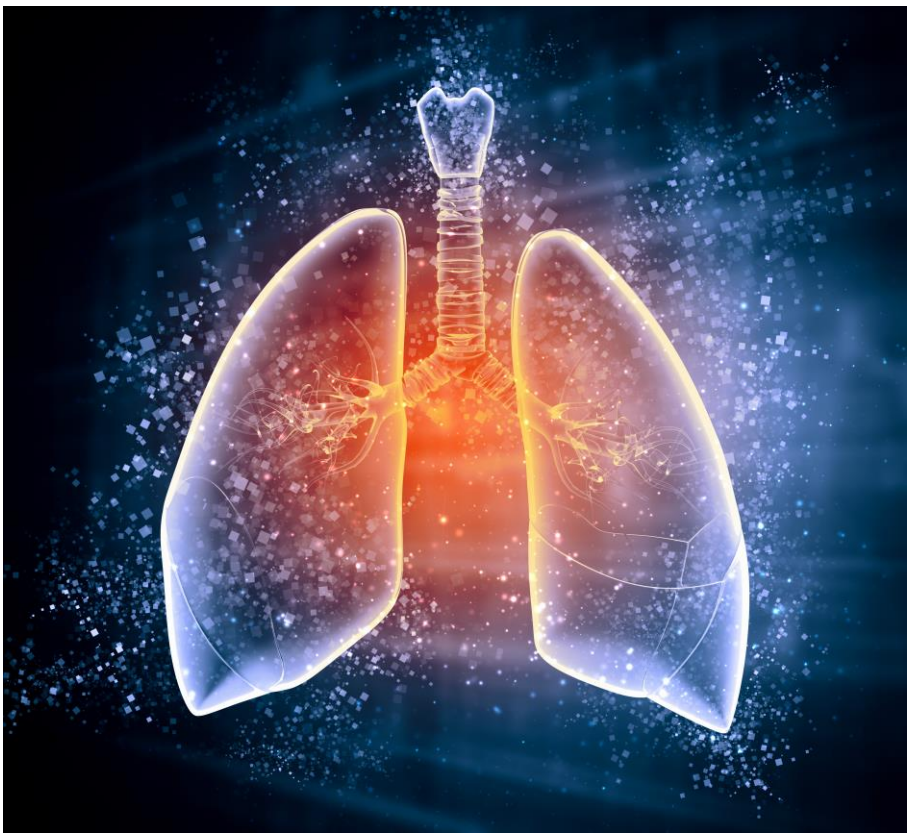
UNIVERSITY OF COPENHAGEN
FACULTY OF HEALTH AND MEDICAL SCIENCES

# Neonatal Airway Immunology: Influence of Early Life Exposures and Prediction of Later Respiratory Health



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This PhD thesis is based on four scientific papers, referred to by their roman numerals:

**Paper I**

**Wolsk, HM**; Chawes, BL; Thorsen, J; Stokholm, J; Bønnelykke, K; Brix, S; Bisgaard, H. Non-invasive sampling of mucosal lining fluid for *in situ* quantification of upper airway immune mediators, in Review in *Journal of Visualized Experiments*

**Paper II**

**Wolsk, HM**; Følsgaard, NV; Birch, S; Brix, S; Hansel, TT; Johnston, SL; Kebadze, T; Chawes, BL; Bønnelykke, K; Bisgaard, H. Picornavirus-induced Airway Mucosa Immune Profile in Asymptomatic Neonates, published in *Journal of Infectious Disease, 2016 Apr 15;213(8):1262-70*

**Paper III**

**Wolsk, HM**; Chawes, BL; Følsgaard, NV; Rasmussen, MA; Brix, S; Bisgaard, H. Siblings Promote a Type 1/Type 17-oriented immune response in the airways of asymptomatic neonates, published in *Allergy, 2016 Jun;71(6):820-8.*

**Paper IV**

**Wolsk, HM**; Rasmussen, MA; Chawes, B; Brix, S; Bisgaard, H: Asymptomatic neonates display an up regulated airway immune profile prior to development of sensitization. *Ready for submission*

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## Acknowledgements

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## Summary and conclusions

From the moment of birth, the airway immune system is exposed to airborne pathogens and other environmental triggers. The failure of the immune system to mediate a balanced immune response in the airways towards such irritants is thought to be fundamental for development of asthma and allergy, which are the most common chronic childhood diseases. Even though it is broadly believed that early life subclinical immune changes precede the clinical manifestation of asthma and allergy, there are few published studies on that topic. In particular, no longitudinal studies have been performed, where the airway topical immune signature in neonates has been combined with outcomes of asthma and allergy later in childhood.

In this thesis, I present a method to assess the unstimulated upper airway immune signature in neonates and explore if early environmental exposures affect the immune signature in healthy neonates and whether such airway immune profile is related to later development of asthma and allergy. My hypotheses were that early exposures, including presence of airway viruses and siblings at birth, have immune programming effects; and that the neonatal immune signature would predict development of allergy and asthma-related diseases in childhood.

In **paper I**, we describe the method, which is the foundation of this thesis. The mucosal lining fluid sampling method has the advantage of being non-invasive and undiluted. This has enabled us to measure *in vivo* and unstimulated levels of cytokines and chemokines longitudinally - from the neonatal period and into childhood.

In **paper II**, we explore the effect of viruses on the immune signature in asymptomatic neonates. We found that asymptomatic neonates with picornavirus in the airways had a topical up-regulation of 20 key immune mediators related to the Type 1, Type 2, Type 17 and regulatory (Treg) immune pathways. This indicates that early exposure to picornavirus may have an early immune programming effect in asymptomatic neonates.

In **paper III**, we explore the effect of siblings on the neonatal immune signature at the time of birth. We observed that the presence of siblings mediated a Type 1/Type 17-related immune-stimulatory effect in the airways of asymptomatic neonates, independent of presence of pathogenic bacteria and viruses. This indicates that presence of siblings in the home exert early immune modulatory effect. Furthermore, we found that the effect was attenuated by the time since previous pregnancies. Hence, this may indicate an *in utero* immune priming of the fetal immune system.

In **paper IV**, we examine the association between the neonatal immune signature and later development of asthma, allergy and lower respiratory tract infections between 0-5 years of age. For allergic sensitization, we found evidence of an airway immune signature characterized by a down regulation of TGF-β1, whereas asthma-related disorders, were characterized by a down-regulation of mediators important for neutrophilic recruitment (CXCL8, IL-1β and TNF-α). Hence, the immunological basis for the subsequent chronic inflammatory component of asthma and allergy seems to originate in pre- and perinatal life.

In conclusion, we observed that neonatal environmental exposures are mirrored in the airway immune profile; and that distinct immune profiles are evident in children with subsequent development of asthma and allergy.

The mucosal lining fluid method, which is the backbone of this thesis, holds promising implications for studying the longitudinal airway immune response through childhood. This will aid our understanding of how changes in the compositions of the airway immune response are associated with, and possibly precede diseases such as asthma and allergy.

## Danish Summary

Fra det øjeblik vi fødes, udsættes luftvejene for patogener og andre luftbårne stoffer. Derfor er evnen til at mobilisere et immunrespons i luftvejene essentielt for overlevelse. Evnen til at mønstre et afbalanceret immunrespons er en af de afgørende brikker i at bibeholde sunde luftveje, hvorimod et immunforsvar der reagerer inadekvat, kan føre til udvikling af de mest almindelige kroniske sygdomme i barndommen, som astma og allergi. Samtidig er det velkendt at tidlige miljømæssige exponeringer lader til at være involveret i udvikling af astma og allergi, men baggrunden herfor er ikke velbelyst. Viden om hvordan miljømæssige eksponeringer påvirker luftvejenes immunrespons i neonatal perioden og sammenhængen med senere udvikling af astma og allergi, er mangelfuld.

I denne afhandling præsenterer jeg en *in vivo* metode til at måle det ustimulerede immunrespons i luftvejene hos raske nyfødte børn fra Copenhagen Prospective Studies of Asthma in $Childhood_{2010}$ ($COPSAC_{2010}$) kohorten. Ydermere udforsker jeg om tidlige eksponeringer påvirker immunresponset hos raske nyfødte børn og om en specific luftvejsimmunprofil er associeret til senere udvikling af astma og allergi. Mine hypoteser er, at tidlige eksponeringer, herunder tilstedeværelse af virus i luftvejene og ældre søskende kan ændre den neonatale immunsignatur; og at en distinkt neonatal immunsignatur forudgår senere udvikling af astma og allergi i barndommen.

I **artikel I**, beskrives metoden, som er grundlaget for denne afhandling. Mucosal lining fluid sampling metoden har den fordel, at den er non-invasiv og ustimuleret. Dette har gjort det muligt for os at kvantificere de fysiologiske *in vivo* niveauer af cytokiner og kemokiner, allerede i den

neonatale periode, hvilket giver et mere retvisende billede af immun signaturen sammenlignet med nasal lavage, som er den mest anvendte metode.

I **artikel II**, undersøges det om forekomsten af luftvejsvirus hos asymptomatiske nyfødte børn har betydning for immunresponset i luftvejene. Vi fandt, at picornavirus i luftvejene hos asymptomatiske børn var associeret med opregulering af cytokiner og chemokiner relateret til type 1, type 2, type 17 og T regulerende (Treg) immunerespons. Dette peger på, at tidlig eksponering for picornavirus kan have en immun programmerende effekt i luftvejene hos asymptomatiske nyfødte børn.

I **artikel III**, undersøges det om tilstedeværelsen af søskende i hjemmet var associeret med en specific immun-profil hos raske nyfødte børn. Tilstedeværelsen af ældre søskende var associeret med en opregulering af Type 1/Type 17-relaterede immunmediatorer i luftvejene hos asymptomatiske nyfødte, uafhængig af tilstedeværelsen af bakterier og picornavirus i luftvejene. Vi konkluderer at forekomsten af søskende påvirker immunsignaturen hos nyfødte børn. Denne påvirkning af immunsignaturen blev svagere med tiden siden sidste graviditet, tydende på at tidligere graviditeter er forbundet med en *in utero* immunstimulerende effekt af det føtale immunforsvar.

I **artikel IV** udforsker vi sammenhængen mellem den neonatale immunprofil og udvikling af allergi og astma relaterede lidelser over en opfølgningsperiode på 5 år. Udvikling af allergi var associeret med en specifik immun-profil karakteriseret ved en nedregulering af TGF-β1, en T celle regulatorisk mediator, hvorimod astma relaterede lidelser var kendetegnet ved en nedregulering af mediatorer vigtige for rekruttering af neutrofile celler (CXCL8, IL-1β og TNF-

α). Dette tyder på at noget af den inflammatoriske komponent af astma og allergi i barndommen stammer fra den præ- og perinatale periode.

I denne afhandling har vi beskrevet at neonatale eksponeringer afspejles i luftvejenes immun-profil; og at distinkte immun profiler ses hos børn med senere udvikling af allergi og astma-relaterede lidelser.

Mucosal lining fluid sampling metoden, som denne afhandling er baseret på, er en metode til at forstå den immunologiske udvikling i luftvejene, fra den neonatale periode og igennem barndommen. Dette vil give os en øget forståelse for de lokale mekanismer i luftvejene der er associeret med, og forudgår sygdomme som astma og allergi, og på sigt øge vores muligheder for at udvikle forebyggende foranstaltninger til disse meget hyppigt forekommende sygdomme.

## Abbreviations

COPSAC – Copenhagen Prospective Studies on Asthma in Childhood

IL – Interleukin

Interferon-gamma – IFN-γ

Tumor necrosis factor-alpha – TNF-α

Transforming growth factor beta1 – TGF-β1

PLS – Partial Least Square

PCA – Principal Component Analysis

PC – Principal Component

GMR – Geometric Mean Ratio

HR – Hazard Ratio

OR – Odds Ratio

CI – Confidence Intervals

sIgE – specific IgE

AR – Allergic Rhinoconjunctivitis

SPT – Skin Prick Test

# 1. **Introduction**

Asthma and allergy are the most common diseases in childhood[1] with great socioeconomic costs due to health care utilization, productivity loss and impact on quality of life[2]. Asthma and allergy development is not fully understood but are thought to arise from a complex interaction between the genetic composition and early pre- and postnatal environmental encounters[3]. This results in a disease-promoting skewing of the immune response, leading to development of chronic airway inflammation[4]. With the increased incidence of asthma and allergy over the last decades[5], it is of utmost importance to define events that lead to asthma and allergic sensitization and identify predictive markers that can distinguish susceptible individuals.

## 1.1 The in utero immune response

The immune system is developed during fetal life[6,7] with a complex network of cells comprising the mucosal immune system of the airways. The newborn child is exposed to potentially harmful substances from the moment of birth and the immediate ability to mount an immune response is crucial for survival. Apart from protection against pathogens such as bacteria and viruses, the mucosal surface of the respiratory tract is an important route of exposure for environmental airborne agents and antigens; and may be important for balancing the immune maturation.
Until recently the newborn child was considered to be sterile, and the immune system to be inactive until the moment of birth, but in recent years, several studies have shown that the child is born with the capacity to mount an immune response; the infants gut is already colonized at the time of birth[8] and a unique microbiome has been found in the placenta, mainly resembling the mature oral microbiome[9]. Since some environmental factors can cross the placenta it may be possible to alter the fetal immune system by dietary interventions. The data on this is limited, but we have previously reported that a randomized trial of Vitamin D intervention in pregnancy was associated with an up-regulated immune response in healthy neonates[10]. This supports the notion of an *in utero* interaction between mother and fetus, which may be important for neonatal airway immune activation and the postnatal protection against invading pathogens. However, the importance of different *in utero* environmental exposures and how these affect the neonatal airway immune system is not well described.
During the neonatal period, the immune response primarily relies on innate and passive immunity[7,11]. Age-dependent changes in the innate immune response has been found to be dependent on which microbes the neonate is exposed to; hence, exposure to a diverse composition of microbes early in life has been found to accelerate the maturation of a Type 1 based response, protective against allergy development later in life[12,13]. Similarly, lack of

exposure to microbes has been associated with an increased risk of allergy, due to a skewing of the immune response towards an overweight of Type 2 related mediators[14].

## 1.2 Development of methods for *in vivo* quantification of proteins from mucosal surfaces

When studying the immune system of the airways, a clear limitation is that most knowledge is derived from quantification of *ex vivo* stimulated peripheral blood and *in vitro* airway models. These methods may not convey a representative picture of the *in vivo* immune system in the airways - the target organ of asthma and allergen exposure. Data is scarce on the immune expression in the airways of neonates and there is a need to develop non-invasive methods to sample tissue lining fluid from the respiratory system already from the neonatal period. The nose is more accessible than the airways and it has been shown that there is a close immunological relationship between the nose and bronchi[15,16]. It is possible to obtain samples of nasal lining fluid and mucosal cells in a relatively non-invasive manner by techniques such as nasal lavage, filter paper adsorption, and nasal brushing and scraping[17].

To sample nasal exudates for airway inflammatory mediators, nasal lavage has been used[18–21] and is often preceded by a nasal challenge test, introducing an allergen in high levels in order to stimulate an inflammatory response[22,23]. There are several problems with this technique, first, it is not easily performed in young children; second, the results are confounded by an unknown dilution factor that might dilute the mediators to below the detection limit[18]; third, because of the dilution factor, the measured immune mediators from the nasal challenge tests are not comparable between individuals, and this limits the usefulness of the nasal lavage technique in a cohort setting. Fourth, introducing an allergen may not necessarily mimic a physiological interaction. In contrast to the nasal challenge test, the nasal filter paper method has the advantage of sampling undiluted nasal secretions and therefore lower levels of mediators can generally be

detected[24]. It is non-invasive and can be applied to children of all ages. Therefore, in order to increase our understanding of these underlying pathophysiological mechanisms *in vivo*, we developed a non-invasive method (Mucosal Lining Fluid filter paper) to assess local airway immune signatures by cytokine/chemokine profiles in 4 week old children.

The aim of **paper I** was to describe the method of nasal mucosal lining fluid sampling by Synthetic Absorptive Matrix and assess whether unstimulated levels of cytokines and chemokines could be detected. We studied the $COPSAC_{2010}$ birth cohort at one month of age, without prior nasal allergen challenge.

### 1.3 Early exposure to respiratory viruses

Viral respiratory tract infections in children are frequent and usually self-limiting. However, in susceptible individuals viral infections and especially infections caused by rhinovirus and respiratory syncytial virus (RSV) are the most common triggers of wheezing and asthma exacerbations[25–27]. In addition to inducing asthmatic symptoms such as wheezing, coughing and breathlessness, viral infections in the first year of life have been associated with later development of asthma[28,29].

Infections requiring hospitalization early in life are often caused by RSV, whereas from around 12 months of age the causal agent is more often rhinovirus[30].

RSV is from the genus Orthopneumovirus and the family of Pneumoviridae[31]; it is the most common pathogen for radiographically diagnosed pneumonia in children in the United States[32] and the most common cause of acute respiratory tract illness in infants[31,33]. RSV has significant impact on mortality in the pediatric population worldwide with an estimated 160.000 children

dying from RSV infections or complications related to RSV infections in 2005[34]. No efficient treatment or prophylaxis is commercially available, although there has been some success with treatment with ribavirin for immune-compromised children[35].

Picornavirus is a part of the picornaviridae family and comprises the genus of enterovirus which includes the species of rhinovirus. Rhinovirus is responsible for one-half of the common cold illnesses worldwide with high health care system utilization and days lost at work. There is no approved antiviral therapy or vaccine for rhinovirus infections. It still remains to be established if the clinical symptoms of a rhinovirus infection is due to direct impact of the virus or if it is caused by the secondary host immune response[36]. A particular immune response has been reported from a rhinovirus infection[37,38]; furthermore, earlier studies have shown that especially infections at an early age with rhinovirus but also RSV may have long term consequences in regards to later asthma development[39–41]. Because of the influences of respiratory viruses on asthma development, it is important to understand the mechanisms and risk factors by which these airway infections cause changes in airway immunology.

Presence of viruses can alter the cytokine response *in vitro*, in blood mononuclear cells[42], but it is not known if airway viruses have immune modulating effects in the airway epithelium, in the asymptomatic state. Since the target organ of respiratory diseases is the airway epithelium and many airway infections are cleared locally, assessment of the *in vivo* activity of the immature immune system of the airway is important. It has been shown that asymptomatic presence of pathogenic airway bacteria has immune stimulatory effects; and that these differ depending on the type of bacteria detected. An up-regulated Type 1 and Type 17-based airway immune profile was observed in neonates colonized with intracellular bacteria, whereas presence of extracellular bacteria was associated with a Type 17-based profile[43].

In **Paper II** we investigate the immature immune signature *in vivo*, in the airway mucosa of asymptomatic neonates in response to presence of common respiratory viruses[44]. We quantified the topical immune response in the airway mucosal lining fluid of one month old asymptomatic healthy neonates[45] from the unselected Copenhagen Prospective Studies of Asthma in $Childhood_{2010}$ ($COPSAC_{2010}$) birth cohort in relation to simultaneous detection of airway viruses.

### 1.4 The hygiene hypothesis and the sibling-effect

The term "sibling-effect" was first proposed based on the British Birth Survey[46] showing that exposure to siblings protected against eczema and hay-fever at age 5[46,47]; in line with this, the "hygiene hypothesis" was first proposed by David Strachan in 1989[14] based on observations that a large household size protected against development of hay fever. This effect was suggested to be mediated through an increased exposure to microbes. Following the publication of the stated "sibling-effect" and "hygiene hypothesis" there have been a number of observational studies showing that the presence of siblings in the household alter the risk of allergy, asthma, and eczema[14,48,49]. A possible mechanism behind these findings could be that the presence of siblings conveys immune modulation through an increased exposure of virus and bacteria in very early childhood thereby inducing a Type 1 related immune response, whereas children exposed to fewer microbes would have a Type 2 dominated immune response. Earlier studies have shown that children living on traditional dairy farms had a decreased risk of asthma and allergy[50] supporting the hypothesis that exposure to microbes is protective towards asthma and allergy development. The impact of environment and genetic susceptibility on the immune response was recently described, showing that traditional farming conveys protection of asthma development primarily through innate immunity when compared to industrialized farming traditions[13]. The

proposed sibling effect could also be caused by *in utero* immune priming related to events induced by previous pregnancies. In line with this, a decrease in cord blood IgE as well as an increase in anti-inflammatory T cell activation has been reported with increasing birth-order[47,51].

In **Paper III** we explore the background for the findings in the "hygiene hypothesis" and the "sibling-effect"[52]. The aim was to study if the presence of siblings at birth was associated with a skewing of the neonatal immune signature in the airways, as early as one month of age. We analysed immune mediators *in vivo* in the nasal mucosal lining fluid in one month old neonates from the unselected Copenhagen Prospective Studies on Asthma in $Childhood_{2010}$ ($COPSAC_{2010}$) birth cohort[43,45] in relation to presence of siblings while adjusting for coexisting airway viruses and bacteria. For multiparous women, we examined a possible *in utero* immune priming effect of siblings by studying the association between mediator levels and time since last childbirth.

## 1.5 The mucosal immune response as early predictor of asthma and allergy development

Allergy and asthma-related diseases are complex multifactorial disorders, with interactions of genetic and environmental factors determining different disease phenotypes. Many of the children have IgE-mediated reactions, but not all children with a positive skin prick test or specific IgE develop clinical symptoms. Hence, the relationship between the symptoms of allergy and positive IgE-sensitization are not fully understood[53,54].

The immunologic mechanism behind the development of allergy and asthma-related diseases, has been described as an early skewing of the immune response towards the production of Type 2 related immune cells[55]. In a Type 2 response, cytokines and chemokines, characterized by their ability to promote airway inflammation, are released, whereas an immune response dominated

by Type 1 and T regulatory immune cells will promote the production of anti-inflammatory cytokines and chemokines.

Early exposures are important for shaping the developing immune system towards health or disease[6], but whether the neonatal airway immune response is predictive of later development of allergy and asthma-related diseases is not established. Using Synthetic Absorptive Matrix measurements we measured the neonatal immune signature *in vivo*. Using the same methodology it was shown that, in the $COPSAC_{2010}$ cohort, children born to a mother with asthma, allergy or eczema, had an early suppression of the immune signature[56].

In **paper IV** we explore if a specific airway immune signature in healthy neonates from the COPSAC2010 cohort precedes the development of allergy and asthma-related diseases.

## 2. Aims and objectives

The aim of this PhD thesis was to perform a thorough description of the mucosal lining fluid method, used for *in vivo* assessment of the neonatal airway immune profile, in relation to early exposures, such as viruses and siblings. Furthermore, we explored if an association existed between the neonatal airway immune signature and respiratory health later in childhood.
Such insight could aid our understanding of the pathogenesis behind asthma and allergy development, and help future efforts for the development of preventive measures.

The specific objectives were:

- To describe the method of mucosal lining fluid sampling.
- To study the influence of airway viruses and siblings on the airway immune signature in neonates.
- To study if a specific airway immune signature in the neonatal airway precedes development of allergy and asthma-related diseases later in childhood.

