

Low-grade disease activity in early life precedes childhood asthma and allergy

Bo Lund Krogsgaard Chawes

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Official opponents: Peter Lange, John Henderson and Johan de Jongste.

Correspondence: COPSAC; Copenhagen Prospective Studies on Asthma in Childhood, Herlev and Gentofte Hospital, Ledreborg Allé 34, 2820 Gentofte, Denmark

E-mail: b_chawes@hotmail.com

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THE 7 PREVIOUSLY PUBLISHED PAPERS ARE

- I. Chawes BL, Bønnelykke K, Jensen PF, Schoos AM, Heickendorff L, Bisgaard H. Cord Blood 25(OH)-Vitamin D Deficiency and Childhood Asthma, Allergy and Eczema: The COPSAC₂₀₀₀ Birth Cohort Study. *PLoS One*. 2014 Jun 12;9(6):e99856
- II. Følsgaard NV, Chawes BL, Bønnelykke K, Jenmalm MC, Bisgaard H. Cord blood Th2-related chemokine CCL22 levels associate with elevated total-IgE during preschool age. *Clin Exp Allergy*. 2012 Nov;42(11):1596-603
- III. Chawes BL, Bønnelykke K, Bisgaard H. Elevated eosinophil protein X in urine from healthy neonates precedes development of atopy in the first 6 years of life. *Am J Respir Crit Care Med*. 2011 Sep 15;184(6):656-61
- IV. Chawes BL, Buchvald F, Bischoff AL, Loland L, Hermansen M, Halkjaer LB, Bønnelykke K, Bisgaard H. Elevated exhaled nitric oxide in high-risk neonates precedes transient early but not persistent wheeze. *Am J Respir Crit Care Med*. 2010 Jul 15;182(2):138-42
- V. Chawes BL, Bischoff AL, Kreiner-Møller E, Buchvald F, Hakonarson H, Bisgaard H. DENND1B gene variants associate with elevated exhaled nitric oxide in healthy high-risk neonates. *Pediatr Pulmonol*. 2015 Feb;50(2):109-17
- VI. Chawes BL, Poorisrisak P, Johnston SL, Bisgaard H. Neonatal bronchial hyperresponsiveness precedes acute severe viral bronchiolitis in infants. *J Allergy Clin Immunol*. 2012 Aug;130(2):354-61
- VII. Chawes BL, Stokholm J, Bønnelykke K, Brix S, Bisgaard H. Neonates with Reduced Neonatal Lung Function Have Systemic Low-grade Inflammation. *J Allergy Clin Immunol*. 2015 Jun;135(6):1450-1456

In the following review the papers are referred to by their roman numerals.

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 (22). 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 (23) and emerging evidence also pinpoints a role of T helper 17 cells (Th17) characterizing steroid non-responsive neutrophilic airway inflammation (24).

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 (25). 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 (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 (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 (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 (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 (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 (30) and personalized medical care (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 (COPSAC₂₀₀₀) 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 (32). 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 (33,34). 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) (35). 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 (36).

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

Table 1: Baseline characteristics of the COPSAC₂₀₀₀ birth cohort

Baseline characteristics	
Mothers enrolled, N	452
Number of newborns, N	411
Birthdate, range	02.08.1998 – 29.02.2001
Boys	49.4%
Twins pairs	2%
Sibling pairs	2%
Caucasian	97%
Mother's age at birth, mean (SD), years	30.0 (4.5)
Father's age at birth, mean (SD), years	32.0 (5.2)
Season of birth	
Winter	23%
Spring	21%
Summer	27%
Fall	29%
Pregnancy and birth	
Gestational age, mean (SD), weeks	39.9 (1.6)
Birth weight, mean (SD), kg	3.52 (0.52)
Birth length, mean (SD), cm	52.3 (2.3)
Head circumference at 1 week, mean (SD), cm	35.2 (1.6)
Apgar score at 5 min., mean (SD)	9.8 (0.6)
Mode of delivery, Caesarean section	21%
Exposures	
Older children in household	
0	64%
1	24%
2	9%
>2	3%
Mother's smoking during pregnancy, any	24%
Mother's alcohol use during pregnancy, any	26%
Mother's antibiotics use during pregnancy, any	30%
Furred pets at home, any	30%
Duration of solely breastfeeding, mean (SD), days	113 (62)
Age at start in daycare, mean (SD), days	349 (147)
Hair nicotine level at age 1 yr, mean (SD), ng/mg	3.28 (7.98)
Socioeconomics	
Household annual income	
<53.000 Euro	29%
53.000 – 80.000 Euro	47%
>80.000 Euro	24%
Mother with university education (>3yrs)	13%
Father with university education (>3yrs)	17%
Mother without occupation (unemployed or student)	19%
Father without occupation (unemployed or student)	7%
Atopic disposition (diagnosed by doctor)	
Mother with asthma	100%
Mother with allergic rhinitis	73%
Mother with eczema	46%
Father with asthma	15%
Father with allergic rhinitis	30%
Father with eczema	11%
Genetics	
ORMDLR3, TT genotype (rs7216389)	29%
DENND1B (rs2786098)	
AA	4%
AC	27%
CC	69%
Filaggrin mutation (R501X or 2282del4 null mutation)	11%

