LOW-GRADE DISEASE ACTIVITY IN EARLY LIFE PRECEDES CHILDHOOD ASTHMA AND ALLERGY

DMSc Thesis by

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The Faculty of Health and Medical Sciences at the University of Copenhagen has accepted this dissertation for public defence for the doctoral degree in medicine.

Copenhagen, 10 March 2016.
Ulla Wewer,
Head of Faculty

The public defence will take place 3 June 2016 at 13:00 in the large auditorium at Gentofte Hospital, Kildegårdsvej 28, 2900 Hellerup.
Papers included in the thesis

The following published papers are referred to by their roman numerals in the thesis:

I. **Cord Blood 25(OH)-Vitamin D Deficiency and Childhood Asthma, Allergy and Eczema: The COPSAC2000 Birth Cohort Study.**
   Chawes BL, Bønnelykke K, Jensen PF, Schoos AM, Heickendorff L, Bisgaard H.

II. **Cord blood Th2-related chemokine CCL22 levels associate with elevated total-IgE during preschool age.**
    Følsgaard NV, Chawes BL, Bønnelykke K, Jenmalm MC, Bisgaard H.
    *Clin Exp Allergy.* 2012 Nov;42(11):1596-603

III. **Elevated eosinophil protein X in urine from healthy neonates precedes development of atopy in the first 6 years of life.**
     Chawes BL, Bønnelykke K, Bisgaard H.
     *Am J Respir Crit Care Med.* 2011 Sep 15;184(6):656-61

IV. **Elevated exhaled nitric oxide in high-risk neonates precedes transient early but not persistent wheeze.**

V. **DENND1B gene variants associate with elevated exhaled nitric oxide in healthy high-risk neonates.**
   Chawes BL, Bischoff AL, Kreiner-Møller E, Buchvald F, Hakonarson H, Bisgaard H.
   *Pediatr Pulmonol.* 2015 Feb;50(2):109-17

VI. **Neonatal bronchial hyperresponsiveness precedes acute severe viral bronchiolitis in infants.**
    Chawes BL, Poorisrisak P, Johnston SL, Bisgaard H.

VII. **Neonates with Reduced Neonatal Lung Function Have Systemic Low-grade Inflammation.**
     Chawes BL, Stokholm J, Bønnelykke K, Brix S, Bisgaard H.
# Table of Contents

Abstract ................................................................. 4  
Abbreviations .............................................................. 5  
Introduction ................................................................. 6  
  The Disease Burden ...................................................... 6  
  The Pathophysiology .................................................... 7  
  Exploring The Origins .................................................. 8  
Objective .................................................................... 9  
  The COPSAC\textsubscript{2000} Birth Cohort.......................... 10  
  Neonatal Biomarkers .................................................... 12  
  Neonatal Lung Function ................................................ 13  
  Clinical Outcomes ...................................................... 13  
Cord Blood Biomarkers .................................................. 16  
  Specific and Unspecific IgE Antibodies .............................. 16  
  Immune Cell Subsets, Proliferation and Mediators .................. 17  
  Vitamin D ................................................................. 21  
  Other Cord Blood Biomarkers ......................................... 28  
Urinary Biomarkers ...................................................... 29  
  Inflammatory Biomarkers .............................................. 29  
  Metabolomic Profiling ................................................ 33  
Biomarkers in Exhaled Breath ......................................... 35  
  FeNO ................................................................. 35  
  Exhaled Breath Condensate .......................................... 41  
  Volatile Organic Compounds (VOCs) ................................ 42  
Neonatal Lung Function ................................................ 44  
  Bronchiolitis, Recurrent Wheeze and Asthma ....................... 44  
  Systemic Low-grade Inflammation .................................... 48  
Conclusions and Future Directions ................................... 53  
Summary ................................................................. 57  
Danish Summary ........................................................... 60  
Acknowledgements ....................................................... 64  
References ............................................................... 65  
Appendix A: Paper I-VII ............................................... 85
Abstract

Epidemiological data suggest that asthma and allergy originate in early life, where many cases become clinically manifest and the diseases are highly prevalent. An improved insight into the subtle antecedent pathophysiological steps leading to penetrance of symptoms is warranted to improve prevention and treatment.

The objective of this thesis is to investigate the presence of an early life disease activity before symptoms emerge. The thesis is built on seven studies from the Copenhagen Prospective Studies on Asthma in Childhood (COPSAC2000) birth cohort examining markers of disease activity in asymptomatic neonates prior to development of asthma and allergy-related disorders.

First, it is explored how studies of biomarkers in cord blood, urine and exhaled breath support the theory of a pre-symptomatic early life low-grade disease activity. Second, it is explored how studies of neonatal lung function and bronchial responsiveness further corroborate this theory. Third, it is discussed how these findings could represent a common systemic low-grade inflammation, which is part of the trajectory to develop asthma and allergy, but possibly also several other non-communicable welfare diseases of modernity. Last, it is discussed how these findings could be enforced and refined by applying novel biomarker omics technologies, which may provide a novel avenue for improved prevention and treatment of the asthma and allergy pandemic.
ABBREVIATIONS
- CCL17 = C-C motif ligand 17 (previously TARC)
- CCL22 = C-C motif ligand 22 (previously MDC)
- COPSAC = Copenhagen Prospective Studies on Asthma in Childhood
- CXCL10 = C-X-C motif ligand chemokine 10 (previously IP-10)
- CXCL11 = C-X-C motif ligand chemokine 11 (previously I-TAC)
- CV = Coefficient of Variation
- C\textsubscript{rs} = Airway conductance
- EBC = Exhaled Breath Condensate
- FEF\textsubscript{50} = Forced Expiratory Flow at 50% of
- FeNO = Fractional exhaled Nitric Oxide
- FEV\textsubscript{0.5} = FVC Forced Expiratory Volume at 0.5s
- FRC = Functional Residual Capacity
- FVC = Forced Vital Capacity
- IgE = Immunoglobulin E
- IL = Interleukin
- LCPUFA = Long chain polyunsaturated fatty acids
- LRTI = Lower respiratory tract illness
- NCD = Non-communicable diseases
- NOS = Nitric oxide synthases
- PCA = Principal component analysis
- PD\textsubscript{15} = The provocative dose causing a 15% drop in PtcO2
- PtcO2 = Transcutaneous oxygen saturation
- ppb = Parts per billion
- R\textsubscript{rs} = Airway resistance
- RSV = Respiratory syncytial virus
- Th2 = T helper type 2 cell
- Th17 = T helper 17 cell
- Treg = T regulatory cell
- TROLS = Troublesome lung symptoms
- u-EPX = Urinary eosinophil protein X
- u-LT\textsubscript{C4/D4/E4} = Urinary leukotriene C4/D4/E4
- u-11\beta-PG\textsubscript{2\alpha} = Urinary 11\beta-prostaglandin F2\alpha
- V\textsubscript{maxFRC} = Maximum flow at FRC
- VOC = Volatile organic compounds
- WHO = World Health Organization
- 25(OH)-Vitamin D = 25-hydroxyvitamin D
INTRODUCTION

THE DISEASE BURDEN
Asthma and allergy are the most common chronic diseases found in childhood\(^1\)-\(^3\). The prevalence of these diseases has increased dramatically with a more than doubling of the prevalence in developed societies worldwide over the recent decades\(^4\). The World Health Organization (WHO) estimates that there are a total of 300 million asthma and allergy sufferers globally, for most of whom the disease originated in early childhood\(^5\). WHO assumes that the disease prevalence will continue to rise, in particular in developing countries\(^6\), involving further 100 million patients till the year 2025 (www.WHO.int).

In westernized cultures, where the highest disease burden is seen\(^7\), approximately half of young children will experience wheezing in relation to respiratory infections\(^8\) and one out of five preschool children will develop recurrent asthma-like symptoms\(^1\). At school age, approximately 8-10\% will suffer from asthma\(^4\) and 10-15\% will have symptoms characteristic of allergic rhinitis\(^9\)-\(^11\). Asthma and allergy are now the main reasons for hospitalization during childhood, chronic medication usage, and repeated contact with health care providers, with an associated immense direct public healthcare expenditure\(^3\) and a large indirect societal cost due to parents’ loss of work days.

Although asthma and allergies are usually not considered severe diseases, they have a major impact on quality of life for the affected children and their families. Asthma and allergy in childhood can result in a range of psychosocial impairments\(^12\),\(^13\): Children with asthma are less physically active\(^14\) and may be unable to play like their peers and participate in sports\(^15\). Sleep disturbances are common, which result in daytime fatigue and negatively affect the child’s social activities and interactions\(^16\),\(^17\). School-aged children with asthma and allergies have increased school absenteeism\(^18\); they may experience learning impairment\(^19\) and have reduced performance at school exams during the pollen season\(^20\). In general, living with asthma and allergy causes stress and anxiety due to physical discomfort and limitations and due to the unpredictable occurrence of asthma attacks and allergic reactions\(^21\).
Obviously, improved preventive strategies are warranted to alleviate the large global burden of these common childhood disorders. However, despite decades of intensive research this clinical need has not been met, which is presumably due to a lack of knowledge into responsible pathophysiological mechanisms.

**THE PATHOPHYSIOLOGY**

Asthma is a heterogeneous disease with divergent temporal presentations of either episodic or more persistent chronic symptoms such as cough, wheezing and breathlessness, which are typically triggered by airway infections, physical exercise, and exposure to aeroallergens or unspecific irritants such as tobacco smoke. Established underlying pathophysiological mechanisms are reversible and variable airway obstruction, bronchial hyperresponsiveness, and airway inflammation.

The asthmatic airway inflammation is traditionally described as a T helper type 2 cell (Th2) mediated eosinophilic inflammation with predominance of eosinophils and mast cells. More recently, a role of T regulatory cells (Treg) has been described in Th2 associated airway inflammation and emerging evidence also pinpoints a role of T helper 17 cells (Th17) characterizing steroid non-responsive neutrophilic airway inflammation.

Clinical allergy manifestations can involve multiple organs such as the skin, the respiratory system, the cardiovascular system, and the gastrointestinal tract, and range from mild to very severe life threatening anaphylactic reactions. The allergy-associated disease entities are allergic rhinoconjunctivitis, food, drug and venom allergies, asthma, and eczema, which can be partially or solely ascribed to exposure to allergens.

The biological mechanism behind allergic reactions is archetypically thought to be a Th2 cell polarized immune response involving the release of a complex cascade of mediators such as interleukin-4 (IL-4), IL-5 and IL-13, which drive immunoglobulin E (IgE) production from B cells and recruits eosinophil granulocytes. When the child is sensitized to allergen specific IgE, symptoms arise upon exposure to the specific
allergen in a dual early- and late-phase reaction\textsuperscript{26}. The early-phase reaction is orchestrated by degranulation of mast cells after surface binding of allergens with release of cysteinyl leukotrienes, prostaglandins, histamine, and cytokines, and subsequently acute symptoms of e.g. allergic rhinoconjunctivitis\textsuperscript{27,28}. The late-phase reaction is characterized by focal influx of inflammatory cells such as mast cells, mononuclear cells, eosinophil, basophil, and neutrophil granulocytes\textsuperscript{27,28}. The eosinophils dominate the chronic late-phase reaction, where the release of e.g. cysteinyl leukotrienes, cationic proteins, major basic proteins and eosinophil peroxidase sustains the inflammatory process\textsuperscript{25}.

**EXPLORING THE ORIGINS**

Evidence suggests that asthma and allergy are programmed already in the pre- or neonatal life as a result of complex gene-environment interactions occurring long before symptoms develop\textsuperscript{29}. However, studies examining the pathophysiology of asthma and allergy are primarily done in subjects with manifest clinical disease in a case-control design. Unfortunately, this approach only adds limited insight into the mechanisms involved in the inception of these diseases. Thus, investigations of the underlying pathophysiological mechanisms must be performed in earliest life in longitudinal birth cohort studies in order to gain thorough insight and ultimately improved preventive strategies, precise\textsuperscript{30} and personalized medical care\textsuperscript{31}. 
OBJECTIVE
The objective of this thesis is to investigate the presence of early life disease activity prior to clinical symptoms to understand the etiology of childhood asthma and allergy. The thesis is built on seven studies (I-VII) originating from the Copenhagen Prospective Studies on Asthma in Childhood (COPSAC 2000) birth cohort investigating markers of disease activity in asymptomatic neonates in relation to subsequent development of asthma, allergy, and their associated intermediate phenotypes.

First, it is explored how studies of biomarkers in cord blood (I-II), urine (III) and exhaled breath (IV-V) have established the theory of an early life low-grade disease activity preceding symptom penetrance. Thereafter, it is explored how studies of neonatal lung function and bronchial responsiveness (VI-VII) further corroborate this theory and suggest that systemic low-grade inflammation is part of the trajectory to develop asthma, allergy, and possibly several other common non-communicable diseases (NCDs). Last, it is discussed how these findings could be enforced and refined utilizing novel biomarker omics technologies, which might prepare the ground for improved prevention and treatment strategies to combat the asthma and allergy pandemic.
THE COPSAC APPROACH

THE COPSAC2000 BIRTH COHORT

The Danish COPSAC2000 birth cohort is an at-risk, single-center prospective study comprising 411 children born to mothers with physician-diagnosed asthma, recruitment of whom is previously described in details. The children were enrolled at age 4 weeks excluding subjects with gestational age <36 weeks, severe congenital abnormality or systemic illness, neonatal mechanical ventilation, and lower airway symptoms at any time prior to inclusion. Baseline characteristics of the participating children are outlined in Table 1.

The children attended the COPSAC clinical research unit at age 4 weeks for assessment of neonatal lung function and collection of exhaled breath and urine for biomarker analyses. Thereafter, the children were seen at scheduled clinical investigations at 6-monthly intervals till age 7 years as well as at acute visits arranged upon occurrence of any respiratory- or allergy-related symptoms. At every visit a full physical examination was performed and medical history was obtained by parental interviews using predefined questions with closed response categories. The medical history was supported by day-to-day diary cards fulfilled from birth, capturing burden of troublesome lung symptoms (TROLS) between visits. TROLS were defined as clinically significant cough or 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 and recorded in the diary chart as a dichotomized daily score (yes/no). The pediatricians employed at the COPSAC research unit, not the general practitioners, were the ones solely responsible for diagnosing and treating asthma and allergy strictly adherent to predefined validated algorithms.
Table 1. Baseline characteristics of the COPSAC\textsubscript{2000} birth cohort.

<table>
<thead>
<tr>
<th>Characteristics of the COPSAC\textsubscript{2000} birth cohort</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Mothers enrolled, N</td>
<td>452</td>
</tr>
<tr>
<td>Number of newborns, N</td>
<td>411</td>
</tr>
<tr>
<td>Birthdate, range</td>
<td>02.08.1998 – 29.02.2001</td>
</tr>
<tr>
<td>Boys</td>
<td>49.4%</td>
</tr>
<tr>
<td>Twins pairs</td>
<td>2%</td>
</tr>
<tr>
<td>Sibling pairs</td>
<td>2%</td>
</tr>
<tr>
<td>Caucasian</td>
<td>97%</td>
</tr>
<tr>
<td>Mother’s age at birth, mean (SD), years</td>
<td>30.0 (4.5)</td>
</tr>
<tr>
<td>Father’s age at birth, mean (SD), years</td>
<td>32.0 (5.2)</td>
</tr>
<tr>
<td>Season of birth</td>
<td></td>
</tr>
<tr>
<td>Winter</td>
<td>23%</td>
</tr>
<tr>
<td>Spring</td>
<td>21%</td>
</tr>
<tr>
<td>Summer</td>
<td>27%</td>
</tr>
<tr>
<td>Fall</td>
<td>29%</td>
</tr>
<tr>
<td>Pregnancy and birth</td>
<td></td>
</tr>
<tr>
<td>Gestational age, mean (SD), weeks</td>
<td>39.9 (1.6)</td>
</tr>
<tr>
<td>Birth weight, mean (SD), kg</td>
<td>3.52 (0.52)</td>
</tr>
<tr>
<td>Birth length, mean (SD), cm</td>
<td>52.3 (2.3)</td>
</tr>
<tr>
<td>Head circumference at 1 week, mean (SD), cm</td>
<td>35.2 (1.6)</td>
</tr>
<tr>
<td>Apgar score at 5 min., mean (SD)</td>
<td>9.8 (0.6)</td>
</tr>
<tr>
<td>Mode of delivery, Caesarean section</td>
<td>21%</td>
</tr>
<tr>
<td>Exposures</td>
<td></td>
</tr>
<tr>
<td>Older children in household</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>64%</td>
</tr>
<tr>
<td>1</td>
<td>24%</td>
</tr>
<tr>
<td>2</td>
<td>9%</td>
</tr>
<tr>
<td>&gt;2</td>
<td>3%</td>
</tr>
<tr>
<td>Mother smoking during pregnancy, any</td>
<td>24%</td>
</tr>
<tr>
<td>Mother's alcohol use during pregnancy, any</td>
<td>26%</td>
</tr>
<tr>
<td>Mother's antibiotics use during pregnancy, any</td>
<td>30%</td>
</tr>
<tr>
<td>Furred pets at home, any</td>
<td>30%</td>
</tr>
<tr>
<td>Duration of solely breastfeeding, mean (SD), days</td>
<td>113 (62)</td>
</tr>
<tr>
<td>Age at start in daycare, mean (SD), days</td>
<td>349 (147)</td>
</tr>
<tr>
<td>Hair nicotine level at age 1 yr, mean (SD), ng/mg</td>
<td>3.28 (7.98)</td>
</tr>
<tr>
<td>Socioeconomics</td>
<td></td>
</tr>
<tr>
<td>Household annual income</td>
<td></td>
</tr>
<tr>
<td>&lt;53.000 Euro</td>
<td>29%</td>
</tr>
<tr>
<td>53.000 – 80.000 Euro</td>
<td>47%</td>
</tr>
<tr>
<td>&gt;80.000 Euro</td>
<td>24%</td>
</tr>
<tr>
<td>Mother with university education (&gt;3yrs)</td>
<td>13%</td>
</tr>
<tr>
<td>Father with university education (&gt;3yrs)</td>
<td>17%</td>
</tr>
<tr>
<td>Mother without occupation (unemployed or student)</td>
<td>19%</td>
</tr>
<tr>
<td>Father without occupation (unemployed or student)</td>
<td>7%</td>
</tr>
<tr>
<td>Atopic disposition (diagnosed by doctor)</td>
<td></td>
</tr>
<tr>
<td>Mother with asthma</td>
<td>100%</td>
</tr>
<tr>
<td>Mother with allergic rhinitis</td>
<td>73%</td>
</tr>
<tr>
<td>Mother with eczema</td>
<td>46%</td>
</tr>
<tr>
<td>Father with asthma</td>
<td>15%</td>
</tr>
<tr>
<td>Father with allergic rhinitis</td>
<td>30%</td>
</tr>
<tr>
<td>Father with eczema</td>
<td>11%</td>
</tr>
<tr>
<td>Genetics</td>
<td></td>
</tr>
<tr>
<td>ORMDL3, TT genotype (rs7216389)</td>
<td>29%</td>
</tr>
<tr>
<td>DENND1B (rs2786098)</td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>4%</td>
</tr>
<tr>
<td>AC</td>
<td>27%</td>
</tr>
<tr>
<td>CC</td>
<td>69%</td>
</tr>
<tr>
<td>Filaggrin mutation (R501X or 2282del4 null mutation)</td>
<td>11%</td>
</tr>
</tbody>
</table>
**NEONATAL BIOMARKERS**

**Cord blood**: The midwives of the participating COPSAC mothers were given written instructions to collect 14 ml cord blood by needle puncture from the umbilical cord vein. The samples were sent by mail to the COPSAC research unit, centrifuged for 10 min at 4300 rpm to separate serum and plasma, and subsequently frozen at -80°C (I-II).

The chemokines C-X-C motif ligand chemokine 10 (CXCL10), CXCL11, C-C motif ligand 17 (CCL17), and CCL22 were analysed in duplicates utilizing an in-house multiplexed Luminex assay (II) and re-analysing samples if the coefficient of variation (CV) was >15%.

Serum 25-hydroxyvitamin D (25(OH)-Vitamin D) levels were measured in duplicates by isotope dilution liquid chromatography-tandem mass spectrometry using calibrators traceable to NIST SRM 972 (Chromsystems Instruments and Chemicals©, Munich, Germany) (I). If both 25(OH)-Vitamin D2 and D3 were below the detection limit, the combined value was set to 10 nmol/L.

Urine was collected at the COPSAC clinic at age 4 weeks into a sterile plastic bag adherent to the skin and stored without addition of preservatives at -80°C. Urinary eosinophil protein X (u-EPX) level was measured utilizing a double-antibody immunoassay (RIA - Pharmacia Upjohn®, AB, Uppsala, Sweden) and urinary leukotriene C4/D4/E4 (u-LTC4/D4/E4) and 11β-prostaglandin F2α (u-11β-PGF2α) by ELISA test kits (Neogen Corporation©, Lexington, USA) (III) adjusting for creatinine excretion.

Exhaled breath was collected at age 4 weeks into an impermeable bag (750 ml, Quintron Instrument©, Milwaukee, USA) at stable tidal breathing after completion of neonatal lung function testing during sedation. Concentration of fractional exhaled nitric oxide (FeNO) was measured in duplicates using an off-line technique with a chemiluminescence analyzer (EcoPhysics CLD 77 AM, Duernten, Switzerland) cancelling measurement if ambient NO exceeded 10 parts per billion (ppb) (IV-V).
NEONATAL LUNG FUNCTION
Forced volumes and flows were measured by spirometry at age 4 weeks from three to five acceptable curves obtained by the raised volume rapid thoraco-abdominal compression technique. In brief, repeated ventilations to a predefined mouth-pressure were applied to assure expansion of the lung volume before an instant inflation of the “squee” jacket caused a forced exhalation where the flow was measured by a pneumotachograph with an aircushion facemask. The software identified the Forced Vital Capacity (FVC), the Forced Expiratory Volume at 0.5 s (FEV0.5), and the Forced Expiratory Flow at 50% of FVC (FEF50) from the obtained volume-time curve (VI-VII).

Bronchial responsiveness to methacholine was assessed after an initial saline inhalation by administering methacholine in quadrupling dose-steps via a dosimeter attached to a nebulizer (SPIRA 08 TSM 133; Respiratory Care Center; Hämeenlinna, Finland). The responsiveness was determined by continuous assessment of transcutaneous oxygen saturation (PtcO2) (TCM3; Radiometer; Copenhagen, Denmark) calculating the provocative dose causing a 15% drop in PtcO2 (PD15) from baseline (VI-VII).

CLINICAL OUTCOMES
Recurrent wheeze at age 0-7 years was diagnosed according to a quantitative algorithm from the lung symptom diaries reviewed by the COPSAC pediatricians in conjunction with the parents at the scheduled or acute visits to the research clinic. Recurrent wheeze was defined as five diary-verified episodes of TROLS lasting at least three consecutive days within six months or daily TROLS for four consecutive weeks. Children with such a symptom burden were prescribed a 3-month trial of inhaled budesonide 200 mcg twice daily.

Asthma at age 7 years was diagnosed according to recognized international guidelines and was based on (1) recurrent wheeze as defined above, (2) typical asthma symptomatology such as exercise-related symptoms, prolonged nocturnal cough, recurrent cough outside common cold, symptoms causing wakening at night, (3) intermittent need
of rescue inhaled β2-agonist, and (4) responding to a 3-month trial of inhaled corticosteroids and relapsing upon cessation. 

Acute bronchiolitis was defined irrespective of viral trigger as an acute respiratory illness with coryza progressing over a few days to cough, tachypnea, chest retractions and auscultative wide spread crepitation and/or rhonchi in a child below 2 years either diagnosed at the COPSAC clinic or from retrieved hospital records.

Allergic sensitization: Levels of specific IgE antibodies were measured at ages ½, 1½, 4, and 6 years against a range of common inhalant allergens (cat, dog, horse, birch, timothy grass, mugwort, house dust mites, or molds) and food allergens (hen’s egg, cow’s milk, fish, wheat, peanut, soybean, or shrimp) by ImmunoCAP assay (Pharmacia Diagnostics AB, Uppsala, Sweden). Allergic sensitization was defined as specific IgE levels ≥0.35kU/L.

Skin prick tests were performed at the same age-points against the same allergen panel as specific IgE assessments. A positive test was defined as a wheal diameter ≥2 mm larger than the negative control at age ½ and 1½ year and ≥3 mm at age 4 and 6 years.

Allergic rhinitis was diagnosed at age 7 years by the COPSAC pediatricians based on clinical interviews (not questionnaires) of the parents on history of symptoms in the child’s 7th year of life. Rhinitis was defined as bothersome sneezing or blocked or runny nose in the past 12 months outside periods with common cold or flu.

Figure 1 summarizes the COPSAC investigator-diagnosed clinical endpoints, intermediate phenotypes, and neonatal biomarkers and lung function incentives utilized in the studies presented in this thesis.
**Figure 1.** Overview and temporal collection of biomarkers and endpoints from the COPSAC2000 birth cohort presented in the thesis.

<table>
<thead>
<tr>
<th>COPSAC2000 Neonatal Biomarkers and Clinical Endpoints</th>
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<tbody>
<tr>
<td></td>
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<tr>
<td><strong>Birth</strong></td>
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<tr>
<td></td>
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<tr>
<td><strong>Neonatal Biomarkers</strong></td>
</tr>
<tr>
<td>Cord blood</td>
</tr>
<tr>
<td>Chemokines (CXCL10, CXCL11, CCL17, CCL22)</td>
</tr>
<tr>
<td>25(OH)-Vitamin D3</td>
</tr>
<tr>
<td>Urine</td>
</tr>
<tr>
<td>u-EPX, u-LTC4/D4/E4, u-11β-PGF2α</td>
</tr>
<tr>
<td>Exhaled breath</td>
</tr>
<tr>
<td>FeNO</td>
</tr>
<tr>
<td><strong>Neonatal Lung Function</strong></td>
</tr>
<tr>
<td>Spirometry</td>
</tr>
<tr>
<td>Airway reactivity, metacholine</td>
</tr>
<tr>
<td><strong>Clinical Endpoints</strong></td>
</tr>
<tr>
<td>Respiratory symptoms, infections and eczema</td>
</tr>
<tr>
<td>Daily diary on symptoms and medication</td>
</tr>
<tr>
<td>Prospective diagnosis by research staff</td>
</tr>
<tr>
<td>Physical Examination</td>
</tr>
<tr>
<td><strong>Allergy</strong></td>
</tr>
<tr>
<td>Skin Prick Test</td>
</tr>
<tr>
<td>Specific IgE</td>
</tr>
<tr>
<td>Nasal eosinophilia</td>
</tr>
<tr>
<td>Allergic Rhinitis</td>
</tr>
<tr>
<td><strong>Intermediate Phenotypes</strong></td>
</tr>
<tr>
<td>Blood</td>
</tr>
<tr>
<td>hs-CRP, IL-1β, IL-6, TNF-α, CXCL8</td>
</tr>
<tr>
<td>Total IgE</td>
</tr>
<tr>
<td>Eosinophil count</td>
</tr>
<tr>
<td>Lung Function</td>
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<tr>
<td>Spirometry</td>
</tr>
<tr>
<td>Airway reversibility</td>
</tr>
<tr>
<td>Airway reactivity, metacholine</td>
</tr>
</tbody>
</table>
CORD BLOOD BIOMARKES

SPECIFIC AND UNSPECIFIC IgE ANTIBODIES
Cord blood is an easily accessible biomaterial to sample and investigate for the presence of low-grade disease activity already at birth, which would support the hypothesis of fetal programming of childhood asthma and allergy.

Priming of the developing immune system starts in utero and it has been shown that the fetus is capable of producing IgE already during gestational week 11. Furthermore, it is a general belief that IgE antibodies do not cross the placenta barrier and, therefore, cord blood IgE is assumed to be of fetal origin. Based on this, a large amount of studies have investigated the role of cord blood IgE for determining the child’s propensity to develop asthma and allergy later in childhood.

The relevance of cord blood IgE as a marker of predisposition to allergic disease has been suggested by studies showing association between supposed prenatal risk factors such as allergen exposure during pregnancy, maternal allergy status, maternal age, birth order, the child’s gender and elevated cord blood total IgE. In addition, some studies have shown that both high total IgE levels in cord blood predict subsequent development of allergic sensitization, wheezing, and asthma. These findings suggest that elevated cord blood IgE might be a surrogate marker of allergic disease propensity and that reduced exposure to e.g. allergenic foods such as peanut during pregnancy could alter the child’s risk of allergy. However, clinical trials of avoiding either aeroallergens or food allergens during pregnancy have not shown a beneficial effect on sensitization in childhood. The reason for these disappointing results is presumably that a large proportion of detected IgE in cord blood is not a result of fetal de novo synthesis, but merely a reflection of maternofetal transfer and thus maternal IgE levels.

Although some studies have proposed mechanisms for intrauterine sensitization of the fetus, there are several reasons to believe that allergen specific IgE in cord blood is predominantly acquired from the
mother. First, a range of recent studies have consistently shown a linear association between maternal and fetal levels of specific IgE. Second, data from the COPSAC cohort showed that cord blood specific IgE was only detected when the mother had the same specific IgE, there was a strong fingerprinting between the types of specific IgE detected in cord blood and maternal blood, and there was no association with paternal IgE or specific IgE level in the cord blood and at 6 months of age. Third, cellular studies of cord blood immune cells pinpoint that putative T cell memory is not caused by allergen specific priming and that such specific Th2 polarization is first acquired after birth.

Cord blood unspecific IgE may also largely be a result of maternofetal transfer through e.g. placental bleedings during pregnancy or labor, or by contamination with maternal blood during cord venopuncture as illustrated by increased cord blood IgA. Another plausible mechanism is transplacental transfer suggested by normal cord blood IgA level, but detectable specific IgE mirroring maternal specific IgE. However, in some samples with elevated total IgE (>0.5 IU/mL) there are no indicia of maternal contamination, which suggests fetal IgE production and is further supported by association with IgE levels later in childhood. Thus, despite the discussed restrictions and precautions, high level of cord blood total IgE, but not specific IgE, is in some cases compatible with a low-grade disease activity in early life before symptoms develop.

**Immune Cell Subsets, Proliferation and Mediators**

At birth, the fetal immature immune system is thought to be dominated by a default low-level Th2 skewed T cell response. During early childhood, normal T cell maturation leads to the adult-like Th1 oriented immune constitution whereas continuation of the fetal Th2 pattern is seen in children developing asthma and allergy.

In line with this, a stimulation study of cord blood and peripheral blood mononuclear cells from 31 children with house dust mite, cat allergen, and tetanus toxoid showed a suppression of the inborn Th2 response in healthy children contrasting a persistent Th2 response in terms of T cell proliferation and cytokine release in children developing atopy-related disorders at age 2 years. Another similar
study showed a significantly increased proliferative response upon stimulation of cord blood mononuclear cell with inhaled (house dust mite) and food (betalactoglobulin and ovalbumin) allergens in children, who developed allergic disease by one year of age compared to healthy children.85

Treg cell responses are assumed to play a key role in such early life skewing of the immature plastic immune system as they are capable of inhibiting allergen-specific T cell proliferation and secretion of Th2-type cytokines with the ability to suppress IgE production and activity of effector cells in the allergic inflammatory cascade.86 This has been demonstrated in a study examining T cell responses to innate (lipid A/peptidoglycan) and adaptive (Dermatophagoides pteronyssinus) immune stimulation of cord blood from the offspring of 161 atopic and non-atopic mothers.87 In addition to a decreased secretion of the classical Th1-type cytokine, interferon-gamma, cord blood from children of atopic mothers showed a reduced Treg cell number, expression and function, which may be an important step in the inception of asthma and allergies. Furthermore, the same group showed an increased Treg cell count and an associated decreased level of IL-5 after peptidoglycan stimulation of cord blood cells from mothers with farming exposure during pregnancy,88 which is believed to protect against development of allergic disorders.89

Apparently, several studies of cord blood immune cell subsets and their associated mediator release suggest a distinct response to innate and adaptive stimuli in children with a predisposition to asthma and allergy. However, even though these studies are intriguing and hypothesis generating, they should be interpreted with caution as such stimulation induces an unphysiological, exaggerated response. Thus, a clinical follow-up on one of those studies was not able to demonstrate an association between the perinatal immune response and allergic diseases at 6 years of age,90 and another study found no association between cord blood reactivity to house dust mites and later development of dust mite specific IgE.79

An approach to overcome the limitations of challenge models could be to measure unstimulated, circulating levels of cord blood cytokines.
representative of T cell polarizations characteristic of manifest asthma and allergy. However, cord blood cytokines are difficult to quantify as the circulating levels are very low and close to the detection limit of available assays, whereas chemokines, representing another family of immune signaling proteins primarily with chemoattractant effects, are more feasible to measure. Inflammatory chemokines manage the migration of immune cells in inflammatory processes in a distinct Th1/Th2 oriented manner as the receptors of e.g. CCL17 and CCL22 are expressed on eosinophils and Th2 lymphocytes, whereas the receptors of e.g. CXCL10 and CXCL11 are expressed on the surface of Th1 lymphocytes and natural killer cells. Inflammatory chemokines are as relevant as cytokines to examine in this context as they have been shown to express specific Th1/Th2 immunity patterns in children with ongoing asthma, allergy and eczema. However, there is limited knowledge of cord blood chemokine patterns preceding asthma, allergy, and related conditions.

We aimed to address this gap in knowledge in our current report investigating unstimulated levels of selected inflammatory cord blood Th1-associated chemokines (CXCL10 and CXCL11), Th2-associated chemokines (CCL17 and CCL22) and their ratios in 223 samples in relation to the longitudinal development of allergic sensitization, asthma, allergic rhinitis, and associated intermediary phenotypes during preschool age. The study showed a strong positive correlation between levels of the Th2-associated chemokine CCL22, the Th2/Th1 ratio of CCL22/CXCL10 and total IgE levels. CCL22 also showed a trend of association with increased risk of allergic sensitization, but this was not significant after Bonferroni correction for multiple testing (Table 2). Amongst the very few other published reports, comparable results have been shown in smaller cohorts with significant correlations between cord blood CCL22 and development of elevated total IgE and specific IgE levels in the offspring of mixed allergic and non-allergic mothers. These and our findings are compatible with the presence of unchallenged traces of Th2 deviation in the immature immune system of newborns developing elevated IgE antibodies during early childhood, which is a well-established intermediary phenotype in asthma and allergy.
Table 2. Associations between cord blood chemokines and development of clinical endpoints during preschool age (modified from II). Results are odds ratios with 95% CI in brackets.

<table>
<thead>
<tr>
<th>Association between cord blood chemokines and clinical endpoints</th>
<th>Total IgE</th>
<th>Specific IgE</th>
<th>Allergic Rhinitis</th>
<th>Asthma</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCL22</td>
<td>1.54***</td>
<td>1.35</td>
<td>0.55</td>
<td>0.75</td>
</tr>
<tr>
<td>[1.25-1.89]</td>
<td>[0.94-1.95]</td>
<td>[0.2-1.5]</td>
<td>[0.4-1.5]</td>
<td></td>
</tr>
<tr>
<td>CCL17</td>
<td>1.02</td>
<td>0.97</td>
<td>1.07</td>
<td>0.97</td>
</tr>
<tr>
<td>[0.90-1.15]</td>
<td>[0.76-1.24]</td>
<td>[0.61-1.9]</td>
<td>[0.7-1.5]</td>
<td></td>
</tr>
<tr>
<td>CXCL10</td>
<td>1.05</td>
<td>1.15</td>
<td>1.01</td>
<td>0.73</td>
</tr>
<tr>
<td>[0.83-1.32]</td>
<td>[0.76-1.72]</td>
<td>[0.4-2.5]</td>
<td>[0.4-1.3]</td>
<td></td>
</tr>
<tr>
<td>CXCL11</td>
<td>0.93</td>
<td>0.94</td>
<td>0.95</td>
<td>0.87</td>
</tr>
<tr>
<td>[0.80-1.10]</td>
<td>[0.66-1.33]</td>
<td>[0.4-2.1]</td>
<td>[0.52-1.5]</td>
<td></td>
</tr>
<tr>
<td>CCL22/CXCL10</td>
<td>1.22*</td>
<td>1.08</td>
<td>0.7</td>
<td>1.1</td>
</tr>
<tr>
<td>[1.03-1.43]</td>
<td>[0.80-1.45]</td>
<td>[0.35-1.5]</td>
<td>[0.7-1.7]</td>
<td></td>
</tr>
<tr>
<td>CCL22/CXCL11</td>
<td>1.31***</td>
<td>1.23</td>
<td>0.7</td>
<td>0.97</td>
</tr>
<tr>
<td>[1.13-1.51]</td>
<td>[0.90-1.68]</td>
<td>[0.38-1.55]</td>
<td>[0.6-1.5]</td>
<td></td>
</tr>
</tbody>
</table>

*p<0.05; **p<0.01; ***p<0.001

It is unknown whether CCL22 is directly involved in the pathogenesis of asthma and allergies or is just secondary to a general immune imbalance, but recent findings suggest that CCL22 has a crucial role for the recruitment of Th2 lymphocytes into the airways during allergic inflammation\textsuperscript{101}. However, we were not able to detect any association with asthma or allergic rhinitis. The lack of association with asthma at age 6 years is not unexpected as preschool asthmatic symptoms are more closely related to viral than allergen triggers, whereas the classical Th2-type allergic airway inflammation is a more common feature of asthma during school age and later in life\textsuperscript{102}. In line with this, an in vivo study of CCL22 and CCL17 levels in 56 cord blood samples showed elevated levels in children with asthma by age 6 years, which were most pronounced among and primarily driven by children who had comorbid allergic sensitization\textsuperscript{97}. In contrast, another study measuring the same chemokines in 61 samples found no differences for CCL22 levels, but increased CCL17 in children developing recurrent wheeze during the first two years of life. However, the study was on infants enrolled in a placebo-controlled trial of Lactobacillus reuteri during the last month of gestation and the first year of life, which may have impacted the findings\textsuperscript{37}.
The lack of association with allergic rhinitis, despite a trend of association with sensitization, may be attributable to the relative low number of cases in our cohort or the fact that the complex nature of the involved immune imbalance is not sufficiently described by the selected panel of chemokines; e.g. not encompassing markers of Treg or Th17 responses. Thus, apart from applying assays with improved sensitivity, future cord blood mediator studies should aim to assess a broader panel of mediators representing both Th1, Th2, Treg, and Th17 lymphocyte subsets. Furthermore, additional information of underlying immune patterns could be accomplished by applying pattern recognition analyses (e.g. principal component analyses (PCA)) unbiased from preconceived assumptions of pathophysiological pathways and grouping of mediators.