# 3. Methods

## 3.1 The $COPSAC_{2010}$ birth cohort

The studies are based on the Copenhagen Prospective Studies on Asthma in $Childhood_{2010}$ ($COPSAC_{2010}$) birth cohort. $COPSAC_{2010}$ is an ongoing, prospective, mother and child cohort study of 700 unselected children recruited in Zealand, Denmark, during 2009–2010[57]. Pregnant women were recruited by utilizing a monthly surveillance of reimbursement to general practitioners for the mandatory first pregnancy visit. Exclusion criteria were gestational age above week 26, any cardiac, endocrinological, nephrological or lung disease other than asthma.

The families attended the research unit for 2 planned visits during pregnancy and 11 planned visits from 0 – 5 years of age. A thorough clinical examination was performed at all visits to detect any early manifestations of asthma, allergy and eczema, as defined in section 3.6.
We followed the guidelines for "Good Clinical Practice" for data validation and quality control. The data collected during visits to the clinical research unit was stored in a dedicated online database. This database was double-checked against source data and subsequently locked to ensure data consistency. An audit trail was run routinely.

## 3.2 Ethics

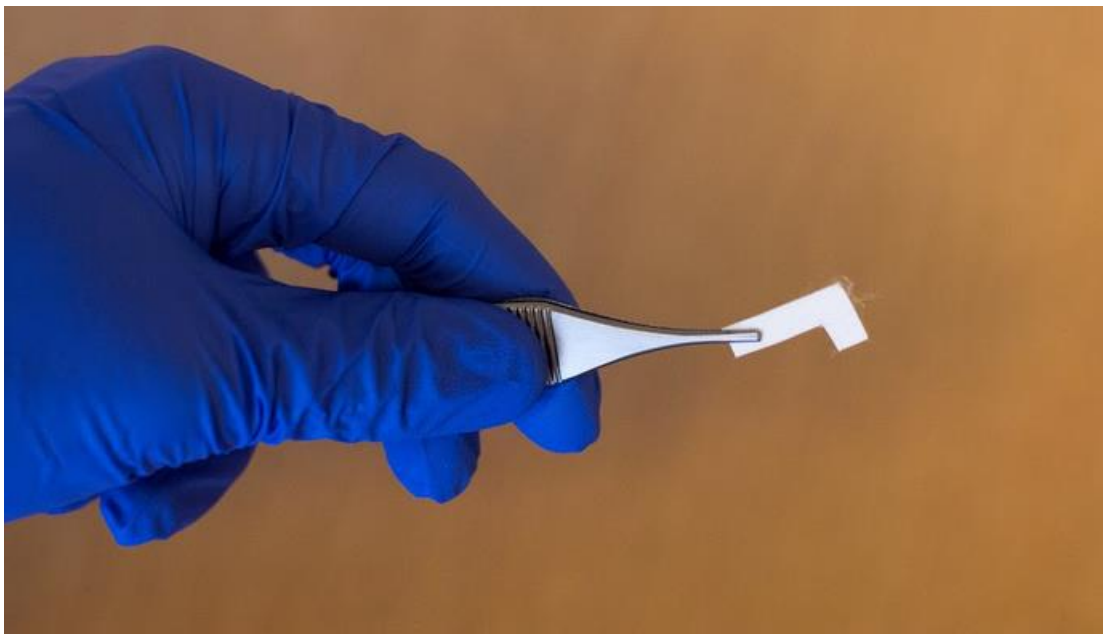
The study was conducted in accordance with the guiding principles of the Declaration of Helsinki. Approval by the Ethics Committee for Copenhagen (H-B-2008–093) and the Danish

Data Protection Agency was achieved. Informed written and oral consent was obtained from both parents before enrollment.

### 3.3 The mucosal lining fluid method

Unstimulated airway mucosal lining fluid was sampled during the visit to the COPSAC clinic at one month of age[43,45]. Strips of filter paper (Accuwik Ultra, fibrous hydroxylatedpolyester sheets, cat no.SPR0730, Pall Life Sciences, Portsmouth, Hampshire, UK) were inserted bilaterally into the anterior part of the inferior turbinate of the nasal cavity. After 2 minutes of adsorption, the filter papers were removed and immediately frozen at -80°C until analysis (**Figure 3.1**).

**Figure 3.1:** Filter paper used for mucosal lining fluid sampling



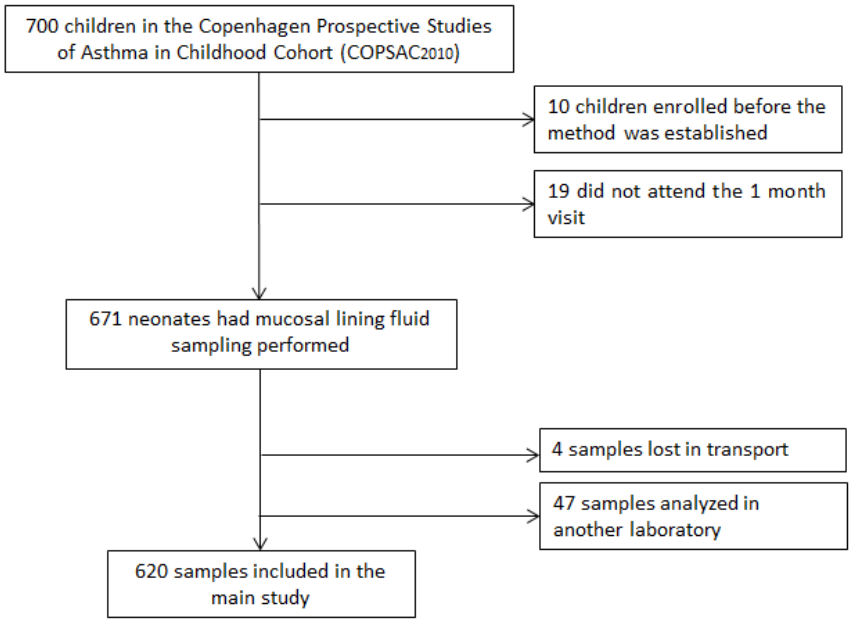
The samples were analysed for IL (interleukin)-12p70, CXCL10/IP-10, Interferon-gamma (IFN-γ), Tumor necrosis factor(TNF)-α, CCL4/MIP-1β, CCL2/MCP-1, CCL13/MCP-4, IL-4, IL-5, IL-13, CCL11/eotaxin-1, CCL26/eotaxin-3, CCL17/TARC, CCL22/MDC, IL-17A, IL-1β, CXCL8/IL-8, Transforming growth factor (TGF)-β1, IL-10, and IL-2[45,56] by MesoScale

Discovery multiplexed array system (MesoScale Discovery, Gaithersburg, MD), a high-sensitivity electrochemiluminescence-based ELISA-type assay[45]. See section 4.3 (**Table 4.1)** for information about detection limits, samples below detection limit, and median (IQR) level for all 20 mediators.

## 3.4 Study design

Complete data on upper airway mucosal immune mediator levels at one month of age was available in 620 (89%) of the 700 children enrolled in the $COPSAC_{2010}$ cohort. Ten neonates were enrolled before the method was established, and 19 did not attend the one month visit. An additional 47 samples were excluded because they were extracted and measured in another laboratory or used in a pilot study, and 4 samples were lost in transportation (See study flow chart, **Figure 3.2**). Baseline characteristics of the cohort are described under the respective studies (section 5.3, **Table 5.1 and 5.2**, and section 6.3, **Table 6.1**).

**Figure 3.2:** Study Flow chart



### 3.5 Nasopharyngeal sampling for virus detection

Nasopharyngeal sampling for airway viruses was obtained at age one month. The sampling was performed after the sampling of mucosal lining fluid. For the first 107 neonates, nasopharyngeal sampling was performed by a nasopharyngeal swab, but due to discomfort, the method of collection was changed to a nasopharyngeal aspirate. The nasopharyngeal swab was performed with "ESWAB flock" swab kit with transport medium (pernasal) SSI No. 65745 (482-CE). It was obtained via one of the nostrils, from the bottom of the nasal cavity at the posterior nasopharynx. The swab was rotated a few times within the nasopharynx, and put directly into the virus transport medium. The nasopharyngeal aspirate was also collected via one nostril from the bottom of nasopharynx posterior; subsequently the aspirate was diluted in 1 ml of virus transport media.

Specimens were frozen and stored at -80°C until shipment to Imperial College, London, UK, for RNA extraction and further analysis with reverse transcriptase polymerase chain reaction (RT-PCR). Following extraction, the RNA was reverse transcribed to produce cDNA representative of all RNA species in the original clinical sample[58]. This cDNA was then used in a panel of PCR assays specific for respiratory syncytial viruses (RSV) A & B[59], influenza A (H1 & H3) & B[60], and picornavirus[61]. Differentiation of rhinoviruses from enteroviruses was achieved by restriction enzyme digestion of the PCR product from all picornavirus positive tests with BglI[61] and subsequent gel-electrophoresis.

### 3.6 Validation of the endpoints

*Skin prick test* was performed at 6 and 18 months of age. A positive test was defined as any skin prick test (SPT) reaction larger than 2 mm (ALK-Abello, Hørsholm, Denmark)

*Specific IgE (sIgE)* was determined at 6 and 18 months of age. A positive sIgE was defined as a concentration of sIgE ≥0.35 kUa/L of one or more the following allergens: milk, egg, dog and/or cat (ImmunoCAP; Thermo Fischer Scientific, Allerød, Denmark)[57]. Children were classified as "non-sensitized" if both SPT and sIgE were negative for all tested allergens.

*Total IgE* level was determined at age 6 months using the ImmunoCAP assay (Phadia AB, Uppsala, Sweden)[62].

*Allergic rhinoconjunctivitis (0-5 years of age)* was diagnosed at the discretion of the COPSAC pediatrician based on significant symptoms during the previous year of 1) sneezing or a runny or congested nose and/or 2) red, swollen or watery eyes in periods when the child did not have a cold or flu, as previously described[45,63,64].

*Asthma/persistent wheeze (0-5 years of age)* was diagnosed based on a detailed daily diary of symptoms observed by the parent. The previously validated diagnostic algorithm required all of the following criteria: (1) five episodes of troublesome lung symptoms within 6 months, each lasting at least 3 consecutive days. Troublesome lung symptoms was defined as symptoms of wheeze or whistling sounds, breathlessness, or a persistent cough severely affecting the well-being of the child[65,66]; (2) symptoms typical of asthma including exercise-induced symptoms, prolonged nocturnal cough, and/or persistent cough outside periods with common cold; (3) need for intermittent rescue use of inhaled β2-agonist; and (4) clinical improvement following a 3 month course of inhaled corticosteroids and relapse upon cessation of treatment[65,67].

*Wheeze exacerbation* was defined as either: 1) treatment with inhaled β2-agonist in a pediatric admission ward or during hospitalization, and/or 2) treatment with oral or high-dose inhaled corticosteroid prescribed by a general practitioner or the COPSAC clinical research unit.

*Lower respiratory tract infection (LRTI)* was defined as a diagnosis of pneumonia or bronchiolitis from age 0 to 3 years. Pneumonia was diagnosed when a persistent cough severely

affecting the well-being of the child was accompanied by tachypnea, fever, and abnormal auscultation, whereas bronchiolitis was defined as cough, tachypnea, chest retractions, and auscultation with widespread crepitation and/or rhonchi in a child below 1 year of age[68–70].

### 3.7 Specific methods of the papers

Additional specific methods used in this thesis are described in details together with the studies.

## 4. Non-invasive sampling of mucosal lining fluid for *in situ* quantification of upper airway immune mediators

Helene M Wolsk, Bo L Chawes, Jonathan Thorsen, Jakob Stokholm, Klaus Bønnelykke, Susanne Brix, Hans Bisgaard. In review in *Journal of Visualized Experiments.*

## 4.1 Introduction

In this paper we describe the use of a novel non-invasive method for sampling mucosal lining fluid from the nasal cavity[71].

The method can be used for obtaining *in situ* immune signatures from the airway of healthy and diseased individuals. We first implemented the technique in the Copenhagen Prospective Studies on Asthma in Childhood$_{2000}$ (COPSAC$_{2000}$) cohort to determine the airway immune profile in 7-year old children with allergic rhinitis and healthy controls[43,45]. Subsequently, we have applied this technique to the COPSAC$_{2010}$ cohort and assessed changes in the airway immune profile from one month of age, through 2 and 6 years [10,43,44,52,56,71,72].

## 4.2 Methods

*Experimental setup for mucosal lining fluid sampling:*

Sheets of filter paper (fibrous hydroxylatedpolyester sheets from Accuwik Ultra [cat no. SPR0730, Pall Life Sciences, Portsmouth, Hampshire, UK])[43,45] were cut into 3 x 15 mm pieces for the one month old children, and into an L-shape for all latter sample times (size 5 x 20 x 10 mm [short arm of the L]) (**Figure 3.1**).

One filter paper was inserted in each nostril, placed at the anterior part of the inferior turbinate, using the long arm of the L-shaped filter paper. A nose clip was applied to minimize the discomfort and avoid accidental rejection of the filter paper. After 2 minutes of adsorption the filter papers were removed, placed in Eppendorf tubes and immediately frozen at -80°C until further analysis. We recorded if the child had symptoms of an airway infection on the day of sampling, and we recorded if there were any adverse events (sneezing, persistent crying or epistaxis during sampling)[45,71].

*Measurements of airway cytokines and chemokines*

After thawing on ice, ten random samples were chosen and immersed in 300 µL freshly prepared Milliplex Assay Buffer (Millipore, Cat no. L-AB, Billerica, MA, USA) containing 1 complete Protease Inhibitor Tablet (Roche) per 25 mL buffer. The volume of buffer was adjusted according to the size of the filter paper; 300 µL buffer was used for filter papers with the size of 3 x 15 mm and if only one filter paper was available, we used half the buffer volume (150 µL).

The moist filter papers and assay buffer were transferred into a cup of a cellulose acetate tube filter (0.22 µm pore size), within an Eppendorf tube (Spin-X Centrifuge Tube Filter, Cat no. CLS8161, Sigma-Aldrich, St Louis, MO, USA). They were centrifuged for five minutes in a cooled centrifuge at 16,000 *g*. The supernatant was then transferred into Eppendorf tubes on ice, and on to low-protein binding storage plates (cat no. 249944, Thermo Scientific, Rochester, NY, USA). Concentrations of cytokines and chemokines in supernatants were determined by use of the high-sensitivity electrochemiluminescence-based MesoScale Discovery multiplexed array system (Human 10-plex $T_H1/T_H2$ cytokine assay and 9-plex chemokine assay, and singleplex IL-17A, TGF-β1and TSLP). The levels of IL-1β, IL-2, IL-4, IL-5, IL-10, IL-12p70, IL-13, IL-17A, IFN-γ, TNF-α, CXCL8/IL-8, TGF-β1, CCL11, CCL26, CXCL10, CCL2, CCL13, CCL22, CCL4, CCL17 and TSLP in the eluates were quantitated. Assays were conducted according to standard manufacturer's protocols except for incubation with eluates overnight at 4°C. Samples were read with the Sector Imager 6000. The MesoScale Discovery immunoassays typically have a high dynamic measurement range for concentrations ranging between 1 and 10,000 pg/mL, but for some assays it even expands to 100,000 pg/mL. This means that samples can be run at the same dilution, hence limiting the influence from dissimilar dilution of healthy and diseased subjects, as is the case with other similar immunoassays. The lower limit of determination for all cytokines were 1 pg/mL or less, and for chemokines the measuring range was between 1 and 50 pg/mL (**Table 4.1**). TSLP

was not detectable in 98% of samples at 1 month of age and has been excluded[71].

*Statistics*

Mediator values with a level between zero and the lower determination limit were not changed; whereas samples with zero values were set to half of the lowest concentration detected in any sample of the particular mediator, in order to be able to perform a log-transformation, without loss of informative low range concentration data. Due to collection of only one filter paper, a few samples contained missing data; we did not include samples with missing data in the data analysis.

We performed the laboratory analysis in two batches, and corrected the cytokine and chemokine data for batch, by batch-wise centering on log-transformed data. The data was summarized by calculating the median and interquartile range (IQR) for each mediator. A heat map was constructed to study the overall correlation between the 20 immune mediators. The immune mediator levels were log-transformed prior to this, in order to obtain normally distributed residuals.

Analyses were carried out using R version 3.3.2 and the package "pheatmap".

## 4.3 Main results

*Baseline characteristics of the airway immune profiles*

Complete data on upper airway mucosal immune mediator levels at age one month was obtained in 620 (89%) of the 700 children enrolled in the $COPSAC_{2010}$ cohort (**Figure 3.2**).

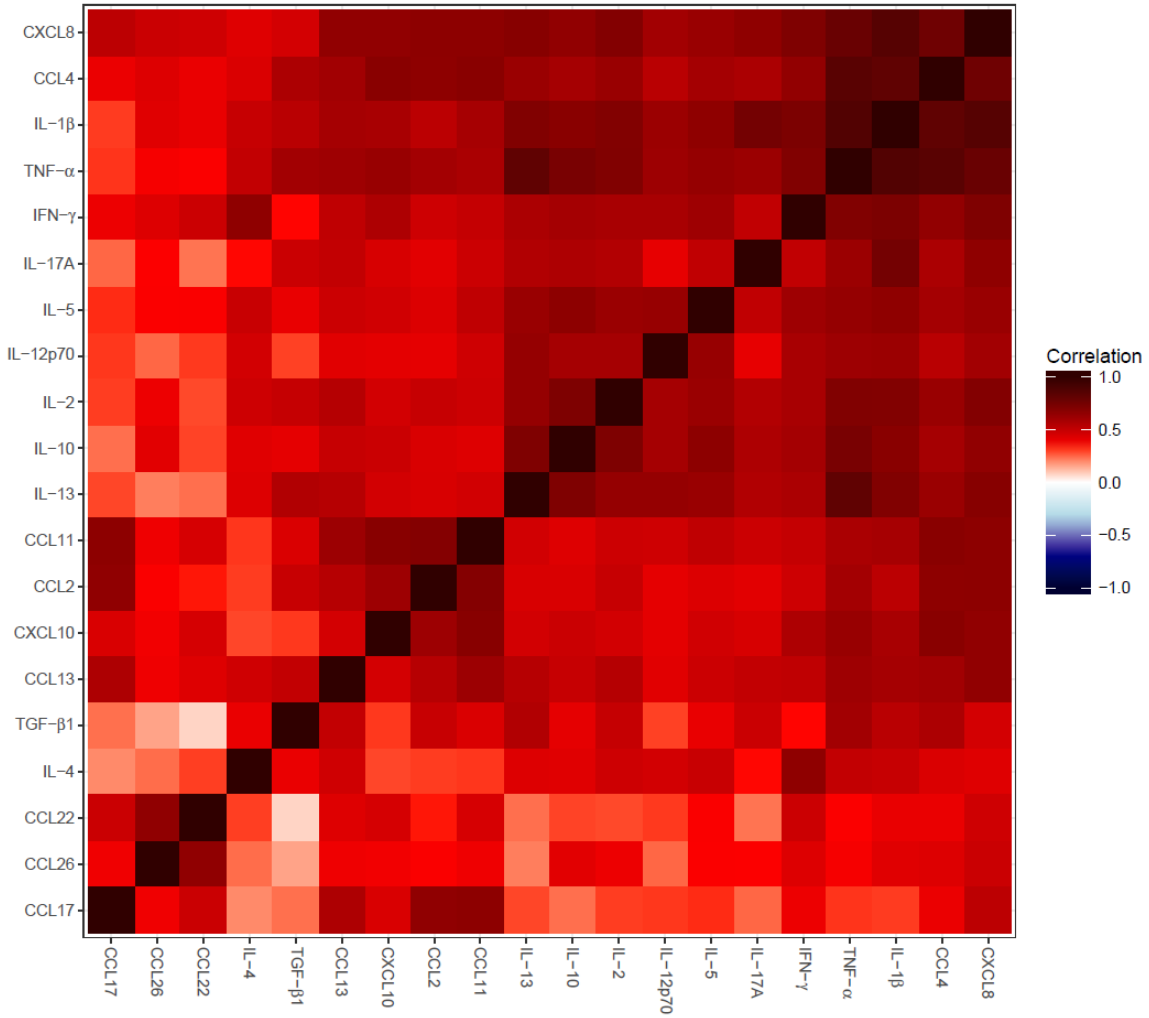
The median (IQR) number of samples below detection limit (i.e. from 0 to detection limit) for each mediator was: 29 (7.25 – 119.5). IFN-γ and IL-17A levels were below the lower detection limit in almost half (46%) of the samples, whereas CXCL8 and IL-1β levels were detectable in all samples and TGF-β1, CCL4 and TNF-α levels were below the lower detection limit in <1% of the samples(**Table 4.1**).

**Table 4.1:** Mucosal lining fluid samples from one month old neonates from the $COPSAC_{2010}$ cohort.

| Mediator | Detection limit pg/ml | Samples below detection limit N (%) | Median pg/ml | IQR pg/ml |
|---|---|---|---|---|
| IL-12p70 | 1.4 | 102 (16%) | 10.80 | 4.99 - 22.22 |
| CXCL10 | 31 | 8 (1%) | 1787.78 | 722.8 - 5423 |
| IFN-γ | 4.1 | 285 (46%) | 20.14 | 7.63 - 48.51 |
| TNF-α | 0.6 | 5 (<1%) | 28.33 | 10.06 - 88.55 |
| CCL4 | 4.3 | 4 (<1%) | 194.90 | 71.22 - 655.8 |
| CCL2 | 16.0 | 9 (1%) | 256.11 | 134.4 - 468.5 |
| CCL13 | 2.8 | 13 (2%) | 15.17 | 10.35 - 22.80 |
| IL-4 | 1.1 | 196 (32%) | 1.37 | 0.42 - 2.86 |
| IL-5 | 1.2 | 172 (28%) | 3.72 | 1.42 - 8.10 |
| IL-13 | 2.7 | 48 (8%) | 14.49 | 6.84 - 27.51 |
| CCL11 | 22.0 | 39 (6%) | 139.54 | 85.00 – 236.0 |
| CCL26 | 45.0 | 252 (41%) | 164.93 | 56.33 - 350.02 |
| CCL17 | 7.2 | 90 (15%) | 41.30 | 26.83 - 60.68 |
| CCL22 | 25.0 | 78 (13%) | 398.31 | 243.4 - 575.7 |
| IL-1 | 0.4 | 0 (0%) | 225.49 | 50.07 - 1169 |
| IL-17A | 1.5 | 285 (46%) | 1.480 | 0.43 - 4.79 |
| CXCL8 | 3 | 0 (0%) | 19465 | 6321 - 38274 |
| TGF-β1 | 5.2 | 1 (<1%) | 28.66 | 20.76 - 39.50 |
| IL-10 | 1 | 13 (2%) | 22.32 | 9.28 - 49.16 |
| IL-2 | 1.5 | 19 (3%) | 18.72 | 8.29 - 37.33 |

A strong inter-correlation was found for all 20 immune mediators, as is visualized in a heat map illustrating that all the mediators were positively correlated (**Figure 4.1**).