re-analysing samples if the coefficient of variation (CV) was >15% (37).

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 (38,39).

Urine

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 (40).

Exhaled breath

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 (41,42). Concentration of fractional exhaled nitric oxide (FeNO) was measured in duplicates using an off-line technique (43) 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 (44). In brief, repeated ventilations to a predefined mouth-pressure were applied to assure expansion of the lung volume before an instant inflation of the “squeeze” jacket caused a forced exhalation where the flow was measured by a pneumotachograph with an aircushion facemask (41,42). 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) (42). 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

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 (33,34,45). Children with such a

	Birth	Mo	Years							Acute symp.
	0	1 6	1	2	3	4	5	6	7	
Neonatal Biomarkers										
Cord blood										
Chemokines (CXCL10, CXCL11, CCL17, CCL22)	▼									
25(OH)-Vitamin D3	▼									
Urine										
u-EPX, u-LTC4/D4/E4, u-11β-PGF2α		▼								
Exhaled breath										
FeNO		▼								
Neonatal Lung Function										
Spirometry		▼								
Airway reactivity, metacholine		▼								
Clinical Endpoints										
Respiratory symptoms, infections and eczema										
Daily diary on symptoms and medication			←-----→							▼
Prospective diagnosis by research staff			←-----→							▼
Physical Examination		▼ ▼	▼ ▼ ▼ ▼ ▼ ▼ ▼ ▼ ▼ ▼ ▼ ▼ ▼ ▼ ▼ ▼ ▼							▼
Allergy										
Skin Prick Test		▼	▼			▼		▼		▼
Specific IgE		▼	▼			▼		▼		▼
Nasal eosinophilia								▼		
Allergic Rhinitis									▼	▼
Intermediary Phenotypes										
Blood										
hs-CRP, IL-1β, IL-6, TNF-α, CXCL8		▼								
Total IgE		▼	▼			▼		▼		▼
Eosinophil count		▼	▼			▼		▼		▼
Lung Function										
Spirometry								▼ ▼ ▼ ▼ ▼ ▼		▼
Airway reversibility								▼ ▼ ▼ ▼ ▼ ▼		▼
Airway reactivity, metacholine									▼	

Figure 1: Overview and temporal collection of biomarkers and endpoints from the COPSAC₂₀₀₀ birth cohort presented in the thesis.

symptom burden were prescribed a 3-month trial of inhaled budesonide 200 mcg twice daily.

Asthma

Asthma at age 7 years was diagnosed according to recognized international guidelines (22,46) 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 (33,34,45).

Acute bronchiolitis

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 (47,48) 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) (49). Allergic sensitization was defined as specific IgE levels ≥0.35kU/L (50,51).

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 (49).

Allergic rhinitis

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 (11,36,52). Rhinitis was defined as bothersome sneezing or blocked or runny nose in the past 12 months outside periods with common cold or flu (53).

Figure 1 summarizes the COPSAC₂₀₀₀ investigator-diagnosed clinical endpoints, intermediate phenotypes, and neonatal biomarkers and lung function incentives utilized in the studies presented in this thesis.

CORD BLOOD BIOMARKERS

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 (54).

Priming of the developing immune system starts in utero (55) and it has been shown that the fetus is capable of producing IgE already during gestational week 11 (56,57). Furthermore, it is a general belief that IgE antibodies do not cross the placenta barrier (58) 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 (59,60), maternal allergy status (61), maternal age (62), birth order (63), the child's gender (62,64) and elevated cord blood total IgE. In addition, some studies have shown that both high total IgE (61,65–67) and specific IgE (68) 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 (69). However, clinical trials of avoiding either aeroallergens (70) or food allergens (71,72) 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 (73), 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 (62,74–76). Second, data from the COPSAC₂₀₀₀ 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 (77). Third, cellular studies of cord blood immune cells pinpoint that putative T cell memory is not caused by allergen specific priming (78) and that such specific Th2 polarization is first acquired after birth (79).