Another important issue to consider is whether maternofetal transfer of inflammatory chemokines is apparent and thus a potential source of bias as demonstrated for cord blood IgE studies\textsuperscript{77,81}. However, inflammatory chemokine levels are typically higher in cord blood than in maternal blood, and maternofetal transfer may, therefore, be less important compared to specific IgE levels, which are often 1000 times higher in maternal blood than in cord blood\textsuperscript{81}. Despite this, future studies should investigate and subsequently adjust for maternofetal transfer as it has been shown that inflammatory chemokines such as CCL17 are capable of passing the blood placenta barrier\textsuperscript{103}.

Still, our finding of an imbalance in unstimulated circulating levels of cord blood Th1- and Th2-associated chemokines in children developing elevated total IgE, underpins the presence of a low-grade disease activity in early life. We recently demonstrated an aberrant immune signature in the airways of neonates born to atopic vs. non-atopic mothers suggesting that such early life immune deviation is a hereditary trait\textsuperscript{104}. However, non-heritable factors such as microflora, diet composition, and other lifestyle associated influences are thought to explain a large proportion of the variation in the human immune system\textsuperscript{105}.

**Vitamin D**

Vitamin D status is highly dependent on lifestyle as production of the biologically active form of vitamin D, 1,25(OH)\textsubscript{2}-vitamin D, depends on
dietary intake and exposure to sunlight\textsuperscript{106}. Only 10–20\% of vitamin D is obtained from foods such as oily fish, fortified products and dietary supplements\textsuperscript{107}, whereas the main contributor in humans is synthesis from UVB light, which facilitates the conversion of cutaneous 7-dehydrocholesterol to vitamin D\textsubscript{3} that subsequently enters the circulation. Vitamin D\textsubscript{3} from this source, together with ingested vitamin D, is thereafter hydroxylated in the liver to 25(OH)-vitamin D, the storage form of vitamin D, which is converted to 1,25(OH)\textsubscript{2} vitamin D predominantly in the kidneys, but also in the respiratory epithelium and in certain immune cells\textsuperscript{108}.

Vitamin D serves an important function for calcium absorption and bone homeostasis and hypovitaminosis D can lead to disorders such as rickets. However, more recently it has been shown that vitamin D also possesses a range of immune regulatory properties which, if distorted, may constitute a fetal programming effect towards asthma and allergy development\textsuperscript{109,110}. This hypothesis is supported by the recent decades’ global surge of vitamin D deficiency induced by a westernized more sedentary indoor lifestyle and decreased dietary vitamin D intake\textsuperscript{111} occurring in parallel with the arising asthma and allergy pandemic\textsuperscript{4}. Of note, vitamin D deficiency is especially prevalent among pregnant and lactating mothers, whose vitamin D levels are highly correlated with levels in their offspring\textsuperscript{112}. In addition, some studies have shown significant associations between polymorphisms in the vitamin D receptor gene\textsuperscript{113} and in genes involved in vitamin D metabolism and signaling pathways\textsuperscript{114} and increased susceptibility to childhood asthma and allergy.

Murine models of allergic asthma have revealed a general downregulating effect of vitamin D on the inflammatory response with decreased IL-4 level in bronchoalveolar lavage fluid\textsuperscript{115}. Further experimental data from murine models have demonstrated that vitamin D through binding to the vitamin D receptor on the surface of immune cells such as T lymphocytes has the ability to shift the balance of Th1 and Th2-type cytokines towards the allergic prototypic Th2 predominance\textsuperscript{116,117}. This is supported by a human cord blood study showing that vitamin D enhances interferon-gamma production and reduces secretion of IL-4 and IL-13\textsuperscript{118} and by an
additional longitudinal study showing inhibited IL-5 and IL-13 production upon house dust mite stimulation at age 6 months in infants with sufficient cord blood vitamin D levels. However, timing, duration and amount of vitamin D exposure seem crucial for the direction of the resulting immune deviation.

Vitamin D is also believed to promote induction of Treg cells, which may inhibit allergen-specific T cell activation and subsequently reduce production of specific IgE in B lymphocytes. In line with this, a recent human cord blood study of 568 newborns showed an association between 25(OH)-vitamin D level and number of Treg cells, and downregulated expression of the Treg cell transcription factor FOXP3 has been demonstrated in placental tissue of vitamin D deficient pregnant women. In vitro studies have suggested that vitamin D is also involved in a range of other immunologic pathways including increased macrophage production of the antimicrobial polypeptides cathelicidin and β-defensin, inhibited monocyte Toll-like receptor production, and the promotion of tolerogenic dendritic cells. The first has important innate immunity functions in the defense towards bacteria and may impact the constitution of the early life airway microbiome, which has been related to an increased propensity to asthma in childhood. The latter, which was demonstrated by association between increased cord blood mRNA transcripts from antigen-presenting tolerogenic dendritic cells and vitamin D supplementation during pregnancy among 927 European children, may impact the trajectory towards allergy-related illnesses.

Apart from an immune modulating effect, studies in rodents have shown that vitamin D has important functions for differentiation of fetal type II alveolar cells, which are important for lung maturation, structure and surfactant production. Cellular studies of human fetal lung tissue have shown presence of the vitamin D receptor and confirmed that in utero vitamin D deficiency may interfere with fetal lung cell maturation and subsequent lung function development originating as early as 2nd trimester of pregnancy. Thus, there is a growing amount of indirect evidence linking vitamin D to mechanisms with a potential role in the inception of asthma and allergies.
Further hints for a protective role of a sufficient vitamin D exposure in utero for development of asthma and allergies in childhood have been provided from epidemiological studies. In 2007, two articles based on independent mother-child cohorts for the first time demonstrated an inverse association between maternal dietary vitamin D intake during pregnancy and risk of wheezing in the offspring\textsuperscript{128,129}. The studies were based on 1,194 mother-child pairs from Boston, MA\textsuperscript{128}, and 1,212 mother-child pairs from Aberdeen, Scotland\textsuperscript{129}, and both showed a more than 60% reduced risk of recurrent wheeze among children born to mothers with the highest vitamin D intake. These findings were replicated in a Finnish cohort of 1,669 mother-child pairs\textsuperscript{130}, whereas a similarly sized Spanish study observed a protective effect on respiratory infections, but no effect on wheezing or asthma development\textsuperscript{131}. Additionally, a reduced risk of allergic rhinitis at age 5 years has been reported\textsuperscript{130}, whilst a large register based Danish study of 32,456 pregnant mothers found no relationship between predicted maternal vitamin D status and allergic diseases in the offspring\textsuperscript{132}. However, a major limitation of these epidemiological studies is that maternal vitamin D status is approximated from questionnaires on food sources, which only contribute with 10-20% of vitamin D status and are thus not a direct measure of circulating levels available for the developing fetus.

Recent studies\textsuperscript{133–143} including our own (I) circumvent estimating fetal exposure from maternal dietary intake by measuring 25(OH)-Vitamin D level in cord blood. This is much more direct, but still an approximation as cord blood levels predominantly reflect exposure during late pregnancy. The findings from these 12 cord blood studies are summarized in Figure 2.
**Figure 2.** Overview of findings from published cord blood 25(OH)-Vitamin D3 studies summarizing reduced risk, increased risk or no effect on endpoints by increasing Vitamin D3 levels.

<table>
<thead>
<tr>
<th>Overview of cord blood 25(OH)-Vitamin D3 studies</th>
<th>Wheeze</th>
<th>Asthma</th>
<th>Lung Function</th>
<th>Respiratory Infections</th>
<th>Allergic Sensitization</th>
<th>Rhinitis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baiz et al., 2014 (n=239)</td>
<td>Reduced risk (0-3yrs)</td>
<td>No effect (5yrs)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>No effect (5yrs)</td>
</tr>
<tr>
<td>Belderbos et al., 2011 (n=156)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Reduced risk* (0-1yrs)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Camargo et al., 2011 (n=922)</td>
<td>Reduced risk (0-5yrs)</td>
<td>No effect (5yrs)</td>
<td>-</td>
<td>Reduced risk (0-3mo)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Chawes et al., 2014 (n=257)</td>
<td>Reduced risk (0-7yrs)</td>
<td>No effect (7yrs)</td>
<td>No effect (1mo and 7yrs)</td>
<td>No effect (0-3yrs)</td>
<td>No effect (0-6yrs)</td>
<td>No effect (7yrs)</td>
</tr>
<tr>
<td>Chiu et al., 2014 (n=186)</td>
<td>-</td>
<td>No effect (4yrs)</td>
<td>-</td>
<td>-</td>
<td>No effect** (0-4yrs)</td>
<td>No effect (4yrs)</td>
</tr>
<tr>
<td>Jones et al., 2012 (n=231)</td>
<td>No effect (0-1yr)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>No effect (1yr)</td>
<td>-</td>
</tr>
<tr>
<td>Liu et al., 2011 (n=649)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>No effect*** (2yrs)</td>
<td>-</td>
</tr>
<tr>
<td>Łuczyńska et al., 2014 (n=777)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Reduced risk (0-1yrs)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Mohamed et al., 2013 (n=266)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Reduced risk (0-2yrs)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Rother et al., 2011 (n=219)</td>
<td>-</td>
<td>No effect (5yrs)</td>
<td>-</td>
<td>-</td>
<td>Dual effect**** (0-5yrs)</td>
<td>No effect (5yrs)</td>
</tr>
<tr>
<td>Stelmach et al., 2015 (n=240)</td>
<td>Reduced risk (0-2yrs)</td>
<td>-</td>
<td>-</td>
<td>No effect (0-2yrs)</td>
<td>No effect (0-2yrs)</td>
<td>-</td>
</tr>
<tr>
<td>Weisse et al., 2013 (n=378)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Increased risk (0-2yrs)</td>
<td>-</td>
</tr>
</tbody>
</table>

*ONLY RSV lower respiratory tract infections were investigated.

**Reduced risk of sensitization ONLY to cow milk at age 2yrs.

***Reduced risk ONLY among children carrying the CC/CT (rs2243250) IL-4 genotype.

****Levels <50nmol/l AND >100nmol/l increased risk of aeroallergen sensitization.

The COPSAC<sub>2000</sub> cord blood study (N=257) is currently the only published work with a full 7-year clinical follow-up by research pediatricians diagnosing wheezing, asthma, allergy and related disorders based on a predefined algorithm including symptoms captured from a day-to-day respiratory diary (I), which is a major advantage compared to other studies utilizing cross-sectional unspecific diagnoses based on reporting from community doctors and parents obtained from questionnaires<sup>140-142</sup>. The main observation in the COPSAC<sub>2000</sub> cord blood study was a 2.7-fold increased risk of recurrent wheeze at age 0-7 years among children with deficient cord blood 25(OH)-Vitamin D levels (<50nmol/L) (see Figure 3). This aligns with findings from all other published cord blood studies with such endpoint<sup>138,141,144</sup> except from one
null study, which only assessed the children at 1 year of age where a diagnosis of recurrent wheeze is quite infrequent in unselected populations\textsuperscript{140}.

**Figure 3.** Kaplan Meier curve showing the association between cord blood 25(OH)-Vitamin D levels and risk of recurrent wheeze (modified from I).

The increased propensity to develop recurrent wheeze in early childhood could be ascribed to an inborn lung function deficit or hyperresponsiveness among children with too low in utero vitamin D exposure. This has hitherto only been investigated in our study (I), where we were unable to demonstrate a relationship between cord blood levels and neonatal lung function indices or lung function trajectories in childhood, which argues against such hypothesis. It has also been suggested that intrauterine vitamin D deficiency through immune modulation predominantly increases frequency of respiratory infections\textsuperscript{137,141} including RSV bronchiolitis\textsuperscript{143} and thus leads to viral-
induced transient early wheezing. However, we observed no effect on the frequency of either upper or lower respiratory tract infections (I), which aligns with a Polish study of 190 children followed till 2 years of age\textsuperscript{138}, but is in contrast to a large study of 777 mother-infant pairs from Ulm, Germany\textsuperscript{136}. Interestingly, the increased risk observed in the latter study was most profound in the strata of children born to mothers without allergy suggesting genetic effect modification\textsuperscript{136}, which may explain the contradicting results. We found no effect on current asthma at age 7 years (I) fully comparable to the univocal null findings from all other published cord blood studies analyzing asthma as a cross-sectional endpoint at age 4-5 yrs\textsuperscript{133,134,141,142}.

The results derived from cord blood studies on allergic sensitization and clinical allergy manifestations are much more diverging compared to wheezing and asthma. A recent study from Taiwan investigated inhalant and food allergen specific IgE levels from 186 children at ages 0.5, 1, 1.5, 2, 3, and 4 years and found that low cord blood levels generally increased the risk of food sensitization, but only significantly for milk at age 2 yrs\textsuperscript{133}. Conversely, a German study of 378 mother-child pairs showed that higher maternal levels during pregnancy and in cord blood conferred a higher risk for food allergy at age 2 yrs\textsuperscript{139}. These disparate findings could be explained by a non-linear relationship, which is suggested by a study founded in the desert climate of Tucson showing a U-shaped relationship with increased risk of aeroallergen sensitization from both low and high cord blood 25(OH)-Vitamin D levels\textsuperscript{134}. In the COPSAC\textsubscript{2000} cohort we did not detect an association with either inhalant or food sensitization (I), which is in line with null reports from three other cohorts\textsuperscript{135,138,140}. However, in one of those studies vitamin D deficiency did increase the risk of food sensitization, but only among individuals with a certain IL-4 genotype suggesting presence of gene-vitamin D interaction\textsuperscript{135}.

The lesson learned from cord blood studies seems to be that vitamin D deficiency is associated with increased risk of wheezing, whereas there is no effect on asthma and no clear conclusions derived concerning allergic sensitization. However, a major limitation of all these
Observational studies is that vitamin D levels are influenced by a multitude of factors such as altitude, latitude, age at delivery, season of birth, skin color, exposure to sun, skin coverage, time spent outdoors, physical activity, tobacco smoke exposure, diet, supplement use, etc. Although most researchers try to account for lifestyle in vitamin D studies there is still a risk of residual confounding and the question of causality can only be answered by randomized controlled high-dose vitamin D supplementation trials during pregnancy (currently: NCT00856947 and NCT00920621). Regardless of the outcomes of such studies and the pathophysiological role(s) of vitamin D, deficient cord blood level is an early life biomarker of disease activity prior to symptom debut.

**Other Cord Blood Biomarkers**

The dietary exposures in prenatal life are crucial for organogenesis and fetal growth and may have a programming effect for asthma and allergy. Apart from vitamin D, there are studies pinpointing a possible role of other nutrients in mother’s diet such as glutathione, zinc, cobber, selenium, iron, vitamin A and E, which may serve antioxidant and immune modulating activities. Particularly, a pregnancy diet deprived of n-3 polyunsaturated fatty acids (LCPUFA), which are known to influence immune regulation, has been associated with increased risk of asthma and allergies in the offspring. However, randomized controlled trials of n-3 LCPUFA supplementation during pregnancy have shown ambiguous results.
Urine is an easy biofluid to sample from children of all ages without the need for stressful or invasive sampling procedures. Despite this there has been a limited search for urinary biomarkers of asthma and allergy in children and there is a striking paucity of studies investigating young children before symptoms emerge.

Inflammatory Biomarkers

The most commonly studied urinary biomarkers in relation to asthma and allergy are the cationic granules proteins of eosinophil granulocytes such as eosinophil protein X (u-EPX), leukotrienes including C4, D4, E4 (u-LT_{C4/D4/E4}), and major metabolites of prostaglandin D2 such as 11β-prostaglandin F2α (u-11β-PG_{F2α}).

Eosinophil cationic protein (ECP) and eosinophil protein X/eosinophil-derived neurotoxin\textsuperscript{155} are both members of the ribonuclease A superfamily and contain a range of properties including neurotoxicity. They are solely released after degranulation of activated eosinophils in the chronic late-phase allergic reaction inducing and sustaining inflammation and symptoms from the nose and lungs such as nasal congestion, bronchial irritability and coughing. EPX is the only of the 4 basic eosinophil granules proteins that can be reliably detected in urine\textsuperscript{156}, it is correlated to eosinophil count in blood and bronchoalveolar lavage fluid\textsuperscript{157} as well as serum ECP levels\textsuperscript{158} proposing a usage as a marker of eosinophilic activation.

Leukotrienes and prostaglandins are released after degranulation of mast cells and basophils during the immediate early-phase allergic reaction caused by allergen induced cross-linking of surface anchored IgE-Fc receptor (FceRI) complexes, but they are also released from mononuclear cells, mast cells and basophil during the first hours of the late-phase reaction\textsuperscript{27}. The cysteinyl leukotrienes (C4/D4/E4) and prostaglandin D2 recruit inflammatory cell types, are potent triggers of smooth muscle contraction in the bronchioles, increase mucus secretion, and induce vasodilation and increased vascular permeability, which leads to the classical acute symptoms of asthma and rhinitis such as bronchoconstriction and rhinorrhea. u-LT_{C4/D4/E4} represents an established
measure of total body cysteinyl leukotriene production, whereas the u-11β-PGF\(_{2\alpha}\) level is a stable measure of prostaglandin D2 production by activated mast cells\(^{159}\).

Clinical studies of urinary inflammatory biomarkers have predominantly investigated: (1) differences between children with manifest asthma, wheezing or allergy vs. healthy controls, (2) the predictive value for persistence of disease among symptomatic children, and (3) whether biomarker levels can predict treatment response. Quite consistently, elevated u-EPX has been reported in children of different ages with current allergic sensitization compared to non-sensitized controls\(^{160,161}\).

The longitudinal English Manchester Asthma and Allergy Study (MAAS) of 903 children found elevated u-EPX at age 3 years in children with aeroallergen and cow’s milk sensitization, which was most pronounced for subjects sensitized both at age 1 and 3 years\(^{160}\). In the COPSAC\(_{2000}\) study of 369 children we also observed elevated u-EPX levels at age 6 months among sensitized children (III). Similarly, increased u-EPX levels were seen among Austrian schoolchildren (N=877) sensitized to common inhaled allergens in particular for perennial allergens\(^{161}\). Eosinophil activity and u-EPX is also influenced by presence of eczema (III) and depends on eczema severity scores\(^{158}\), but the effect of concurrent sensitization is stronger than the observed eczema effects and yields higher u-EPX levels\(^{162}\).

The findings for wheezing and asthma are less univocal compared to sensitization. Some studies found elevated u-EPX among wheezy preschoolers\(^{160}\), whereas we did not detect differences between 6-month-old children with current wheezing and healthy peers (III), which is in line with another study of 1-year-old children with ongoing respiratory symptoms\(^{163}\). In addition, u-EPX level measured in 105 children hospitalized with severe wheezing during their 1\(^{st}\) year of life was unable to predict recurrent wheeze two years later, but high levels were associated with skin prick test reactivity towards food and inhalant allergens\(^{164}\). In populations of children >5 years of age with asthma plus sensitization u-EPX is raised compared to healthy children\(^{156,165,166}\); it is associated with declining lung function (FEV\(_1\)) over time\(^{167}\), and levels
decrease at commencement of inhaled corticosteroids\textsuperscript{156,168}. Despite these promising findings, the usage of u-EPX in clinical practice for diagnosing and monitoring childhood asthma is significantly hampered by low sensitivity and specificity\textsuperscript{169}. Another marker of eosinophil activity, urinary bromotyrosine, which is a marker of eosinophil-catalyzed protein oxidation, has been suggested to reflect asthma control in children\textsuperscript{170}, but this finding still awaits replication.

Urinary leukotriene E4 (u-LT\textsubscript{E4}) was explored in 108 German 10-year-old children showing higher levels in children diagnosed with moderate-severe atopic asthma compared to controls\textsuperscript{171}. Although excretion of u-LT\textsubscript{E4} was correlated with lung function, there were non-significant differences between mild steroid-naïve asthmatics vs. moderate-severe cases and a great overlap in levels between controls and mild cases\textsuperscript{171}. A study of children <3 years found that u-LT\textsubscript{E4} could separate non-atopic children with RSV bronchiolitis (N=32) from controls (N=23) and reported even higher levels among recurrent wheezers with coexisting allergic sensitization (N=35)\textsuperscript{172}. In line with this, two similarly sized studies of preschool children observed increased u-LT\textsubscript{E4} levels during acute viral wheeze, which was exaggerated among children with high total-IgE levels\textsuperscript{173} and sensitization\textsuperscript{174}. In contrast, a study of 1-year-old children with atopic predisposition saw no differences in u-LT\textsubscript{E4} in children with a history of wheezy breathing or any other respiratory symptoms\textsuperscript{163}.

Pediatric studies of u-11\beta-PGF\textsubscript{2α} in relation to asthma and allergy are scarce and solely related to challenges or exacerbations. A brief communication showed that u-11\beta-PGF\textsubscript{2α} rose significantly in 31 children with food sensitization after a positive oral allergen challenge, whereas there were no differences at baseline compared to non-sensitized children (N=16)\textsuperscript{175}. Another small study of 30 children demonstrated elevated levels upon admission to hospital with an acute asthma attack, which declined during convalescence\textsuperscript{176}. Additionally, elevated u-11\beta-PGF\textsubscript{2α} after exercise challenge testing compared to baseline has been demonstrated in two childhood studies with 86\textsuperscript{177} and 14 children\textsuperscript{176}, respectively, whereas rising levels after inhaled allergen challenge and aspirin challenge are documented solely in adult settings\textsuperscript{178,179}.
The COPSAC2000 high-risk birth cohort study is the first and hitherto only study investigating levels of inflammatory biomarkers in the urine of healthy asymptomatic neonates before development of any symptoms (III). We demonstrated that elevated u-EPX at age 4 weeks significantly increased the risk of allergic sensitization during preschool age, presence of nasal eosinophilia at age 6 years, and eczema development in early childhood (Figure 4). We did not detect an association with development of any wheezy phenotype (recurrent, episodic viral, early transient, late onset, persistent) nor asthma at age school age, but we did not investigate the combined endpoint of wheezing/asthma plus sensitization. The risk of such combined endpoint might have been increased, but as allergy is seldom the trigger of respiratory symptoms in this age group, a possible effect of u-EPX would, therefore, presumably be driven by the tendency to produce specific IgE antibodies and not the wheeze propensity. Neonatal levels of u-LT$_{C4/D4/E4}$ and u-11β-PGF$_{2a}$ were not associated with subsequent development of any of the studied endpoints (III).

The study design investigating asymptomatic neonates is of utmost importance to unravel whether elevated biomarkers herald onset of asthma and allergy as levels are confounded by concurrent eczema$^{158}$, respiratory symptoms and infections, and use of anti-asthmatic drugs$^{156}$. Furthermore, the narrow age range at urine sampling, the equal gender distribution, and collection of samples consecutively during a 3-year period accounted for variation caused by those factors$^{161}$, whereas the effect of the circadian rhythm represents a possible residual confounder$^{180}$. Interestingly, u-EPX was a better predictor of allergy development during preschool age than the blood eosinophil count at age 6 months suggesting that the low-grade disease activity in neonates characterized by elevated u-EPX is an increased degranulation liability of eosinophils rather than increased amounts of cells. This may be caused by a dysfunctional eosinophil granulocyte phenotype and/or genetically determined variation in the activation of eosinophils such as deviations in immune regulation by e.g. IL-5, IL-10, IL-13, and IFN-gamma$^{181,182}$. In support of the latter, a recent Danish twin study showed that genetic factor accounted for 57% of the variation in serum eosinophil cationic
protein levels\(^\text{183}\). Thus, in order to further explore how increased u-EPX contributes to a trajectory to develop childhood allergies, future studies should assess functional and regulatory aspects of eosinophils.

**Figure 4.** Odds ratio plot illustrating the associations between neonatal u-EPX and development of atopic endpoints (modified from III).

**Associations between u-EPX and atopic endpoints**

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**Metabolomic Profiling**

Metabolomics is an omics approach to study the human systemic metabolism applied to disentangle complex molecular foundations of diseases or metabolic consequences of environmental effects\(^\text{184}\). The approach includes assessment of the dynamic metabolome, which is the complete set of small-molecule metabolites (e.g. cholesterols, triglycerides, fatty acids, metabolic substrates, amino acids, and other signaling molecules) in a biological sample to identify metabolic phenotypes\(^\text{185}\).
is unique for investigating the pathophysiological transition zone between health and disease by representing the far end from gene expression to systemic metabolism and might, therefore, be able to unmask an altered homeostasis in early life prior to symptom onset.

Metabolomic profiling of urine has recently been utilized in asthma research, but studies are few, have small sample sizes, account inconsistent for race, medication and diet, and apply different profiling platforms. The first childhood study published in 2011 showed that nuclear magnetic resonance (NMR) profiling of 70 metabolites in urine was capable of separating 4-16 year-old children with stable asthma (N=73) and asthma exacerbations (N=20) from healthy controls (N=42)186. Subsequently, a liquid chromatography mass spectrometry (LC-MS) study of 41 children with atopic asthma and 12 controls showed that asthmatics had reduced excretion of metabolites correlated with immune modulation187. Lastly, another LC-MS based study of asthmatic adolescents reported signs of metabolic derangements associated with oxidative stress among severe uncontrolled cases (N=35) vs. mild-moderate cases (N=22)188. Hitherto, no negative studies have been published raising a concern for publication bias, and no study has yet investigated the early life metabolome in serum or urine of healthy neonates before symptoms emerge. Currently, additional urine samples from the COPSAC biobank collected at age one month is undergoing LC-MS metabolomic profiling.
**BIOMARKERS IN EXHALED BREATH**

**FeNO**
Nitric oxide was first discovered in human exhaled breath in 1991\(^{189}\) and was for the first time shown to be elevated in asthmatics in 1993\(^ {190}\). Nitric oxide is produced from L-arginine by the nitric oxide synthases (NOS), where the inducible iNOS activity is particularly enhanced in epithelial cells like eosinophil granulocytes during asthmatic airway inflammation\(^ {191}\). Therefore, FeNO is proposed as a noninvasive marker of eosinophilic airway inflammation – an inflammometer – and elevated levels have been reported in preschool\(^ {192-194}\) and school-aged children\(^ {195}\) with asthma-like symptoms as well as in children with stable asthma prior to exacerbations\(^ {196}\). We, therefore, hypothesized that elevated FeNO in healthy neonates could be a marker of a low-grade disease activity prior to symptom penetrance.

Children from approximately 5 years of age can cooperate adequately to assessment of FeNO by an online chemiluminescence technique at a constant exhalation flow of 50 ml/s\(^ {194,197}\). It is also feasible and reproducible to measure FeNO in younger children and infants, but for such purpose an offline technique is applied where expired air is sampled into a reservoir and subsequently connected to an analyzer\(^ {193,198}\). The sampling procedure in the offline technique is important in order to obtain an accurate measurement, as FeNO is flow dependent with higher values at lower flow rates and vice versa. Two techniques have been proposed in infants to standardize offline FeNO measurements and account for the flow dependency: the single-breath\(^ {199}\) and the tidal-breathing techniques\(^ {200}\).

The single-breath technique is used in sedated infants in relation to spirometric testing by the raised volume rapid thoracoabdominal compression “squeeze” technique\(^ {41}\), where a constant forced expiratory flow rate during sampling can be achieved by regulating the squeeze jacket pressure\(^ {199}\). In the tidal-breathing technique, which can be performed in sedated or unsedated infants, exhaled air is sampled at repeated steady breathing cycles through a face mask attached to a two-way valve with a resistor interposed between the valve and the bag.
assuring a fixed expiratory resistance. The repeated cycles and fixed resistance diminish breath-to-breath flow variability and limit nasal nitric oxide contamination of the sample. Whereas FeNO values obtained sequentially from forced expiration maneuvers and tidal breathing have been compared in school-aged children with allergic asthma (mean age 11.7 years, N=101), no previous large scale study has compared the techniques in neonates. We, therefore, measured FeNO by both techniques in 253 healthy neonates from the COPSAC cohort and showed that levels were highly correlated, but the single-breath technique yielded slightly higher FeNO values than the tidal-breathing technique with increasing differences conditional on increasing FeNO values. It is recommended to refrain from lung function testing prior to FeNO measurement, and our data was obtained in sedated neonates after spirometry, which may have transiently altered the FeNO values. However, we did not detect association between FeNO and the concomitantly measured neonatal lung function incentives, which aligns with a study of 45 1-year-old children showing no FeNO difference before and after sedation or pre vs. post lung function testing. Based on that, we suggest measuring FeNO in unanaesthetized infants by the least invasive tidal-breathing technique for future studies.

Currently, there are quite few studies of FeNO in neonates due to the methodological obstacles inherent to the technique and determinants of neonatal FeNO are largely unknown. Tobacco smoking is believed to lower FeNO in adults due to airway epithelial changes, but the relationship between neonatal FeNO levels and smoke exposure in pre- and early postnatal life is not fully elucidated. One study of 2-month-old infants (N=187) found lower FeNO in infants exposed pre- and postnatally compared to infants exposed only postnatally and never-exposed infants, whilst another study of 1-month-olds (N=98) showed higher FeNO in infants exposed postnatally, and a third study of unselected children aged 2 to 6 months (N=110) found no association between FeNO and concurrent tobacco-smoke exposure. We did not detect an association between neonatal FeNO and maternal smoking during pregnancy or with the child’s hair nicotine level at age 1 year. This negative finding may be due to the at-risk nature of the COPSAC cohort as others have
demonstrated an interaction between prenatal tobacco exposure, presence of maternal asthma and neonatal FeNO\textsuperscript{207}. Previous studies have not shown influence from father’s history of asthma or allergies\textsuperscript{198,207,208}, whereas we detected significantly elevated FeNO in infants with atopic fathers (V); e.g. children predisposed from both their father and mother. Some studies have shown gender differences with higher FeNO in baby boys\textsuperscript{207,209}, whereas we did not observe such difference (V), which could also be ascribed to all mothers having a history of asthma. Data from COPSAC\textsubscript{2000} and other cohorts have not revealed relationships between other environmental factors such as antibiotic and acetaminophen consumption during pregnancy, socioeconomics, older siblings, furred pet exposure, breastfeeding or deviations in the airway microbiome (V) and neonatal FeNO\textsuperscript{204,207}.

It is plausible that neonatal FeNO is mainly an inherited trait and that well-known childhood asthma genes such as Filaggrin\textsuperscript{210,211}, ORMDL3\textsuperscript{45}, and DENND1B\textsuperscript{212} influence FeNO levels in early life. Accordingly, we investigated and discovered that children carrying the DENND1B rs2786098 C allele have elevated neonatal FeNO with increasing levels per risk allele (V) (Figure 5). It is unknown how DENND1B gene variants may influence nitric oxide production, but DENND1B is expressed by immune cells such as dendritic cells, which take part in the linkage of innate and adaptive immune responses in the process of developing tolerability or immunity\textsuperscript{213}. Thus, DENND1B gene variants may induce a skewing of the immature immune response towards a proinflammatory state, which could up-regulate iNOS and result in elevated FeNO levels very early in life. Interestingly, the DENND1B single nucleotide polymorphism has also been shown to confer a risk of other complex inflammatory diseases such as Chrons disease\textsuperscript{214} and primary biliary cirrhosis\textsuperscript{215} indicating that the DENND1B associated childhood asthma endotype may have communalities with other NCDs characterized by immune dysregulation.
Recently, a large meta-GWAS study identified that genetic variants in rs8069176, which are associated with ORMDL3 expression, influenced FeNO levels in children aged 5-15 years\textsuperscript{216}. We found no association between neonatal FeNO and ORMDL3 variants or Filaggrin null-mutations (V) highlighting the dissimilar etiology of FeNO in neonatal life vs. later in childhood. No other previous studies have investigated the association between childhood asthma genes and neonatal FeNO levels. In addition, genetic studies of the nitric oxide synthesis pathway in relation to neonatal FeNO have not yet been performed, but variants in NOS2\textsuperscript{216} (encoding iNOS) and arginases (ARG2), which compete for L-arginine, have been shown to correlate with FeNO level in a large sample of American children aged 6-11 years, with stronger influences in the subset of children with asthma\textsuperscript{217}. Subsequently, it was shown that DNA methylation of iNOS contributed to FeNO level and interacted with the degree of particulate air pollution\textsuperscript{218}. Together, these and our findings suggest that infant FeNO concentration is largely influenced by the child’s genetic makeup and gene-environment interactions.

Studies investigating the clinical value of FeNO in early life for distinguishing between different respiratory diseases are scarce and typically include children within a wide age range where a diagnosis is
already established and treatment initiated. Thus, a Dutch study of 218 infants aged 1 to 25 months showed that FeNO levels differentiated between children with recurrent wheezing, cystic fibrosis, bronchopulmonary dysplasia and healthy children, with the highest levels amongst recurrent wheezers. Similarly, a study conducted in Switzerland including 391 children aged 3 to 47 months showed significantly raised FeNO in children with frequent recurrent wheezing compared to children with recurrent cough without wheezing.

Recently, data from the Generation R birth cohort showed that elevated FeNO measured by the tidal breathing technique in 294 6-month-old infants predicted development of wheezing in the 2nd year of life. This is an interesting finding, but it does not unravel whether subclinical airway inflammation precedes wheezing as the same study showed that a previous history of upper or lower respiratory tract infection was negatively associated with FeNO, which has also been demonstrated in other infant FeNO studies. In addition, the initiation of inhaled corticosteroids is also well-known to lower FeNO values.

Currently, only two studies have examined FeNO in neonates with naïve airways prior to any respiratory symptoms or prescription of anti-asthmatic drugs: a study by Latzin et al and our study. The Latzin study prospectively monitored severe respiratory symptoms at age 0-1 year in 164 infants and showed that neonatal tidal breathing FeNO values were positively associated with symptom development, but only among children of atopic and/or smoking mothers. In the COPSAC at-risk cohort, we also observed that raised neonatal FeNO preceded recurrent wheeze, episodic viral wheezing and number of wheezy episodes in the 1st year of life, but not at age 1-6 years. The study is limited by only 4% of the cohort having recurrent wheeze at age 0-1 year, whilst the additional associations with episodic viral wheeze and number of wheezy episodes increase confidence in the findings. These results, supported by the Latzin study, suggest that a low-grade airway disease process in early life characterized by elevated FeNO heralds onset of a distinct transient wheeze endotype in at-risk children.
The identified wheeze endotype was unrelated to atopy since FeNO was not associated with the development of increased levels of total IgE, specific IgE or blood eosinophil count (IV). This aligns with a study showing similar FeNO concentration in sensitized and non-sensitized infants suggesting that elevated FeNO in this age group is not a marker of eosinophilic inflammation, which is further supported by bronchial biopsy findings revealing very few eosinophils in the airways of symptomatic infants. It has been proposed that congenitally small airway dimensions predispose to an increased propensity of early...
transient wheezing\textsuperscript{222}. However, we found no association between neonatal FeNO and indices of infant spirometry suggesting that the wheeze endotype defined by elevated FeNO is independent of lung function and airway caliber. Further studies are needed to characterize the FeNO related disease mechanism and target this particular wheezy endotype.

**Exhaled Breath Condensate**

The discovery of FeNO as a marker of airway inflammation has led to further research into the development of novel noninvasive techniques to explore the composition of exhaled breath in relation to respiratory illnesses. One of those techniques is exhaled breath condensate (EBC), where expired air is sampled through a cooling system at tidal breathing and subsequently condenses\textsuperscript{223}. The breath condensate is believed to constitute aerosolized airway lining fluid and contains water vapor and microdroplets, where a large range of mediators can be determined\textsuperscript{224}. A recent systematic review summarized the findings from EBC pediatric asthma trials\textsuperscript{225}, which were purely cross-sectional in nature comparing healthy vs. asthmatic children, different degrees of asthma severity, and acute vs. stable asthma. In general, the EBC of asthmatic children had lower pH and showed signs of increased oxidative stress with elevated hydrogen peroxide ($\text{H}_2\text{O}_2$) and nitric oxide products (NOx) and decreased antioxidant glutathione suggesting a homeostatic imbalance between oxidants and antioxidants in the airways. Studies of more complex EBC molecules yielded ambiguous results, but overall found elevated eicosanoids (e.g. 8-isoprostane and cysteinyll leukotrienes) and Th2-related cytokines (e.g. IL-4) in particular amongst children with coexisting sensitization and during exacerbations. Although these results seem promising several methodological issues such as cooling temperature, collection time, condenser material, noseclip, saliva trap, resistor, filter, dilution marker, deaeration, assay sensitivity, within subject reproducibility, etc.\textsuperscript{223}, hamper comparisons and validity of the results. Thus, two recent longitudinal trials did not find that EBC biomarker analysis identified children progressing from preschool wheezing to asthma\textsuperscript{226} or predicted forthcoming asthma exacerbations\textsuperscript{227}.

Additional potential biomarkers have been sought by applying metabolic profiling to EBC – “breathomics”. An NMR based metabolomics analysis
showed that the metabolic biochemical fingerprint was slightly better than the combination of FeNO and FEV\textsubscript{1} to discriminate between 25 children with asthma and 11 controls (7-15 years).\textsuperscript{228} A mass spectrometry based approach was also able to distinguish between 8-17 year-old children with asthma (N=42) vs. controls (N=15) and between severe (N=11) vs. non-severe asthma (N=31).\textsuperscript{229}

It is feasible and safe to collect EBC in infants as early as one month of age,\textsuperscript{230} but hitherto no longitudinal study has examined the early life composition of EBC in relation to asthma and allergies later in childhood. There is a need for refined EBC collection techniques in neonates and improved and validated chemical analytical platforms. Thereafter, EBC studies in healthy symptom-free neonates are warranted to investigate whether a deficient antioxidant capacity, a proinflammatory state and/or a distinct metabolic phenotype characterizes children on a trajectory towards asthma and allergy.