**Figure 4.1:** Heat map of the correlation between the 20 cytokines and chemokines. A darker red color represents a stronger positive correlation and a blue color represents a negative correlation (none seen).




## 4.4 Discussion

*Principle findings*

With the mucosal lining fluid sampling method we have been able to determine the physiological unstimulated upper airway mucosal immune profile in children from as early as one month of age; something which has not been possible in this age group before.

This method has yielded results that associate the presence of specific airway bacteria as well as other pre- and perinatal exposures with changes in the airway immune profile of neonates[10,43,56]. The method was also utilized to study the effect of a high-dose vitamin D intervention in

pregnant women, in relation to the airway immune profile and possible effects on asthma in the offspring; thereby underlining the value of the mucosal lining fluid method as a non-invasive sampling technique for subsequent *in situ* quantification of airway immune mediators.

*Other studies:*

The unstimulated airway immune profile in neonates has not been described before. Earlier studies of the airway immune response have mainly used the nasal challenge test, where an allergen is introduced and an unknown dilution factor is present[18,22,23]. Since the nasal allergen test is not applicable in young children and not comparable between individuals, the baseline airway immune response has not previously been reported.

The systemic immune response in neonates has been thoroughly described and earlier *ex vivo* studies have found that early exposures are imprinted in the neonatal immune response; studies of cord blood cells have shown that atopic heredity is important for the cord blood immune response[73,74] and vitamin D supplementation promoted a balanced immune response in the offspring[75]. Furthermore, reduced level of IL-13 and IFN-γ in cord blood was found in children with later wheeze[42,76]. These studies support that cytokine levels at birth are an important marker for later disease, but it should be noted that the results from studies of systemic mediators are not easily compared, due to different stimulation regimens being used, problems harvesting cells and differences in laboratory practices etc.

*Meaning of the study:*

This method enables longitudinal assessment of the airway immune response onwards through childhood, which will help our understanding of the interaction between different exposures in pre- and perinatal life with changes in the immune response. Until now, we have analysed the airway immune signature sampled at one month of age. We found a strong inter-correlation of the 20 measured immune mediators at age one month, despite the fact that we measured different

pathways of the immune system. The reason for this finding is not fully understood, but tells us that the airway immune response seems to act in concert and when certain stimuli are encountered a general increase or suppression of the immune signature is evident.

We found varying numbers of mediator levels below the determination range. Despite being below determination range, we still used the measured levels in our analyses, as we assessed that omission of these data would affect the precision of the geometric mean ratio estimates even more.

*Strengths and limitations:*

The described method has the advantage of being non-invasive and easy to perform. This enables repeated sampling of the mucosal airway immune signature from the neonatal period and onwards. The sampling technique is performed in an *in situ* unstimulated manner, thereby enabling us to quantify the physiological *in vivo* concentrations of biomarkers that would have been impossible with other methods such as the nasal lavage technique, which introduces an unknown dilution factor[24]. The Accuwik Ultra Medium system was used for obtaining the mucosal lining fluid. This is a commercially available synthetic, fibrous, hydroxylated polyester medium, designed for sample collection, storage, and conjugate release. The filter paper is highly adsorbent, and has been modified to enhance fluid wettability.

In the $COPSAC_{2010}$ study, we only studied a panel of 21 cytokines and chemokines which is a limitation, and we were not able to get detectable level of the immune mediator TSLP, at one month of age. In theory, the Mesoscale Discovery multiplex immunoassay platform can measure all types of mediators for which a pair of monoclonal antibodies can be obtained for development of an immunoassay. The platform has high precision, with little sensitivity for pH and temperature variations or variations in viscosity of the fluids to be analysed. Standard ELISA

solutions are not applicable to quantitate the protein eluates because of the sensitivity of the viscous matrices.

We measured the airway mediators with targeted quantitative high-sensitivity immunoassays, alternatively, targeted or untargeted nuclear magnetic resonance (NMR) spectroscopy, or liquid chromatography–mass spectrometry (LC-MS) based metabolomics techniques could be applied. The LC-MS approach has already been implemented to some extent with the exhaled breath condensate method, however, in contrast to the mucosal lining fluid method, some methodological difficulties remains, such as sampling of biofluid[71,77].

### 4.5 Conclusion and perspectives

The described method has the advantage of being non-invasive and easy to perform. This enables repeated sampling of the mucosal airway immune signature from the neonatal period and onwards. The method has the potential to help us understand the longitudinal interaction between heredity, environmental exposures, and the underlying immunological factors preceding or acting concomitantly with airway mediated diseases such as asthma and allergy. This may enable development of novel therapeutics in a personalized manner, where specific disease endotypes can be targeted.

*Future studies:*

Since mucosal lining fluid sampling is applicable for all surfaces of the body there are many possible applications for this method. For example, it could be applied to bronchoscopic microsampling for assessment of lower airway immunology. Bronchoscopic microsampling collects local bronchial epithelial lining fluid by a sheathed polyester fibre probe[78]. Bronchoscopic microsampling has been used in acute respiratory distress syndrome[78–80] and

chronic obstructive pulmonary disease[81]. In asthma, the probe could cause bleeding due to disease-induced epithelial fragility and increased vascularization; this problem could be solved by applying the mucosal lining fluid method to the bronchoscopic microsampling. This has the potential to increase our understanding of the pathophysiology, monitoring, treatment, and outcome of several acute and chronic pulmonary diseases in both children and adults.

In future studies, expansion of the panel of immune mediators would provide an even more detailed perspective on the immune processes at any given body site. This could be measurement of a broad range of mediators such as antimicrobial peptides, total and specific IgA; acute phase reactants such as C-reactive protein; lipid mediators such as prostaglandins, and neurological mediators such as brain-derived neurotrophic factor (BDNF).

With the longitudinal data from the mucosal lining fluid method, detailed insight into the developing immune system could be gained; combining these data with our extensive phenotyping of the $COPSAC_{2010}$ cohort could provide us with a unique overview of the developing airway immune response in health and disease.

## 5. Picornavirus-induced airway mucosa immune profile in asymptomatic neonates

Helene M Wolsk, Nilofar V Følsgaard, Sune Birch, Susanne Brix, Trevor T Hansel, Sebastian L Johnston, Tatiana Kebadze, Bo L Chawes, Klaus Bønnelykke, Hans Bisgaard. Published in *Journal of Infectious Diseases, 2016 Apr 15;213(8):1262-70*

## 5.1 Introduction

Following testing of the applicability and feasibility of the mucosal lining fluid sampling method in paper I, we next investigated if airway viruses was modulated the airway immune signature of asymptomatic neonates.

The presence of viruses is well-known to alter the cytokine response in *ex vivo* studies of blood mononuclear cells[37,42]. Furthermore, we have shown that bacterial colonization alters the airway immune response in asymptomatic neonates using the mucosal lining fluid sampling method[43]; but it is not known if the presence of airway viruses in asymptomatic healthy neonates also triggers a topical immune response.

The aim of this study was to investigate the neonatal airway immune signature *in vivo* in response to presence of common respiratory viruses. Assessment of the airway immune signature was performed in one month old asymptomatic healthy neonates from the unselected Copenhagen Prospective Studies of Asthma in Childhood$_{2010}$ (COPSAC$_{2010}$) birth cohort and related to simultaneous detection of common airway viruses.

## 5.2 Methods

*The COPSAC$_{2010}$ birth cohort*

The COPSAC$_{2010}$ birth cohort is an ongoing, prospective, clinical mother-child cohort study of 700 unselected children recruited in Zealand, Denmark, during 2009–2010[57]. See section 3.1 for more details.

*Measurements of airway cytokines and chemokines*

Sampling of mucosal lining fluid is described in details in section 3.2. Briefly, un-stimulated airway mucosal lining fluid was sampled at one month of age with 3 x 15-mm strips of filter-paper[43,45] and analysed for levels of IL-12p70, CXCL10, Interferon-gamma (IFN-γ), Tumor

necrosis factor-alpha (TNF-α), CCL4 (MIP-1β), CCL2 (MCP-1), CCL13 (MCP-4), IL-4, IL-5, IL-13, CCL11 (eotaxin-1), CCL26 (eotaxin-3), CCL17 (TARC), CCL22 (MDC), IL-17A, IL-1β, CXCL8/IL-8, Transforming growth factor-β1 (TGF-β1), IL-10, and IL-2[45,56].

*Detection of Airway Viruses in Nasopharyngeal Aspirates*

Nasopharyngeal sampling is described in section 3.5. This was performed after the mucosal lining fluid sampling. The samples were analysed at Imperial College, London, UK, by PCR for respiratory syncytial viruses (RSV) A & B[59], influenza A (H1 & H3) & B[60] and picornavirus[61].

*Statistics*

Mediator values with a level between zero and the lower determination limit were not changed; whereas samples with zero values were set to half of the lowest concentration detected in any sample of the particular mediator, in order to be able to perform a log-transformation, without loss of informative low range concentration data.

Data was log transformed prior to analyses in order to obtain normally distributed residuals of the mediator levels.

Differences in the baseline characteristics of children with picornavirus and no picornavirus were analysed using a chi-squared test or Fisher's exact test.

An analysis of variance (ANOVA) was used for the univariate associations between mediator levels and presence of any of the respiratory viruses; the outcome variable was the transformed mediator levels and the explanatory variables were presence of respiratory viruses as well as possible confounders. Results were reported as geometric mean ratios (GMR) of the mean mediator levels, with 95% confidence intervals (CI), for neonates with a virus detected versus no virus, as well as for neonates with and without pathogenic bacteria detected.

Partial Least Squares Discriminant Analysis (PLS-DA) was employed in order to unravel the cytokine to cytokine covariance structure relevant for discriminating the children with-, and without picornavirus. PLS regression was used to investigate the difference in patterns of mediator levels associated with virus. First, mediator variables were imputed using probabilistic principal component analysis. The first latent PLS component was tested for any association with the viruses detected, using permutation test adjusted for the identified covariates and an ANOVA with viruses and covariates as explanatory variables and the first latent component as outcome[44].

Analyses were carried out using SAS version 9.3 (SAS Institute, Cary, NC, USA) and MATLAB R2013a v. 8.1.0.604 (MathWorks Inc, Natick, MA, USA).

### 5.3 Main results

*Baseline*

Complete information about mediator levels was available for 620 neonates (**Flowchart, Figure 3.1**). Additionally, 5 children were excluded as they did not have information on airway viruses and 44 children were excluded due to an airway infection on the day of sampling leaving 571 (82%) of the neonates in the cohort.

In a drop-out analysis of baseline characteristics between the 571 included children and the 129 excluded children, we found that the two groups were identical except for a higher household income ($p = 0.02$) and a lower gestational age ($p = 0.004$) among the excluded children(**Table 5.1**)[44].

**Table 5.1:** Drop-out table of baseline characteristics (571 included children vs 129 children excluded). P-values < 0.05 are shown in bold.

| | Included % (n=571) | Excluded % (n = 129) | p-value |
|---|---|---|---|
| History of maternal asthma, allergy, or eczema | 53 (303) | 54 (69) | 0.91 |
| Caucasian | 96 (548) | 94 (121) | 0.28 |
| High income (above 130.000 euro) | 13 (77) | 22 (28) | **0.02** |
| Maternal consumption of antibiotics in 3rd trimester | 27 (35) | 20 (112) | 0.06 |
| Maternal smoking in 3rd trimester* | 4 (21) | 3 (4) | 1.00 |
| Maternal alcohol consumption (> 1 u/week in 3rd trimester) | 5 (26) | 6 (8) | 0.42 |
| Low gestational age (< 37 weeks) | 3 (20) | 8 (12) | **0.004** |
| Male | 51 (292) | 52 (68) | 0.75 |
| Low apgar score (< 7 at 1 min.) | 4 (25) | 5 (7) | 0.61 |
| Caesarian section | 21 (122) | 22 (29) | 0.78 |
| Older siblings | 57 (324) | 56 (72) | 0.85 |
| Exclusively breastfeeding at one month of age | 92 (525) | 95 (109) | 0.37 |
| Furred pets in the home | 21 (118) | 19 (25) | 0.74 |

*Fisher's exact test

A total of 12% (n = 68) of the 571 included neonates had a positive sample for any of the airway viruses examined. Of these 68 samples, 85% (n=58) were picornavirus, 4% (n=3) RSV, and 10% (n=7) any influenza virus. No children had more than one virus detected. Of the 58 samples with picornavirus, 81% (n=47) were rhinovirus and 14% (n=8) were "other picornavirus", in the remaining 5% (n=3) no further classification was possible. We restricted the analyses to looking at picornavirus, since few samples were positive for influenza virus and RSV[44].

Baseline characteristics of the included children are depicted in **Table 5.2**. Sampling was performed at age 32 days (Standard deviation 5.4 days).

**Table 5.2**: Baseline characteristics of all children included, grouped according to presence of picornavirus or controls. P-values ≤ 0.05 are shown in bold.

| | Picornavirus % (n=58) | Controls % (n = 513) | p-value |
|---|---|---|---|
| History of maternal asthma, allergy, or eczema | 47 (27) | 54 (276) | 0.34 |
| Caucasian | 98 (57) | 96 (491) | 0.35 |
| High income (above 130.000 euro) | 12 (7) | 14 (70) | 0.74 |
| Maternal consumption of antibiotics in 3rd trimester | 29 (17) | 19 (95) | **0.05** |
| Maternal smoking in 3rd trimester* | 9 (5) | 3 (16) | **0.05** |
| Maternal alcohol consumption (> 1 u/week in 3rd trimester) | 7 (4) | 4 (22) | 0.35 |
| Low gestational age (< 37 weeks) | 2 (1) | 4 (19) | 0.44 |
| Male | 59 (34) | 50 (258) | 0.23 |
| Low apgar score (< 7 at 1 min) | 7 (4) | 4 (21) | 0.32 |
| Caesarian section | 21 (12) | 21 (110) | 0.89 |
| Older siblings | 83 (48) | 54 (276) | **<0.0001** |
| Exclusively breastfeeding at one month of age | 91 (53) | 93 (472) | 0.75 |
| Furred pets in the home | 17 (10) | 21 (108) | 0.50 |

*Fisher's exact test

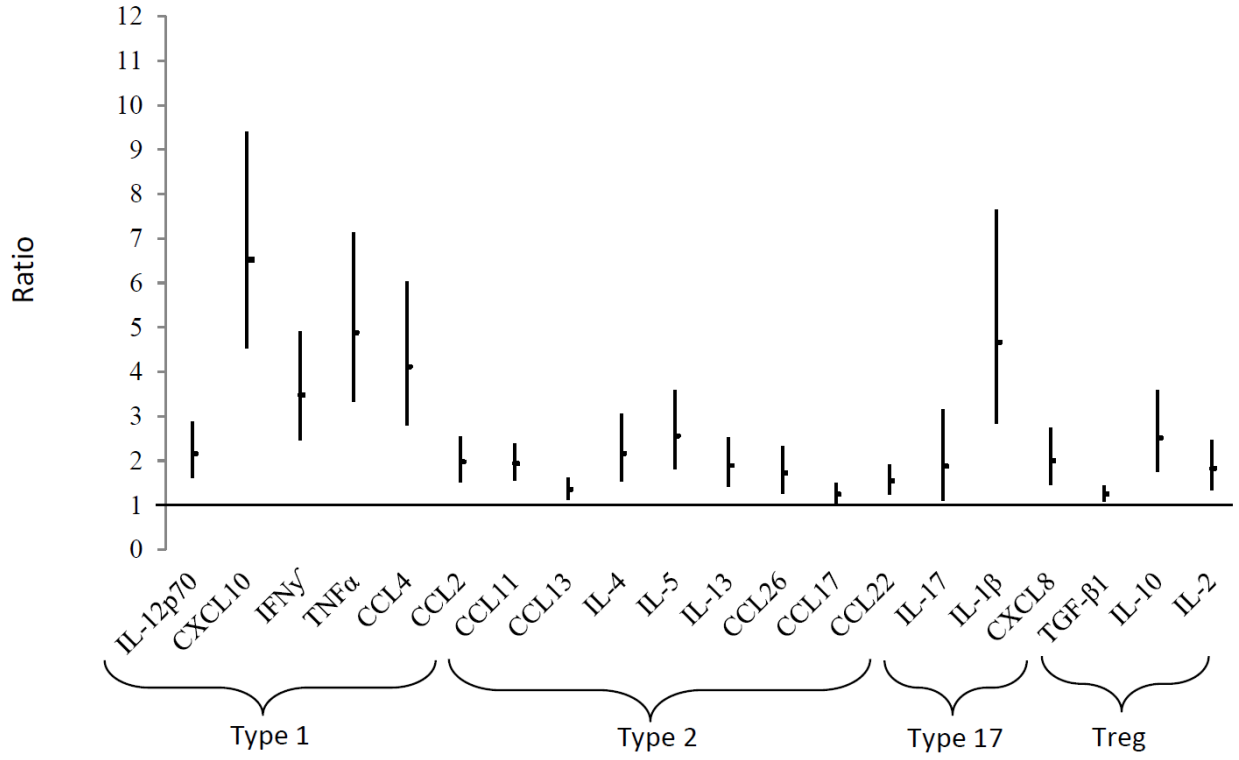
The variables significantly associated with having picornavirus were further tested in a multivariable backward selection analysis for association with the immune mediator levels. Older siblings and maternal smoking in the 3rd trimester associated with the level of immune mediators and were included as covariates in the final models. Using backward selection with the first principal component as the response variable, sampling site, sampling season, method of virus sampling, and batch of immune mediator analysis were also found to affect the immune mediator level and were included as covariates in the models. Based on our previous studies[42,82], a maternal history of asthma, allergy, or eczema and detection of any of the pathogenic airway bacteria *S. pneumoniae*, *H. influenzae* or *M. catarrhalis* were also included as forced covariates in all statistical models[44].

*Effect of Picornavirus on Airway Immune Mediator Profiles*

We found a uniform up-regulation of all mediator levels in neonates with picornavirus compared to neonates without picornavirus, adjusted for all confounders (maternal history of asthma, allergy, or eczema, older siblings in the home, method of virus sampling, sampling site, season

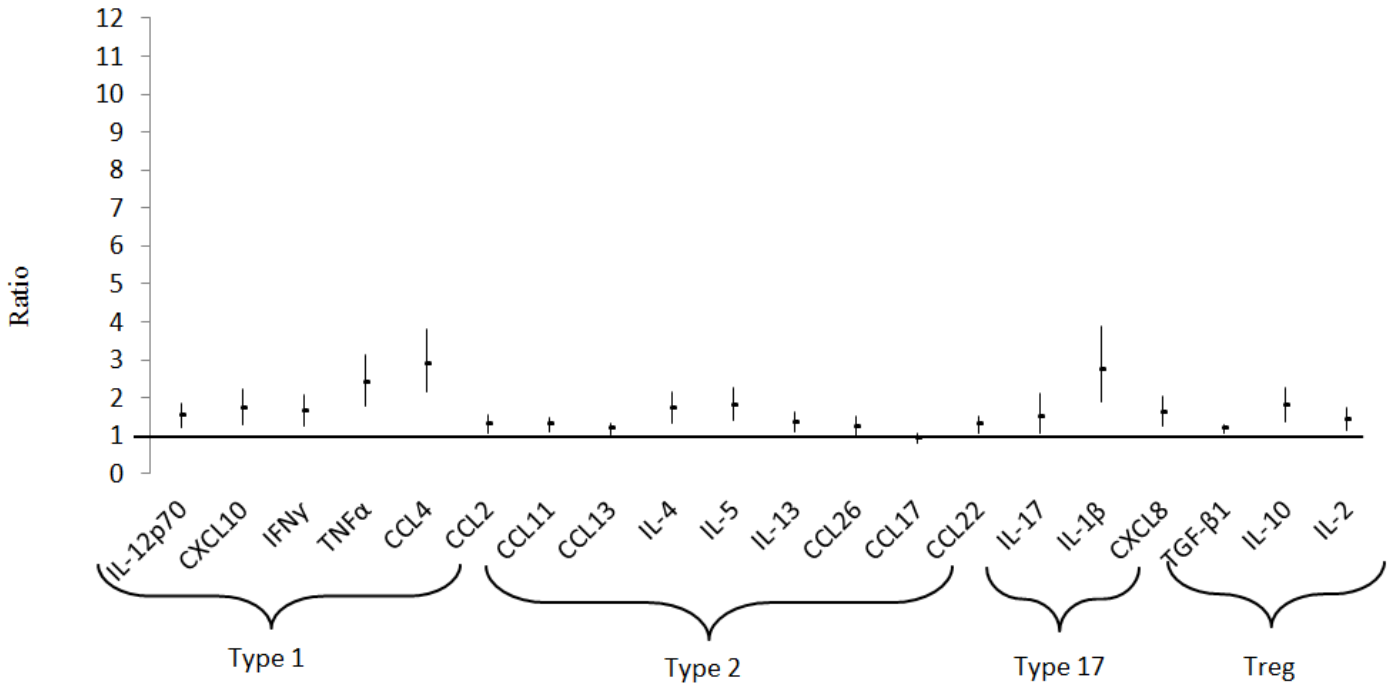
of sampling, batch of immune mediator analyses, maternal consumption of antibiotics in 3rd trimester, and picornavirus), with significantly elevated geometric mean ratios (GMR) for 19 out of 20 mediators; this was especially seen for the Type 1 related mediators (CXLC10, CCL4 and TNF-α), as well as IL-1β involved in an expansion of Th17 cells when produced by dendritic cells (**Figure 5.1**).

**Figure 5.1**: Adjusted geometric mean ratios with 95% confidence interval of cytokines and chemokines in the airway mucosal lining fluid of asymptomatic one month old neonates with picornavirus detected in the airway versus controls.



For neonates with bacterial airway colonization we found an overall elevated geometric mean ratio of the measured mediators, although the upregulation was quantitatively smaller than the effect of picornavirus (**Figure 5.2**). Detection of both picornavirus and bacterial colonization resulted in an additively increased level of all the immune mediators, with no interaction between bacteria and picornavirus (p = 0.91).