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 (80). Another plausible mechanism is transplacental transfer suggested by normal cord blood IgA level, but detectable specific IgE mirroring maternal specific IgE (81). However, in some samples with elevated total IgE (>0.5IU/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 (81). 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 (82). 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 (83).

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 (84). 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 (91). Inflammatory chemokines manage the migration of immune cells in inflammatory processes (92,93) 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 (74). 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 (94–96). However, there is limited knowledge of cord blood chemokine patterns preceding asthma, allergy, and related conditions (94,97).

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.

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

* $p < 0.05$; ** $p < 0.01$

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 (II). 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 (97) and specific IgE levels (94,98) 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 (99,100).

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 (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 (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 (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 (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 (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 (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 (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 (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 (105).

Table 3: 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.

	Wheeze	Asthma	Lung Function	Respiratory Infections	Allergic Sensitization	Rhinitis
Bañ et al., 2014 N=239	Reduced risk (0-3yrs)	No effect (5yrs)	-	-	-	No effect (5yrs)
Belderbos et al., 2011, N=156	-	-	-	Reduced risk* (0-1yrs)	-	-
Camargo et al., 2011, N=922	Reduced risk (0-5yrs)	No effect (5yrs)	-	Reduced risk (0-3mo)	-	-
Chawes et al., 2014, N=257	Reduced risk (0-7yrs)	No effect (7yrs)	No effect (1mo and 7yrs)	No effect (0-3yrs)	No effect (0-6yrs)	No effect (7yrs)
Chiu et al., 2014, N=186	-	No effect (4yrs)	-	-	No effect** (0-4yrs)	No effect (4yrs)
Jones et al., 2012, N=231	No effect (0-1yr)	-	-	-	No effect (1yr)	-
Liu et al., 2011, N=649	-	-	-	-	No effect*** (2yrs)	-
Łuczyńska et al., 2014, N=777	-	-	-	Reduced risk (0-1yr)	-	-
Mohamed et al., 2013, N=206	-	-	-	Reduced risk (0-2yrs)	-	-
Rothers et al., 2011, N=219	-	No effect (5yrs)	-	-	Dual effect**** (0-5yrs)	No effect (5yrs)
Stelmach et al., 2015, N=240	Reduced risk (0-2yrs)	-	-	No effect (0-2yrs)	No effect (0-2yrs)	-
Weisse et al., 2013, N=378	-	-	-	-	Increased risk (0-2yrs)	-

*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.

VITAMIN D

Vitamin D status is highly dependent on lifestyle as production of the biologically active form of vitamin D, 1,25(OH)₂-vitamin D, depends on dietary intake and exposure to sunlight (106). Only 10-20% of vitamin D is obtained from foods such as oily fish, fortified products and dietary supplements (107), whereas the main contributor in humans is synthesis from UVB light, which facilitates the conversion of cutaneous 7-dehydrocholesterol to vitamin D₃ that subsequently enters the circulation. Vitamin D₃ 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)₂-vitamin D predominantly in the kidneys, but also in the respiratory epithelium and in certain immune cells (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 (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 (111) occurring in parallel with the arising asthma and allergy pandemic (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 (112). In addition, some

studies have shown significant associations between polymorphisms in the vitamin D receptor gene (113) and in genes involved in vitamin D metabolism and signaling pathways (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 (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 (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 (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 (119). However, timing, duration and amount of vitamin D exposure seem crucial for the direction of the resulting immune deviation (117).

Vitamin D is also believed to promote induction of Treg cells (120), 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 (121), and downregulated expression of the Treg cell transcription factor FOXP3 has been demonstrated in placental tissue of vitamin D deficient pregnant women (122). 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 (123), inhibited monocyte Toll-like receptor production, and the promotion of tolerogenic dendritic cells (124).