**Volatile Organic Compounds (VOCs)**

The EBC technique is constrained to assessment of soluble volatile components and non-volatile components in expired air, whereas an analysis of the entire fraction of exhaled volatile organic compounds (VOCs) requires alternative approaches. The electronic nose contains a panel of semi-selective sensors, which upon adsorption of volatile molecules to the surface change their electrical properties. The physical changes of the sensors are recorded by an electronic interface resulting in a unique breath-o-gram fingerprint of the child, but without information on the specific VOCs recorded. Identification of the spectrum and amount of specific VOCs involved can be determined by sampling exhaled air in a resistance-free bag system, which is subsequently emptied in a stainless steel sorption tube and analyzed by thermal desorption GC-time-of-flight-MS.\textsuperscript{231}

Studies of VOC profiles obtained by GC-MS in childhood wheezing and asthma are still sparse and all originate from the same research group. In 2010, the first published childhood study measured ~900 different VOCs and showed that 8 selected components discriminated between 63 asthmatic and 57 healthy children with a 92% correct classification.
(sensitivity 89%, specificity 95%)\textsuperscript{232}. Subsequently, a larger case-control study of 3-year-old children with (N=202) and without (N=50) recurrent wheeze also determined ~900 different VOCs and found that 28 selected VOCs correctly classified 83% of the children (84% sensitivity, 80% specificity)\textsuperscript{233}. A small 1-year prospective study of 40 children with asthma analyzed VOC profiles consecutively with 2-months intervals showed that 6 VOCs discriminated between children with and without exacerbations with 96% correct classification (sensitivity 100%, specificity 93%)\textsuperscript{234}. Finally, a prospective questionnaire-based study of 252 children aged 2-4 years, who had experienced >2 episodes of wheezing ever, showed that 17 amongst ~3,000 determined VOCs related to oxidative stress and lipid peroxidation, was able to correctly classify 80% of the children as transient wheeze vs. asthma by age 6 years\textsuperscript{235}. This finding is supported by a similarly sized preschool cohort of recurrent wheezers, where VOC profiles were shown to improve asthma prediction at school age\textsuperscript{226}.

Even fewer studies have evaluated the value of breath-o-grams obtained by electronic noses to distinguish between healthy and asthmatic subjects. A small study found that the electronic nose could discriminate between young adults with (N=10) and without asthma (N=10), but with a poor accuracy for separating mild vs. severe cases\textsuperscript{236}. Only one childhood study has been published so far, which showed that breath-o-grams from 178 preschool children could differentiate between children suffering acute wheeze from asymptomatic controls\textsuperscript{237}.

These results seem promising and stimulating for further research into the diagnostic and predictive value of VOC profiles in childhood respiratory disorders, but further validation studies and consensus on data modeling modalities are warranted. In the unselected COPSAC\textsubscript{2010} cohort of 700 children\textsuperscript{238}, we have repetitively sampled expired air for breathomics by electronic nose and GC-MS profiling from as early as 1 week of age enabling analyses of whether a distinct smellprint characterizes asymptomatic neonates who go on to develop wheeze and asthma later in childhood.
**NEONATAL LUNG FUNCTION**

Pulmonary function in infants and neonates can be determined by a range of techniques including spirometry, plethysmography, the interrupter technique, the forced oscillation technique, and the multiple-breath inert gas washout technique\(^{239}\), among which spirometry is the best validated and standardized test for neonates\(^44\) also permitting bronchial challenge testing\(^{41,42}\). Assessments of lung function and bronchial responsiveness in healthy neonates enable exploring whether a low-grade disease process is active in the target organ already in the pre-symptomatic era.

**BRONCHIOLITIS, RECURRENT WHEEZE AND ASTHMA**

Respiratory infections with RSV, Rhinovirus and other viruses result in common colds in most infants, while a minority develops acute bronchiolitis, which is a leading cause of hospitalization and respiratory insufficiency during infancy\(^47\). The wide dispersion of the severity of clinical manifestations in response to common airway pathogens could be due to underlying host factors such as diminished neonatal lung function and bronchial hyperresponsiveness.

In the COPSAC\(_{2000}\) cohort, neonatal spirometry was performed prior to any respiratory symptoms in 402 children and analyzed in relation to prospectively diagnosed acute severe bronchiolitis, which was present before age 2 years in 8.5% of the children (\(N=34\)) (VI). The prevalence of bronchiolitis in COPSAC\(_{2000}\) is higher than the prevalence of 1-3% reported in unselected populations\(^{240,241}\), but comparable the 7% diagnosed cases in a cohort of 253 infants where 71% had a family history of asthma or allergy\(^242\). Our data revealed that children experiencing acute severe bronchiolitis compared to controls had a significant 2.5-fold increased bronchial responsiveness to methacholine as neonates as well as indica of diminished baseline FEV\(_{0.5}\) and FEF\(_{50}\), which was however not significant (Figure 7). Interestingly, the findings were largely unchanged in post-hoc subgroup analyses restricted to RSV cases (2/3 of the cases), non-RSV cases (1/3 of the cases), hospitalized cases (~60%), and cases encountered before age 1 year (~64%), suggesting that a low-grade disease activity characterized by neonatal bronchial
hyperresponsiveness precedes acute severe bronchiolitis irrespective of the viral trigger or age at infection.

**Figure 7.** Neonatal lung function indices and bronchial responsiveness in children developing acute bronchiolitis vs. healthy controls (modified from VI).

The association between premorbid pulmonary function in early infancy and subsequent development of severe viral lower respiratory tract illness (LRTI)/bronchiolitis is only investigated in a limited amount of studies, which are heterogeneous in nature with respect to populations, case definitions and applied lung function technique. In premature infants born <32 weeks gestation, a small study using the single occlusion technique showed increased airway resistance ($R_{rs}$), but no difference in compliance ($C_{rs}$) or functional residual capacity (FRC) at 36 weeks postmenstrual age in 15 of 39 premature neonates, who experienced RSV-LRTI in their first year of life\textsuperscript{243}. These findings were sought replicated by the same research group after enrolling 159 similar preterm infants, but $R_{rs}$ was only significantly higher among severe cases (both RSV and non-RSV), who were admitted to hospital\textsuperscript{244}. An older study of term infants reported trends of reduced maximum flow at FRC ($V_{max,FRC}$), whilst no differences in $R_{rs}$, $C_{rs}$ or bronchial responsiveness to histamine at age 5 weeks in children developing bronchiolitis before age 2 years (N=17) compared to controls (N=236)\textsuperscript{242}. However, the study had several limitations including the use of a not volume-anchored infant spirometry methodology, a retrospective questionnaire defined “doctor-diagnosed bronchiolitis” at age 2 years, and very mild cases only requiring
hospitalization in 2/17 cases\textsuperscript{242}. More recently, a Dutch study of 417 healthy 2-month-old infants measured passive respiratory mechanics by the single occlusion technique and showed increased $R_{rs}$ and decreased $C_{rs}$ in RSV-positive children hospitalized with bronchiolitis (N=18) vs. non-hospitalized RSV-positive children (N=84) representing both symptomatic and asymptomatic cases\textsuperscript{245}. Thus, in line with our study, others have shown impaired pulmonary capacity in infants subsequently developing severe LRTI/bronchiolitis, but our finding that bronchial hyperresponsiveness associates with an increased propensity to severe bronchiolitis during common viral infections still needs replication.

Apart from premorbid lung function, asthmatic heredity, environmental risk factors, and occurrence of asthma-like symptoms in early life have all been shown to increase the risk of subsequent RSV-hospitalization during infancy\textsuperscript{246,247}. These findings were also present in our study as children suffering acute severe bronchiolitis were characterized by increased prevalence of wheezy episodes before the bronchiolitis incident, genetic and environmental asthma risk factors including male gender, the ORMDL3 risk allele, intrauterine tobacco exposure, early age at daycare start, and stigmata such as elevated total IgE and blood eosinophil count (VI). Adjusting the analyses for all of these potential confounders did not modify the association between preexisting bronchial hyperresponsiveness and development of bronchiolitis.

The abovementioned association between asthma predisposition, asthma comorbidity and bronchiolitis along with the increased prevalence of bronchiolitis in asthma high-risk populations proposes that small airway caliber and/or bronchial hyperresponsiveness is a shared phenotypic trait in early life for both bronchiolitis and asthma. In support of a shared topical low-grade disease activity, children of the COPSAC\textsubscript{2000} cohort with asthma by age 7 years were also characterized by diminished forced flow, volume, and increased bronchial responsiveness as neonates, which progressed during childhood\textsuperscript{248}. These findings argue against a causal role of RSV, Rhinovirus and other common respiratory viruses in the inception of childhood asthma and suggest that acute bronchiolitis
during infancy may purely represent a severe early debut of asthma persisting into school-age.

Previous studies of pre-illness infant lung function have shown that children experiencing a wheezing LRTI in their first year of life had preexisting lower respiratory conductance ($t_{ptef}/t_E$) in one study (N=124)\textsuperscript{222} and lower forced expiratory flow at FRC in another study (N=97)\textsuperscript{249}. In addition, children suffering recurrent wheezing episodes in their first 1-2 years of life have been shown to have altered neonatal lung function demonstrated in both high-risk cohorts (reduced $V_{maxFRC}$, N=69)\textsuperscript{250} and in unselected populations (reduced $V_{maxFRC}$ and airway hyperresponsiveness, N=253)\textsuperscript{251}. Furthermore, follow-up of the Tucson birth cohort study showed that wheezing by age 3 years was still characterized by altered neonatal $t_{ptef}/t_E$\textsuperscript{252}. Finally, data obtained from a large Norwegian cohort utilizing infant tidal breathing flow-volume loops (N=802) and passive respiratory mechanics (N=664) showed that lung function abnormalities at birth increased the risk of asthma at age 10 years\textsuperscript{253}. Together, evidence from the COPSAC\textsubscript{2000} studies and other cohorts pinpoints the existence of a low-grade disease activity in the newborn naïve lung increasing the risk of an exaggerated airway response to respiratory viruses and continuation of obstructive airway symptoms throughout childhood.

Small airway caliber in early life characterized by altered forced flow and volume is speculated to origin from anatomical differences, reduced elastic recoil pressure of the lung, increased airway wall compliance or subclinical inflammation\textsuperscript{222}. Further narrowing of the peripheral airways during respiratory infections in such susceptible individuals is thought to result in exaggerated obstructive airway symptoms typical of acute bronchiolitis as well as recurrent wheezing. It is unknown how increased bronchial responsiveness predisposes and contributes to wheezing and bronchiolitis; it may be driven by subclinical airway inflammation, which in our cohort seems unrelated to elevated FeNO as neonatal FeNO and PD_{15} were not associated (IV), or an increased airway sensitivity driven by other pathophysiological mechanisms.
SYSTEMIC LOW-GRADE INFLAMMATION

C-reactive protein (CRP) is an acute-phase reactant with important innate immunity functions, which is released from the liver triggered by pro-inflammatory cytokines such as interleukin IL-6, IL-1β, and TNF-α during acute and chronic inflammatory disorders\textsuperscript{254}. Newer assays with increased sensitivity\textsuperscript{255} have enabled measurements of CRP levels in the blood previously below the limit of detection. This biomarker is termed high sensitivity CRP (hs-CRP) and is now well established as a sensitive marker of systemic low-grade inflammation.

Elevated hs-CRP has been demonstrated in adult steroid naïve asthmatics (N=22) compared to healthy peers (N=14)\textsuperscript{256} suggesting that current asthma in adults has a low-grade systemic inflammatory component. In addition, a cross-sectional analysis of 259 adults showed that raised hs-CRP was associated with lower FEV\textsubscript{1} and increased prevalence of bronchial hyperresponsiveness\textsuperscript{257} indicating that the degree of airflow obstruction and inflammation contributes to the systemic inflammatory process. Two large community based prospective studies of 531\textsuperscript{258} and 2,442\textsuperscript{259} adults confirmed the cross-sectional reciprocal relationship between hs-CRP and lung function, but only the former showed an association between increasing hs-CRP and declining FEV\textsubscript{1} over a 10-year period\textsuperscript{258}. Low-grade inflammation has also been demonstrated in chronic obstructive pulmonary disease, where transient elevations of hs-CRP were shown to predict imminent exacerbations\textsuperscript{260}.

Currently, only very few published studies have investigated the interrelationship between low-grade inflammation and pulmonary function outcomes in children\textsuperscript{261-263}. All of these studies are cross-sectional, have low numbers, and are conducted among children with a diagnosis of asthma without a healthy control group. Two studies including 63 children aged 2–12 years with and without concurrent exacerbations\textsuperscript{263} and 60 steroid naïve and steroid treated school-aged children\textsuperscript{261}, respectively, showed an inverse relationship between hs-CRP and FEV\textsubscript{1}. A third study investigating 62 school-aged children with controlled and uncontrolled asthma\textsuperscript{262} did not detect such relationship, but reported higher hs-CRP levels in uncontrolled cases, presumably reflecting an aggravated airway inflammation.
Thus, the existing literature on adults and children with symptomatic asthma supports presence of low-grade systemic inflammation, which seems dependent on the degree of disease related respiratory impairment. However, it is unknown whether the inflammatory process predates onset of symptoms in early life or if such systemic disease component is related to reduced neonatal lung function. To address this gap in knowledge, we measured serum levels of hs-CRP, the pro-inflammatory cytokines IL-1β, IL-6, TNF-α, and the neutrophil chemotactic CXCL8 at the early age of 6 months and investigated the possible association with neonatal lung function indices (VII). We detected a strong linear inverse association between FEV$_{0.5}$ at age 4 weeks prior to any respiratory morbidity and hs-CRP level at age 6 months suggesting increasing grade of systemic inflammation by diminished neonatal forced volume. Additionally, a PCA variable reduction approach including all the inflammatory biomarkers showed that reduced FEV$_{0.5}$ was associated with an up-regulated blood inflammatory profile. Identical trends were seen for FEF$_{50}$, whereas the PD$_{15}$ values were unrelated to any biomarkers of systemic inflammation (Table 3).

**Table 3.** Associations between neonatal lung function indices and low-grade inflammation at age 6 months (modified from VII). Results are β-coefficients with 95% CI in brackets. The PCA includes hs-CRP, IL-6, TNF-α and CXCL8.

<table>
<thead>
<tr>
<th>Neonatal lung function and inflammatory biomarkers at age 6 months</th>
<th>hs-CRP</th>
<th>PCA analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>z-score FEV$_{0.5}$</td>
<td>-0.12**</td>
<td>-0.10*</td>
</tr>
<tr>
<td>(95% CI: [-0.21 to -0.04])</td>
<td>[-0.19 to -0.01]</td>
<td></td>
</tr>
<tr>
<td>z-score FEF$_{50}$</td>
<td>-0.06</td>
<td>-0.06</td>
</tr>
<tr>
<td>(95% CI: [-0.15 to 0.02])</td>
<td>[-0.14 to 0.03]</td>
<td></td>
</tr>
<tr>
<td>Log-PD$_{15}$</td>
<td>0.04</td>
<td>0.03</td>
</tr>
<tr>
<td>(95% CI: [−0.12 to 0.21])</td>
<td>[-0.14 to 0.19]</td>
<td></td>
</tr>
</tbody>
</table>

*p<0.05; **p<0.01

These unparalleled data from the COPSAC$_{2000}$ cohort indicate that diminished neonatal lung function is part of an asymptomatic low-grade disease process with a measurable systemic component from the beginning of life. Still, the findings should be interpreted with caution as
causality cannot be determined by this study and several factors may account for the observed relationship by affecting either the neonatal lung function, the biomarker levels or both. Neonatal spirometry assessments before any respiratory symptoms are unbiased from previous or concurrent airway symptoms, whereas a recent history of asthma-like symptoms\textsuperscript{263} or infections\textsuperscript{264}, even mild viral upper respiratory infections\textsuperscript{265}, could result in elevated hs-CRP levels. Bacterial colonization of the neonatal airway is associated with increased risk and number of pneumonia during preschool-age\textsuperscript{266}, showed a trend of increased hs-CRP at age 6 months (\textbf{VII}), and could, therefore, also act as effect modifier. In addition, the observed association could have been affected by factors such as tobacco smoke exposure and high BMI, which is known to negatively impact both neonatal lung function\textsuperscript{267} and hs-CRP levels\textsuperscript{268}. Finally, established genetic and environmental determinants of childhood wheezing and asthma such as parental asthma and allergy, male gender, socioeconomics, sibship size, breastfeeding, childcare attendance, etc., may have influenced both the pulmonary function tests and inflammatory biomarker levels. It is, therefore, difficult to preclude residual confounding even though the association between FEV\textsubscript{0.5} and hs-CRP persisted with a largely unchanged effect estimate after adjusting for a multitude of covariates including father’s history of asthma, eczema or allergy; maternal smoking during pregnancy; caesarean section; gender; anthropometrics; household income; older siblings; furred pets; neonatal bacterial airway colonization; breastfeeding length; age at daycare start; any troublesome lung symptoms or viral wheezing before biomarker assessment; and any infections 14 days prior to biomarker assessment (\textbf{VII}). Overall, it is a major limitation that evaluations of low-grade inflammation and neonatal lung function were not done concomitantly as we can only speculate whether the association concurs with an underlying disorder or reflects an inflammatory disorder of the mother during pregnancy.

A possible explanation favoring a causal link between neonatal lung function and hs-CRP is that diminished forced flow and volume represent an asymptomatic airway inflammation with a detectable systemic component. In support of this theory, cytokines and chemokines involved
in asthmatic airway inflammation have been shown to eventuate recruitment of inflammatory progenitor cells from the bone marrow as a possible systemic immune-inflammatory pathway. Local production of IL-6 and TNF-α triggering CRP release from the liver and/or IL-1β-related inflammasome activation in airway macrophages could also contribute to sustained elevation of CRP and systemic low-grade inflammation. Persistently elevated CRP in early life may originate from or precipitate an increased susceptibility to changes in the exposome through its actions as a general scavenger protein with important immune functions recognizing and eliminating bacteria and damaged human cells through opsonization, phagocytosis, and cell-mediated cytotoxicity. The reduced lung function in asymptomatic neonates may, therefore, reflect an altered airway microbiome and subclinical airway inflammation, which precedes the debut of clinical symptoms and systemic low-grade inflammation.

An alternative explanation to the causal link between reduced neonatal lung function and systemic inflammation is that the conditions are indirectly connected through shared genetic and environmental risk factors. The observed association could be due to pleiotropic gene effects and not the low-grade inflammation per se as some genetic loci might confer an increased risk of both asthma and elevated CRP. Maternal stress and inflammation during pregnancy reflected in raised CRP levels have been shown to increase the risk of eczema, respiratory infections, and recurrent wheezing in early childhood. In addition, elevated CRP during pregnancy is associated with fetal growth restriction, which may result in smaller lungs and airways in the newborn and thereby explain the increased susceptibility to respiratory infections and wheezing. The biological mechanisms may also encompass other fetal developmental adaptations such as induction of a pro-inflammatory state and immune dysregulation possibly influencing both neonatal airway inflammation, the development of systemic low-grade inflammation, wheezing and asthma. Such inefficient immune-regulation and sustained systemic inflammation probably arise from a complex interplay between the newborn’s genetic makeup and the intrauterine and early life environment, where alterations of the human microbiome and
changing dietary habits\textsuperscript{278} are thought to play a key role. Independent of the underlying pathobiology, our findings demonstrate the presence of a low-grade systemic inflammatory disease process in early life, which is associated with asymptomatic impaired neonatal lung function (\textit{VII}).
CONCLUSIONS AND FUTURE DIRECTIONS

The research presented in this thesis (I-VII) piggybacks on data collected from the Danish COPSAC2000 high-risk birth cohort, which is unique due to the extensive biobanking and lung function testing in the neonatal period prior to any clinical signs of disease\textsuperscript{32}. The series of papers show evidence of a pre-symptomatic low-grade disease activity measurable in several body compartments including cord blood, urine and exhaled breath as well as reduced lung function and bronchial hyperresponsiveness. Interestingly, we observed that each biomarker showed distinct associations with different disease trajectories: low cord blood 25(OH)-vitamin D was associated with development of recurrent wheeze, but not asthma or allergies (I); high cord blood CCL22 was associated with increased total IgE, but not specific IgE, allergic rhinitis or asthma (II); elevated u-EPX was associated with allergic sensitization and eczema, but not with wheezing, asthma or allergic rhinitis (III); and elevated FeNO increased the risk of wheezing in early childhood, but not thereafter, and was unrelated to allergic endpoints (IV-V). These findings indicate that low-grade disease activity before the emergence of symptoms is a generic trait in childhood asthma and allergies, which implicates, that primary preventive initiative should be launched in earliest life or even during fetal life to work properly. Furthermore, our findings can be interpreted in support of asthma and allergies constituting a heterogeneous syndrome of several specific endotypes with distinct clinical features, divergent underlying molecular causes, and different prevention and treatment options\textsuperscript{279}. The discovery and elucidation of such disease endotypes is essential for improved understanding of the biological pathways leading to symptoms, for the development of novel therapeutics, and for achieving and practicing precision medicine\textsuperscript{39}.

The presented findings are intriguing and for some part supported by other studies, but much work remains to be done to describe the disease processes and phenotypes characterized by the identified biomarkers and reduced lung function. For instance, we are unable to determine whether the bronchial hyperresponsiveness, which increases the susceptibility for acute bronchiolitis (VI), is caused by subclinical airway
inflammation. This would require concomitant assessments of neonatal lung function and invasive bronchoscopy with biopsies and retrieval of bronchoalveolar lavage fluid. An alternative and less invasive approach could be sampling of epithelial lining fluid with a synthetic absorptive matrix from the respiratory system to examine the immune-inflammatory profile by e.g. a quantitative multiplexed assay for immune mediators\textsuperscript{280}. This can be done from the lower airways by bronchoscopic microsampling\textsuperscript{281} or noninvasive with little discomfort from the nasal mucosa\textsuperscript{104}, which is known to share both functional and immunological properties with the bronchial mucosa\textsuperscript{26}. Applying the latter methodology in our novel unselected COPSAC\textsubscript{2010} mother-child cohort\textsuperscript{238}, we recently demonstrated an altered topical immune response in the upper airways of asymptomatic neonates colonized with pathogenic bacteria\textsuperscript{282}, but these inflammatory blueprints remain to be analyzed in relation to respiratory morbidity later in childhood.

The list of biomarkers presented in this thesis is not exhaustive as biomedical literature databases contain a wealth of articles investigating other biomarkers in asthma and allergic disorders measured in various body fluids. Additional biomarkers such as chitinase-like protein YKL-40 would be interesting to exploit due to the apparent association with airway inflammation, lung function and severe asthma presentations in childhood\textsuperscript{283,284}. However, new biomarkers are constantly being proposed but unfortunately no noninvasive easily interpretable clinical biomarker has yet been discovered to assesses the nature, progression or treatment response of childhood asthma and allergy\textsuperscript{285,286}. This is predominantly due to lack of specificity and overlap between disease subtypes as exemplified by FeNO, which is a well-established biomarker of eosinophilic airway inflammation, but an unreliable tool for gauging asthma control and tailoring treatment in clinical practice\textsuperscript{219}. Therefore, biomarker research has recently drifted from quantification of single biomarkers towards more global omics approaches combining various markers for added clinical value. Metabolomic analyses of serum\textsuperscript{287}, urine\textsuperscript{186} and EBC\textsuperscript{228} as well as VOC profiling of exhaled breath\textsuperscript{232} are all promising advances in pediatric biomarker research, which allow detection of suspected metabolites, unknown metabolites and
biomarkers, and may ultimately unravel novel disease-related pathways. Applying these technologies on biobank material collected from carefully characterized longitudinal birth cohorts such as the COPSAC\textsubscript{2000}\textsuperscript{32} and COPSAC\textsubscript{2010}\textsuperscript{238} would facilitate a broader understanding of the early life low-grade disease activity preceding clinical symptoms, which are proposed in this thesis. In addition to sophisticated metabolomic fingerprints, multiple data layers including genomics, epigenomics, transcriptomics, proteomics, and deep clinical phenotyping data should be integrated in a systems biology multiparametric approach in order to disentangle the origins of asthma and allergies.

Furthermore, the discovery that children with reduced neonatal lung function are characterized by systemic low-grade inflammation very early in life (VII) holds the promise that exploring the origins of asthma and allergy may also shed light on disease mechanisms involved in other NCDs of modern times. The chronic NCDs encompass disorders such as cardiovascular diseases, metabolic diseases, and chronic lung diseases, which have been shown to rise in parallel in prevalence among westernized cultures during the previous decades\textsuperscript{288}. Recent studies pinpoint that chronic low-grade inflammation is a common nominator of virtually all NCDs\textsuperscript{29}, which is reflected by elevated levels of hs-CRP in highly endemic societies\textsuperscript{289} and accompanying the specific disorders like cardiovascular disease\textsuperscript{290}, diabetes mellitus\textsuperscript{291}, chronic obstructive pulmonary disease\textsuperscript{260}, and asthma\textsuperscript{256}. In our study (VII), we demonstrated that such common immune distortion initiating a vicious cycle of sustained low-grade inflammation including elevated hs-CRP is already prevalent in the early life course before any clinical symptoms among children at increased risk of asthma and allergies, which are the earliest debuting NCDs\textsuperscript{292}. Strategies to restore the early life immunological health status may, therefore, not only have the capacity to prevent development of childhood asthma and allergy, but could potentially also prevent a range of other frequent NCDs debuting later in life. A greater understanding of the low-grade disease activity occurring before symptoms are established is the only solid step stone for conducting successful randomized controlled trials to promote immune
health during pregnancy and early childhood to combat the major global challenge of asthma, allergy, and other NCDs.
SUMMARY

Asthma and allergies are today the most common chronic diseases in children and the leading causes of school absences, chronic medication usage, emergency department visits and hospitalizations, which affect all members of the family and represent a significant societal and scientific challenge. These highly prevalent disorders are thought to originate from immune distortion in early childhood, but the etiology and heterogeneity of the disease mechanisms are not understood, which hampers preventive initiatives and makes treatment inadequate.

The objective of this thesis is to investigate the presence of an early life disease activity prior to clinical symptoms to understand the anteceding pathophysiological steps towards childhood asthma and allergy. The thesis is built on seven studies from the Copenhagen Prospective Studies on Asthma in Childhood (COPSAC2000) birth cohort examining biomarkers of disease activity in 411 asymptomatic neonates in cord blood (I-II), urine (III), exhaled breath (IV-V) and infant lung function (VI-VII) in relation to the subsequent development of asthma and allergy during the first 7 years of life.

In papers I-II, we studied cord blood chemokines and 25(OH)-vitamin D, which represent a proxy of the inborn immature immune system, the intrauterine milieu, and the maternal immune health during pregnancy. High levels of the Th2-related chemokine CCL22 and high CCL22/CXCL11 ratio were positively correlated with total IgE level during preschool age (II). This suggests an inborn Th2 skewing of the immune system in healthy newborns subsequently developing elevated total IgE antibodies, which is considered to increase the risk of asthma and allergies later in life. Additionally, deficient cord blood 25(OH)-vitamin D levels were associated with a 2.7-fold increased risk of recurrent wheeze at age 0-7 years (I). Together, these findings support the concept that early life immune programming in the pre-symptomatic era plays an essential role for promotion of or protection against asthma and allergies. Therefore, preventive initiatives to restore immune health, such as vitamin D supplementation, should be directed to the fetus and the earliest postnatal life.
The eosinophil granulocyte has a major role in the allergic inflammatory cascade and eosinophilia is considered a hallmark of many allergic phenotypes. In paper III, we examined neonatal urinary biomarkers including eosinophil protein X (u-EPX), which is contained in the eosinophil granules. Elevated u-EPX in asymptomatic neonates was associated with development of allergic sensitization and nasal eosinophilia, but not with wheezing or asthma (III). These findings suggest the presence of an ongoing low-grade disease process in early life characterized by eosinophil activation prior to appearance of allergy-related conditions.

In papers IV-V, we investigated perinatal and genetic predictors of neonatal fractional exhaled nitric oxide (FeNO) and the relationship between neonatal FeNO and wheezing later in childhood. The a priori selected determinants encompassed asthma genetic risk variants, anthropometrics, demographics, socioeconomics, parental asthma and allergy, maternal smoking, paracetamol and antibiotic usage during pregnancy, and neonatal bacterial airway colonization. Among those, only the DENND1B risk allele and paternal history of asthma and allergy were associated with increased FeNO values (V) suggesting that raised FeNO in neonatal life is primarily an inherited trait. The neonatal FeNO levels were widely dispersed (1-67ppb) and children with values in the upper quartile were at increased risk of recurrent wheezing in early childhood, but not persistent wheezing, reduced lung function or allergy-related endpoints (IV). This suggests that elevated neonatal FeNO represents an early asymptomatic low-grade disease process other than congenitally small airway caliber contributing to a transient wheezing phenotype.

Reduced lung function in neonates is associated with wheezing and asthma proneness, but it is unknown if such host factor also confers a risk of acute bronchiolitis, which is considered an index event of asthma persisting into school age. In paper VI, we investigated neonatal forced flow, volume, and responsiveness to methacholine in relation to occurrence of acute severe bronchiolitis at age 0-2 years. Children developing bronchiolitis had a 2.5-fold increased bronchial
responsiveness as neonates (VI) suggesting a preexisting joint propensity of the airways to react adversely to common respiratory viruses and to develop asthma. This finding proposes airway hyperresponsiveness as yet another marker of low-grade disease activity among asymptomatic neonates on a trajectory towards childhood asthma.

In paper VII, we examined whether neonates with impaired pulmonary capacity also had signs of systemic inflammation prior to clinical symptoms. Reduced FEV$_{0.5}$ was significantly associated with elevated serum hs-CRP and other blood inflammatory markers (VII) suggesting presence of systemic low-grade inflammation from the beginning of life. Chronic low-grade inflammation is a common nominator of virtually all the major non-communicable welfare diseases (NCDs) of modernity whereof asthma and allergies are the earliest debuting disorders. The novel finding of systemic low-grade inflammation among neonates at increased risk of asthma and allergy, therefore, implies that exploring the origins of asthma and allergy may also unravel disease mechanisms involved in other NCDs.

In conclusion, the series of papers presented in this thesis (I-VII) evidence the presence of a pre-symptomatic disease process measurable in several body compartments, which supports the notion of low-grade disease activity in early life as a generic trait among neonates developing asthma and allergy. This hypothesis piggybacking on single biomarker assessments could be enforced and refined by applying novel global omics approaches. In particular, metabolomic analyses of serum, urine, and airway lining fluid from neonates as well as neonatal VOC profiling of exhaled breath may facilitate a broader understanding of the early low-grade disease activity preceding clinical symptoms. Disentangling the introductory pathophysiological mechanisms and underlying endotypes of disease is paramount for generating successful preventive measures to alleviate the major global burden of asthma, allergy, and other NCDs of modern time.
**Danish Summary**

Astma og allergi er i dag de hyppigste sygdomme i barndommen, de hyppigste årsager til skolefravær, kronisk medicinering, lægekontakter og indlæggelser. Sygdommene påvirker såvel barnet som alle øvrige familiemedlemmer og repræsenterer en stor samfundsmæssig og videnskabelig udfordring. Disse hyppigt forekommende sygdomme opstår formodentlig på baggrund af en skævvedrøndning af den immunologisk udvikling i den tidlige barndom, men ætiologien og de heterogene sygdomsmekanismer er ufuldstændigt forstået, hvilket kompromitterer sufficient forebyggelse og behandling.

Formålet med denne afhandling er at undersøge tilstedeværelsen af sygdomsaktivitet tidligt i livet, før kliniske symptomer udvikles, for at kunne forstå de indledende patofysiologiske mekanismer, der fører til astma og allergi i barndommen. Afhandlingen er baseret på syv studier fra Copenhagen Prospective Studies on Asthma in Childhood (COPSAC2000) fødselskohorten, der alle omhandler analyser af sygdomsaktivitet hos 411 raske neonatale børn i navlesnorsblod (I-II), urin (III), udåndingsluft (IV-V) samt neonatal lungefunktion (VI-VII) i relation til udvikling af astma og allergi i førskolealderen.

I I-II undersøgte vi niveauet af navlesnors chemokiner og 25(OH)-vitamin D, som er et mål for det medfødte immature immunsystem, det intrauterine miljø, samt moderens immunologiske helbred under graviditeten. Høje niveauer af det Th2-relaterede chemokin CCL22 og høj ratio af CCL22/CXCL11 var positivt korreleret med niveauet af total IgE i førskolealderen (II). Dette antyder en medfødt Th2 skævvedrøndning af immunsystemet hos raske nyfødte, der udvikler forhøjede total IgE antistoffer, som antages at øge risikoen for astma og allergi senere i livet. Ligeledes observerede vi, at for lavt niveau af 25(OH)-vitamin D i navlesnorsblodet var associeret med en 2,7-fold forøget risiko for at udvikle tilbagevendende astmaligne symptomer i alderen 0-7 år (I). Sammenholdt støtter disse fund det koncept, at immunprogrammering tidligt i livet i den præsymptomatiske æra spiller en afgørende rolle for, om barnet får en forøget eller formindsket risiko for at udvikle astma og allergi. Dette betyder, at forebyggende tiltag, der sigter mod
at forbedre immunstatus, såsom vitamin D supplement, skal initieres allerede i graviditeten og den tidligste barndom.

Den eosinofile granulocyt har en afgørende rolle i den allergiske inflammatoriske kaskade, og eosinofili er et vigtigt karakteristika ved flere allergiske fænotyper. I III undersøgte vi neonatale biomarkører i urinen inkluderende eosinofil protein X (u-EPX), der findes i de eosinofile granula. Forhøjet u-EPX hos de asymptotiske neonatale børn var associeret med udvikling af allergisk sensibilisering og nasal eosinofili, men ikke med astmaligne symptomer eller astma (III).

Disse fund indikerer tilstedeværelsen af en igangværende lavgrads sygdomsmæssige karakteristik ved eosinofili aktivering ganske tidligt i livet, før der udvikles tegn på allergisk sygdom.

I IV-V analyserede vi perinatale og genetiske prædiktorer for niveauet af fraktioneret ekshaleret nitrogen oxid (FeNO) hos neonatale børn og sammenhængen mellem neonatal FeNO og astmaligne symptomer senere i barndommen. De a priori udvalgte prædiktorer var velkendte astma risiko genvarianter, antropometri, demografi, socioøkonomi, forældrenes astma, allergi og eksem status, mors rygning samt forbrug af paracetamol og antibiotika i graviditeten, og neonatal bakteriel kolonisering af luftvejene. Blandt disse prædiktorer var det kun risiko allellen DENND1B og faderens sygdomshistorik, der var associeret med forhøget FeNO (V), hvilket antyder, at forhøjet FeNO hos neonatale primært er et medfødt karaktertræk. Der var stor spændvidde på de målte neonatale FeNO niveauer (1-67 ppb), hvor børn med niveauer i den øvre kvartil havde en øget risiko for at udvikle astmaligne symptomer i det første leveår, men ikke mere persistente symptomer, nedsat lungefunktion eller allergirelaterede tilstande (IV). Dette antyder, at forhøjet neonatal FeNO niveau repræsenterer en tidlig asymptomatisk lavgrads sygdomsmæssig forskellig fra medfødt nedsat lungefunktion, der har betydning for udvikling af en fænotype karakteriseret ved tidlige forbigående astmaligne symptomer.

Nedsat neonatal lungefunktion er associeret med en øget risiko for astmaligne symptomer og astma, men det er ikke belyst, hvorvidt det også øger risikoen for akut bronkiolitis, der antages at være et event,
der karakteriserer børn, som udvikler persistente astma. I VI studerede vi neonatale forcerede flow, volumen, og reaktivitet overfor metacholin i forhold til udvikling af akut svær bronkiolitis i alderen 0-2 år. Børn, der udviklede bronkiolitis, havde en 2,5-fold øget reaktivitet som neonatale (VI), hvilket indikerer en præksisterende fælles tilbøjelighed i luftvejene til at reagere uhensigtsmæssigt overfor almindelige luftvejsvira og til at udvikle astma. Dette fund antyder, at bronkial hyperreaktivitet er endnu en markør for en lavgrads sygdomsaktivitet hos asymptomatiske neonatale børn, der senere udvikler børneastma og astmarelaterede sygdomme.

I VII undersøgte vi, om neonatale børn med reduceret lungefunktion også har tegn på systemisk inflammation, før de udvikler kliniske symptomer. Vi fandt, at lav FEV_{0.5} var signifikant associeret med forhøjet serum hs-CRP og andre inflammatoriske markører i blodet (VII), hvilket indikerer tilstedeværelse af systemisk lavgrads inflammation fra livets begyndelse. Kronisk lavgrads inflammation er en fællesnævner for stort set alle de store hyppigt forekommende ikkesmitsomme livsstilssygdomme, af hvilke astma og allergi er de sygdomme, der debuterer tidligst i livet. Den nye opdagelse af systemisk lavgrads inflammation blandt neonatale med en øget risiko for at udvikle astma og allergi indebærer derfor, at undersøgelser af ophavet til astma og allergi måske også kan afdække sygdomsmekanismer involveret i andre livsstilssygdomme, der debuterer senere i livet.

Studierne i denne afhandling (I-VII) påviser eksistensen af en præsymptomatisk sygdomsproces, som kan måles i flere forskellige kropssystemer, hvilket understøtter, at lavgrads sygdomsaktivitet tidligt i livet er et generisk karakteristika hos neonatale børn, der udvikler astma og allergi i barndommen. Denne hypotese, som baserer sig på måling af single biomarkører, kunne udbygges og raffineres ved at anvende nyere globale omics teknologier. Metabolomics analyser af serum, urin og luftvejssekret samt VOC profilering af uødandningsluft fra neonatale børn kunne formodentlig føre til en bredere forståelse af den lavgrads sygdomsproces, der foregår tidligt i livet, lang tid før børnene udvikler symptomer. En dybere forståelse af disse indledende
patofysiologiske mekanismer og de underliggende sygdoms subtyper er afgørende for at kunne udvikle succesfulde forebyggende tiltag for at afhjælpe den store globale byrde, som astma, allergi og andre moderne livsstilssygdomme udgør.
ACKNOWLEDGEMENTS

The work presented in this thesis is based on investigations performed at the COPSAC research clinic, Danish Pediatric Asthma Center, Copenhagen University Hospital, Gentofte, from 2007 to 2015. This work would not have been possible without the invaluable support and guidance from Professor Hans Bisgaard. Hans has been my mentor, friend, and a great source of inspiration for more than a decade. He believed in me, challenged me, and has, with his uncompromised ambitious approach to pediatric research, lit a lifetime passion for research in me.

I would like to thank all the co-authors of the included manuscripts for lots of fruitful discussions and constructive input. I owe thanks to Johannes Waage for helping me with the figures included in the thesis and to my mother for proofreading the text. I also wish to thank all my dear colleagues at the COPSAC research unit - it would never have been the same without you.

A special thank to all the children and parents in the COPSAC cohort whose participation and dedication made these studies possible, and to all funding agencies, who supported the studies financially (acknowledged on www.copsac.com).