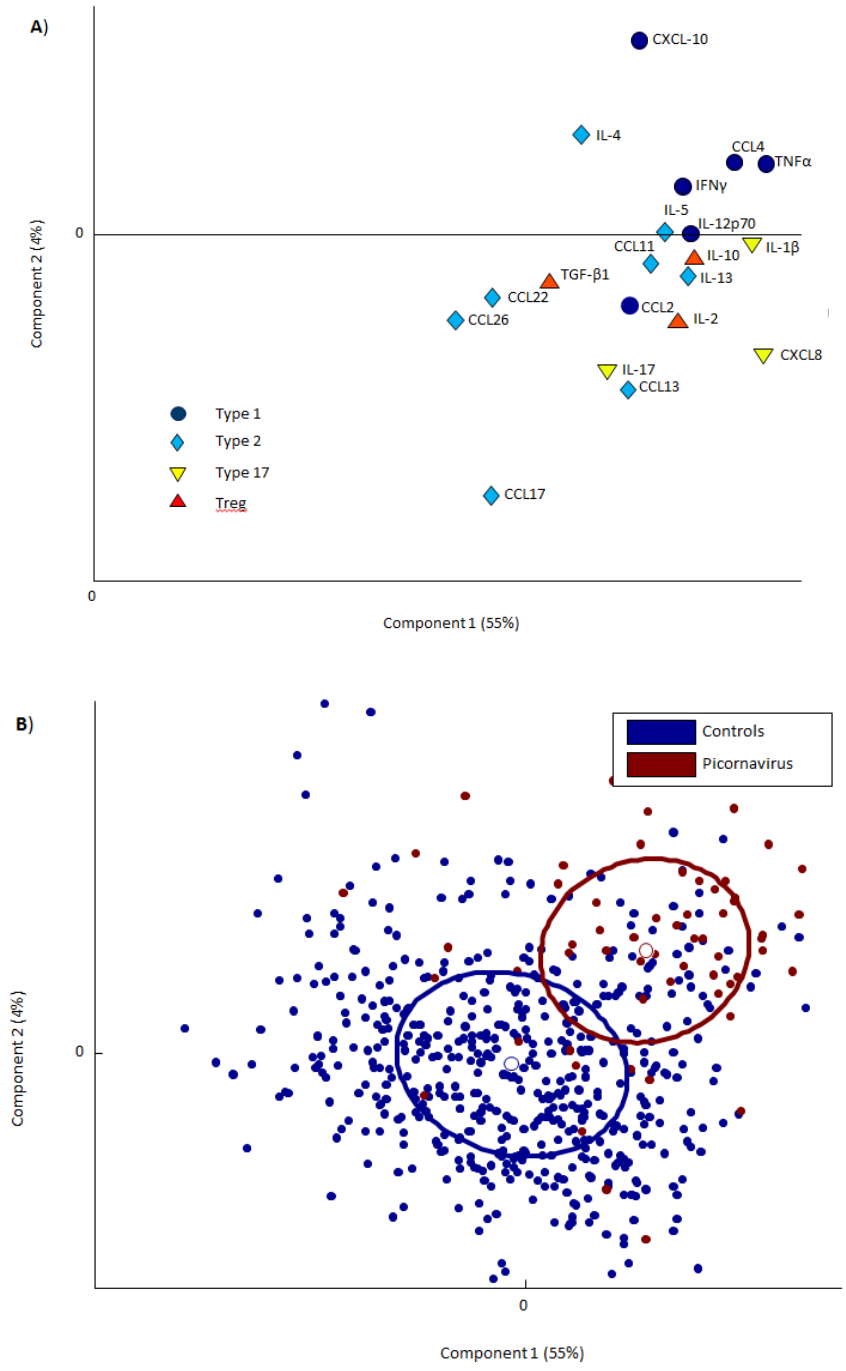
**Figure 5.2**: Unadjusted geometric mean ratios with 95% confidence intervals of cytokines and chemokines in the airway mucosal lining fluid of healthy one month old neonates with pathogenic airway bacteria detected in the airway versus controls.




We excluded neonates with a reported current airway infection on the day of sampling; and from the parent-recorded daily diary records we found an additional 15 children with troublesome lung symptoms (cough/wheeze and/or breathlessness) one week prior to and/or after the sampling day. Excluding these children as a sensitivity analysis did not modify our findings (data not shown)[44].

From the multivariate Partial Least Square-Discriminant Analysis (PLS-DA) we observed that a clustering of the immune mediators was evident in the first component of the loading plot (**Figure 5.3A**), suggesting strong inter-correlation. In the first component, a significant separation was found between children with picornavirus versus children without picornavirus, suggesting that presence of picornavirus in the airway of an asymptomatic neonate is immune-stimulating (**Figure 5.3B**). This was supported by a PLS regression analysis showing a highly significant difference in immune mediator levels in children with picornavirus compared to controls (confounder adjusted $p < 0.001$). From the second component, we observed that this was primarily driven by the Type 1-related immune mediators CXCL10, CCL4, and TNF-$\alpha$[44].

**Figure 5.3**: A Partial Least Square Discriminate Analysis model of immune mediators and picornavirus versus no picornavirus. **A**) Loading plot of the 20 different immune mediators; the cytokines are marked and colored according to their immune pathway. **B**) Score plot. Each point corresponds to one child and the distribution of picornavirus cases versus controls are shown as ellipses. Differences are registered between the blue ellipse (controls) and the red ellipse (picornavirus).



## 5.4 Discussion

*Principle Findings*

Presence of picornavirus in the airways of asymptomatic neonates is associated with an upregulation of the upper airway mucosal immune signature, dominated by Type 1-related mediators important for clearing intracellular pathogens, such as picornavirus. These findings suggest that asymptomatic presence of picornavirus promotes early airway immune activation. A concomitant rise in Type 2-related immune mediators was also found, which may be involved in the progression of asthma and allergic sensitization[44].

*Other studies*

The airway immune response has not previously been studied in asymptomatic neonates in regards to presence of airway viruses, limiting the comparability with other studies. There are studies of stimulated cord blood mononuclear cells; and although the translation from the airway immune response is not easily performed, there are reports of a Type 1-oriented immune response in cord blood mononuclear cells *ex vivo* in response to viral infections[37,42,83]. Studies of the respiratory system have been performed *in vitro* in respiratory cell lines, showing that the presence of virus induces an innate immune response[84]. How this compares to the *in vivo* situation in the airway mucosa is not clear since study on bronchial cells performed both *in vitro* and *in vivo* have shown that these designs are not comparable[85]. Symptomatic picornavirus-infections in the first year of life, predominantly studied as rhinovirus infections, have previously been found to be a pivotal external trigger for wheezing and later asthma development[29,40].

It has been well described that children exposed early to a diverse microbial milieu, which can be found in traditional farming sites, is associated with a Type 1- or regulatory oriented immune activation in cord blood mononuclear cells, and these children display lower incidence rates of asthma and allergy compared to peers exposed to a less diverse microbial environment[13,86–88].

*Meaning of the study*

We found an overall immune stimulatory effect of having picornavirus in the airway of asymptomatic neonates, with a predominant enhancement of Type 1-based inflammatory mediators; driven primarily by CXCL10 but also by CCL4, and TNF-α. CXCL10 is a key Type 1-related chemokine produced in response to both Type 1 and Type 2 interferon stimulation[89]. The observed immune response seen from exposure to picornavirus is biologically plausible, since the Type 1 immune response is known to be important for intracellular clearance of pathogens such as viruses[37,42,83,90], which strengthens the confidence in our findings.

The observed response of the immune system to picornavirus in the airways of asymptomatic newborns, with an overweight of Type 1-related immune cells, may play an important role in programming the specific type of memory response to picornavirus during first encounter in early life; and may be involved in the skewing of the Th1:Th2 balance in favor of Th1 in healthy children. It should be noted that the reported immune signature could merely reflect a normal immune response against virus.

Type 17-related mediators as well as regulatory factors were enhanced by picornavirus. However, we also observed an increased Type 2 related mediators, which may have long term consequences for childhood health as enhanced production of Type 2-related mediators predisposes to asthma and allergy development[91,92]. In support, we have previously shown that elevated levels of Type 2-associated chemokines within cord blood are associated with increased total-IgE production in preschool children[93]. This would also explain the previously described association between early respiratory viral infections and increased asthma propensity[29,40].

In children with both pathogenic airway bacteria and picornavirus we saw an additive increase in the immune mediators, which is in line with a previously described enhanced disease promoting effect in animal models with both virus and bacterial infections[94,95]; importantly, no synergistic

effect was found in this study which underlines that pathogenic airway bacteria and picornavirus do not seem to interact.

*Strengths and Limitations*

The strength of the study is the method of mucosal lining fluid sampling, enabling data on levels of immune mediators of the adaptive and innate immune response *in vivo* in the target organ of respiratory viruses. Combined with concomitant detection of respiratory viruses and bacteria as well as other exposures, these data gives insight into the developing immune response of neonates[43,45]. The respiratory viruses were detected by PCR technique and our finding of 12% of the samples being virus-positive, with the majority being picornavirus, is comparable to other studies of asymptomatic children[96,97].

We cannot quantify the load of virus based on the applied method; and we only tested for the presence of the most common pathogenic respiratory viruses. It is also a potential limitation that the nasopharyngeal virus sampling for detection of airway viruses was obtained with two different methods. However, adjusting for sampling method did not modify our findings.

Another limitation of the study is that we restricted our mucosal lining fluid analyses to 20 different cytokines and chemokines. These were chosen in order to provide a representative view of mediators produced by the different airway immune cells, of both innate and adaptive mediators involving activation of Type 1, Type 2, Type 17 and regulatory type responses, but should not be viewed as an exhaustive characterization of the immune response

To circumvent the issue of multiple testing we included a multivariate approach involving a partial least square model. We found comparable results from conventional statistics and the data driven approach, thereby enhancing the confidence in our findings. In our cohort we found a low incidence of colonization with the other viruses examined (influenza A and B and RSV), and

these cases were omitted from the analyses. Hence, we do not know if asymptomatic neonates with these viruses display similar or different changes in their immune profile.

## 5.5 Conclusion and perspectives

In this study we found that presence of picornavirus in the airway of asymptomatic neonates activates the mucosal immune system in the upper airways. An enhancement of key pro-inflammatory mediators of Type 1 origin was found, as well as an activated Type 2 response. Our findings are important for understanding the immune programming effects of early life exposure to picornavirus.

*Future research:*

Being able to investigate which exposures are important for the neonatal airway immune signature is a key component to understand human immune development. We have previously shown that maternal atopy and pathogenic airway bacteria are associated with a specific neonatal airway immune profile[43,56]. With the current study we expand this to also be true for picornavirus. Combined with the knowledge that children born in traditional farming environments display an enhanced Type 1 based immune profile protective of later asthma and allergy[13,86–88]; this suggests that exposure to microbes early in life is important for an optimal and healthy immune maturation. However, studies of the impact of early life immune activation in the airways by external exposures, and its relation to later development of asthma, are missing.

## 6. Siblings promote a Type 1/Type 17-oriented immune response in the airways of asymptomatic neonates

Helene M Wolsk, Bo L Chawes, Nilofar V Følsgaard, Morten A Rasmussen, Susanne Brix, Hans Bisgaard. Published in *Allergy, 2016 Jun;71(6):820-8.*

## 6.1 Introduction

In this paper we studied the association between siblings and the neonatal airway immune response at one month of age.

Our hypothesis was that older siblings convey an activation of the neonatal airway immune response. We analysed key immune mediators *in vivo*, in the airway mucosal lining fluid of one month old neonates from the unselected Copenhagen Prospective Studies on Asthma in $Childhood_{2010}$ ($COPSAC_{2010}$) birth cohort[43,45] and associated levels with presence and number of siblings. A possible *in utero* immune priming effect of siblings was investigated by studying the association between mediator levels and time since last childbirth, for multiparous women[52].

## 6.2 Methods

*The $COPSAC_{2010}$ birth cohort*

See section 3.1 for a description and characterization of the cohort.

*Measurements of airway cytokines and chemokines*

The method of mucosal lining fluid is described in detail in section 3.2.

The MesoScale Discovery multiplexed array system (MesoScale Discovery, Gaithersburg, MD, USA)[45] was used for analysing the mucosal lining fluid samples for the following immune mediators: IL(interleukin)-12p70, CXCL10, Interferon-gamma (IFN-γ), Tumor necrosis factor-alpha (TNF-α), CCL4, CCL2, CCL13, IL-4, IL-5, IL-13, CCL11, CCL26, CCL17, CCL22, IL-17, IL-1β, CXCL8, Transforming growth factor beta1 (TGF-β1), IL-10, and IL-2[45,56].

*Siblings*

At one week of age, the neonate and their families visited the clinic and detailed information about the presence of siblings in the household was obtained by a standardized interview of the parents.

*Statistics*

Differences in baseline characteristics between neonates with and without siblings were analysed with chi-squared test or Fisher's exact test. Significant univariate variables were included as covariates in all analyses. A p-value of $< 0.05$ was considered significant.

Concentrations of mediator levels were right-skewed and log transformed in order to obtain normally distributed residuals. Immune mediator concentrations between zero and the respective detection limits were not changed, while the zero-values were set to half the minimum of the non-zero values, in order to perform a log transformation, without loss of data. Missing data on immune mediator levels were excluded.

We sub-categorized sibling data for analysis according to 1) any siblings (biological and half-siblings), 2) any biological siblings (excluding half-siblings), 3) quantitatively as 0 vs. 1 vs. >1 sibling and 4) time since last childbirth of the youngest older sibling for multiparous women.

Univariate associations between siblings in the home and the mediator levels were analysed with an analysis of variance (ANOVA); results are reported as geometric mean ratios (GMR) of the mean mediator levels with 95% confidence intervals (CI) for neonates with/without siblings. A principal component analysis (PCA) was applied for capturing the overall trends in the immune signal from the 20 mediators into a few independent components[52]. "Time since last childbirth" was analysed from the immune signal captured in Principal Component (PC) 1.

*Covariates*

*Parental characteristics:* "maternal asthma, allergy, or eczema" (Yes/No), "Caucasian" (both parents of Caucasian descent, Yes/No), "high income" (annual household income > 130.000 euro, Yes/No), "maternal antibiotic consumption in 3rd trimester" (Yes/No), "maternal smoking in 3rd trimester" (Yes/No) and "maternal alcohol consumption in 3rd trimester" (> one unit of alcohol per week, Yes/No).

*Characteristics of the child: "*gestational age < 37 weeks" (Yes/No), "sex" (Male/Female), "Apgar score < 7 at 1 minute" (Yes/No), "caesarean section" (planned or acute caesarean section, Yes/No), "exclusively breastfeeding at one month" (Yes/No), "pets" (cats and/or dogs in the home, Yes/No), "picornavirus" (Yes/No) and "airway bacteria" (presence of the pathogenic airway bacteria *Haemophilus influenzae, Streptococcus pneumonia* and/or *Moraxella catarrhalis*, Yes/No).

Sampling site, season of sampling, batch of immune mediator analysis, maternal asthma, allergy or eczema, and maternal consumption of antibiotics in 3rd trimester have been associated with the levels of immune mediators[43,56,98]. Along with significant univariate variables identified from the baseline characteristics, and covariates listed above, they were included as forced covariates in the models.

Analyses were carried out using SAS version 9.3 (SAS Institute, Cary, NC) and the PCA were conducted in MATLAB R2013a v. 8.1.0.604 (MathWorks Inc, Natick, MA, USA) utilizing the PLStoolbox 7.8.2 (Eigenvector Research Inc. Wenatchee, WA, USA) for building the PCA model.

## 6.3 Main results

*Baseline characteristics*

A total of 571 neonates were included in the main study. See description in section 5.3 for more details. Of the included neonates, 57% (N = 324) had siblings; of these 324, 4% (N = 13) had half-siblings, 67% (N = 216) had 1 sibling, and 33% (N = 108) had > 1 sibling. In **Table 6.1**, baseline characteristics of neonates with and without siblings are presented.

**Table 6.1**: Baseline characteristics of neonates with/without siblings. P-values ≤ 0.05 are shown in bold.

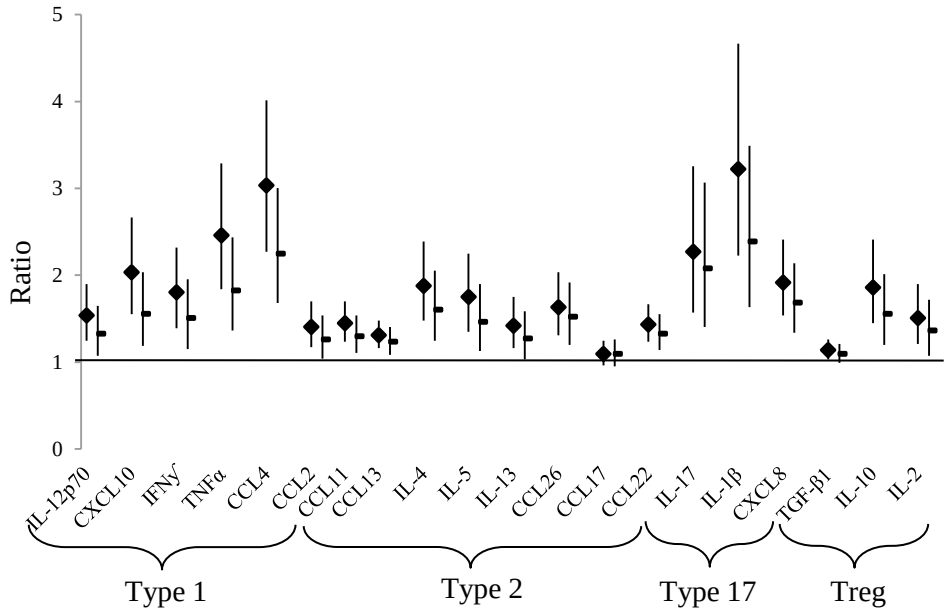
| | Siblings % (n = 324) | No siblings % (n = 247) | p-value |
|---|---|---|---|
| Maternal asthma, allergy or eczema | 50 (161) | 58 (142) | 0.08 |
| Caucasian | 97 (314) | 95 (234) | 0.19 |
| High Income (above 130,000 euro) | 17 (55) | 9 (22) | **0.01** |
| Maternal antibiotic consumption in 3rd trimester | 22 (70) | 17 (42) | 0.17 |
| Maternal smoking in 3rd trimester | 3 (11) | 4 (10) | 0.68 |
| Maternal alcohol consumption in 3rd trimester* | 7 (21) | 2 (5) | **0.01** |
| Gestational age < 37 weeks | 2 (8) | 5 (12) | 0.12 |
| Male | 51 (164) | 52 (128) | 0.78 |
| Apgar score < 7 at 1 min | 5 (17) | 3 (8) | 0.25 |
| Caesarian section | 20 (66) | 23 (56) | 0.51 |
| Exclusively breastfeeding at 1-month | 94 (303) | 90 (222) | **0.04** |
| Furred pets | 23 (73) | 18 (45) | 0.21 |
| Picornavirus | 15 (48) | 4 (10) | **<0.0001** |
| Pathogenic Airway Bacteria | 40 (128) | 13 (31) | **<0.0001** |

Neonates with siblings had more mothers with alcohol consumption in the 3rd trimester (7% vs. 2%, p = 0.01), higher household income (17% vs. 9%, p = 0.01), and more breastfeeding at one month of age (94% vs. 90%, p = 0.04). Furthermore, neonates with siblings had increased presence of pathogenic airway bacteria (40% vs. 13%, p < 0.0001) and picornavirus (15% vs. 4%, p < 0.0001). These variables were included as confounders in the adjusted model.

*Siblings and the neonatal airway immune response*

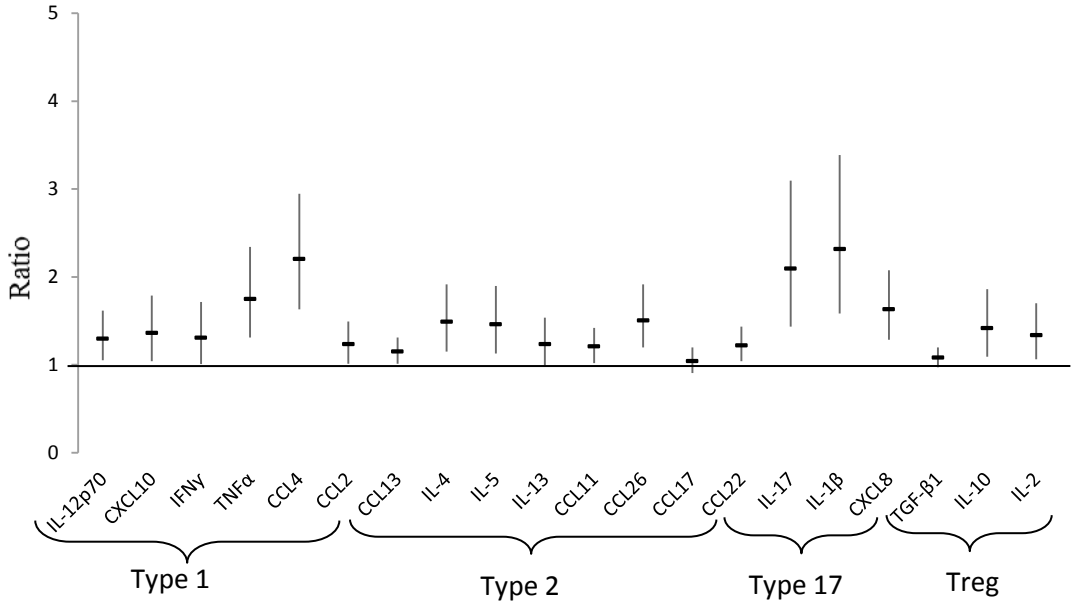
In the univariate analysis, neonates with siblings had a significant up-regulation of 19 out of 20 mediators, compared to neonates with no siblings, with a 2-3.5 fold increase for specific Type 1- (TNF-α, CCL4) and Type 17- (IL-17, IL-1β) associated mediators. Adjusting the analysis for all confounders, except pathogenic airway bacteria and picornavirus, did not modify the results substantially (**Figure 6.1**). Further adjustment for pathogenic airway bacteria and picornavirus resulted in a marginal decrease in the levels of the immune mediators, but still with an overall increased ratio of the immune mediators in neonates with siblings (**Figure 6.1**).

**Figure 6.1**: Geometric mean ratios (GMR) with 95% confidence interval of cytokines and chemokines in the airway mucosal lining fluid of healthy one month old neonates with/without siblings in the home. The bar with a rhombus shape is adjusted for maternal asthma, allergy, or eczema, sampling clinic, season of sampling, batch of immune mediator analyses, breastfeeding at one month of age, high income, and maternal alcohol consumption in 3rd trimester; the bar with a horizontal line represents GMR adjusted for the same variables and presence of bacteria and picornavirus.



When only neonates with biological siblings were included (n = 310) (**Figure 6.2**) a significant up-regulation was found in 18 of 20 immune-mediators (except for CCL17 and TGFβ1).

**Figure 6.2**: Geometric mean ratios with 95% confidence interval of cytokines and chemokines in the airway mucosal lining fluid of healthy one month old neonates with/without biological siblings present in the home at time of birth (n = 310). Adjusted for maternal asthma, allergy, or eczema, sampling clinic, season of sampling, batch of immune mediator analyses, breastfeeding at one month of age, high income, maternal alcohol consumption in 3rd trimester and presence of bacteria and picornavirus.




*Multivariate interrelations between siblings and the neonatal airway immune response*

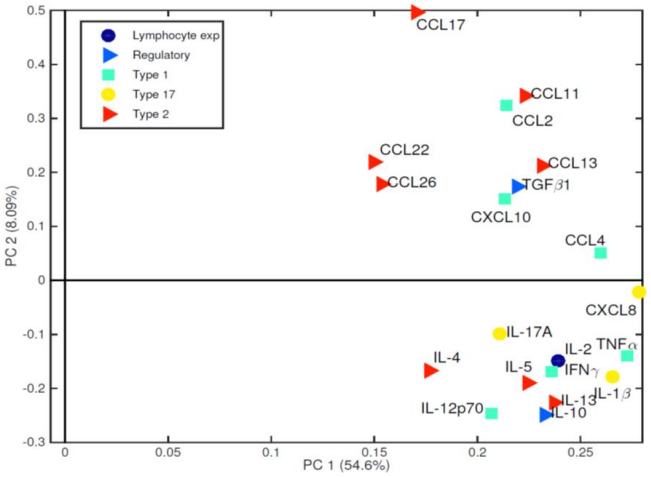
In **Figure 6.3A** the loading plot is shown for the 20 immune mediators. From the score plot (**Figure 6.3B**), a clear separation was found between neonates with siblings and those without siblings (adjusted $p < 0.001$).