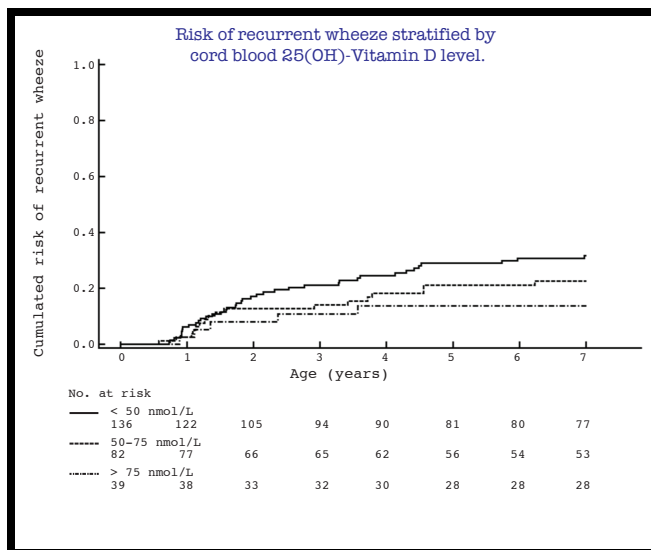


Figure 2: Kaplan Meier curve showing the association between cord blood 25(OH)-Vitamin D levels and risk of recurrent wheeze (modified from I).

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 (34). 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 (125), 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 (126). 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 (127). 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 (128,129). The studies were based on 1,194 mother-child pairs from Boston, MA (128), and 1,212 mother-child pairs from Aberdeen, Scotland (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 (130), whereas a similarly sized Spanish study observed a protective effect on respiratory infections, but no effect on wheezing or asthma development (131). Additionally, a reduced risk of allergic rhinitis at age 5 years has been reported (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 (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 (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 Table 3.

The COPSAC₂₀₀₀ 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 (140–142). The main observation in the COPSAC₂₀₀₀ 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 (≤ 50 nmol/L) (see Figure 2). This aligns with findings from all other published cord blood studies with such endpoint (138,141,144) 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 (140).

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 (137,141) including RSV bronchiolitis (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 (138), but is in contrast to a large study of 777 mother-infant pairs from Ulm, Germany (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 (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 (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 (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 (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 (134). In the COPSAC₂₀₀₀ 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 (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 (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. (106). 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 (145), selenium (146), iron (147), vitamin A and E (148), 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 (149–151). However, randomized controlled trials of n-3 LCPUFA supplementation during pregnancy have shown ambiguous results (152–154).

URINARY BIOMARKERS

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-LTC₄/D₄/E₄), and major metabolites of prostaglandin D₂ such as 11 β -prostaglandin F₂ α (u-11 β -PGF₂ α).

Eosinophil cationic protein (ECP) and eosinophil protein X/eosinophil-derived neurotoxin (155) are both members of the ribonuclease A superfamily and contain a range of properties including neurotoxicity. They are solely released after degranula-

tion 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 (156), it is correlated to eosinophil count in blood and bronchoalveolar lavage fluid (157) as well as serum ECP levels (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 (Fc ϵ R1) complexes, but they are also released from mononuclear cells, mast cells and basophil during the first hours of the late-phase reaction (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-LTC₄/D₄/E₄ represents an established measure of total body cysteinyl leukotriene production, whereas the u-11 β -PGF₂ α 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 COPSAC₂₀₀₀ 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 1st 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 (FEV₁) over time (167), and levels decrease at commencement of inhaled corticosteroids (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

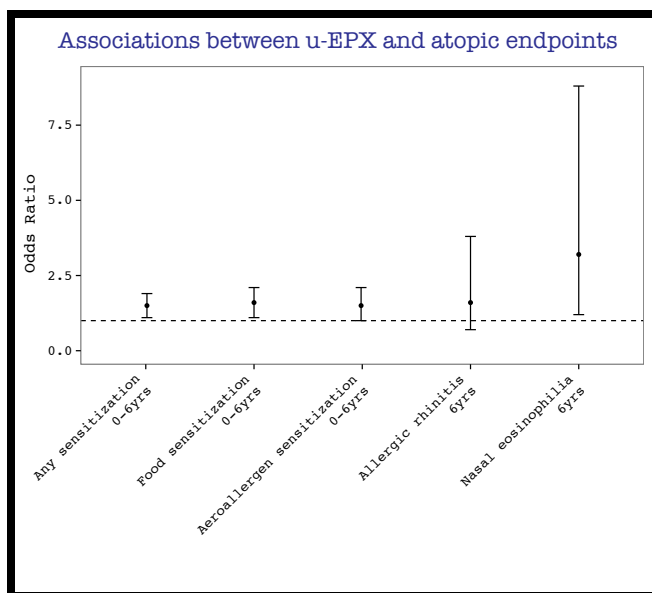


Figure 3: Odds ratio plot illustrating the associations between neonatal u-EPX and development of atopic endpoints (modified from III).

specificity (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 (170), but this finding still awaits replication.

