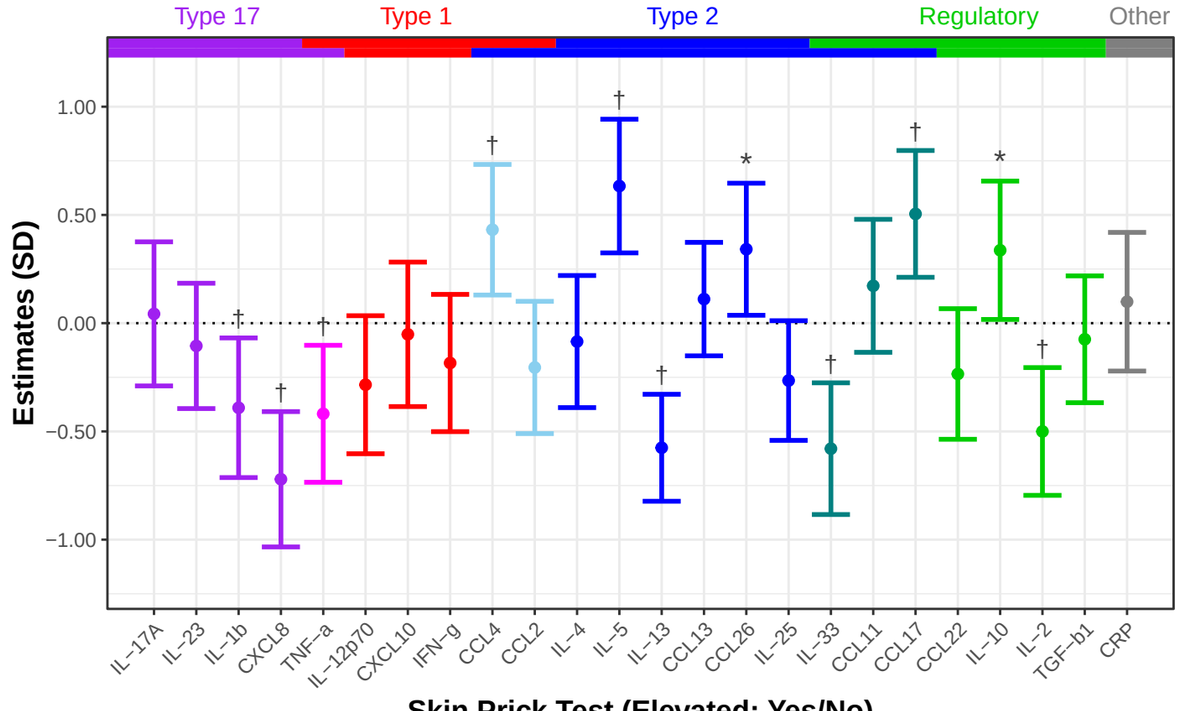
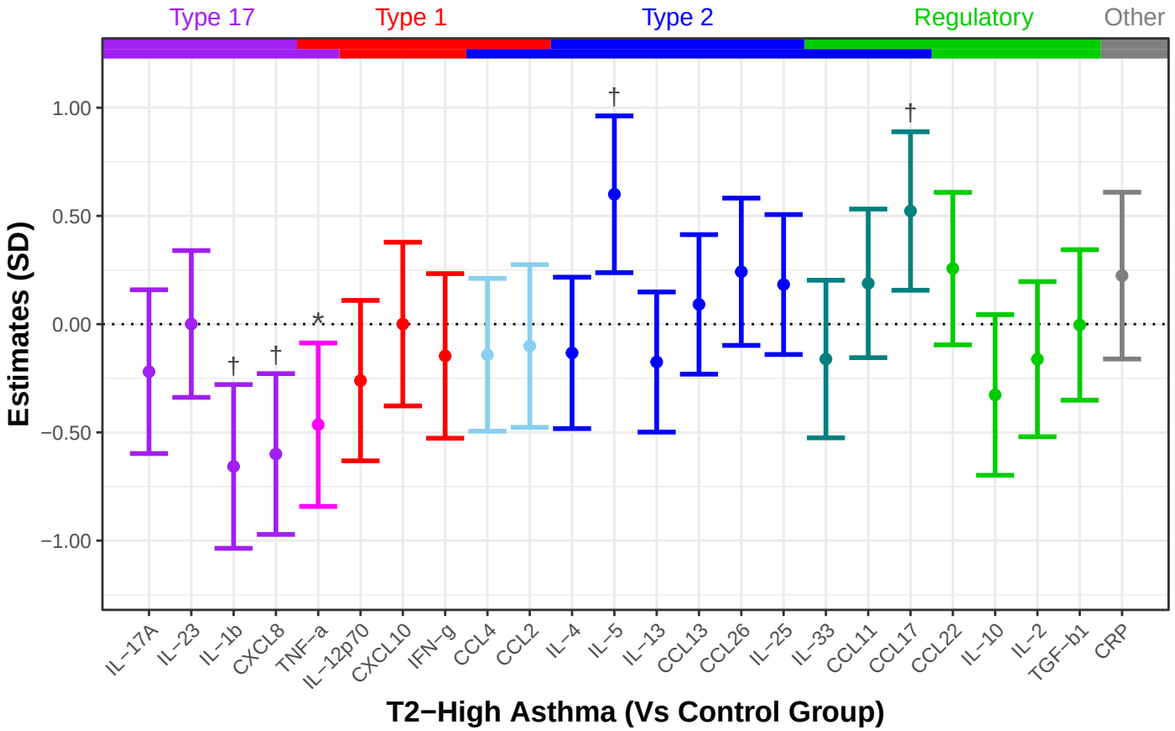
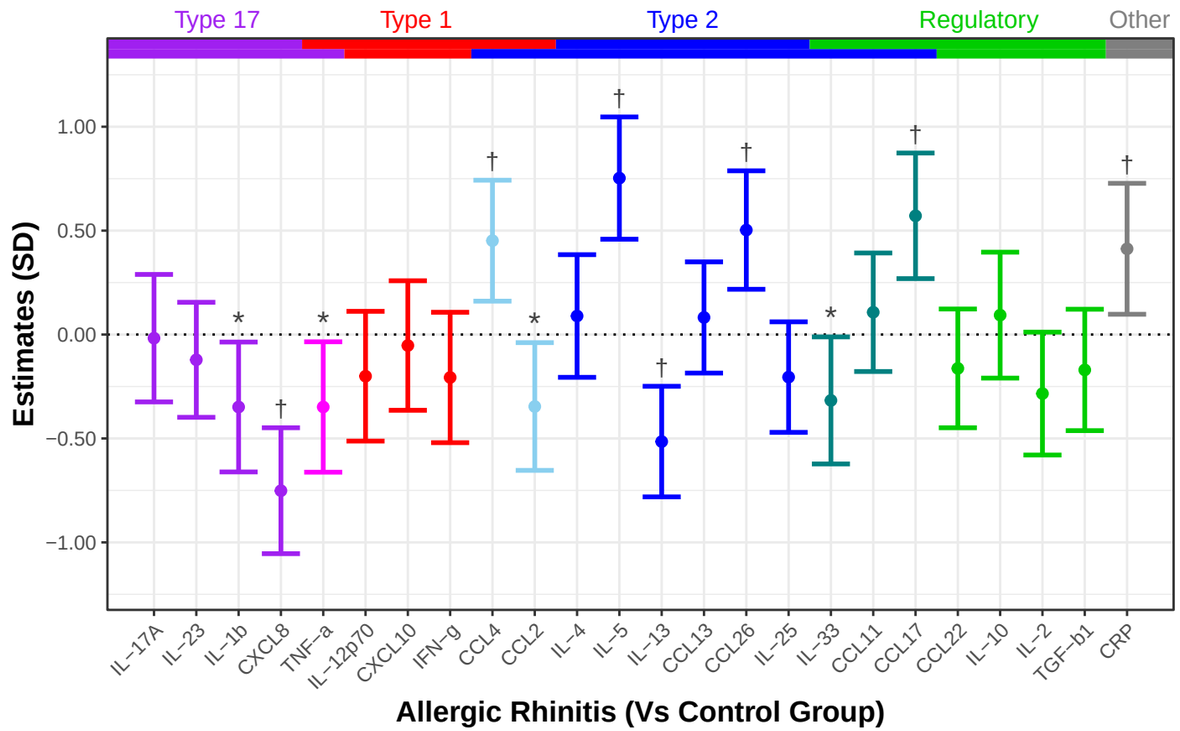

Type 17
Type 1
Type 2
Regulatory
Other
Estimates (SD)
1.00
0.50
0.00
−0.50
−1.00
IL−17A
IL−23
IL−1b
CXCL8
TNF−a
IL−12p70
CXCL10
IFN−g
CCL4
CCL2
IL−4
IL−5
IL−13
CCL13
CCL26
IL−25
IL−33
CCL11
CCL17
CCL22
IL−10
IL−2
TGF−b1
CRP
Skin Prick Test (Elevated: Yes/No)

Figure E6. Sensitivity analysis: CLR-transformed nasal cytokines and type 2 outcomes. *Forest plots depicting the association between centered log-ratio (CLR) transformed airway cytokines and type 2 diseases and biomarkers at age 6 years. Linear regression models were adjusted for asthma, age, sex, sampling season, sampling plate, analysis date, extraction date, airway infection less than one week prior to sampling, and prior use of nasal and inhalation steroids before sampling. P < 0.05 (*), FDR-adjusted p < 0.05 (†). T2-high N=569 (35/534), T2-low N=553 (19/534), AR N=571 (43/528), NAR N=561 (33/528), allergic conjunctivitis N=606 (45/561), atopic dermatitis N=602 (75/527). Per biomarker panels, complete cases: blood eosinophils N=480 (216/264), FeNO N=366 (18/348), specific-IgE N=523 (106/417), SPT N=529 (41/488).*

(See next page)


Type 17
Type 1
Type 2
Regulatory
Other
Estimates (SD)
1.00
0.50
0.00
−0.50
−1.00
IL−17A
IL−23
IL−1b
CXCL8
TNF−a
IL−12p70
CXCL10
IFN−g
CCL4
CCL2
IL−4
IL−5
IL−13
CCL13
CCL26
IL−25
IL−33
CCL11
CCL17
CCL22
IL−10
IL−2
TGF−b1
CRP
T2−High Asthma (Vs Control Group)


Type 17
Type 1
Type 2
Regulatory
Other
Estimates (SD)
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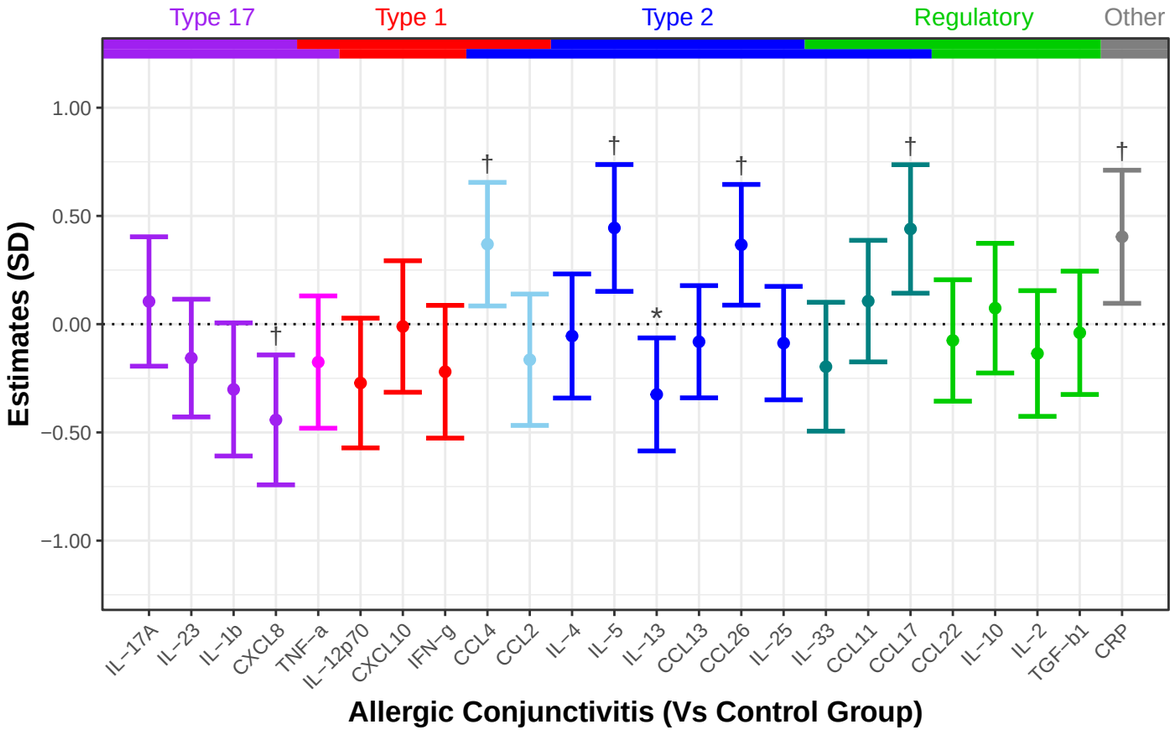
0.50
0.00
−0.50
−1.00
IL−17A
IL−23
IL−1b
CXCL8
TNF−a
IL−12p70
CXCL10
IFN−g
CCL4
CCL2
IL−4
IL−5
IL−13
CCL13
CCL26
IL−25
IL−33
CCL11
CCL17
CCL22
IL−10
IL−2
TGF−b1
CRP
Allergic Rhinitis (Vs Control Group)


Type 17
Type 1
Type 2
Regulatory
Other
Estimates (SD)
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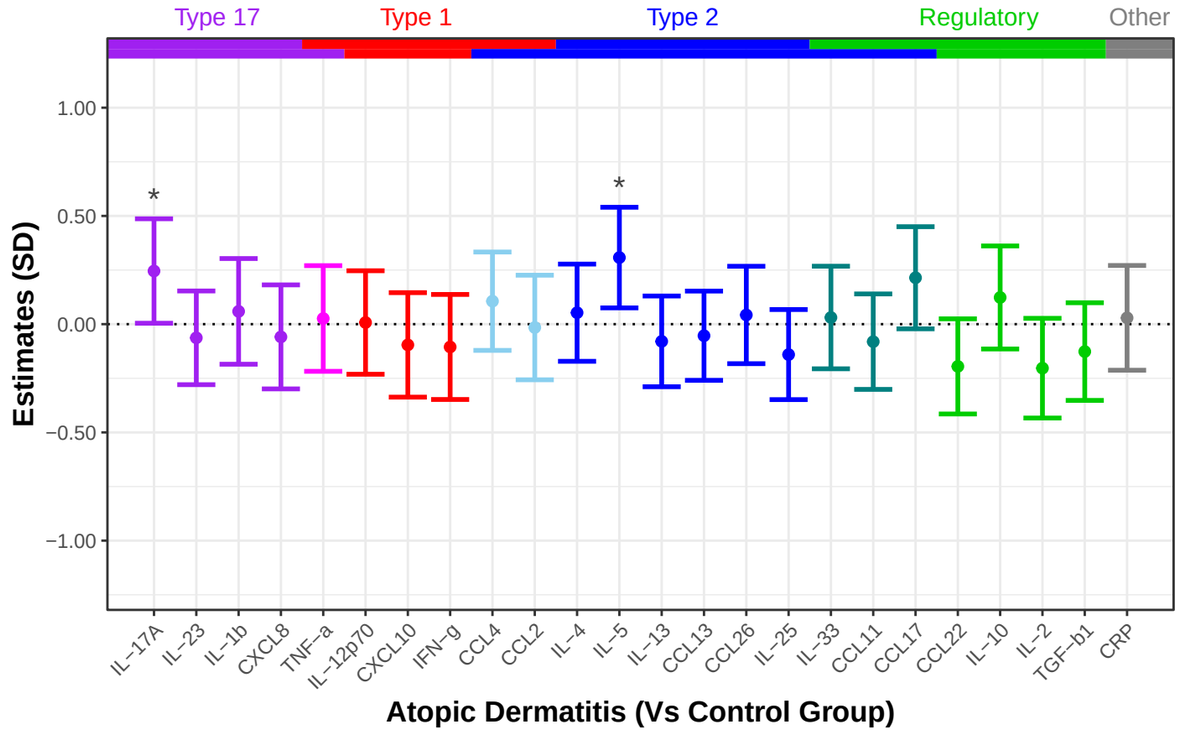
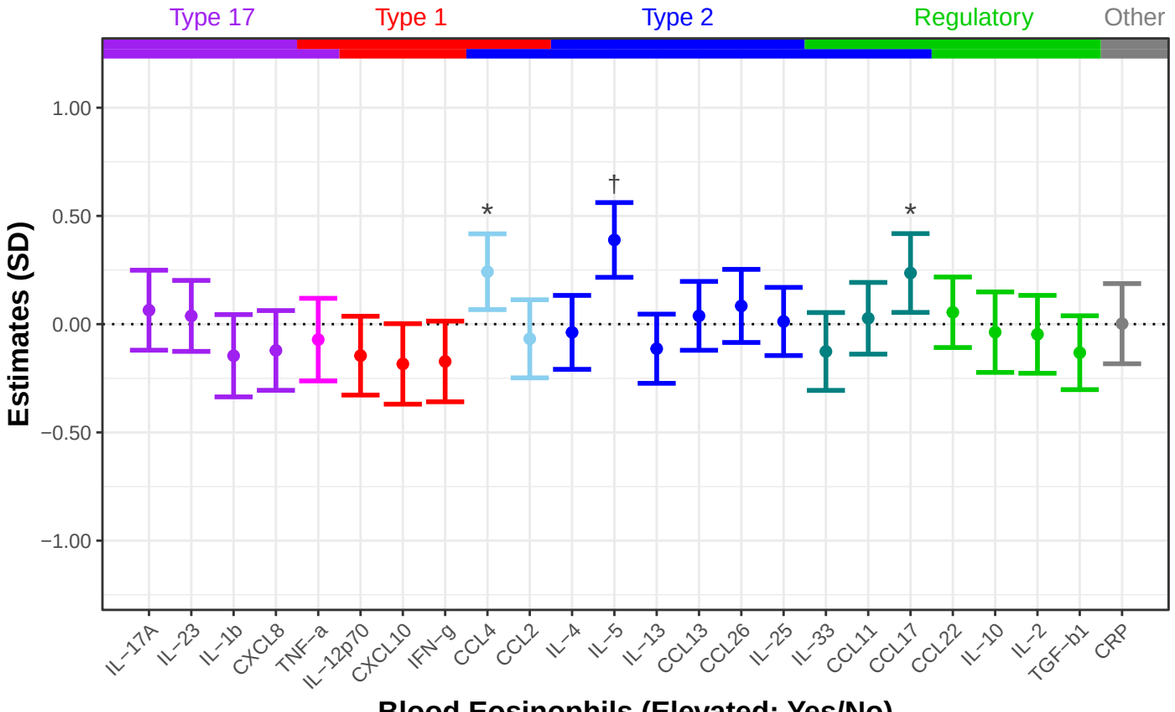
0.00
−0.50
−1.00
IL−17A
IL−23
IL−1b
CXCL8
TNF−a
IL−12p70
CXCL10
IFN−g
CCL4
CCL2
IL−4
IL−5
IL−13
CCL13
CCL26
IL−25
IL−33
CCL11
CCL17
CCL22
IL−10
IL−2
TGF−b1
CRP
Allergic Conjunctivitis (Vs Control Group)


Type 17
Type 1
Type 2
Regulatory
Other
Estimates (SD)
1.00
0.50
0.00
−0.50
−1.00
IL−17A
IL−23
IL−1b
CXCL8
TNF−a
IL−12p70
CXCL10
IFN−g
CCL4
CCL2
IL−4
IL−5
IL−13
CCL13
CCL26
IL−25
IL−33
CCL11
CCL17
CCL22
IL−10
IL−2
TGF−b1
CRP
Atopic Dermatitis (Vs Control Group)


Type 17
Type 1
Type 2
Regulatory
Other
Estimates (SD)
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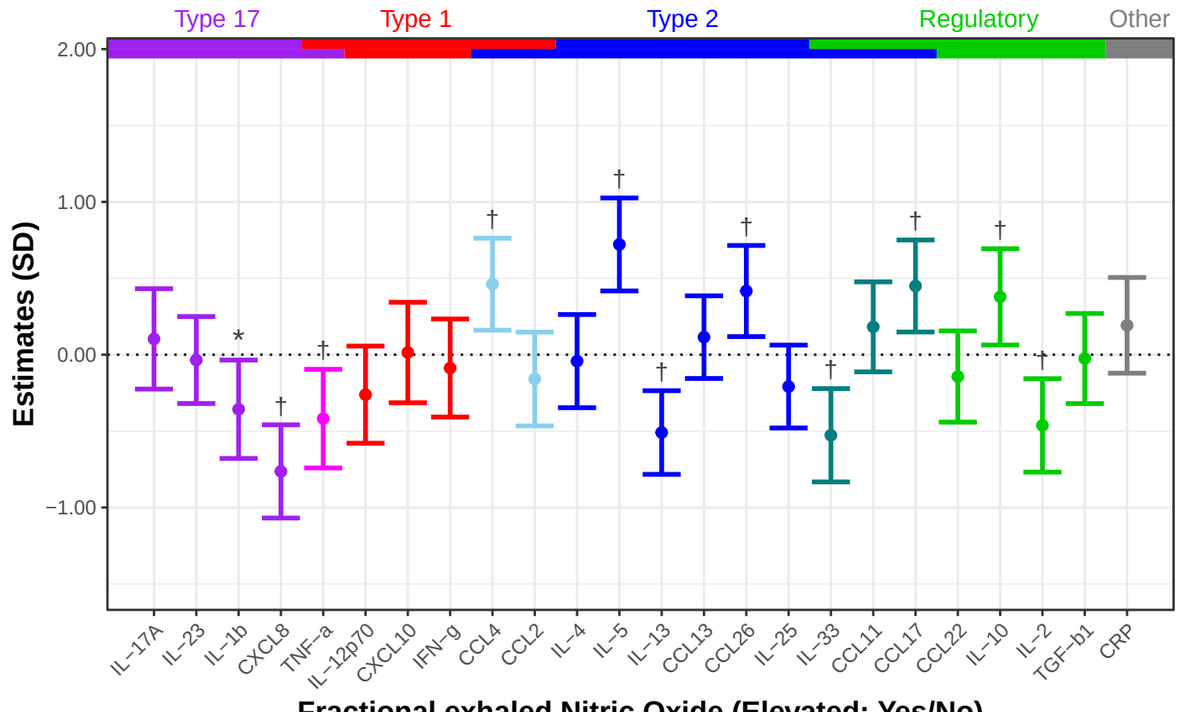
0.00
−0.50
−1.00
IL−17A
IL−23
IL−1b
CXCL8
TNF−a
IL−12p70
CXCL10
IFN−g
CCL4
CCL2
IL−4
IL−5
IL−13
CCL13
CCL26
IL−25
IL−33
CCL11
CCL17
CCL22
IL−10
IL−2
TGF−b1
CRP
Blood Eosinophils (Elevated: Yes/No)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Type 1
Type 2
Regulatory
Other
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0.00
−1.00
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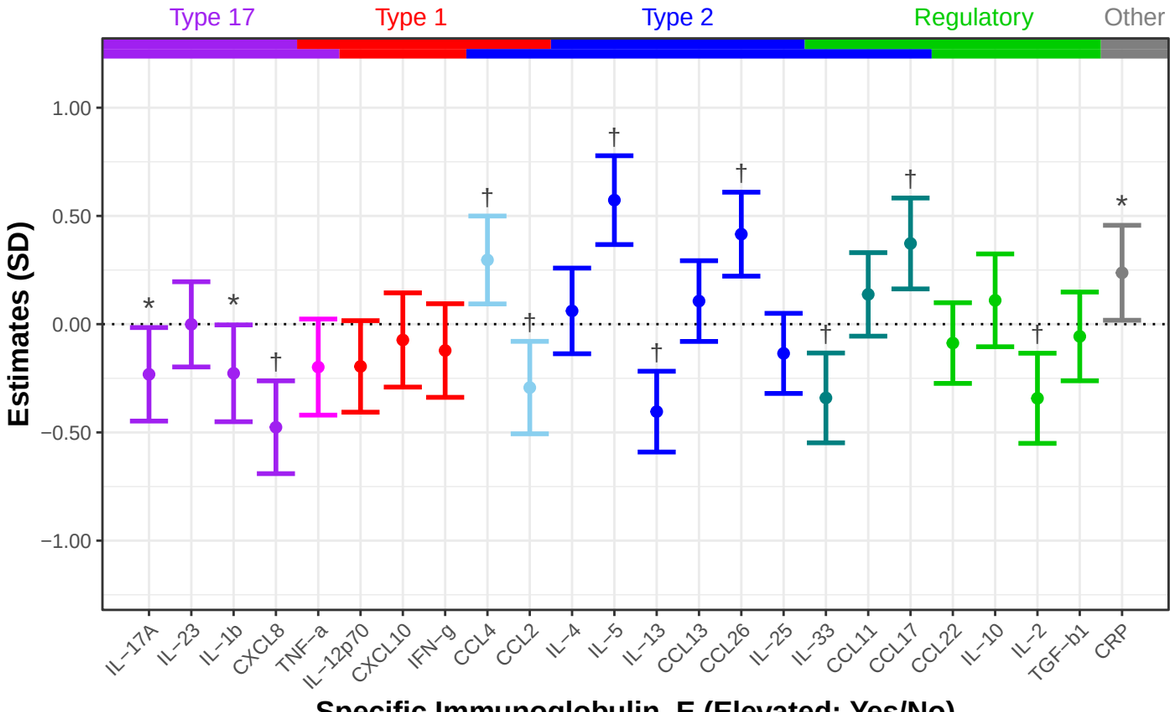
IL−23
IL−1b
CXCL8
TNF−a
IL−12p70
CXCL10
IFN−g
CCL4
CCL2
IL−4
IL−5
IL−13
CCL13
CCL26
IL−25
IL−33
CCL11
CCL17
CCL22
IL−10
IL−2
TGF−b1
CRP
Fractional exhaled Nitric Oxide (Elevated: Yes/No)


Type 17
Type 1
Type 2
Regulatory
Other
Estimates (SD)
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0.00
−0.50
−1.00
IL−17A
IL−23
IL−1b
CXCL8
TNF−a
IL−12p70
CXCL10
IFN−g
CCL4
CCL2
IL−4
IL−5
IL−13
CCL13
CCL26
IL−25
IL−33
CCL11
CCL17
CCL22
IL−10
IL−2
TGF−b1
CRP
Specific Immunoglobulin E (Elevated: Yes/No)