Finally, I wish to thank my love, wife, and best friend Anne for her deep support, never ending patience, encouragement and understanding, and our children Asbjørn, Ellen and Jens for being there - thank you for letting me know what is important in life.
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APPENDIX A: PAPER I-VII
Cord Blood 25(OH)-Vitamin D Deficiency and Childhood Asthma, Allergy and Eczema: The COPSAC2000 Birth Cohort Study

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Abstract

Background: Epidemiological studies have suggested an association between maternal vitamin D dietary intake during pregnancy and risk of asthma and allergy in the offspring. However, prospective clinical studies on vitamin D measured in cord blood and development of clinical end-points are sparse.

Objective: To investigate the interdependence of cord blood 25-hydroxyvitamin D (25(OH)-Vitamin D) level and investigator-diagnosed asthma- and allergy-related conditions during preschool-age.

Methods: Cord blood 25(OH)-Vitamin D level was measured in 257 children from the Copenhagen Prospective Studies on Asthma in Childhood (COPSAC2000) at-risk mother-child cohort. Troublesome lung symptoms (TROLS), asthma, respiratory infections, allergic rhinitis, and eczema, at age 0–7 yrs were diagnosed exclusively by the COPSAC pediatricians strictly adhering to predefined algorithms. Objective assessments of lung function and sensitization were performed repeatedly from birth.

Results: After adjusting for season of birth, deficient cord blood 25(OH)-Vitamin D level (<50 nmol/L) was associated with a 2.7-fold increased risk of recurrent TROLS (HR = 2.65; 95% CI = 1.02–6.86), but showed no association with respiratory infections or asthma. We saw no association between cord blood 25(OH)-Vitamin D level and lung function, sensitization, rhinitis or eczema. The effects were unaffected from adjusting for multiple lifestyle factors.

Conclusion: Cord blood 25(OH)-Vitamin D deficiency associated with increased risk of recurrent TROLS till age 7 years. Randomized controlled trials of vitamin D supplementation during pregnancy are needed to prove causality.

Introduction

Vitamin D deficiency caused by adaption of a more sedentary indoor lifestyle and changing dietary habits has become a common health problem in developed and developing countries worldwide[1] which has occurred in parallel with the “asthma epidemic”[2]. It is now evident that vitamin D possesses a panoply of immune-regulatory functions that may protect against asthma and allergy [3] and recent findings also suggest a role of vitamin D for fetal lung cell maturation and subsequent lung function development [4]. These findings lend support to the hypothesis that fetal vitamin D deficiency may promote a trajectory to develop asthma and allergy [5]. A growing amount of studies have attempted to prove this hypothesis but with inconsistent findings.

Some epidemiological studies have shown an association between low maternal vitamin D intake during pregnancy and increased risk of wheezy phenotypes in the offspring [6,7] whereas others showed association with lower respiratory tract infections but not with wheezy symptoms [8] or asthma [8,9]. Likewise, some studies have shown that fetal vitamin D deficiency increases the risk of food allergy [10] and eczema [11,12] whereas others found no association with allergic sensitization [12]. Thus, it remains uncertain whether fetal vitamin D deficiency contributes to the development of childhood asthma, allergy and eczema. This may be a result from the inaccuracy of determining fetal Vitamin D...
exposure by questionnaire-based estimations of maternal dietary intake of vitamin D and/or poorly defined case definitions.

The aim of the current study was to investigate the programming effect of cord blood vitamin D deficiency on the subsequent development of troublesome lung symptoms (TROLS), asthma, lower respiratory tract infections, lung function, allergy, and eczema during preschool age. We studied these aspects in the children from the Copenhagen Prospective Study on Asthma in Childhood (COPSAC2000) high-risk birth cohort [13–15] with longitudinal clinical data determined from strict predefined algorithm-based clinical case definitions and repeated objective assessments of intermediary end-points.

Materials and Methods

Study Design

The COPSAC2000 birth cohort is a single-center prospective clinical study of 411 children born to mothers with physician verified asthma recruited between 1998 and 2001 as previously described [13–15]. The children were enrolled at one month of age and subsequently attended the clinical research unit for scheduled clinical investigations at six-monthly intervals as well as immediately upon onset of any respiratory-, allergy- or skin-related symptom. The pediatricians employed at the clinical research unit (not the family practitioners) were solely responsible for diagnosis and treatment of asthma, allergy, and eczema. At every visit a full physical examination was performed including lung function testing and history was obtained by parental interviews using predefined questions with closed response categories. History information was collected online during the visits and the objective measurements were double checked against source data and subsequently locked.

The study was conducted in accordance with the Declaration of Helsinki and was approved by The Copenhagen Ethics Committee (KF 01-289/96) and The Danish Data Protection Agency (2008-41-1754). Written informed consent was obtained from both parents before enrollment.

Cord Blood Vitamin D Measurement

Cord blood was collected by the midwives by needle puncture from the umbilical cord vein obtaining an aliquot of approximatively 14 mL, which was subsequently sent to the COPSAC research unit, centrifuged for 10 min at 4300 rpm to separate serum, and thereafter frozen at −80°C until analysis.

The serum samples were transported on dry ice for duplicate analyses for 25-hydroxyvitamin D2 (25(OH)-Vitamin D2) and 25(OH)-Vitamin D3 at the Dept. of Clinical Biochemistry, Aarhus University Hospital, Denmark. Serum 25-hydroxyvitamin D levels were analyzed by isotope dilution liquid chromatography-tandem mass spectrometry (LC-MS/MS) [16,17]. Calibrators traceable to NIST SRM 972 (Chromsystems, DE) were used. Mean coefficients of variation (CV) for 25(OH)-Vitamin D3 were 6.4% and 9.1% at levels of 66.5 and 21.1 nmol/L and for 25(OH)-Vitamin D2 the CV values were 8.8% and 9.4% at levels of 41.2 and 25.3 nmol/L. The average of the combined 25(OH)-Vitamin D values was calculated and used in the analysis. If both 25(OH)-Vitamin D2 and 25(OH)-Vitamin D3 were under the detection level, the combined value was defined as equal to 10 nmol/L.

Clinical Investigator-diagnosed End-points

Troublesome lung symptoms (TROLS) were defined as significant cough or wheezing or dyspnea and were explained to the parents as wheeze or whistling sounds, breathlessness, or recurrent troublesome cough severely affecting the well-being of the child and were recorded by the parents in a day-to-day diary chart as a dichotomized daily score (yes/no) from birth till age 7 yrs [18].

Recurrent TROLS was defined from the diaries at the scheduled or acute visits to the research clinic as five episodes within 6 months, each episode lasting at least three consecutive days, or daily symptoms for four consecutive weeks [19,20]. Children meeting these criteria were prescribed a 3-month trial of budesonide 200 mcg bid increasing to 6 and 12 months at subsequent relapses.

Asthma at age 7 yrs was diagnosed according to international guidelines and was based on recurrent TROLS as defined above, symptoms judged by the COPSAC pediatricians to be typical of asthma (e.g. exercise induced symptoms, prolonged nocturnal cough, recurrent cough outside common cold, symptoms causing wakeining at night); in need of intermittent rescue use of inhaled β₂-agonist; responding to a 3-month trial of inhaled corticosteroids and relapsing when stopping treatment [14,15].

Lower respiratory tract infections (LRTI) included occurrence of pneumonia and/or acute bronchiolitis at age 0–3 yrs where such disorders are most prevalent. The diagnoses were established at the acute visits to the clinic by the research pediatricians based on clinical appearance regardless of identified pathogen(s) in accordance with predefined standard procedures [21] or if the child had been hospitalized for such disorders.

Allergic rhinitis was diagnosed at age 7 yrs based on clinical interviews of the parents on history of symptoms in the child's 7th year of life [22–24]. Rhinitis was defined as troublesome sneezing or blocked or runny nose in the past 12 months in periods without accompanying cold or flu [25].

Eczema was diagnosed utilizing the Hanifin-Rajka criteria as previously detailed [26,27] obtaining age at onset data. Skin lesions were described at both scheduled and acute visits according to pre-defined morphology and localization.

Lung Function

Infant spirometry was performed during sedation at age 1 month by applying the raised volume rapid thoraco-abdominal compression technique as previously detailed [28–30]. The forced expiratory volume at 0.5 seconds (FEV₅₀) and forced expiratory flow at 50% of the forced vital capacity (FEF₅₀) were used as lung function indices.

Spirometry at age 7 yrs was performed as previously detailed [31] using a pneumotachograph Masterscope Pneumoscreen, system 754,916 spirometer (Erich Jaeger, Wurtzburg, Germany) for assessing FEV₁ and maximal mid-expiratory flow (MMEF).

Bronchial responsiveness at age 1 month was assessed as previously detailed [29] by continuous measurements of transcutaneous oxygen saturation (PtCO₂) during quadrupling methacholine-dose-steps and was defined as the provocative dose causing a 15% drop in PtCO₂ (PD₁₅). Bronchial responsiveness at age 7 yrs was defined as the provocative dose of methacholine causing a 20% drop in FEV₁ from baseline (PD₂₀) [31].

Allergy Intermediary End-points

Specific-IgE was measured at age ½, 1½, 4, and 6 yrs against 16 common inhalant and food allergens (cat, dog, horse, birch, timothy grass, mugwort, house dust mites, moulds, hen’s egg, cow’s milk, fish, wheat, peanut, soybean, or shrimp) by ImmunoCAP assay (Pharmacia Diagnostics AB, Uppsala, Sweden). Allergic sensitization was defined as specific-IgE ≥0.35 kU/L [32,33] for (1) any of the tested allergens, (2) any inhaled allergen, and (3) any food allergen.
Results are reported with 95% confidence intervals (CI) in multivariable models including all covariates significantly associated with analyses.

Statistical Analysis

The combined cord blood 25(OH)-Vitamin D value was analyzed as a continuous variable per 100 nmol/L decrease as well as categorized as: deficient (<50 nmol/L), insufficient (50–75 nmol/L), sufficient (>75 nmol/L), based on biologically relevant levels according to recent studies on multiple health outcomes [1].

Distribution of baseline characteristics within the study group and the drop-out analysis was done with univariable parametric and non-parametric tests such as $\chi^2$, Fischer’s exact test, t-test, and Kruskal-Wallis rank sum test.

The association of cord blood 25(OH)-Vitamin D level with age at onset end-points (recurrent TROLS, LRTI, eczema) was estimated as hazard ratios by Cox regression and visualized by Kaplan-Meier curves for the categorized 25(OH)-Vitamin D levels. The effect of cord blood 25(OH)-Vitamin D level on the cross-sectional end-points, e.g., asthma and allergic rhinitis, was estimated as odds ratios by logistic regression. The association between 25(OH)-Vitamin D and cross-sectional continuous outcomes such as lung function incentives was explored by general linear models (GLM).

The effect on outcome measures with repetitive assessments throughout childhood was measured objectively as hair nicotine level at age 1yr [34].

Covariates

Blood sampling season was categorized as: winter (Dec-Feb), spring (March-May), summer (June-Aug) and fall (Sep-Nov). Demographics included child gender, birth BMI, maternal age at birth of proband, and household income (low: $<$50,000€, medium: 50,000–80,000€, high: $>$80,000€). Postnatal exposures were older siblings (yes/no), length of solely breastfeeding, age at start in daycare, and environmental tobacco exposure measured objectively as hair nicotine level at age 1yr [34].

The core data of the manuscript is available online in Data S1.

Results

Baseline Characteristics

Cord blood was available for 25(OH)-Vitamin D analysis in 257 (63%) of the 411 children in the COPSAC2000 cohort. Characteristics of the children with and without available cord blood samples are given in Table 1. Children in the drop-out group without available cord blood came from families with significantly lower household income (p = 0.01), but did not differ from the study group in any other baseline characteristics studied.

The median cord blood 25(OH)-Vitamin D level in the study group was 47.6 nmol/L (range, 10–145 nmol/L). The distribution of cord blood 25(OH)-Vitamin D concentrations was: 132 (33%) children with deficient levels, 82 (20%) with insufficient, and 39 (15%) with sufficient levels. Concentration of cord blood 25(OH)-Vitamin D varied significantly with season of birth (p = 0.01) with most cord blood 25(OH)-Vitamin D deficient children born during winter and most sufficient children born during summer. Children with deficient vs. sufficient levels were born to younger mothers (median age at birth, 29.5 yrs vs. 30.8 yrs; p = 0.02), had fewer siblings (children with older siblings, 44% vs. 50%; p = 0.02), and had a higher exposure to environmental tobacco smoke (median hair nicotine level at age 1yr, 0.84 vs. 0.47 ng/mg; p = 0.03).

Association between Cord Blood 25(OH)-Vitamin D Level and Asthma-related Outcomes and LRTI

Cord blood 25(OH)-Vitamin D level vs. Asthma-related Outcomes and LRTI

Recurrent TROLS was diagnosed in 24% (N = 61) of the children at age 0–7 yrs. The cumulative prevalence rates were 4% (N = 11) at age 0–1 yrs, 14% (N = 35) at 0–2 yrs, 16% (N = 42) at 0–3 yrs, 19% (N = 50) at 0–4 yrs, 22% (N = 57) at 0–5 yrs, and 23% (N = 59) at 0–6 yrs. Cord blood 25(OH)-Vitamin D levels were lower in children developing recurrent TROLS as illustrated in Figure 1. Having deficient vs. sufficient cord blood 25(OH)-Vitamin D level was associated with a 2.7-fold increased risk of recurrent TROLS (hazard ratio (HR), 2.65; 95% CI = 1.02–6.86; p = 0.04). The association was borderline significant when adjusting for multiple confounding factors but with unchanged effect estimates (aHR, 2.50; 95% CI, 0.95–6.57; p = 0.06).

Cord blood 25(OH)-Vitamin D level was not suppressed in the subgroup of children experiencing recurrent TROLS who were subsequently diagnosed with asthma at age 7 yrs (Table 2). In addition, cord blood 25(OH)-Vitamin D level was not associated with an increased risk of allergic rhinitis diagnosed in 12% (N = 23) at age 7 yrs. Cord blood 25(OH)-Vitamin D deficiency did not modify time to first LRTI (Figure 2) nor was the number of episodes affected by cord blood 25(OH)-Vitamin D level (Table 2).

Association between Cord Blood 25(OH)-Vitamin D Level and Lung Function

Cord blood 25(OH)-Vitamin D level was not associated with forced flows assessed at age 1mo (FEV$_{15}$, FEV$_{45}$, MMEF) or 7 yrs (FEV$_{1}$, MMEF) nor bronchial responsiveness to methacholine at any age point (PD$_{20}$, PD$_{30}$) (Table 2).

Relationship between Cord Blood 25(OH)-Vitamin D and Allergic Outcomes (Table 3)

Specific-IgE.

Allergic sensitization at any of the four measuring points (1/6, 1/4, 4, 6 yrs) was present in 33% (N = 88) for any of the investigated allergens, in 26% (N = 64) for any food allergens, and in 21% (N = 52) for any inhaled allergens. There was no significant association between cord blood 25(OH)-Vitamin D level and allergic sensitization throughout childhood although there was a tendency of Vitamin D deficiency being associated with decreased risk of sensitization.

Total-IgE.

Cord blood 25(OH)-Vitamin D level was not associated with level of total-IgE throughout childhood.

Allergic rhinitis was diagnosed in 12% (N = 23) at age 7 yrs. Deficient vs. sufficient cord blood 25(OH)-Vitamin D level seemed to be associated with a reduced risk of allergic rhinitis at age 7 yrs but this was not significant.
Table 1. Baseline characteristics of children with and without available cord blood for 25(OH)-Vitamin D analysis.

<table>
<thead>
<tr>
<th>Vitamin D cord blood (N)</th>
<th>Deficient: &lt;50</th>
<th>Insufficient: 50–75</th>
<th>Sufficient: &gt;75</th>
<th>P*</th>
<th>P**</th>
</tr>
</thead>
<tbody>
<tr>
<td>No cord blood available, N = 154</td>
<td></td>
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<tr>
<td>Demographics</td>
<td></td>
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</tr>
<tr>
<td>Maternal age at birth¹, median (range)</td>
<td>29.5 yrs (20.9–38.9)</td>
<td>29.2 yrs (22.6–41.1)</td>
<td>30.8 yrs (22.1–37.8)</td>
<td>0.02</td>
<td>30.0 (19.2–41.1)</td>
</tr>
<tr>
<td>Household income² (N)</td>
<td>25% (136)</td>
<td>32% (82)</td>
<td>15% (39)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Low, &lt;50.000E</td>
<td>28% (35)</td>
<td>25% (19)</td>
<td>13% (5)</td>
<td>37% (33)</td>
<td>0.25</td>
</tr>
<tr>
<td>Medium, 50.000–80.000E</td>
<td>52% (65)</td>
<td>55% (42)</td>
<td>53% (20)</td>
<td>38% (55)</td>
<td>0.01</td>
</tr>
<tr>
<td>High, &gt;80.000E</td>
<td>1% (6)</td>
<td>20% (15)</td>
<td>34% (13)</td>
<td>25% (36)</td>
<td>0.01</td>
</tr>
<tr>
<td>Boy² (N)</td>
<td>45% (61)</td>
<td>55% (45)</td>
<td>46% (18)</td>
<td>51% (79)</td>
<td>0.55</td>
</tr>
<tr>
<td>Birth BMI1, median (range)</td>
<td>12.8 kg/m² (7.0–16.3)</td>
<td>12.7 kg/m² (10.0–16.6)</td>
<td>12.8 kg/m² (9.6–15.1)</td>
<td>0.98</td>
<td>12.6 kg/m² (8.4–16.5)</td>
</tr>
<tr>
<td>Birth season¹ (N)</td>
<td>&lt;0.01</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Winter</td>
<td>30% (41)</td>
<td>15% (12)</td>
<td>18% (7)</td>
<td>23% (35)</td>
<td>0.39</td>
</tr>
<tr>
<td>Spring</td>
<td>24% (33)</td>
<td>21% (17)</td>
<td>15% (6)</td>
<td>19% (30)</td>
<td>0.25</td>
</tr>
<tr>
<td>Summer</td>
<td>21% (28)</td>
<td>33% (27)</td>
<td>49% (19)</td>
<td>24% (37)</td>
<td>0.01</td>
</tr>
<tr>
<td>Fall</td>
<td>25% (34)</td>
<td>32% (26)</td>
<td>18% (7)</td>
<td>34% (52)</td>
<td>0.01</td>
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<tr>
<td>Postnatal Exposures</td>
<td></td>
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<tr>
<td>Older siblings¹ (N)</td>
<td>44% (55)</td>
<td>26% (20)</td>
<td>50% (19)</td>
<td>40% (58)</td>
<td>0.87</td>
</tr>
<tr>
<td>Breastfeeding³, median (range)</td>
<td>123 (0–243)</td>
<td>121 (0–274)</td>
<td>137 (0–266)</td>
<td>0.14</td>
<td>120 (0–244)</td>
</tr>
<tr>
<td>Daycare⁴, median (range)</td>
<td>339 (140–1074)</td>
<td>308 (156–674)</td>
<td>286 (179–578)</td>
<td>0.46</td>
<td>335 (127–1003)</td>
</tr>
<tr>
<td>Nicotine in hair 1 yr⁵, median (range)</td>
<td>0.84 ng/mg (0.06–30.9)</td>
<td>0.53 ng/mg (0.03–43.5)</td>
<td>0.47 ng/mg (0.1–12.9)</td>
<td>0.03</td>
<td>0.95 (0.03–103.9)</td>
</tr>
</tbody>
</table>

*P-value for the distribution of characteristics within the groups of 25(OH)-Vitamin D.
**P-value for the distribution of characteristics between children with and without available 25(OH)-Vitamin D data.
¹Linear regression; ²Chi-Square test; ³Days solely breastfed; ⁴Kruskal-Wallis rank sum test; ⁵Age at start in daycare.
doi:10.1371/journal.pone.0099856.t001

Figure 1. Kaplan Meier survival curve showing the risk of developing recurrent troublesome lung symptoms (TROLS) at age 0–7 yrs stratified by cord blood 25(OH)-Vitamin D level.
doi:10.1371/journal.pone.0099856.g001
<table>
<thead>
<tr>
<th>Cord blood 25(OH)-Vitamin D</th>
<th>&lt;50 vs &gt;75 nmol/L</th>
<th>P</th>
<th>&lt;50 vs &gt;75 nmol/L Adjusted*</th>
<th>P</th>
<th>Per 100 nmol/L decrease</th>
<th>P</th>
<th>Per 100 nmol/L decrease Adjusted*</th>
<th>P</th>
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<tbody>
<tr>
<td><strong>Asthma-related outcomes</strong></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Recurrent TROLS, 0–7 yrs (^{2})</td>
<td>2.65 (1.02 to 6.86)</td>
<td>0.04</td>
<td>2.50 (0.95 to 6.57)</td>
<td>0.06</td>
<td>2.65 (0.83 to 8.50)</td>
<td>0.10</td>
<td>2.27 (0.65 to 7.90)</td>
<td>0.20</td>
</tr>
<tr>
<td>Asthma, 7 yrs (^{3})</td>
<td>160 (0.49 to 5.22)</td>
<td>0.31</td>
<td>163 (0.47 to 5.61)</td>
<td>0.38</td>
<td>1.18 (0.25 to 5.58)</td>
<td>0.84</td>
<td>1.01 (0.18 to 5.80)</td>
<td>0.99</td>
</tr>
<tr>
<td><strong>LRTI</strong> (^{4})</td>
<td></td>
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<tr>
<td>Time to first LRTI, 0–3 yrs</td>
<td>108 (0.63 to 1.85)</td>
<td>0.78</td>
<td>1.08 (0.62 to 1.87)</td>
<td>0.79</td>
<td>1.17 (0.54 to 2.52)</td>
<td>0.69</td>
<td>1.05 (0.46 to 2.39)</td>
<td>0.91</td>
</tr>
<tr>
<td>No. of LRTI, 0–3 yrs (^{5})</td>
<td>107 (0.66 to 1.75)</td>
<td>0.78</td>
<td>0.94 (0.57 to 1.55)</td>
<td>0.81</td>
<td>1.08 (0.53 to 2.20)</td>
<td>0.83</td>
<td>0.75 (0.35 to 1.58)</td>
<td>0.69</td>
</tr>
<tr>
<td><strong>Lung function</strong></td>
<td></td>
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<tr>
<td>z-FEV(_{0.5}), 1mo (^{6})</td>
<td>0.02 (−0.10 to 0.15)</td>
<td>0.73</td>
<td>−0.01 (−0.14 to 0.13)</td>
<td>0.94</td>
<td>0.01 (−0.02 to 0.04)</td>
<td>0.40</td>
<td>0.01 (−0.02 to 0.04)</td>
<td>0.70</td>
</tr>
<tr>
<td>z-FEV(_{1}), 7 yrs</td>
<td>0.04 (−0.09 to 0.18)</td>
<td>0.54</td>
<td>0.07 (−0.08 to 0.22)</td>
<td>0.37</td>
<td>0.02 (−0.02 to 0.06)</td>
<td>0.28</td>
<td>0.02 (−0.01 to 0.06)</td>
<td>0.24</td>
</tr>
<tr>
<td>z-FEF(_{50}), 1mo</td>
<td>−0.03 (−0.15 to 0.09)</td>
<td>0.63</td>
<td>−0.06 (−0.18 to 0.07)</td>
<td>0.38</td>
<td>0.01 (−0.02 to 0.04)</td>
<td>0.42</td>
<td>0.00 (−0.03 to 0.03)</td>
<td>0.82</td>
</tr>
<tr>
<td>z-MMEF, 7 yrs</td>
<td>−0.04 (−0.17 to 0.09)</td>
<td>0.55</td>
<td>−0.05 (−0.18 to 0.09)</td>
<td>0.50</td>
<td>0.00 (−0.03 to 0.03)</td>
<td>0.90</td>
<td>−0.01 (−0.04 to 0.02)</td>
<td>0.62</td>
</tr>
<tr>
<td>logPD(_{15}), 1mo (^{8})</td>
<td>0.02 (−0.05 to 0.09)</td>
<td>0.62</td>
<td>0.01 (−0.07 to 0.03)</td>
<td>0.92</td>
<td>0.01 (−0.01 to 0.02)</td>
<td>0.66</td>
<td>0.00 (−0.01 to 0.02)</td>
<td>0.82</td>
</tr>
<tr>
<td>logPD(_{20}), 7 yrs (^{9})</td>
<td>0.02 (−0.08 to 0.12)</td>
<td>0.68</td>
<td>0.03 (−0.07 to 0.14)</td>
<td>0.52</td>
<td>0.01 (−0.02 to 0.04)</td>
<td>0.41</td>
<td>0.01 (−0.02 to 0.04)</td>
<td>0.40</td>
</tr>
</tbody>
</table>

*Adjusted for mother’s age at birth, blood sampling season, older siblings, and hair nicotine level at age 1 yr.

\(^{1}\)TROLS = Troublesome lung symptoms; \(^{2}\)Cox regression: hazards ratio (95% CI); \(^{3}\)Logistic regression: odds ratio (95% CI); \(^{4}\)Lower respiratory tract infections; \(^{5}\)Poisson regression: incidence risk ratio (95% CI); \(^{6}\)All lung function analyses are done with general linear models: \(\beta\)-coefficients (95% CI); \(^{7}\)Z refers to the calculated z-score; \(^{8}\)PD\(_{15}\) refers to the provocative dose of methacholine resulting in a 15% decrease in transcutaneous oxygen saturation (Ptco\(_{2}\)) from baseline; \(^{9}\)PD\(_{20}\) refers to the provocative dose of methacholine resulting in a 20% decrease in FEV\(_{1}\) from baseline.

doi:10.1371/journal.pone.0099856.t002
Cord Blood 25(OH)-Vitamin D Level and Risk of Eczema

Eczema was diagnosed in 42% (N = 109) of the study group. Cord blood 25(OH)-Vitamin D level was not significantly associated with the risk of eczema during preschool age although the effect estimates suggested an increased risk by deficient cord blood levels (See Table 3 and Figure 3).

Discussion

Principal Findings

Deficient cord blood 25(OH)-Vitamin D level was associated with a more than doubled risk of developing TROLS during preschool age in children of the Danish COPSAC 2000 high-risk birth cohort study. This finding supports the theory that deficient 25(OH)-Vitamin D exposure in utero has a programming effect on the immune maturation thus predisposing to development of chronic inflammation.

Strengths and Limitations of the Study

The primary strength of the study is the seven years of clinical assessments of our birth cohort with longitudinal clinical deep phenotyping and standardized diagnoses solely performed by the COPSAC research pediatricians. Repeated objective measurements along with day-to-day respiratory symptom diary recordings through the first seven years of life provided strong age at onset data which is a major advantage compared to studies using cross-sectional unspecific community-based diagnoses [6,7,11].

Another significant strength is the objective assessment of fetal 25(OH)-Vitamin D exposure by measuring the concentration in the cord blood. Even though cord blood 25(OH)-Vitamin D level primarily reflects recent exposure during 3rd trimester and may be contaminated by maternal blood, this biological measure is a more accurate estimate of fetal 25(OH)-Vitamin D exposure compared to approximations from questionnaires on maternal dietary and supplementary intake since only 10% of vitamin D is obtained through diet [55].

It is also a strength of the analyses that environmental exposure assessments were comprehensive including hair nicotine level [34] as a validated objective measure of tobacco smoke exposure allowing for robust confounder adjustment. In agreement with other studies we found a marked seasonal variation in cord blood 25(OH)-Vitamin D level [12] as well as significant influence of various lifestyle factors such as passive smoking, maternal age at delivery, and older siblings [6,7]. We adjusted for season of blood sampling (i.e. sunlight exposure) which is a major determinant of serum 25(OH)-Vitamin D level [12]. We did not adjust our analyses for ethnicity [1] as 98% of the study group is of Caucasian origin.

The study is limited from the at-risk nature of the cohort as all mothers have a history of asthma which may limit the generalizability of our findings to other populations. However, this should not affect our ability to analyze the relationship between 25(OH)-Vitamin D levels and development of asthma and allergy-related traits within the cohort, and our findings are in line with studies of unselected populations [7,11].

We expected to see the largest effects in children with deficient compared to sufficient cord blood 25(OH)-Vitamin D levels. An essential limitation of the study is therefore the study sample size as only 15% had sufficient cord blood levels, equivalent to 39 participants, which results in lack of statistical power and a likelihood of overlooking the true effect of 25(OH)-Vitamin D exposure in fetal life on development of childhood asthma and allergy.

Interpretation

Our finding that deficient cord blood 25(OH)-Vitamin D level was associated with a more than doubled risk of developing recurrent TROLS during preschool age is in line with a range of epidemiological studies utilizing maternal dietary vitamin D intake during pregnancy as a surrogate measure of fetal vitamin D exposure [6,7,36]. However, studies of cord blood 25(OH)-Vitamin D are still sparse [9,11,12] and our study is the only one with a full clinical follow-up till age 7 yrs at a single research
Table 3. Cord blood 25(OH)-Vitamin D vs. allergic outcomes and eczema at 0–7 yrs.

<table>
<thead>
<tr>
<th>Cord blood 25(OH)-Vitamin D</th>
<th>Per 100 nmol/L decrease</th>
<th>Adjusted*</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Allergic outcomes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Any sensitization, spec-IgE, 0–6 yr</td>
<td>0.96 (0.43–2.16)</td>
<td>0.93</td>
<td>0.96 (0.64–2.31)</td>
</tr>
<tr>
<td>Inhaled sensitization, spec-IgE, 0–6 yr</td>
<td>0.97 (0.47–2.01)</td>
<td>0.94</td>
<td>1.20 (0.54–2.67)</td>
</tr>
<tr>
<td>Total-IgE, 0–6 yr</td>
<td>0.40 (0.12–1.26)</td>
<td>0.32</td>
<td>0.44 (0.13–1.50)</td>
</tr>
<tr>
<td>Allergic rhinitis, 7 yr</td>
<td>0.97 (0.56–1.69)</td>
<td>0.97</td>
<td>1.13 (0.63–2.01)</td>
</tr>
<tr>
<td>Eczema, 7 yr</td>
<td>0.97 (0.56–1.69)</td>
<td>0.97</td>
<td>1.13 (0.63–2.01)</td>
</tr>
</tbody>
</table>

*Adjusted for mother's age at birth, sex, smoking, alcohol, age, siblings, and hair nicotine level at age 1 yr.

The immune-regulatory properties of vitamin D exerted through e.g. promotion of pro-inflammatory cytokines and induction of regulatory T cells include an ability to shift the Th1/Th2 balance with capacity to inhibit both Th1 and Th2-type responses leading to opposite effects on disease development, i.e. enhancing vs. reducing the risk [35, 36]. The complex interplay between the genetic makeup, the early life milieu, and the timing of sufficient vitamin D exposure during maturation of the immature immune system may determine the direction of the immune-modulatory properties of Vitamin D. Additional programming effects of fetal vitamin D exposure apart from immune-modulatory mechanisms might be alterations of the airway microbiome from e.g. induction of the endogenous antimicrobial peptide cathelicidin in bronchial epithelial cells [39].

Overall, our study suggests an association between deficient fetal 25(OH)-Vitamin D exposure and development of recurrent TROLS during preschool age with a sizable effect estimate. However, despite the rigorously clinically determined diagnoses in this study and the objective assessment of cord blood 25(OH)-Vitamin D, the question of causality cannot be answered by an observational study. Thus, acknowledging that and the possibility of residual lifestyle confounding, this study prompts the performance of randomized controlled trials of maternal supplementation of vitamin D during pregnancy to shed light on the possible causative and thus modifiable effect of fetal vitamin D deficiency for development of asthma, allergy and eczema. Several randomized controlled trials are currently being conducted by our group and others (clinicalTrials.gov identification numbers: NCT00856947, NCT00920621).

Conclusion

This study shows an interdependence of 25(OH)-Vitamin D deficiency in fetal life and occurrence of recurrent TROLS during preschool in children of the at-risk COPSAC2000 cohort. Further research is required to establish whether vitamin D supplementation during pregnancy can prevent development and severity of such disorders.
Supporting Information

Data S1
Core data of the manuscript.

(XLS)

Acknowledgments
We gratefully express our gratitude to the children and families of the COPSAC2000 cohort study for all their support and commitment. We acknowledge and appreciate the unique efforts of the COPSAC research team.

Author Contributions
Conceived and designed the experiments: BLC KB HB. Performed the experiments: BLC KB PFJ AMS. Analyzed the data: BLC KB PFJ AMS HB. Contributed reagents/materials/analysis tools: LH. Wrote the paper: BLC KB PFJ AMS LH HB. The guarantor of the study who has been responsible for the integrity of the work as a whole, from conception and design to conduct of the study and acquisition of data, analysis and interpretation of data and writing of the manuscript: HB. Responsible for data analysis, interpretation and writing the manuscript: BLC KB PFJ AMS LH. Wrote the first draft of the manuscript: BC. No honorarium, grant, or other form of payment was given to anyone to produce the manuscript. All co-authors have contributed substantially to the analyses and interpretation of the data, and have provided important intellectual input and approval of the final version of the manuscript.

References


Cord blood Th2-related chemokine CCL22 levels associate with elevated total-IgE during preschool age

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Summary

Background Early-life immune deviation is suspected in the inception of atopic disease.

Objective To investigate the association between cord blood chemokines and the subsequent development of atopic biomarkers and clinical end-points during the first 6 years of life.

Methods The Th1-associated chemokines CXCL10 and CXCL11 and the Th2-associated chemokines CCL17 and CCL22 were assessed in cord blood of asymptomatic at-risk newborn children from the Copenhagen Prospective Study on Asthma in Childhood (COPSAC2000) birth cohort and associated with the longitudinal development of biomarkers and clinical end-points of asthma, eczema, and allergic rhinitis during the first 6 years of life.

Results Cord blood CCL22 levels were significantly associated to total-IgE levels measured at four time-points during the first 6 years of life; overall odds ratio, 1.54 [CI, 1.25–1.89; P < 0.0001]. CXCL10 and CXCL11 were not associated with development of any atopic disorders or biomarkers.

Conclusion and Clinical Relevance High cord blood levels of the Th2 related chemokine CCL22 were significantly associated with high total-IgE levels during the first 6 years of life, but not with specific sensitization, asthma, eczema or allergic rhinitis. This suggests an inborn skewing of the immune system in healthy newborns developing elevated total-IgE later in life.

Keywords cord blood, chemokine, CCL22, CCL17, CXCL11, CXCL10, preschool age, atopic disorders, total-IgE, Th2 skewing

Submitted 02 March 2012; revised 30 April 2012; accepted 25 May 2012

Introduction

The ‘atopic diseases’ asthma, eczema, and allergic rhinitis origin early in life, presumably as a result of a Th2 skewing of the immune system [1, 2]. It is important to understand early-life immune mechanisms responsible for the inception of atopic disease to unravel disease pathogenesis towards preventive measures and possibly customized therapies.

Cord blood is easily accessible at birth and provides a unique opportunity to investigate the immunity of the newborn prior to disease onset. Circulating levels of Th1 and Th2 cytokines are difficult to measure in cord blood due to low levels close to the detection limit of the currently available assays [3] and challenge models generate an exaggerated and possibly un-physiological response. Chemokines are simple to measure with current technology and are equally robust markers of Th1/Th2 immunity [4–8] in manifest atopic disorders. However, little is known about cord blood Th1- and Th2-related chemokine levels [4, 9]. We hypothesized that levels of Th2 related chemokines in the cord blood are associated with the development of atopic disease and biomarkers during preschool age.

The aim of this study was to investigate the possible association between the cord blood chemokines Thymus and activation regulated chemokine, TARC (CCL17), Macrophage-derived chemokine, MDC (CCL22), interferon gamma-induced protein 10 kDa, IP-10 (CXCL10), and Interferon-inducible T cell alpha chemoattractant,
I-TAC (CXCL11) in asymptomatic at-risk newborn children from the Copenhagen Prospective Study on Asthma in Childhood (COPSAC2000) birth cohort in relation to the subsequent development of atopic biomarkers and clinical end-points during the first 6 years of life.

Methods
The study is reported in accordance with the STROBE guidelines for observational studies [10].

Study population
Participants comprised children from the COPSAC2000 cohort of 411 children born of mothers with doctor-verified asthma, the recruitment of whom was previously described in detail [11]. The pregnant women visited the COPSAC research centre during pregnancy for information on the study procedures. Consenting mothers brought their 1-month-old child to the research clinic and subsequently attended the clinic at 6-monthly intervals and immediately upon onset of any allergy-, respiratory- or skin-related symptom until age 7 years. Key exclusion criteria were gestational age < 36 weeks, severe congenital abnormality, neonatal mechanical ventilation or symptoms of lower airway infection before 1 month of age.

Ethics
The study was conducted in accordance with the guiding principles of the Declaration of Helsinki and approved by the Ethics Committee for Copenhagen (KF01-289/96) and the Danish Data Protection Agency (2008/41-1754). Data validity was assured by compliance with Good Clinical Practice guidelines and quality control procedures.

Cord blood collection and analyses
The midwives received written instructions on the collection of cord blood using needle puncture from the umbilical cord vein: 14 mL cord blood was collected (7 mL in an EDTA tube and 7 mL whole blood) and subsequently send to the COPSAC research unit, where the blood was centrifuged for 10 min at 2800 g to separate serum and plasma, and thereafter frozen at –80°C until analysis at the Department of Clinical and Experimental Medicine, Linköping University, Sweden. The chemokines CXCL10, CXCL11, CCL17, and CCL22 were analysed in plasma with an in-house multiplexed Luminex assay, as described in detail previously [4]. The limit of detection was 6 pg/mL for CXCL10, 28 pg/mL for CXCL11, and 2 pg/mL for CCL17 and CCL22. All samples were analysed in duplicates and the sample was re-analysed if the coefficient of variation (CV) was > 15%.

Atopic biomarkers
Blood was collected from the children at ages 6 months, 18 months, 4 years, and 6 years during visits at the research unit. All samples were stored at –80°C until analysis.

Total-IgE levels were measured at ages 6 months, 18 months, 4 years, and 6 years using ImmunoCAP (Pharmacia Diagnostics AB, Uppsala, Sweden) with a detection limit of 2 kU/L.

Specific IgE levels against 15 common inhalant and food allergens (cat, dog, horse, birch, timothy grass, mugwort, house dust mites, moulds, hen’s egg, cow’s milk, fish, wheat, peanut, soyabean, or shrimp) were measured at ages 6 months, 18 months, 4 years, and 6 years using ImmunoCAP (Pharmacia Diagnostics AB) [12, 13].