Urinary leukotriene E4 (u-LTE4) was explored in 108 German 10-year-old children showing higher levels in children diagnosed with moderate-severe atopic asthma compared to controls (171). Although excretion of u-LTE4 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 (171). A study of children <3 years found that u-LTE4 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) (172). In line with this, two similarly sized studies of preschool children observed increased u-LTE4 levels during acute viral wheeze, which was exaggerated among children with high total-IgE levels (173) and sensitization (174). In contrast, a study of 1-year-old children with atopic predisposition saw no differences in u-LTE4 in children with a history of wheezy breathing or any other respiratory symptoms (163).

Pediatric studies of u-11 β -PGF2 α in relation to asthma and allergy are scarce and solely related to challenges or exacerbations. A brief communication showed that u-11 β -PGF2 α 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) (175). Another small study of 30 children demonstrated elevated levels upon admission to hospital with an acute asthma attack, which declined during convalescence (176). Additionally, elevated u-11 β -PGF2 α after exercise challenge testing compared to baseline has been demonstrated in two childhood studies with 86 (177) and 14 children (176), respectively, whereas rising levels after inhaled allergen challenge and aspirin challenge are documented solely in adult settings (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 develop-

ment 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 3). 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-LTC4/D4/E4 and u-11 β -PGF2 α 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 (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.

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 (184). The approach includes assessment of the dynamic metabolome, which is the complete set of small-molecule metabolites (e.g. cholesterol, triglycerides, fatty acids, metabolic substrates, amino acids, and other signaling molecules) in a biological sample to identify metabolic phenotypes (185). Metabolomics 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 modulation (187). 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 un-sedated 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 (200). The repeated cycles and fixed resistance diminish breath-to-breath flow variability and limit nasal nitric oxide contamination of the sample (201). 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) (202), no previous large scale study has compared the techniques in neonates. We, therefore, measured FeNO by both techniques in 253 healthy neonates from the COPSAC₂₀₀₀ 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 (V). It is recommended to refrain from lung function testing prior to FeNO measurement (203), 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 (IV), which aligns with a study of