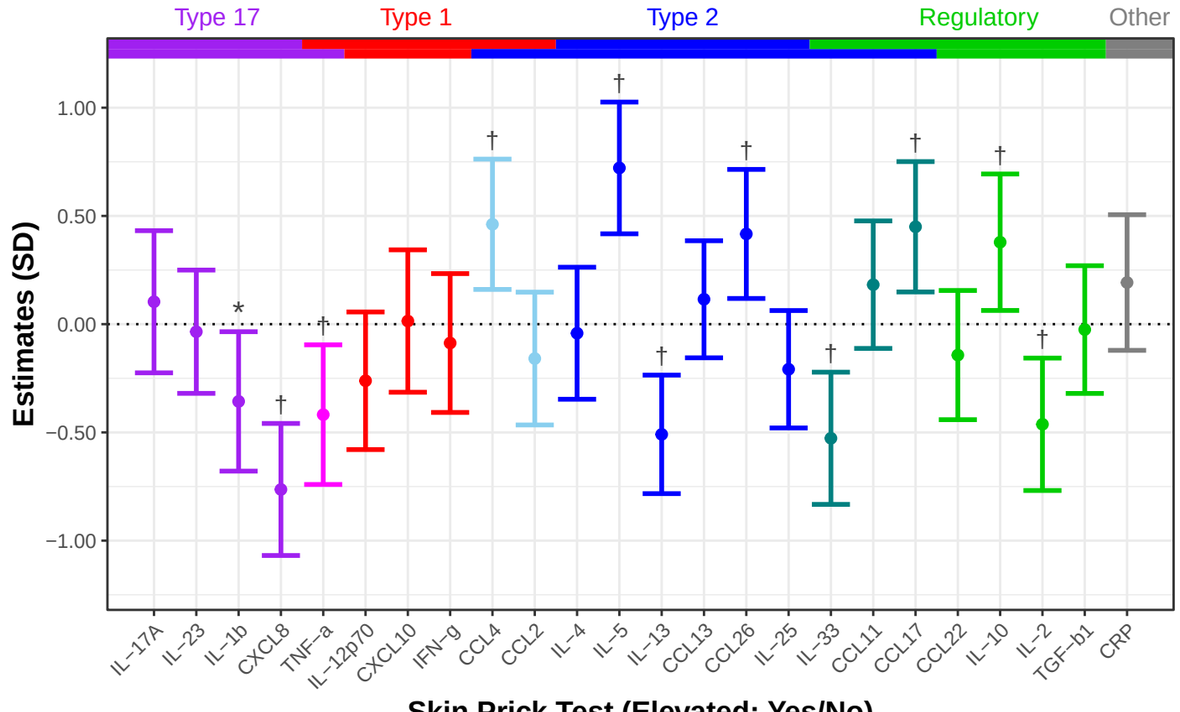
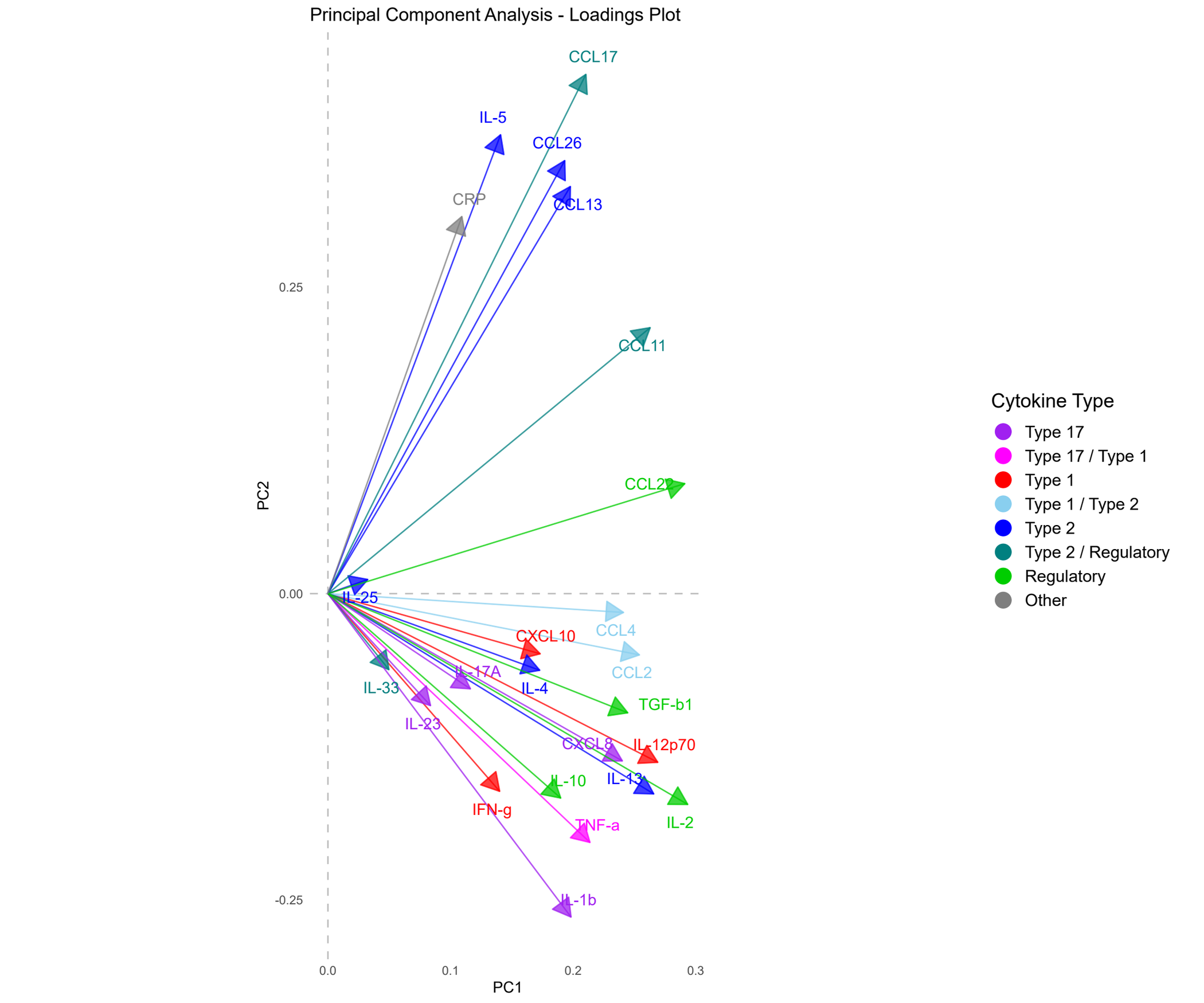

Type 17
Type 1
Type 2
Regulatory
Other
Estimates (SD)
1.00
0.50
0.00
−0.50
−1.00
IL−17A
IL−23
IL−1b
CXCL8
TNF−a
IL−12p70
CXCL10
IFN−g
CCL4
CCL2
IL−4
IL−5
IL−13
CCL13
CCL26
IL−25
IL−33
CCL11
CCL17
CCL22
IL−10
IL−2
TGF−b1
CRP
Skin Prick Test (Elevated: Yes/No)

Figure E7. Sensitivity analysis: Principal component loadings of raw nasal cytokines. *Loadings plot of raw nasal epithelial lining cytokine levels from principal component analysis (PCA) loadings plot of raw nasal epithelial lining cytokine levels. The PC2 axis demonstrates the same directionality of Type 2 inflammation-associated cytokines (IL-5, CCL17, CCL13, CCL26) observed in the transformed data, while also showing an inverse relationship to cytokines such as IL-13, IL-1β, CXCL8, TNF-α, and IL-2. This confirms the inherent signal of cytokine associated with Type 2 inflammation in the raw data. N=612.*

(See next page)


Principal Component Analysis - Loadings Plot
CCL17
IL-5
CCL26
CRP
CCL13
CCL11
CCL22
IL-25
CCL4
CXCL10
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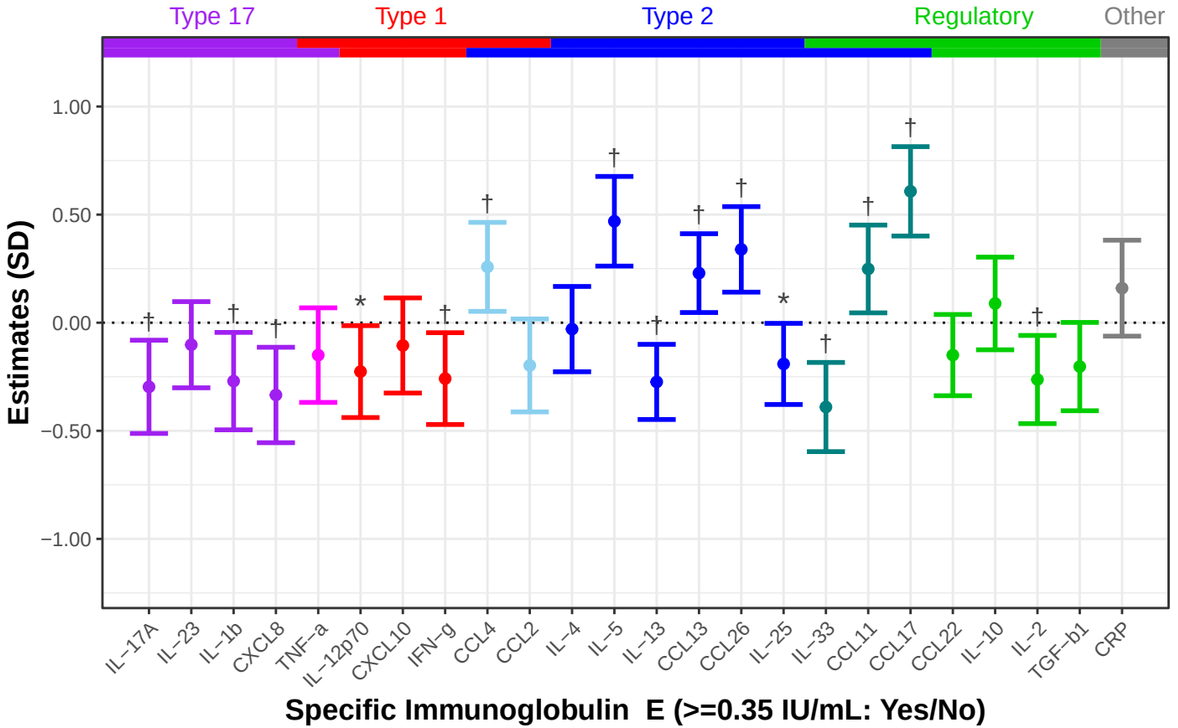
IL-33
IL-4
TGF-b1
IL-23
CXCL8
IL-12p70
IL-10
IL-13
IFN-g
TNF-a
IL-2
IL-1b
0.25
0.00
-0.25
PC2
0.0
0.1
0.2
0.3
PC1
Cytokine Type
Type 17
Type 17 / Type 1
Type 1
Type 1 / Type 2
Type 2
Type 2 / Regulatory
Regulatory
Other

Figure E8. Sensitivity analysis: Higher specific-IgE cutoff (≥0.70 IU/mL). *Forest plots show cytokine associations with sensitization status at age 6 years using both standard (≥0.35 IU/mL) and an elevated cutoff for specific-IgE (≥0.70 IU/mL). Linear regression models adjusted for age, sex, sampling season, sampling plate, analysis date, extraction date, and steroid use. Cytokine signals were consistent and slightly stronger than with the conventional cutoff. P < 0.05 (*), FDR-adjusted p < 0.05 (†). Per biomarker panel, complete cases: specific-IgE ≥0.35 IU/mL N=523 (106/417); specific-IgE ≥0.70 IU/mL N=523 (92/431).*

(See next page)


Type 17
Type 1
Type 2
Regulatory
Other
Estimates (SD)
1.00
0.50
0.00
−0.50
−1.00
IL−17A
IL−23
IL−1b
CXCL8
TNF−a
IL−12p70
CXCL10
IFN−g
CCL4
CCL2
IL−4
IL−5
IL−13
CCL13
CCL26
IL−25
IL−33
CCL11
CCL17
CCL22
IL−10
IL−2
TGF−b1
CRP
Specific Immunoglobulin E (>=0.35 IU/mL: Yes/No)

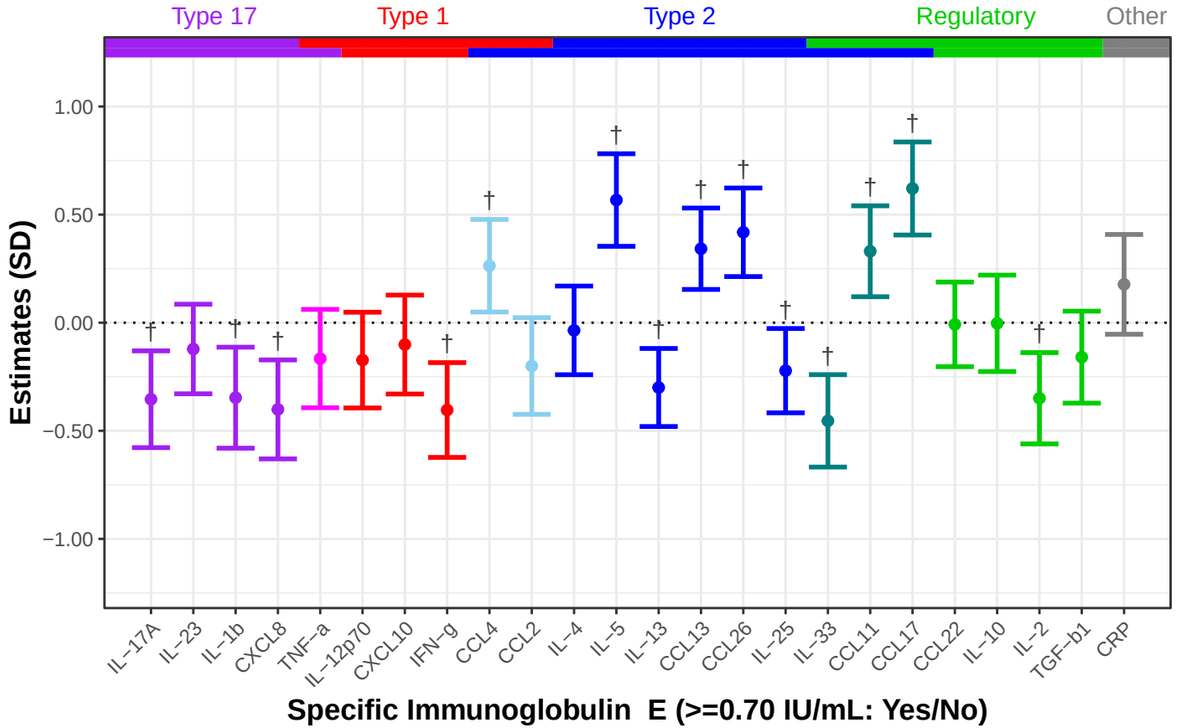
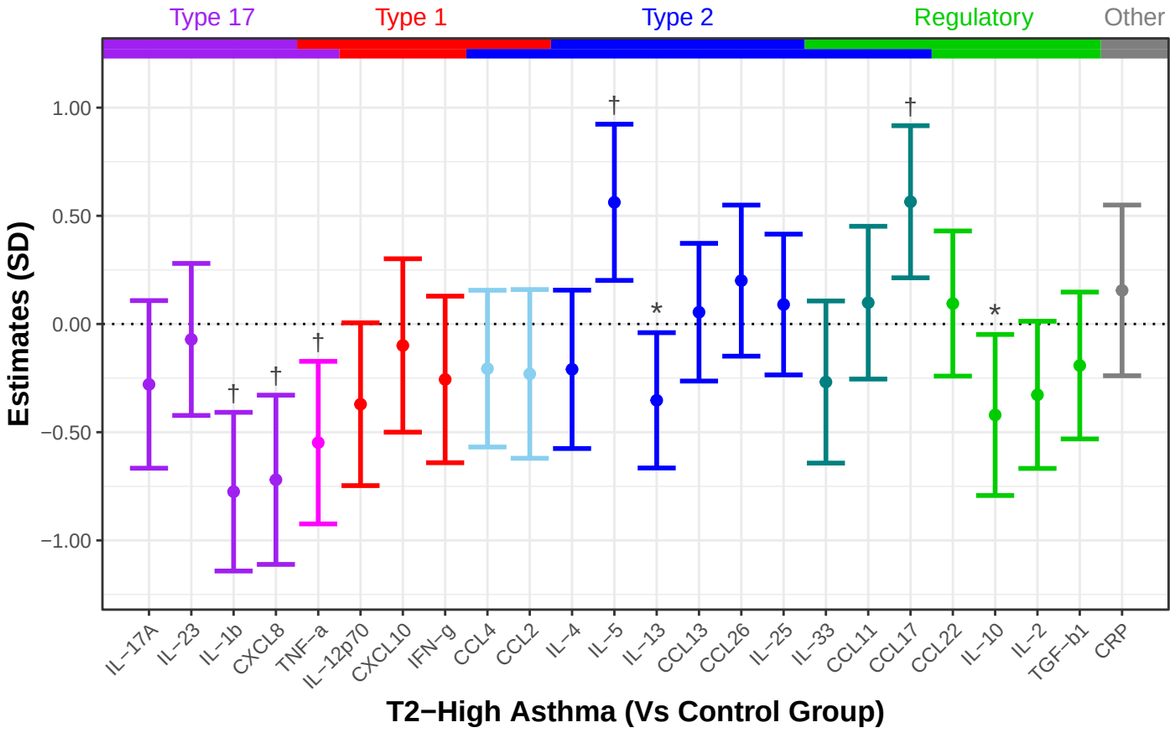
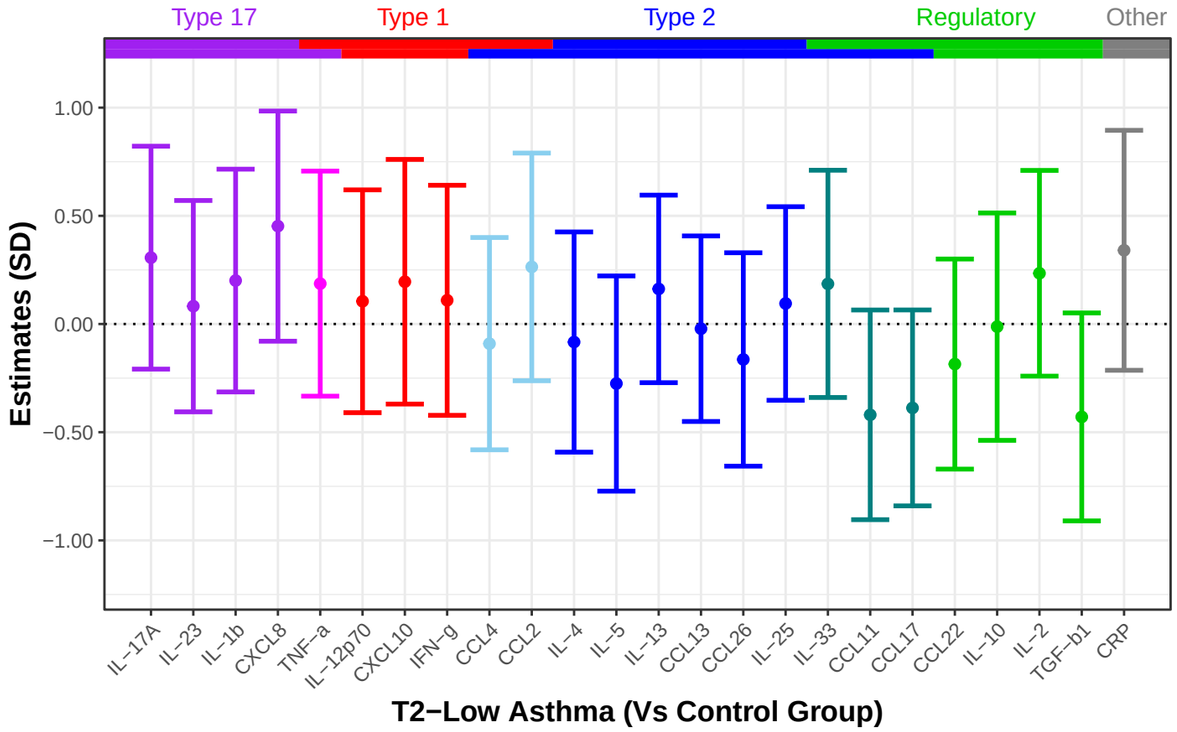

Type 17
Type 1
Type 2
Regulatory
Other
Estimates (SD)
1.00
0.50
0.00
−0.50
−1.00
IL−17A
IL−23
IL−1b
CXCL8
TNF−a
IL−12p70
CXCL10
IFN−g
CCL4
CCL2
IL−4
IL−5
IL−13
CCL13
CCL26
IL−25
IL−33
CCL11
CCL17
CCL22
IL−10
IL−2
TGF−b1
CRP
Specific Immunoglobulin E (>=0.70 IU/mL: Yes/No)

Figure E9. Sensitivity analysis: Disease groups vs. controls without other type 2 diseases. *Cytokine associations with type 2 diseases are shown using a restricted control group free from any other type 2 disease. Forest plots display covariate-adjusted regression results with preserved directionality of key cytokine signals. P < 0.05 (*), FDR-adjusted p < 0.05 (†). Per disease panel, complete cases: T2-high N=470 (35/435), T2-low N=454 (19/435), AR N=475 (43/432), NAR N=465 (33/432), allergic conjunctivitis N=477 (45/432), atopic dermatitis N=505 (75/430).*

(See next page)


Type 17
Type 1
Type 2
Regulatory
Other
Estimates (SD)
1.00
0.50
0.00
−0.50
−1.00
IL−17A
IL−23
IL−1b
CXCL8
TNF−a
IL−12p70
CXCL10
IFN−g
CCL4
CCL2
IL−4
IL−5
IL−13
CCL13
CCL26
IL−25
IL−33
CCL11
CCL17
CCL22
IL−10
IL−2
TGF−b1
CRP
T2−High Asthma (Vs Control Group)


Type 17
Type 1
Type 2
Regulatory
Other
Estimates (SD)
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0.00
−0.50
−1.00
IL−17A
IL−23
IL−1b
CXCL8
TNF−a
IL−12p70
CXCL10
IFN−g
CCL4
CCL2
IL−4
IL−5
IL−13
CCL13
CCL26
IL−25
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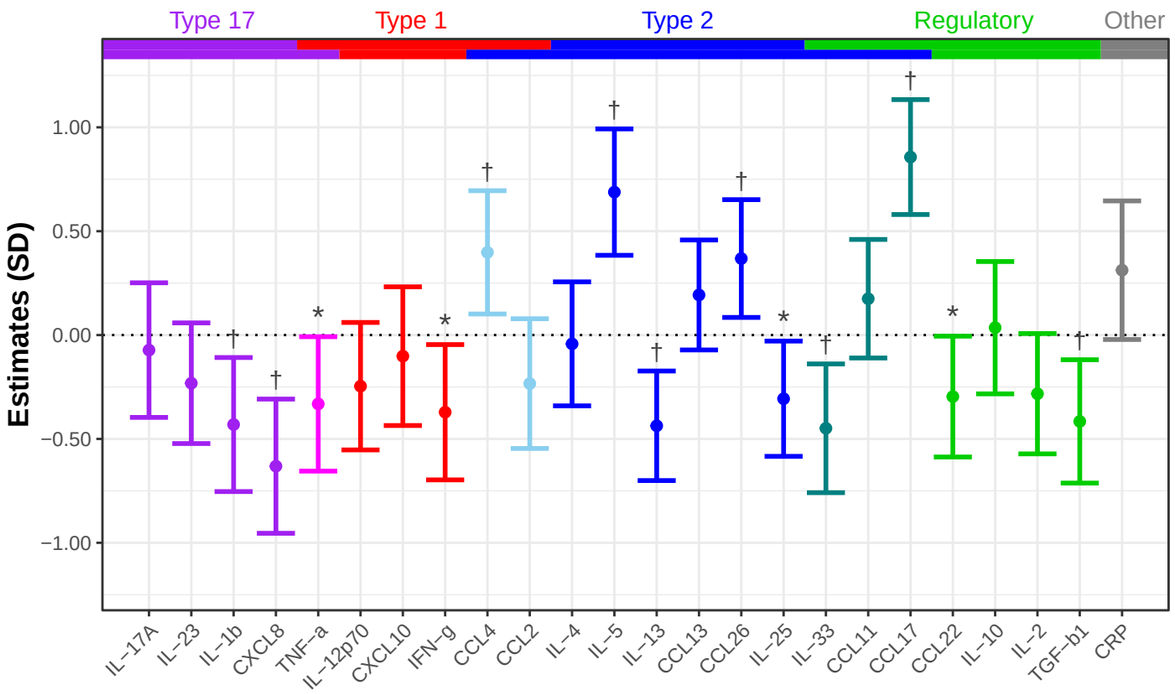
CCL11
CCL17
CCL22
IL−10
IL−2
TGF−b1
CRP
T2−Low Asthma (Vs Control Group)


Type 17
Type 1
Type 2
Regulatory
Other
Estimates (SD)
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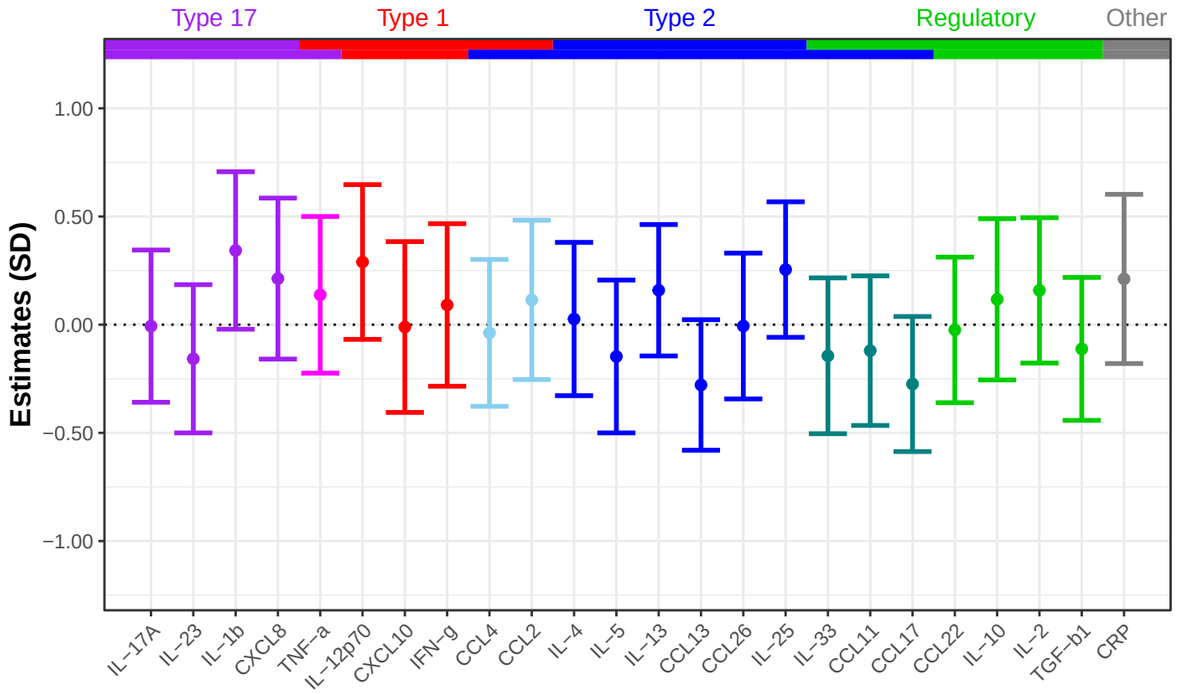
0.50
0.00
−0.50
−1.00
IL−17A
IL−23
IL−1b
CXCL8
TNF−a
IL−12p70
CXCL10
IFN−g
CCL4
CCL2
IL−4
IL−5
IL−13
CCL13
CCL26
IL−25
IL−33
CCL11
CCL17
CCL22
IL−10
IL−2
TGF−b1
CRP
Allergic Rhinitis (Vs Control Group)