Skin Prick Test was performed at 6 months, 18 months, 4 years, and 6 years against 18 common inhalant and food allergens (birch, timothy grass, mugwort, horse, dog, cat, house dust mites, moulds, hen’s egg, cow’s milk, fisk, wheat, rye, oat, peanut, soyabean, beef, and pork) with standard allergen solutions from ALK-Abello® (Bøge Alle 1, DK-2970 Hørsholm, Denmark), and in addition with pasteurised, fresh products for cow’s milk and hen’s egg.

Allergic sensitization was defined as specific IgE ≥ 0.35 kU/L [12, 13] for any of the tested allergens and/or a positive skin prick test defined as a weal ≥ 2 mm at 6 and 18 months or ≥ 3 mm at 4 or 6 years. Allergic sensitization was analysed as a dichotomized measurement.

Blood eosinophil count (10⁹ cells/L) was assessed at ages 6 months, 18 months, 4 years, and 6 years.

Nasal eosinophilia was examined by nasal scrapings in the child’s 6th year of life and rated according to Meltzer’s semi-quantitative scale [14] as previously detailed [15].

Investigator-diagnosed clinical end-points
Allergic rhinitis was diagnosed at age 6 by the COPSAC doctors, based on parent interviews on symptom history in the child’s 6th year of life [15]. Rhinitis was defined as troublesome sneezing or blocked or runny nose in the past 12 months in periods without accompanying virus symptoms [16]. Allergic rhinitis was diagnosed in children sensitized to inhaled allergens of relevance to the symptomatic periods. Asthma at age 7 years was diagnosed as previously detailed, [17, 18] and was based on a history of recur-
rent wheeze; symptom character judged by the study physicians to be typical of asthma; response to trials of inhaled corticosteroids; and relapse when stopping treatment.

Eczema during the first 6 years of life was diagnosed using the Hanifin–Rajka criteria as previously detailed [19]. Skin lesions were described at both scheduled and acute visits according to pre-defined morphology and localization.

Statistical analysis

We investigated the association between cord blood levels of the chemokines CXCL10, CXCL11, CCL17, CCL22 and the Th2/Th1 ratios CCL22/CXCL10, CCL17/CXCL10, CCL22/CXCL11, CCL17/CXCL11, and the development of atopic biomarkers and atopic clinical end-points during the first 7 years of life. Models using general estimating equations (GEE; PROC GENMOD in SAS version 9.1; SAS Institute, Inc., Cary, NC, USA) were applied to compute the overall odds ratio for allergic sensitization, blood eosinophil count, and total-IgE using compiled data from all four measuring points (6 months, 18 months, 4 years, and 6 years). Logistic regression was used to calculate odds ratios of the cross-sectional end-points allergic rhinitis, nasal eosinophilia, and asthma. Hazard ratios of recurrent wheeze and eczema at ages 0–1 and 0–6 year were calculated using Cox regression. Age at onset was modelled as a function of chemokine levels and ratios; the children were retained in the analysis from birth until age at onset of disease, drop-out, or age at end of the observation period (i.e. 1 year for 0–1 year), whichever first. Levels of chemokines, total-IgE, and blood eosinophil count were log-transformed prior to analysis to obtain normal distribution of data.

Results are reported with 95% CI in brackets. Robustness of results was investigated by adjusting for multiple testing according to the method by Bonferroni correction for control of false discovery rate. All analyses were performed in SAS version 9.1 for Windows (SAS Institute, Inc.).

Results

Baseline

Cord blood samples were available for analyses from 223 of the 411 children from the COPSAC2000 cohort. Children from low-income families were significantly less likely to have cord blood sampled \( P \)-value = 0.008, whereas there were no differences between children with and without cord blood samples with respect to ethnicity, exposure to tobacco, alcohol, and maternal antibiotic use during 3rd trimester, mode of delivery, gestational age, gender or anthropometrics (Table 1).

Levels of chemokines, total-IgE, blood eosinophil count, and frequency of nasal eosinophilia, allergic rhinitis, asthma, eczema, and allergic sensitization in the study group are outlined in Table 2.

### Table 1. Baseline demographics

<table>
<thead>
<tr>
<th>Baseline demographics</th>
<th>Entire COPSAC cohort ( N = 411 )</th>
<th>Cord blood</th>
<th>( P )-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( N = 223 )</td>
<td>( N = 188 )</td>
<td></td>
</tr>
<tr>
<td>Gender male</td>
<td>203 (49.4%)</td>
<td>112 (55.2%)</td>
<td>91 (44.8%)</td>
</tr>
<tr>
<td>Birth length(^1)</td>
<td>133 (32.0%)</td>
<td>75 (33.6%)</td>
<td>58 (30.9%)</td>
</tr>
<tr>
<td>Birth weight(^2)</td>
<td>129 (31.4%)</td>
<td>69 (30.9%)</td>
<td>60 (31.9%)</td>
</tr>
<tr>
<td>Mode of delivery(^3)</td>
<td>85 (20.7%)</td>
<td>43 (19.3%)</td>
<td>42 (22.3%)</td>
</tr>
<tr>
<td>Occupation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-professional</td>
<td>128 (33.5%)</td>
<td>69 (33.7%)</td>
<td>59 (33.3%)</td>
</tr>
<tr>
<td>Professional</td>
<td>174 (45.6%)</td>
<td>100 (48.8%)</td>
<td>74 (41.8%)</td>
</tr>
<tr>
<td>Student</td>
<td>42 (10.9%)</td>
<td>22 (10.7%)</td>
<td>20 (11.3%)</td>
</tr>
<tr>
<td>Unemployed</td>
<td>14 (6.8%)</td>
<td>14 (6.8%)</td>
<td>24 (13.6%)</td>
</tr>
<tr>
<td>Household income</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>112 (29.2%)</td>
<td>47 (22.8%)</td>
<td>65 (36.5%)</td>
</tr>
<tr>
<td>Average</td>
<td>182 (47.4%)</td>
<td>110 (53.4%)</td>
<td>72 (40.5%)</td>
</tr>
<tr>
<td>High</td>
<td>90 (23.4%)</td>
<td>49 (23.8%)</td>
<td>41 (23.0%)</td>
</tr>
<tr>
<td>Exposions</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smoking during third trimester</td>
<td>63 (15.3%)</td>
<td>36 (16.1%)</td>
<td>27 (14.4%)</td>
</tr>
<tr>
<td>Alcohol during third trimester</td>
<td>73 (17.8%)</td>
<td>40 (17.9%)</td>
<td>33 (17.6%)</td>
</tr>
<tr>
<td>Antibiotics third trimester</td>
<td>125 (30.4%)</td>
<td>67 (53.6%)</td>
<td>58 (46.4%)</td>
</tr>
</tbody>
</table>

\(^1\)Lowest birth length tertile.

\(^2\)Lowest birth weight tertile.

\(^3\)Caesarian section.

\(P\)-values were calculated using chi-square test.
Association between cord blood chemokine levels and atopic biomarkers

**Total-IgE.** Cord blood CCL22 levels were significantly associated with total-IgE levels at 6 months, 18 months, 4 years, and 6 years (GEE model including all four measuring points): odds ratio, 1.54 [CI, 1.25–1.89; P < 0.0001]. In addition, the Th2/Th1 ratios were also associated with Total-IgE levels: CCL22/CXCL11: odds ratio, 1.31 [CI, 1.13–1.51; P = 0.0002] and CCL22/CXCL10 odds ratio, 1.22 [CI, 1.03–1.43; P = 0.02]. However, the CCL22/CXCL10 ratio did not pass the Bonferroni threshold for significance (Table 3). There was no association between CCL17, CXCL11 or CXCL10 and Total-IgE levels. Figure 1 shows the individualized association between cord blood CCL22 and total-IgE at each measured time-point.

**Specific IgE.** No association was found between specific IgE and any cord blood chemokine levels (Table 3).

**Blood eosinophil count.** Cord blood CCL22 and CXCL11 levels were associated with development of blood eosinophilia in the overall GEE model: Odds ratio, 1.15 [CI, 1.00–1.32; P = 0.05] and odds ratio, 1.09 [CI, 1.00–1.20; P = 0.05], respectively. However, this was not significant after correction for multiple testing. There was no association between CCL22/CXCL10, CCL22/CXCL11, CCL17/CXCL10, CCL17/CXCL11 ratios, CXCL10 or CCL17, and blood eosinophilia (Table 3).

**Nasal eosinophilia at age 6 years** was not associated with any of the tested chemokines or Th2/Th1 ratios (Table S1 online).

### Discussion

**Principle findings**

Cord blood levels of the Th2 related chemokine CCL22 and Th2/Th1 chemokine ratios in healthy newborns from the Danish COPSAC2000 birth cohort of asthmatic mothers were significantly associated with elevated total-IgE levels during the first 6 years of life. This suggests an inborn/intrauterine Th2 skewing of the immune system in children subsequently developing high levels of total-IgE.

**Strengths and limitations**

The major strength of this study is the 6-year longitudinal, stringent, and uniform clinical follow-up of a birth cohort. COPSAC is a single-centre study, where the COPSAC doctors act as general practitioners for the children, diagnosing and treating all airway-, allergy- and skin-related symptoms according to pre-defined algorithms thus assuring consistency in diagnoses and reducing the risk of misclassification.

In addition, the atopic biomarkers; blood eosinophil count, specific-, and total-IgE were assessed repeatedly...

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**Table 2. Baseline levels of chemokines, atopic biomarker levels and occurrence of clinical end-points**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Birth (Cord blood)</th>
<th>6 Months</th>
<th>18 Months</th>
<th>4 Years</th>
<th>6 Years</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCL22 pg/mL; median (range)</td>
<td>441 (57–3581)</td>
<td>0.27 (0.06–1.45)</td>
<td>0.21 (0.04–1.19)</td>
<td>0.24 (0.01–3.0)</td>
<td>0.32 (0.0–3.5)</td>
</tr>
<tr>
<td>CCL17 pg/mL; median (range)</td>
<td>211 (8.6–3949)</td>
<td>4 (0.13–71)</td>
<td>7.7 (0.03–90)</td>
<td>27 (0.13–71)</td>
<td>46 (1.9–1564)</td>
</tr>
<tr>
<td>CXCL11 pg/mL; median (range)</td>
<td>215 (35–2657)</td>
<td>6.3 (14)</td>
<td>9.3 (21)</td>
<td>19 (42)</td>
<td>25 (56)</td>
</tr>
<tr>
<td>CXCL10 pg/mL; median (range)</td>
<td>35 (6.4–294)</td>
<td>36 (77)</td>
<td>36 (77)</td>
<td>36 (77)</td>
<td>36 (77)</td>
</tr>
<tr>
<td>Blood eosinophil count (× 10^9/L) median (range)</td>
<td>8 (13)</td>
<td>8 (13)</td>
<td>8 (13)</td>
<td>8 (13)</td>
<td>8 (13)</td>
</tr>
<tr>
<td>Total-IgE (kU/L); median (range)</td>
<td>5 (8)</td>
<td>5 (8)</td>
<td>5 (8)</td>
<td>5 (8)</td>
<td>5 (8)</td>
</tr>
<tr>
<td>Allergic sensitization*; % (N)</td>
<td>17 (12)</td>
<td>17 (12)</td>
<td>17 (12)</td>
<td>17 (12)</td>
<td>17 (12)</td>
</tr>
<tr>
<td>Sensitization ever; % (N)</td>
<td>17 (12)</td>
<td>17 (12)</td>
<td>17 (12)</td>
<td>17 (12)</td>
<td>17 (12)</td>
</tr>
<tr>
<td>Allergic rhinitis; 6 years; % (N)</td>
<td>17 (12)</td>
<td>17 (12)</td>
<td>17 (12)</td>
<td>17 (12)</td>
<td>17 (12)</td>
</tr>
<tr>
<td>Nasal eosinophilia† 6 years; % (N)</td>
<td>17 (12)</td>
<td>17 (12)</td>
<td>17 (12)</td>
<td>17 (12)</td>
<td>17 (12)</td>
</tr>
<tr>
<td>Asthma 7 years; % (N)</td>
<td>17 (12)</td>
<td>17 (12)</td>
<td>17 (12)</td>
<td>17 (12)</td>
<td>17 (12)</td>
</tr>
</tbody>
</table>

*Allergic sensitization = any sensitization for cat, dog, horse, birch, timothy grass, mugwort, house dust mites, moulds, hen’s egg, cow’s milk, fish, wheat, peanut, soybean, or shrimp.

†Nasal eosinophilia (≥ + 1 on Meltzer’s semi-quantitative scale).
at four time-points till 6 years of age, increasing the statistical power to detect true associations in GEE models accounting for repeated within subject measurements.

A further strength of the study is the sensitivity of the chemokine assays allowing assessment of chemokine levels in all available cord blood samples without stimulation, thus avoiding excessive un-physiological responses accompanying challenge models. Circulating Th1- and Th2-associated chemokine levels can be used as markers for the Th1/Th2 balance [4–8] making immune-regulatory chemokines potential biomarkers of current and future disease [20]. Th1 responses increase the levels of CXCR3 ligands CXCL10 and CXCL11, whereas Th2 responses enhance the levels of CCR4 ligands CCL17 and CCL22 [21, 22]. CXCR3 is preferentially expressed on Th1 cells and CCR4 on Th2 cells, thus further amplifying Th1 and Th2 immunity, respectively [22].

Finally, the large cord blood sample size of 223 samples increases the validity of the data.

Cord blood biomarkers may be biased by transfer from maternal blood. This has been demonstrated for specific- and total-IgE where maternal levels are often 1000 times higher than cord blood levels and even minimal amount of maternal blood may significantly...

Table 3. Association between cord blood chemokine levels and allergic sensitization, blood eosinophil count and total-IgE

<table>
<thead>
<tr>
<th>Chemokines</th>
<th>Allergic sensitization*</th>
<th>Blood eosinophil count</th>
<th>Total-IgE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Odds ratio [95% CI]</td>
<td>P-value</td>
<td>Odds ratio [95% CI]</td>
</tr>
<tr>
<td>CCL22</td>
<td>1.35 [0.94–1.95]</td>
<td>0.10</td>
<td>1.15 [1.00–1.32]</td>
</tr>
<tr>
<td>CCL22/CXCL10</td>
<td>1.08 [0.80–1.45]</td>
<td>0.62</td>
<td>1.07 [0.97–1.18]</td>
</tr>
<tr>
<td>CCL22/CXCL11</td>
<td>1.23 [0.90–1.68]</td>
<td>0.19</td>
<td>0.99 [0.91–1.09]</td>
</tr>
<tr>
<td>CXCL10</td>
<td>1.15 [0.76–1.72]</td>
<td>0.51</td>
<td>1.01 [0.90–1.13]</td>
</tr>
<tr>
<td>CXCL11</td>
<td>0.94 [0.66–1.33]</td>
<td>0.71</td>
<td>1.09 [1.00–1.21]</td>
</tr>
<tr>
<td>CCL17</td>
<td>0.97 [0.76–1.24]</td>
<td>0.82</td>
<td>1.06 [0.98–1.13]</td>
</tr>
<tr>
<td>CCL17/CXCL10</td>
<td>0.95 [0.77–1.16]</td>
<td>0.61</td>
<td>1.03 [0.97–0.99]</td>
</tr>
<tr>
<td>CCL17/CXCL11</td>
<td>1.02 [0.72–1.44]</td>
<td>0.91</td>
<td>0.99 [0.90–1.10]</td>
</tr>
</tbody>
</table>

Results are based on GEE analysis; According to Bonferroni correction for multiple testing, only P-values < 0.006 are considered significant and highlighted in bold.

*Allergic sensitization = any sensitization to cat, dog, horse, birch, timothy grass, mugwort, house dust mites, moulds, hen’s egg, cow’s milk, fish, wheat, peanut, soybean, or shrimp.
affect cord blood levels [23, 24]. Such bias from maternal transfer seems not to be an issue for cord blood chemokines that are often found in higher level in cord blood than maternal blood.

A limitation of our study is the high-risk nature of the COPSAC-2000 cohort where all mothers have a history of asthma, which requires replication in an unselected population. However, the analyses were based on within subject associations between cord blood chemokine levels and the subsequent development of atopic disease and atopic biomarkers unlikely to be affected by the increased risk of atopic diseases. This is supported by a previous study where elevated cord blood CCL22 levels preceded development of sensitisation independent of atopic heredity [9].

A further limitation is that cord blood samples were only available from half of the cohort. However, the selected cohort was representative of the main cohort for the majority of population characteristics.

Interpretation

Elevated cord blood levels of the CCL22 chemokine preceding development of raised total-IgE during the first 6 years of life suggests a triggering role of CCL22 in the inception of elevated total-IgE in children on an atopic trajectory. The mechanism responsible for the linkage between elevated CCL22 and raised total-IgE remains unknown, although CCL22 is described to play a central role in enhancing the Th2 responses. Thus, our data support the general hypothesis of an early-life Th2 skewing of the immune response [1, 3].

Cord blood CCL22 levels have previously been proposed as a predictor of elevated IgE levels and clinically manifest allergy development [6, 25]. However, only a few others have studied un-stimulated cord blood chemokines. Our findings are supported by a previous report from 56 children where cord blood CCL22 was associated with elevated IgE levels at age 6 [9]. We found no association between cord blood chemokine levels and development of atopic symptoms of asthma, rhinitis or eczema. It is possible that CCL22 is involved in a specific immunological mechanism only causing elevated total-IgE, but not specific sensitization or disease. Cord blood CCL17 and CCL22 have been associated with atopic dermatitis and asthma development in cohorts of mixed atopic and non-atopic parental predisposition [9, 26]. As this study is the largest to date, it is possible that previously reported association with atopic disease were the result of a type 1 error. The discrepancy between these findings and our data might also be due to the high-risk nature of the COPSAC-2000 cohort.

The mechanism behind elevated cord blood CCL22 remains unclear. However, such pronounced Th2/Th1 imbalance in the newborn may be driven by genetic variants and/or caused by unknown intrauterine environmental triggers impacting the fetal development.

We have previously shown that urine eosinophil protein X in neonates precedes development of allergic sensitization and eczema, [27] and that raised exhaled nitric oxide in neonates associates with development of early transient wheeze [28]. Importantly, these findings and the current study supports the concept that early immunological programming of immune function plays an essential role in the skewing of the immune response. Together such fingerprints of atopic disease development in newborns may contribute to construction of clinically feasible prediction models in the future.

Conclusion

High cord blood levels of the Th2 related chemokine CCL22 were significantly associated with high total-IgE levels during the first 6 years of life. This suggests an inborn Th2 skewing of the immune system in healthy newborns developing elevated total-IgE later in life.

Acknowledgement

The authors thank the children and parents participating in the COPSAC cohort as well as the COPSAC study team. We also thank Mrs Anne-Marie Fornander, Linköping University, for excellent technical assistance.

Source of funding and conflict of interest: COPSAC is funded by private and public research funds listed on http://www.copsac.com. The Lundbeck Foundation; The Danish Strategic Research Council; the Pharmacy Foundation of 1991; Augustinus Foundation; the Danish Medical Research Council and The Danish Pediatric Asthma Centre provided the core support for COPSAC research center. This study was also supported by the Swedish Research Council (K2011-56X-21854-01-06), the Cancer and Allergy Association and the Olle Engkvist Foundation to MJ. No pharmaceutical company was involved in the study. 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.

Authors Contribution: The guarantor of the study is HB who has been responsible for the integrity of the study as a whole, from conception and design to acquisition of data, analysis, and interpretation of data and writing of the manuscript. NVF contributed to data analyses, statistical analysis, interpretation, and writing of the manuscript. MJ supervised the chemokine analyses, data interpretation, and writing of the manuscript. BLKC and KB contributed to data analyses, interpretation, and writing of the manuscript. All authors made important intellectual contributions and critical final revision of the manuscript.

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References

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Association between chemokine levels at birth and development of nasal eosinophilia at age 6. Nasal eosinophilia is measured by nasal scraping twice in the sixth year of life and defined as more than +1 on Meltzer’s semi-quantitative scale.

Table S2. Association between chemokines at birth and the development of eczema in the first 6 years of life.

Table S3. Association between chemokine levels at birth and recurrent wheeze.

Table S4. Association between chemokine levels at birth and development of asthma at age 7.

Table S5. Association between Chemokine levels at birth and development of allergic rhinitis at age 6.

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Elevated Eosinophil Protein X in Urine from Healthy Neonates Precedes Development of Atopy in the First 6 Years of Life

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Rationale: Biomarkers predicting development of atopic disease are needed for targeted preventive measures and to study if disease pathology may be active before onset of symptoms.

Objectives: To investigate whether eosinophil protein X, leukotriene C4/D4/E4, and 11β-prostaglandin (PG) F2α (PGD2 metabolite) assessed in urine from healthy at-risk neonates precede development of atopic disease during the first 6 years of life.

Methods: We measured eosinophil protein X (n = 369), leukotriene C4/D4/E4 (n = 367), and 11β-PGF2α (n = 366) in urine from 1-month-old children participating in the Copenhagen Prospective Studies on Asthma in Childhood birth cohort. Clinical data on development of allergic sensitization, allergic rhinitis, nasal eosinophilia, blood eosinophilia, eczema, troublesome lung symptoms (significant cough or wheeze or dyspnea), and asthma were collected prospectively until age 6 years. Associations between urinary biomarkers and development of atopic traits were investigated using general estimating equations, logistic regression, and Cox regression.

Measurements and Main Results: Eosinophil protein X in the urine of the asymptomatic 1-month-old neonates was significantly associated with development of allergic sensitization (odds ratio, 1.49; 95% confidence interval [CI], 1.08–1.89), nasal eosinophilia (odds ratio, 3.2; 95% CI, 1.2–8.8), and eczema (hazard ratio, 1.4; 95% CI, 1.0–2.0), but not with allergic rhinitis, asthma, or blood eosinophilia. Neither leukotriene-C4/D4/E4 nor 11β-PGF2α was associated with any of the atopic phenotypes.

Conclusions: Eosinophil protein X in urine from asymptomatic neonates is a biomarker significantly associated with later development of allergic sensitization, nasal eosinophilia, and eczema during the first 6 years of life. These findings suggest activation of eosinophil granulocytes early in life before development of atopy-related symptoms.

Keywords: atopy; blood eosinophils; eczema; neonates; urinary eosinophil protein X

Identification of children at high risk of developing atopic disorders is a major research challenge. Biomarkers predicting development of atopic disease before the onset of symptoms are needed for targeted prevention and individualized intervention and treatment. Furthermore, it is an important research question if the disease process may be active before development of symptoms as we previously reported on increased fraction of exhaled nitric oxide in neonates later developing early wheeze (1) and pathogenic bacterial colonization of the airways in neonates later developing asthma (2).

Urinary eosinophil protein X (u-EPX) released from activated eosinophil granulocytes is seen in association with current allergic sensitization (3) and has been associated with severity of eczema (4) and acute and chronic childhood asthma (3, 5, 6). Urinary leukotriene (LT) C4/D4/E4 (u-LTC4/D4/E4), a measure of total body cysteinyl LT production, is elevated in subjects with symptomatic allergic rhinitis (7) and in preschoolers with acute wheeze illnesses (8, 9). Prostaglandin (PG) D2, the major cyclooxygenase product from activated mast cells, has been associated with allergen-induced asthma illustrated by elevated urinary level of 11β-PGF2α (u-11β-PGF2α), which is a major stable PGD2 metabolite (10). Thus, u-EPX, u-LTC4/D4/E4, and u-11β-PGF2α are all well-known markers of established atopic inflammation, but it is unknown whether levels are elevated very early in life preceding symptom debut.

We measured u-EPX, u-LTC4/D4/E4, and u-11β-PGF2α in healthy high-risk 1-month-old asymptomatic neonates to investigate whether elevated level of these biomarkers precede subsequent development of atopic disease during the first 6 years of life. We used the comprehensive longitudinal clinical data on troublesome lung symptoms, asthma, eczema, rhinitis, allergic sensitization, blood, and nasal eosinophils in the Copenhagen Prospective Study on Asthma in Childhood (COPSAC) birth cohort of high-risk babies.
We hypothesized that elevated levels of u-EPX, u-LT\textsubscript{C4/D4/E4}, and u-11\beta-PGF\textsubscript{2a} very early in life reflects presymptom disease activity preceding later development of atopic disease.

METHODS

Study Design

The COPSAC birth cohort is a prospective clinical study of 411 children born to mothers with asthma previously described in detail (2, 11, 12). The symptom-free children were enrolled at 1 month of age and subsequently attended the clinical research unit for scheduled clinical investigations at 6-monthly intervals and immediately on onset of any respiratory or skin-related symptom. Key exclusion criteria were gestational age less than 36 weeks, severe congenital abnormality, neonatal mechanical ventilation and symptoms of lower airway infection before 1 month of age.

The study was conducted in accordance with the Declaration of Helsinki and was approved by The Copenhagen Ethics Committee (KF 01–289/96) and The Danish Data Protection Agency (2008–41–1754) (11). Informed consent was obtained from both parents before enrollment.

Urinary Inflammatory Markers

Urine was collected during visits to the COPSAC research unit at ages 1 and nasal swabs in a sterile plastic bag adherent to the skin. The urine samples were immediately transferred to 3.6-mL Nunc tubes, and the aliquots were stored without addition of any preservatives at −80°C until analysis.

After thawing, levels of EPX, LT\textsubscript{C4/D4/E4}, and 11\beta-PGF\textsubscript{2a} were measured in the urine samples. u-EPX concentration was determined by a double-antibody immunosay (radioimmunoassay; Pharmacia Upjohn, AB, Uppsala, Sweden) in the both 1- and 6-month samples. The combined quantitative value of u-LT\textsubscript{C4/D4/E4} levels was measured by ELISA test kits (Neogen Corporation, Lexington, KY). Urinary level of 11\beta-PGF\textsubscript{2a} was also assessed by ELISA test kits (Neogen Corporation).

To standardize for variations in renal excretion, levels of all urinary markers were adjusted for creatinine excretion (13). Urinary creatinine levels were measured by buffered kinetic Jaffe reaction (Cobas Integra 700 analyzer; Roche Diagnostics, Hvidovre, Denmark). All urine laboratory analyses were performed at Phadia AbS, Allerød, Denmark.

Atopic Intermediary End Points

Allergic sensitization was determined at ages 6 and 18 months and 4 and 6 years by measurement of serum-specific IgE against 15 common inhalant and food allergens (cat, dog, horse, birch, timothy grass, mugwort, house dust mites, molds, hen’s egg, cow’s milk, fish, wheat, peanut, soybean, and shrimp) by ImmunoCAP assay (Pharmacia Diagnostics AB, Uppsala, Sweden). Sensitization was defined as specific IgE greater than or equal to 0.35 kU/L (14, 15) for any of the tested allergens and was analyzed as a dichotomized measurement.

In a secondary analysis we stratified presence of allergic sensitization as (1) food sensitization, being any sensitization for hen’s egg, cow’s milk, fish, wheat, peanut, soybean, or shrimp; (2) sensitization for inhaled allergens, being any sensitization for cat, dog, horse, birch, timothy grass, mugwort, house dust mites, or molds; (3) sensitization for outdoor aeroallergens, being any sensitization for birch, timothy grass, or mugwort; and (4) sensitization for indoor aeroallergens, being any sensitization for cat, dog, house dust mites, or molds.

Blood eosinophil count (10\textsuperscript{9} cells per liter) was assessed at ages 6 and 18 months and 4 and 6 years. Nasal eosinophilia was investigated by nasal scraping in the child’s sixth year of life and rated according to Meltzer’s semiquantitative scale (16) as previously detailed (17).

Clinical Investigator-diagnosed End Points

Allergic rhinitis was diagnosed at age 6 by the COPSAC doctors based on clinical interviews of the parents (not questionnaires) on history of symptoms in the child’s sixth year of life (17–19). Rhinitis was defined as troublesome sneezing or blocked or runny nose in the past 12 months in periods without accompanying cold or flu (20). Significance of symptoms was judged by severity, length of periods, and time of year. Allergic rhinitis was diagnosed in children with rhinitis and sensitization to aeroallergens (cat, dog, horse, birch, timothy grass, mugwort, house dust mites, or molds) clearly related to the symptomatic periods.

Troublesome lung symptoms (significant cough or wheeze or dyspnea) were recorded by the parents as a dichotomized daily score (yes or no) from birth until age 7 years (21). Recurrent troublesome lung symptoms (recently termed “multitrigger wheezing” [22]) were defined from the diaries as five episodes within 6 months, each episode lasting at least 3 consecutive days, or daily symptoms for 4 consecutive weeks (1, 23). Children meeting these criteria were prescribed a 3-month trial of budesonide, 200 μg twice a day in a pressurized metered dose inhaler with a spacer. Episodic viral troublesome lung symptoms (22) characterized children suffering discrete symptomatic episodes, with the child being well between episodes, not fulfilling the criteria for recurrent troublesome lung symptoms, and treated intermittently solely with inhaled β₂-agonist. Early transient troublesome lung symptoms was diagnosed in children with troublesome lung symptoms at age 0–3 years but without symptoms at age 3–6 years; late-onset troublesome lung symptoms in children with troublesome lung symptoms with an age at onset at age 3–6 years; and persistent troublesome lung symptoms in children who had symptoms both at age 0–3 and 3–6 years.

Asthma at age 7 years was diagnosed by the COPSAC doctors according to international guidelines and was based on recurrent troublesome lung symptoms, symptoms characteristic, and response to trials of inhaled corticosteroids, as previously detailed (2, 12) (see www.copsac.com and the online supplement for further details on diagnosis of recurrent troublesome lung symptoms and asthma).

Eczema was diagnosed using the Hanifin-Rajka criteria, as previously detailed (24, 25). Skin lesions were described at both scheduled and acute visits according to predefined morphology and localization.

Statistical Analyses

Quantitative levels of u-EPX, u-LT\textsubscript{C4/D4/E4}, and u-11\beta-PGF\textsubscript{2a} at age 1 month and u-EPX and blood eosinophil count at age 6 months were used as explanatory variables. Models using general estimating equations (GEE) (PROC GENMOD in SAS version 9.2; SAS Institute, Inc., Cary, NC) were applied to compute the overall odds ratio for allergic sensitization and the overall β-coefficient for blood eosinophil count using compiled data from all four time points (6 and 18 mo, 4 and 6 yr). Logistic regression was used to calculate odds ratios of the cross-sectional end points allergic rhinitis, nasal eosinophilia, asthma, and the patterns of troublesome lung symptoms from 0–6 years. Hazard ratios of recurrent troublesome lung symptoms and eczema at age 0–1 year and 0–6 years were calculated by Cox regression. Age at onset was modeled as a function of the urinary biomarkers; the children were retained in the analysis from birth until age at onset of disease, dropout, or age at end of the observation period (i.e., 1 yr for 0–1 yr), whichever came first. Levels of urinary biomarkers and blood eosinophil count were log-transformed before analysis.

Results are reported with 95% confidence interval (CI) in brackets; a P value less than or equal to 0.05 was considered significant. All analyses were done in SAS version 9.2 for Windows (SAS Institute, Cary, NC). Further details of the methods are outlined in the online supplement.

RESULTS

Baseline Characteristics

This protocol for urinary inflammatory markers was initiated after enrolling 42 of the 411 children of the cohort leaving 369 for urine analysis at age 1 month. Measurement of u-EPX was completed for all 369 children, u-LT\textsubscript{C4/D4/E4} for 367, and u-11\beta-PGF\textsubscript{2a} for 366, respectively; u-EPX was available for 337 children at age 6 months.

The study group consisted of 51% boys (n = 187). In the first year of life, 4% of the children developed recurrent troublesome lung symptoms and 27% were diagnosed with eczema. A further
17% developed recurrent troublesome lung symptoms and 15% eczema before age 6 years. Level of urinary markers, blood eosinophil count, and frequency of allergic sensitization, allergic rhinitis, nasal eosinophilia, and asthma are presented in Table 1.

There were no significant differences among the 42 subjects without urine samples and the 369 subjects in the study group with respect to sex, allergic sensitization, allergic rhinitis, nasal eosinophilia, asthma, eczema, or blood eosinophilia at age 6 years (see Table E1 in the online supplement).

**u-EPX at Age 1 Month**

**Allergic sensitization.** u-EPX at age 1 month was significantly associated with increased odds of allergic sensitization (GEE model including the four time points of 6 and 18 mo and 4 and 6 yr; odds ratio, 1.49; 95% CI, 1.08–1.89; P = 0.02) (Table 2), as illustrated by the plot of age-dependent odds ratios in Figure 1. Adjusting the analysis for maternal allergic sensitization assessed by specific-IgE measurements did not substantially modify the association (odds ratio, 1.45; 95% CI, 1.03–1.88; P = 0.04). Analyses stratified for type of sensitization: food allergens, inhaled allergens, and outdoor and indoor aeroallergens showed similar results (Table E2). Elevated u-EPX at age 1 month was associated with an increased odds of sensitization irrespective of the type of sensitization; the analysis was significant for food sensitization (P = 0.01), borderline significant for aeroallergen sensitization (P = 0.06) and sensitization to indoor inhaled allergens (P = 0.08), but not significant for outdoor inhaled allergens (P = 0.38) (Table E2).

**Allergic rhinitis and nasal eosinophilia.** u-EPX at age 1 month was strongly and significantly associated with nasal eosinophilia at age 6 years (odds ratio, 3.2; 95% CI, 1.2–8.8; P = 0.02). In addition, there was a tendency of increased odds for allergic rhinitis by age 6 years (Table 2).

**Blood eosinophilia.** u-EPX at 1 month was significantly associated with blood eosinophil count at age 6 years (β-coefficient, 0.19; 95% CI, 0.03–0.38; P = 0.05) (Table E3), but was not overall significantly associated with blood eosinophil count in the GEE model including all four measuring points (Table 2).

**Eczema.** The cumulative risk of eczema during the first 6 years of life for neonates with u-EPX level above and below the third quartile of the study group (149 ng/L) is illustrated in Figure E1, suggesting that elevated u-EPX is associated with development of eczema early in life. Accordingly, the risk of eczema during the first year of life increased significantly with increasing levels of u-EPX at age 1 month (hazard ratio, 1.4; 95% CI, 1.0–2.0; P = 0.05), but not thereafter (Table 3).

**Troublesome lung symptoms and asthma.** u-EPX was not associated with an increased risk of developing either recurrent, episodic viral, early transient, late-onset, or persistent troublesome lung symptoms during the first 6 years of life (Tables E3 and E4), or with asthma by age 7 (Table 2, Table E4).

**u-EPX at Age 6 Months**

In the overall GEE model including all four time points (6 and 18 mo and 4 and 6 yr), we found an increased odds of allergic sensitization, which was borderline significant (odds ratio, 1.21; 95% CI, 0.98–1.54; P = 0.10). Log (u-EPX/u-creatinine) ratio at age 6 months was significantly higher in subjects with current sensitization at age 6 months (mean difference, +0.28; 95% CI, 0.00–0.56; P = 0.05).

Elevated u-EPX at age 6 months was significantly associated with development of allergic rhinitis at age 6 years (odds ratio, 2.3; 95% CI, 1.0–3.5; P = 0.05). Apart from this, the analyses of u-EPX at age 6 months showed similar association to the atopic end points as described for u-EPX at age 1 month (Tables 2 and 3). In particular, we found no association with any patterns of troublesome lung symptoms or asthma (Tables E3 and E4), and log (u-EPX/u-creatinine) ratio at age 6 months was not significantly increased in children with current troublesome lung symptoms at age 6 months (mean difference, +0.10; 95% CI, −0.25 to 0.46; P = 0.58).

**u-LTα4/D4/E4 and u-11β-PGF2α at Age 1 Month**

No associations were found between these biomarkers and any of the end points studied (Tables 2 and 3).

**Blood Eosinophil Count at Age 6 Months**

Blood eosinophil count at age 6 months was associated with sensitization at ages 4 and 6 years, but not significantly in the overall GEE model (Table 2). Blood eosinophil count at 6 months was strongly overall associated with eosinophil counts at ages 18 months and 4 and 6 years (β, 0.41; 95% CI, 0.31–0.51; P < 0.0001). There was no association of blood eosinophil count at age 6 months with the development of allergic rhinitis, nasal eosinophilia, eczema, troublesome lung symptoms, or asthma (Tables 2 and 3).

**DISCUSSION**

Principal Findings

u-EPX in healthy 1-month-old neonates was significantly associated with development of allergic sensitization, nasal eosinophilia, and eczema during the first 6 years of life in children of the Danish COPSAC birth cohort suggesting early life eosinophilic activation before symptom debut in children developing atopy-related conditions.

### TABLE 1. BASELINE CHARACTERISTICS

<table>
<thead>
<tr>
<th>Variable</th>
<th>1 Mo</th>
<th>6 Mo</th>
<th>18 Mo</th>
<th>4 Yr</th>
<th>6 Yr</th>
</tr>
</thead>
<tbody>
<tr>
<td>u-EPX, µg/L, median (range)</td>
<td>83.9 (5.4–1,298)</td>
<td>131 (14.8–2,293)</td>
<td></td>
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<tr>
<td>u-LTα4/D4/E4, µg/L, median (range)</td>
<td>0.05 (0–19.4)</td>
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<tr>
<td>u-11β-PGF2α, µg/L, median (range)</td>
<td>0.20 (0.03–1.5)</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Blood eosinophil count, ×10^9/L, median (range)</td>
<td>0.29 (0.02–1.6)</td>
<td>0.21 (0.02–1.2)</td>
<td>0.24 (0.01–3)</td>
<td>0.33 (0–3.5)</td>
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</tr>
<tr>
<td>Allergic sensitization, prevalence, % (n)</td>
<td>7.1 (24)</td>
<td>11.6 (38)</td>
<td>26.6 (77)</td>
<td>36.3 (97)</td>
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</tr>
<tr>
<td>Allergic rhinitis, prevalence, % (n)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>9.3 (24)</td>
</tr>
<tr>
<td>Nasal eosinophilia, prevalence, % (n)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>6.7 (17)</td>
</tr>
<tr>
<td>Asthma, prevalence, % (n)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>13.6 (41)</td>
</tr>
</tbody>
</table>

*Definition of abbreviations: PG = prostaglandin; u-EPX = urinary eosinophil protein X; u-LT = urinary leukotriene.

* Allergic sensitization — any sensitization for cat, dog, horse, birch, timothy grass, mugwort, house dust mites, molds, hen’s egg, cow’s milk, fish, wheat, peanut, soybean, or shrimp.