We did not find a difference in immune profile from having no siblings vs. one sibling vs. more than one sibling (**Figure 6.4**). A possible *in utero* immune priming effect was examined by analysing the time since last childbirth of the youngest older sibling (**Figure 6.5**). An inverse association between time since last childbirth and the airway immune response in the neonate

was observed (p = 0.0015, adjusted p = 0.02); we found that the immune response was attenuated towards the level found in neonates with no siblings with increasing time since last childbirth. No effect of previous miscarriage was found on the immune mediators (data not shown).

**Figure 6.3**: A) Loading plot from principal component analysis showing the 20 different immune mediators colored according to immune classification. B) Score plot, where each point corresponds to one child. The distributions of neonates with siblings (blue) versus no siblings (red) are shown as ellipses.

A)




B)

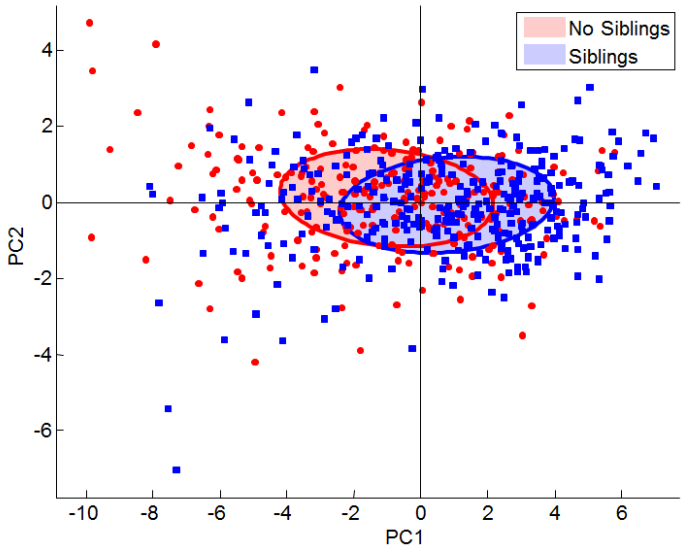


**Figure 6.4** Principal component analysis of the distribution of the neonates with no siblings (red circle) versus one sibling (green circle) versus more than one sibling (blue circle).

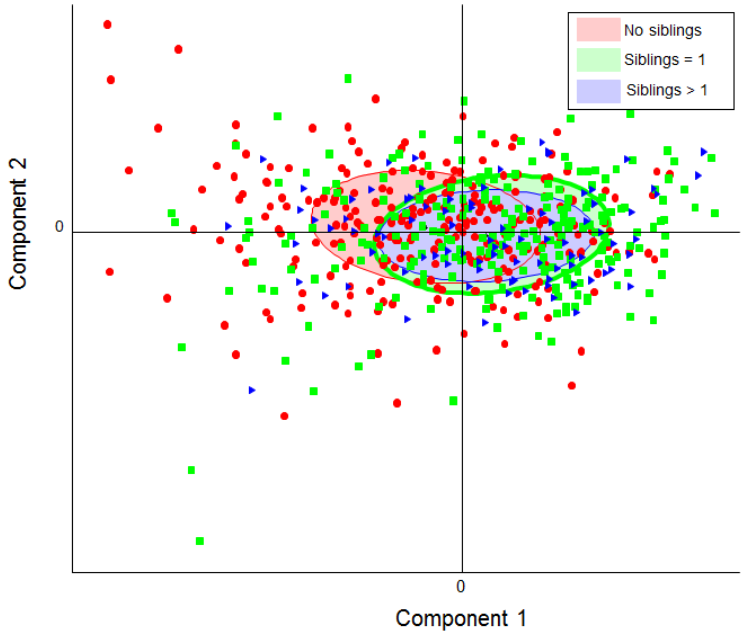
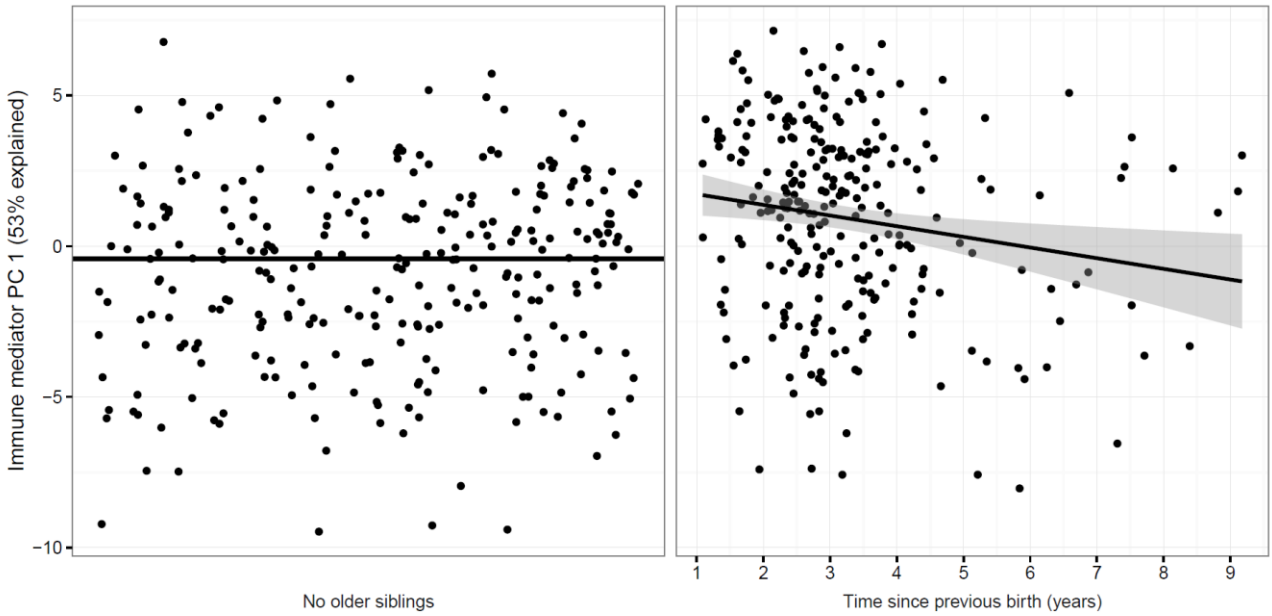



**Figure 6.5**: Airway immune signature (PC1) in neonates with and without siblings at birth. Left panel: Neonates without siblings, Right panel: Scatterplot of the inverse association between the airway immune signature (PC1) and time since previous birth, for neonates with siblings.



## 6.4 Discussion

*Principle findings*

We found an overall up-regulated airway immune signature in asymptomatic neonates with siblings present in the home at birth. This association was present after multivariable adjustment, including concomitant presence of airway picornavirus and pathogenic airway bacteria. With these findings we question the explanation of the observations termed the "sibling-effect" and "hygiene-hypothesis", being mediated solely through a spread of microbes. Additionally, our data suggests that the "sibling-effect" may represent *in utero* immune priming initiated by events during previous pregnancies, since the immune response was attenuated with time since last childbirth, but further studies are needed to confirm our findings[52].

*Other studies*

The "hygiene hypothesis" stated that a large household size protected against development of hay fever, supposedly mediated through increased exposure to infectious agents in early childhood[14,99]. Separately, a "sibling-effect" was found in the British Birth Survey, where it was shown that exposure to siblings protected against eczema and hay fever at age 5[46,47]. The "hygiene hypothesis" and "siblings-effect" are thought to be mediated through early exposure to a diverse microbial flora. An increased Type 1- and/or T regulatory related response has been observed in children living in traditional farming communities[13,86–88]; similarly, presence of older siblings in the home as well as early daycare attendance are associated with a reduction in asthma and wheezy symptoms during childhood[14,48]. Daycare attendance and older children in the household are the reservoirs for common respiratory viruses[100], and together these findings support the "hygiene-hypothesis" of exposure to microbes early in life being important for an optimal immune maturation and protection against later disease. It should be noted that the results for asthma are ambiguous. Most studies support the association between the presence of

siblings and protection against asthma [48,99,101–103], but a recent study did not find any association between siblings and asthma through childhood[104].

Along with the microbial milieu, birth-order may be important for immune maturation, and differences have been reported in immune activation of cord blood mediators and immune cell distributions according to birth-order[47,51]. Kragh et al. found a reduced anti-inflammatory profile in T cells at the birth of first-born infants, compared to second and later born[51], and Karmaus et al. found cord blood IgE levels associated with birth-order[47]. The mechanism behind this finding is not known, one hypothesis is that immune regulatory mechanisms occur during pregnancy, inducing materno-fetal tolerance in the offspring. This is supported by a study showing that anti-inflammatory, but not pro-inflammatory, cytokines correlate between mother and child[105]. Alternatively, the differences in immune activation found between first and later-born children may be induced by the older sibling in the home being a reservoir for infections, thereby exposing the pregnant mother and/or newborn child to bacteria and viruses. It has been shown in adults, that the distribution of pathogens in relation to respiratory infections, differed according to age[32]. This may also apply for children, and having younger siblings compared to older siblings could convey a higher risk of transmitting infections to which the neonates are more vulnerable.

*Meaning of the study*

A significant immune stimulatory effect from having siblings was found in this study. As there was a sibling effect independent of concomitant presence of airway pathogens, these findings challenge the well-known "hygiene hypothesis" and the "sibling-effect", which are believed to be mediated by an increased exposure to airway pathogenic microbes.

We did find an attenuation of the effect estimates after adjustment for bacteria and viruses; and the observed immune profile in neonates with siblings, dominated by Type 1- and Type 17-

related mediators does indicate that enhanced stimulation with environmental microbes may be the underlying factor for this specific immune signature in neonates with siblings.

We found an increased level of the Type 1-related mediator CCL4. This mediator is important for fighting off intracellular pathogens such as viruses and some bacteria, when coupled to the receptor CCR5[106]. Together with the observed Type 17-related response, this maybe an important part of a shift the immune system away from Type 2 dominance, in the neonates with siblings. Although not as pronounced as the Type 1 and Type 17 response, we did also find an increased level of Type 2 associated mediators. The univocal up-regulation of all types of immune responses in the neonatal airway shows that siblings convey direct activation of the neonatal airway immune response.

Another possible explanation could be an *in utero* programming of the fetal immune system due to events occurring during previous pregnancies. The effect did not differ in respect to birth-order, but we found an inverse association between the airway immune response and time since last childbirth, also when adjusting the analysis for confounders including pathogenic airway bacteria and picornavirus. Hence, pregnancies and childbirths may induce immunological effects *in utero* which are passed on in later pregnancies, and from our data it seems that these effects gradually decline over time. The reported effect could also be mediated by younger siblings having more infections, thereby exposing the vulnerable neonate to more pathogens. And it has been shown that the incidence is higher and intervals between airway infections are shorter with younger age[107]. Comparing the immune signal between the first born children of mothers with or without an earlier miscarriage revealed no significant difference, and since changes in the maternal immune system after a miscarriage should have similar effects as those occurring in a normal pregnancy, this supports that our finding are not mediated by *in utero* changes but rather

by pathogenic microbes transmitted by the siblings. It should be noted that the power for this analysis was low.

*Strength and limitations*

We assessed the immune response in the target organ of airway disease, which is an advantage compared to measurements of systemic mediators in blood. Information about presence of siblings was obtained by interviewing the parents and not from questionnaires.

The data was analysed utilizing two statistical methods. Comparable results were obtained from these, which increased the validity of our findings. Furthermore, the multivariate PCA model has the advantage of evading the problem of multiple testing.

Information about the immune response was only obtained at a single time point, limiting our knowledge about the chronicity of our findings. We only examined the most pathogenic and prevalent viruses and bacteria in childhood, not having the knowledge about if other viruses and bacteria were present and these could represent a residual confounding effect. Importantly, the investigated bacteria and viruses are the main pathogens affecting the immune response during early childhood, and they predispose to development of asthma[43,44,66,108]. Although we did multivariable adjustment to isolate the effects of siblings in the household, we cannot exclude the possibility that other important confounders were not included in the analysis.

## 6.5 Conclusion and perspectives

The presence of siblings at birth was associated with an up-regulated neonatal airway mucosal immune signature, not mediated solely through an increased occurrence of airway pathogens, and may be related to *in utero* immune priming. With these findings we suggest that siblings

exert early Type 1/Type 17-immune priming effects that may modulate development of asthma and allergy.

*Future studies:*

The suggested *in utero* effect needs further exploration. Future studies may need a wider approach to understand the mechanisms of how previous pregnancies, siblings, and risk of asthma and allergy are interconnected.

We speculate that our findings may highlight an important immune programming event in relation to later development of asthma and allergy; and future studies will need to associate the neonatal immune response to later development of asthma and allergy.

## 7. Neonatal airway immune profiles and childhood allergy and asthma-related diseases

Helene M Wolsk, Morten A Rasmussen, Jakob Stokholm, Klaus Bønnelykke, Susanne Brix, Bo L Chawes, Hans Bisgaard, *Ready for submission.*

## 7.1 Introduction

The airways represent the target organ of allergies and asthma, which are among the most common chronic childhood disorders[109,110]. We wanted to examine whether the development of childhood asthma and allergy-related diseases was associated with specific neonatal airway immune profiles.

Our hypothesis was that specific in neonatal airway immune profiles could be a possible risk marker of subsequent asthma and allergy-related diseases later in childhood. We studied the association between the airway immune profiles in neonates from the unselected Copenhagen Prospective Studies on Asthma in Childhood$_{2010}$ (COPSAC$_{2010}$) birth cohort[43,45] and data on development of allergy and asthma-related disease during a maximum of 5 years of follow-up.

## 7.2 Methods

*The COPSAC$_{2010}$ birth cohort*

See a description of the cohort in section 3.1.

*Measurements of airway cytokines and chemokines*

Unstimulated airway mucosal lining fluid was sampled when the neonates visited the COPSAC clinic at one month of age as described in details in section 3.3[43,45]. The samples were analysed for IL (interleukin)-12p70, CXCL10, Interferon-gamma (IFN-γ), Tumor necrosis factor-alpha (TNF-α), CCL4, CCL2, CCL13, IL-4, IL-5, IL-13, CCL11, CCL26, CCL17, CCL22, IL-17A, IL-1β, CXCL8, Transforming growth factor beta1 (TGF-β1), IL-10, and IL-2[45,56].

*End-points*

*Allergic sensitization*: Screening of allergic sensitization was done at visits to the clinic at 6 and 18 months of age. This was defined as any skin prick test (SPT) reaction larger than 2 mm or

specific IgE (sIgE) ≥ 0.35 kUa/L against milk, egg, dog and/or cat [57]. See section 3.6 for more details.

*Total IgE:* This was measured at 6 months of age using the ImmunoCAP assay (Phadia AB, Uppsala, Sweden)[62].

*Allergic rhinoconjunctivitis (AR) (0-5 years of age):* The diagnosis of allergic rhinoconjunctivitis was established by the COPSAC pediatrician, based on significant symptoms during the previous year of 1) sneezing or a runny/blocked nose and/or 2) red, swollen or watery eyes in periods when the child did not have a cold or flu[45,63,64].

*Asthma/persistent wheeze (0-5 years of age):* This was diagnosed based on a detailed daily diary of symptoms observed by the parents, as previously described[65,67]. For more details, see section 3.6.

*Wheeze exacerbations:* A wheeze exacerbation was defined as one or more of the following criteria; 1) treatment with inhaled β2-agonist in a pediatric admission ward or during hospitalization, or 2) treatment with oral or high-dose inhaled corticosteroid prescribed by a general practitioner or the COPSAC clinical research unit.

*Lower respiratory tract infections (LRTI):* Defined as the occurrence of pneumonia and/or bronchiolitis from age 0 to 3 years[68–70]. See section 3.6 for more details.

*Statistics*

A number of reads in each sample were below the detection limit; levels between zero and detection limit have not been changed, while the zero values were set to half of the minimum of the non-zero values per mediator. This was done to be able to perform log transformations due to right-skewed data, without loss of data. We measured the mediators in two batches and data was corrected for batch, by batch-wise centering on log transformed data, followed by addition of the overall mean and anti-log transformation to get data in original units. Subsequently, we scaled

and centered the individual mediator levels, based on the sum of all quantitated mediators to obtain a profile of mediators being independent of the individual response level. This strategy has proved more useful than normalizing to a total protein measure, which is 500 - 1,000-fold higher than the sum of mediators, and therefore includes many other proteins than the low level cytokine and chemokines of interest.

The airway immune composition was analysed with a multivariate principal component analysis (PCA). This model was chosen as it decomposes the immune signal from the 20 mediators into a few independent components, capturing the overall immunological trends in the data. These independent principle components (PCs) were used as data-driven surrogate representations of the neonatal airway immune profile.

The following dichotomous covariates were used to adjust for confounding, as they have previously been associated with the immune mediator levels[10,43,44,52,56]: "Maternal asthma, allergy, or eczema" (Yes/No), "Vitamin D intervention" (vitamin D intervention/placebo), "Siblings" (children in the home at birth, Yes/No), "Pathogenic microbes at one month of age" (*Haemophilus influenza* or *Streptococcus pneumonia* or *Moraxella catarrhalis* or *Staphylococcus aureus*, Yes/No) and/or "Picornavirus at one month of age" (Yes/No) and technical "Batch of mucosal lining fluid analysis" (first/second batch)".

Using Cox proportional hazard models with time to event, we analysed the association between the neonatal airway immune profile represented in PC1-4 and each of the clinical endpoints asthma/persistent wheeze, wheeze exacerbations, LRTI and AR. The dichotomized endpoints, sIgE and SPT were analysed by logistic regression. Total IgE was modeled as a continuous outcome as well as a dichotomized outcome using the median as the cut-off. To further explore the specific immune signals we also conducted univariate analyses using an Analysis of Variance

(ANOVA) between mediator levels and each end-point. We summarized the data using geometric mean ±95 Confidence intervals (CI) to display data in original units.
A p value of <0.05 was considered statistically significant.

Analyses were carried out with R version 3.2.4, using the packages "survival" for Cox proportional hazard models and "gglot2" for visualization of data and results.

### 7.3 Main results

*Baseline characteristics*
We included 620 (89%) of the 700 neonates in $COPSAC_{2010}$ in this study (see study flow chart, **Figure 3.2**)

In our cohort, 43 (9%) had a positive sIgE (126 missing samples), 38 (6%) were diagnosed with allergic rhinitis, 125 (20%) were diagnosed with asthma, 151 had one or more LRTI and 49 (8%) had one or more wheeze exacerbations.

In the PCA model we studied the first four PCs, explaining a total of 55.9% of the variation in immune mediator data, distributed as PC1 explaining 25.8%, PC2 11.5%, PC3 10.8% and PC4 7.8% of the variation.

*Neonatal airway immune composition and allergy endpoints*
*Specific IgE:* An association was found between elevated sIgE at 6 and/or 18 months and the neonatal airway immune profile represented by PC1: odds ratio (OR) 1.44 (95% CI 1.03–2.01), p = 0.03. Adjustment for confounders as previously listed, did not alter this substantially: adjusted OR (aOR) 1.77 (1.14–2.76), p = 0.01 (**Table 7.1**).

**Table 7.1:** Overview of clinical end-points and associations with the airway immune profile, analysed by principal component analyses (PCA); shown as unadjusted and adjusted for pathogenic airway microbes, siblings, maternal asthma, allergy or eczema and the vitamin D randomized controlled trial. Odds ratios and hazard ratios (95% CI) of principal component (PC) 1-4 are calculated for every endpoint. P < 0.05 is shown in bold.

| End-point | PC1 | PC1 adjusted | PC2 | PC2 adjusted | PC3 | PC3 Adjusted | PC4 | PC4 adjusted |
|---|---|---|---|---|---|---|---|---|
| Specific IgE | **1.44 (1.03 – 2.01) p = 0.03** | **1.77 (1.14 – 2.76) p = 0.01** | 0.98 (0.79 – 1.22) p = 0.87 | 0.99 (0.77 – 1.26) p = 0.93 | 0.86 (0.70 – 1.05) p = 0.13 | 0.92 (0.73 – 1.17) p = 0.51 | 1.18 (0.91 – 1.52) p = 0.21 | 1.12 (0.83 – 1.51) p = 0.45 |
| Total IgE | **1.26 (1.07 – 1.49) p = 0.005*** | **1.36 (1.12 – 1.87) p = 0.002** | 0.97 (0.87 – 1.07) p = 0.52 | 0.97 (0.86 – 1.08) p = 0.57 | 0.97 (0.87 – 1.08) p = 0.55 | 0.97 (0.86 – 1.09) p = 0.57 | 0.95 (0.83 – 1.08) p = 0.40 | 0.99 (0.86– 1.14) p = 0.87 |
| Allergic rhinitis | **1.42 (1.01 – 1.99) p = 0.04** | **1.71 (1.12 – 2.61) p = 0.01** | 0.92 (0.74 – 1.13) p = 0.41 | 0.96 (0.76 – 1.21) p = 0.71 | 0.88 (0.72 – 1.07) p = 0.19 | 0.93 (0.73 – 1.18) p = 0.54 | 1.11 (0.87 – 1.43) p = 0.40 | 1.02 (0.77 – 1.35) p = 0.89 |
| Skin prick test | 0.96 (0.83 – 1.13) p = 0.65 | 0.94 (0.78 – 1.13) p = 0.51 | 0.96 (0.75 – 1.21) p = 0.71 | 0.87 (0.67 – 1.13) p = 0.30 | **0.63 (0.46 – 0.87) p = 0.005** | **0.57 (0.40 – 0.82) p = 0.002** | 1.08 (0.81 – 1.45) p = 0.60 | 1.00 (0.72 – 1.37) p = 0.99 |
| Asthma | 0.98 (0.90 – 1.05) p = 0.52 | 0.99 (0.90 – 1.08) p = 0.80 | 0.97 (0.86 – 1.09) p =0.61 | 0.98 (0.87 – 1.11) p =0.75 | 0.95 (0.84 – 1.07) p = 0.40 | 0.96 (0.85 – 1.08) p = 0.49 | **1.21 (1.03 – 1.45) p = 0.02** | 1.15 (0.96 – 1.38) p = 0.12 |
| Wheeze Exacerbation | 0.94 (0.84 – 1.06) p = 0.34 | 0.94 (0.82 – 1.08) p = 0.39 | **0.73 (0.55 – 0.96) p = 0.03** | 0.75 (0.58 – 1.00) p = 0.05 | 1.05 (0.87 – 1.28) p = 0.60 | 1.05 (0.86 – 1.29) p = 0.61 | 1.11 (0.89 – 1.38) p = 0.36 | 1.09 (0.87 – 1.37) p = 0.45 |
| LRTI | 1.06 (0.98 – 1.14) p = 0.12 | 1.05 (0.97 – 1.15) p = 0.19 | **0.81 (0.68 – 0.96) p = 0.02** | **0.79 (0.66 – 0.96) p = 0.01** | 0.99 (0.89 – 1.10) p = 0.88 | 0.96 (0.85 – 1.08) p = 0.48 | 1.08 (0.95 – 1.23) p = 0.24 | 1.09 (0.95 – 1.25) p = 0.22 |

*P = 0.001 as continuous variable in a linear model

We modelled a PCA biplot of the immune mediators and children with no sIgE vs. children with positive sIgE (**Figure 7.1A**). Here we identified that the profile related to positive sIgE was characterized by decreased TGF-β1, CCL2, CCL11, CCL13, CCL17, CCL22 and CCL26. For three of these mediators (TGF-β1, CCL22 and CCL26) this was replicated in the univariate analysis showing a significantly reduced level in children with later positive sIgE (**Table 7.2A**).