Type 17
Type 1
Type 2
Regulatory
Other
Estimates (SD)
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0.00
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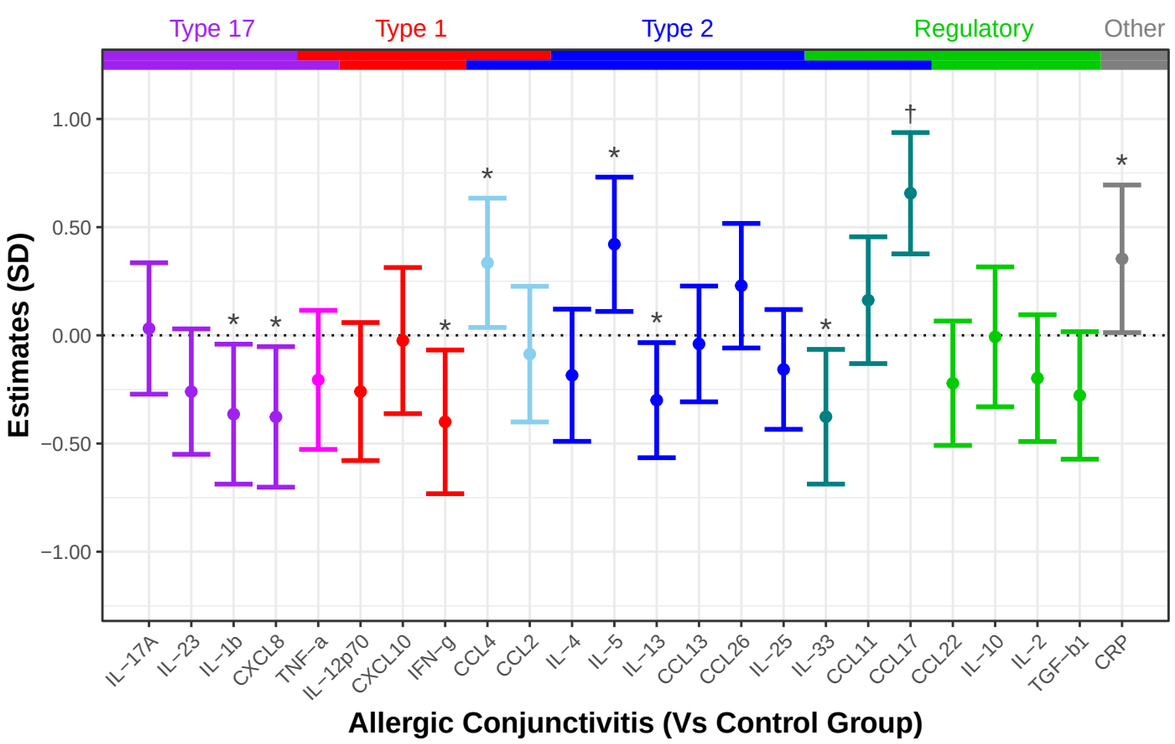
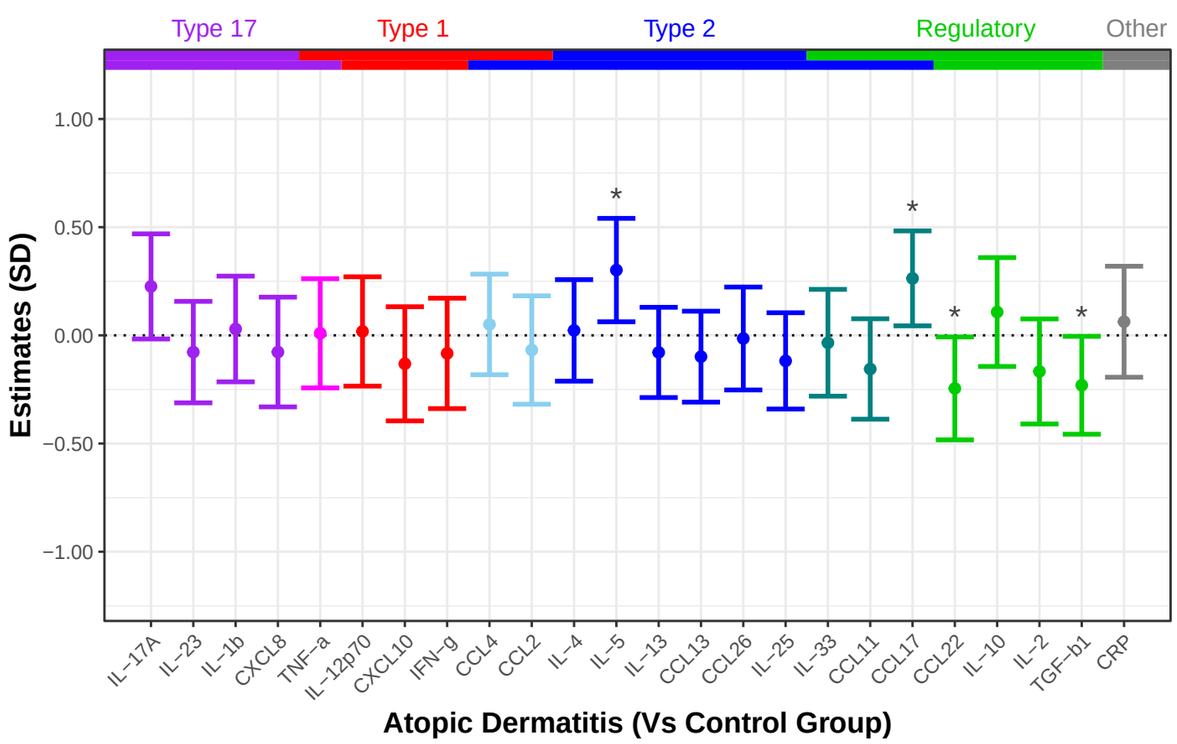
−1.00
IL−17A
IL−23
IL−1b
CXCL8
TNF−a
IL−12p70
CXCL10
IFN−g
CCL4
CCL2
IL−4
IL−5
IL−13
CCL13
CCL26
IL−25
IL−33
CCL11
CCL17
CCL22
IL−10
IL−2
TGF−b1
CRP
Non−Allergic Rhinitis (Vs Control Group)


Type 17
Type 1
Type 2
Regulatory
Other
Estimates (SD)
1.00
0.50
0.00
−0.50
−1.00
IL−17A
IL−23
IL−1b
CXCL8
TNF−a
IL−12p70
CXCL10
IFN−g
CCL4
CCL2
IL−4
IL−5
IL−13
CCL13
CCL26
IL−25
IL−33
CCL11
CCL17
CCL22
IL−10
IL−2
TGF−b1
CRP
Allergic Conjunctivitis (Vs Control Group)


Type 17
Type 1
Type 2
Regulatory
Other
Estimates (SD)
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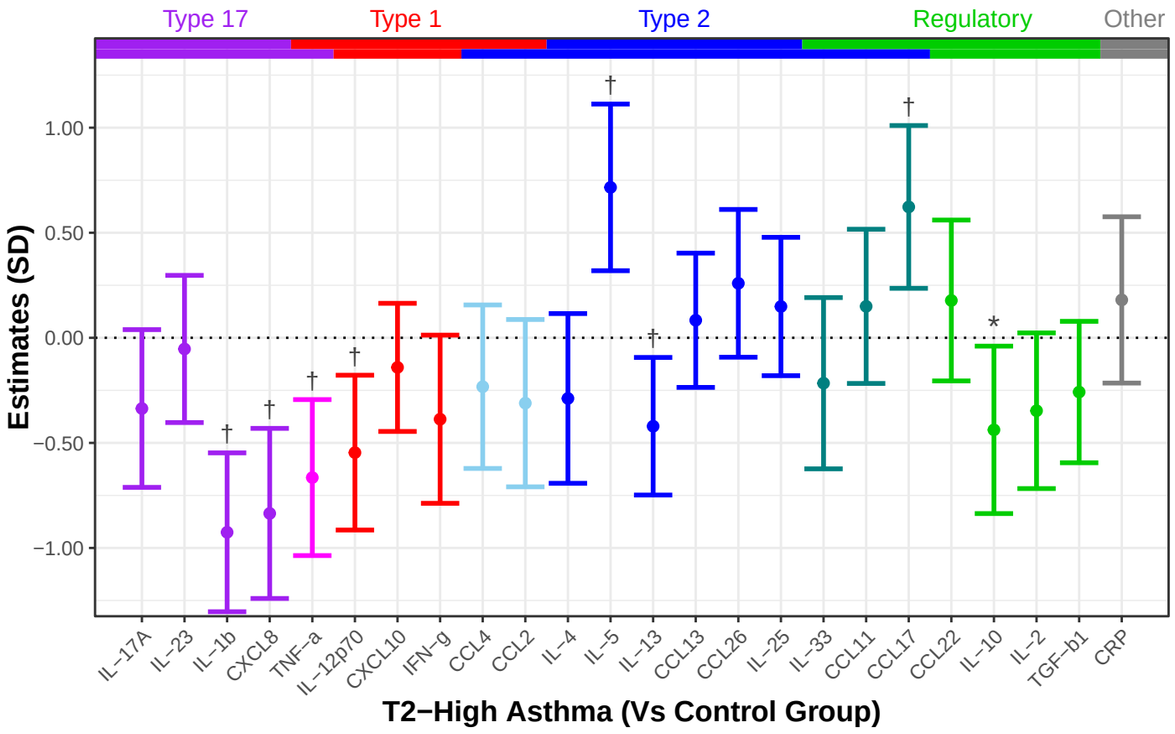
0.00
−0.50
−1.00
IL−17A
IL−23
IL−1b
CXCL8
TNF−a
IL−12p70
CXCL10
IFN−g
CCL4
CCL2
IL−4
IL−5
IL−13
CCL13
CCL26
IL−25
IL−33
CCL11
CCL17
CCL22
IL−10
IL−2
TGF−b1
CRP
Atopic Dermatitis (Vs Control Group)

Figure E10. Sensitivity analysis: Disease groups vs. controls without type 2 biomarker elevations. *Forest plots illustrate nasal cytokine levels among children with type 2 diseases compared to children without elevated blood eosinophils, FeNO, specific-IgE, or skin prick test. Missing biomarker values were coded as non-elevated for this analysis. Linear models adjusted for relevant covariates. Cytokine signal remained, though modestly attenuated. P < 0.05 (*), FDR-adjusted p < 0.05 (†). Per disease panel, complete cases: T2-high N=339 (35/304), T2-low N=323 (19/304), AR N=362 (43/319), NAR N=352 (33/319), allergic conjunctivitis N=377 (45/332), atopic dermatitis N=383 (75/308).*

(See next page)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Type 2
Regulatory
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−1.00
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IL−23
IL−1b
CXCL8
TNF−a
IL−12p70
CXCL10
IFN−g
CCL4
CCL2
IL−4
IL−5
IL−13
CCL13
CCL26
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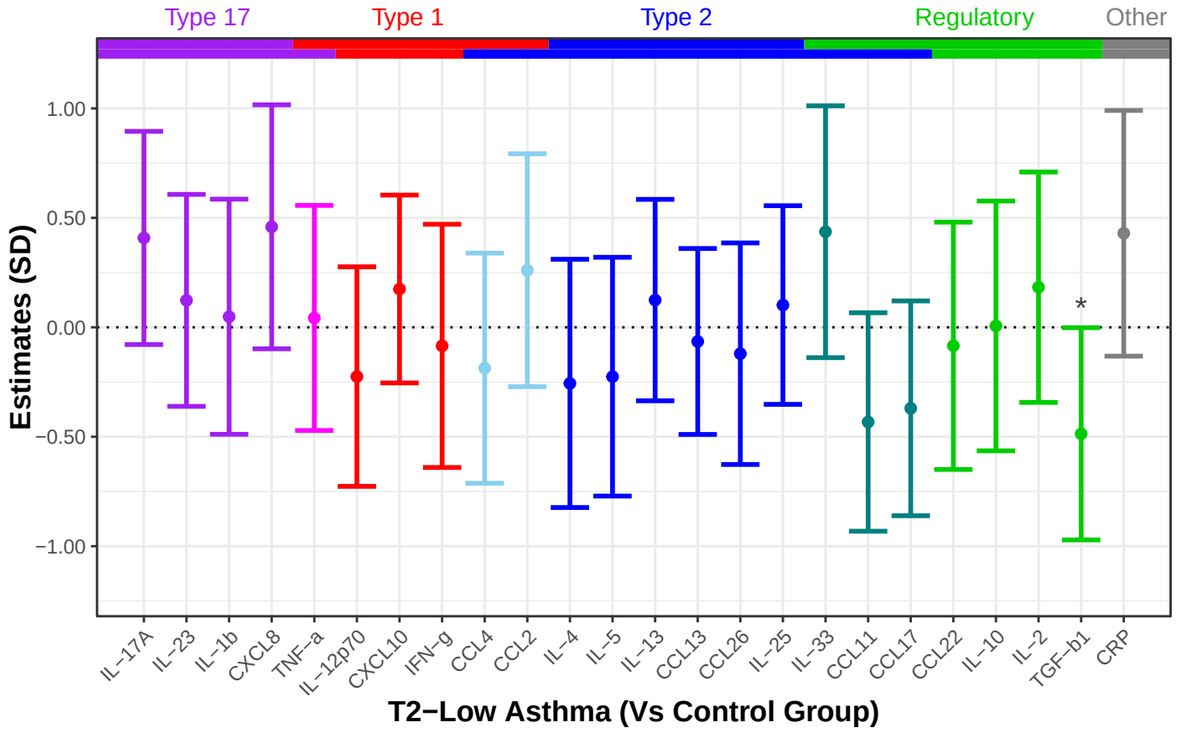
CCL11
CCL17
CCL22
IL−10
IL−2
TGF−b1
CRP
T2−High Asthma (Vs Control Group)


Type 17
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Type 2
Regulatory
Other
Estimates (SD)
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0.00
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−1.00
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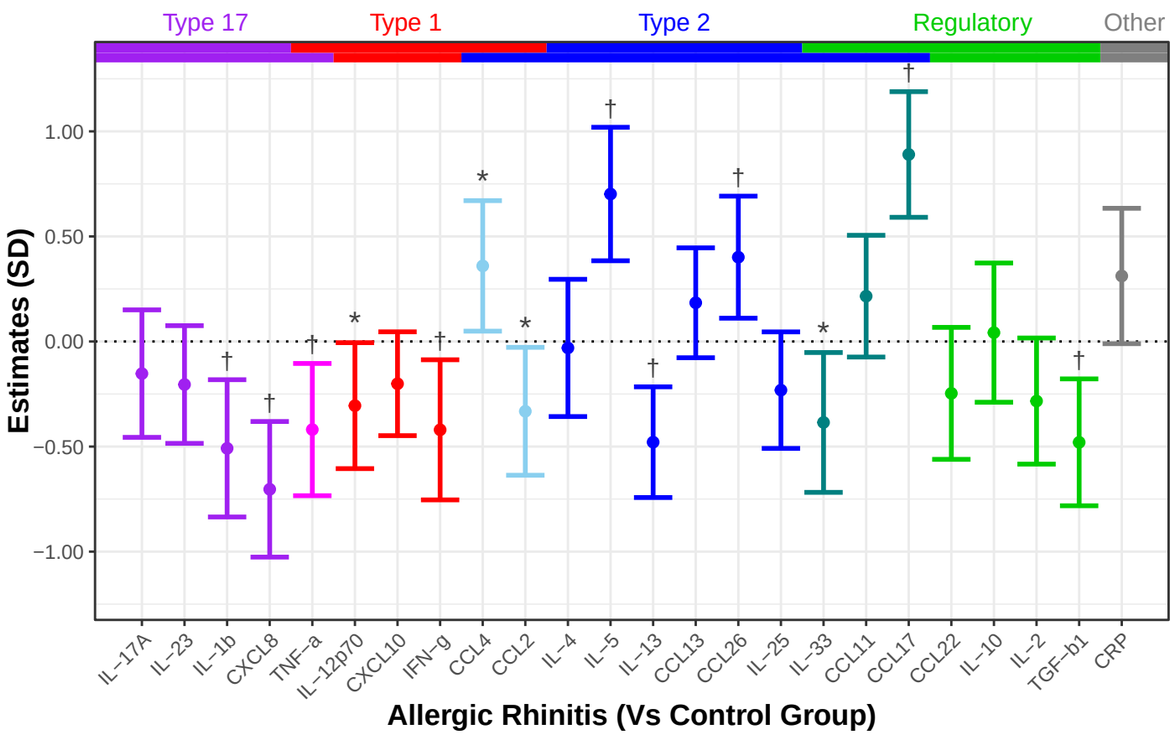
IL−23
IL−1b
CXCL8
TNF−a
IL−12p70
CXCL10
IFN−g
CCL4
CCL2
IL−4
IL−5
IL−13
CCL13
CCL26
IL−25
IL−33
CCL11
CCL17
CCL22
IL−10
IL−2
TGF−b1
CRP
T2−Low Asthma (Vs Control Group)


Type 17
Type 1
Type 2
Regulatory
Other
Estimates (SD)
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0.50
0.00
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−1.00
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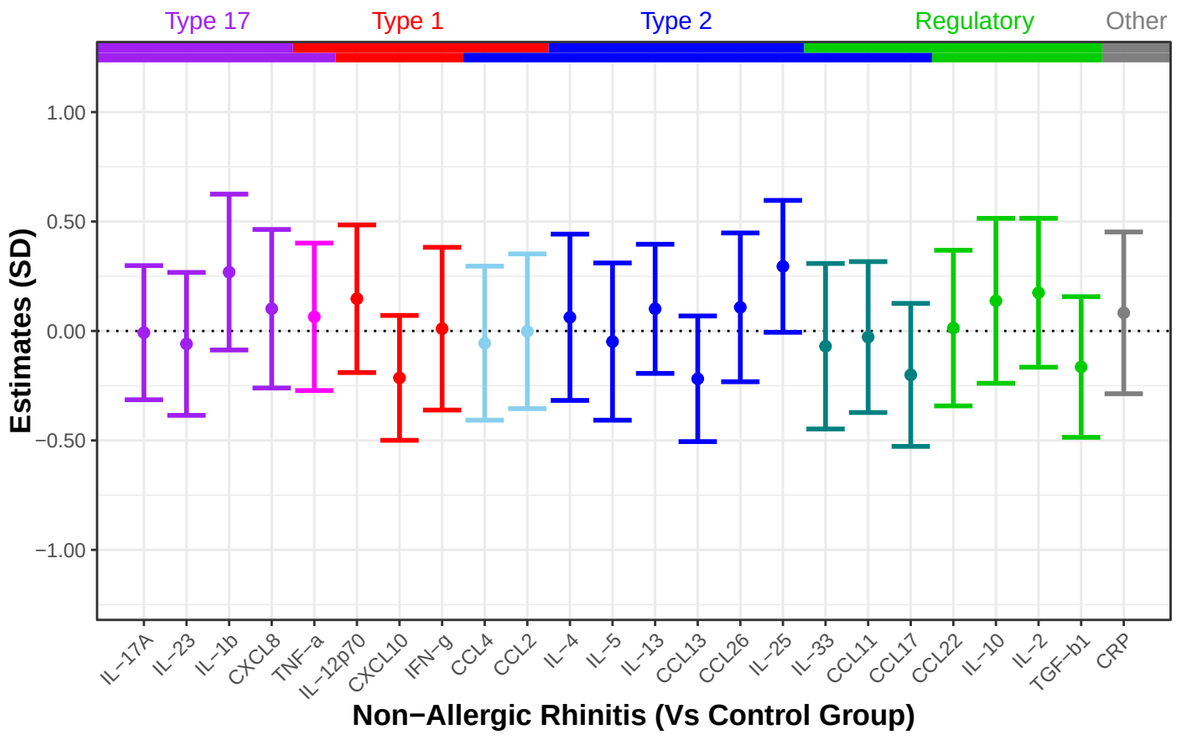
IL−23
IL−1b
CXCL8
TNF−a
IL−12p70
CXCL10
IFN−g
CCL4
CCL2
IL−4
IL−5
IL−13
CCL13
CCL26
IL−25
IL−33
CCL11
CCL17
CCL22
IL−10
IL−2
TGF−b1
CRP
Allergic Rhinitis (Vs Control Group)


Type 17
Type 1
Type 2
Regulatory
Other
Estimates (SD)
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−1.00
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IL−23
IL−1b
CXCL8
TNF−a
IL−12p70
CXCL10
IFN−g
CCL4
CCL2
IL−4
IL−5
IL−13
CCL13
CCL26
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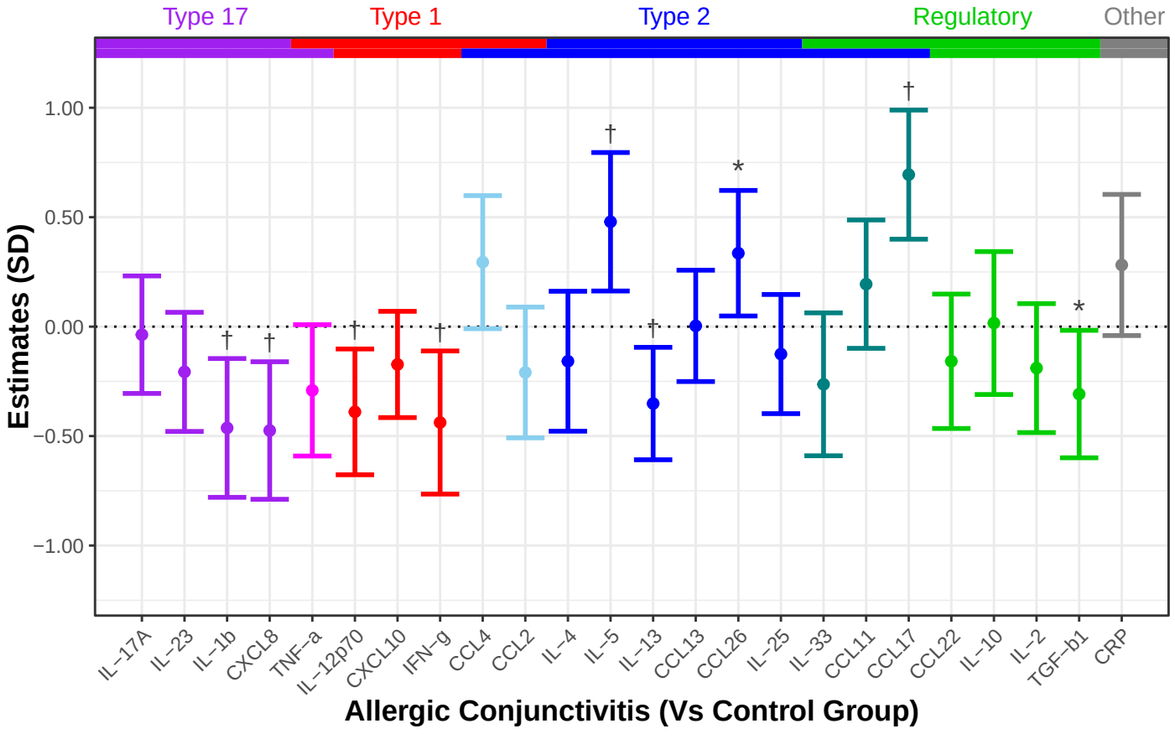
CCL11
CCL17
CCL22
IL−10
IL−2
TGF−b1
CRP
Non−Allergic Rhinitis (Vs Control Group)


Type 17
Type 1
Type 2
Regulatory
Other
Estimates (SD)
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−1.00
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IL−23
IL−1b
CXCL8
TNF−a
IL−12p70
CXCL10
IFN−g
CCL4
CCL2
IL−4
IL−5
IL−13
CCL13
CCL26
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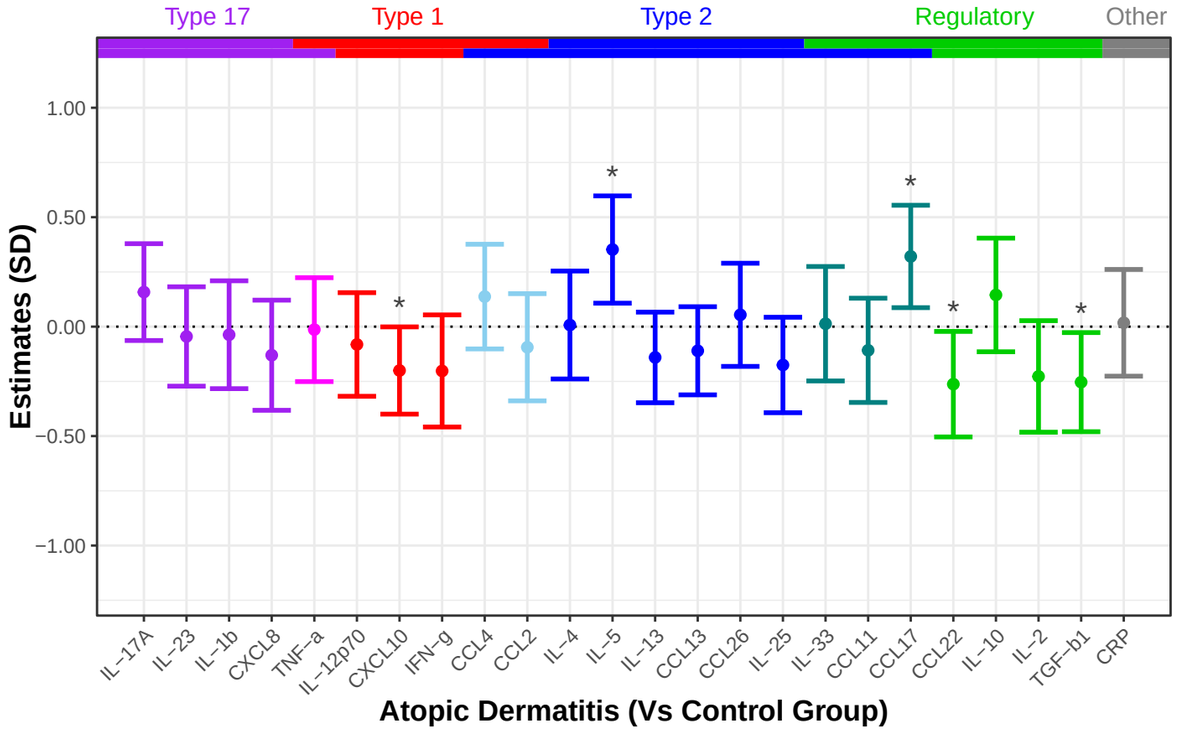
CCL11
CCL17
CCL22
IL−10
IL−2
TGF−b1
CRP
Allergic Conjunctivitis (Vs Control Group)


Type 17
Type 1
Type 2
Regulatory
Other
Estimates (SD)
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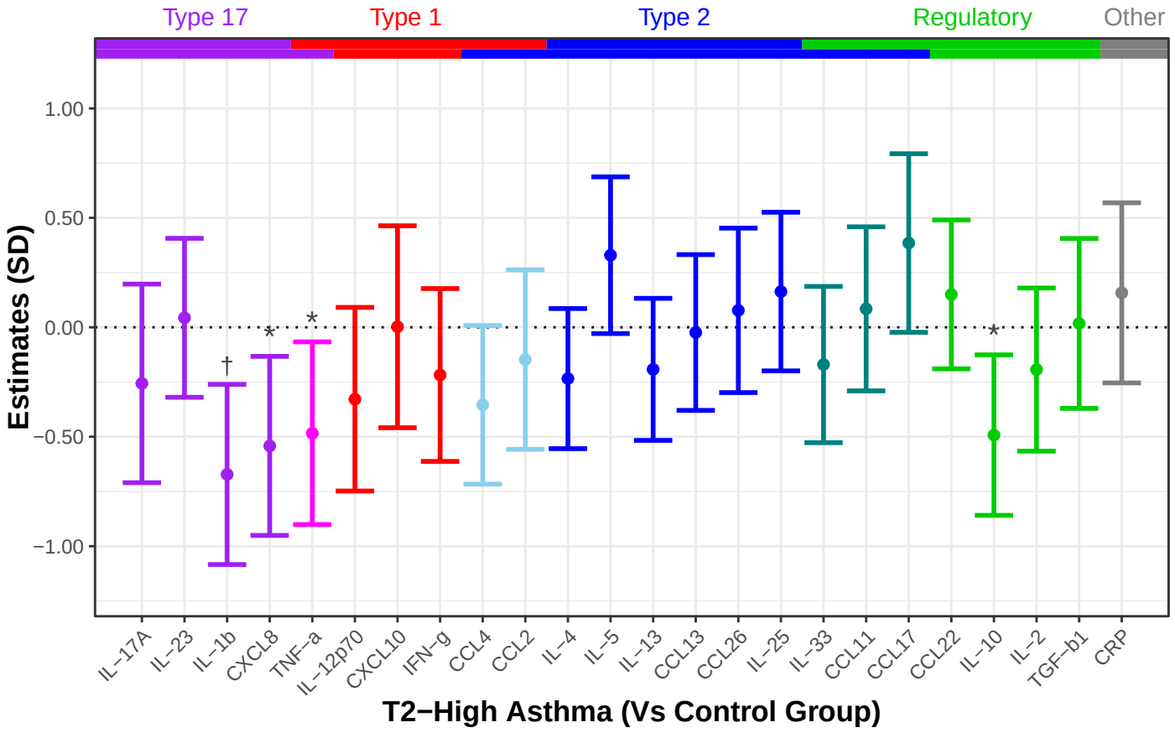
IL−23
IL−1b
CXCL8
TNF−a
IL−12p70
CXCL10
IFN−g
CCL4
CCL2
IL−4
IL−5
IL−13
CCL13
CCL26
IL−25
IL−33
CCL11
CCL17
CCL22
IL−10
IL−2
TGF−b1
CRP
Atopic Dermatitis (Vs Control Group)

Figure E11. Sensitivity analysis: Disease groups vs. biomarker-positive, asymptomatic children. *Forest plots compare cytokine levels in children with type 2 diseases to biomarker-positive but asymptomatic children. Covariate-adjusted linear models show maintained cytokine trends, suggesting disease-specific signal beyond biomarker elevation alone. P < 0.05 (*), FDR-adjusted p < 0.05 (†). Per disease panel, complete cases: T2-high N=265 (35/230), T2-low N=249 (19/230), AR N=252 (43/209), NAR N=242 (33/209), allergic conjunctivitis N=274 (45/229), atopic dermatitis N=294 (75/219).*