† Nasal eosinophilia ≥ +1 on Meltzer’s semiquantitative scale.

† Asthma at age 7 yr.
This study provides the first data of urinary inflammatory markers measured in healthy neonates before development of any atopic symptom. Previous studies have solely investigated subjects with established atopy (3–10).

The differential associations with u-EPX associating with several atopic traits during preschool age and u-LT_C4/D4/E4 and u-11′-PGF_2 alpha without association with any of the atopic endpoints make the risk of a chance finding unlikely.

It is a major strength of this study that the clinical end points were collected in a closely monitored single-center birth cohort study with comprehensive longitudinal data on development of atopic disorders. The risk of misclassification and variability in the clinical orders. The risk of misclassification and variability in the clinical
diagnosis (21). The diagnostic specificity was ensured by the COPSAC doctors who acted as general practitioners for the cohort. Risk of recall bias was minimized because the families attended the research unit at half-yearly sessions and at acute symptomatic periods.

Parental misinterpretation of symptoms was minimized because all mothers have a history of asthma and high prevalence of related atopic disorders, such as eczema and rhinitis, and clinical assessments were made at all visits by the COPSAC doctors.

The symptom diaries on troublesome lung symptoms (significant cough or wheeze or dyspnea) maintained by the parents day-to-day from birth until age 7 years is another significant strength of the study increasing the sensitivity of the asthma diagnosis (21). The diagnostic specificity was ensured by the research doctors making all diagnoses at the clinical research center minimizing the risk of misclassification by different doctors. Furthermore, the asthma diagnoses were strictly based on an algorithm including only children responding to inhaled corticosteroids and currently in need of this treatment after repeated attempts to withdraw the treatment (i.e., we have not included children with intermittent asthma in this end point definition). Other studies with less specific diagnostic criteria inevitably led to a higher prevalence of asthma. However, our approach with a strong emphasis on diagnostic specificity has proved powerful in both genetic (23, 26, 27) and clinical association studies (1, 2).

The high-risk nature of the cohort is a limitation to the generalizability of our findings and replication is needed in an unselected population-based cohort.

Meaning of the Study

It is intriguing that elevation of the biomarker u-EPX in healthy symptom-free newborns precedes the development of atopy later in childhood. u-EPX is a biomarker known to correlate well with eosinophil count in blood and bronchoalveolar lavage fluid (28). Therefore, our findings suggest eosinophilic activation before atopy-related symptoms develop.

Elevated level of u-EPX in healthy asymptomatic neonates was associated with later development of allergic sensitization and eczema during the first 6 years of life. Previous studies have consistently reported increased u-EPX in preschoolers with established allergic sensitization (3, 5) and atopic dermatitis (4, 29), and in children more than 5 years of age with a diagnosis of atopic asthma (5, 6, 30, 31). We found no association between u-EPX at ages 1 or 6 months and the later development of troublesome lung symptoms or asthma, nor was u-EPX elevated in 6-month-old children with current troublesome lung symptoms, which is in agreement with a previous report of 1-year-old wheezy infants (32) and probably reflects the fact that airway...
esinophils are scarce in this age group (33). In contrast, elevated EPX in nasal lavage samples from a population of healthy 4-week-old infants at high risk of atopy was significantly associated with the development of wheezing at age 6 months (34). However, this was a questionnaire-based survey with “a doctor’s diagnosis of wheezy bronchitis” as the main outcome measure and the authors found no association between EPX and parental observations of wheeze (34).

We also found a significant association between u-EPX at age 1 month and presence of nasal eosinophilia at 6 years of age, which has not been reported previously. Elevated u-EPX at ages 1 and 6 months showed increased odds of allergic rhinitis at age 6 years and the association was significant for the 6-month sample. A previous study found no association between u-EPX and allergic rhinitis (3), but this was based on questionnaire diagnosis in children at age 3 years (35 cases among 903 children) where a diagnosis of rhinitis is infrequent and difficult to establish (35).

The mechanism behind this presumptive eosinophilic activation is unknown. The origin of an early life eosinophilic activation might be dermal or nondermal (29). A dermal contribution to elevated u-EPX is suggested by increasing levels of u-EPX in children with increasing severity of atopic dermatitis (4). However, the gut mucosa has been proposed as a nondermal focus of eosinophilic inflammation in infants with food allergy (36), which is plausible because food sensitization prevails during infancy. Neonates with elevated u-EPX may host a distinct dysfunctional eosinophil cell immune phenotype with altered surface antibodies resulting in an increased liability to degranulate EPX and other eosinophil basic proteins. In addition, genetic variants in the eosinophil pathway activation genes might modulate immunoregulation (e.g., via IL-5, IL-10, IL-13, and IFN-γ) of eosinophil granulocytes (37, 38) and contribute to increased degranulation of EPX.

Blood eosinophil count at age 6 months was strongly associated with persistent blood eosinophilia until age 6 years, but interestingly, it showed poorer association with all other atopic end points than u-EPX at 1 and 6 months of age. This suggests that u-EPX is a better assessment of eosinophil activity than the blood cell count.

We found no association between u-LTα,β,γ,δ and u-11β-PGF₂α at age 1 month and any of the studied atopic end points. LTs and PGs are released during the immediate early phase allergic reaction by degranulation of IgE-sensitized mast cells, but are also released within the first hours of the late-phase reaction from several inflammatory cells types, such as mononuclear cells and basophilic granulocytes (39). In contrast, EPX is solely secreted by activated eosinophils, which are the predominant inflammatory cell type in the chronic late-phase reaction (16). The different cellular origin and temporal release pattern of EPX, LTα,β,γ,δ, and 11β-PGF₂α in the inflammatory cascade might explain why only EPX seems to play a role in the inception of atopy very early in life. Future cellular studies of functional and regulatory aspects of the eosinophil granulocyte may help understand initiation of atopic disease early in life.

The clinical implication of our findings is that elevated u-EPX early in life seems to herald the onset of atopy-related conditions, such as allergic sensitization to food and inhaled allergens, allergic rhinitis, nasal eosinophilia, and eczema, but not troublesome lung symptoms or asthma during preschool age. The lack of association with preschool troublesome lung symptoms may reflect the heterogeneity of symptom triggers in this age group where allergens serve a quantitatively minor role compared with triggers of troublesome lung symptoms and asthma later in life.

Conclusions

u-EPX measured in healthy asymptomatic 1-month-old neonates was associated with the development of allergic sensitization, nasal eosinophilia, and eczema during preschool age. This suggests ongoing disease pathology including activation of eosinophil granulocytes preceding the development of childhood atopy-related disorders.

Author Disclosure: B.L.K.C. does not have a financial relationship with a commer- cial entity that has an interest in the subject of this manuscript. K.B. was a consultant for Merck and GlaxoSmithKline (GSK) and was on the Advisory Board for Merck. He received lecture fees from Merck and GSK. H.B. was a consultant for Chiesi and was on the Advisory Board for Merck. He received lecture fees from Merck and AstraZeneca (AZ). He was an expert witness for NeoLab and received grant support from Novartis and Chiesi. He received grant support from the Lundbeck Foundation and the Danish Medical Research Foundation.

Acknowledgment: The authors gratefully express their gratitude to the children and families of the COPSAC cohort study for all their support and commitment. The authors acknowledge and appreciate the unique efforts of the COPSAC research team. The authors thank Phadis ApS, and especially Bjarne Kristensen and all laboratory crew at Phadia ApS, Allerød, Denmark, for assistance with analyses of the urine samples.

References


Elevated Exhaled Nitric Oxide in High-Risk Neonates Precedes Transient Early but Not Persistent Wheeze

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Rationale: Elevated fractional exhaled nitric oxide (FE\textsubscript{NO}) concentration has been suggested to predict early childhood wheeze and sensitization.

Objectives: To investigate the association between FE\textsubscript{NO} in asymptomatic neonates and the development of wheeze patterns and atopic intermediary phenotypes in the first 6 years of life.

Methods: We measured FE\textsubscript{NO} in 253 healthy 1-month-old neonates from the Copenhagen Prospective Study on Asthma in Childhood birth cohort and monitored prospectively wheezy episodes by daily diary cards during the first 6 years of life. Total IgE, specific IgE, and blood eosinophil count were assessed at age 6 months, 4 years, and 6 years. Associations were studied by Cox regression, logistic regression, and generalized linear models.

Measurements and Main Results: Increased neonatal FE\textsubscript{NO} level was significantly associated with the development of recurrent wheeze in the first year of life (hazard ratio, 2.63; 95% confidence interval, 1.1 to 6.2; \(P = 0.026\)) but not thereafter. The association was unaffected by environmental tobacco smoke exposure. FE\textsubscript{NO} was not associated with elevated levels of total IgE, specific IgE, or blood eosinophil count at any age point and was unrelated to neonatal lung function.

Conclusions: An elevated FE\textsubscript{NO} level in asymptomatic neonates born to mothers with asthma preceded the development of transient early wheezing, but not persistent wheezing during preschool age, and was unrelated to atopy. This suggests an early disease process other than small airway caliber contributing to the transient wheezing phenotype.

Keywords: atopy; children; fractional exhaled nitric oxide; infants; wheeze

Fraction of exhaled nitric oxide (FE\textsubscript{NO}) has been proposed as a noninvasive marker of eosinophilic airway inflammation (1), which can be measured in both school-aged (2) and preschool-aged children (3, 4), and in infants (5, 6). FE\textsubscript{NO} is increased in chronic asthma (7), before exacerbations in children with asthma (8, 9), and in preschool children with recurrent wheeze (3, 4, 10). Increased FE\textsubscript{NO} values in neonates have been suggested to predict severe respiratory symptoms in the first year of life, but only in a particular subgroup of newborns with atopic and/or smoking mothers (6).

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject
Elevated exhaled nitric oxide has been suggested to predict early childhood wheeze and sensitization.

What This Study Adds to the Field
Increased exhaled nitric oxide level in asymptomatic neonates born to mothers with asthma precedes transient early wheezing, but not persistent wheezing during preschool age, and is unrelated to elevated levels of total IgE, specific IgE, and blood eosinophil count.

The aim of our study was to analyze the association between FE\textsubscript{NO} in asymptomatic neonates and the development of wheezy phenotypes and intermediary atopic end-points during the first 6 years of life. We studied the association between FE\textsubscript{NO} in 253 healthy 1-month-old neonates and subsequent development of wheeze patterns, blood eosinophil count, total immunoglobulin E (IgE), and allergic sensitization in the first 6 years of life. The children were included from the Copenhagen Prospective Study on Asthma in Childhood (COPSAC) birth cohort born of mothers with a history of asthma.

None of the results in this study has been previously reported in abstract or any other form.

METHODS

Design
The study was nested in COPSAC: a single-center, prospective clinical birth cohort study of 411 children born to mothers with asthma, enrollment of which was previously described in detail (11–13). Key exclusion criteria included the following: gestational age less than 36 weeks, severe congenital abnormality, neonatal mechanical ventilation, and symptoms of lower airway infection before inclusion.

All subjects participated in a randomized controlled clinical trial of intermittent treatment with inhaled budesonide versus placebo for 2-week periods during wheezy episodes in the first 3 years of life, showing no short-term or long-term treatment effect (11).

Oral and written informed consent was obtained from all parents of participating children. The study followed the guiding principles of the Declaration of Helsinki, and was approved by the Ethics Committee for Copenhagen (KF 01-289/96) and the Danish Data Protection Agency (2008-41-1754).

Neonatal FE\textsubscript{NO} Measurements
FE\textsubscript{NO} was measured at 1 month of age by an offline technique (14), after the completion of neonatal lung function testing during sedation (15, 16). FE\textsubscript{NO} measurement was performed in asymptomatic neonates, that is, before any respiratory symptoms or prescription of inhaled corticosteroid.

A mask covering the mouth and nose attached to a two-way valve was gently placed on the infant’s face during sedation. A respiratory resistance of at least 5 cm H\textsubscript{2}O was achieved by a resistor fitted into the
expiratory side of the valve. The infant inhaled ambient air, but measurements were canceled if ambient NO exceeded 10 parts per billion (ppb). When stable tidal breathing was assured, an impermeable bag (Quintron Instrument, Milwaukee, WI) was attached to the expiratory side of the valve and 750 ml of mixed expired air was collected. Within 15 minutes, the bags were attached to the inlet of the NO analyzer and air was continuously sampled from the bags at a flow of 110 ml/minute. The $F_{\text{NO}}$ level was measured with a chemiluminescence analyzer (CLD 77 AM; EcoPhysics, Duernten, Switzerland). The sensitivity was 0.1 ppb and rise time (0–90%) was 0.1 second. The analyzer was calibrated at least once daily with certified NO gas (100 ppb) (AGA, Gothenburg, Sweden). $F_{\text{NO}}$ levels were calculated as the mean of duplicate measurements in each infant.

Wheezy Phenotypes

Wheezy episodes. Wheeze was explained to the parents as wheeze or whistling sounds, breathlessness, or cough severely affecting the well-being of the child, and was recorded by the parents in daily diaries as composite dichotomized scores from birth until age 6 years. Doctors at the COPSAC research unit reviewed symptom definition and the diary entries with the parents at 6-monthly clinical sessions and at acute clinic visits for wheezy episodes defined from the diaries as three consecutive days with wheeze.

Recurrent wheeze. Recurrent wheeze was defined as five wheezy episodes within 6 months, or daily symptoms for four consecutive weeks. The symptom character was judged by the COPSAC doctor to be typical of discrete wheezy episodes and included symptoms between episodes, such as exercise-induced symptoms; prolonged nocturnal cough; persistent cough not associated with a common cold; symptoms causing waking at night; and need for intermittent rescue use of inhaled $\beta_2$-agonist. Age at onset of recurrent wheeze was used as the primary endpoint.

Episodic viral wheeze. Episodic viral wheeze (17) characterized children suffering discrete wheezy episodes, with the child being well between episodes and treated intermittently with inhaled $\beta_2$-agonist.

Atopic Intermediary Phenotypes

Blood was sampled at age 6 months, 4 years, and 6 years for measurements of eosinophil count, total IgE, and specific IgE (18). Sensitization was defined as specific IgE equal to or greater than 0.35 kU/L for at least 1 of 15 tested airborne and food allergens. Blood was sampled at age 6 months, 4 years, and 6 years for measurements in each infant.

RESULTS

COPSAC enrolled 411 infants and performed neonatal lung function testing in 402 at 1 month of age by the raised volume rapid thoracoabdominal compression technique (15, 16). This current protocol was enacted approximately 1 year into the enrollment period, leaving 286 infants eligible for $F_{\text{NO}}$ measurement. Measurements were canceled if the child woke up before expired air was collected (n = 5), ambient NO exceeded 10 ppb (n = 21) (14), or technical problems arose (n = 7), leaving 253 infants for analysis.

Baseline Characteristics

The study group consisted of 122 males (48%). Thirty-seven numbers (15%) smoked during the third trimester of their pregnancy. In the first year of life, 138 children suffered wheezy episodes (median number of episodes, 2; quartile 1 [Q1]–Q3, 1–4; range, 1–8), whereas 172 children had at least one wheezy episode until age 6 years (median, 6; Q1–Q3, 3–12; range, 1–59). Recurrent wheeze was diagnosed in 4% of the children before age 1 year, and in a further 17% before age 6 years. Anthropometrics, lung function data, total IgE levels, eosinophil counts, age at child care start, and hair nicotine levels at age 1 year are given in Table 1.

Infants not included in this nested study were comparable to the study group with respect to anthropometrics at study date, mothers smoking during the third trimester, previous miscarriages, number of siblings, and age at child care start (univariate tests; data available on request).

$F_{\text{NO}}$ and Wheezy Phenotypes

The cumulative risk of recurrent wheeze during the first 6 years of life for neonates with $F_{\text{NO}}$ concentration above and below the third quartile of the study group (22 ppb) is illustrated in Figure 1, suggesting that neonatal $F_{\text{NO}}$ is associated with a transient early wheezy phenotype. Accordingly, increased $F_{\text{NO}}$ concentration measured at age 1 month was significantly associated with the development of recurrent wheeze in the first year of life (hazard ratio, 2.63; 95% CI, 1.1–6.2; P = 0.026), but not thereafter (Table 2). Adjusting the analyses for sex, age, maternal smoking in the third trimester, nicotine in hair at age 1 year, previous miscarriages, number of siblings, child care attendance, minute volume, and ambient NO level did not alter the results substantially (Table 2).

Elevated neonatal $F_{\text{NO}}$ level was also associated with the development of episodic viral wheeze in the first year of life.

### TABLE 1. STUDY GROUP ANTHROPOMETRICS, LUNG FUNCTION DATA, TOTAL IGE, BLOOD EOSINOPHIL COUNT, CHILD CARE ATTENDANCE, AND HAIR NICOTINE LEVELS

<table>
<thead>
<tr>
<th>Age at study date, d</th>
<th>n</th>
<th>Median</th>
<th>Interquartile Range</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight at study date, kg</td>
<td>253</td>
<td>5.0</td>
<td>4.5–5.5</td>
<td>3.0–8.2</td>
</tr>
<tr>
<td>Total IgE at age 6 yr, kU/L</td>
<td>189</td>
<td>42.7</td>
<td>18.7–110.0</td>
<td>0.8–2,423.7</td>
</tr>
<tr>
<td>Blood eosinophil count at age 4 yr, 10/L</td>
<td>220</td>
<td>0.3</td>
<td>0.2–0.4</td>
<td>0.1–1.5</td>
</tr>
<tr>
<td>Blood eosinophil count at age 6 mo, 10/L</td>
<td>190</td>
<td>0.2</td>
<td>0.2–0.4</td>
<td>0.05–1.7</td>
</tr>
<tr>
<td>Blood eosinophil count at age 4 yr, 10/L</td>
<td>178</td>
<td>0.3</td>
<td>0.2–0.5</td>
<td>0.3–3.5</td>
</tr>
<tr>
<td>Total IgE at age 6 mo, kU/L</td>
<td>222</td>
<td>4.0</td>
<td>2.2–9.3</td>
<td>0.1–8.07</td>
</tr>
<tr>
<td>Total IgE at age 4 yr, kU/L</td>
<td>198</td>
<td>25.8</td>
<td>12.6–64.7</td>
<td>2.1–722.0</td>
</tr>
<tr>
<td>Total IgE at age 6 yr, kU/L</td>
<td>189</td>
<td>12.5</td>
<td>8.7–110.0</td>
<td>0.8–2,423.7</td>
</tr>
<tr>
<td>Age at child care start, d</td>
<td>238</td>
<td>342</td>
<td>244–411</td>
<td>140–1,074</td>
</tr>
<tr>
<td>Total IgE at age 6 yr, 10/L</td>
<td>229</td>
<td>0.7</td>
<td>0.3–2.2</td>
<td>0.03–41.5</td>
</tr>
</tbody>
</table>

Definition of abbreviations: $F_{\text{NO}}$ = fraction of exhaled nitric oxide; ppb = parts per billion.
Figure 1. Cumulative risk of recurrent wheeze during the first 6 years of life stratified by neonatal exhaled nitric oxide concentrations above and below the third quartile of the study group.

(OR, 2.24; 95% CI, 1.3–3.9; P = 0.010), but not until age 6 years (OR, 0.90; 95% CI, 0.5–1.6; P = 0.701) (Table 3).

In addition, \( F_{\text{NO}} \) concentration was associated with the number of wheezy episodes during the first year of life, but not until age 6 years. The incidence risk ratio of having wheezy episodes during the first year of life was 1.36 (95% CI, 1.1–1.7; P = 0.011) and 0.93 (95% CI, 0.8–1.1; P = 0.135) until age 6 years (Table 4).

\( F_{\text{NO}} \) and Environmental Tobacco Smoke Exposure

There was no effect modification from environmental tobacco smoke exposure on the association between neonatal \( F_{\text{NO}} \) levels and the development of recurrent wheeze in the first 6 years of life; neither from mothers smoking as assessed by interview in the third trimester of pregnancy nor when assessed by nicotine in the hair by age 1 year (OR, 2.24; 95% CI, 1.3–3.9; P = 0.010), but not until age 6 years (OR, 0.90; 95% CI, 0.5–1.6; P = 0.701) (Table 3).

Table 3. Association Between Neonatal Fraction of Exhaled Nitric Oxide and Development of Recurrent Wheeze Until Age 6 Years

<table>
<thead>
<tr>
<th>Recurrent Wheeze</th>
<th>F(_{\text{NO}}) per 20 ppb, Crude</th>
<th>F(_{\text{NO}}) per 20 ppb, Adjusted*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hazard Ratio (95% CI)</td>
<td>P Value</td>
<td>Hazard Ratio (95% CI)</td>
</tr>
<tr>
<td>-------------------</td>
<td>-------------------</td>
<td>-------------------</td>
</tr>
<tr>
<td>0–1 yr</td>
<td>2.63 (1.1–6.2)</td>
<td>0.026</td>
</tr>
<tr>
<td>0–2 yr</td>
<td>1.37 (0.7–2.7)</td>
<td>0.381</td>
</tr>
<tr>
<td>0–3 yr</td>
<td>0.90 (0.4–1.8)</td>
<td>0.752</td>
</tr>
<tr>
<td>0–4 yr</td>
<td>0.68 (0.3–1.3)</td>
<td>0.263</td>
</tr>
<tr>
<td>0–5 yr</td>
<td>0.63 (0.3–1.2)</td>
<td>0.171</td>
</tr>
<tr>
<td>0–6 yr</td>
<td>0.63 (0.3–1.2)</td>
<td>0.159</td>
</tr>
</tbody>
</table>

* \( F_{\text{NO}} \) per 20 ppb adjusted for sex, age, maternal smoking in third trimester, nicotine in hair at age 1 year, number of previous miscarriages, number of siblings, age at start of child care, minute volume, and ambient NO.

DISCUSSION

Principal Findings

\( F_{\text{NO}} \) level in symptom-free 1-month-old neonates was associated with the development of transient early wheeze, but not persistent wheeze, in the COPSAC birth cohort of mothers with asthma. There was no association between infant \( F_{\text{NO}} \) and neonatal lung function or lung function at age 6 years, or with the development of total IgE, blood eosinophil count, or allergic sensitization, and no effect modification from environmental tobacco exposure.

Strength and Limitations

The major strength of our study is the 6-year-long clinical follow-up of a birth cohort with prospective monitoring of respiratory symptoms in daily diary cards and 6-monthly symptom review by doctors at the research unit. Risk of misclassification was minimized as recurrent wheeze was diagnosed from the diaries according to predefined algorithms and the families solely attended the doctors employed at the clinical research unit for diagnosis and treatment of any respiratory symptom rather than their family practitioner.

The major limitation of our study is the setting of a high-risk cohort as all mothers have a history of asthma, which limits the external validity of the study. However, the analyses are based on within-subject associations between \( F_{\text{NO}} \) and subsequent development of wheezy phenotypes, which are unlikely to be affected by the increased risk of atopic diseases.

Table 3. Association Between Neonatal Fraction of Exhaled Nitric Oxide and Episodic Viral Wheeze

<table>
<thead>
<tr>
<th>Episodic Viral Wheeze</th>
<th>Odds Ratio (95% CI)</th>
<th>P Value</th>
<th>Odds Ratio (95% CI)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>0–1 yr</td>
<td>2.24 (1.3–3.9)</td>
<td>0.010</td>
<td>2.11 (1.3–3.7)</td>
<td>0.014</td>
</tr>
<tr>
<td>0–2 yr</td>
<td>0.90 (0.5–1.6)</td>
<td>0.701</td>
<td>0.91 (0.5–1.7)</td>
<td>0.751</td>
</tr>
</tbody>
</table>

* \( F_{\text{NO}} \) per 20 ppb adjusted for sex, age, maternal smoking in third trimester, nicotine in hair at age 1 year, number of previous miscarriages, number of siblings, age at start of child care, minute volume, and ambient NO.
The accuracy of the method may be limited as the expiratory flow was not controlled. This was partly compensated by FENO assessment in mixed exhaled air, which diminished breath-to-breath variations. In addition, sedation stabilized respiration and the expiratory resistance limited flow range and reduced nasal contribution to the exhaled air. FENO levels in our study group were comparable to other studies despite different methodology (6, 24).

Meaning of the Study

Wheezy disorders are highly prevalent in early childhood (25) and one of the main reasons for hospitalization of preschool children in westernized countries (26). Early age at onset (27) and recurrent severe symptoms (28) are important determinants of persistent wheeze throughout childhood and subsequent development of asthma later in life. Thus, the challenge is to develop noninvasive identification strategies applicable to early childhood before onset of first wheeze to improve prevention and treatment. Our findings suggest that neonatal FENO measurement cannot provide such risk assessment because it does not predict persistent wheeze.

The underlying mechanism causing raised FENO in neonates before the development of persistent wheeze is still unknown. This may not reflect atopic inflammation of the airways because there are few eosinophils in the airways even in symptomatic infants (29). Accordingly, neonatal FENO was not associated with the development of increased levels of total IgE, specific IgE, or increased blood eosinophil count at the ages of 6 months, 4 years, or 6 years. Cross-sectional studies have shown elevated FENO levels in older children with eosinophilia and allergic sensitization (30), but no difference in FENO level in sensitized as compared with nonsensitized infants (31), suggesting that elevated FENO in this age group is not a marker of atopic inflammation. In line with these findings, we observed a similar association between neonatal FENO and preschool recurrent wheeze in neonates with and without raised total IgE, specific IgE, or blood eosinophil count. Therefore, it may be speculated that other inflammatory processes are driving transient early wheeze and increased FENO.

Latzin and colleagues also reported that high FENO values after birth in a subgroup of neonates born of atopic and smoking mothers were associated with an increased risk of severe respiratory symptoms in the first year of life (6), but did not monitor their study population after age 1 year. We found that elevated FENO in 1-month-old neonates born to mothers with asthma was associated with transient early wheezing, but not persistent wheezing in our 6-year follow-up with daily symptom recordings. In contrast to the findings of Latzin and colleagues, we saw no effect from mothers smoking as assessed by interview in the third trimester of pregnancy, or when assessed by nicotine in the hair by age 1 year.

### TABLE 4. ASSOCIATION BETWEEN NEONATAL FRACTION OF EXHALED NITRIC OXIDE AND NUMBER OF WHEEZY EPISODES IN FIRST 6 YEARS OF LIFE

<table>
<thead>
<tr>
<th>No. of Wheezy Episodes</th>
<th>FENO per 20 ppb, Crude</th>
<th>FENO per 20 ppb, Adjusted*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Incidence Risk (95% CI)</td>
<td>P Value</td>
</tr>
<tr>
<td>0–1 yr</td>
<td>1.36 (1.1–1.7)</td>
<td>0.011</td>
</tr>
<tr>
<td>0–6 yr</td>
<td>0.93 (0.8–1.1)</td>
<td>0.135</td>
</tr>
</tbody>
</table>

**Definition of abbreviations:** CI = confidence interval; FENO = fraction of exhaled nitric oxide; ppb = parts per billion.

* FENO per 20 ppb adjusted for sex, age, maternal smoking in third trimester, nicotine in hair at age 1 year, number of previous miscarriages, number of siblings, age at start of child care, minute volume, and ambient NO.

### Conclusion

An elevated FENO level measured in healthy neonates of mothers with asthma before any wheezy symptom precedes the onset of transient early wheezing, but not persistent wheezing, and is unrelated to underlying lung function changes or the development of increased total IgE, specific IgE, and blood eosinophil count. These findings suggest an early disease process other than small airway caliber contributing to transient early wheeze.

**Conflict of Interest Statement:** B.L.K.C. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; A.L.B. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; L.L. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; M.H. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; L.B.H. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; K.B. received $10,001–$50,000 from Merck as a website consultant and $1,001–$5,000 from GlaxoSmithKline as a consultant on educational material, $1,001–$5,000 from Merck and $10,001–$50,000 from NeoLab in consultancy fees, $10,001–$50,000 from Merck and $10,001–$50,000 from AstraZeneca in lecture fees, $1,001–$5,000 from NeoLab, and $1,001–$5,000 from Chiesi for serving as an expert witness, more than $100,001 from Novartis and more than $100,001 from NeoLab in industry-sponsored grants.

**Acknowledgment:** The authors gratefully acknowledge all children and families participating in the COPSAC cohort study. The authors thank research assistants Lena Vinding, Kirsten Hiby Mathiesen, and Lotte Klaasen.

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DENND1B Gene Variants Associate With Elevated Exhaled Nitric Oxide in Healthy High-Risk Neonates

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Summary. Rationale of the Study: Increased neonatal fraction of exhaled nitric oxide (FeNO) is associated with lung symptoms early in life, while predictors of neonatal FeNO levels are unknown. The objective of this study was to investigate perinatal and genetic predictors of FeNO in healthy at-risk neonates. Methods: FeNO was measured during sedation by single-breath and tidal-breathing techniques in 253 one-month-old neonates from the Copenhagen Prospective Study on Asthma in Childhood (COPSAC2000) birth cohort. The risk factor analyses included genetic variants in DENND1B, Filaggrin, and ORMDL3; anthropometrics; demographics; socioeconomics; paternal atopy; maternal smoking, and mother’s consumption of paracetamol and antibiotics during 3rd trimester; and neonatal bacterial airway colonization. Results: FeNO values measured by the single-breath versus tidal-breathing technique yielded slightly higher values (median, 21.0 ppb; range, 2.0–74.0 ppb vs. 16.0 ppb; 1.0–67.0 ppb; P < 0.0001) with increasing differences conditional on increasing FeNO values (P < 0.0001). The multivariable analysis including all risk factors showed that the DENND1B rs2786098 C allele was associated with increasing levels of FeNO (additive model; +2.30 ppb per C allele; 95% CI, 0.10–5.00 ppb; P = 0.04) and that children of atopic fathers had elevated FeNO (+2.90 ppb; 95% CI, 0.38–5.43 ppb; P = 0.02). We did not detect association between the remaining risk factors and neonatal FeNO levels. Conclusion: Increased FeNO in healthy newborns seems strongly influenced by genetics including father’s atopy and child’s variants in the DENND1B locus at chromosome 1q31.3.


Key words: asthma; genetics; nitric oxide; infant.

Funding source: Lundbeck Foundation; Pharmacy Foundation of 1991; Augustinus Foundation; Danish Medical Research Council and The Danish Pediatric Asthma Centre

INTRODUCTION

Fraction of exhaled nitric oxide (FeNO) is measured as a part of the routine clinical work up in many clinical pediatric pulmonology centers to diagnose1 and monitor childhood asthma.2,3 While assessment of FeNO level is standard in children from approximately 5 years of age,4,5 it is also feasible and reproducible to measure FeNO in neonates.6,7

In a recent report from the Copenhagen Prospective Study on Asthma in Childhood (COPSAC2000) birth cohort born to asthmatic mothers8 we observed a wide inter-subject variation in neonatal FeNO values (1–67 ppb) and subsequently showed that raised FeNO measured in asymptomatic healthy neonates prior to any respiratory symptoms is associated with the development of specific childhood wheezy endotypes.9 Whereas FeNO during school age is interpreted as a marker of eosinophilic airway inflammation,10 the predictors of elevated neonatal FeNO are poorly understood.

The purpose of the current study was to explore the previously reported wide range of neonatal FeNO levels by investigating genetic and early life environmental factors.
predictors of the FeNO levels measured in 253 neonates from the COPSAC2000 birth cohort. Environmental risk factors were selected a priori based on the existing literature on predictors of neonatal FeNO, for example, parental atopic status and environmental tobacco smoke exposure,7,11,12 but also including well-know pre- and perinatal factors influencing development of wheezy disorders, for example, anthropometrics, socioeconomic factors, antibiotic and paracetamol consumption during pregnancy, and bacterial colonization of the neonatal airway.13–16 Genetic analyses were performed according to a candidate gene approach including only well-known robust early-onset-asthma risk variants, that is, Filaggrin,17 ORMDL3,18 and DENND1B.19

MATERIALS AND METHODS

Study Design

Participants were included from the COPSAC2000 single center prospective clinical birth cohort study recruited in 1998–2001. This birth cohort included 411 one-month-old high-risk neonates born to mothers with a history of asthma. Exclusion criteria were gestational age <36 weeks; any congenital abnormality and systemic illness; requirement of neonatal mechanical ventilation; and symptoms of lower airway infection prior to inclusion. Enrollment and baseline characteristics of the COPSAC2000 cohort were previously described in detail.8,13,20

Ethics

The study followed the guiding principles of the Declaration of Helsinki, and was approved by the Ethics Committee of Copenhagen (KF 01-289/96) and The Danish Data Protection Agency (2008-41-1754).8 Oral and written informed consent was obtained from all parents of participating children.

Neonatal FeNO Measurements

FeNO level was measured at 1 month of age after completion of neonatal spirometry testing utilizing raised volume rapid thoracoabdominal compression (RVRTC) technique during sedation with an oral dose of chloral hydrate 90 mg/kg.21–24 Measurements and bag sampling were performed in asymptomatic neonates prior to any respiratory symptoms, and by two separate techniques in all neonates.

Single-Breath Technique25. After completion of baseline lung function measurement, a mask attached to a two-way valve was placed on the infants face covering the mouth and nose. A 250 ml impermeable bag (Quintron Instrument, Milwaukee, WI) was connected to the expiratory side of the valve with a resistor interposed assuring an expiratory resistance of at least 5 cm H2O.

Thereafter, a compression force, similar to the one used for the previous spirometry assessment by inflation of a squeeze jacket wrapped around the infants thorax and abdomen, was applied, that is, leading to a standardized forced expiration into the bag with flow similar to the one obtained during spirometry measurements. The squeeze maneuver was repeated 10 times collecting five expirations into two bags.

Tidal-Breathing Technique26. After completion of the last squeeze maneuver, steady tidal breathing was assured in the sedated infant. Using the same mask and two-way valve as for the single-breath technique, a 750 ml impermeable bag (Quintron Instrument) was attached to the expiratory side of the valve, and two bags of expired air were sampled during steady tidal breathing. The respiratory frequency and the collection time were registered to calculate respiratory minute volume.

During both techniques, the infant inhaled ambient air, but if ambient nitric oxide (NO) exceeded 10 ppb, the FeNO measurement was cancelled.

FeNO Measurements. Within 15 min, the bags were attached to the inlet of a NO-analyzer and the expired air was sampled continuously from the bags with a constant flow of 110 ml/min. Concentration of FeNO was measured by a chemiluminescence analyzer (EcoPhysics CLD 77 AM, Duernen, Switzerland). The sensitivity was 0.1 ppb and rise time (0–90%) was 0.1 sec. The analyzer was calibrated once daily using certified NO gas (100 ppb) (AGA, Gothenburg, Sweden).

FeNO levels were analyzed and calculated as the mean of duplicate measurements in each infant separate for each sampling technique.

Pre- and Perinatal Risk Factors

Anthropometrics: Height and weight were assessed prior to neonatal FeNO measurement. Body mass index (BMI) was calculated and categorized as 7–12, 12–13, 13–14, and 14–17 kg/m2 as previously reported.14

Socioeconomic status was evaluated from yearly household income at birth of the infant classified into three groups as low (<53,000€), medium (53,000–80,000€), and high (>80,000€).
Demographics comprised sex and older siblings at birth (yes/no).

Paternal atopy was any history of asthma, allergic rhinitis and/or atopic dermatitis (yes/no).

Exposures during 3rd trimester of pregnancy included smoking (yes/no), paracetamol [acetaminophen] (yes/no), and antibiotic usage (yes/no).

Bacterial colonization of the airway was assessed at age 1 month from hypopharyngeal aspirates performed immediately after the FeNO sampling procedure as previously reported. Denend1b, filaggrin, and ORMdl3 genotyping:

Filaggrin genotyping for the two common independent null-mutations R501X and 2282del4 was performed as previously described. Children were assigned as having a filaggrin mutation if they carried at least one of the mutations.

ORMDL3 genotyping: Allelic discrimination at the rs7216389 single nucleotide polymorphism on chromosome 17q12–21 was performed as previously detailed. DENND1B genotyping: Allelic discrimination at the rs2786098 on chromosome 1q31.3 was performed as described by Sleiman et al.

Statistical Analysis Strategy

First, we investigated the distribution of the neonatal FeNO values by quantile–quantile plots (QQ-plot) and Shapiro–Wilks test for normality.

Second, we performed a Box-Cox transformation analysis in the lambda-range from −3 to 3 investigating lambda at 0.5 subsequent steps.

Third, we tested agreement between the two FeNO measuring techniques (single-breath vs. tidal-breathing) by calculating the mean difference between the measured FeNO values, standard deviation of the difference, paired t-tests, Bland–Altman plots and by fitting a regression model for the difference between the methods conditional on the average using both raw data and transformed data according to the results of the Box-Cox transformation analysis. We used an additive model when analyzing rs2786098 in the DENND1B gene using the A allele as reference and the C allele as risk factor (AA = 0; AC = 1; CC = 2). Association with the ORMdl3 T allele was investigated in a recessive model using the CC and CT genotypes as reference. All analyses were adjusted for respiratory minute volume (750 ml) estimated from the bag volume (750 ml/time) × 60.

Fifth, a multivariable analysis was performed with complete cases only including all variables in the initial GLM model with subsequent backward elimination of non-significant variables using a cut-off at P < 0.10. We challenged the multivariable model by performing a forward selection procedure based on the largest value of adjusted R² with a P < 0.10 cut off, that is, when all candidate effects for entry at a step have a statistical significance level <0.10.

In addition, we calculated coefficients of variation (CV) between the duplicate measurements of FeNO in the two bags utilizing the raw data in the formula: CV = standard deviation of the difference between bags/mean of the difference.

RESULTS

Baseline Characteristics

This current protocol was initiated 1 year after starting the recruitment of the Copsac cohort leaving 286 of 411 recruited infants eligible for neonatal FeNO measurements. FeNO levels were assessed with both techniques at one month of age in 253 of the 286 infants (Fig. E1).

FeNO values, lung function data, bacterial airway colonization, gender distribution, anthropometrics, demographics, older siblings at birth, paternal atopy, exposures during 3rd trimester of pregnancy, and genetics are outlined in Table 1.

Infants enrolled in the study compared to the rest of the Copsac cohort had lower FEV0.5, less paracetamol consumption in 3rd trimester, and higher household income (Table 1). Since we did not detect association between these variables and FeNO levels, we do not assume this selection bias affected the main findings of the study.