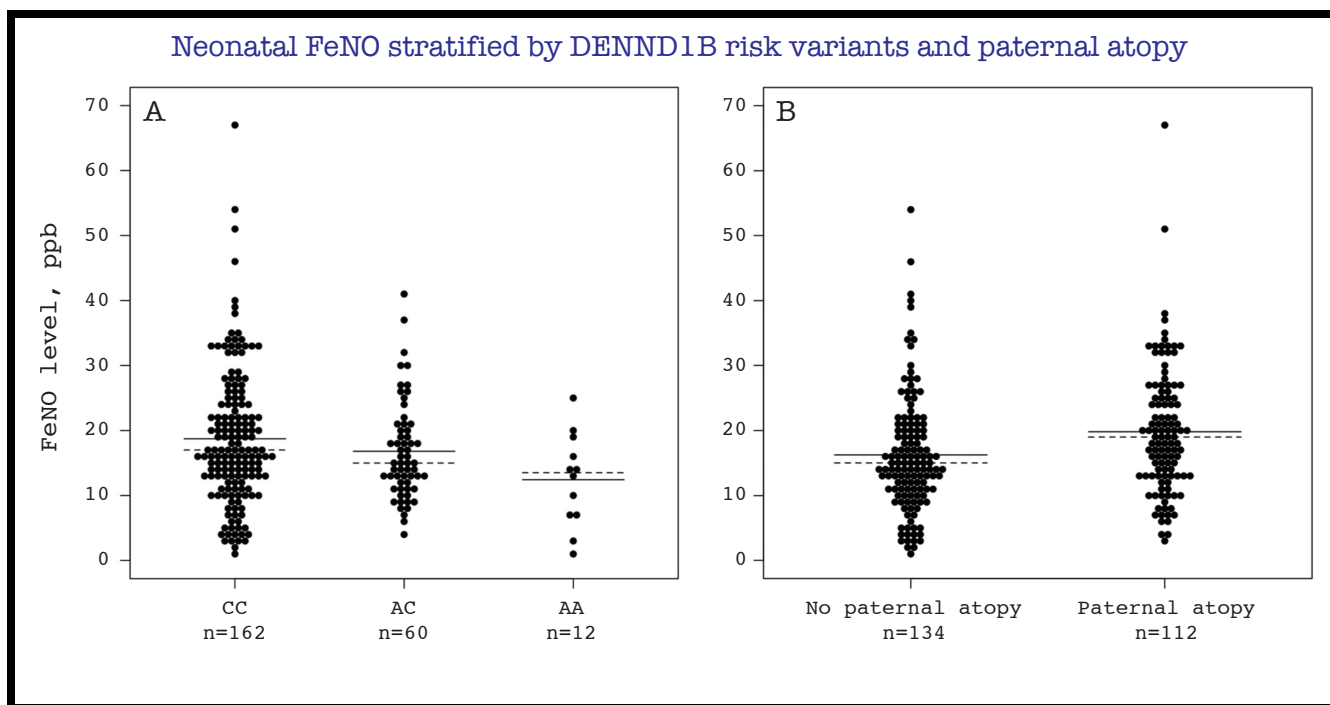


Figure 4: Relationship between neonatal FeNO levels, DENND1B risk variants (A), and paternal atopic diseases (B) (modified from V).

45 1-year-old children showing no FeNO difference before and after sedation or pre vs. post lung function testing (204). Based on that, we suggest measuring FeNO in unanaesthetized infants by the least invasive tidal-breathing technique for future studies (IV-V).

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 (205), 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 (206), whilst another study of 1-month-olds (N=98) showed higher FeNO in infants exposed postnatally (207), and a third study of unselected children aged 2 to 6 months (N=110) found no association between FeNO and concurrent tobacco-smoke exposure (208). 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 (V). This negative finding may be due to the at-risk nature of the COPSAC₂₀₀₀ cohort as others have demonstrated an interaction between prenatal tobacco exposure, presence of maternal asthma and neonatal FeNO (207). Previous studies have not shown influence from father's history of asthma or allergies (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 (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 COPSAC₂₀₀₀ and other cohorts have not revealed relationships between other environmental factors such as antibiotic and acetaminophen consumption during pregnancy, socioeconomic, older siblings, furred pet exposure, breastfeeding or deviations in the airway microbiome (V) and neonatal FeNO (204,207).

It is plausible that neonatal FeNO is mainly an inherited trait and that well-known childhood asthma genes such as Filaggrin (210,211), ORMDL3 (45), and DENND1B (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 4). 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 (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 (214) and primary biliary cirrhosis (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 (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 NOS216 (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 (217). Subsequently, it was shown that DNA methylation of iNOS contributed to FeNO level and interacted with the degree of particulate air pollution (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 (193). 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 (194).

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 (209). 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 (206,208,209). In addition, the initiation of inhaled corticosteroids is also well-known to lower FeNO values (219).

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 (198) and our study (IV). 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 (198). In the COPSAC₂₀₀₀ at-risk cohort, we also observed that raised neonatal FeNO preceded recurrent wheeze (Figure 5), episodic viral wheezing and number of wheezy episodes in the 1st year of life, but not at age 1-6 years (IV). 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 (198), 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 (220) 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

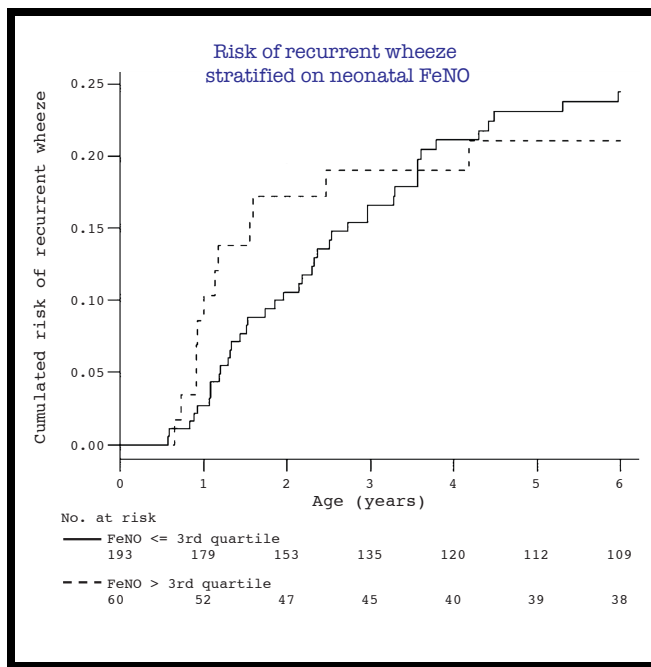


Figure 5: Kaplan Meier curve showing the association between neonatal FeNO and risk of recurrent wheeze (modified from IV).