(See next page)


Type 17
Type 1
Type 2
Regulatory
Other
Estimates (SD)
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0.50
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−0.50
−1.00
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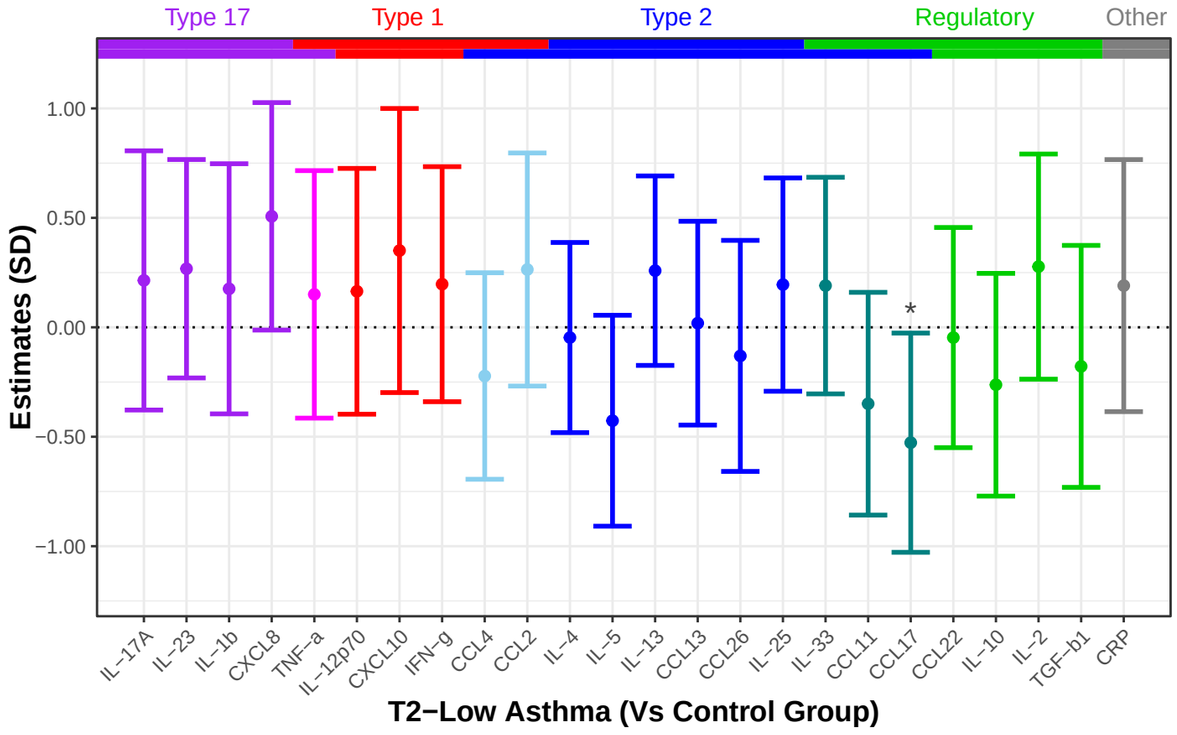
IL−23
IL−1b
CXCL8
TNF−a
IL−12p70
CXCL10
IFN−g
CCL4
CCL2
IL−4
IL−5
IL−13
CCL13
CCL26
IL−25
IL−33
CCL11
CCL17
CCL22
IL−10
IL−2
TGF−b1
CRP
T2−High Asthma (Vs Control Group)


Type 17
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Type 2
Regulatory
Other
Estimates (SD)
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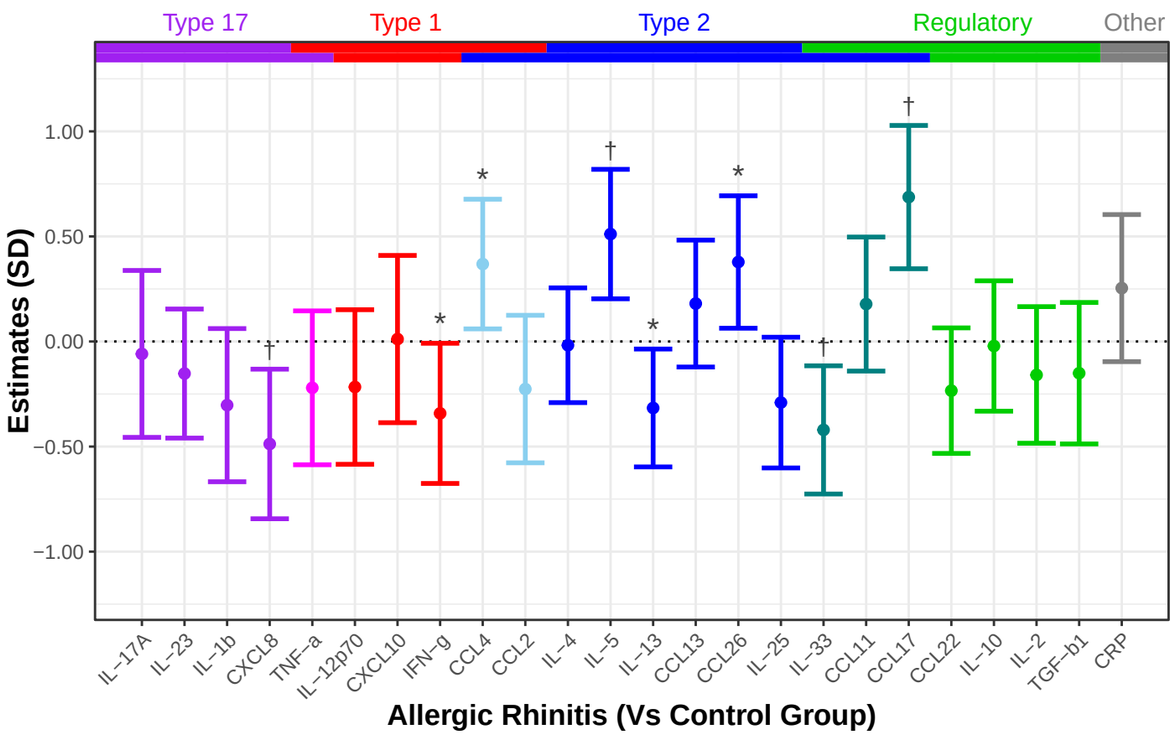
0.00
−0.50
−1.00
IL−17A
IL−23
IL−1b
CXCL8
TNF−a
IL−12p70
CXCL10
IFN−g
CCL4
CCL2
IL−4
IL−5
IL−13
CCL13
CCL26
IL−25
IL−33
CCL11
CCL17
CCL22
IL−10
IL−2
TGF−b1
CRP
T2−Low Asthma (Vs Control Group)


Type 17
Type 1
Type 2
Regulatory
Other
Estimates (SD)
1.00
0.50
0.00
−0.50
−1.00
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IL−23
IL−1b
CXCL8
TNF−a
IL−12p70
CXCL10
IFN−g
CCL4
CCL2
IL−4
IL−5
IL−13
CCL13
CCL26
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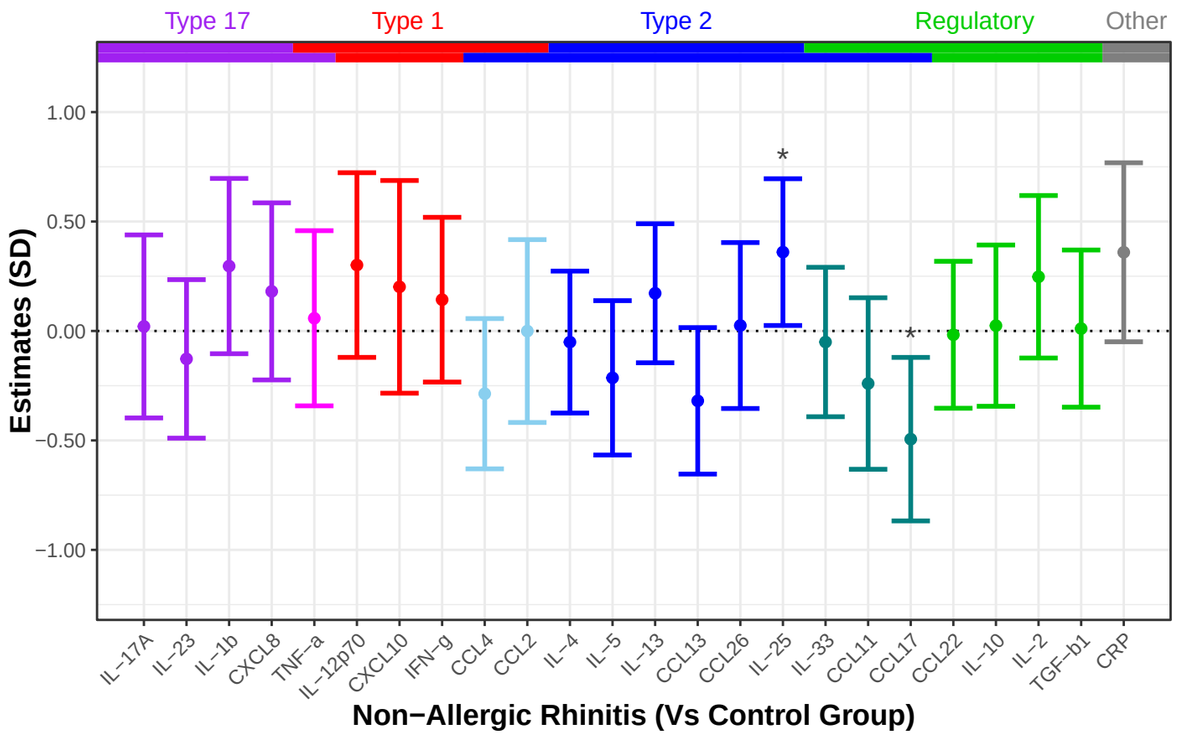
CCL11
CCL17
CCL22
IL−10
IL−2
TGF−b1
CRP
Allergic Rhinitis (Vs Control Group)


Type 17
Type 1
Type 2
Regulatory
Other
Estimates (SD)
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IL−12p70
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CCL4
CCL2
IL−4
IL−5
IL−13
CCL13
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CCL11
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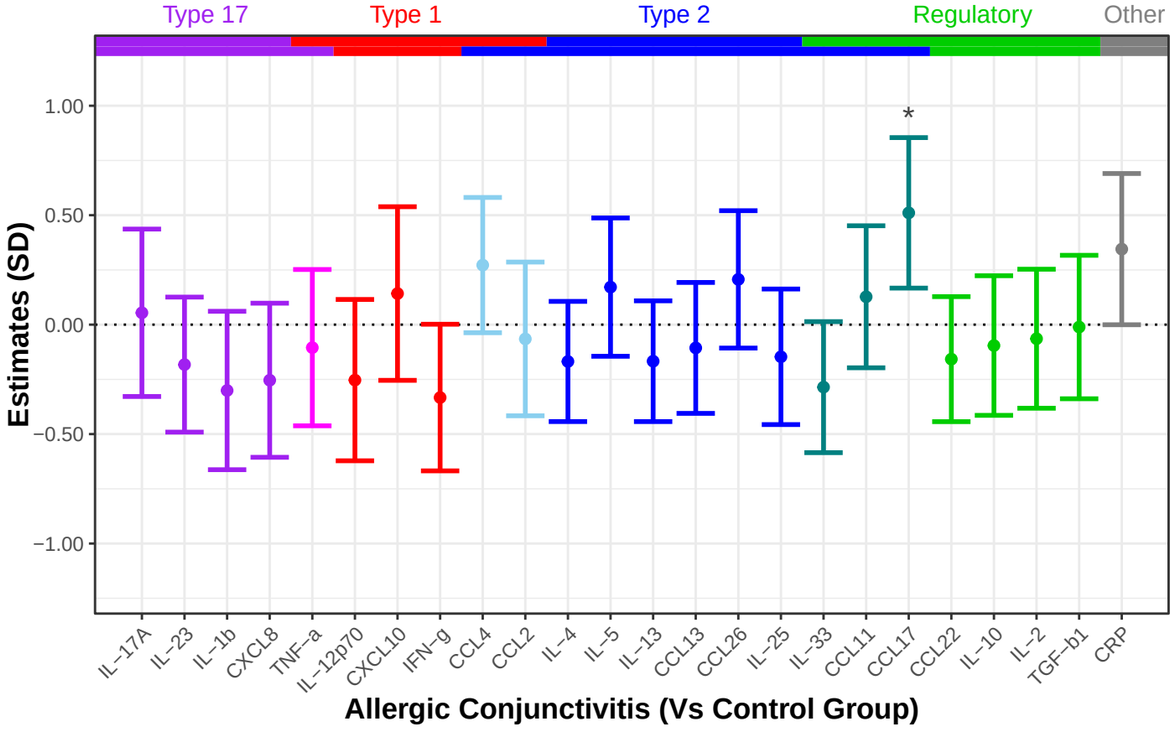
CCL22
IL−10
IL−2
TGF−b1
CRP
Non−Allergic Rhinitis (Vs Control Group)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Regulatory
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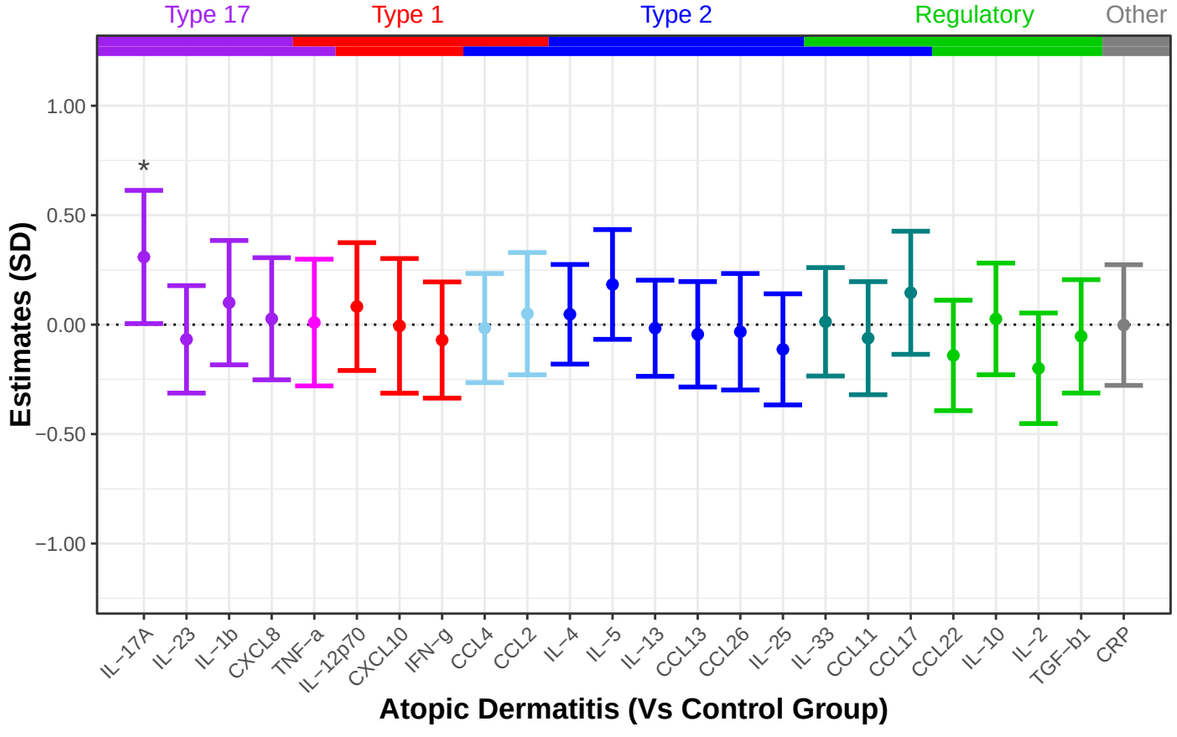
TGF−b1
CRP
Allergic Conjunctivitis (Vs Control Group)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CCL11
CCL17
CCL22
IL−10
IL−2
TGF−b1
CRP
Atopic Dermatitis (Vs Control Group)

PAPER III

Coughing and Wheezing in Early Life Define Distinct Asthma and Lung Function Trajectories

Tamo Sultan, Nicklas Brustad, Julie Nyholm Kyvsgaard, Signe Kjeldgaard Jensen, Liang Chen, Tingting Wang, Casper Emil Tingskov, Anders Ulrik Eliasen, Yang Luo, Karen Angeliki Krogfelt, Benjamin McDonald Smith, Ann-Marie Malby Schoos, Nilo Vahman, Jonathan Thorsen, Morten Arendt Rasmussen, Jakob Stokholm, Nicole Prince, Jessica Lasky-Su, Bo Chawes, Klaus Bønnelykke.

Under review in The European Respiratory Journal

Coughing and Wheezing in Early Life Define Distinct Asthma and Lung Function Trajectories

Objective

A) Investigate independent associations of cough and wheeze burden with later asthma and obstructive lung function

B) Characterize phenotypes of children with cough-only and cough+wheeze, compared with healthy controls, focusing on later asthma risk, obstructive lung function, infections, and mechanistic asthma-related signatures

Methods

Danish prospective birth cohort

COPSAC

Daily recordings of symptoms, medication, and infections during first 3 years of life

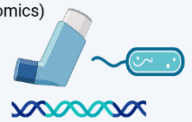
Coughing (n=556) | Wheezing (n=556) | Symptom diary (>5 million)

Early-Life phenotypes by recurrent troublesome lung symptoms

Healthy (n=381) | Cough-Only (n=46) | Cough+Wheeze (n=129)

Early life (0-3 years)

- Infection burden and pathogens
- Asthmatic signatures (gut and airway-microbiome, airway cytokines, and genomics)

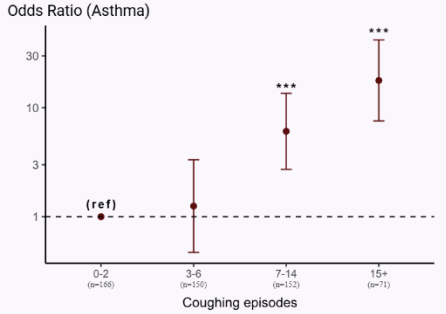


Later life (6-10 years)

- School-age asthma
- Type 2 biomarkers
- Lung function

Results

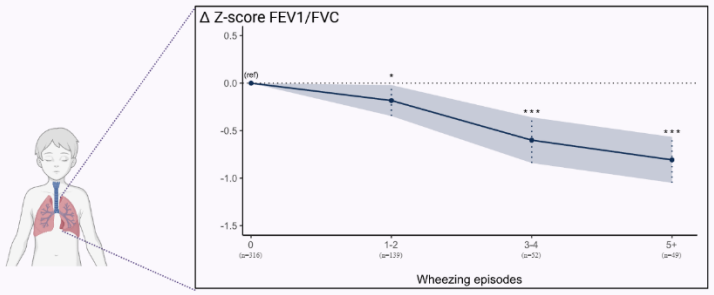
Later Asthma Risk



Only coughing was independently associated with school-age asthma, and its predictive performance was higher than wheezing (AUC 0.79 vs 0.70)

Coughing (n=556)

Obstructive Lung Function



Only wheezing was independently associated with later obstructive lung function

Wheezing (n=556)

Phenotypes

Cough-Only (n=46):
Higher asthma risk, normal lung function, asthma-related airway signatures (airway cytokines and airway microbiome)

Cough+Wheeze (n=129):
Higher asthma risk, obstructive lung function, asthma-related airway signatures (airway cytokines and airway microbiome), gut microbiome immaturity, stronger genetic risk and more rhinovirus-associated episodes and pneumonia

Conclusion

Only early-life cough burden was independently associated with later asthma and showed stronger predictive value than wheeze, whereas only early-life wheeze burden was independently associated with later obstructive lung function

** p-value < 0.05,*
*** p-value < 0.01,*
**** p-value < 0.001*

Created with BioRender.com.

Abbreviations:
AUC: Area under curve,
FEV1/FVC: Ratio of forced expiratory volume in 1 second to forced vital capacity

Created in BioRender. Bønnelykke, K. (2026) https://BioRender.com/lfke58m

Title:

Coughing and Wheezing in Early Life Define Distinct Asthma and Lung Function Trajectories

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Conflict of Interest: AMS has been a paid speaker for ALK and Thermo Fisher Scientific and is on an advisory board for ALK. All other authors declare no potential, perceived, or real conflict of interest regarding the content of this manuscript. The funding agencies did not have any role in the design and conduct of the study; collection, management, and interpretation of the data; or preparation, review, or approval of the manuscript. No pharmaceutical company was involved in the study.

Governance: We are aware of and comply with recognized codes of good research practice, including the Danish Code of Conduct for Research Integrity. We comply with national and international rules on the safety and rights of patients and healthy subjects, including Good Clinical Practice (GCP) as defined in the EU's Directive on Good Clinical Practice, the International Conference on Harmonisation's (ICH) good clinical practice guidelines and the Helsinki Declaration. Privacy is important to us which is why we follow national and international legislation on General Data Protection Regulation (GDPR), the Danish Act on Processing of Personal Data and the practice of the Danish Data Inspectorate.

Abbreviations:

AUC: Area under the curve

β_2-agonists: Beta-2-agonists

CI: Confidence interval

COPD: Chronic obstructive pulmonary disease

FeNO: Fractional exhaled nitric oxide

FEV1: Forced expiratory volume in 1 second

FEV1/FVC: Ratio of forced expiratory volume in 1 second to forced vital capacity

FVC: Forced vital capacity

GWAS: Genome-wide association study

ICS: Inhaled corticosteroids

IQR: Interquartile range

IRR: Incidence rate ratio

MAZ: Microbiota-by-age z-score

MMEF: Maximal mid-expiratory flow

OR: Odds ratio

PCR: Polymerase chain reaction

PD20: Provocative dose causing a 20% fall in FEV1

PRS: Polygenic risk score

ROC: Receiver operating characteristic

RSV: Respiratory syncytial virus

RV: Rhinovirus

RV-C: Rhinovirus C

SD: Standard deviation

SNP: Single nucleotide polymorphism

SPT: Skin prick test

sRaw: Specific airway resistance

T2: Type 2 (inflammation)

ABSTRACT

Background: Early-life wheezing is a risk factor for later asthma, whereas the role of early coughing is less defined.

Objective: To investigate whether early-life coughing and wheezing independently relate to school-age asthma, lung function and mechanistic signatures.

Methods: In COPSAC2010 (n=556), parents completed prospective symptom and infection diaries during the first 3 years. Cough and wheeze episode burden and symptom-dominant phenotypes were related to asthma and lung function at ages 6–10 years. Mechanistic profiling included type-2 biomarkers, airway cytokines, airway and gut microbiome, genetics and polygenic scores.

Results: In mutually adjusted analyses, only early-life coughing, not wheezing, was independently associated with asthma at 6–10 years (P<0.0001). Coughing predicted later asthma better (area under curve: coughing=0.79 vs wheezing=0.70, P=0.001). Children with the highest cough burden (≥15 episodes) had an odds ratio (OR) of 17.8 (95% confidence interval [CI]: 7.6–42.0, P<0.0001) for later asthma compared to the lowest burden (0–2 episodes). In contrast, only wheeze burden, not cough burden, was independently associated with later airway obstruction. Highest wheeze burden (≥5 vs 0 episodes) was associated with lower FEV1/FVC (-0.81 z-score, CI: -1.05–-0.57, P<0.0001) and higher odds of obstruction (lowest decile; OR=9.5, CI: 4.2–21.7, P<0.0001). By phenotype, cough-only (n=46) and cough+wheeze (n=129) had higher asthma risk than controls (n=381), but reduced lung function was confined to cough+wheeze. The cough+wheeze phenotype was also characterized by higher risk of rhinovirus infections, pneumonia and genetic risk.

Conclusion: Early coughing and wheezing show distinct associations to later asthma and obstructive lung function. Assessment of cough burden may improve prediction and early management of asthma.

INTRODUCTION

Childhood asthma is a common but heterogeneous disease that presents with varying combinations of wheeze, cough, and breathlessness[1]. The majority of cases debut in preschool years.[2,3] At this age, symptoms of wheeze have historically been used to identify children at higher risk of later asthma and guide clinical diagnosis and treatment decisions[4–6]. Wheezing phenotypes are well characterized and show greater disease severity and poorer lung function trajectories compared with children without wheeze, and they typically co-occur with early atopic sensitization[7,8]. In contrast, cough-dominant asthma-like phenotypes remain understudied and frequently misclassified[9,10].

Persistent cough in early childhood affects up to 10% of children and remains challenging to diagnose and manage in clinical practice[11,12]. Around 30-50% of children with cough consult a doctor[13,14], and in referral-based cohorts more than 80% of families report at least five doctor visits in the prior year,[15] and therefore represents a major disease burden. Furthermore, persistent cough is highly disruptive for children and families and is consistently linked to reduced quality of life[15,16].

In this study, we aimed to shed light on the "black box" of early childhood asthma by investigating whether cough and wheeze symptoms in the first 3 years of life were independently associated with later asthma and lung function status at age 6-10 years. Secondly, we aimed to determine whether persistent cough constitutes a distinct phenotype relative to wheeze in terms of prognostic and biological profile. For this purpose, we leveraged more than 5 million entries from structured symptom diaries from age 0-3 years in the Copenhagen Prospective Studies on Asthma in Childhood 2010 ($COPSAC_{2010}$) birth cohort. We defined early-life cough- and wheeze-dominant phenotypes based on these symptom diaries and linked them to later asthma and lung function

development from age 6-10 years, microbiome-, immune-, and genotype profiling. The perspective was to improve understanding of the early development of childhood asthma and lung function impairment, and provide the basis for improved diagnosis and treatment of this clinically challenging disease entity.

METHODS

Study population

Children from the ongoing Danish $COPSAC_{2010}$ mother-child cohort were used for this study (N=700). The primary objective of the cohort was to understand the mechanisms of asthma and atopic diseases in an unselected population and prevent the development of asthma[17]. Within this cohort, pregnant mothers were randomized to vitamin D and/or fish oil supplementation. Clinical visits were conducted by trained COPSAC physicians starting at age 1 week, frequently during the first three years, and subsequently at regular intervals until age 10 years. Additional visits were conducted during episodes of acute symptoms or respiratory illnesses[18].

Daily symptom recordings

During the first 3 years of life, parents completed daily recordings on structured diary cards with dichotomous (yes/no) responses on troublesome lung symptoms (coughing, wheezing, or breathlessness), use of asthma medication (e.g. beta-2-agonists [β_2-agonists]), and signs of common childhood infections (cold, fever, pneumonia, acute otitis media, tonsillitis, or acute gastrointestinal infection defined as vomiting and/or diarrhea)[19]. Parents received thorough instructions and a written manual at study entry to ensure consistent symptom reporting. Diary entries were reviewed with parents at scheduled biannual clinical visits. All infections were doctor-verified either at the time of the episode or retrospectively. An episode was defined as ≥3 consecutive symptom days with ≥3 symptom-free days between episodes. Cough and wheeze burden was categorized by episode frequency using empirically chosen cutoffs based on the observed distribution.

Early-life cough-only and cough+wheeze phenotypes

Children were assigned to symptom-based phenotypes if they had recurrent episodes of troublesome lung symptoms during the first 3 years of life. Recurrent troublesome lung symptoms were defined as ≥5 episodes, lasting at least 3 days, within 6 months and were prospectively diagnosed by COPSAC clinicians using standardized procedures, and used as entry criterion for a 3-month inhaled corticosteroid (ICS) trial[20].

Among children with recurrent troublesome lung symptoms, phenotypes were defined using all daily symptom recordings from the first 3 years of life. The cough-only phenotype comprised children with coughing but no recorded days of wheezing during the entire period, while the cough+wheeze phenotype comprised children with any recorded wheeze. Children without recurrent troublesome lung symptoms during the first 3 years of life were classified as healthy controls.