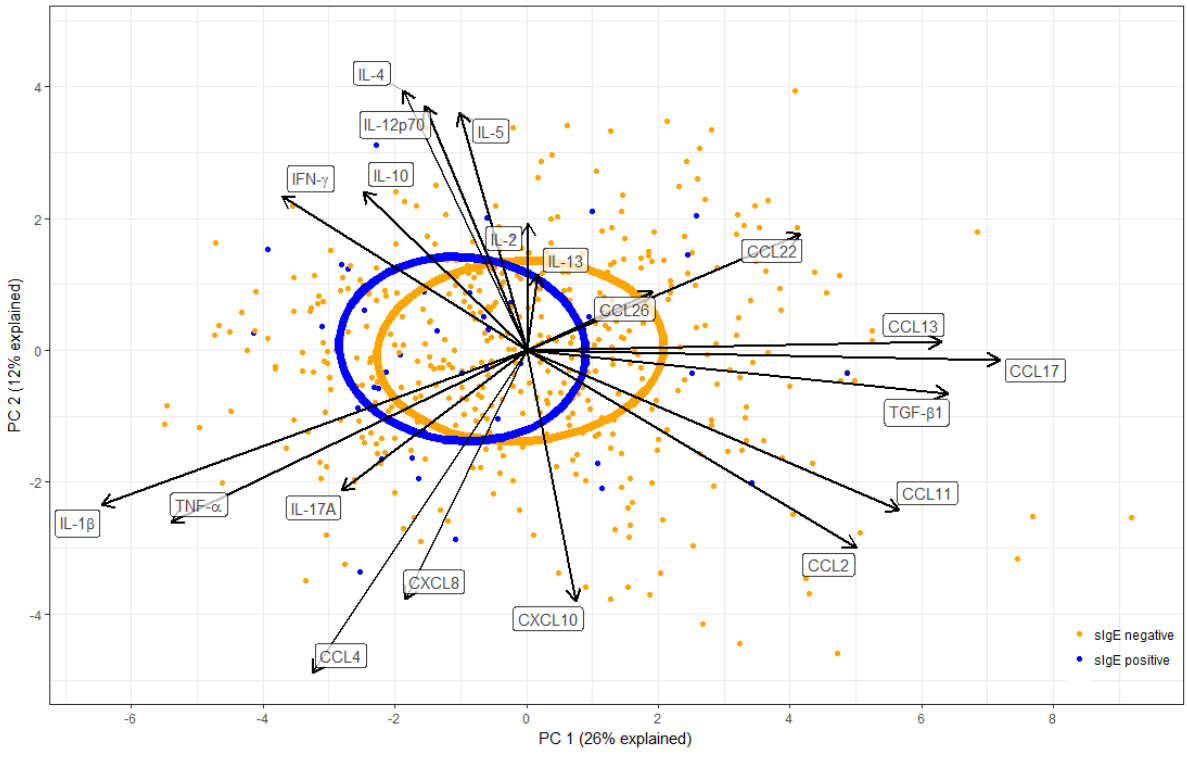
*Allergic rhinoconjunctivitis*: An association between the immune profile represented by PC1 and AR (until age 5 years) was found: adjusted HR 1.71 (1.12 – 2.61), p = 0.01 (**Table 7.1**). In the

PCA biplot we identified a pattern similar to that of positive sIgE with decreased airway levels of TGF-β1, CCL2, CCL11, CCL13, CCL17, CCL22 and CCL26 in children with later rhinoconjunctivitis (**Figure 7.1B**); CCL13 and CCL22 were found to be significantly reduced in the univariate analysis (**Table 7.2A**).

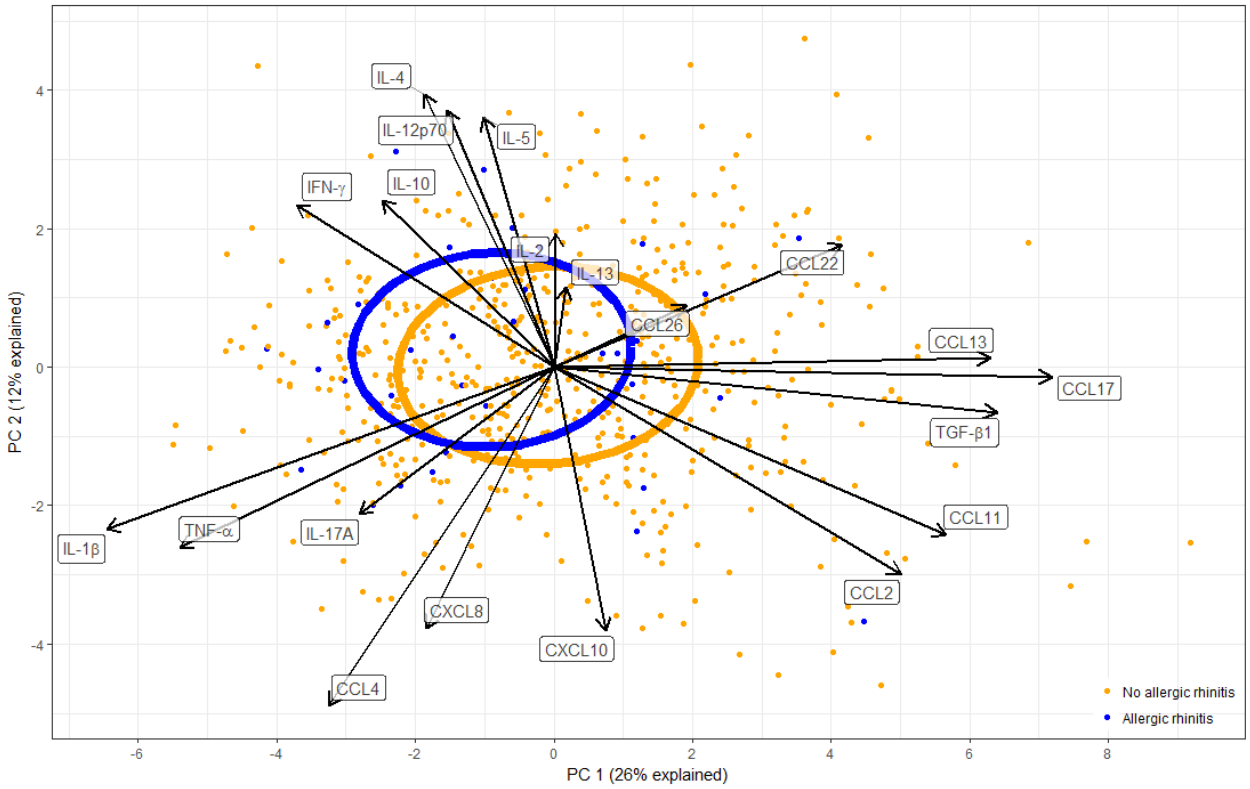
*Total IgE:* Elevated total IgE (> median value of 4.73 kU/L) was associated with the airway immune profile represented by PC1: adjusted OR 1.36 (1.12–1.87), p = 0.002 (**Table 7.1**). This immune profile (PC1) was characterized by significantly reduced levels of the key Treg-related mediator TGF-β1 and the chemokines CCL2 and CCL17, as well as elevated levels of IL-1β and IL-17A (**Table 7.2B).** Analysing total IgE as a continuous vs. a dichotomized variable yielded comparable results (not shown).

**Figure 7.1**: Neonatal airway immune profiles of healthy and later allergic neonates. The biplots show the principal components analysis of the distribution of children with A) positive specific IgE versus the controls (children with levels below the threshold for specific IgE and a negative SPT), and B) children with allergic rhinitis versus controls; shown as ellipses. The blue ellipse represents neonates with a positive sIgE/allergic rhinitis, and the red ellipse represents healthy controls.

A)



B)




*Skin prick test*: The neonatal airway immune profile in PC3 was associated with a positive SPT: aOR 0.57 (0.40 – 0.82) p= 0.002). (**Table 7.1**), this was characterized by a down-regulation of CCL26 (**Table 7.2B**).

*Neonatal airway immune composition and asthma/wheeze endpoints*

*Asthma/persistent wheeze:* The immune profile represented by PC4 was most strongly associated with development of asthma/persistent wheeze until age 5 years, although no significance was reached in the adjusted model: adjusted HR 1.15 (0.96–1.38), p = 0.12 (**Table 7.1**). This difference was mainly driven by reduced IL-17A and IL-1β and enhanced CCL2, as visualized in a biplot of PC3 vs PC4 (**Figure 7.2**). In the univariate analysis, the ratio of IL-1β and CCL2 were significantly different in neonates with later development of asthma/persistent wheeze (**Table 7.2C**).

*Wheeze exacerbations:* The airway immune profile (PC2) was associated with subsequent wheeze exacerbations: adjusted HR 0.75 (0.58–1.00), p = 0.05 (**Table 7.1**). The PCA biplot of PC1 vs PC2 (**Figure 7.3**) showed that the difference was mainly driven by reduced levels of TNF-α, CXCL8, IL-1β, IL-17A and CCL4. In the univariate analysis we found reduced TNF-α, CXCL8 and IL-1β to be associated with subsequent wheeze exacerbations (**Table 7.2C**).

*LRTI:* The immune profile in PC2 was associated with development of LRTI: adjusted HR 0.81 (0.68–0.96), p = 0.01 (**Table 7.1**).

**Figure 7.2** Neonatal airway immune profiles of healthy and later asthmatic neonates. The biplot (PC3 vs. PC4) shows the principal components analysis of the distribution of children with asthma versus controls (p = 0.03).

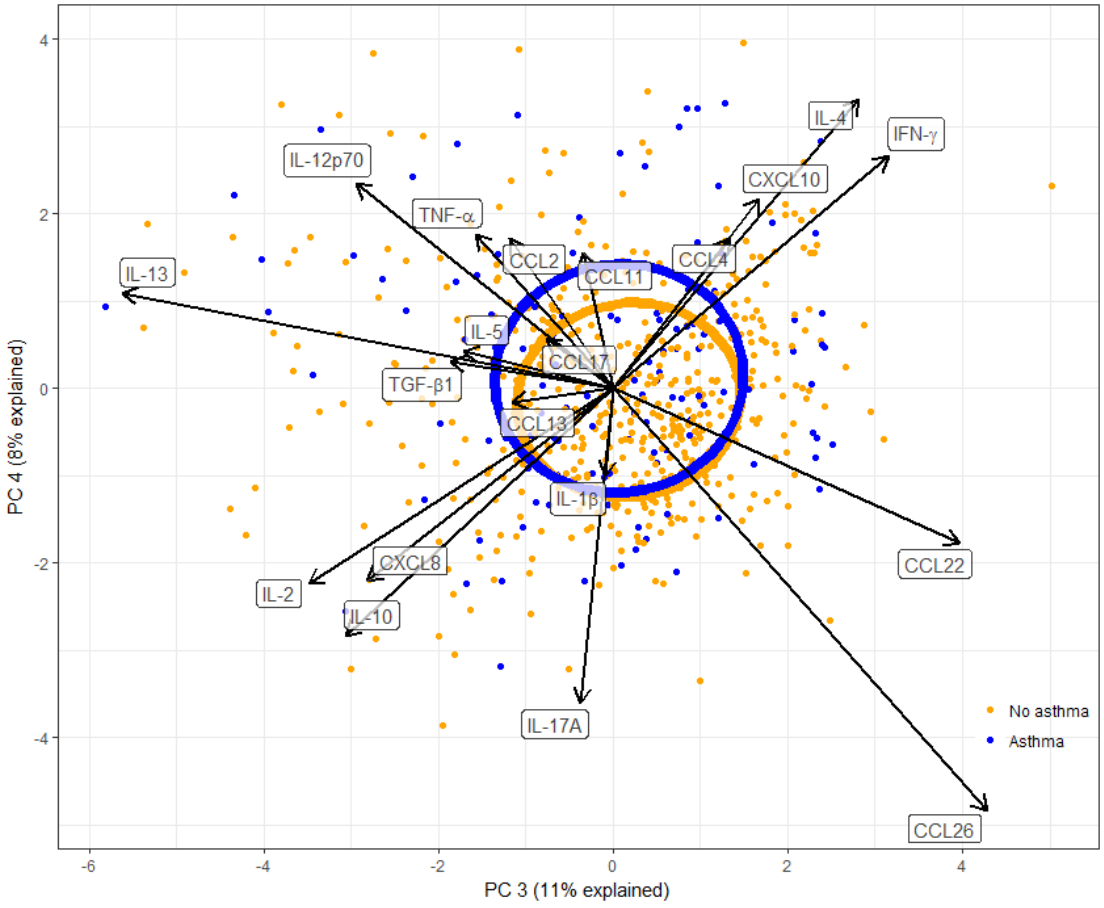


**Figure 7.3** Neonatal airway immune profiles of healthy vs neonates with later development of one or more wheeze exacerbations from 0-5 years of age. The biplot shows the principal components analysis of the distribution of children with wheeze exacerbations (blue circle) versus controls (red circle).

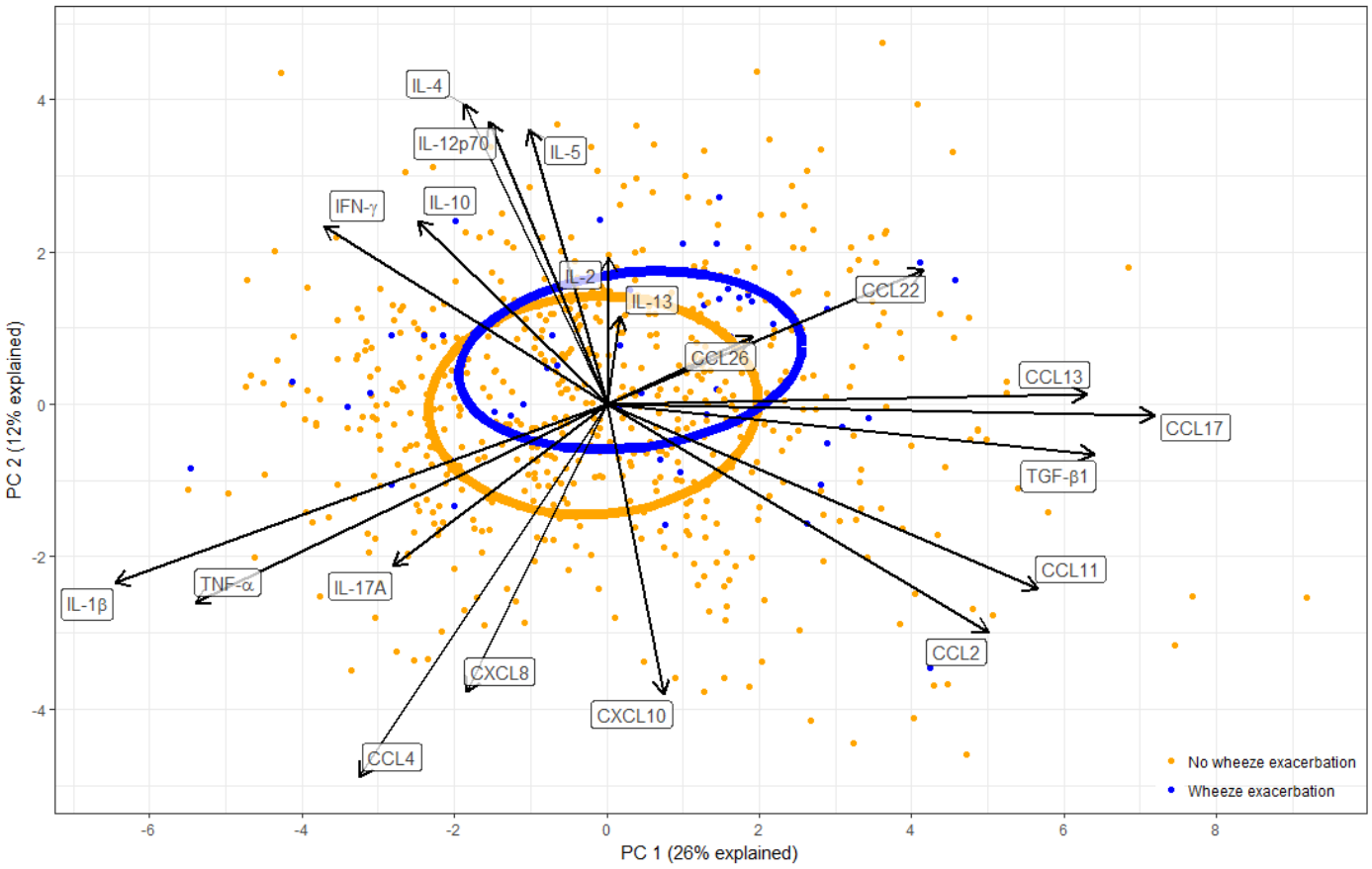



**Table 7.2** Unadjusted geometric mean ratios (GMR) of A) specific IgE (sIgE) and allergic rhinitis (AR), B) Total IgE and positive skin prick test (SPT) and C) Asthma and wheeze exacerbations. P values < 0.05 are shown in bold. GMR > 1 indicates that neonates with a positive endpoint had a higher immune mediator ratio. Conversely, GMR < 1 indicates that neonates with a positive endpoint had a lower ratio of immune mediator concentrations.

## A)

| **Immune mediator** | **sIgE** GMR | Confidence interval (CI) | p-value | **Total IgE*** GMR | Confidence interval (CI) | p-value |
|---|---|---|---|---|---|---|
| IL-12p70 | 1.35 | 0.92 - 1.96 | 0.12 | 1.02 | 0.88 – 1.18 | 0.79 |
| CXCL10 | 1.08 | 0.80 – 1.46 | 0.60 | 1.01 | 0.86 – 1.17 | 0.94 |
| IFN-Ɣ | 1.31 | 0.90 – 1.93 | 0.16 | 1.09 | 0.94 -1.27 | 0.25 |
| TNF-α | 1.29 | 0.89 – 1.87 | 0.17 | 1.16 | 0.95 – 1.41 | 0.14 |
| CCL4 | 1.14 | 0.82 – 1.59 | 0.42 | 0.95 | 0.80 – 1.12 | 0.52 |
| CCL2 | 0.92 | 0.62 – 1.36 | 0.67 | ***0.79*** | ***0.65 – 0.97*** | ***0.02*** |
| CCL13 | 0.68 | 0.42 – 1.08 | 0.10 | 0.81 | 0.64 - 1.02 | 0.08 |

| | | | | | | |
|---|---|---|---|---|---|---|
| IL-4 | 1.03 | 0.81 – 1.31 | 0.80 | 1.02 | 0.92 – 1.14 | 0.71 |
| IL-5 | 1.13 | 0.77 – 1.65 | 0.53 | 1.17 | 0.97 – 1.41 | 0.11 |
| IL-13 | 1.31 | 0.85 – 2.03 | 0.22 | 1.05 | 0.84 – 1.32 | 0.65 |
| CCL11 | 0.74 | 0.47 – 1.17 | 0.20 | 0.77 | 0.61 - 0.97 | 0.03 |
| CCL26 | ***0.77*** | ***0.63 – 0.94*** | ***0.01*** | 0.95 | 0.84 – 1.06 | 0.35 |
| CCL17 | 0.75 | 0.54 – 1.04 | 0.09 | ***0.82*** | ***0.68 – 0.98*** | ***0.03*** |
| CCL22 | ***0.75*** | ***0.57 – 0.98*** | ***0.03*** | 0.90 | 0.77 – 1.05 | 0.16 |
| IL-1β | 1.08 | 0.83 – 1.41 | 0.56 | ***1.18*** | ***1.03 – 1.35*** | ***0.02*** |
| IL-17A | 1.18 | 0.93 – 1.50 | 0.18 | ***1.13*** | ***1.00 – 1.28*** | ***0.05*** |
| CXCL8 | 1.09 | 0.59 – 2.01 | 0.78 | 1.18 | 0.87 – 1.60 | 0.29 |
| TGF-β1 | ***0.58*** | ***0.36 – 0.92*** | ***0.02*** | ***0.73*** | ***0.58 – 9.91*** | ***0.01*** |
| IL-10 | 1.23 | 0.86 – 1.76 | 0.25 | 1.04 | 0.86 – 1.25 | 0.67 |
| IL-2 | 1.21 | 0.78 – 1.88 | 0.39 | 1.17 | 0.93 – 1.47 | 0.17 |

**B)**

| **Immune mediator** | **AR** GMR | Confidence interval (CI) | p-value | **SPT** GMR | Confidence interval (CI) | p-value |
|---|---|---|---|---|---|---|
| IL-12p70 | 1.20 | 0.84 - 1.72 | 0.32 | 1.16 | 0.80 - 1.68 | 0.44 |
| CXCL10 | 0.93 | 0.68 - 1.27 | 0.63 | 0.88 | 0.62 - 1.25 | 0.48 |
| IFN-Ɣ | 1.13 | 0.80 - 1.59 | 0.50 | 0.85 | 0.64 - 1.12 | 0.23 |
| TNF-α | 1.29 | 0.88 - 1.90 | 0.19 | 1.12 | 0.73 - 1.71 | 0.60 |
| CCL4 | 1.05 | 0.75 – 1.48 | 0.77 | 0.76 | 0.50 - 1.14 | 0.18 |
| CCL2 | 0.75 | 0.50 – 1.14 | 0.18 | 1.24 | 0.81 - 1.91 | 0.33 |
| CCL13 | ***0.48*** | ***0.29 – 0.79*** | ***0.00*** | 1.31 | 0.79- 2.18 | 0.30 |
| IL-4 | 1.14 | 0.89 – 1.46 | 0.31 | 0.96 | 0.76 - 1.21 | 0.71 |
| IL-5 | 1.29 | 0.88 – 1.89 | 0.19 | 1.28 | 0.84 - 1.96 | 0.25 |
| IL-13 | 1.24 | 0.77 – 1.99 | 0.37 | 1.56 | 0.93 - 2.59 | 0.09 |
| CCL11 | 0.83 | 0.53 – 1.28 | 0.39 | 1.06 | 0.64 - 1.75 | 0.83 |
| CCL26 | 0.82 | 0.67 – 1.01 | 0.06 | 0.77 | 0.61 - 0.96 | **0.02** |
| CCL17 | 0.84 | 0.59 – 1.19 | 0.32 | 1.24 | 0.83 - 1.85 | 0.30 |
| CCL22 | ***0.73*** | ***0.56 – 0.96*** | ***0.02*** | 0.84 | 0.62 - 1.14 | 0.25 |
| IL-1β | 1.24 | 0.94 – 1.64 | 0.12 | 0.99 | 0.74 - 1.34 | 0.96 |
| IL-17A | 1.11 | 0.87 – 1.43 | 0.39 | 1.01 | 0.78 - 1.32 | 0.92 |
| CXCL8 | 0.93 | 0.51 – 1.71 | 0.82 | 1.15 | 0.58 - 2.28 | 0.69 |
| TGF-β1 | 0.86 | 0.55 – 1.34 | 0.50 | 1.38 | 0.85 - 2.23 | 0.20 |
| IL-10 | 1.13 | 0.77 – 1.65 | 0.54 | 1.18 | 0.79 - 1.78 | 0.41 |
| IL-2 | 1.43 | 0.93 – 2.21 | 0.10 | 1.36 | 0.82 - 2.26 | 0.23 |