(221). It has been proposed that congenitally small airway dimensions predispose to an increased propensity of early transient wheezing (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 (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 (224). A recent systematic review summarized the findings from EBC pediatric asthma trials (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 (H₂O₂) and nitric oxide products (NO_x) 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 cysteinyl 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. (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 (226) or predicted forthcoming asthma exacerbations (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 FEV1 to discriminate between 25 children with asthma and 11 controls (7-15 years) (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) (229).

It is feasible and safe to collect EBC in infants as early as one month of age (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 (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%) (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) (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%) (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

(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 (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 (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 (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 COPSAC₂₀₁₀ cohort of 700 children (238), we have repeatedly 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 neonates⁴⁴ 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 COPSAC₂₀₀₀ 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 COPSAC₂₀₀₀ 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 indicia of diminished baseline FEV_{0.5} and FEF₅₀, which was however not significant (Figure 6). 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.

The association between premonitory 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 (Rrs), but no difference in compliance (Cr) 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 (243). These findings were sought replicated by the same research group after enrolling 159 similar preterm infants, but Rrs was only significantly higher among severe cases (both RSV and non-RSV), who were admitted to hospital (244). An older study of term infants reported trends of reduced maximum flow at FRC (V_{max}FRC), whilst no differences in Rrs, Crs or bronchial responsiveness to histamine at age 5 weeks in children developing bronchiolitis before age 2 years

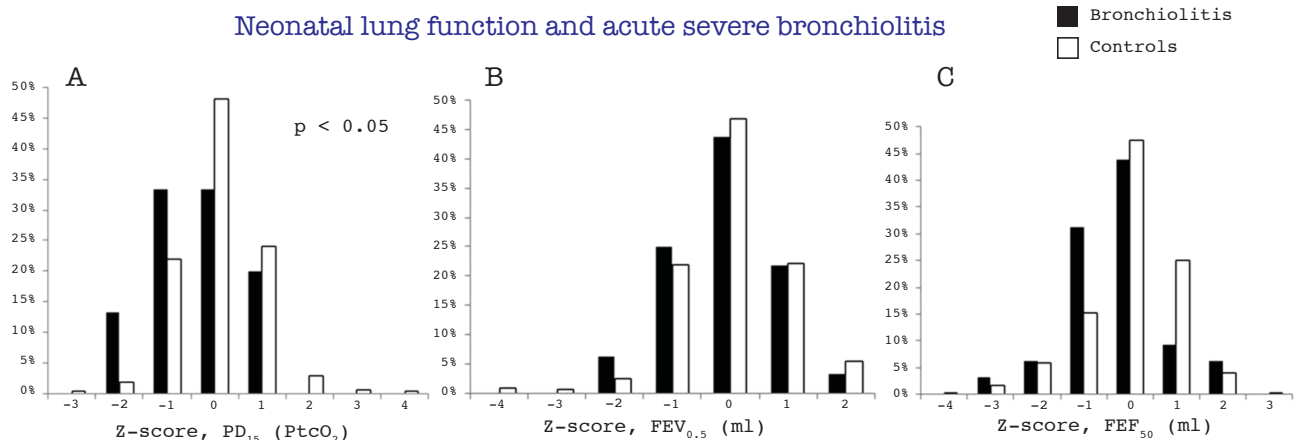


Figure 6: Neonatal lung function indices and bronchial responsiveness in children developing acute bronchiolitis vs. healthy controls (modified from VI).

(N=17) compared to controls (N=236) (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 (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 Rrs and decreased Crs in RSV-positive children hospitalized with bronchiolitis (N=18) vs. non-hospitalized RSV-positive children (N=84) representing both symptomatic and asymptomatic cases (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 (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 COPSAC₂₀₀₀ 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 (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 (tptef/tE) in one study (N=124) (222) and lower forced expiratory flow at FRC in another study (N=97) (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 VmaxFRC, N=69) (250) and in unselected populations (reduced VmaxFRC and airway hyperresponsiveness, N=253) (251). Furthermore, follow-up of the Tucson birth cohort study showed that wheezing by age 3 years was still characterized by altered neonatal tptef/tE (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 (253). Together, evidence from the COPSAC₂₀₀₀ 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 (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 PD15 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-1 β , and TNF- α during acute and chronic inflammatory disorders (254). Newer assays with increased sensitivity (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) (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 FEV1 and increased prevalence of bronchial hyperresponsiveness (257) indicating that the degree of airflow obstruction and inflammation contributes to the systemic inflammatory process. Two large community based prospective studies of 531 (258) and 2,442 (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 FEV1 over a 10-year period (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 (260).