Asthma in school-age

School-age asthma required an ongoing diagnosis between ages 6 and 10 years and was defined according to a standardized treatment algorithm including 1) recurrent episodes of symptoms typical of asthma (≥5 within 6 months), 2) sporadic symptoms typical of asthma (>2 days per week for 1 month) with response to β2-agonists prompting a 3-month ICS trial, and 3) relapse after stopping ICS[20–22]. The diagnosis was terminated after 12 months without need for ICS.

A sensitivity analysis was performed using objective asthma at school age, defined as meeting at least 2 of the following criteria: elevated fractional exhaled nitric oxide (FeNO), bronchodilator reversibility, obstructive lung function, and/or bronchial hyperreactivity, in accordance with the European Respiratory Society criteria[23].

Acute respiratory illnesses

During episodes of acute respiratory illnesses, children were seen at the COPSAC clinic for biosampling of bacterial culture and viral PCR[24].

Lung function measurements

Spirometry and plethysmography were assessed at ages 6, 8, and 10 years using the MasterScope system (*Erich Jäeger, Würzburg, Germany*)[25,26]. Measurements included forced expiratory volume in 1 second (FEV_1), forced vital capacity (FVC), FEV_1/FVC, maximal mid-expiratory flow (MMEF), and specific airway resistance (sRaw). The best of multiple technically acceptable measurements was used for analysis. All lung function values were calibrated by regressing raw measurements on age, height, and sex in children never diagnosed with asthma (0-10 years), and residuals were scaled using the standard deviation (SD) from this reference group to derive z-scores. Methacholine challenge testing at age 6 assessed bronchial hyperresponsiveness as cumulative dose causing a 20% fall in FEV_1 (PD_{20}) and bronchodilator reversibility was assessed at age 10.

Type 2 biomarkers

Type 2 biomarkers were assessed at multiple timepoints, including blood eosinophils (6, 10 years), fractional exhaled nitric oxide (FeNO; 6, 8, 10 years), total immunoglobulin E (IgE; 10 years), and aeroallergen sensitization by specific IgE and skin prick testing (6, 10 years)[27,28].

Mechanism-related signatures

We included early-life asthmatic signatures derived from multi-omics data collected within the $COPSAC_{2010}$ cohort.

A microbiota-by-age z score (MAZ) at age 1 year was derived to quantify gut maturity using 16S rRNA sequencing of stool samples collected at 1 week, 1 month, and 1 year of age, and has previously been associated with protection against asthma[29].

Two independent early-life airway scores were derived using supervised modeling approaches: (i) an airway bacterial score, based on hypopharyngeal microbiota composition at 1 month and trained to predict asthma by age 6 years, capturing a microbial signature of later asthma risk; and (ii) an airway immune score, trained on 18 nasal cytokines at 1 month using the bacterial score as input[30,31].

A subset of single nucleotide polymorphisms (SNPs) in *TSLP*, *IL1RL1*, *IL13*, and *IL33* were used to construct a composite genetic risk score for type 2 inflammation (T2 genetic score), and SNPs in *CDHR3* and *GSDMB* were used to construct a score for non-type 2 inflammation (non-T2 genetic score). The scores were based on risk allele counts weighted by effect sizes reported in a genome-wide association study (GWAS) of childhood asthma, as previously described[32,33]. Polygenic risk scores (PRS) for childhood asthma exacerbations (asthma exacerbation genetic score) at ages 0-6 years, chronic obstructive pulmonary disease (COPD)[34] and dysanapsis[35] were computed using summary statistics from referenced GWAS, and the PRS were standardized before analysis.

A detailed description of all methodology is provided in the **Online Repository**.

Statistical analysis

Descriptive statistics were frequencies (%) and mean (SD) or median (IQR). Analyses included children with ≥90% diary completeness; those with rare diseases or unreliable diaries were excluded. Count outcomes used quasi-Poisson log-link models with exposure offsets (total valid

diary days) to estimate incidence risk ratios (IRR). Lung function was analyzed with linear mixed-effects models with child ID as a random intercept and fixed effect adjustment for age/visit. Generalized estimating equations (GEE) estimated odds ratios (OR) for asthma from 6 to 10 years (logit link, robust standard errors) accounting for repeated asthma status within each child. Standardized continuous outcomes (biomarkers and composite scores) used linear regression. Phenotype as outcome used multinomial regression with healthy as reference. Predictive performance was assessed by receiver operating characteristic (ROC) analysis quantified by the Area Under the Curve (AUC) metric using the DeLong t-test for comparison, and Youden's Index for optimal cutoff[36]. All analyses were two-tailed with a significance level of α=0.05. Statistical analyses were conducted in R version 4.2.1 (R Core Team, 2022).

Ethics

This study was approved by the Danish Ethical Committee (H-B-2008-093) and Data Protection Agency (2015-41-3696).

RESULTS

Of the 700 children in $COPSAC_{2010}$, 144 were excluded due to < 90% symptom diary completion. A baseline comparison of the 556 (79.4%) included versus 144 (21.6%) excluded children showed differences in cesarean delivery, maternal smoking during pregnancy and household income in the first year of life (all P≥0.027; **E-Table 1**).

Coughing and wheezing in the first 3 years of life as continuous traits

Coughing episodes were common and more widely distributed compared to wheezing episodes (**E-Figure 1**). The frequency of coughing was several times higher than wheezing in the first 3 years of life but with similar age and seasonal patterns, showing peaks around 1 year of age and in winter seasons (**E-Figure 2**).

For each symptom, symptom days and symptom episodes were strongly correlated (rho=0.94), supporting that either metric captures symptom burden similarly (**E-Figure 3**). Episodes were used as the primary metric. The number of coughing and wheezing episodes were moderately correlated (rho=0.51), while the number of coughing episodes was almost identical to the total number of episodes with any troublesome lung symptoms (rho=0.99).

Relationship to later asthma

87/539 (16.1%) children had later asthma between 6-10 years. In mutually adjusted models, the association between coughing episodes and later asthma was largely unchanged after adjustment for wheezing (OR 1.17, 95% CI 1.12; 1.22, P<0.0001). In contrast, there was no evidence of association for wheezing episodes after adjustment for coughing (OR 1.04, P=0.32) (**Table 1**). Coughing episodes were also associated with later asthma in the subset of children without any wheezing (OR 1.14, P=0.0004).

Finding for coughing episodes were consistent in sensitivity analysis using a stricter, objectively validated asthma definition and when analyzing T2-high and T2-low asthma groups separately (**E-Table 2**).

Additionally, AUC for prediction of asthma by age 6-10 was higher for cough burden than wheeze burden (AUC coughing: 0.79 vs wheezing: 0.70, P=0.001), and the cutoffs with best sensitivity-specificity balance were ≥8 coughing episodes and ≥1 wheezing episode (**Figure 1)**. By year, discrimination was higher in years 2 and 3 than year 1 for both symptoms (**E-Figure 4A–C**).

Analyses of categories of cough burden showed a dose-response relationship between early-life coughing and later asthma with increasing risk for children in the highest episode category (≥15 episodes) compared to children in the lowest category (0-2 episodes) in the first three years (OR 17.84, CI 7.58; 41.96, P<0.0001) (**Figure 2**). Asthma prevalence at ages 6–10 was 4.8% in children with 0–2 episodes compared to 48.0% in those with ≥15 episodes (**Figure 2**).

Relationship to later lung function

In mutually adjusted analyses, a higher burden of wheezing episodes in the first 3 years was strongly associated with all measures of obstructive lung function, including lower FEV1, FEV1/FVC, and MMEF, and sRaw (**Table 2**). These results were largely unchanged in analyses restricted to children without asthma at 6-10 years. Contrary, the cough burden showed no independent association with any lung function parameters.

Findings were unchanged after adjustment for genetic predisposition (COPD and dysanapsis genetic scores, **E-Table 3**).

A similar but attenuated effect was observed for post-bronchodilator at 10 years for FEV1, FEV1/FVC, and MMEF (**E-Table 4**). Higher wheeze burden was associated with greater

bronchodilator reversibility in FEV1, FEV1/FVC, MMEF, and sRAW. Results were similar in the no-asthma subset.

By year, wheeze burden showed larger effect estimates in year 3 (**E-Table 5**).

In analyses of wheezing categories, there was a dose-response relationship between the number of wheezing episodes and obstructive lung function (**E-Tables 6-7**), with children in the highest burden category (≥5 episodes) showing a reduced FEV1/FVC z-score compared with no wheezing (-0.81, CI -1.05; -0.57, P<0.0001) (**Figure 3**).

The risk of having an FEV1/FVC below the lowest decile in the population increased from 4.4% in children with no wheezing episodes to 30.6% in children with ≥5 episodes (**Figure 3)** with a corresponding increased OR of 9.52 (CI 4.23; 21.65, P<0.0001). Similar dose-response relationships were seen for the other obstructive lung function measures, including FEV1, MMEF, and sRaw (**E-Figure 5**).

Phenotypes of cough-only and cough+wheeze

Among included children, 175 (31.5%) met the early-life definition of recurrent troublesome lung symptoms in the first 3 years of life. In total, 46 children (26.3%) were classified as the cough-only phenotype, defined by no recorded wheeze days, and 129 children (73.7%) as the cough+wheeze phenotype.

The symptom burden in the first three years by phenotype generally showed a higher symptom burden in the cough+wheeze phenotype (**E-Table 8**). The median (IQR) number of coughing episodes was 3 (1; 6) in healthy children, 12 (9; 15) in the cough-only phenotype, and 14 (9; 18) in the cough+wheeze phenotype. Median number of wheezing episodes were 0 (0; 1) in healthy children, 0 (0; 0) in the cough-only phenotype and 3 (1; 6) in the cough+wheeze phenotype.

Median days of β2-agonist use in the first three years of life were lower in the cough-only phenotype compared to the cough+wheeze phenotype, 68 (23; 91) vs 95 (48; 160).

Asthma in school-age

Both cough-only (GEE OR: 6.26, CI 3.11; 12.61, P<0.001) and cough+wheeze (GEE OR: 10.47 CI 6.02; 18.21, P<0.001) phenotypes in the first three years of life were associated with higher odds of asthma between ages 6-10, with no difference between phenotypes (GEE OR: 0.53, CI 0.24; 1.16, P=0.11) (**E-Figure 6**).

Findings were similar when analyzing T2-high and T2-low asthma groups separately (**E-Figure 7**).

Lung function

The cough-only phenotype had similar lung function compared to the healthy group by age 6-10 years. In contrast, the cough+wheeze phenotype had reduced FEV1, FEV1/FVC (both pre- and post-bronchodilator) and MMEF, and increased sRaw compared to both the healthy and cough-only phenotype, and also had higher bronchial responsiveness than the healthy group (**E-Table 9**). Differences were stable over time (**Figure 4**) with no group×age interaction (all P≥0.17).

Type 2 biomarkers

There were no significant differences in blood eosinophils, FeNO, total IgE, or specific IgE between phenotypes at ages 6, 8, or 10 years (**E-Table 10**).

Early-life risk factors

Relative to healthy controls, the cough-only phenotype was more often male and less likely to have an older sibling at birth. Relative to healthy controls, the cough+wheeze phenotype was associated with parental asthma, prenatal and early-life smoke exposure, and adverse birth characteristics

(lower gestational age, fewer term births, and lower birthweight). In direct comparison, the cough-only phenotype was less likely to have an older sibling at birth than the cough+wheeze phenotype (**E-Table 11**).

Burden of common childhood infections

Both the cough-only and cough+wheeze phenotype had more episodes of cold and fever than the healthy group, and the cough+wheeze phenotype in addition had more episodes of pneumonia, acute otitis media, and acute gastroenteritis. Direct comparison of the cough-only and cough+wheeze phenotypes showed less pneumonia episodes in the former (IRR 0.47, P=0.007) (**Table 3**).

Microbial triggers

Bacterial and viral triggers were evaluated per sampled acute episode (i.e., the IRRs reflect pathogen detection per sampled acute episode, not the overall number of illnesses). After adjustment for sampling intensity, RV was more common in the cough+wheeze phenotype than in healthy children (IRR 1.33, P=0.044) and than in the cough-only phenotype (IRR 0.72, P=0.066). In contrast, RSV was less frequent in cough-only compared to both healthy and cough+wheeze (**E-Table 12**).

Gut and airway microbiome and immune profiles

The gut immaturity score was higher in cough+wheeze compared with healthy controls, and with no difference between phenotypes (**Figure 5A**). Both phenotypes showed similar associations with the asthma-associated airway immune score and airway bacterial score compared with healthy controls (**Figure 5B-C**).

Genetics

In all three genetic scores, cough+wheeze had higher scores than both healthy controls and cough-only, while cough-only did not differ from healthy controls (**Figure 5D-F**).

The COPD genetic score showed a borderline increase in cough+wheeze vs healthy (OR 1.23, CI 0.99; 1.54, P=0.065), while the dysanapsis score was not associated (OR 1.16, CI 0.94; 1.44, P=0.17), with no differences for cough-only vs healthy or cough+wheeze (all P≥0.14).

Sensitivity Analyses

Findings remained consistent when adjusting for vitamin D and fish oil supplementation during pregnancy (*data not shown*).

DISCUSSION

Main findings:

In this study, using daily symptom diary data from the first 3 years of life and long-term follow-up into school-age in more than 500 children, we found that early-life cough and wheeze burdens showed independent and distinct associations with later asthma development and lung function impairment. When mutually adjusted, only cough burden remained independently associated with later asthma, whereas wheeze burden remained independently associated with impaired lung function, specifically with an obstructive pattern.

Next, symptom-based phenotypes were defined, cough-only vs cough+wheeze, to test whether these patterns reflect qualitatively different disease entities. As expected from the symptom burden analyses, children with the cough-only phenotype (0-3 years) had increased risk of later asthma but normal lung function at school-age, while children with the cough+wheeze phenotype also had obstructive lung function. Both phenotypes had increased infection burden but the cough+wheeze phenotype had a higher burden of pneumonia, and rhinovirus was a distinct trigger of acute symptoms specifically for this phenotype.

Interpretation:

We found that cough burden in the first 3 years of life was the main determinant of school-age asthma, independent of wheeze burden, and cough burden remained associated with asthma at 6-10 years even among children who never wheezed in the first 3 years of life. On contrast, wheezing did not improve prediction beyond cough burden and accordingly, cough burden outperformed wheeze burden for asthma prediction, with an AUC of approximately 0.80.

These results are in line with a previous study from the high-risk $COPSAC_{2000}$ cohort (n=411) where the total number of acute visits for respiratory illnesses at 0-3 years predicted asthma at age 7, whereas wheeze on auscultation at those visits added no independent information[37]. However, in $COPSAC_{2000}$, diary data on specific symptoms was not available to assess the total symptom quantity in early life. Likewise, a study from a U.S. cohort (n=393) with questionnaire data at 6 and 9 months of age found that a first-year total symptom intensity score, using both respiratory and non-respiratory symptoms, predicted school age asthma beyond wheeze[38]. Complementing this, an earlier study based on yearly questionnaires suggested that chronic coughing by ages 3-4 years predicted later asthma independently of wheezing, although this was done as a small subgroup analysis (n=35)[39]. More recently, cough studies demonstrated prolonged coughing and coughing without cold in infancy to predict later asthma independent of infant wheezing[40], and that persistent trajectories of coughing without colds are strongly linked to asthma[41]. Our study refines earlier observations by showing that within prospectively recorded early-life symptom diaries, the link to later asthma is specifically driven by coughing episodes with a clear dose-response relationship, importantly demonstrating that a sole focus on early wheezing will lead to suboptimal prediction of asthma.

In contrast, the wheeze burden in the first 3 years of life seems to be the main driver of impaired lung function at 6-10 years, with no independent contribution from the cough burden. The wheeze-associated lung function deficits were characterized by an obstructive lung function pattern. The obstructive deficits persisted in part after bronchodilation, suggesting a partially fixed obstructive component. We also observed an apparent dose-response relationship with the largest lung function deficit for children with the highest number of wheezing episodes (e.g. -0.81 z-score deficit and 31% risk of being in the lowest population decile).

Importantly, these associations were equally strong in analyses restricted to children, who did not develop asthma, indicating that early wheezing symptoms relate to lung development rather than operating through an asthma pathway. This pattern is compatible with dysanapsis, meaning disproportionate growth of airways relative to lung size, and suggests wheezing is a marker of altered lung function development. Given that reduced lung function in early life is a recognized route to chronic obstructive pulmonary disease (COPD)[42,43], and that dysanapsis is associated with obstructive spirometry[44] and COPD[45], early wheezing may mark elevated risk for obstructive disease trajectories[46,47]. In line with this, our data showed directionally higher COPD and dysanapsis genetic scores in the wheeze-driven phenotype of cough+wheeze. Still, lower baseline lung function may also predispose to wheezing, consistent with neonatal and infant studies where early deficits predict subsequent wheezing[48–51]. This substantial increased risk of later obstructive lung function in children with the highest burden of wheezing emphasizes the importance of understanding the early life processes involved in development of early lung function impairment to prevent permanent airflow obstruction and COPD. Therefore, a quantitative wheeze burden measure might provide a useful screening tool for this purpose.

In the first three years of life, a cough-only phenotype was defined by recurrent cough without any wheeze, while the cough+wheeze phenotype included both symptoms. In school-age, cough-only was associated with increased asthma risk, whereas cough+wheeze was also linked to obstructive lung function deficits. Both phenotypes showed increased burden of respiratory infections in terms of colds, but the cough+wheeze phenotype was characterized by a higher risk of lower respiratory tract infections. This higher pneumonia burden in the cough+wheeze phenotype may contribute to later lung function deficits, as early-life pneumonia is consistently linked to reduced later lung function[52–56].

Another specific association for the cough+wheeze phenotype was a higher risk of respiratory illnesses triggered by rhinoviruses. This supports the hypothesis that rhinovirus infections play a specific role in asthma and lung function development through airway remodeling[57–59]. The cough+wheeze phenotype also seemed to be more driven by genetic susceptibility to asthma exacerbations and impaired lung function. Both phenotypes also showed similar early-life upper-airway cytokine profiles that differed from the non-asthmatic control group. This aligns with an Australian bronchoalveolar lavage study showing that both preschool wheeze (n=12) and chronic suppurative lung disease with persistent wet cough (n=29) differ from controls (n=22), with comparable hyper-inflammatory patterns, including cytokines, identified in both conditions[60].

Based on our findings, it can be speculated that cough burden reflects susceptibility to respiratory infections, which is a main risk factor and trigger of asthma symptoms in all ages, and therefore is the main symptom defining risk for still having asthma in school age. In contrast, only wheezing was independently associated with later airflow limitation. This could reflect that only the children where infections result in more severe inflammation of the small to medium-sized bronchi, due to genetics, other host susceptibility, or the nature of the viral trigger, will experience airway remodeling leading to persistent airflow obstruction[61,62].

Strengths and limitations:

The strengths of our study include extensive phenotype data available from daily symptom diaries surveying the first three years of life, enabling high-resolution, symptom-based phenotyping of persistent troublesome lung symptoms during a critical developmental window. Our main analyses were based on symptom-based asthma algorithm, with an inherent risk of misclassification. However, this algorithm is very strict and includes response to ICS and subsequent relapse, and

our conclusions were further supported by sensitivity analyses using objectively verified asthma diagnoses.

CONCLUSION

Early-life coughing and wheezing symptoms are key indicators of later respiratory outcomes in children. Cough burden is independently associated with later asthma, whereas wheeze burden is associated with later impaired lung function. This implies that coughing and wheezing symptoms reflect qualitatively distinct traits that both needs to be considered to understand early asthma and lung function development. Furthermore, a symptom-based assessment including cough burden might improve asthma prediction and clinical management of early asthma.

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TABLES & FIGURES

	Single-symptom model vs later asthma		Mutually adjusted model vs later asthma	
Exposure	OR (95% CI)	P-value	OR (95% CI)	P-value
Coughing episodes	1.18 (1.14; 1.23)	**<0.0001**	1.17 (1.12; 1.22)	**<0.0001**
Coughing episodes (no-wheezing subset)	1.14 (1.06; 1.22)	**0.0004**	—	—
Wheezing episodes	1.20 (1.12; 1.29)	**<0.0001**	1.04 (0.96; 1.13)	0.32

Table 1. Association of early symptom burden with later asthma. *Odds ratios (ORs) with 95% confidence intervals (CIs) and P-values from logistic regression for asthma at 6–10 years(n=87). Predictors are counts of cough and wheeze episodes from 0–3 years, modeled per one additional episode. All models are adjusted for sex. Mutually adjusted models are adjusted for both coughing and wheezing episodes in the same model.*

Cough burden 0-3 years and later lung function						
	Unadjusted		**Wheezing-adjusted**		**No-asthma subset (Wheezing-adjusted)**	
Outcome	β (95% CI)	P-value	β (95% CI)	P-value	β (95% CI)	P-value
FEV1	-0.02 (-0.03; 0.00)	**0.026**	0.00 (-0.02; 0.01)	0.91	-0.01 (-0.03; 0.01)	0.47
FVC	0.00 (-0.01; 0.01)	0.67	0.00 (-0.01; 0.01)	0.96	-0.01 (-0.03; 0.01)	0.36
FEV1/FVC	-0.02 (-0.03; -0.01)	**0.0003**	0.00 (-0.01; 0.01)	0.86	0.00 (-0.01; 0.02)	0.71
MMEF	-0.02 (-0.04; -0.01)	**0.0001**	0.00 (-0.02; 0.01)	0.64	0.00 (-0.02; 0.02)	0.88
sRAW	0.01 (0.00; 0.02)	*0.052*	0.00 (-0.02; 0.01)	0.52	0.00 (-0.02; 0.02)	0.93
Wheeze burden 0-3 years and later lung function						
	Unadjusted		**Coughing-adjusted**		**No-asthma subset (Coughing-adjusted)**	
Outcome	β (95% CI)	P-value	β (95% CI)	P-value	β (95% CI)	P-value
FEV1	-0.06 (-0.09; -0.03)	**<0.0001**	-0.06 (-0.09; -0.03)	**0.0004**	-0.07 (-0.11; -0.03)	**0.0007**
FVC	-0.01 (-0.04; 0.01)	0.36	-0.01 (-0.04; 0.02)	0.41	-0.02 (-0.06; 0.01)	0.21
FEV1/FVC	-0.08 (-0.11; -0.06)	**<0.0001**	-0.08 (-0.11; -0.06)	**<0.0001**	-0.08 (-0.11; -0.05)	**<0.0001**
MMEF	-0.09 (-0.12; -0.07)	**<0.0001**	-0.09 (-0.12; -0.06)	**<0.0001**	-0.10 (-0.14; -0.06)	**<0.0001**
sRAW	0.06 (0.04; 0.08)	**<0.0001**	0.06 (0.04; 0.09)	**<0.0001**	0.06 (0.02; 0.09)	**0.001**

Table 2. Coughing and wheezing episodes in early life in relation to later lung function outcomes. *Linear mixed models with a child random intercept and fixed effects for time (6/8/10 year). Exposures were number of coughing and wheezing episodes. Three models: Unadjusted, mutually adjusted for both symptom episodes, and mutually adjusted among no-asthma 6-10 subset. Beta (β) represents the change in lung function z-score per 1-episode increase in coughing or wheezing (95% confidence interval (CI)).*

FEV1, forced expiratory volume in 1 second; FVC, forced vital capacity; MMEF, mid-expiratory flow; sRAW, specific airway resistance.

Daily Diary Outcomes	Healthy (n=381)	Cough-Only (n=46)	Cough-Only vs Healthy		Wheeze (n=129)	Cough+Wheeze vs Healthy		Cough-Only vs Cough+Wheeze	
(Episodes)	(Median, IQR)	(Median, IQR)	(IRR, 95% CI)	P-value	(Median, IQR)	(IRR, 95% CI)	P-value	(IRR, 95% CI)	P-value
Cold	9 (6; 14)	17 (10; 20)	1.58 (1.36; 1.83)	**<0.0001**	15 (11; 20)	1.54 (1.39; 1.70)	**<0.0001**	1.03 (0.87; 1.20)	0.76
Fever	5 (3; 7)	6 (4; 9)	1.24 (1.04; 1.48)	**0.019**	6 (4; 9)	1.25 (1.11; 1.41)	**0.0002**	0.99 (0.81; 1.20)	0.91
Pneumonia	0 (0; 1)	0 (0; 1)	1.38 (0.79; 2.40)	0.25	1 (0; 2)	2.95 (2.22; 3.92)	**<0.0001**	0.47 (0.27; 0.81)	**0.007**
Acute Otitis Media	1 (0; 2)	1 (0; 3)	1.30 (0.91; 1.87)	0.15	1 (0; 3)	1.41 (1.12; 1.78)	**0.0004**	0.92 (0.63; 1.36)	0.69
Tonsillitis	0 (0; 0)	0 (0; 1)	0.75 (0.32; 1.78)	0.52	0 (0; 1)	1.31 (0.84; 2.04)	0.24	0.58 (0.23; 1.42)	0.23
Acute Gastroenteritis	1 (1; 2)	1 (1; 3)	1.19 (0.90; 1.58)	*0.22*	2 (1; 3)	1.35 (1.13; 1.60)	**0.0007**	0.88 (0.66, 1.19)	0.42

Table 3. Early-life infection burden by phenotype. *Values are median (IQR) from daily diary recordings during the first 3 years of life. Episodes of infections were defined as ≥3 consecutive symptom days with ≥3 symptom-free days between episodes verified by a physician. Incidence rate ratios (IRRs) with 95% confidence intervals (CIs) were estimated using quasi-Poisson regression with the log of completed diary days as an offset, adjusted for sex, older sibling at birth and age at daycare start.*

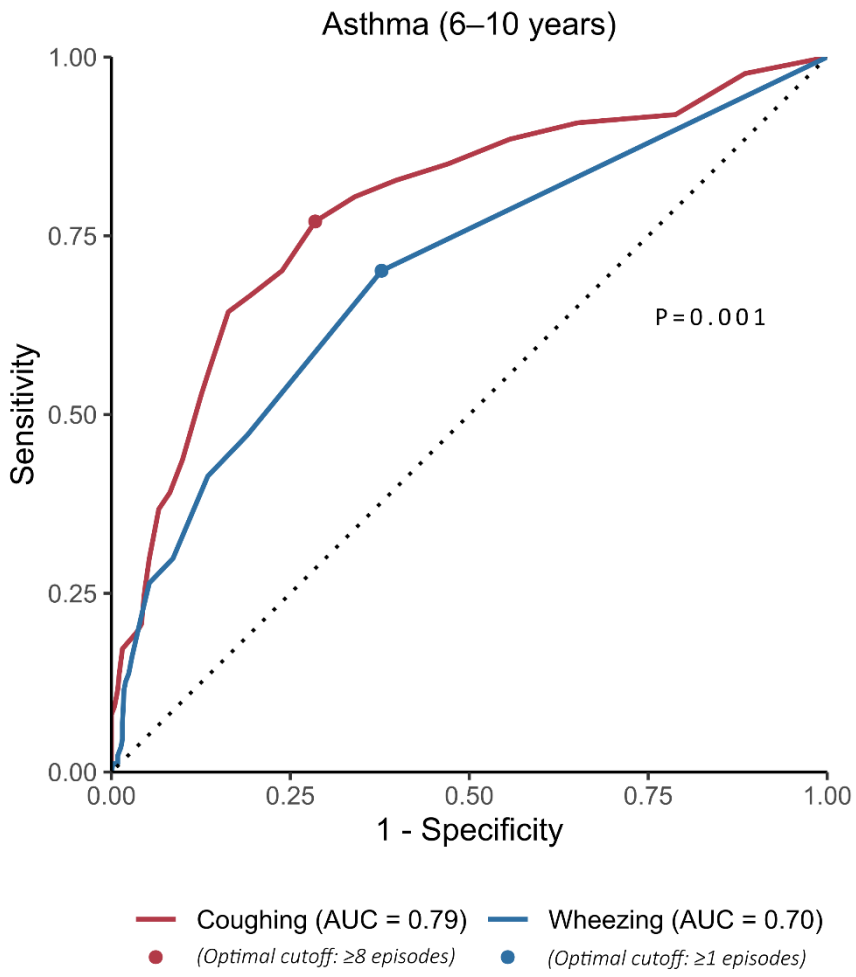

Figure 1. Predictive performance of early-life symptom burden for later asthma. *Receiver operating characteristic (ROC) curves for asthma between 6 and 10 years based on cumulative coughing (red) and wheezing (blue) episodes in the first three years of life. The area under the curve (AUC) was compared using the DeLong test. The cutoff with best sensitivity and specificity balance was identified by maximizing Youden's index (J = sensitivity + specificity − 1) and is shown as a dot on each curve.*