**As binary variable (above median (4.69 kU/L))*

**C)**

| **Immune mediator** | **Asthma** GMR | Confidence interval (CI) | p-value | **Wheeze exacerbation** GMR | Confidence interval (CI) | p-value |
|---|---|---|---|---|---|---|
| IL-12p70 | 1.15 | 0.94 - 1.40 | 0.18 | 1.16 | 0.85 - 1.58 | 0.35 |
| CXCL10 | 0.98 | 0.82 - 1.18 | 0.87 | 1.11 | 0.85 - 1.46 | 0.45 |
| IFN-ϒ | 1.09 | 0.90 - 1.33 | 0.38 | 1.07 | 0.80 - 1.44 | 0.64 |
| TNF-α | 0.92 | 0.72 - 1.16 | 0.47 | ***0.65*** | ***0.45 - 0.93*** | ***0.02*** |
| CCL4 | 1.03 | 0.84 - 1.26 | 0.78 | 0.76 | 0.55 - 1.05 | 0.10 |
| CCL2 | ***1.28*** | ***1.00 - 1.63*** | ***0.05*** | 1.17 | 0.81 - 1.67 | 0.40 |
| CCL13 | 1.07 | 0.81 - 1.43 | 0.63 | 0.97 | 0.64 - 1.49 | 0.90 |
| IL-4 | 1.04 | 0.91 - 1.19 | 0.54 | 1.21 | 0.95 - 1.53 | 0.12 |
| IL-5 | 1.04 | 0.83 - 1.31 | 0.72 | 1.12 | 0.80 - 1.58 | 0.51 |
| IL-13 | 1.02 | 0.77 - 1.34 | 0.90 | 0.90 | 0.60 - 1.36 | 0.62 |
| CCL11 | 1.10 | 0.84 - 1.44 | 0.51 | 1.02 | 0.68 - 1.53 | 0.91 |
| CCL26 | 0.93 | 0.81 - 1.06 | 0.27 | 0.96 | 0.78 - 1.17 | 0.66 |
| CCL17 | 1.14 | 0.92 - 1.42 | 0.24 | 1.07 | 0.78 - 1.48 | 0.67 |
| CCL22 | 0.91 | 0.76 - 1.09 | 0.31 | 1.16 | 0.87 - 1.55 | 0.31 |
| IL-1β | ***0.84*** | ***0.71 - 0.99*** | ***0.04*** | ***0.75*** | ***0.58 - 0.96*** | ***0.02*** |
| IL-17A | 0.88 | 0.76 - 1.02 | 0.10 | 0.89 | 0.72 - 1.11 | 0.31 |
| CXCL8 | 1.04 | 0.72 - 1.50 | 0.85 | ***0.59*** | ***0.35 - 0.98*** | ***0.04*** |
| TGF-β1 | 1.05 | 0.81 - 1.37 | 0.71 | 1.02 | 0.69 - 1.51 | 0.92 |
| IL-10 | 1.00 | 0.80 - 1.26 | 0.99 | 1.15 | 0.81 - 1.62 | 0.43 |
| IL-2 | 0.97 | 0.74 - 1.27 | 0.83 | 1.18 | 0.79 - 1.76 | 0.41 |

## 7.4 Discussion

*Principle findings*

Distinct immune profiles were evident in neonates with later development of allergy and asthma-related diseases from 0 to 5 years of age. These findings suggest that the composition of the neonatal airway immune response predicts allergy and asthma development and that the trajectory towards respiratory diseases is evident already in pre- and perinatal life.

*Other studies*

The neonatal immune response has mainly been studied in stimulated cord blood. A recent study found that children developing allergic sensitization by age 12 months had a deficient cord blood Treg response[111], supporting the important role of the Treg response in the pathogenesis of

childhood allergy. There are, however, problems with using stimulated cord blood. First, different stimulation regimens and laboratory practices complicates the interpretation and comparability between studies; second, correct sampling of the cord blood by needle puncture is important in order to decrease the contamination of cord blood with maternal blood[98]. This is mainly examined for IgE measurements in cord blood, but may also be true when studying cytokine concentrations in cord blood.

Studies of nasal secretion have shown that individuals suffering from allergic rhinoconjunctivitis have an increased production of pro-inflammatory markers and a down-regulated Treg response[112]. Also, we have shown that neonates developing elevated specific IgE and rhinoconjunctivitis until age 5 years had reduced levels of CCL22 in cord blood, a chemokine mainly attracting Treg cells to the mucosa[93]. To our knowledge, airway immune profile changes prior to development of disease have not been examined.

*Meaning of the study*

Our study is the first to show that distinct immune profiles in the airways of healthy neonates precede development of allergy- and asthma-related diseases later in childhood; with these results we gain insight into the pathogenesis of these very common childhood disorders. Children with elevated specific and total IgE at 6 and 18 months were characterized by a neonatal airway immune profile with down-regulation of the T regulatory related mediator, TGF-β1. Down-regulation of TGF-β1 will lead to an increased production of Th1 and Th2 related cells from naïve CD4+ T-cells, through an increase in the transcription factors T-bet and GATA-3[113]. Especially, GATA-3 has been found to be a master transcription factor for a Type2 based immune response, early T-cell development, and inducer of inflammation[114–116]. Hence, a reduction of TGF-β1 in the children with a positive specific IgE will ultimately lead to an up-

regulation of Type 2 cells. This is in line with previous findings of deficient Treg responses being involved in the development of childhood allergy[111].

Children developing a positive SPT did not display down-regulation of TGF-β1, as seen for children with elevated specific IgE. This suggests a different basic mechanism of elevated blood specific IgE and skin reactivity towards allergens and is in accordance with a previous report showing a poor overlap between a positive SPT and elevated sIgE in young children[117].

We observed another specific immune profile in neonates who developed asthma, wheeze exacerbations and LRTI later in childhood. These immune profiles were characterized by a down-regulation of IL-1β, involved in immune activation and neutrophilic recruitment via Th17 expansion[118]. In wheeze exacerbations, we also saw a down-regulated TNF-α and CXCL8. These mediators are important for combating respiratory bacterial infections via neutrophilic recruitment and activation[119], and these findings are in line with studies showing a role of early bacterial airway colonization in the origins of asthma[66]. Asthma development was also associated with upregulated CCL2, which is involved in recruitment of CCR1- and CCR2-expressing immune cells, including basophils, Th2, Th1, CD8+ T cells, monocytes and immature dendritic cells[120]. The dissimilarities between the immune profiles associated with allergy vs. asthma/wheeze outcomes conforms with the observation of most asthma/wheeze during preschool age being non-atopic[53].

*Strengths and limitations*

Since the airways represent the target organ of allergic rhinoconjunctivitis and asthma, as well as first line of defense against airway exposures, it is an advantage that the sampling was performed

in the airways, compared to measurements of systemic mediators in blood. Furthermore, the immune response in the airway mucosal lining fluid was assessed *in vivo* by a validated method of collecting and measuring cytokines and chemokines[45].

We analysed 20 cytokines and chemokines; these were chosen *a priori* to represent major pathways of the immune system, which represented both innate and adaptive mediators. This was done in order to provide a representative view of mediators produced by the different airway epithelial and immune cells.

In this study, we performed a total mediator normalization approach of the cytokine and chemokine mediator levels (sample centering). This was done to minimize the impact of individual differences in fluidic excretion dynamics. A PCA model was applied to the normalized immune mediator data, since the mediators are highly correlated. The multivariate PCA model can be used for unravelling the cytokine to cytokine covariance structure, as well as circumventing the issue of multiple testing, raised by the high number of mediators.

We collected information about the clinical endpoints from parent reported diary records and from acute visits to the research unit at the onset of respiratory or allergic symptoms. Compared to questionnaire-based diagnostics, we were able to obtain more comprehensive and precise information about the outcomes with this approach. It is also a strength that the objective assessments and measurement of specific IgE and SPT were done repetitively during childhood.

The major limitation of this study is that it is observational; and although the reported associations are biologically plausible, we cannot establish causality between the neonatal airway immune composition and respiratory health and disease. Furthermore, the cross-sectional design of our immune characterization does not allow us to speculate whether the profiles associated with the endpoints were dynamic over time or represented stationary immune profiles.

## 7.5 Conclusion and perspectives

We found that distinct neonatal airway immune profiles are associated with clinical symptoms of allergy and asthma later in childhood with plausible altered immune profiles suggestive of a pathophysiological role in the inception of disease. These findings further support the hypothesis that childhood asthma and allergy originate in early life; and in susceptible individuals, environmental exposures are involved in initiating early immune perturbation and a trajectory towards chronic airway inflammation.

*Future studies*

Our data suggest that distinct airway immune profiles exist, especially for key T regulatory and Type 2 related mediators. It would be of interest to expand the numbers of analysed mediators to gain incremental knowledge of the intricate changes in immune function observed. Furthermore, studies on how these distinct immune profiles track through childhood could provide further knowledge of the relationship between immune mediators and respiratory disease. Being able to identify, from the neonatal period and onwards, by a non-invasive method, children on the trajectory towards development of disease would mean a great progress in our understanding and ability to predict and possibly prevent these very common diseases. We have previously shown that a number of pre- and perinatal exposures are imprinted in the neonatal airway immune response[10,43,52,56], but the programming effect of each exposure should be explored further. Additionally, common traits exists between immune mediated diseases such as asthma, connective tissue disorders, inflammatory bowel disease, and juvenile idiopathic arthritis[121]. Future studies may need a wider approach, e.g. mucosal/fluid sampling from different sites, to understand this interrelationship.

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# Appendix

Paper I - IV

**Paper I:**

# Non-invasive sampling of mucosal lining fluid for *in situ* quantification of upper airway immune mediators

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**KEYWORDS:**

Cytokines; chemokines; mucosal lining fluid; asthma; allergy; electrochemoluminescence-based immunoassay

**SHORT ABSTRACT:** (40 words)

This protocol describes a non-invasive technique for sampling of undisturbed mucosal lining fluid from the upper airways, by which quantification of *in situ* levels of protein mediators such as cytokines and chemokines can be performed in subjects of all ages.

**LONG ABSTRACT:** (296 words)

This protocol describes a non-invasive technique for sampling of undisturbed upper airway mucosal lining fluid in subjects of all ages and the following extraction procedure used prior to analysis of immune mediators in the fluid eluates. The purpose of such technique is to study the *in situ* airway topical immune signature without the need for unphysiological stimulation procedures used by other techniques.

The mucosal lining fluid is sampled on a strip of filter paper placed at the anterior part of the inferior turbinate and left for 2 min of absorption. Analytes are eluted from the filter papers after addition of identical volumes of buffer to all samples. Thereafter, the extracted protein-based eluates are analyzed by an electro-chemoluminescence-based immunoassay, allowing for high-sensitivity quantification of low-level analytes within a complex matrix. We measured *in situ* levels of 20 preselected immune mediators related to specific immune signaling pathways in the upper airway mucosa, but the technique is not limited to that specific panel or sampling site.

The technique was first implemented in 7-year-old children from the Copenhagen Prospective Studies on Asthma in Childhood$_{2000}$ (COPSAC$_{2000}$) cohort with allergic rhinitis, and thereafter used in the longitudinal COPSAC$_{2010}$ birth cohort, where sampling was performed at 1 month, 2 years and 6 years of age and at acute respiratory symptoms. We were able to obtain and

analyze samples from 620 (89%) of 700 children at age 1 month, with few samples below the detection limit: median (IQR), 29 (7.25 – 119.5) for the respective analytes.

This technique enables *in situ* quantification of the airway mucosal immune profile from birth and can be applied longitudinally, which has important applications for studying the effect of genetics and early life environmental exposures, the pathophysiology, endotyping and monitoring of respiratory diseases and for developing and evaluating novel therapeutics.

**INTRODUCTION:** (608 words)

The mucosal lining fluid of the nose comprises the liquid part of the upper airway system and consists of a complex matrix of mediators derived from the interplay between epithelium and immune cells that make up the first line of defense against invading microorganisms. The nasal mucosa is easily accessible, and there is a strong functional and immunological relationship between the nose and bronchi[1]. This compartment is of special interest in relation to airway diseases such as asthma and allergic rhinitis that are common in childhood, but also in a range of other respiratory disorders more prevalent later in life.

We here describe the implementation of a method to sample undisturbed mucosal lining fluid from the nasal cavity by a filter paper-based non-invasive technique, and the following extraction procedure used to elute protein-based analytes from the filter papers prior to their quantification. Such technique can for example be used to obtain an *in situ* immune signature of both healthy individuals and in different respiratory diseases. Furthermore, it is possible to examine exposures of importance for a specific immune signature and whether this is a predictor or mediator of later disease development.

Mucosal lining fluid has previously been obtained by nasal lavage[2] that is often preceded by a nasal challenge test, where an allergen is introduced in high levels to stimulate an inflammatory response[3,4]. However, the nasal lavage technique is not feasible for young children and it introduces an unknown dilution factor, which makes up a significant confounder that might dilute the mediators to below the detection limit of the following assay[5]. Moreover, due to the uncontrollable dilution factor, the measured analyte responses from the nasal challenge tests are not comparable between individuals, thereby limiting the usefulness of the nasal lavage technique in a cohort setting. This problem is circumvented in the presented filter paper-based

technique for nasal lining fluid collection, where the individual secretion of fluids and analyte levels are the only factors accounting for inter-individual variance.

During the extraction procedure, analytes are eluted from the filter papers after addition of identical volumes of buffer to all samples. This favors similar *ex vivo* dilution of all samples. A sample buffer of albumin-based isotonic salt solution is employed for the extraction step that enables extraction of protein-based mediators. To avoid protein degradation during the extraction phase, we added a cocktail of protease inhibitors to the extraction buffer.

Implementation of techniques that allows for quantification of undisturbed *in situ* generated immune mediators at mucosal sites is of utmost importance. First of all, the mucosal site makes up the largest immunological organ in the body. Secondly, the nasal location is the primary site of airborne exposures and is tightly connected to the respiratory immunological compartment of the lungs[1]. Thirdly, the possibility of surveying this important organ by a non-invasive technique will open up a plethora of information on the important microbe-immune interaction axis in relation to health and disease issues in the airways. Fourthly, there are many other possible applications of this technique such as studying local immunological alterations in randomized controlled trials of drugs and micronutrients.

We initially implemented the technique in the Copenhagen Prospective Studies on Asthma in Childhood$_{2000}$ (COPSAC$_{2000}$) cohort, where we determined the immune profile of the nasal lining fluid in 7-year-old children with allergic rhinitis vs. healthy controls[13]. Following that, we have applied this technique with great success to the longitudinal COPSAC$_{2010}$ cohort and assessed the airway immune profile as early as 1 month of age, 2 years, 6 years, and at acute respiratory symptoms. Results from the 1 month old neonates have demonstrated important associations between the immune signature and early life environmental exposures[7–12].

**PROTOCOL:**

*1. Experimental setup:*

1.1 Use sheets of filter paper (fibrous hydroxylatedpolyester sheets from Accuwik Ultra (cat no. SPR0730, Pall Life Sciences, Portsmouth, Hampshire, UK)[6,7]) to cut out strips in the size of 3 x 15-mm for the 1 month old child, and in an L-shape for all later samples (size 5 x 20 mm x 10 mm (short arm of the L) (**Figure 1**).

1.2 Insert one filter paper in each nostril, using the long arm of the L-shaped filter paper. The

filter paper should be placed at the anterior part of the inferior turbinate.

1.3 Apply a nose clip to minimize the discomfort and avoid accidental rejection of the filter paper.

1.4 After 2 minutes of absorption the filter papers can be removed.

1.5 Place the filter papers in Eppendorf tubes and freeze immediately at -80°C.

1.6 Record if there are any symptoms of airway infection on the day of sampling.

1.7 Record if there was sneezing, persistent crying or epistaxis during the two minutes of sampling.

*2. Quantification of airway immune mediators:*

2.1 Take up 10 random samples at a time, and record the identification numbers. Keep the samples at ice during the whole working process.

2.2 After thawing on ice, immerse the filter papers from both nostrils in 300 µL freshly prepared Milliplex Assay Buffer (Millipore, Cat no. L-AB, Billerica, Mass) containing 1 complete Protease Inhibitor Tablet (Roche) per 25 mL buffer. The volume of buffer can be adjusted according to the size of the filter paper. 300 µL buffer was used for filter papers with the size of 3 x 15-mm; if only one filter paper is available, then use half the buffer volume (150 µL).

2.3 Transfer the moist filter papers and assay buffer in the cup of a cellulose acetate tube filter (0.22 µm pore size) placed within an Eppendorf tube (Spin-X Centrifuge Tube Filter, Cat no. CLS8161, Sigma-Aldrich, St Louis, Mo).

2.4 Centrifuge for 5 minutes in a cooled centrifuge at 16,000 *g*.

2.5 Transfer the supernatant into Eppendorf tubes on ice and aliquot 100 µL nasal extract per well into low-protein binding storage plates (cat no. 249944, Thermo Scientific, Rochester, NY).

2.6 Store at -80°C until analysis.

2.7 Concentrations of cytokines and chemokines in supernatants were determined by use of the high-sensitivity electrochemoluminesce-based MesoScale Discovery multiplexed array system (Human 10-plex $T_H1/T_H2$ cytokine assay and 9-plex chemokine assay, and singleplex IL-17A, TGF-β1and TSLP). The levels of IL-1β, IL-2, IL-4, IL-5, IL-10, IL-12p70, IL-13, IL-17A, IFN-γ, TNF-α, CXCL8 (IL-8), Transforming growth factor beta1 (TGF-β1), CCL11 (eotaxin-1), CCL26 (eotaxin-3), CXCL10 (IP-10), CCL2 (MCP-1), CCL13 (MCP-4), CCL22 (MDC), CCL4 (MIP-1β), CCL17 (TARC) and TSLP in the eluates were quantitated. Assays were conducted according to standard manufacturer's protocols except for incubation with eluates overnight at 4°C. Samples were read with the Sector Imager 6000. The MesoScale Discovery immunoassays has typically a high dynamic range for measurement ranging between 1 and 10,000 pg/mL, but for some assays it even expands to 100,000 pg/mL. This means that samples can be run at the same dilution, hence limiting the influence from dissimilar dilution of healthy and diseased subjects, as is the case with other similar immunoassays. The sensitivities for all cytokines were 1 pg/mL or less, and for chemokines the sensitivity ranged between 1 and 50 pg/mL. TSLP was not detectable in 98% of samples at 1 month of age.

3. Statistics

3.1 In order to obtain normally distributed residuals of the mediator levels, we log transformed data prior to analyses. For every immune mediator, a number of samples were below the detection limit. We did not change the mediator values for samples with a read-out concentration between zero and the assay detection limit, but samples with zero values were set to half of the minimum concentration detected. This strategy was applied in order to be able to perform a log transformation, without loss of informative low range data. A few samples contained missing data due to collection of only one filter paper, limiting the number of assays that could be performed on the eluate. We decided not to include samples with missing data in the data analyses.

3.2 We performed the laboratory analysis of the 1 month samples in two batches, and corrected the cytokine and chemokine data for batch by batch-wise centering on log transformed data. This was followed by addition of the overall mean and anti-log transformation to get data in original units.

3.3 Analyses of the univariate associations between mediator levels and different explanatory variables were performed using linear regression with the transformed mediator levels as the outcome variables and the pre- and perinatal exposures as the explanatory variables. Results were reported as geometric mean ratios (GMR) of the mean mediator levels for children with the exposure versus no exposure (± 95% confidence intervals (CI)).

3.4 A multivariate model is also a good approach to analyze the multiple immune mediator data. A Partial Least Squares Discriminant Analysis (PLS-DA) or a Principal Component Analysis (PCA) can be employed in order to unravel the cytokine to cytokine covariance structure relevant for discriminating children with and without a given exposure. The multivariate approach is especially useful to handle immune mediator data as these are highly correlated.

The studies were conducted in accordance with the guiding principles of the Declaration of Helsinki. Approval by the Ethics Committee for Copenhagen (KF 01-289/96 – for $COPSAC_{2000}$ and H-B-2008–093 for $COPSAC_{2010}$) and the Danish Data Protection Agency was achieved, and written informed consent was obtained from both parents before enrollment.

**REPRESENTATIVE RESULTS:**

*Baseline characteristics of the airway immune profiles*

Complete data on upper airway mucosal immune mediator levels at age one month was obtained in 620 (89%) of the 700 children enrolled in the $COPSAC_{2010}$ cohort. Ten neonates were enrolled before the technique was established, and 19 did not attend the 1-month visit. Additionally, 47 samples were excluded because they were extracted and measured in another laboratory used in a pilot study, and 4 samples were lost in transportation (See study flow chart, **Figure 2**).

Most of the mediators had a low level of detection < 10 pg/ml (IL-1β, IL-2, IL-4, IL-5, IL-10, IL-12p70, IL-13, IL-17A, TGF-β1, IFN-γ, TNF-α, CXCL8, CCL13, CCL4, CCL17), whereas 5 had a detection level >10 pg/mL (CXCL10, CCL2, CCL11, CCL26, CCL22) (**Table 1**). The median (inter quartile range (IQR)) number of samples below detection limit (i.e. from 0 to detection limit) for each mediator was: 29 (7.25 – 119.5) (**Table 1**). IFN-γ levels were below the lower detection limit in almost half (46%) of the samples, whereas CXCL8 and IL-1β levels were detectable in all samples and TGF-β1, CCL4 and TNF-α levels were below the lower detection limit in <1% of the samples. One of the mediators, TSLP, was only detected in 2% of samples and TSLP data was therefore not used for consecutive data analyses.

A strong inter-correlation was evident for the 20 detected immune mediator levels, as is visualized in a heat map illustrating that all the mediators are positively correlated (**Figure 3**).