Currently, only very few published studies have investigated the interrelationship between low-grade inflammation and pulmonary function outcomes in children (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 (263) and 60 steroid naïve and steroid treated school-aged children (261), respectively, showed an inverse relationship between hs-CRP and FEV1. A third study investigating 62 school-aged children with controlled and uncontrolled asthma (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-1 β , 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 FEV0.5 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 FEV0.5 was associated with an up-regulated blood inflammatory profile. Identical trends were seen for FEF50, whereas the PD15 values were unrelated to any biomarkers of systemic inflammation (Table 4).

Table 4: 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.

	hs-CRP	PCA analysis
z-score FEV0.5	-0.12** [-0.21 to -0.04]	-0.10* [-0.19 to -0.01]
z-score FEF50	-0.06 [-0.15 to 0.02]	-0.06 [-0.14 to 0.03]
Log-PD15	0.04 [-0.12 to 0.21]	0.03 [-0.14 to 0.19]

* $p < 0.05$; ** $p < 0.01$

These unparalleled data from the COPSAC2000 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 (263) or infections (264), even mild viral upper respiratory infections (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 (266), showed a trend of increased hs-CRP at age 6 months (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 (267) and hs-CRP levels (268). Finally, established genetic and environmental determinants of childhood wheezing and asthma such as parental asthma and allergy, male gender, socioeconomic, 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 FEV0.5 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 (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 (269). Local production of IL-6 and TNF- α triggering CRP release from the liver and/or IL-1 β -related inflammasome activation in airway macrophages could also contribute to sustained elevation of CRP and systemic low-grade inflammation (270). 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 (264). 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 (271). Maternal stress and inflammation during pregnancy reflected in raised CRP levels have been shown to increase the risk of eczema (272), respiratory infections, and recurrent wheezing in early childhood (273). In addition, elevated CRP during pregnancy is associated with fetal growth restriction (274,275), 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 (276). 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 (277) and changing dietary habits (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 (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 (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 hyper-responsiveness. 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 (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 (30).

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 hyper-responsiveness, 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 (280). This can be done from the lower airways by bronchoscopic microsampling (281) or noninvasive with little discomfort from the nasal mucosa (104), which is known to share both functional and immunological properties with the bronchial mucosa (26). Applying the latter methodology in our novel unselected COPSAC₂₀₁₀ mother-child cohort (238), we recently demonstrated an altered topical immune response in the upper airways of asymptomatic neonates colonized with pathogenic bacteria (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 (283,284). However, new biomarkers are constantly being proposed but unfortunately no noninvasive easily interpretable clinical biomarker has yet been discovered to assess the nature, progression

or treatment response of childhood asthma and allergy (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 (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 (287), urine (186) and EBC (228) as well as VOC profiling of exhaled breath (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 COPSAC₂₀₀₀ (32) and COPSAC₂₀₁₀ (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 (288). Recent studies pinpoint that chronic low-grade inflammation is a common nominator of virtually all NCDs (29), which is reflected by elevated levels of hs-CRP in highly endemic societies (289) and accompanying the specific disorders like cardiovascular disease (290), diabetes mellitus (291), chronic obstructive pulmonary disease (260), and asthma (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 (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.

LIST OF 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

Crs = Airway compliance
 EBC = Exhaled Breath Condensate
 FEF50 = Forced Expiratory Flow at 50% of
 FeNO = Fractional exhaled Nitric Oxide
 FEV0.5 = 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
 PD15 = The provocative dose causing a 15% drop in PtcO₂
 PtcO₂ = Transcutaneous oxygen saturation
 ppb = Parts per billion
 Rrs = 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-LTC₄/D₄/E₄ = Urinary leukotriene C₄/D₄/E₄
 u-11 β -PGF₂ α = Urinary 11 β -prostaglandin F₂ α
 VmaxFRC = Maximum flow at FRC
 VOC = Volatile organic compounds
 WHO = World Health Organization
 25(OH)-Vitamin D = 25-hydroxyvitamin D

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 antecedent 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 FEV0.5 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.

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