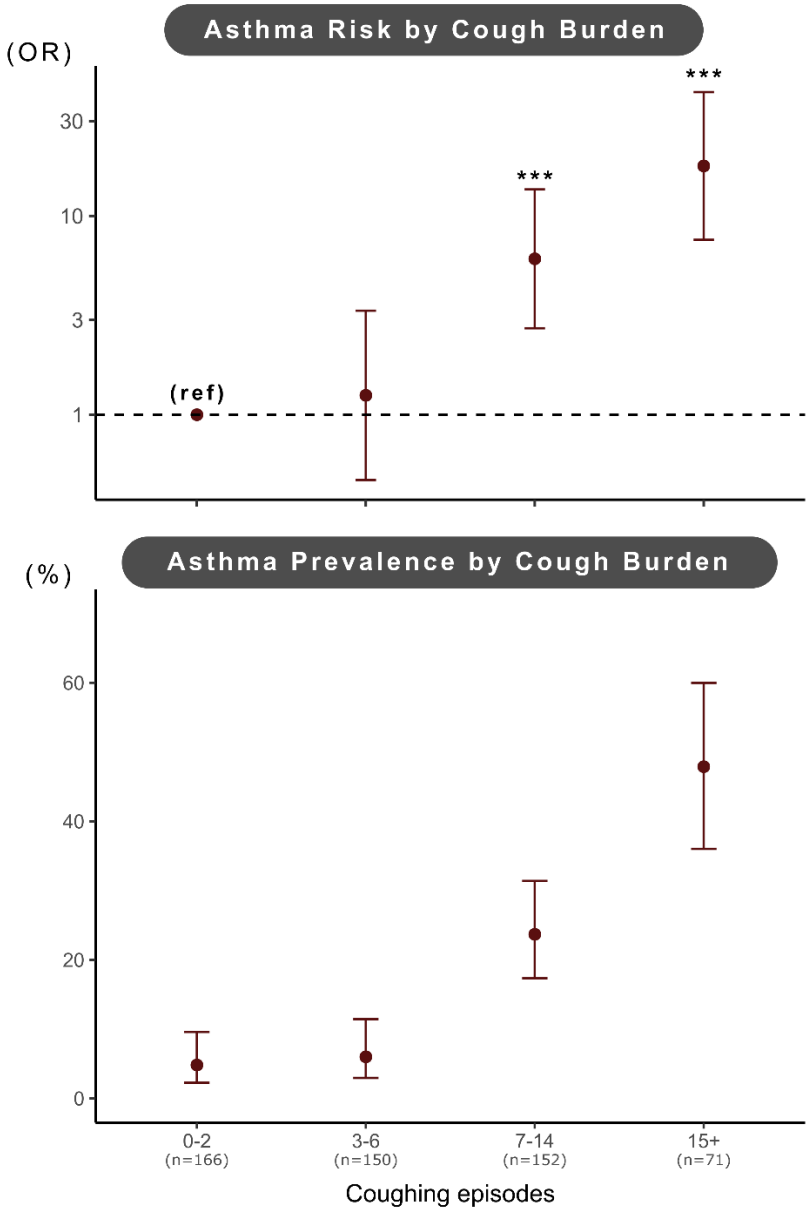

Figure 2. Early-life cough burden and later asthma risk. *Asthma risk and prevalence by cumulative number of coughing episodes during the first three years of life. Top panel shows odds ratios (OR) with 95% confidence intervals for asthma at 6-10 years by logistic regression model adjusted for sex and with lowest category as reference (0-2 episodes). Bottom panel shows corresponding asthma prevalence within each ordered coughing episode category with 95% Wilson confidence intervals (shown as error bars). Children were categorized as: 0–2 episodes, 3–6 episodes, 7–14 episodes, and ≥15 episodes. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.*

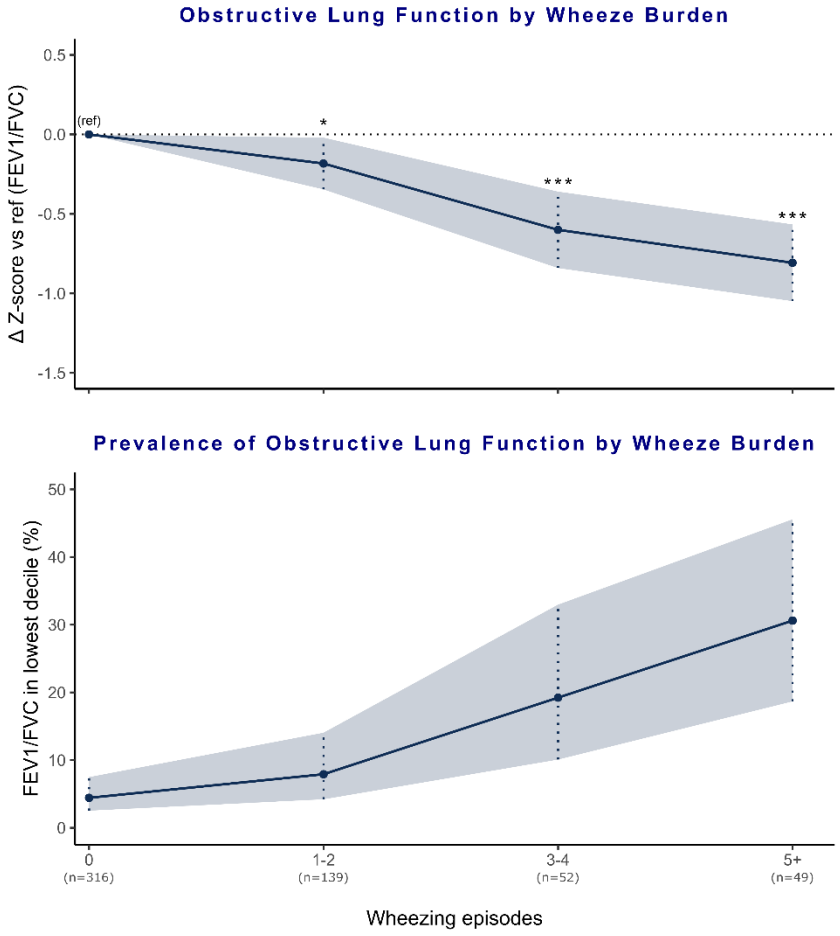

Figure 3. Early-life wheeze burden and later obstructive lung function. *Estimated mean differences in lung function (FEV_1/FVC z-scores) and prevalence of obstructive lung function (FEV_1/FVC in the lowest decile) at ages 6–10 years by cumulative number of wheezing episodes during the first three years of life. Top panel shows estimated mean differences with 95% confidence intervals from a linear mixed-effects model with a child random intercept and fixed effects for time at 6, 8, and 10 years. Bottom panel shows corresponding prevalence of obstructive lung function by wheezing category, defined using the child-specific mean FEV_1/FVC z-score across ages 6, 8, and 10 years, with 95% Wilson confidence intervals (shown as error bars). Obstructive lung function was defined as FEV_1/FVC below the 10th percentile based on the healthy reference population. Lines connect category means to aid visual interpretation. Children were categorized as: 0 episodes (reference, n=316), 1–2 episodes (n=139), 3–4 episodes (n=52), and ≥5 episodes (n=49). * p<0.05, ** p<0.01, *** p<0.001.*

FEV1, forced expiratory volume in 1 second; FVC, forced vital capacity.

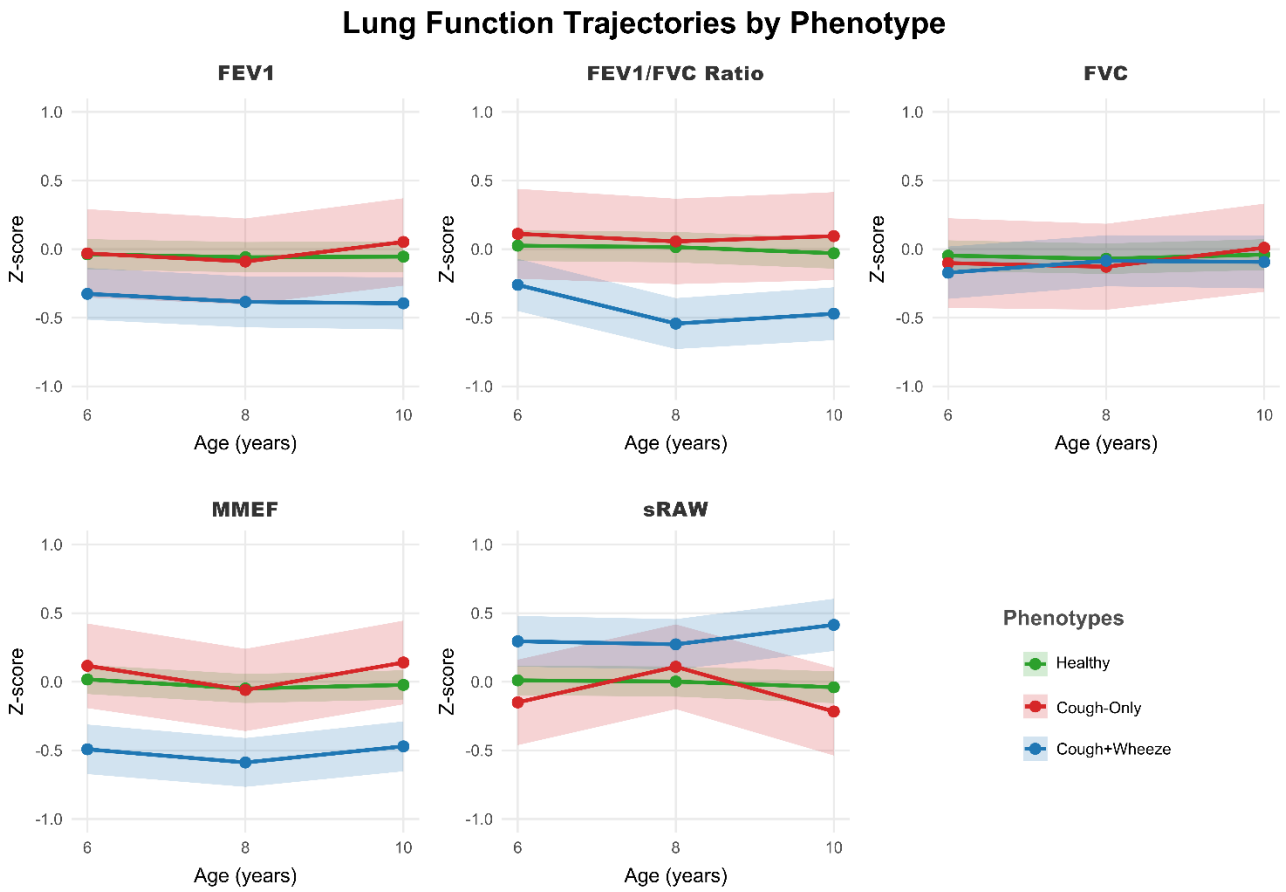

Figure 4. ***Longitudinal lung function trajectories by phenotype.*** *Estimated marginal means (z-scores) of FEV_1, FEV_1/FVC ratio, FVC, MMEF, and sRaw at ages 6, 8, and 10 years in healthy controls (n=369) and children with early-life cough-only (n=44) and cough+wheeze (n=126) phenotypes. Trajectories were derived from linear mixed models with repeated measures. Shaded areas indicate 95% CI.*

FEV1, forced expiratory volume in 1 second; FVC, forced vital capacity; MMEF, mid-expiratory flow; sRAW, specific airway resistance.

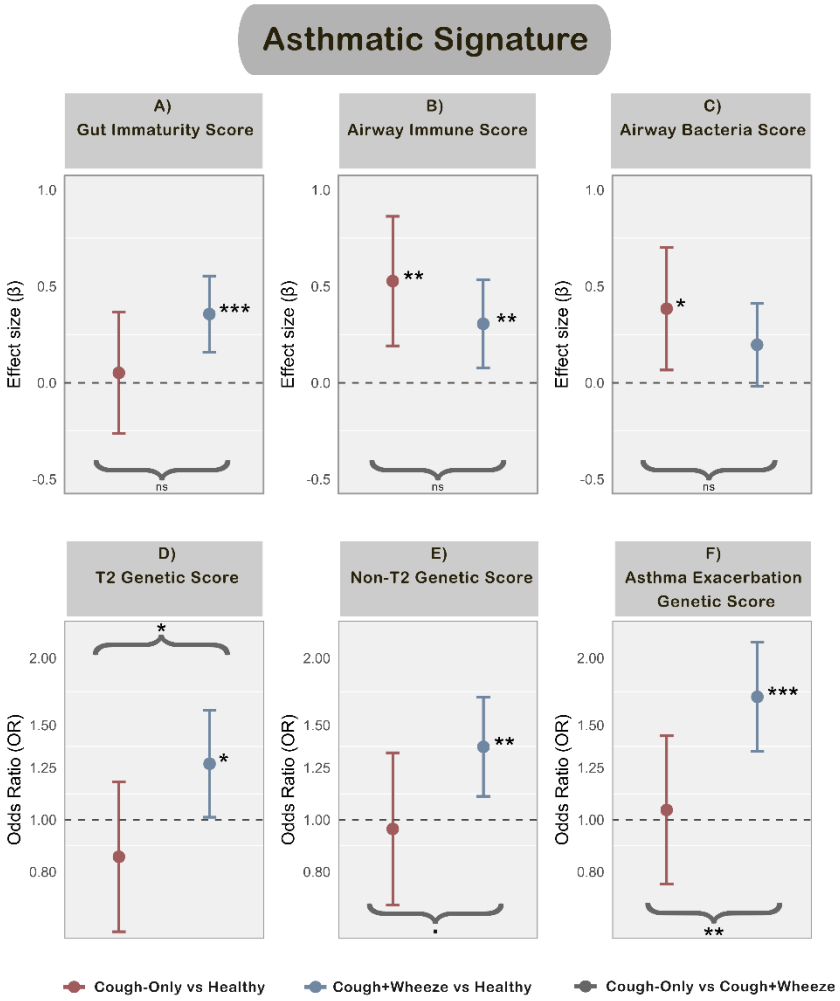

Figure 5. Asthmatic signature of phenotypes. *Gut microbiome was derived from stool 16S at 1 week, 1 month, and 1 year and summarized as inversed Microbiota-by-Age Z-scores (MAZ). Airway immune- and bacteria scores were computed at 1 month from nasal cytokines and hypopharyngeal microbiota using supervised models trained on later asthma. Genetic risk scores summarize asthma-associated variants (weighted by published childhood asthma genome-wide association study effect sizes): the T2 genetic score combines selected SNPs in IL33, IL1RL1, IL13 and TSLP, and the non-T2 genetic score combines selected SNPs in CDHR3 and GSDMB. The asthma exacerbation genetic score was a polygenic risk score based on a genome-wide association study on severe early-life asthma. Panels A–C display effect sizes (β) from linear models, panels D–F display odds ratios (OR). All models are adjusted for sex.*

** $p<0.05$, ** $p<0.01$, *** $p<0.001$.*

Title:

Coughing and Wheezing in Early Life Define Distinct Asthma and Lung Function Trajectories

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Document: Online repository (Supplementary material)

Abbreviations:

AUC: Area under the curve

β_2-agonists: Beta-2-agonists

CI: Confidence interval

COPD: Chronic obstructive pulmonary disease

FeNO: Fractional exhaled nitric oxide

FEV1: Forced expiratory volume in 1 second

FEV1/FVC: Ratio of forced expiratory volume in 1 second to forced vital capacity

FVC: Forced vital capacity

GWAS: Genome-wide association study

ICS: Inhaled corticosteroids

IQR: Interquartile range

IRR: Incidence rate ratio

MAZ: Microbiota-by-age z-score

MMEF: Maximal mid-expiratory flow

OR: Odds ratio

PCR: Polymerase chain reaction

PD20: Provocative dose causing a 20% fall in FEV1

PRS: Polygenic risk score

ROC: Receiver operating characteristic

RSV: Respiratory syncytial virus

RV: Rhinovirus

RV-C: Rhinovirus C

SD: Standard deviation

SNP: Single nucleotide polymorphism

SPT: Skin prick test

sRaw: Specific airway resistance

T2: Type 2 (inflammation)

ONLINE REPOSITORY

SUPPLEMENTARY METHODS

1.a: Daily Symptom Diary Recordings

Parents completed daily symptom recordings consisting of no symptoms, no medication, cough, breathlessness, wheeze, blue spray (β2-agonist), brown spray (ICS), asthma tablet (leukotriene receptor antagonists), cold, pneumonia, inflammation of the throat, middle ear infection (otitis media), fever, gastric or intestinal infection, child home from institution, eczema, treatment with cutaneous steroid. These were validated by COPSAC physicians either during infection, by medical records, or later during scheduled visits.

Two children were excluded due to congenital or genetic conditions (cleft lip/palate or fragile X syndrome), and 35 were excluded due to unreliable diary recordings. Of the remaining 663 children, only those with >90% diary completeness from birth to 3 years were retained for analysis, resulting in a final study population of 556 children.

1.b: Diagnosis of school-age asthma and other type 2 diseases

Asthma in the main analysis was defined as ≥5 episodes of asthma symptoms lasting at least 3 consecutive days within 6 months, or spontaneous symptoms (including exercise-induced) occurring more than twice per week for 1 month with documented β2-agonist response. In these cases, inhaled corticosteroid (ICS) treatment was continued for at least 3 months. Asthma was defined as relapse of airway symptoms after discontinuation of ICS, with relapse defined as ≥2 episodes of ≥3 consecutive days within 3 months, symptoms more than twice per week for 2 consecutive weeks, or a severe acute exacerbation requiring hospital admission or physician assessment. Children meeting this definition received ICS treatment for at least 6 months. **Objective asthma** was validated according to European Respiratory Society guidelines, requiring

at least two concurrent objective measures. Airway inflammation was defined as fractional exhaled nitric oxide (FeNO) ≥25 ppb. Bronchial reversibility was defined as an increase in FEV1 of at least 12% or a decrease in specific airway resistance of at least 30% after inhalation of a short-acting β2-agonist. Obstructive lung function was defined as FEV1 <80%, FEV1/FVC <80%, or specific airway resistance >1.6 kPa s. Bronchial hyperreactivity was defined as a decrease in FEV1 of more than 10% during an exercise challenge or a decrease of at least 20% during a methacholine challenge with a provocation dose ≤8 mg/ml.

T2-high asthma was defined as a two-step case definition of asthma, as previously explained[1], accompanied by at least one elevated type 2 biomarker of b-eos (≥ 0.30 10^9/L), FeNO (≥20 ppb), tIgE (≥ 85 kU/L). sIgE (≥ 0.35 kUA/L), or SPT between 6-10 years of age. **T2-low asthma** was then defined similarly but without any type 2 biomarker elevation. [2–4]

1.c: Acute respiratory illnesses

During episodes of acute respiratory illnesses, children were seen at the COPSAC clinic for biosampling of bacterial culture and viral PCR[5]. Hypopharyngeal aspirates were cultured within 24 hours with species-level identification of *Haemophilus influenzae*, *Streptococcus pneumoniae*, and *Moraxella catarrhalis,* among others[6]. Nasopharyngeal aspirates underwent PCR for respiratory viruses, with analyses prespecified to respiratory syncytial virus (RSV) and rhinovirus (RV), including rhinovirus C (RV-C), given their role in asthma development[7].

1.d: Lung function measurements

Spirometry was performed using a MasterScope Pneumoscreen spirometer (Erich Jäeger, Würzburg, Germany), and the airway resistance was measured using a MasterScope Body plethysmograph (Erich Jäeger, Würzburg, Germany) at the ages of 6, 8, and 10 years. For

spirometry, a minimum of three technically acceptable expiratory efforts were required, with a maximum within-test variation in FEV_1 of 10%. The expiration curve had to show a steep and distinct rise, a well-defined peak flow, and a smooth, reproducible shape with a duration of more than 1 second (preferably >2 seconds). The best FEV_1 value from these measurements was selected, and the corresponding FVC and MMEF values from the same blow were used. For sRaw, at least two technically acceptable plethysmography assessments with a within-test variation of ≤0.3 kPa's were required, and the average was used for further analysis. Tests were excluded if flagged as defective in the system or if cooperation was insufficient based on technician evaluation by testing personnel.

Bronchial hyperresponsiveness (BHR) at 6 years was assessed by methacholine challenge. Recordings were required to have an expiration phase longer than 1 second, a clearly defined peak flow on the expiratory curve, a sharp and well-defined start of forced expiration with a smooth curve, and FEV_1 values within 10% (around 0.1 L) of each other. No short-acting β2-agonist use was permitted within 6 hours prior to testing. BHR was expressed as PD_{20}, the cumulative methacholine dose causing a 20% fall in FEV_1 from baseline, calculated from the individual dose-response curve; lower PD_{20} values indicate greater airway responsiveness. At 10 years of age, after the baseline assessment, a bronchodilator was administered either as salbutamol (Ventoline) 0.1 mg per dose via spacer device (four inhalations, each followed by 10 breaths) or terbutaline (Bricanyl) 0.25 mg per dose via Turbuhaler device (four inhalations). Lung function testing was then repeated after 15 minutes to achieve post-bronchial testing.

All lung function values were calibrated by regressing raw measurements on age, height, and sex among children without any asthma between ages 0-10 (recurrent troublesome lung symptoms in

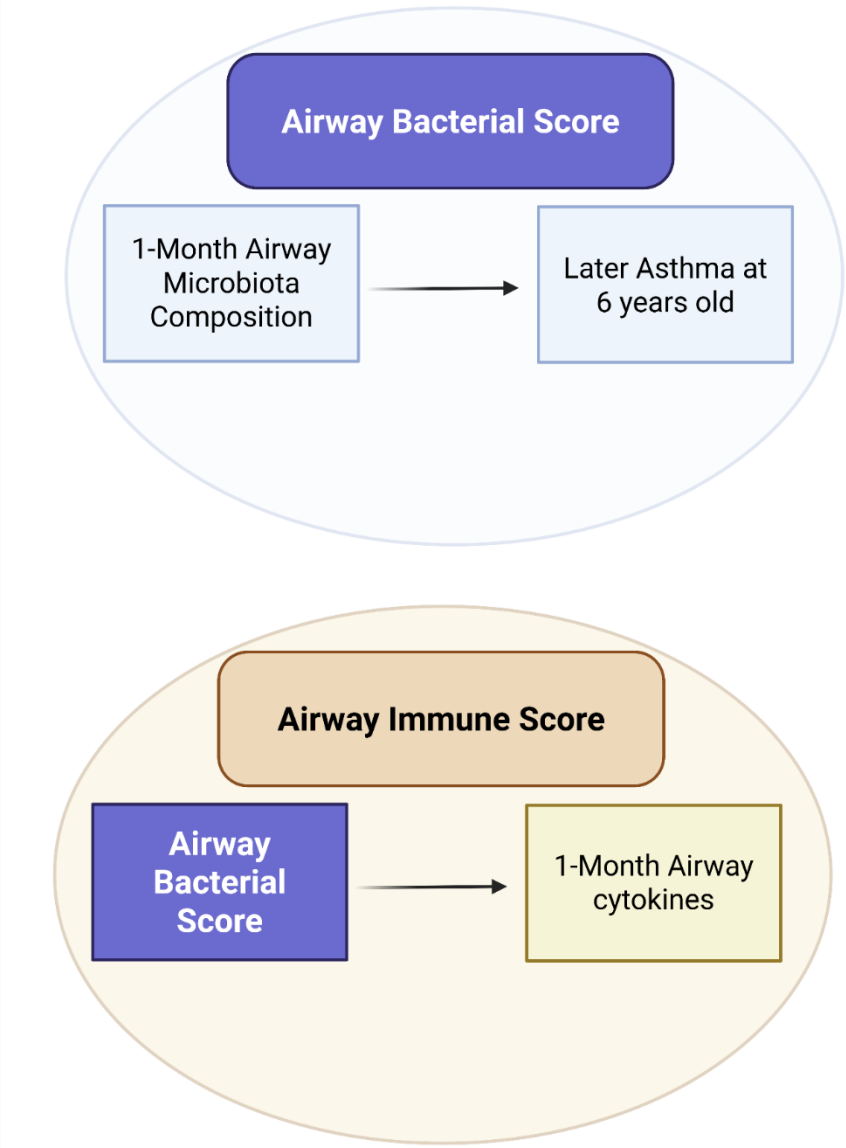
the first 3 years of life). Residuals were scaled using the standard deviation from this healthy reference group to derive z-scores.

1.e: Early life gut immaturity

Gut microbiome maturity was assessed from stool samples collected at 1 week, 1 month, and 1 year of age. Bacterial community composition was profiled by 16S rRNA gene amplicon sequencing of the V4 hypervariable region, using a two-step PCR protocol with 515F/806R primers and dual-indexing, followed by 2 × 250 bp paired-end sequencing on the Illumina MiSeq platform. Sequence data were quality filtered, screened for chimeras, and assigned taxonomy against a curated reference database. A measure of maturation was derived from this: the microbiota-by-age z-score (MAZ), estimating the predicted microbial age from taxonomic profiles relative to a reference cohort and expressing deviations in standard deviation units[8]. The inverse value of this measure s used as a gut immaturity score.

1.f: sPLS supervised airway bacteria score and airway immune score

Two early-life composite scores were generated using supervised sparse partial least squares (sPLS) modeling. The airway bacterial score was derived from the relative abundances of seven bacterial genera in hypopharyngeal aspirates collected at age 1 month, selected for their joint ability to predict asthma by age 6 years. This score represents a microbial composition associated with increased asthma risk. The airway immune score was generated from 18 nasal cytokines measured at age 1 month, using the bacterial score as the predictor. It reflects a cytokine pattern linked to both the bacterial composition and later asthma, but with only ~10% shared variance with the bacterial score, indicating that it captures a partially independent immune signature[9].