Neonatal FeNO Measurements

Median neonatal FeNO level was 21.0 ppb (range: 2.0–74.0 ppb) measured with the single-breath technique and
16.0 ppb (range, 1.0–67.0 ppb) with the tidal-breathing technique. The coefficient of variation between the duplicate measurements of FeNO in the two bags was 0.99 for the single-breath technique and 1.05 for the tidal-breathing technique.

QQ-plots of the raw neonatal FeNO values (Fig. E2 Online) showed a skewed distribution of FeNO measured by both techniques and the Shapiro–Wilk test indicated that the FeNO data was not normally distributed ($P < 0.0001$). A Box-Cox transformation analysis showed that the best lambda value for normalizing data from both measuring techniques was 0.5 corresponding to a square root transformation.

A paired $t$-test of the square root transformed FeNO values showed slightly higher values measured by the single-breath versus tidal-breathing technique (mean difference, 0.54; standard deviation of the difference, 0.39; $P < 0.0001$). Bland–Altman plots of the raw data and the square root transformed data indicated that the difference was dependent on the average of measured FeNO levels with increasing difference conditional on increasing average (Fig. E3 Online), which was confirmed by fitting regression models for the raw data: estimate, 0.27; 95% CI, 0.23–0.31; $P < 0.0001$, and the square root transformed data: estimate 0.13; 95% CI, 0.09–0.16; $P < 0.0001$).
We chose the raw untransformed FeNO values from the least invasive tidal-breathing technique to investigate possible risk factors influencing neonatal FeNO levels in our primary analyses and added auxiliary analyses with square root transformed FeNO values.

**Risk Factor Analysis of Neonatal FeNO**

**Univariable Analyses.** The univariable risk factor analyses showed higher levels of FeNO in children of atopic fathers (+3.57 ppb; 95% CI, 1.20 to 5.97; \( P < 0.01 \)) and in children with the DENND1B C allele (+2.55 ppb per C allele; 95% CI, 0.38 to 4.71; \( P = 0.02 \)) (Figs. 1 and 2). FeNO was higher in children of fathers with asthma and eczema \( (P < 0.01) \), borderline higher in children of fathers with rhinitis and allergy \( (P = 0.08 \) for both), whereas we did not detect association between neonatal FeNO values and maternal rhinitis, allergy or eczema (Table E1 Online). We did not detect association between neonatal FeNO levels and sex; BMI; older siblings at birth; tobacco, paracetamol, and antibiotic consumption in 3rd trimester; household income; bacterial colonization of the airway; Filaggrin mutations or ORMDL3 gene variants (Table 2).

Ancillary analysis with square root transformed FeNO values confirmed the results of the primary analyses with higher FeNO estimates in children with atopic fathers (estimate, 0.45; 95% CI, 0.17–0.73; \( P < 0.01 \)) and in children with the DENND1B allele (estimate per C allele, 0.28; 95% CI, 0.03–0.54; \( P = 0.03 \)). Also, utilizing FeNO values measured by the single-breath method yielded comparable results with higher values in children of atopic fathers (+4.81 ppb; 95% CI, 2.06 to 7.56 ppb; \( P < 0.01 \)) and in children with the DENND1B C allele (+2.98 ppb per C allele; 95% CI, 0.42 to 5.54 ppb; \( P = 0.02 \)).

**TABLE 2—Univariate Risk Factor Analysis of Neonatal FeNO**

<table>
<thead>
<tr>
<th>Neonatal FeNO, tidal-breathing technique, ppb</th>
<th>( \beta )-coefficient</th>
<th>95% CI</th>
<th>( P )-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female sex</td>
<td>1.05</td>
<td>−1.32 to 3.43</td>
<td>0.38</td>
</tr>
<tr>
<td>Household income</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low &lt;53,000€</td>
<td>Reference</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Medium 53,000–80,000€</td>
<td>−1.63</td>
<td>−4.75 to 1.49</td>
<td>0.30</td>
</tr>
<tr>
<td>High &gt;80,000€</td>
<td>0.05</td>
<td>−3.50 to 3.60</td>
<td>0.98</td>
</tr>
<tr>
<td>Father, atopy</td>
<td>3.58</td>
<td>1.20 to 5.97</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Bacterial colonization at age 1 mo</td>
<td>−1.99</td>
<td>−4.97 to 1.00</td>
<td>0.19</td>
</tr>
<tr>
<td>Smoking, 3rd trimester</td>
<td>−1.46</td>
<td>−4.82 to 1.91</td>
<td>0.39</td>
</tr>
<tr>
<td>Paracetamol, 3rd trimester</td>
<td>−1.84</td>
<td>−4.21 to 0.54</td>
<td>0.13</td>
</tr>
<tr>
<td>Antibiotics, 3rd trimester</td>
<td>0.38</td>
<td>−2.21 to 2.98</td>
<td>0.77</td>
</tr>
<tr>
<td>Older siblings at birth</td>
<td>0.81</td>
<td>−1.71 to 3.33</td>
<td>0.53</td>
</tr>
<tr>
<td>BMI, kg/m(^2), 4 weeks</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7–12</td>
<td>Reference</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12–13</td>
<td>6.29</td>
<td>−13.92 to 26.49</td>
<td>0.54</td>
</tr>
<tr>
<td>13–14</td>
<td>7.33</td>
<td>−12.20 to 26.86</td>
<td>0.46</td>
</tr>
<tr>
<td>14–17</td>
<td>3.52</td>
<td>−15.42 to 22.47</td>
<td>0.71</td>
</tr>
<tr>
<td>DENND1B(^1) (additive model)</td>
<td>2.55</td>
<td>0.38 to 4.71</td>
<td>0.02</td>
</tr>
<tr>
<td>Filaggrin mutation</td>
<td>−0.50</td>
<td>−4.77 to 3.76</td>
<td>0.82</td>
</tr>
<tr>
<td>ORMDL3</td>
<td>0.30</td>
<td>−2.39 to 2.99</td>
<td>0.83</td>
</tr>
</tbody>
</table>

\(^{1}\)Per C allele.

Variables significantly associated with neonatal FeNO are in bold \( (P < 0.05) \).

Pediatric Pulmonology
Multivariable Analysis. In the multivariable analysis with backward selection, including all pre- and perinatal risk factors and genetics, DENND1B gene variants (+2.30 ppb per C allele; 95% CI, 0.10 to 5.00 ppb; \( P = 0.04 \)) and paternal atopy (+2.90 ppb; 95% CI, 0.38 to 5.43 ppb; \( P = 0.02 \)) remained predictors of increased neonatal FeNO. The forward selection procedure stopped at step 2 with paternal atopy \( (P = 0.01) \) and DENND1B \( (P = 0.05) \) included in the model resulting in \( R^2 = 0.037 \) thus confirming the results from the backward selection procedure.

DISCUSSION

Principle Findings

Neonatal FeNO measured prior to any respiratory symptoms exhibited a wide dispersion ranging between 1 and 67 ppb. Neonatal FeNO levels were increased from paternal atopy and DENND1B gene variants, suggesting that the wide range of FeNO values in healthy high-risk newborns is mainly driven by genetics.

Strengths and Limitations of the Study

It is a strength of this study that one study physician performed all FeNO measurements following standard operating procedures for both methods including criteria for accepting and rejecting data. FeNO measurements were completed in a high proportion of the eligible infants (88.4%) and measurements were done sequentially by both techniques in 99.6% of the children. All FeNO values were double checked against source data and the database was subsequently locked for further editing.

This is the first study to measure and compare the two different measuring techniques of neonatal FeNO and the largest study of FeNO in neonates measured prior to any respiratory symptoms.

The assessment of FeNO in neonates is complicated by requirements of steady breathing at a constant flow. A methodological limitation is that the exhaled flow was not controlled during FeNO measurements by the tidal-breathing technique, but this was partially compensated by the fact that the children were sedated ensuring steady tidal breathing which together with the fixed respiratory resistance limited the flow range. The resistor fitted into the expiratory side of the valve also ensured that the soft palate closed during sampling and thereby, limited nasal contamination of the breath sample. Finally we applied the alternative technique of FeNO measurements during fixed flow repeating the RVRTC technique used for the previous spirometric assessments assuring standardized flow similar to the one obtained during spirometry.

The absolute FeNO values measured may also have been influenced by the previous infant spirometry that could have altered expiratory flows during gas sampling. However, these conditions were the same for all the infants and should not compromise our ability to study risk factors driving the inter-subject variation in the cohort.

A limitation of the study is the low number of subjects in the study cohort which precludes a Genome-wide association study (GWAS) approach to investigate genetic predictors of neonatal FeNO. Instead we applied a candidate gene approach including only three well-known risk variants of childhood asthma. The novel finding of association between DENND1B gene variants and raised neonatal FeNO is biologically plausible and was identified in the univariable and in the multivariable analysis. The set of ancillary analyses showing comparable results including the untransformed FeNO values obtained by both techniques as well the square root transformed FeNO values increase our confidence in the findings. Adjustments for the multiplicity of tests performed were not made; consequently, the apparent association with both DENND1B and paternal atopy may be chance findings. Replication in a large population based cohort is therefore needed.

Another limitation of the study is the setting of a high-risk cohort which limits the ability to generalize the findings from the risk-factor analysis. Still, the median FeNO levels of our study group are comparable to findings from population based cohorts.

Interpretation

It was an interesting observation that healthy neonates presented a very wide range of FeNO values ranging between 1 and 67 ppb that led us to study what possible pre- or perinatal factors could explain such variability. The risk factor analyses showed that neonates of atopic fathers and neonates carrying the DENND1B C allele have increased neonatal FeNO levels, but the analysis was inconclusive in regard to association between FeNO and other pre- and perinatal environmental risk factors studied.

The C allele of the DENND1B single nucleotide polymorphism has recently been found to confer an increased risk of childhood asthma, but other complex inflammatory diseases such as Crohn’s disease and primary biliary cirrhosis have also been associated with variants in this gene suggesting a common immune dysregulation. The mechanism by which DENND1B gene variants may influence NO production is unknown, but DENND1B is expressed by natural killer cells and dendritic cells which play an important role linking innate and adaptive immune responses in the process of developing tolerability or immunity. DENND1B gene variants may therefore induce a skewing of the immune...
response and a dysregulated inflammatory response leading to elevated FeNO levels very early in life.

We have previously shown that raised neonatal FeNO is associated with wheezing illnesses early in life but is unrelated to development of other asthma- and allergy related traits such as sensitization, allergic rhinitis, elevated total-IgE, and blood eosinophil count during pre-school age. Neonatal FeNO is not associated with infant pulmonary function suggesting that DENND1B risk variants induce an early inflammatory disease process independent of small airway caliber contributing to the development of a specific endotype of childhood asthma.

Previous studies failed to detect association between FeNO and paternal atopy; the discrepancy between that and our finding could be explained by the COPSAC cohort being born to mothers with asthma as previous studies showed that maternal atopy modifies the prenatal risk of elevated FeNO. We conjecture that in our COPSAC cohort association between neonatal FeNO and maternal atopic disorders (e.g., eczema, allergic sensitization, and rhinitis) may not be evident due to all mothers having a history of asthma.

Evidence of association between pre- and postnatal tobacco exposure and neonatal FeNO was inconclusive. In contrast, others have reported that neonatal FeNO is affected by both perinatal tobacco exposure. However, these results are ambiguous as one study showed that infants exposed pre- and postnatally to tobacco smoke had lower FeNO than infants exposed only after birth and never-exposed infants whereas another study reported higher FeNO in infants exposed to maternal smoking after birth.

Studies of FeNO in infants are scarce because most measuring techniques are complicated and difficult to perform. The two techniques used in the current study have previously been shown to be feasible. The single-breath technique ensures a high relatively constant FeNO and paternal atopy; and our finding could be explained by the discrepancy between that and our finding could be explained by the COPSAC cohort being born to mothers with asthma as previous studies showed that maternal atopy modifies the prenatal risk of elevated FeNO. We conjecture that in our COPSAC cohort association between neonatal FeNO and maternal atopic disorders (e.g., eczema, allergic sensitization, and rhinitis) may not be evident due to all mothers having a history of asthma.

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Studies of FeNO in infants are scarce because most measuring techniques are complicated and difficult to perform. The two techniques used in the current study have previously been shown to be feasible. The single-breath technique ensures a high relatively constant expiratory flow for the 10 exhalations used to sample air in each child, but not a constant inter-child expiratory flow, which is considered a prerequisite in FeNO measurements as FeNO is flow-dependent. The tidal-breathing technique partly compensates this issue by sampling exhaled air in a bag during several breathing cycles producing roughly similar results compared to measurements done with fixed expiratory flow. Our data showed that the single-breath technique yielded slightly higher FeNO values than the tidal-breathing technique with increasing differences by increasing FeNO values. However, ancillary analyses utilizing FeNO obtained from both techniques showed comparable associations to paternal atopy and DENND1B variants and we therefore suggest usage of the least invasive tidal-breathing technique for future studies.

CONCLUSION

Variants in the DENND1B locus of chromosome 1q31.3 and paternal atopy were found to be associated with elevated FeNO in healthy Danish high-risk neonates. Associations were not detected between FeNO and other pre- and perinatal environmental factors studied suggesting that raised FeNO early in life is primarily an inherited trait. These findings however need replication in large population-based GWAS cohorts.

ACKNOWLEDGMENTS

We gratefully express our gratitude to the children and families of the COPSAC cohort study for all their support and commitment. We acknowledge and appreciate the unique efforts of the COPSAC research team. COPSAC is funded by private and public research funds all listed on www.copsac.com. The Lundbeck Foundation; the Pharmacy Foundation of 1991; Augustinus Foundation; the Danish Medical Research Council and The Danish Pediatric Asthma Centre provided core support for COPSAC. The funding agencies did not have any role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Contributions

The guarantor of this study is H.B. who is responsible for the integrity of the work as a whole, from conception and design to conduct of the study and acquisition of data, analysis and interpretation of data and writing of the manuscript. A.L.B., B.C., E.K.M., F.B., H.H. and H.B. are responsible for data acquisition, analysis, interpretation, and writing the manuscript. All co-authors have contributed substantially to the analyses and interpretation of the data, and have provided important intellectual input and approval of 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.

REFERENCES


Pediatric Pulmonology


SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher’s web-site.
Asthma and lower airway disease

Neonatal bronchial hyperresponsiveness precedes acute severe viral bronchiolitis in infants

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Copenhagen and Gentofte, Denmark, and London, United Kingdom

Background: Respiratory syncytial virus and other respiratory tract viruses lead to common colds in most infants, whereas a minority develop acute severe bronchiolitis often requiring hospitalization. We hypothesized that such an excessive response to respiratory tract viral infection is caused by host factors reflected in pre-existing increased bronchial responsiveness.

Objective: We sought to compare bronchial responsiveness and lung function in 1-month-old neonates who later develop acute severe bronchiolitis with those who do not.

Methods: We measured infant lung function (n = 402) and bronchial responsiveness to methacholine (n = 363) using the raised-volume rapid thoracoabdominal compression technique before any respiratory symptoms in 1-month-old neonates from the Copenhagen Prospective Study of Asthma in Childhood birth cohort born to mothers with asthma. The children were prospectively monitored for respiratory symptoms and given a diagnosis of acute severe bronchiolitis according to a fixed algorithm.

Results: Thirty-four (8.5%) infants had acute severe bronchiolitis before 2 years of age, 21 (62%) were hospitalized, and 23 (67%) of the cases were associated with respiratory syncytial virus. Children who later had acute severe bronchiolitis irrespective of viral species had a 2.5-fold increased responsiveness to methacholine (provocative dose of methacholine producing a 15% decrease in transcutaneous oxygen pressure [PD15]) at age 1 month compared with control subjects (median PD15 in cases vs control subjects, 0.13 vs 0.33 μmol; P = .01), whereas differences in baseline airflow were not significant for forced expiratory volume at 0.5 seconds (mean z score for cases vs control subjects, −0.18 vs −0.01; P = .36) and forced expiratory flow at 50% of forced vital capacity (mean z score for cases vs control subjects, −0.37 vs −0.09; P = .13).

Conclusion: Bronchial hyperresponsiveness in at-risk neonates precedes acute severe bronchiolitis in response to infections with respiratory tract virus. (J Allergy Clin Immunol 2012;130:354-61.)

Key words: Bronchial responsiveness, infants, lung function measurements, viral bronchiolitis

Children with acute severe bronchiolitis in relation to airway virus infections, such as respiratory syncytial virus (RSV) and rhinovirus, have an increased risk of asthma and allergy at school age. This has led to the suggestion of a causal role of RSV and rhinovirus on the risk of asthma. On the other hand, asthma heredity and troublesome asthma-like symptoms in early infancy are significant risk factors for subsequent RSV-related hospitalization during infancy, suggesting that host factors might determine a shared predisposition to viral bronchiolitis and childhood asthma. We hypothesized that pre-existing abnormal infant spirometry and bronchial hyperresponsiveness in neonates precedes later development of acute severe bronchiolitis.

We recently demonstrated that neonatal pulmonary function was associated with asthma by age 7 years in the Copenhagen Prospective Studies on Asthma in Childhood (COPSAC2000). The present study investigates whether neonatal pulmonary function is also associated with acute severe bronchiolitis in infants (ie, whether neonatal lung function is a shared host factor for both asthma and acute severe bronchiolitis).

This study was nested in COPSAC2000, a prospective clinical study of a birth cohort of 411 neonates born of mothers with a history of asthma. The study included measurements of infant spirometry and bronchial responsiveness to methacholine at 1 month of age in 402 of the 411 cohort participants. The deep phenotyping with close clinical, single-center follow-up of the birth cohort by the COPSAC physicians allowed prospective identification of infants who developed acute severe bronchiolitis.
Abbreviations used
COPSAC: Copenhagen Prospective Studies on Asthma in Childhood
FEF\textsubscript{25-75}: Forced expiratory flow at 50% of forced vital capacity
FE\textsubscript{VOC}: Forced expiratory volume at 0.5 seconds
FVC: Forced vital capacity
IQR: Interquartile range
\(\text{PD}_{2.5}\): Provocative dose of methacholine producing a 15\% decrease in transcutaneous oxygen pressure
\(\text{PCO}_2\): Transcutaneous oxygen pressure
RSV: Respiratory syncytial virus

METHODS
Study population
Four hundred eleven infants born at term of mothers with a history of physician-diagnosed asthma were enrolled at 1 month of age in the COPSAC\textsubscript{2000} prospective birth cohort study.\textsuperscript{11-13} Key exclusion criteria were symptoms of lower airway infection or neonatal mechanical ventilation before inclusion, gestational age of less than 36 weeks, and any congenital abnormality or systemic illness.

Ethics
The study was conducted in accordance with the guiding principles of the Declaration of Helsinki and was approved by the local ethics committee (KF 01-227/97) and the Danish Data Protection Agency (2008-41-1754). Both parents provided written informed consent before enrollment.

Neonatal lung function measurement
Infant spirometry was assessed during sedation with an oral dose of 90 mg/kg chloral hydrate at age 1 month by applying the raised-volume rapid thoracoabdominal compression technique in agreement with the American Thoracic Society and European Respiratory Society standards.\textsuperscript{17} An inflatable “squeeze” jacket was wrapped around the sedated infant’s chest and abdomen. Repeated ventilations to predefined mouth pressures ensured expansion of lung volume before an instant inflation of the jacket caused a full exhalation during which the flow was measured by using a pneumotachograph with an air-cushion facemask.\textsuperscript{10,15}

The software identified forced vital capacity (FVC) as the first plateau on the volume-time curve; only measurements with an FVC appearing after 0.5 seconds and with the forced expiratory volume at 0.5 seconds (FE\textsubscript{VOC}) being less than or equal to the FVC were accepted. Three to 5 acceptable curves were obtained at each measurement; the curve containing the median value of FE\textsubscript{V0.5} was used for the analyses of both volume (FE\textsubscript{VOC}) and flow (forced expiratory flow at 50\% of forced vital capacity [FE\textsubscript{FV0.5}]) parameters.

Baseline lung function measurement was repeated after a saline inhalation. Subsequently, methacholine was administered in quadrupling dose steps administered through a dosimeter attached to a nebulizer (SPIRA 08 TSM 133; Respiratory Care Center, Hameenlinna, Finland), as previously detailed.\textsuperscript{15} Bronchial responsiveness was assessed by means of repeated measurements of infant spirometry and continuous assessment of transcutaneous oxygen pressure (\(\text{PCO}_2\); TCM3; Radiometer, Copenhagen, Denmark). The provocative dose of methacholine producing a 15\% decrease in transcutaneous oxygen pressure (\(\text{PD}_{2.5}\)) was estimated from the dose-response curves fitted with a logistic function. Our previous sensitivity analyses showed \(\text{PCO}_2\) determined bronchial responsiveness with greater sensitivity than any of the forced flow indices of infant spirometry,\textsuperscript{15,16} therefore \(\text{PCO}_2\) (\(\text{PD}_{2.5}\)) was used to assess bronchial responsiveness in the present analyses.

Acute severe bronchiolitis cases
The birth cohort was prospectively monitored closely for respiratory symptoms with daily diary cards from 1 month of age and clinical examinations performed by the COPSAC physicians at the research clinic every 6 months and in cases of acute respiratory symptoms. Acute severe bronchiolitis was defined as an acute respiratory tract illness with onset before the age of 2 years based on symptoms of coughing progressing over a few days to cough, tachypnea, chest retractions, and auscultative widespread crepitation and/or rhonchi.\textsuperscript{17-19}

In cases in which the infants had been brought directly to the local pediatric emergency department for admission, hospital records were retrieved and carefully reviewed to verify respiratory symptoms compatible with the abovementioned criteria for diagnosing acute severe bronchiolitis.

Viruses were detected by means of PCR analysis of nasopharyngeal aspirate samples for RSV; rhinoviruses; influenza viruses AH1, AH3, and B; parainfluenza viruses 1 to 3; coronaviruses 229E and OC43; picornaviruses; bocavirus; adenoviruses; and human metapneumovirus, as previously detailed.\textsuperscript{20}

Bronchiolitis occurring before age 2 years irrespective of the viral trigger (ie, any bronchiolitis) was used as the primary end point, excluding children with acute asthma-like exacerbations. Secondary end points were (1) RSV-induced bronchiolitis, (2) non–RSV-induced bronchiolitis, (3) acute asthma-like exacerbations, (4) bronchiolitis before age 1 year, and (5) any bronchiolitis, including acute asthma-like exacerbations.

Atopic comorbidity
Recurrent episodes of troublesome lung symptoms were defined as 3 consecutive days recorded with significant cough and/or wheeze and/or dysnea.\textsuperscript{21} The number of episodes of troublesome lung symptoms before development of acute severe bronchiolitis among cases was compared with the number of episodes in the control group occurring before the median age at diagnosis of acute severe bronchiolitis in the cases.

Allergic sensitization was determined at age 18 months by means of measurement of serum specific IgE levels against 15 common inhalant allergens and food allergens (cat, dog, horse, birch, timothy grass, mugwort, house dust mite, molds [Penicillium notatum, Cladosporium herbarum, Aspergillus fumigatus, and Alternaria tenus], hen’s egg, cow’s milk, cod, wheat, peanut, soybean, or shrimp) using the ImmunoCAP assay (Pharmacia Diagnostics AB, Uppsala, Sweden). Sensitization was defined as a specific IgE level of 0.35 kU/L or greater\textsuperscript{22,23} for any of the tested allergens and was analyzed as a dichotomized measurement.

Total IgE levels were measured at age 18 months by using ImmunoCAP (Pharmacia Diagnostics AB), with a detection limit of 2 kU/L.\textsuperscript{22}

Blood eosinophil counts (10\textsuperscript{3} cells per liter) were assessed at age 18 months.

Eczema was diagnosed by using the Hanifin-Rajka criteria, as previously detailed.\textsuperscript{24} Skin lesions were described at both scheduled and acute visits according to predefined morphology and localization.

Genetics
The study population was genotyped for filaggrin-null mutations and ORMDL3 and DENND1B variants, as described in detail in the Methods section in this article’s Online Repository at www.jacionline.org.

Statistical analysis
Lung function data (FE\textsubscript{VOC}, FEF\textsubscript{25-75}, and \(\text{PD}_{2.5}\) [\(\text{PCO}_2\)]) were adjusted for lifespan and birth length by using a generalized linear model, as previously detailed.\textsuperscript{16} Lifespan at examination date was calculated as the sum of estimated gestational age in weeks at birth and weeks since birth. The lung function measurements were log transformed before analysis to obtain normal distribution of data.

Generalized linear models were used to compare lung function data in subjects with and without acute severe bronchiolitis, assuming Gaussian distribution of the data and using the identity link function as in multiple regression analyses. Adjustment for significant and borderline-significant
baseline characteristics and intermediary atopic markers was done by adding the variables as covariates in the model.

The comparison of baseline characteristics and the development of atopic stigmata in cases versus control subjects were done by using univariate analyses (ie, t tests and $\chi^2$ tests). The number of episodes of troublesome lung symptoms in cases versus control subjects was analyzed by using Poisson regression.

Analyses were done with SAS version 9.1 software for Windows (SAS Institute, Inc, Cary, NC). A $P$ value of .05 or less was considered significant.

TABLE I. Baseline characteristics of the study group

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Control subjects (n = 366)</th>
<th>Bronchiolitis cases* (n = 34)</th>
<th>RSV-induced bronchiolitis cases (n = 23)</th>
<th>Non-RSV-induced bronchiolitis cases (n = 9)</th>
<th>Asthma-like cases (n = 11)</th>
<th>Bronchiolitis cases vs control subjects</th>
<th>$P$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Baseline characteristics</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Male (no.)</td>
<td>64.7% (22)</td>
<td>48.4% (177)</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td></td>
<td>.07</td>
</tr>
<tr>
<td>Anthropometrics</td>
<td></td>
<td></td>
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<td></td>
<td></td>
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</tr>
<tr>
<td>BMI at birth (cm/kg$^2$), median (IQR)</td>
<td>12.5 (11.6-13.7)</td>
<td>12.8 (12.0-13.7)</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td></td>
<td>.25</td>
</tr>
<tr>
<td>Environment</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smoking, 3rd trimester (no.)</td>
<td>35.3% (12)</td>
<td>13.1% (48)</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td></td>
<td>.005</td>
</tr>
<tr>
<td>Alcohol, 3rd trimester (no.)</td>
<td>14.7% (5)</td>
<td>18.6% (68)</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td></td>
<td>.58</td>
</tr>
<tr>
<td>Cat at birth (no.)</td>
<td>17.7% (6)</td>
<td>14.8% (52)</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td></td>
<td>.65</td>
</tr>
<tr>
<td>Dog at birth (no.)</td>
<td>14.7% (5)</td>
<td>13.6% (48)</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td></td>
<td>.86</td>
</tr>
<tr>
<td>Older siblings at birth (no.)</td>
<td>52.9% (18)</td>
<td>42.4% (155)</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td></td>
<td>.23</td>
</tr>
<tr>
<td>Household income at birth (no.)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>.50</td>
</tr>
<tr>
<td>Low, &lt;53,000€</td>
<td>30.3% (10)</td>
<td>29.6% (101)</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Medium, 53,000€-80,000€</td>
<td>54.6% (18)</td>
<td>46.6% (159)</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td></td>
<td></td>
</tr>
<tr>
<td>High, &gt;80,000€</td>
<td>15.2% (5)</td>
<td>25.8% (81)</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td></td>
<td></td>
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<tr>
<td>Paternal history of asthma (no.)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>.005</td>
</tr>
<tr>
<td>Age at start of day care (d), median (IQR)</td>
<td>296 (230-349)</td>
<td>341 (240-417)</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td></td>
<td>.08</td>
</tr>
<tr>
<td>Solely breast-fed (d), median (IQR)</td>
<td>120 (72-179)</td>
<td>122 (89-155)</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td></td>
<td>.47</td>
</tr>
<tr>
<td>Lung function at age 1 mo</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FEV$\text{_{0.5}}$ ($z$ score), mean (SD)</td>
<td>-0.18 (0.91)</td>
<td>-0.01 (0.99)</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td></td>
<td>.36</td>
</tr>
<tr>
<td>FEF$\text{_{50}}$ ($z$ score), mean (SD)</td>
<td>-0.37 (1.05)</td>
<td>-0.09 (1.00)</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td></td>
<td>.13</td>
</tr>
<tr>
<td>PD$\text{<em>{15}}$ (PtcO$</em>\text{2}$ [μmol]), median (IQR)</td>
<td>0.13 (0.07-0.60)</td>
<td>0.33 (0.12-0.88)</td>
<td>—</td>
<td>—</td>
<td>—</td>
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<td>.01</td>
</tr>
<tr>
<td>Genetics</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Filaggrin mutation</td>
<td>18.2% (6)</td>
<td>9.7% (34)</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td></td>
<td>.13</td>
</tr>
<tr>
<td>$ORMDL3$ (TT)</td>
<td>48.5% (16)</td>
<td>26.7% (92)</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td></td>
<td>.008</td>
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<tr>
<td>$DENND1B$ genotype (rs2786098)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>.17</td>
</tr>
<tr>
<td>AA</td>
<td>3.5% (1)</td>
<td>4.2% (14)</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AB</td>
<td>13.8% (4)</td>
<td>27.5% (91)</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BB</td>
<td>82.7% (24)</td>
<td>68.3% (226)</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td></td>
<td></td>
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<tr>
<td>Atopic intermediary markers</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Allergic sensitization* (18 mo [no.])</td>
<td>9.1% (3)</td>
<td>12.0% (38)</td>
<td>0% (0)</td>
<td>22.2% (2)</td>
<td>20.0% (2)</td>
<td></td>
<td>.78</td>
</tr>
<tr>
<td>Blood eosinophil count (18 mo [10$^6$/μL]), median (IQR)</td>
<td>0.27 (0.18-0.43)</td>
<td>0.21 (0.13-0.32)</td>
<td>0.26 (0.18-0.43)</td>
<td>0.21 (0.15-0.38)</td>
<td>0.41 (0.22-0.48)</td>
<td></td>
<td>.03</td>
</tr>
<tr>
<td>Total IgE (18 mo [kU/L]), median (IQR)</td>
<td>15.3 (6.3-29.5)</td>
<td>8.2 (3.9-17.1)</td>
<td>15.5 (6.3-28.0)</td>
<td>10.2 (4.7-30.2)</td>
<td>7.0 (5.2-30.9)</td>
<td></td>
<td>.03</td>
</tr>
<tr>
<td>Episodes of troublesome lung symptoms, median (IQR)</td>
<td>2 (1-6)</td>
<td>1 (1-2)</td>
<td>1 (1-5)</td>
<td>6 (3-7)</td>
<td>2 (1-4)</td>
<td></td>
<td>.005</td>
</tr>
<tr>
<td>Eczema (0-2 y [no.])</td>
<td>38.2% (13)</td>
<td>38.4% (131)</td>
<td>30.4% (7)</td>
<td>66.7% (6)</td>
<td>40.0% (4)</td>
<td></td>
<td>.98</td>
</tr>
<tr>
<td>Episode characteristics</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fever at diagnosis (no.)</td>
<td>—</td>
<td>59% (20)</td>
<td>57% (13)</td>
<td>56% (5)</td>
<td>36% (4)</td>
<td></td>
<td></td>
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<tr>
<td>Auscultation</td>
<td></td>
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</tr>
<tr>
<td>Crepitations (no.)</td>
<td>—</td>
<td>41% (14)</td>
<td>44% (10)</td>
<td>33% (3)</td>
<td>27% (3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rhonchi (no.)</td>
<td>—</td>
<td>85% (29)</td>
<td>78% (18)</td>
<td>100% (9)</td>
<td>91% (10)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hospitalization (no.)</td>
<td>—</td>
<td>62% (21)</td>
<td>83% (19)</td>
<td>22% (2)</td>
<td>36% (4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Days hospitalized, median (range)</td>
<td>—</td>
<td>3 (1-15)</td>
<td>4 (1-15)</td>
<td>2 (2-2)</td>
<td>2 (1-4)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(Continued)
RESULTS

Study group selection

Infant spirometry was completed in 402 children, and bronchial responsiveness to methacholine was assessed in 363 of the 411 children in the cohort at age 1 month before the development of any respiratory symptoms.

Eleven children had an episode of acute asthma-like symptoms not fulfilling all the diagnostic criteria for acute severe bronchiolitis. These children were excluded because of a lack of objective signs of respiratory distress, such as tachypnea, chest retractions, or both (see Table I and Table E1 in this article’s Online Repository at www.jacionline.org for baseline characteristics of the excluded children). These 11 children were excluded from both the case and control groups, leaving 400 evaluable children (see Fig E1 in this article’s Online Repository at www.jacionline.org for the study group flow chart).

Bronchiolitis cases

Thirty-four (8.5%) of the 400 children in the selected cohort had acute severe bronchiolitis before age 2 years (median age at diagnosis, 327 days; interquartile range [IQR], 128-446 days). Of these 34 cases, 65% (n = 22) were boys, 59% (n = 20) had fever at the time of diagnosis (rectal temperature, >38.0°C), 41% (n = 14) had crepitations, and 85% (n = 29) had rhonchi at auscultation. Median time from infant spirometry to diagnosis of acute severe bronchiolitis was 267 days (range, 8-617 days). In 2 of the 34 bronchiolitis cases, there were no available nasopharyngeal aspirates. RSV was identified in 23 of the 32 bronchiolitis cases, whereas the remaining 9 cases were classified as having non-–RSV-induced bronchiolitis (2 with picornavirus; 1 with bocavirus; 1 with rhinovirus; 1 with bocavirus and rhinovirus; 1 with human metapneumovirus, picornavirus, influenza virus, parainfluenza virus, coronavirus, and bocavirus; and 3 with negative nasopharyngeal suction).

Hospitalization was required for 21 (62%) of the 34 bronchiolitis cases, with a median duration of hospitalization of 10 days (IQR, 6-21 days). Half of the admitted children needed treatment with nasal continuous positive airway pressure, one third were prescribed systemic corticosteroid treatment, and 90% received nebulized β2-agonist. Children with acute severe bronchiolitis who were not hospitalized received outpatient treatment with inhaled β2-agonists (92%), inhaled corticosteroids (62%), systemic corticosteroids (31%), and antibiotics (77%).

Baseline characteristics of children with acute severe bronchiolitis versus control subjects

Comparison of baseline characteristics of the bronchiolitis cases (n = 34) and control subjects (n = 366, Table I) showed that maternal smoking during the third trimester of pregnancy was significantly more common among infants with acute severe bronchiolitis compared with control subjects (35% vs 13%, P = .005). The ORMDL3 TT genotype was also significantly associated with the development of acute severe bronchiolitis during the first 2 years of life (cases vs control subjects, 49% vs 27%; P = .008), whereas male sex (cases vs control subjects, 65% vs 49%; P = .07) and young age at the start of day care (median age at start for cases vs control subjects, 296 vs 341 days; P = .08) were borderline significant. We found no association between cases and control subjects with respect to anthropometrics; maternal alcohol consumption during the third trimester of pregnancy; the presence of a cat, a dog, or an older sibling in the home at birth; household income; length of sole breast-feeding; paternal history of asthma; filaggrin-null mutations; and DENND1B genetic variants (Table I).

Children with acute severe bronchiolitis had significantly (P = .0005) more episodes of troublesome lung symptoms before bronchiolitis diagnosis (median, 2; IQR, 1-6) compared with control subjects before the median age at bronchiolitis diagnosis.
among the cases (median, 1; IQR, 0-2). Blood eosinophil counts and total IgE levels at age 18 months were significantly higher in cases versus control subjects (blood eosinophil count: median, 0.27 x 10^6 cells/L [IQR, 0.18-0.43 x 10^6 cells/L] vs 0.21 x 10^6 cells/L [IQR, 0.13-0.32 x 10^6 cells/L]; P = .03; total IgE level: median, 15.3 kU/L [IQR, 6.3-29.5 kU/L] vs 8.2 kU/L [IQR, 3.9-17.1 kU/L]; P = .03). Eczema and allergic sensitization were equally distributed among cases and control subjects.

Children with RSV-induced bronchiolitis compared with those with non-RSV-induced bronchiolitis were younger at diagnosis (199 vs 462 days; P < .01) and were more often admitted to the hospital (83% vs 22%; P < .01). There were no significant differences in the development of atopic stigmata during the first 2 years of life between cases with RSV-induced bronchiolitis and those with non-RSV-induced bronchiolitis (all P > .05).

A detailed description of the 34 bronchiolitis cases and the control group is outlined in Table I.

**Neonatal lung function in bronchiolitis cases versus control subjects**

Distributions of PDq95 (PtcO2), FEV0.5, and FEF50 measured at age 1 month before any respiratory symptoms in children who subsequently had acute severe bronchiolitis versus control subjects are shown in Fig 1. Children with acute severe bronchiolitis reacted to lower doses of methacholine compared with the control group (median PDq95 [PtcO2] dose in cases vs control subjects, 0.13 vs 0.33 mmol; P = .01; Fig 1, A). This phenomenon remained significant (P = .02) after adjustment for significant and borderline-significant baseline characteristics, including atopic intermediary markers (sex, maternal smoking during the third trimester, age at start of day care, ORMDL3 genotype, episodes of troublesome lung symptoms, and total IgE levels and blood eosinophil counts). Increased bronchial responsiveness to methacholine in cases versus control subjects was confirmed in a sensitivity analysis, including the excluded cases of acute asthma-like symptoms not fulfilling all the diagnostic criteria for acute bronchiolitis (P = .01). The distribution of FEV0.5 and FEF50 in Fig 1, B and C, suggested lower airflows in infants with acute severe bronchiolitis compared with control subjects, but these trends were not significant (mean z score for FEV0.5 in cases vs control subjects, −0.18 vs −0.01 [P = .36]; mean z score for FEF50 in cases vs control subjects, −0.37 vs −0.09 [P = .13]).

Subgroup analysis of PDq5 (PtcO2) in cases with RSV-induced bronchiolitis and those with non-RSV-induced bronchiolitis excluding cases with acute asthma-like exacerbations and bronchiolitis occurring before age 1 year suggested pre-existing hyperresponsive airways independent of these case definitions, although the statistical power was lost in some of these small
subgroups, including the specific RSV-induced bronchiolitis subgroup ($P = .12$). Subgroup analyses of baseline FEV$_{1,5}$ and FEF$_{50}$ values suggested similar trends of impaired neonatal airflow related to the subsequent development of acute severe bronchiolitis (see Table II).

**DISCUSSION**

**Principal findings**
Neonatal bronchial hyperresponsiveness increases the risk of acute severe bronchiolitis in early life in the COPSAC-2000 at-risk birth cohort. This demonstrates that neonatal airway hyperresponsiveness is a pre-existing host factor common to later development of both acute severe viral bronchiolitis and childhood asthma. 9

**Strengths and limitations of the study**
This is the first assessment of premorbid neonatal lung function and bronchial responsiveness in a birth cohort of healthy at-risk neonates combined with a close prospective clinical follow-up for subsequent development of acute severe bronchiolitis.

The risk of misclassification of the clinical end point of acute severe bronchiolitis was minimized in this prospective birth cohort with close clinical monitoring, including daily diary card recordings of respiratory symptoms, 6-month visits to the clinic for clinical assessments by pediatricians, and visits to the clinic at the time of acute respiratory symptoms. The clinical research unit was acting as the primary health care resource for the cohort in relation to all respiratory symptoms, ensuring that diagnosis and treatment followed a predefined algorithm, including a standard clinical definition of acute severe bronchiolitis.

In particular, our clinical definition of acute severe bronchiolitis was based on objective assessment of tachypnea, chest retractions, and auscultative wide-spread fine crepitation and/or expiratory rhonchi and not solely based on parental reporting. This approach enabled us to exclude cases of acute severe asthma-like exacerbations from the study group, which is important because impaired pulmonary function early in life has been described in association with subsequent recurrent asthma-like symptoms, and asthma.

**Bronchiolitis is difficult to differentiate from asthma-like symptoms, and indeed, we suspect it to be the same entity. However, the literature suggests that these subtle clinical differences represent different phenotypes. This is precisely the issue we are questioning in our study. We choose a conservative definition of acute bronchiolitis using a strict predefined algorithm, including objective signs of respiratory distress, which is in line with recently published reviews. 17-19**

RSV-induced bronchiolitis has been associated with asthma later in life, but it was our research hypothesis that bronchial hyperresponsiveness is the common cause of both bronchiolitis and asthma symptoms independently of viral species. In line with this, it has been reported that also rhinovirus-induced bronchiolitis increases the risk of asthma later in life. 12 Children with RSV in our study were younger than the non-RSV cases but showed no differences in atopic stigmata. Furthermore, adjusting the analyses for atopic intermediary end points did not alter the associations.

In reverence to the long tradition of emphasizing the unique quality of RSV, we analyzed a number of secondary bronchiolitis end points: (1) RSV-induced bronchiolitis, (2) non-RSV-induced bronchiolitis, (3) acute asthma-like exacerbations, (4) bronchiolitis before age 1 year, and (5) any bronchiolitis, including acute asthma-like exacerbations. All these secondary analyses show similar patterns of pre-existing increased reactivity to methacholine in cases versus control subjects. Some of these subgroup analyses (eg, the RSV group) did not reach statistical significance, probably because of the lack of power from dividing the main study group into smaller subgroups. The similarities of increased bronchial responsiveness in all groups of infants presenting with symptoms of acute severe airway obstruction independently of trigger or age at onset suggest a common deficiency rather than particular trigger attributes. It is our interpretation that bronchial hyperresponsiveness is the common underlying phenotypic trait in infants presenting with acute severe airway obstruction in response to viral infections.

It is our clinical practice only to admit infants if they need support with feeding tubes; nasal continuous positive airway pressure; suction of the upper airways; nebulized inhalations, such as isotonic saline; or $\beta_2$-agonist or systemic steroids, depending on symptom severity. We are aware of the poor evidence of this, but presumably, our clinical tradition builds from a concern over the “bronchiolitis phenotype” might indeed be an asthmatic reaction in which such treatment might be meaningful and in which no alternatives exist.

It is a significant strength of this study that lung function was assessed within a narrow age range around 1 month after birth based on results of infant spirometry with the state-of-the-art raised-volume rapid thoracoabdominal compression technique in agreement with recognized international guidelines. 14 This is the

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**Table II. (Continued)**

<table>
<thead>
<tr>
<th>RSV-induced bronchiolitis (n = 23)</th>
<th>Non-RSV-induced bronchiolitis (n = 9)</th>
<th>Asthma-like cases (n = 11)</th>
<th>Bronchiolitis cases &lt;1 y (n = 20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean difference (95% CI)</td>
<td>P value</td>
<td>Mean difference (95% CI)</td>
<td>P value</td>
</tr>
<tr>
<td>$-0.10$ ($-0.52$ to $0.33$)</td>
<td>.66</td>
<td>$-0.31$ ($-1.01$ to $0.38$)</td>
<td>.37</td>
</tr>
<tr>
<td>$-0.12$ ($-0.56$ to $0.31$)</td>
<td>.58</td>
<td>$-0.71$ ($-1.42$ to $-0.01$)</td>
<td>.05</td>
</tr>
<tr>
<td>$-0.42$ ($-1.06$ to $0.13$)</td>
<td>.12</td>
<td>$-1.37$ ($-2.38$ to $-0.35$)</td>
<td>.01</td>
</tr>
</tbody>
</table>
largest published sample of lung function assessments in neonates performed under standardized conditions comprising measurements in 402 asymptomatic infants.

The relatively rare prevalence of acute severe bronchiolitis is a limitation of the study. Interestingly, we did not find significantly lower airflow in the infants later having bronchiolitis symptoms. This might suggest that bronchial responsiveness is the primary pathology, leading to acute obstruction in response to virus later in life, and that airflow limitation might not be the primary pathology. Alternatively, we might be unable to see differences in baseline airflow because of the low number of cases, which hampers the statistical power.

The high-risk nature of the birth cohort might diminish the generalizability of our findings because the absolute levels of lung function and bronchial responsiveness might not be representative of the general population, although this would not affect the comparison of lung function within the cohort. A recent study of preterm infants reported that palivizumab prophylaxis significantly reduced the risk of recurrent wheeze in children without atopic predisposition, whereas there was no effect in children with an atopic family history, suggesting a differential effect of viral infection depending on atopic predisposition. Therefore our findings cannot be extrapolated beyond a high-risk population.

Interpretation

The significant association between bronchial hyperresponsiveness at 1 month of age in at-risk children and the later development of acute severe bronchiolitis demonstrates a pre-existing propensity for an exaggerated airway response to common respiratory tract viral infections.

An older study reported trends of reduced airflow but no increase in the bronchial responsiveness to histamine in infants subsequently having bronchiolitis (n = 17) compared with control subjects (n = 236). This study was limited from the use of infant spirometry, which was not volume anchored, testing the infants at a wider age range up to 10 weeks, and from a retrospective questionnaire–defined end point of “doctor-diagnosed bronchiolitis” recalled by the parents at follow-up by 2 years of age.

The association between airway hyperresponsiveness and the subsequent development of acute severe bronchiolitis demonstrated in this study and our recent observation of an increased risk of asthma at age 7 years in children with neonatal bronchial hyperresponsiveness, together with observational evidence of an association between acute severe bronchiolitis early in life and the development of asthma, suggests neonatal bronchial hyperresponsiveness as a shared host factor for bronchiolitis and asthma rather than a causal effect of bronchiolitis on asthma development.

Notably, the incidence of acute severe bronchiolitis in our study (8.5%) was higher than reported in some studies (1-3%) but comparable with the incidence of 7% reported in a cohort of 253 infants with a high prevalence (71%) of atopic predisposition. This association between asthma predisposition and increased incidence of acute bronchiolitis in the infants supports our research hypothesis that a host factor is responsible for acute bronchiolitis.

Children with acute severe bronchiolitis had a high prevalence of early troublesome lung symptoms before bronchiolitis, had increased total IgE levels and blood eosinophil counts, were more often exposed to tobacco smoke in utero, were of the male sex, were carrying the ORM DL3 risk variant, and started day care at an early age compared with children not having such acute severe bronchiolitis. We previously demonstrated the ORM DL3 risk allele is associated with neonatal increased bronchial reactivity and increased risk of asthma in early childhood in our cohort.

Together these characteristics are all typical of children at risk of asthma, supporting that children with acute severe bronchiolitis have a premorbid constitution, including bronchial hyperresponsiveness, which predisposes them to both an exaggerated response to respiratory tract viral infection and later development of asthma.

Therefore it might be speculated that acute severe bronchiolitis during infancy is not a specific disease entity but simply a severe early debut of asthma persisting to school age.

Conclusion

Bronchial hyperresponsiveness in asymptomatic at-risk neonates precedes the later development of acute severe bronchiolitis, suggesting a pre-existing common propensity of the airways to develop asthma and to react adversely to common respiratory tract viruses.

We express our gratitude to the children and families of the COPSAC cohort study for all their support and commitment. We acknowledge and appreciate the unique efforts of the COPSAC research team. We thank S. Jensen for statistical support.

Key messages

- RSV and other respiratory tract viruses lead to common colds in most infants, whereas a minority have acute severe bronchiolitis.
- Bronchial hyperresponsiveness in at-risk neonates precedes acute severe bronchiolitis in response to respiratory tract viral infections.

REFERENCES


METHODS

Bronchial responsiveness

Baseline lung function was repeated after a saline inhalation. Subsequently, methacholine was administered in quadrupling dose steps administered through a dosimeter attached to a nebulizer (SPIRA 08 TSM 133, Respiratory Care Center), as previously detailed. Bronchial responsiveness was assessed by using repeated measurements of infant spirometry and continuous assessment of PtcO₂ (TCM3, Radiometer). The PD₁₅ was estimated from the dose-response curves fitted with a logistic function. Our previous sensitivity analyses showed PtcO₂ to be more sensitive than any of the forced flow indices of infant spirometry. Therefore PtcO₂ was used in the analyses.

Genetics

Blood was sampled at age 6 months, centrifuged and separated into serum and serum cells, and immediately stored at −80°C until analysis. After thawing, DNA was purified from the serum cells by using the QIAamp DNA Blood Maxi Kit (Qiagen, Valencia, Calif).

Filaggrin genotyping for the 2 common independent null mutations R501X and 2282del4 was performed, as previously described. Children were defined as having a filaggrin mutation if they carried at least 1 of the mutations.

For ORMDL3 genotyping, allelic discrimination at the rs7216389 single nucleotide polymorphism on chromosome 17q12-21 was performed, as previously detailed. For DENND1B genotyping, allelic discrimination at the rs2786098 on chromosome 1q31.3 was performed, as described by Sleiman et al.

REFERENCES

Children enrolled in the COPSAC cohort
N=411

Children in the selected cohort
N=400

11 children with acute severe asthma-like exacerbations, 0-2yrs

Cases with acute bronchiolitis, 0-2yrs
N=34

Controls without acute bronchiolitis, 0-2yrs
N=366

FIG E1. Study group flow chart.
TABLE E1. Baseline characteristics and lung function results of children with acute severe bronchiolitis during the first 2 years of life, excluding cases with acute severe asthma-like exacerbations but not fulfilling all diagnostic criteria for acute severe bronchiolitis, and control subjects

<table>
<thead>
<tr>
<th>Study group (n = 411)</th>
<th>Acute asthma-like exacerbations (n = 11)</th>
<th>Bronchiolitis (n = 34)</th>
<th>Control subjects (n = 366)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sex</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male sex (no.)</td>
<td>36.4% (4)</td>
<td>64.7% (22)</td>
<td>48.4% (177)</td>
</tr>
<tr>
<td><strong>Anthropometrics</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI at birth (cm/kg²), median (IQR)</td>
<td>12.3 (11.3-14.8)</td>
<td>12.5 (11.6-13.7)</td>
<td>12.8 (12.0-13.7)</td>
</tr>
<tr>
<td><strong>Environment</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smoking, 3rd trimester (no.)</td>
<td>18.2% (2)</td>
<td>35.3% (12)†</td>
<td>13.1% (48)</td>
</tr>
<tr>
<td>Alcohol, 3rd trimester (no.)</td>
<td>0% (0)</td>
<td>14.7% (5)</td>
<td>18.6% (68)</td>
</tr>
<tr>
<td>Cat at birth (no.)</td>
<td>20.0% (2)</td>
<td>17.7% (6)</td>
<td>14.8% (52)</td>
</tr>
<tr>
<td>Dog at birth (no.)</td>
<td>10.0% (1)</td>
<td>14.7% (5)</td>
<td>13.6% (48)</td>
</tr>
<tr>
<td>Older siblings at birth (no.)</td>
<td>45.5% (5)</td>
<td>52.9% (18)</td>
<td>42.4% (155)</td>
</tr>
<tr>
<td>** Household income at birth (no.)**</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low, &lt;53,000€</td>
<td>9.1% (1)</td>
<td>30.3% (10)</td>
<td>29.6% (104)</td>
</tr>
<tr>
<td>Medium, 53,000€-80,000€</td>
<td>45.5% (5)</td>
<td>54.6% (18)</td>
<td>46.6% (159)</td>
</tr>
<tr>
<td>High, &gt;80,000€</td>
<td>36.4% (4)</td>
<td>15.2% (5)</td>
<td>23.8% (81)</td>
</tr>
<tr>
<td>Paternal history of asthma (no.)</td>
<td>11.1% (1)</td>
<td>24.2% (8)</td>
<td>16.5% (59)</td>
</tr>
<tr>
<td>Age at start of day care (d), median (IQR)</td>
<td>252 (194-376)</td>
<td>296 (230-349)</td>
<td>341 (240-417)</td>
</tr>
<tr>
<td>Solely breast-fed (d), median (IQR)</td>
<td>122 (27-123)</td>
<td>120 (72-179)</td>
<td>122 (89-155)</td>
</tr>
<tr>
<td><strong>Lung function at age 1 mo</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FEV₁₀₅ (z score), mean (SD)</td>
<td>−0.16 (1.38)</td>
<td>−0.18 (0.91)</td>
<td>−0.01 (0.99)</td>
</tr>
<tr>
<td>FEF₂₀₀ (z score), mean (SD)</td>
<td>−0.37 (0.96)</td>
<td>−0.37 (1.05)</td>
<td>−0.09 (1.00)</td>
</tr>
<tr>
<td>PD₁₅ (PtcO₂ [μmol]), median (IQR)</td>
<td>0.09 (0.04-0.25)</td>
<td>0.13 (0.07-0.60)†</td>
<td>0.33 (0.12-0.88)</td>
</tr>
<tr>
<td><strong>Genetics</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Filaggrin mutation</td>
<td>20.0% (2)</td>
<td>18.2% (6)</td>
<td>9.7% (34)</td>
</tr>
<tr>
<td>ORMDL3 (TT)</td>
<td>40.0% (4)†</td>
<td>48.5% (16)†</td>
<td>26.7% (92)</td>
</tr>
<tr>
<td><strong>DENND1B genotype (rs2786098)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>11.1% (1)</td>
<td>3.5% (1)</td>
<td>4.2% (14)</td>
</tr>
<tr>
<td>AB</td>
<td>33.3% (3)</td>
<td>13.8% (4)</td>
<td>27.5% (91)</td>
</tr>
<tr>
<td>BB</td>
<td>55.6% (5)</td>
<td>82.7% (24)</td>
<td>68.3% (226)</td>
</tr>
</tbody>
</table>

*Significant difference, children with acute asthma-like exacerbations versus control subjects.
†Significant difference, bronchiolitis cases versus control subjects.
Neonates with reduced neonatal lung function have systemic low-grade inflammation

Bo L. K. Chawes, MD, PhD, a Jakob Stokholm, MD, PhD, a–c Klaus Bennelykke, MD, PhD, a Susanne Brix, MSc, PhD, b and Hans Bisgaard, MD, DMSc a Copenhagen, Denmark

Background: Children and adults with asthma and impaired lung function have been reported to have low-grade systemic inflammation, but it is unknown whether this inflammation starts before symptoms and in particular whether low-grade inflammation is present in asymptomatic neonates with reduced lung function.

Objective: We sought to investigate the possible association between neonatal lung function and biomarkers of systemic inflammation.

Methods: Plasma levels of high-sensitivity C-reactive protein (hs-CRP), IL-1β, IL-6, TNF-α, and CXCL8 (IL-8) were measured at age 6 months in 300 children of the Copenhagen Prospective Study on Asthma in Childhood (COPSAC2000) birth cohort who had completed neonatal lung function testing at age 4 weeks. Associations between neonatal lung function indices and inflammatory biomarkers were investigated by conventional statistics and unsupervised principal component analysis.

Results: The neonatal forced expiratory volume at 0.5 seconds was inversely associated with hs-CRP (β-coefficient, −0.12; 95% CI, −0.21 to −0.04; P < .01) and IL-6 (β-coefficient, −0.10; 95% CI, −0.18 to −0.01; P = .03) levels. The multivariate principal component analysis approach, including hs-CRP, IL-6, TNF-α, and CXCL8, confirmed a uniform upregulated inflammatory profile in children with reduced forced expiratory volume at 0.5 seconds (P = .02). Adjusting for body mass index at birth, maternal smoking, older children in the home, neonatal bacterial airway colonization, infections 14 days before, and asthmatic symptoms, as well as virus-induced wheezing, at any time before biomarker assessment at age 6 months did not affect the associations.

Conclusion: Diminished neonatal lung function is associated with upregulated systemic inflammatory markers, such as hs-CRP. (J Allergy Clin Immunol 2015;135:1450–6.)

Key words: Asthma, children, high-sensitivity C-reactive protein, proinflammatory cytokines, spirometry

C-reactive protein (CRP) is an acute-phase reactant found in the blood in response to acute and chronic inflammatory conditions and has a broad clinical application in screening for infectious and immune-mediated diseases. 1 CRP has important innate immunity properties and is released from the liver after triggering by proinflammatory cytokines, such as IL-6, IL-1β, and TNF-α. 2 CRP assays with increased sensitivity (high-sensitivity C-reactive protein [hs-CRP]) have demonstrated low-grade inflammation in patients with disorders such as cardiovascular disease, 3 obesity, 4 and diabetes mellitus. 5 Increased hs-CRP levels have also been demonstrated during and shortly after viral respiratory tract infections 6 and in patients with symptomatic airway diseases, such as asthma 7 and chronic obstructive pulmonary disease. 8 In addition, impaired lung function in asthmatic children and adults has been associated with the presence of systemic low-grade inflammation. 9–11

We hypothesized that impaired lung function would be associated with the systemic inflammatory process, even before development of any respiratory symptoms. Therefore we measured plasma hs-CRP, IL-1β, IL-6, TNF-α, and CXCL8 (formerly IL-8) levels at the early age of 6 months and related these to neonatal lung function assessed at age 4 weeks in the Copenhagen Prospective Study on Asthma in Childhood (COPSAC2000) birth cohort.

METHODS

Study cohort

The study participants were 411 neonates born of mothers with a history of asthma and enrolled at 4 weeks of age in the COPSAC2000 prospective birth cohort study. 12–14 Exclusion criteria were any respiratory symptoms or respiratory support before inclusion, gestational age of less than 36 weeks, and any congenital abnormality or systemic illness, such as severe neonatal sepsis. The children attended the COPSAC research clinic at age 4 weeks for assessment of neonatal lung function and subsequently at 6-month intervals, as previously detailed. 12–14

Ethics

The study was conducted in accordance with the guiding principles of the Declaration of Helsinki and was approved by the local ethics committee (KF01-289/96) and the Danish Data Protection Agency (2008-41-1754). Both parents provided oral and written informed consent before enrollment.

Inflammatory biomarkers

Blood was drawn in an EDTA tube from a cubital vein at the age of 6 months, centrifuged to separate plasma and plasma cells, and immediately stored at −80°C.
Bronchial responsiveness was determined by means of continuous assessment of transcutaneous oxygen saturation (TCM3; Radiometer, Copenhagen, Denmark). The provocative dose of methacholine causing a 15% decrease in transcutaneous oxygen saturation was log-transformed to obtain normality.

The associations between neonatal lung function indices and inflammatory biomarkers were tested by using conventional statistics with general linear models and by using unsupervised pattern recognition with principal component analysis (PCA). In the PCA analyses we extracted underlying orthogonal components that described the systematic part of the variation across the biomarkers using log-transformed and z score mediators.

All results are presented as raw estimates with 95% CIs and as estimates obtained from partial regression analyses, adjusting for covariates associated with levels of hs-CRP by using a cutoff P value of .10 or less. Birth BMI and maternal smoking during the third trimester were retained in the multivariable models independently of their association with hs-CRP because these are important determinants of neonatal lung function.22 Interaction with bacterial airway colonization, any TROLS, and acute episodes of TROLS with virus detection was tested by adding cross-products to the models. A P value of .05 or less was considered significant. All analyses were done with SAS software, version 9.3 (SAS Institute, Cary, NC).

RESULTS Inflammatory biomarker assessments Measurements of IL-1β, IL-6, TNF-α, and CXCL8 levels were performed on 309 plasma samples collected at age 6 months, and measurements of hs-CRP levels were performed on 301 plasma samples collected at age 6 months. One sample was lost for technical reasons while performing the 4-plex assay, resulting in 300 children (73% of the original 411 cohort children) with available measurements for all 5 biomarkers. We found no significant differences in baseline characteristics between children with and without available biomarker assessments (see Table E1 in this article’s Online Repository at www.jacionline.org).

Median levels were as follows: hs-CRP, 1.39 mg/L (interquartile range [IQR], 0.46–6.61 mg/L); IL-1β, 0.01 ng/L (IQR, 0.001–0.04 ng/L); IL-6, 0.20 ng/L (IQR, 0.11–0.31 ng/L); TNF-α, 2.34 ng/L (IQR, 1.92–2.88 ng/L); and CXCL8, 3.04 ng/L (IQR, 2.19–4.37 ng/L). IL-6 and TNF-α levels were strongly positively correlated with hs-CRP levels (P < .001 for both), whereas IL-1β and CXCL8 levels were not correlated with hs-CRP levels (P ≥ .62). The measured values of hs-CRP, IL-6, TNF-α, and CXCL8 were within the expected range,23 with very few null values, whereas IL-1β levels were much lower than expected,23 with null values for 72 (23%) of 308 children. Because of this and the fact that IL-1β has been shown to significantly degrade over time, even at −80°C,24 IL-1β was not included in further analyses.
Determinants of hs-CRP

Children with older children in the home at birth had significantly higher hs-CRP levels at age 6 months compared with children without older children in the home (median hs-CRP level, 2.20 mg/L [IQR, 0.63-5.05 mg/L] vs 1.16 mg/L [IQR, 0.41-3.40 mg/L], P = .005). In addition, hs-CRP levels were increased in children who experienced an infectious episode within 14 days before biomarker assessment compared with children without apparent infections (4.29 mg/L [IQR, 1.71-5.34 mg/L] vs 0.84 mg/L [IQR, 0.36-2.67 mg/L], P < .0001); in children experiencing TROLS at any time point before biomarker assessment compared with children without TROLS (1.79 mg/L [IQR, 0.50-4.72 mg/L] vs 1.19 mg/L [IQR, 0.46-14.14 mg/L], P = .05); and in children with acute episodes of TROLS with an airway virus detected (3.78 mg/L [IQR, 1.00-5.42 mg/L] vs 1.16 mg/L [IQR, 0.41-3.85 mg/L], P < .0001). Children with bacterial airway colonization at age 4 weeks compared with noncolonized children showed a trend of increased hs-CRP levels (2.68 mg/L [IQR, 0.84-5.17 mg/L] vs 1.31 mg/L [IQR, 0.49-4.64 mg/L], P = .08). We did not detect associations between hs-CRP levels and paternal history of asthma, eczema, or allergy; child’s sex; birth BMI; household income; maternal smoking during the third trimester of pregnancy; birth by means of cesarean section; breast-feeding; day care attendance; and pets in the home (Table I).

Neonatal lung function and systemic low-grade inflammation

hs-CRP. The conventional statistical approach showed a strong linear inverse association between FEV0.5 values at age 4 weeks and hs-CRP levels at age 6 months (β-coefficient, −0.12; 95% CI, −0.21 to −0.04; P = .004), suggesting increasing grade of inflammation by diminished neonatal lung function (Fig 1). The association was unchanged by adjustment for older children in the home, bacterial airway colonization at age 4 weeks, infections 14 days before, and any TROLS, as well as acute virus-related episodes of TROLS at any time before biomarker assessment, birth BMI, and maternal smoking in the third trimester (β-coefficient, −0.12; 95% CI, −0.22 to −0.02; P = .02). Furthermore, we found no interaction with bacterial airway colonization (P = .21), any TROLS (P = .76), or any acute episodes of TROLS with a virus detected (P = .20).

FEF50 values also seemed inversely associated with hs-CRP levels but was not significant (β-coefficient, −0.06; 95% CI, −0.15 to 0.02; P = .14).

IL-6. Increasing FEV0.5 values were also significantly associated with decreasing IL-6 levels (β-coefficient, −0.10; 95% CI, −0.18 to −0.01; P = .03; Fig 2). Confounder adjustment did not substantially change the association (β-coefficient, −0.11; 95% CI, −0.22 to 0.01; P = .07). We did not detect a significant association between FEF50 values and IL-6 levels.

TNF-α and CXCL8. FEV0.5 and FEF50 measurements were not associated with CXCL8 or TNF-α levels, although the β-coefficients suggested an inverse association between lung function indices and TNF-α levels (Table II).

PCA. Unsupervised PCA showed that hs-CRP, IL-6, TNF-α, and CXCL8 levels were positively correlated in the first principal component (PC1), which explained 41% of the total variation in the data. The PCA approach is illustrated in the
Neonatal bronchial responsiveness and systemic low-grade inflammation

Bronchial responsiveness to methacholine in neonatal life was not associated with biomarkers of low-grade inflammation at age 6 months (Table II).

DISCUSSION

Key findings

Infants with reduced pulmonary capacity as neonates are characterized by systemic low-grade inflammation with an upregulated blood inflammatory response, including increased hs-CRP levels. This association suggests that reduced neonatal lung function is part of a condition with an ongoing asymptomatic airway inflammation and a measurable systemic component from the beginning of life.

Strengths and limitations of the study

A major strength of the study is the unique assessment of neonatal lung function with the state-of-the-art raised-volume rapid thoracoabdominal compression technique performed strictly in adherence with recognized guidelines in the full mother-child birth cohort. The neonatal spirometric measurements were obtained in this cohort of asymptomatic children before any respiratory symptoms and are thus unbiased from previous or concurrent airway symptoms.

Another significant strength of the study is the availability of a range of environmental exposure assessments, including bacterial airway colonization and the presence of a virus, enabling robust confounder adjustment for factors with a possible influence on neonatal lung function and low-grade inflammation. However, it is a limitation that we did not assess the presence of bacteria and viruses at both lung function and inflammatory biomarker testing.

There were strong linear correlations between IL-6 and TNF-α levels and hs-CRP levels. Because IL-6 and TNF-α are the main triggers of CRP release from the liver, these expected correlations serve as a biological validation of the data. The lack of correlation between CXCL8 and hs-CRP levels was not surprising because CXCL8 primarily has a neutrophilic chemotactic function in the innate immune system and does not directly induce CRP release. The finding of significantly increased hs-CRP levels in children experiencing an infectious episode within 14 days before biomarker assessment further validates the data because CRP is a reliable biomarker of airway inflammation. Even after adjusting for this confounder, the association between neonatal lung function and hs-CRP levels remained, with largely unchanged effect estimates.

Both the standard statistical approach and the unsupervised data-driven PCA approach showed similar associations, which strengthened confidence in our findings. Still, we did not detect association between neonatal bronchial hyper-responsiveness and low-grade inflammation, which we would have expected given our previous finding of association between methacholine challenge results and subsequent asthma development.

It is a limitation of the study that we were unable to detect a biologically meaningful signal from IL-1β, which is presumably caused in part by the plasma storage time of up to 13 years before analysis, during which samples had been thawed and frozen on several occasions. IL-1β is particularly sensitive to freeze-thaw cycles and degrades by greater than 50% over time, even when samples are stored at −80°C. It is well known that circulating IL-1β levels are approximately 5 times less than TNF-α levels in healthy adults, but in our case the median IL-1β level was 200 times less than the median TNF-α level (0.01 vs 2.34 ng/L), and we were unable to detect an association between IL-1β and hs-CRP levels.

**FIG 1.** Scatter plot illustrating the relationship between neonatal lung function (z-score of FEV₀.₅) and hs-CRP level at age 6 months (log-transformed values). Solid line, Regression line; shaded area, 95% confidence limits.

**FIG 2.** Scatter plot illustrating the relationship between neonatal lung function (z-score of FEV₀.₅) and IL-6 levels at age 6 months (log-transformed values). Solid line, Regression line; shaded area, 95% confidence limits.
Another limitation of the study is the at-risk nature of the cohort because all children were born to mothers with a history of asthma. We demonstrated recently that the offspring of mothers with a history of asthma, allergy, or eczema in an unselected mother-child cohort have a topical downregulated immune signature in the airway mucosa compared with children of mothers without such disorders. Yet even though the at-risk nature of the cohort might have affected the absolute biomarker levels, this should not influence our ability to explore the association between neonatal spirometry and markers of systemic low-grade inflammation within the cohort.

It is another limitation of our study that biomarkers were assessed at 6 months while neonatal lung function was tested at 4 weeks. However, we adjusted for any lung symptoms in the period between based on the daily diary cards filled out by the parents.

### Study implications

We show that impaired lung function in neonates is associated with a systemic inflammatory process, even before the development of any respiratory symptoms. This suggests a link between reduced neonatal lung function and a disorder characterized by a systemic inflammatory component.

A number of recent larger cross-sectional analyses in adults and adolescents have shown that increased hs-CRP levels are associated with respiratory impairment in both population-based settings and in asthmatic and nonasthmatic strata. hs-CRP levels have also been reported in relation to pulmonary function outcomes in studies of children with established asthma. A study of 63 asthmatic children aged 2 to 12 years with and without acute exacerbations and a study of 60 school-aged children treated with inhaled corticosteroids, as well as steroid-naive children, showed a reciprocal relationship between FEV1 values and hs-CRP levels. In contrast, another study of 62 school-aged children with controlled and uncontrolled asthma did not detect an association between hs-CRP levels and FEV1 values but found that hs-CRP levels were greater in patients with uncontrolled versus those with controlled asthma, which might reflect the degree of airway inflammation. These studies might be underpowered and are hampered by the wide age ranges and lack of control groups. Importantly, it is a different research question whether established asthma is associated with detectable systemic inflammation, unlike our aim to study whether systemic inflammation in very early life is associated with neonatal lung function before symptom debut.

---

**TABLE II.** Association between neonatal lung function and inflammatory biomarkers at age 6 months: conventional and PCA approach

<table>
<thead>
<tr>
<th></th>
<th>Log hs-CRP</th>
<th></th>
<th>Log IL-6</th>
<th></th>
<th>Log TNF-α</th>
<th></th>
<th>Log CXCL8</th>
<th></th>
<th>PC1</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>β-Coefficient (95% CI)</td>
<td>P value</td>
<td>β-Coefficient (95% CI)</td>
<td>P value</td>
<td>β-Coefficient (95% CI)</td>
<td>P value</td>
<td>β-Coefficient (95% CI)</td>
<td>P value</td>
<td>β-Coefficient (95% CI)</td>
<td>P value</td>
</tr>
<tr>
<td>Unadjusted analysis</td>
<td>z-FEV0.5</td>
<td>−0.12</td>
<td>(−0.21 to −0.04)</td>
<td>0.04</td>
<td>−0.10</td>
<td>(−0.18 to −0.01)</td>
<td>0.03</td>
<td>−0.11</td>
<td>(−0.38 to 0.17)</td>
<td>0.44</td>
</tr>
<tr>
<td></td>
<td>z-FEV20</td>
<td>−0.06</td>
<td>(−0.11 to 0.06)</td>
<td>0.14</td>
<td>0.02</td>
<td>(−0.37 to 0.18)</td>
<td>0.61</td>
<td>0.09</td>
<td>(−0.22 to 0.11)</td>
<td>0.52</td>
</tr>
<tr>
<td></td>
<td>Log PD15</td>
<td>0.04</td>
<td>(−0.12 to 0.21)</td>
<td>0.60</td>
<td>0.03</td>
<td>(−0.21 to 0.15)</td>
<td>0.75</td>
<td>0.02</td>
<td>(−0.56 to 0.52)</td>
<td>0.94</td>
</tr>
<tr>
<td>Adjusted analysis*</td>
<td>z-FEV0.5</td>
<td>−0.12</td>
<td>(−0.22 to −0.02)</td>
<td>0.02</td>
<td>−0.11</td>
<td>(−0.23 to 0.01)</td>
<td>0.07</td>
<td>−0.21</td>
<td>(−0.58 to 0.17)</td>
<td>0.27</td>
</tr>
<tr>
<td></td>
<td>z-FEV20</td>
<td>−0.07</td>
<td>(−0.18 to 0.03)</td>
<td>0.16</td>
<td>0.05</td>
<td>(−0.17 to 0.07)</td>
<td>0.42</td>
<td>−0.16</td>
<td>(−0.54 to 0.21)</td>
<td>0.40</td>
</tr>
<tr>
<td></td>
<td>Log PD15</td>
<td>0.05</td>
<td>(−0.13 to 0.23)</td>
<td>0.60</td>
<td>0.00</td>
<td>(−0.20 to 0.21)</td>
<td>0.97</td>
<td>−0.07</td>
<td>(−0.73 to 0.59)</td>
<td>0.84</td>
</tr>
</tbody>
</table>

Values in boldface are \( P < .10 \).

*Partial linear regression analyses adjusted for birth BMI, maternal smoking during the third trimester of pregnancy, older children in the home at birth, bacterial airway colonization at age 4 weeks, infections 14 days before, and any TROLS, as well as episodes of virus-induced TROLS at any time point before blood sampling for inflammatory biomarker assessment.

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**FIG 3.** PCA biplot showing the children’s individual scores (gray dots) in the first principal component (PC1) and second principal component (PC2), as well as biomarker loadings for hs-CRP, IL-6, TNF-α, and CXCL8 (red dots) and correlation with lung function indices (blue dots). Percentages in parentheses are the part of the total variation in the data set explained by the components.
A possible explanation of the identified association between reduced neonatal lung function and increased hs-CRP levels is that diminished forced volume is caused by airway inflammation. In vitro murine and human lung cell studies have established a possible role of the proinflammatory cytokines stimulating CRP release, such as IL-6, TNF-α, and IL-1β, in the pathophysiology of obstructive airway inflammation. Persistently increased CRP levels might induce an increased vulnerability to changes in the early-life environment through its actions as a general scavenger protein with important innate immune functions in the recognition and elimination of bacteria and damaged human cells through opsonization, phagocytosis, and cell-mediated cytotoxicity. Therefore reduced neonatal lung function might reflect subclinical bacterial airway colonization and airway inflammation predating detection of clinical symptoms and systemic low-grade inflammation. Such a disease trajectory is well known in patients with cystic fibrosis, for example, in whom reduced lung function has been shown to precede clinical disease penetrance and correlates with Pseudomonas aeruginosa airway colonization before exacerbations.

Alternatively, reduced neonatal lung function does not lead to systemic inflammation but is rather an independent characteristic of neonates with sustained low-grade inflammation in early life. Such inefficient immune regulation might be driven by the newborn’s genotype interacting with the intrauterine and early-life environment, thereby affecting the plasticity of the developing immune system. In support of the latter theory, higher baseline CRP levels have been demonstrated in westernized populations, where obstructive airway disorders are more prevalent compared with rural societies.

We assessed lung function in the 4-week-old asymptomatic neonates and the inflammatory biomarkers at 6 months. We can only speculate whether this concurs with the onset of an underlying disorder or whether this possibly reflects a disorder beginning even earlier in life, perhaps during pregnancy.

Children of the Danish COPSAC2000 at-risk cohort exhibited an association between reduced neonatal lung function and upregulated systemic inflammatory biomarkers before symptom onset, suggesting that reduced lung function reflects an ongoing inflammatory disorder with a measurable systemic component early in life. 

**Key messages**

- Asthmatic children and adults with diminished pulmonary function have increased levels of hs-CRP, a marker of systemic low-grade inflammation. However, it is unknown whether asymptomatic neonates with reduced lung function have signs of systemic inflammation.
- Neonates with impaired respiratory capacity are characterized by an upregulated blood inflammatory profile, including hs-CRP, suggesting the presence of systemic low-grade inflammation in early life before symptom onset.

**REFERENCES**


**TABLE E1.** Comparison of baseline characteristics between children with and without complete assessment of early-life low-grade inflammation

<table>
<thead>
<tr>
<th>Baseline characteristic</th>
<th>Children with biomarker assessment (n = 300)</th>
<th>Children without biomarker assessment (n = 111)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paternal asthma, allergy, or eczema (no.)</td>
<td>47% (135)</td>
<td>46% (50)</td>
<td>.84†</td>
</tr>
<tr>
<td>Male sex (no.)</td>
<td>51% (154)</td>
<td>44% (49)</td>
<td>.20†</td>
</tr>
<tr>
<td>BMI at birth (m/kg²), mean (SD)</td>
<td>12.79 (1.34)</td>
<td>12.84 (1.22)</td>
<td>.63†</td>
</tr>
<tr>
<td>Older children in home at birth (no.)</td>
<td>39% (114)</td>
<td>40% (38)</td>
<td>.91†</td>
</tr>
<tr>
<td>Household income at birth* (no.)</td>
<td></td>
<td></td>
<td>.12†</td>
</tr>
<tr>
<td>Low</td>
<td>27% (77)</td>
<td>38% (35)</td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td>49% (143)</td>
<td>41% (39)</td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>24% (70)</td>
<td>21% (20)</td>
<td></td>
</tr>
<tr>
<td>Maternal smoking during third trimester (no.)</td>
<td>17% (51)</td>
<td>11% (12)</td>
<td>.12†</td>
</tr>
<tr>
<td>Cesarean section (no.)</td>
<td>23% (60)</td>
<td>27% (25)</td>
<td>.45†</td>
</tr>
<tr>
<td>Solely breast-feeding length (d), median (IQR)</td>
<td>122 (90-155)</td>
<td>122 (74-164)</td>
<td>.90†</td>
</tr>
<tr>
<td>Age at start in day care (d), median (IQR)</td>
<td>345 (240-415)</td>
<td>307 (216-412)</td>
<td>.27§</td>
</tr>
<tr>
<td>Cat in home in first year of life (no.)</td>
<td>16% (46)</td>
<td>14% (14)</td>
<td>.61‡</td>
</tr>
<tr>
<td>Dog in home in first year of life (no.)</td>
<td>15% (44)</td>
<td>10% (10)</td>
<td>.16‡</td>
</tr>
</tbody>
</table>

*Yearly household income at birth of neonate: low (<$53,000), medium ($53,000-$80,000), and high (>=$80,000).
† χ² Test.
‡ χ² Test.
§ Wilcoxon rank sum test.