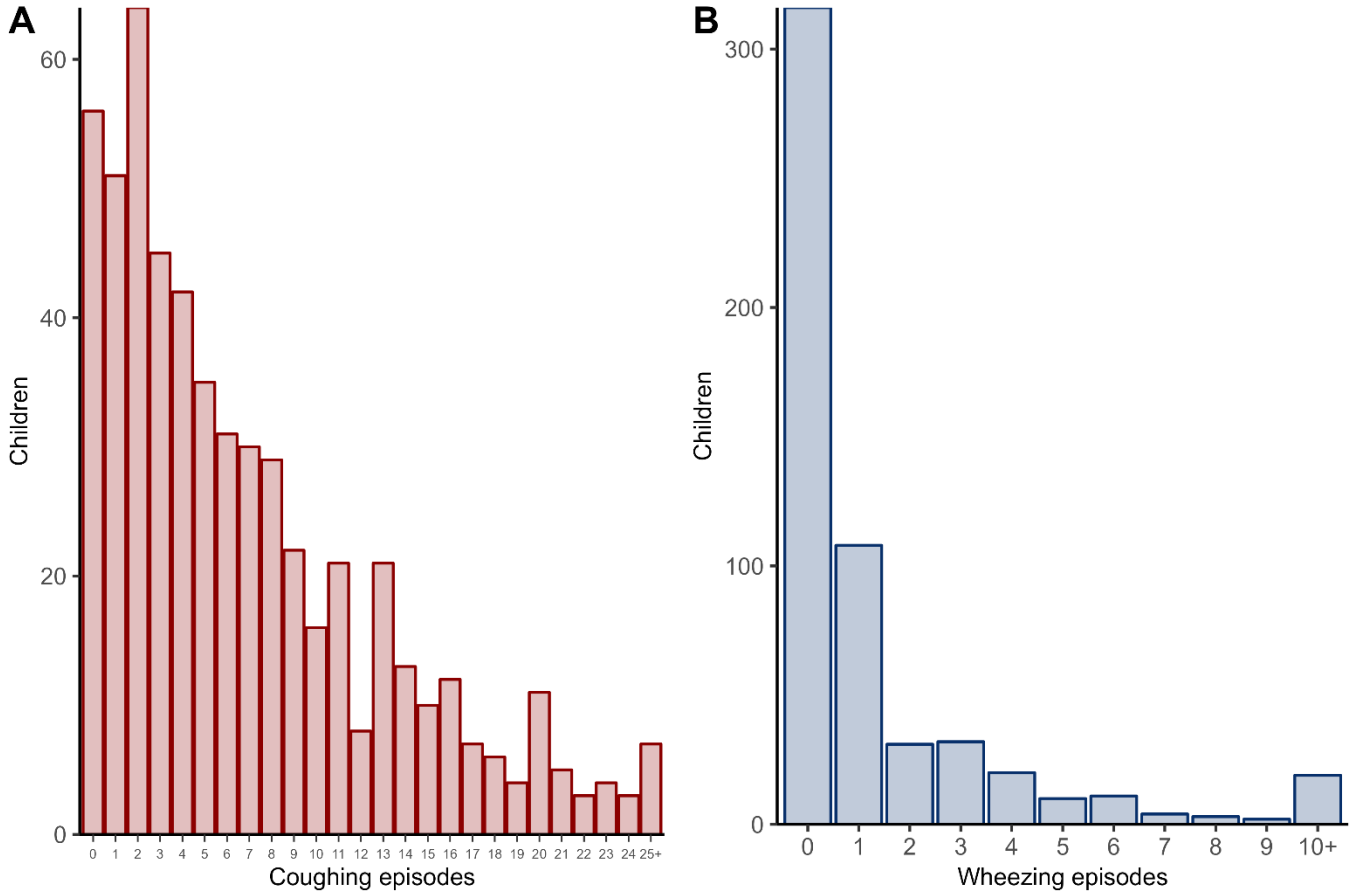


1.g: Genotype and composite SNP scores

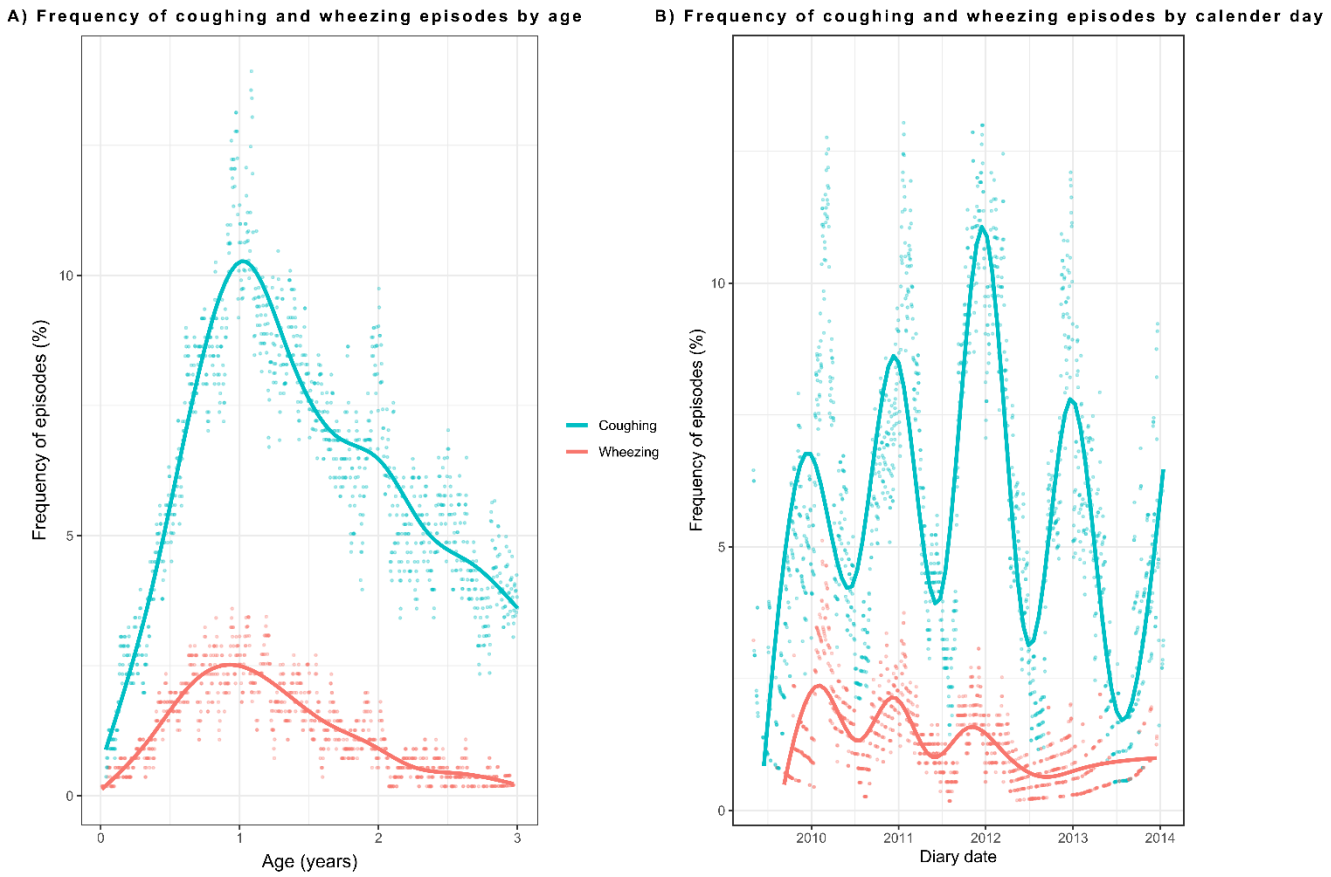
We extracted genotypes for a selection of single-nucleotide polymorphisms (SNPs) previously associated with childhood asthma, covering both non–type 2 (T2) and T2-related loci. Non-T2 SNPs included variants in *GSDMB* (rs2305480) and *CDHR3* (rs6967330). T2 SNPs included variants in *TSLP* (rs1043828), *IL1RL1* (rs10189629), *IL13* (rs20541), and two independent variants in *IL33* (rs1342326, rs340933).

Composite genetic scores were constructed for related loci with shared functional pathways as previously published[10]. A T2 genetic score was defined from variants in *TSLP, IL13, IL1RL1, and IL33*. A non-T2 genetic score was defined from variants in *GSDMB* and *CDHR3*, incorporating their reported epistatic interaction. These genotypes were coded as risk-allele dosages and weighted using published GWAS effect estimates[11]. For each participant, scores were calculated as the sum of weighted risk-allele dosages, with the non-T2 score additionally including a gene-to-gene interaction term. All scores were standardized and centered prior to analysis.

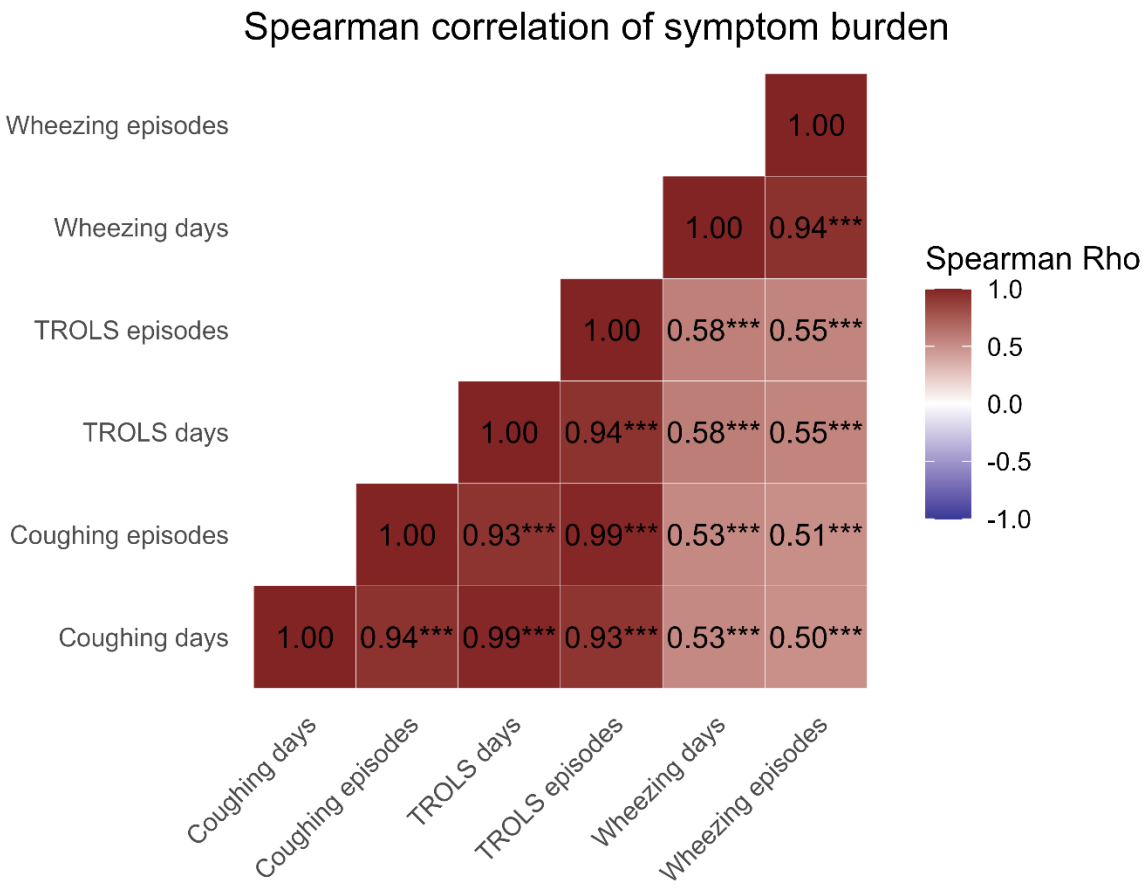
The asthma exacerbation genetic score (PRS-based) was constructed from GWAS summary statistics for severe asthma exacerbations at ages 0–6 years in approximately 70,000 children of European ancestry, combining cohorts of iPSYCH, COPSACsevere (register-based), and Inter99[11]. SNP weights for all polygenic scores were calculated using PRS-CS[12] and then applied in PLINK v1.9[13] to compute a weighted allele-dosage score, which was standardized (mean 0, SD 1), except the dysanapsis score, which used a crude weighted sum based on published SNP weights.



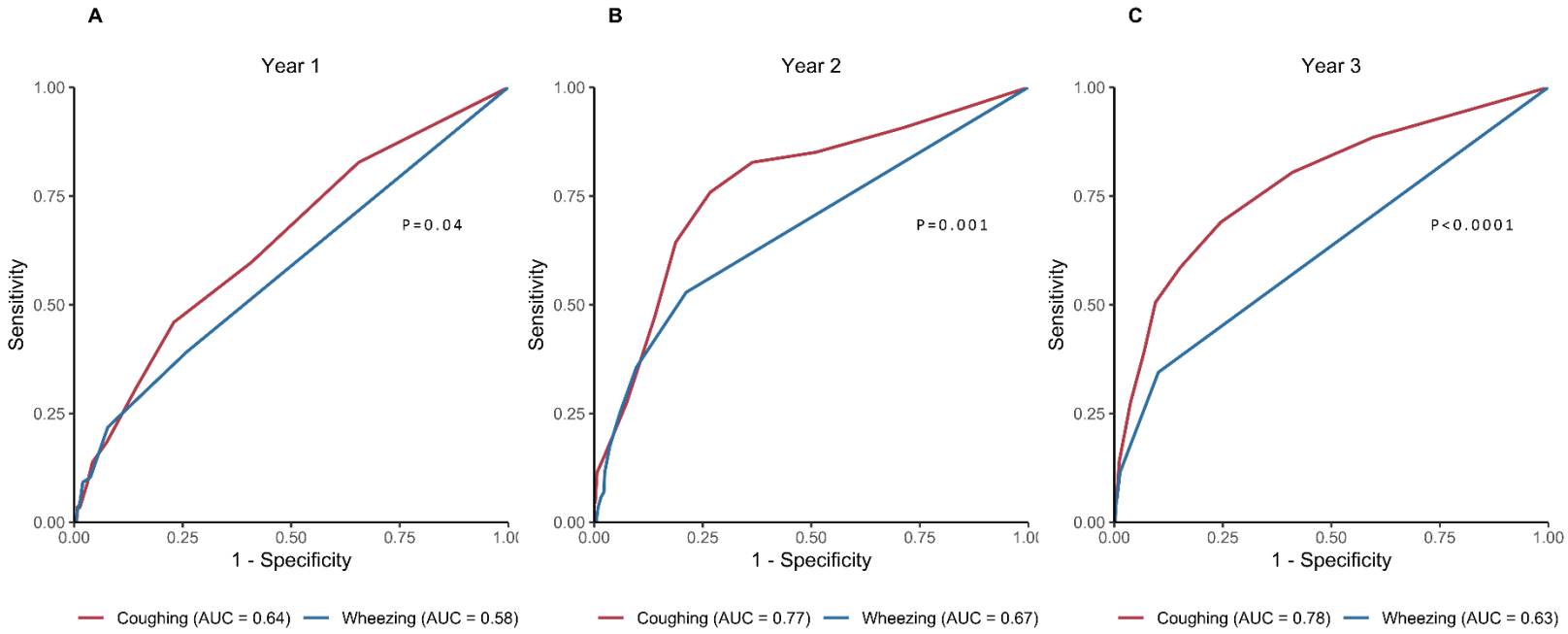
E-Figure 1. Distribution of coughing and wheezing episodes in the first 3 years. *Distribution of coughing (A) and wheezing (B) episodes in the whole study population (n=556).*



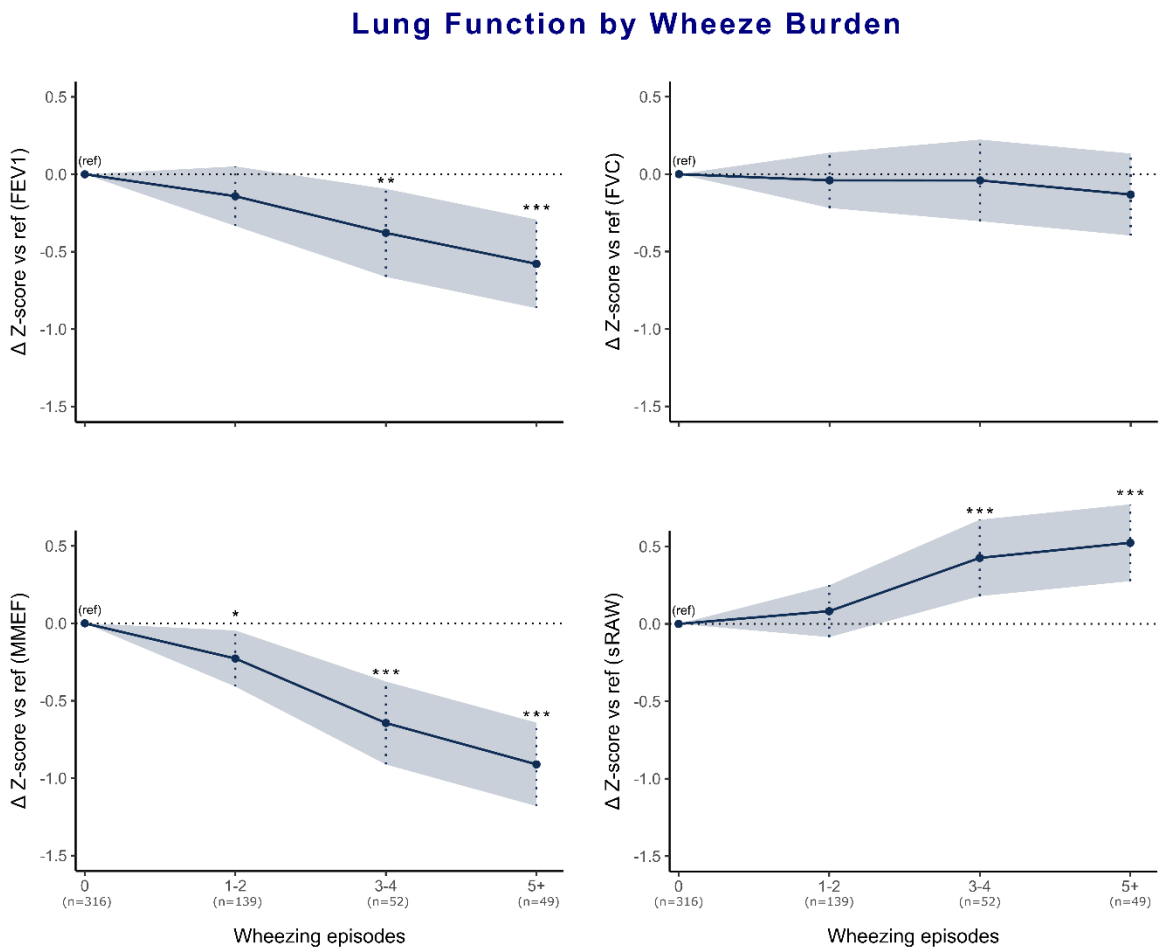
E-Figure 2. Age-wise and seasonal frequency of coughing and wheezing episodes in the first 3 years. ***A)*** *Frequency of coughing and wheezing episodes by age in the first 3 years.* ***B)*** *Temporal distribution of episodes from 2009 to 2014, expressed as the proportion of children with ongoing coughing or wheezing episodes among those with valid diary entries on the corresponding day.*



E-Figure 3. Heatmap of pairwise Spearman rank correlations (rho) among symptom episode and day counts in the first 3 years of life. *Coughing episodes, total of any troublesome lung symptoms (TROLS), and wheezing. Cells display ρ with significance (* p<0.05, ** p<0.01, *** p<0.001). Coughing and total TROLS episodes were highly correlated (ρ=0.99***), whereas wheezing episodes showed only moderate correlations with cough episodes (ρ=0.51***) and total TROLS episodes (ρ=0.55***).*

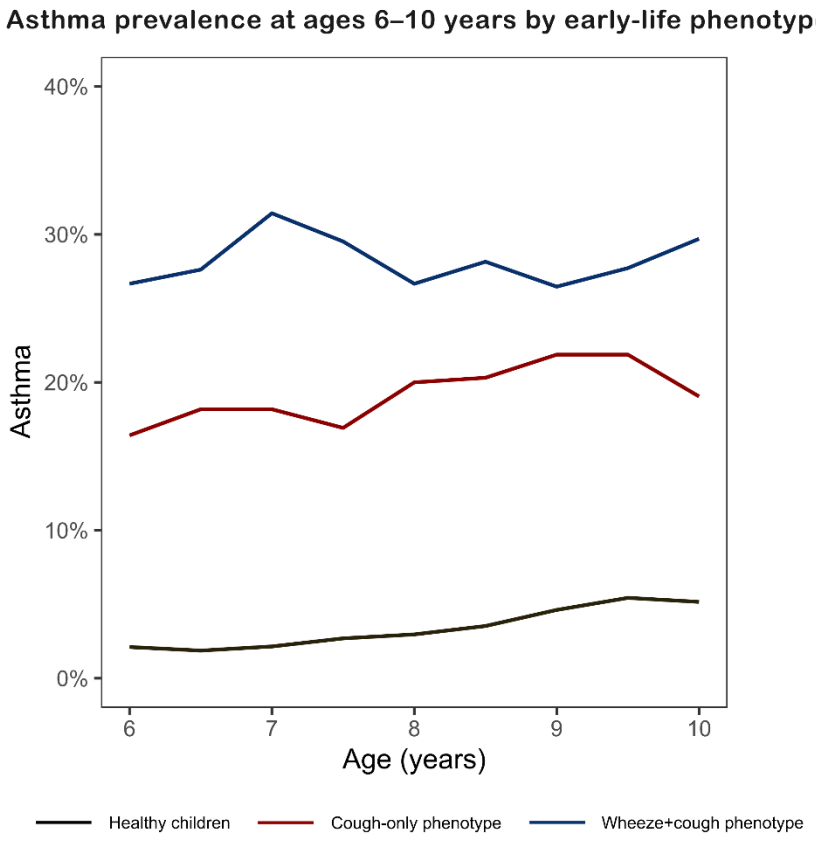


E-Figure 4. Year-specific predictive performance of early-life symptom burden for later asthma. *Receiver operating characteristic (ROC) curves for asthma between 6 and 10 years based on cumulative coughing (red) and wheezing (blue) episode counts within each of the first three years of life (Panels A–C). The area under the curve (AUC) was compared using the DeLong test.*

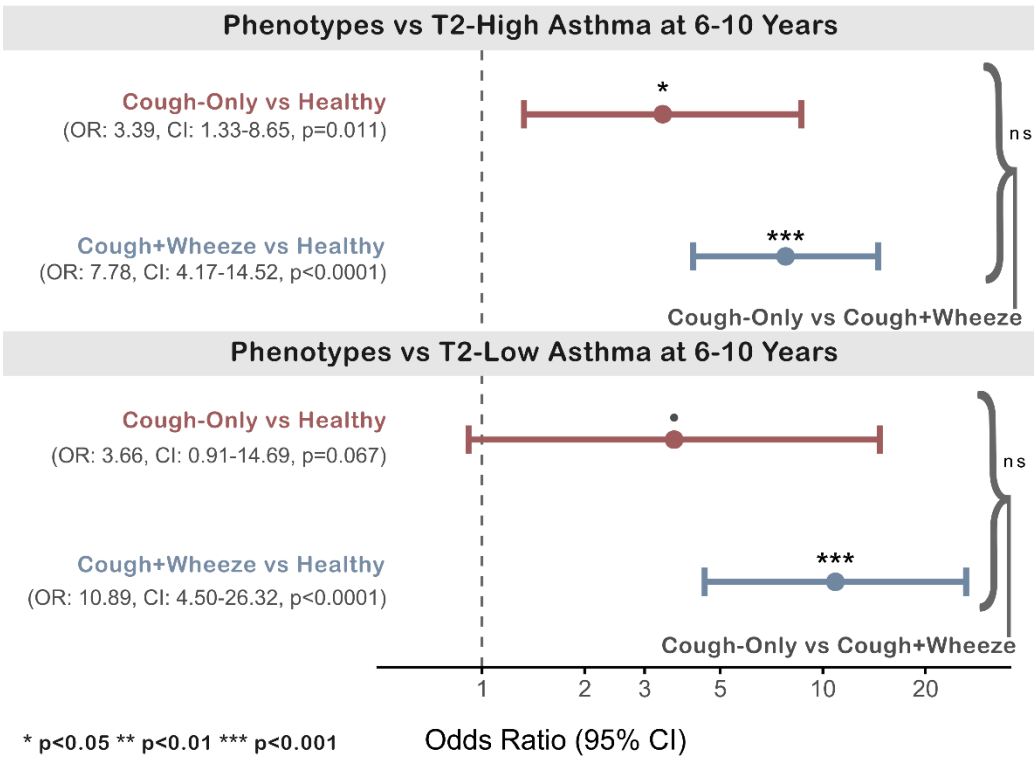


E-Figure 5. Early-life wheeze burden and later lung function. *Estimated mean differences in lung function (FEV_1, FVC, MMEF, and sRaw z-scores) at ages 6-10 years according to cumulative wheeze episodes in the first three years of life. Models include a child random intercept and fixed effects for time at 6, 8, and 10 years. Children were categorized as: 0 episodes (reference, n=316), 1–2 episodes (n=139), 3–4 episodes (n=52), and ≥5 episodes (n=49). Higher sRaw values indicate increased airway resistance. Shaded areas represent 95% confidence intervals. Lines connect category means to aid visual interpretation.*

FEV1, forced expiratory volume in 1 second; FVC, forced vital capacity; MMEF, mid-expiratory flow; sRAW, specific airway resistance.



E-Figure 6. ***Asthma prevalence at ages 6–10 years by early-life phenotype.*** *Asthma prevalence from 6 to 10 years is shown for children with early-life cough-only (n=44), cough+wheeze (n=124), and healthy phenotypes (n=371).*



E-Figure 7. Phenotypes and risk of T2-high/T2-low asthma. *Associations between healthy children (n=365) and early-life cough-only (n=42) and cough+wheeze phenotypes (n=108) and subsequent diagnosis of T2-high (n=58) and T2-low asthma (n=28) at ages 6-10 years. T2-high was defined as ≥1 of: B-eos ≥0.30×10^9/L, FeNO ≥20 ppb, aeroallergen sensitization by specific IgE or SPT, or total IgE ≥85 kU/L, otherwise T2-low.*

$COPSAC_{2010}$ Cohort (Total = 700)	**Included = 556**	**Excluded = 144**	**P-value**
Demographics			
Sex, male	279 (50.18%)	81 (56.25%)	0.19
Ethnicity, Caucasian	534 (96.04%)	136 (94.44%)	0.40
Birth and perinatal characteristics			
Cesarean delivery	111 (19.96%)	40 (27.78%)	**0.042**
Birthweight, g, mean [SD]	3536 (545)	3557 (555)	0.69
Term birth, ≥37 weeks	534 (96.74%)	135 (95.07%)	0.34
Maternal smoking in pregnancy, any	37 (6.65%)	17 (11.81%)	**0.039**
Maternal age at delivery, years, mean [SD]	32.35 (4.17)	32.05 (5.08)	0.46
Parental disposition			
Maternal asthma	145 (26.08%)	46 (31.94%)	0.16
Paternal asthma	116 (21.40%)	26 (18.98%)	0.53
Postnatal characteristics			
Exclusive breastfeeding ≥4 months	318 (57.19%)	73 (50.69%)	0.16
Maternal education at birth* (low/med/high)	37/361/158 (6.65/ 64.93/28.42%)	18/90/36 (12.50/6 2.50/25.00%)	*0.062*
Household income at first year of life** (low/med/high)	42/341/172 (7.57/ 61.4/30.99%)	20/74/40 (14.93 /55.22/29.85%)	**0.027**
Older sibling at birth	314 (56.47%)	81 (56.25%)	0.96

E-Table 1. Baseline characteristics of included versus excluded children in $COPSAC_{2010}$. *Variables (n [%] unless stated). Percentages use non-missing denominators within each group. P-values compare included vs excluded using chi-square tests or Fisher's exact for categorical variables and Student's two-sample t tests for continuous variables (reported as mean [SD]).*

**Maternal education was categorized as Low (elementary school or college graduate), Medium (tradesman or medium length) and High (university candidate).*

***Household income as Low (<55,000 EUR/year), Medium (55,000–110,000 EUR/year) and High (>110,000 EUR/year).*

	Objectively validated asthma (n=67)		T2-high asthma (n=58)		T2-low asthma (n=28)	
Exposure	OR (95% CI)	P-value	OR (95% CI)	P-value	OR (95% CI)	P-value
Coughing episodes	1.19 (1.14; 1.24)	**<0.0001**	1.18 (1.13; 1.23)	**<0.0001**	1.20 (1.13; 1.28)	**<0.0001**
Coughing episodes (no-wheezing subset)	1.13 (1.04; 1.23)	**0.004**	1.15 (1.06; 2.04)	**0.001**	1.15 (1.01; 1.30)	**0.034**

E-Table 2. Association of early-life cough burden with later asthma (sensitivity analyses). *Odds ratios (ORs) with 95% confidence intervals (CIs) and P-values from logistic regression for asthma between 6 and 10 years of age, stratified by objectively validated asthma, T2-high asthma vs healthy and T2-low asthma vs healthy. The predictor is cough burden from 0 to 3 years, modeled per one additional episode. The no-wheeze subset analysis is restricted to children without any wheeze episodes from 0 to 3 years. All models are adjusted for sex. Objectively validated asthma requires at least two of the following: obstructive lung function, bronchial reversibility, bronchial hyperreactivity, or airway inflammation. Likewise, association also association with T2-high vs healthy and T2-low asthma vs healthy at ages 6 and 10 years. T2-high (N=58) was defined as ≥1 of: B-eos ≥0.30×10^9/L, FeNO ≥20 ppb, aeroallergen sensitization by specific IgE or SPT, or total IgE ≥85 kU/L, otherwise T2-low (N=28)*[1].

Wheeze burden 0-3 years and later lung function adjusted for genetic risk (PRS)								
	Unadjusted		**Genetics-adjusted**		**No-asthma subset (unadjusted)**		**No-asthma subset (Genetics-adjusted)**	
Outcome	β (95% CI)	P-value	β (95% CI)	P-value	β (95% CI)	P-value	β (95% CI)	P-value
FEV1	-0.06 (-0.09; -0.03)	**<0.0001**	-0.05 (-0.08; -0.02)	**0.0003**	-0.08 (-0.11; -0.04)	**<0.0001**	-0.07 (-0.10; -0.03)	**0.0001**
FVC	-0.01 (-0.04; 0.01)	0.36	-0.01 (-0.04; 0.01)	0.36	-0.03 (-0.06; 0.01)	*0.056*	-0.03 (-0.06; 0.01)	0.12
FEV1/FVC	-0.08 (-0.11; -0.06)	**<0.0001**	-0.07 (-0.10; -0.05)	**<0.0001**	-0.07 (-0.11; -0.05)	**<0.0001**	-0.07 (-0.10; -0.04)	**<0.0001**
MMEF	-0.09 (-0.12; -0.07)	**<0.0001**	-0.08 (-0.11; -0.06)	**<0.0001**	-0.10 (-0.13; -0.07)	**<0.0001**	-0.09 (-0.12; -0.06)	**<0.0001**
sRAW	0.06 (0.04; 0.08)	**<0.0001**	0.06 (0.03; 0.08)	**<0.0001**	0.06 (0.03; 0.09)	**0.0002**	0.05 (0.02; 0.08)	**0.0004**

E-Table 3. **Wheeze burden 0-3 years and later lung function adjusted by genetic risk (PRS).** *Wheeze burden from 0–3 years in relation to lung function at 6, 8, and 10 years. Linear mixed models with a child random intercept and fixed effects for age (6/8/10 years). Exposure was wheeze burden (0–3 years) as episodes. Four models are shown: unadjusted, adjusted for polygenic risk score (PRS) of COPD and dysanapsis, restricted to the no-asthma subset (unadjusted), and restricted to the no-asthma subset with PRS adjustment. Beta (β) represents the change in lung function z-score per 1-unit increase in wheeze burden (95% confidence interval (CI)).*

FEV1, forced expiratory volume in 1 second; FVC, forced vital capacity; MMEF, mid-expiratory flow; sRAW, specific airway resistance.

Wheeze burden from 0 to 3 years and lung function at 10 years, pre- and post-bronchodilator (Study population)						
	Pre-bronchodilator		**Post-bronchodilator**		**Reversibility (%)**	
Outcome	β (95% CI)	P-value	β (95% CI)	P-value	β (95% CI)	P-value
FEV1	-0.06 (-0.09; -0.03)	**0.0001**	-0.03 (-0.06; 0.00)	**0.024**	0.33 (0.16; 0.50)	**<0.0001**
FVC	-0.01 (-0.04; 0.02)	0.42	-0.01 (-0.04; 0.02)	0.41	-0.04 (-0.22; 0.15)	0.71
FEV1/FVC	-0.08 (-0.11; -0.05)	**<0.0001**	-0.05 (-0.08; -0.01)	**0.006**	0.29 (0.14; 0.44)	**0.0002**
MMEF	-0.09 (-0.12; -0.06)	**<0.0001**	-0.07 (-0.10; -0.04)	**<0.0001**	0.87 (0.28; 1.50)	**0.004**
sRAW	0.06 (0.03; 0.10)	**0.0001**	-0.01 (-0.04; 0.02)	0.62	-1.00 (-1.4; -0.58)	**<0.0001**
Wheeze burden from 0 to 3 years and lung function at 10 years, pre- and post-bronchodilator (No-asthma subset)						
	Pre-bronchodilator		**Post-bronchodilator**		**Reversibility (%)**	
Outcome	β (95% CI)	P-value	β (95% CI)	P-value	β (95% CI)	P-value
FEV1	-0.07 (-0.11; -0.03)	**0.0003**	-0.05 (-0.09; -0.01)	**0.015**	0.29 (0.08; 0.50)	**0.007**
FVC	-0.03 (-0.07; 0.02)	0.22	-0.03 (-0.07; 0.01)	0.19	-0.04 (-0.29; 0.21)	0.73
FEV1/FVC	-0.08 (-0.12; -0.04)	**0.0001**	-0.05 (-0.09; -0.01)	**0.021**	0.26 (0.07; 0.46)	**0.008**
MMEF	-0.09 (-0.13; -0.05)	**<0.0001**	-0.08 (-0.12; -0.04)	**<0.0001**	0.71 (-0.52; 1.47)	*0.068*
sRAW	0.06 (0.02; 0.10)	**0.004**	0.00 (-0.04; 0.04)	1.00	-0.86 (-1.40; -0.40)	**0.001**

E-Table 4 Wheeze burden from 0 to 3 years and lung function at 10 years, pre- and post-bronchodilator. *Wheeze burden was modelled continuously, and β represents the mean change in lung function per 1 additional wheezing episode as 95% confidence interval (CI). Analyses were restricted to children with both pre- and post-bronchodilator measurements at age 10 years. Reversibility outcomes were expressed as percentage change for FEV1, FVC, MMEF, and sRaw, and as absolute change in percentage points for FEV1/FVC. Results are shown for the full study population and for a subset of no-asthma between 6-10 years.*

FEV1, forced expiratory volume in 1 second; FVC, forced vital capacity; MMEF, mid-expiratory flow; sRAW, specific airway resistance.

Cough burden 0-3 years and later lung function by year						
	Year 1		**Year 2**		**Year 3**	
Outcome	β (95% CI)	P-value	β (95% CI)	P-value	β (95% CI)	P-value
FEV1	0.00 (-0.05; 0.05)	0.96	0.00 (-0.03; 0.02)	0.76	-0.02 (-0.05; 0.02)	0.40
FVC	0.01 (-0.03; 0.06)	0.57	0.00 (-0.03; 0.03)	0.94	-0.01 (-0.04; 0.02)	0.53
FEV1/FVC	-0.01 (-0.05; 0.03)	0.52	-0.01 (-0.03; 0.02)	0.45	-0.01 (-0.04; 0.02)	0.68
MMEF	0.00 (-0.05; 0.04)	0.86	-0.01 (-0.04; 0.02)	0.39	-0.02 (-0.06; 0.01)	0.18
sRAW	0.00 (-0.04; 0.04)	0.86	0.00 (-0.03; 0.02)	0.75	0.00 (-0.03; 0.03)	0.96
Wheeze burden 0-3 years and later lung function by year						
	Year 1		**Year 2**		**Year 3**	
Outcome	β (95% CI)	P-value	β (95% CI)	P-value	β (95% CI)	P-value
FEV1	-0.09 (-0.18; -0.01)	**0.038**	-0.11 (-0.17; -0.05)	**0.0001**	-0.14 (-0.24; -0.03)	**0.011**
FVC	-0.01 (-0.09; 0.07)	0.77	-0.03 (-0.09; 0.02)	0.19	-0.01 (-0.11; 0.08)	0.82
FEV1/FVC	-0.15 (-0.22; -0.07)	**0.0001**	-0.13 (-0.18; -0.09)	**<0.0001**	-0.23 (-0.32; -0.14)	**<0.0001**
MMEF	-0.18 (-0.27; -0.10)	**<0.0001**	-0.15 (-0.18; -0.09)	**<0.0001**	-0.22 (-0.32; -0.12)	**<0.0001**
sRAW	0.10 (0.02; 0.18)	**0.01**	0.12 (0.07; 0.17)	**<0.0001**	0.15 (0.06; 0.25)	**0.0008**

E-Table 5. Coughing and wheezing episodes in early life in relation to later lung function outcomes by year. *Linear regression models with outcomes measured at 6, 8, and 10 years. Exposures were the number of coughing and wheezing episodes recorded in each year of the first three years of life. Models were mutually adjusted for both symptom episodes within the same year. Beta (β) represents the change in lung function z-score per 1-episode increase in coughing or wheezing (95% confidence interval (CI)).*

FEV1, forced expiratory volume in 1 second; FVC, forced vital capacity; MMEF, mid-expiratory flow; sRAW, specific airway resistance.

Association of ordered coughing episodes and later lung function (unadjusted)						
	Category 2 vs 1 (ref)		**Category 3 vs 1 (ref)**		**Category 4 vs 1 (ref)**	
Outcome	β (95% CI)	P-value	β (95% CI)	P-value	β (95% CI)	P-value
FEV1	-0.10 (-0.32; 0.11)	0.34	-0.18 (-0.39; 0.03)	0.09	-0.31 (-0.58; -0.05)	**0.021**
FVC	-0.08 (-0.27; 0.12)	0.45	0.01 (-0.18; 0.21)	0.89	-0.11 (-0.35; 0.14)	0.39
FEV1/FVC	-0.05 (-0.24; 0.13)	0.57	-0.34 (-0.52; -0.15)	**0.0003**	-0.36 (-0.60; -0.13)	**0.002**
MMEF	-0.10 (-0.31; 0.10)	0.33	-0.34 (-0.54; -0.14)	**0.001**	-0.44 (-0.70; -0.18)	**0.0008**
sRAW	0.15 (-0.04; 0.34)	0.12	0.29 (0.11; 0.47)	**0.002**	0.20 (-0.03; 0.43)	0.09
Association of ordered coughing episodes and later lung function adjusted for wheezing						
	Category 2 vs 1 (ref)		**Category 3 vs 1 (ref)**		**Category 4 vs 1 (ref)**	
Outcome	β (95% CI)	P-value	β (95% CI)	P-value	β (95% CI)	P-value
FEV1	-0.08 (-0.29; 0.13)	0.47	-0.09 (-0.30; 0.13)	0.42	-0.07 (-0.37; 0.23)	0.66
FVC	-0.07 (-0.27; 0.12)	0.48	0.03 (-0.17; 0.23)	0.75	-0.06 (-0.33; 0.22)	0.69
FEV1/FVC	-0.02 (-0.20; 0.16)	0.85	-0.20 (-0.39; -0.02)	**0.031**	0.00 (-0.25; 0.25)	0.99
MMEF	-0.06 (-0.26; 0.14)	0.55	-0.19 (-0.40; 0.01)	*0.063*	-0.04 (-0.32; 0.24)	0.77
sRAW	0.12 (-0.06; 0.30)	0.20	0.18 (0.00; 0.37)	*0.055*	-0.08 (-0.34; 0.17)	0.52

E-Table 6. **Ordered coughing episode categories and later lung function.** *Associations of ordered cough episode categories with lung function z-scores at ages 6, 8 and 10. Models were linear mixed-effects models including a child random intercept and fixed effects for time. Coughing episode categories were defined by the cumulative number of coughing episodes in the first three years of life: Category 1 (reference): 0–2 episodes (n=171), Category 2: 3–6 episodes (n=153), Category 3: 7–14 episodes (n=160), Category 4: ≥15 episodes (n=72). Beta (β) is the difference vs reference (95% CI).*

FEV1, forced expiratory volume in 1 second; FVC, forced vital capacity; MMEF, mid-expiratory flow; sRAW, specific airway resistance.

Association of ordered wheezing episodes and later lung function (unadjusted)						
	Category 2 vs 1 (ref)		**Category 3 vs 1 (ref)**		**Category 4 vs 1 (ref)**	
Outcome	β (95% CI)	P-value	β (95% CI)	P-value	β (95% CI)	P-value
FEV1	-0.14 (-0.33; 0.05)	0.15	-0.38 (-0.66; -0.09)	**0.009**	-0.58 (-0.87; -0.29)	**<0.0001**
FVC	-0.04 (-0.22; 0.14)	0.66	-0.04 (-0.30; 0.22)	0.76	-0.13 (-0.40; 0.13)	0.33
FEV1/FVC	-0.18 (-0.35; -0.02)	**0.028**	-0.60 (-0.84; -0.36)	**<0.0001**	-0.81 (-1.05; -0.57)	**<0.0001**
MMEF	-0.23 (-0.41; -0.05)	**0.014**	-0.64 (-0.91; -0.38)	**<0.0001**	-0.91 (-1.18; -0.64)	**<0.0001**
sRAW	0.08 (-0.09; 0.26)	0.34	0.43 (0.18; 0.67)	**0.0007**	0.52 (0.28; 0.77)	**<0.0001**
Association of ordered wheezing episodes and later lung function adjusted for coughing						
	Category 2 vs 1 (ref)		**Category 3 vs 1 (ref)**		**Category 4 vs 1 (ref)**	
Outcome	β (95% CI)	P-value	β (95% CI)	P-value	β (95% CI)	P-value
FEV1	-0.15 (-0.34; 0.05)	0.15	-0.39 (-0.70; -0.09)	**0.012**	-0.59 (-0.92; -0.27)	**0.0004**
FVC	-0.04 (-0.23; 0.14)	0.65	-0.05 (-0.33; 0.24)	0.75	-0.14 (-0.44; 0.17)	0.37
FEV1/FVC	-0.19 (-0.36; -0.02)	**0.025**	-0.62 (-0.88; -0.36)	**<0.0001**	-0.84 (-1.12; -0.56)	**<0.0001**
MMEF	-0.23 (-0.42; -0.04)	**0.015**	-0.65 (-0.94; -0.36)	**<0.0001**	-0.92 (-1.23; -0.61)	**<0.0001**
sRAW	0.10 (-0.07; 0.27)	0.27	0.47 (0.20; 0.73)	**0.0006**	0.58 (0.30; 0.87)	**<0.0001**

E-Table 7. Ordered wheezing episode categories and later lung function. *Associations of ordered wheezing episode categories with lung function z-scores at ages 6, 8 and 10. Models were linear mixed-effects models including a child random intercept and fixed effects for time. Wheezing episode categories were defined by the cumulative number of wheeze episodes in the first three years of life: Category 1 (reference): 0 episodes (n=316), Category 2: 1–2 episodes (n=139), Category 3: 3–4 episodes (n=52), Category 4: ≥5 episodes (n=49). Beta (β) is the difference vs reference (95% CI).*

FEV1, forced expiratory volume in 1 second; FVC, forced vital capacity; MMEF, mid-expiratory flow; sRAW, specific airway resistance.

	Healthy (n=381)	Cough-Only (n=46)	Cough+Wheeze (n=129)
Daily Diary Recordings	Median (IQR)	Median (IQR)	Median (IQR)
Days of β2-agonist use	—	68 (23; 91)	95 (48; 160)
Days of cough	23 (8; 50)	128 (97; 158)	144 (86; 202)
Days of coughing without wheezing	20 (7; 45)	128 (97; 158)	103 (59; 163)
Days of wheezing	0 (0; 5)	0 (0; 0)	26 (11; 51)
Days of breathlessness	0 (0; 1)	0 (0; 0)	12 (2; 40)
Days with any symptoms	24 (8; 53)	131 (97; 162)	159 (99; 223)
Episodes of coughing	3 (1; 6)	12 (9; 15)	14 (9; 18)
Episodes of wheezing	0 (0; 1)	0 (0; 0)	3 (1; 6)
Episodes of breathlessness	0 (0; 0)	0 (0; 0)	2 (0; 5)
Episodes with any TROLS	3 (1; 6)	12 (9; 15)	15 (11; 19)
Episode duration of any TROLS	6 (4; 8)	7 (6; 10)	7 (6; 9)
Age (days) at first TROLS episode	264 (151; 389)	296 (155; 372)	175 (100; 277)

E-Table 8. Early-life symptom burden during the first 3 years by phenotype. *Daily diary recordings derived days and episodes of coughing, wheezing, and breathlessness. Troublesome lung symptoms (TROLS) indicates any day with coughing, wheezing or breathlessness. Values are median (IQR). Episodes were defined as ≥3 consecutive symptom days with ≥3 symptom-free days between episodes. Episode duration and age at first episode were calculated among children with ≥1 episode.*

	Cough-Only vs Healthy		Cough+Wheeze vs Healthy		Cough-only vs Cough+Wheeze	
Outcome	β (95% CI)	P-value	β (95% CI)	P-value	β (95% CI)	P-value
FEV1	0.03 (-0.27; 0.33)	0.86	-0.32 (-0.51; -0.13)	**0.001**	0.35 (0.02; 0.67)	**0.038**
FVC	-0.02 (-0.29; 0.25)	0.88	-0.06 (-0.24; 0.11)	0.48	0.04 (-0.26; 0.34)	0.78
FEV1/FVC (pre-BD)	0.08 (-0.17; 0.34)	0.52	-0.43 (-0.59; -0.26)	**<0.0001**	0.51 (0.23; 0.79)	**0.0004**
FEV1/FVC (post-BD)*	0.10 (-0.26; 0.46)	0.59	-0.25 (-0.48; -0.01)	**0.039**	0.34 (-0.05; 0.74)	0.09
MMEF	0.08 (-0.20; 0.37)	0.56	-0.50 (-0.68; -0.31)	**<0.0001**	0.58 (0.27; 0.89)	**0.0003**
sRAW	-0.08 (-0.33; 0.18)	0.56	0.34 (0.17; 0.50)	**0.0001**	-0.41 (-0.70; -0.13)	**0.004**
BHR**	-0.05 (-0.40; 0.31)	0.79	-0.30 (-0.54; -0.06)	**0.016**	0.25 (-0.23; 0.65)	0.21

E-Table 9. Age-averaged group differences in lung function z-scores between phenotypes. *Estimates are mean differences in standard deviation (SD) between phenotypes of cough-only (n=44), cough+wheeze (n=126), and healthy (n=369) averaged over ages 6, 8 and 10. P-values from linear mixed models with child random intercepts and fixed effects of group and age.*

**Post-BD models are single timepoint linear regression analyses at 10 years of age adjusted for sex*

***BHR models are single timepoint linear regression analyses at age 6 years based on log-transformed PD_{20} adjusted for sex, age, and height instead of calibration as done for other lung function outcomes*

BD, bronchodilator; BHR, Bronchial hyperresponsiveness; FEV1, forced expiratory volume in 1 second; FVC, forced vital capacity; MMEF, mid-expiratory flow; PD20, PD 20 (the provocative dose of methacholine that results in a 20% fall in FEV1; sRAW, specific airway resistance; BHR, bronchial hyperresponsiveness

Association between phenotypes and type 2 biomarkers							
		Cough-Only vs Healthy		**Cough+Wheeze vs Healthy**		**Cough-Only vs Cough+Wheeze**	
Time point	**Outcome**	β (95% CI)	P-value	β (95% CI)	P-value	β (95% CI)	P-value
6 years							
	B-eos	1.00 (0.73; 1.37)	0.98	1.00 (0.82; 1.22)	0.98	0.99 (0.71; 1.40)	0.97
	sIgE*	0.56 (0.18; 1.39)	0.25	1.12 (0.65; 1.90)	0.67	0.49 (0.15; 1.33)	0.19
	SPT*	0.69 (0.11; 2.50)	0.62	1.41 (0.64; 2.96)	0.38	0.49 (0.07; 1.96)	0.37
	FeNO	0.98 (0.72; 1.33)	0.89	0.99 (0.82; 1.20)	0.93	0.99 (0.71; 1.38)	0.94
8 years							
	FeNO	0.96 (0.77; 1.19)	0.70	0.94 (0.81; 1.09)	0.39	0.86 (0.67; 1.10)	0.23
10 years							
	B-eos	0.92 (0.70; 1.21)	0.55	1.03 (0.86; 1.23)	0.75	0.89 (0.66; 1.21)	0.47
	sIgE*	1.13 (0.54; 2.31)	0.74	1.49 (0.94; 2.36)	0.09	0.76 (0.34; 1.65)	0.49
	SPT*	0.71 (0.29; 1.58)	0.42	1.24 (0.76; 2.01)	0.38	0.57 (0.22; 1.36)	0.22
	FeNO	0.82 (0.65; 1.03)	0.08	0.95 (0.82; 1.11)	0.52	0.86 (0.67; 1.10)	0.23
	tIgE	0.97 (0.56; 1.68)	0.92	1.06 (0.75; 1.50)	0.74	0.92 (0.50; 1.67)	0.77

E-Table 10. Association between early-life phenotypes and type 2 biomarkers. *Phenotypes of cough-only (n=44), cough+wheeze (n=126) and healthy (n=369) were assessed in relation to type 2 biomarkers. Linear regression models were performed with log-transformed biomarker concentrations as outcomes and adjusted for sex. Results are presented as geometric mean ratios (GMRs) with 95% confidence intervals(CI), derived from exponentiated model coefficients.*

** Indicates binary outcomes, which were analyzed using logistic regression. Results are presented as odds ratios (ORs) with 95% confidence intervals.*

B-eos, blood eosinophils; FeNO, fractional exhaled nitric oxide; sIgE, specific immunoglobulin E; tIgE, total immunoglobulin E; SPT, skin prick test

Risk factors by phenotype									
	Healthy	**Cough-Only**	**Cough-Only vs Healthy**		**Cough+ Wheeze**	**Cough+Wheeze vs Healthy**		**Cough-Only vs Cough+Wheeze**	
Risk factors	n = 381	n = 46	OR (95% CI)	P-value	n = 129	OR (95% CI)	P-value	OR (95% CI)	P-value
Sex, male	178 (46.7%)	29 (63.0%)	1.94 (1.03; 3.66)	**0.039**	72 (55.8%)	1.44 (0.96; 2.15)	0.08	1.35 (0.68; 2.70)	0.40
Ethnicity, caucasian	364 (95.5%)	44 (95.7%)	1.03 (0.23; 4.64)	0.97	126 (97.7%)	1.97 (0.57; 6.84)	0.29	0.52 (0.08; 3.25)	0.49
Cesarean delivery	73 (19.2%)	8 (16.4%)	0.86 (0.38; 1.93)	0.72	30 (23.3%)	1.26 (0.78; 2.04)	0.35	0.69 (0.29; 1.63)	0.39
Gestational age, weeks (mean [SD])	40.0 (1.6)	40.1 (1.4)	1.06 (0.86; 1.29)	0.59	39.5 (2.1)	0.86 (0.77; 0.96)	**0.008**	1.23 (0.99; 1.52)	*0.058*
Birthweight, per 100g (mean [SD])	3571 (527)	3470 (514)	0.96 (0.91; 1.02)	0.18	3457 (601)	0.96 (0.93; 1.00)	**0.031**	1.00 (0.94; 1.06)	0.93
Term birth ≥37 wks	369 (97.9%)	45 (97.8%)	1.00 (0.12; 8.21)	1.00	120 (93.0%)	0.29 (0.11; 0.78)	**0.014**	3.41 (0.42; 27.72)	0.25
Maternal smoking in pregnancy	18 (4.7%)	4 (8.7%)	1.89 (0.61; 5.89)	0.27	15 (11.6%)	2.63 (1.28; 5.41)	**0.008**	0.72 (0.23; 2.29)	0.58
Antibiotics during pregnancy	128 (33.7%)	21 (45.7%)	1.62 (0.87; 3.01)	0.13	49 (38.0%)	1.19 (0.79; 1.80)	0.41	1.36 (0.69; 2.69)	0.38
Maternal age at delivery, y	32.4 (4.0)	32.2 (4.4)	0.99 (0.92; 1.07)	0.85	32.4 (4.47)	1.00 (0.95; 1.05)	0.98	0.99 (0.92; 1.08)	0.87
Exclusive breastfeeding ≥4 mo	223 (58.5%)	25 (54.3%)	0.86 (0.46; 1.59)	0.62	70 (54.3%)	0.85 (0.57; 1.27)	0.42	1.01 (0.51; 1.99)	0.98
Older sibling at birth	219 (57.5%)	18 (39.1%)	0.48 (0.25; 0.89)	**0.021**	77 (59.7%)	1.10 (0.73; 1.65)	0.66	0.43 (0.22; 0.87)	**0.017**
Dog at birth	83 (21.8%)	4 (8.7%)	0.33 (0.11; 0.95)	**0.040**	25 (19.4%)	0.85 (0.51; 1.40)	0.51	0.39 (0.13; 1.19)	0.10
Cat at birth	85 (22.3%)	6 (13.0%)	0.51 (0.21; 1.25)	0.14	26 (20.2%)	0.87 (0.53; 1.42)	0.58	0.59 (0.23; 1.54)	0.28
Maternal asthma	86 (22.6%)	11 (23.9%)	1.10 (0.53; 2.26)	0.80	48 (37.2%)	2.05 (1.33; 3.16)	**0.001**	0.53 (0.25; 1.15)	0.11
Paternal asthma	69 (18.6%)	9 (20.0%)	1.11 (0.51; 2.43)	0.79	38 (29.9%)	1.88 (1.18; 2.99)	**0.007**	0.59 (0.26; 1.35)	0.21

Passive smoking 0–3 y	174 (45.9%)	24 (52.2%)	1.34 (0.72; 2.49)	0.35	73 (56.6%)	1.58 (1.05; 2.36)	**0.028**	0.85 (0.43; 1.68)	0.64
Age at daycare start, y (mean [SD])	0.90 (0.25)	0.89 (0.20)	0.85 (0.23; 3.11)	0.81	0.90 (0.23)	0.93 (0.41; 2.13)	0.86	0.92 (0.22; 3.78)	0.90
Maternal education at birth* (Low/Med/High)	21/245/115 (5.5%, 64.3%, 30.2%)	5/30/11 (10.9%, 65.2%, 23.9%)	Low vs Medium 2.13 (0.74; 6.13); High vs Medium 0.80 (0.39; 1.67)	Low: 0.16; High: 0.56	11/86/32 (8.5%, 66.7%, 24.8%)	Low vs Medium 1.57 (0.72; 3.40); High vs Medium 0.81 (0.51; 1.28)	Low: 0.25; High: 0.36	Low vs Medium 1.36 (0.43; 4.25); High vs Medium 1.00 (0.45; 2.23)	Low: 0.60; High: 1.00
Household income (Low/Med/High)**	31/229/120 (8.1%, 60.3%, 31.6%)	2/28/16 (4.3%, 60.9%, 34.8%)	Low vs Med 0.54 (0.12; 2.41); High vs Med 1.09 (0.57; 2.11)	Low: 0.42; High: 0.79	9/84/36 (7.0%, 65.1%, 27.9%)	Low vs Medium 0.81 (0.37; 1.77); High vs Medium 0.82 (0.52; 1.29)	Low: 0.59; High: 0.39	Low vs Medium 0.68 (0.14; 3.32); High vs Medium 1.34 (0.64; 2.77)	Low: 0.63; High: 0.44

E-Table 11. Risk factors by phenotype with pairwise comparisons. *Categorical values are n (percentage); continuous are mean (SD). Pairwise results are OR (95% CI). Multinomial logistic models adjusted for sex, except the sex row.*

**Maternal education was categorized as Low (elementary school or college graduate), Medium (tradesman or medium length) and High (university candidate).*

***Household income as Low (<55,000 EUR/year), Medium (55,000–110,000 EUR/year) and High (>110,000 EUR/year)*

	Any Bacteria		Any Virus		Rhinovirus		Rhinovirus-C		RS-virus	
Contrast	IRR, 95% CI	P-value	IRR, 95% CI	P-value	IRR, 95% CI	P-value	IRR, 95% CI	P-value	IRR, 95% CI	P-value
Cough-Only vs Healthy	0.96 (0.83; 1.10)	0.56	0.85 (0.72; 0.99)	**0.041**	0.96 (0.65; 1.43)	0.85	1.02 (0.34; 3.06)	0.97	0.30 (0.14; 0.64)	**0.002**
Cough+Wheeze vs Healthy	0.90 (0.81; 0.99)	**0.039**	0.93 (0.84; 1.04)	*0.22*	1.33 (1.01; 1.75)	**0.044**	1.88 (0.87; 4.08)	0.11	0.72 (0.50; 1.05)	0.09
Cough-Only vs Cough+Wheeze	1.07 (0.94; 1.21)	0.33	0.91 (0.79; 1.05)	0.19	0.72 (0.51; 1.02)	*0.066*	0.54 (0.22; 1.33)	0.18	0.41 (0.19; 0.87)	**0.020**

E-Table 12. Bacterial and viral pathogens during acute respiratory illnesses. Phenotypes of cough-only (n=35), cough+wheeze (n=114), and healthy (n=112) were evaluated for pathogen presence during acute respiratory illnesses. *Values are incidence rate ratios (IRRs) with 95% confidence intervals (CIs) and P-values for counts of pathogen-positive acute episodes, estimated using quasi-Poisson models with log link adjusted for sex, older sibling at birth and age at daycare start. Virus models additionally adjust for bacterial presence, and bacteria models for viral presence. Bacterial presence was prespecified as Streptococcus pneumoniae, Haemophilus influenzae or Moraxella catarrhalis. Analysis models were in tested only children (≥1 acute test), and per test with offset log (number of tests).* Acute respiratory illnesses *were defined as ≥3 consecutive days of troublesome lung symptoms leading to an acute visit for sampling.*

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