*The influence of airway bacteria and viruses in neonates*

An association was found between the airway immune profile and colonization with specific airway bacteria sampled concomitantly with mucosal lining fluid at age one month. An up-regulated Type 1 and Type 17-based airway immune profile was observed in neonates colonized with intracellular bacteria, whereas presence of extracellular bacteria was associated with a Type 17-based profile[7]. Presence of picornavirus was associated with an upregulated profile comprised mainly of Type 1 immune mediators (**Figure 4**)[11]. These findings were obtained in children without respiratory symptoms, e.g. asymptomatic, on the day of sampling, suggesting an immune triggering role of colonizing bacteria and viruses in the earliest part of life.

*The influence of pre- and perinatal exposures*

We further observed an association between the level of immune mediators at age one month and a maternal history of atopy[8], where children born to atopic mothers displayed an overall lower level of immune mediators than children of non-atopic mothers. We subsequently identified an association between presence of siblings in the household at birth and a specific Type 1/Type 17-directed mucosal immune response with evidence of this being related to an *in*

*utero* immune priming effect, or a marker of high microbial load in the home, as it was inversely correlated to time since last childbirth (**Figure 5**)[12]. Further evidence for *in utero* immune priming effects was determined from an association between maternal H1N1 influenza vaccination status and upregulation of TGF-β1 levels in the neonatal airway[10].

*Evidence for micronutrients mechanisms of action*

In $COPSAC_{2010}$, we performed a high-dose vitamin D intervention trial during pregnancy, which showed an almost one forth reduced risk of asthma/recurrent wheeze in the offspring at age 3 years[9]. Utilizing the measured neonatal airway immune profile, we observed that children born to mothers receiving high dose vitamin D compared to placebo were characterized by an up-regulation of specific mediators, suggesting that vitamin D exerts specific immunomodulatory actions[9] (**Figure 6**).

**DISCUSSION:**

With the presented technique we were able to determine the *in situ* upper airway mucosal immune profile in children from as early as one month of age, which has not been done previously. We observed that presence of specific airway bacteria and picornavirus[7,11] as well as other pre- and perinatal exposures were mirrored in the airway immune profile of the neonates. Furthermore, we used these airway immune profile data to study the suggested mechanism of action of a randomized micronutrient trial of high-dose vitamin D, thereby underlining the validity and utility of the mucosal lining fluid technique as a non-invasive sampling technique for subsequent *in situ* quantification of airway immune mediators.

The great strength of this technique is that it is non-invasive and easy to perform, enabling longitudinal sampling of the mucosal airway immune signature from the neonatal period and onwards. Such information will provide knowledge of how the local airway immune system matures and develops throughout childhood and into adulthood. Furthermore, the sampling technique is performed in an undisturbed and *in situ* undiluted manner, which enabled us to detect biomarkers that would have been undetectable with the nasal lavage technique, where an unknown dilution factor reduces the validity of findings[13]. We used the Accuwik Ultra Medium, a synthetic, fibrous, hydroxylated polyester medium that is commercially available and designed for sample collection, storage, and conjugate release. The material is hydrophilic

with low biomolecular binding and protein-containing samples are stable during storage at -80°C. The material is highly absorbent, and the fiber surfaces have been modified to enhance water wettability.

Mucosal lining fluid sampling could also be applied to the bronchoscopic microsampling technique to assess lower airway immunology. Bronchoscopic microsampling is a new procedure for bronchoscopic diagnosis, capable of collecting local bronchial epithelial lining fluid, that utilizes a sheathed polyester fiber probe[14]. When the distal airways are reached, the probe is pushed out of the sheath tip and absorbs bronchial epithelial fluid present in the bronchial lumen. Bronchoscopic microsampling have been used in acute respiratory distress syndrome[14–16] and chronic obstructive pulmonary disease[17], whereas in asthma a problem is that the probe causes bleeding due to disease-induced epithelial fragility and increased vascularization. Applying the mucosal lining fluid technique to the bronchoscopic microsampling procedure could have important clinical implications in relation to our understanding of pathophysiology, monitoring, treating, and predicting outcome of several acute and chronic pulmonary diseases in both children and adults.

In the $COPSAC_{2010}$ study, we selected a panel of 21 cytokines and chemokines that were chosen *a priori* to represent major phenotypical pathways of the immune system acting locally in the airways. Only one of these mediators, TSLP, was not detectable at one month of age. However, all types of mediators can theoretically be measured by using the multiplex immunoassay platform by MesoScale Discovery. The platform allows for high sensitivity and accurate quantification of any mediator for which a pair of monoclonal antibodies applicable for development of an immunoassay can be obtained. This platform also brings with it high precision, while being less sensitive for pH and temperature variations, as well as variations in viscosity of the fluids to be analyzed. The use of a standard ELISA solution is not applicable to quantitate the protein eluates due to sensitivity to the viscous matrices.

In future studies it would be of great value to expand the current panel of immune mediators to measurement of e.g. antimicrobial peptides, total and specific IgA, acute phase reactants such as C-reactive protein, lipid mediators such as prostaglandins and neurological mediators such as brain-derived neurotrophic factor (BDNF). This could bring a more detailed view on the nature of the mucosal response system at a body site with extensive airborne exposures. The

suggested mediators may either be measured by means of targeted quantitative high-sensitivity immunoassays such as in the current setup, or by targeted or untargeted nuclear magnetic resonance (NMR) spectroscopy or liquid chromatography–mass spectrometry (LC-MS) based metabolomics techniques. The latter approach has already been implemented to some extent with the exhaled breath condensate method, which has some methodological drawbacks with respect to sampling of the biofluid[18] that are not apparent for the mucosal lining fluid technique.

Longitudinal sampling from the neonatal period into adulthood, as being performed in the $COPSAC_{2010}$ cohort, will greatly increase our understanding of underlying immunological factors preceding or acting concomitantly with airway mediated diseases such as asthma and allergy. Our findings may be important in the development of novel therapeutics, targeting specific disease endotypes, and in order to investigate mechanisms of action of micronutrients and drugs in randomized clinical trials.

**ACKNOWLEDGMENTS:**

We gratefully express our gratitude to the children and families of the $COPSAC_{2010}$ cohort study for all their support and commitment. We acknowledge and appreciate the unique efforts of the COPSAC research team, and the technical help from Technician Lisbeth Buus Rosholm, Technical University of Denmark for measurement of cytokines and chemokines.

**DISCLOSURES:**

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

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**Table 1:** Mucosal lining fluid samples from 1-month-old neonates from the $COPSAC_{2010}$ cohort.

| Mediator | Detection limit pg/ml | < Detection limit N (%) | Median pg/ml | IQR pg/ml |
|---|---|---|---|---|
| IL-12p70 | 1.4 | 102 (16%) | 10.80 | 4.99 - 22.22 |
| CXCL10 | 31 | 8 (1%) | 1787.78 | 722.8 - 5423 |
| IFN-γ | 4.1 | 285 (46%) | 20.14 | 7.63 - 48.51 |
| TNF-α | 0.6 | 5 (<1%) | 28.33 | 10.06 - 88.55 |
| CCL4 | 4.3 | 4 (<1%) | 194.90 | 71.22 - 655.8 |
| CCL2 | 16.0 | 9 (1%) | 256.11 | 134.4 - 468.5 |
| CCL13 | 2.8 | 13 (2%) | 15.17 | 10.35 - 22.80 |
| IL-4 | 1.1 | 196 (32%) | 1.37 | 0.42 - 2.86 |
| IL-5 | 1.2 | 172 (28%) | 3.72 | 1.42 - 8.10 |
| IL-13 | 2.7 | 48 (8%) | 14.49 | 6.84 - 27.51 |
| CCL11 | 22.0 | 39 (6%) | 139.54 | 85.00 – 236.0 |
| CCL26 | 45.0 | 252 (41%) | 164.93 | 56.33 - 350.02 |
| CCL17 | 7.2 | 90 (15%) | 41.30 | 26.83 - 60.68 |
| CCL22 | 25.0 | 78 (13%) | 398.31 | 243.4 - 575.7 |
| IL-1β | 0.4 | 0 (0%) | 225.49 | 50.07 - 1169 |
| IL-17A | 1.5 | 285 (46%) | 1.480 | 0.43 - 4.79 |
| CXCL8 | 3 | 0 (0%) | 19465 | 6321 - 38274 |
| TGF-β1 | 5.2 | 1 (<1%) | 28.66 | 20.76 - 39.50 |
| IL-10 | 1 | 13 (2%) | 22.32 | 9.28 - 49.16 |
| IL-2 | 1.5 | 19 (3%) | 18.72 | 8.29 - 37.33 |

**Figure 1:** Filter paper being inserted into the nostril of a child

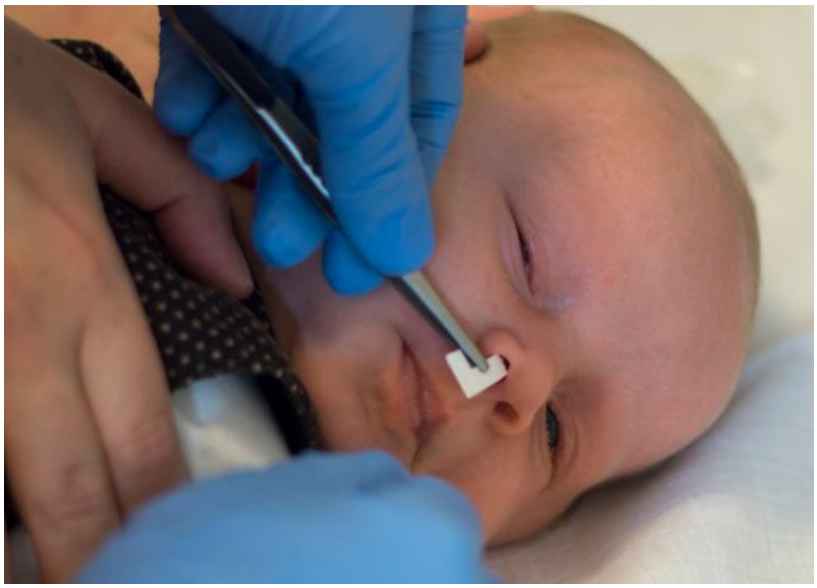


**Figure 2:** Study group flow-chart

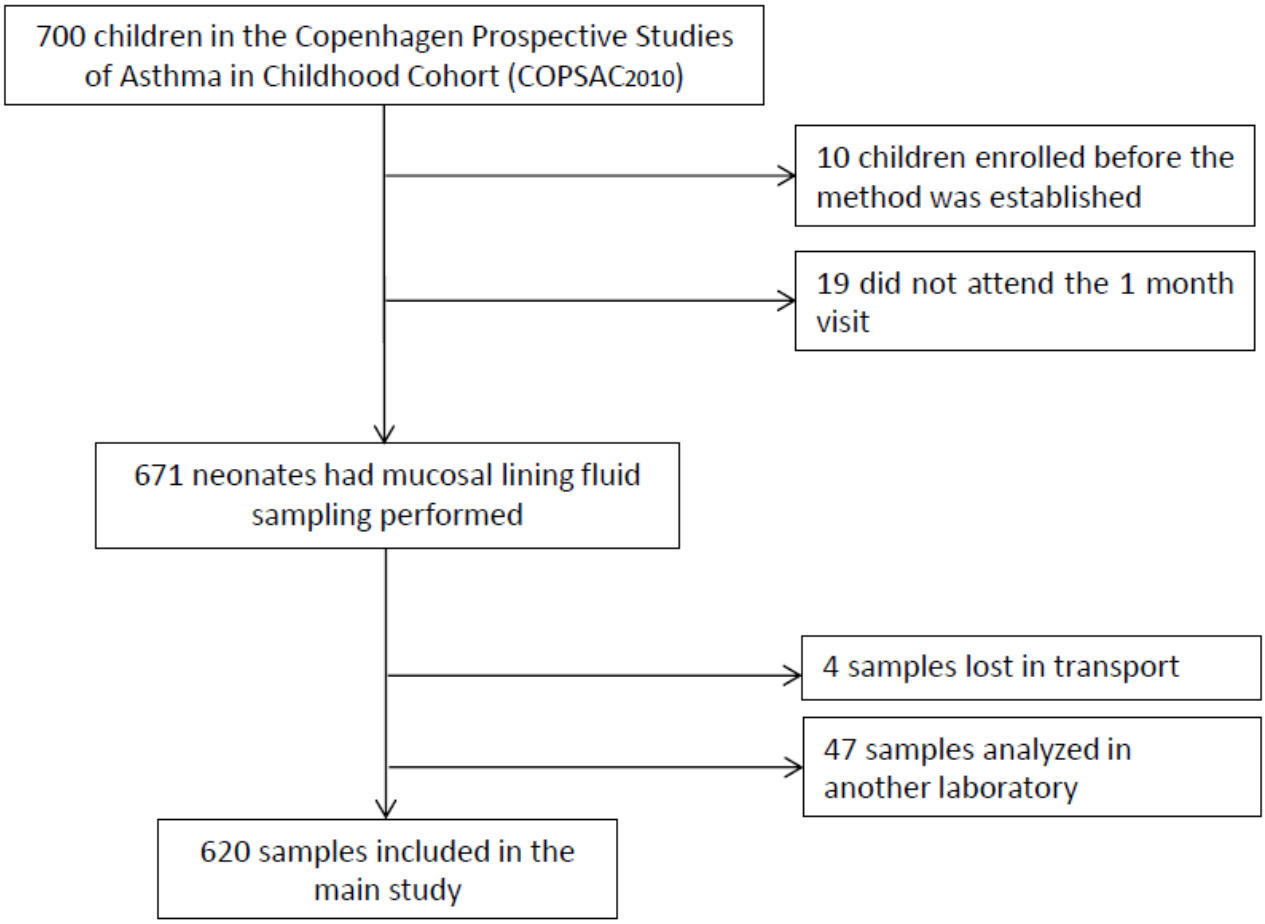


**Figure 3:** Heat map of the correlation between the 20 cytokines and chemokines. A red color represents a positive correlation and a blue color represents a negative correlation. The number inside every square is the R-value between two mediators.

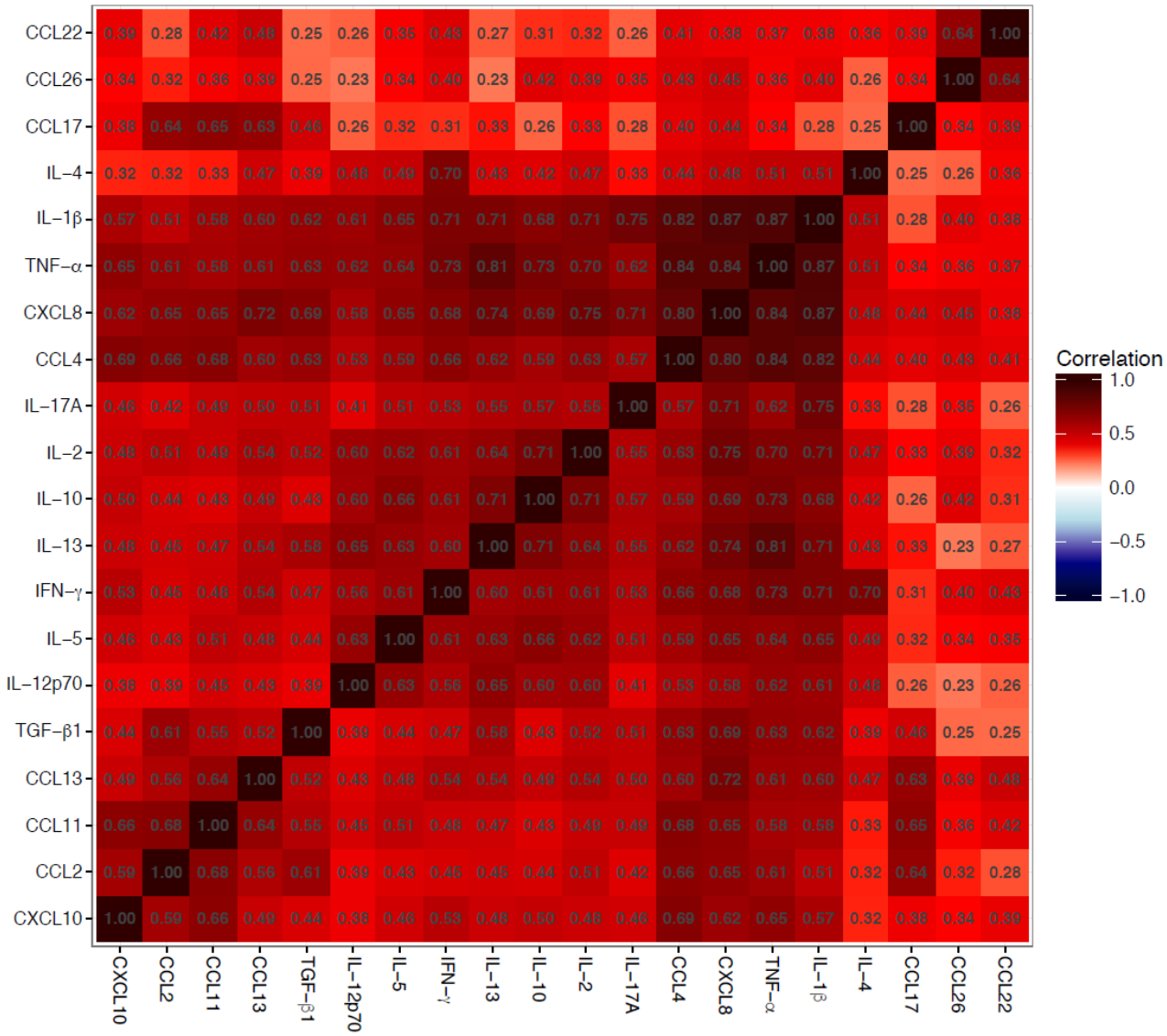


**Figure 4:** Geometric mean ratios (GMR) with 95% confidence intervals (CI) of airway mediators in neonates with/without concomitant presence of picornavirus, measured at one month of age.

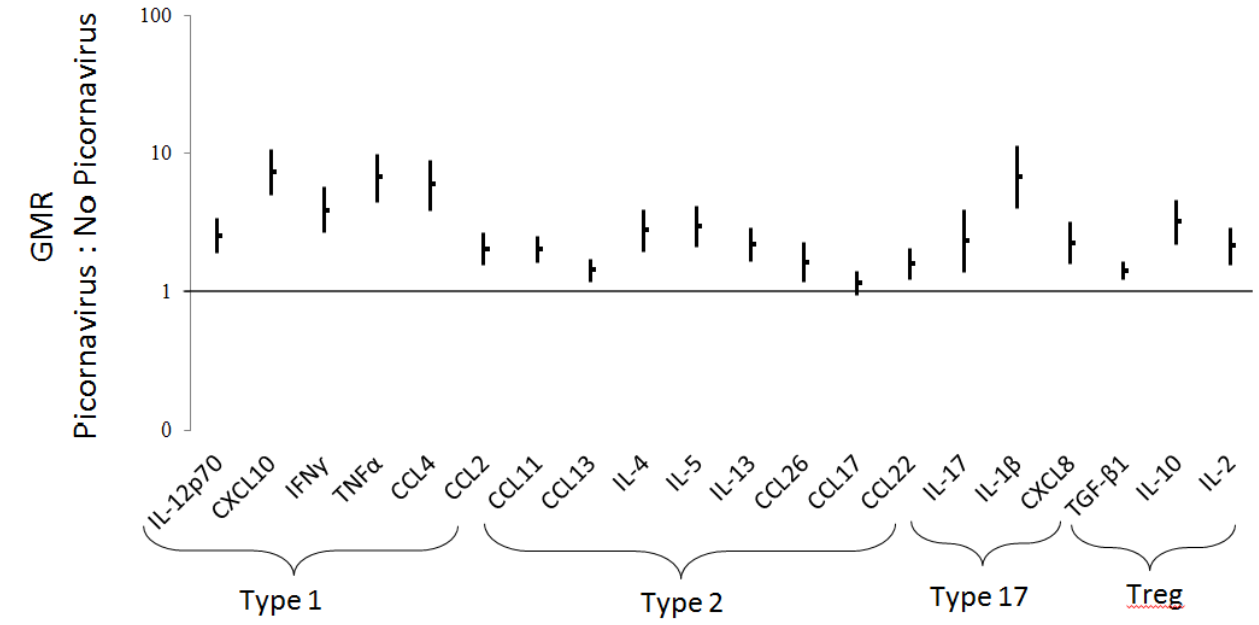



**Figure 5:** A) Airway immune signature (PC1) in neonates with and without siblings at birth. B) Scatterplot showing an inverse association between the airway immune signature (PC1) and time since previous birth for children with siblings.

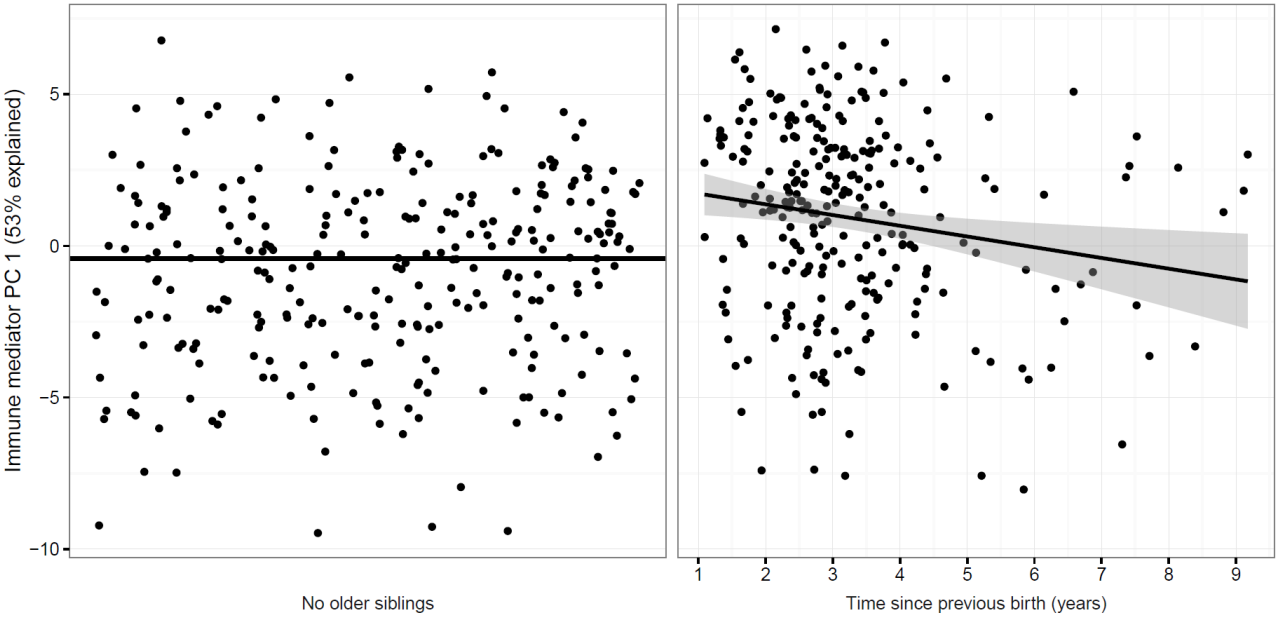
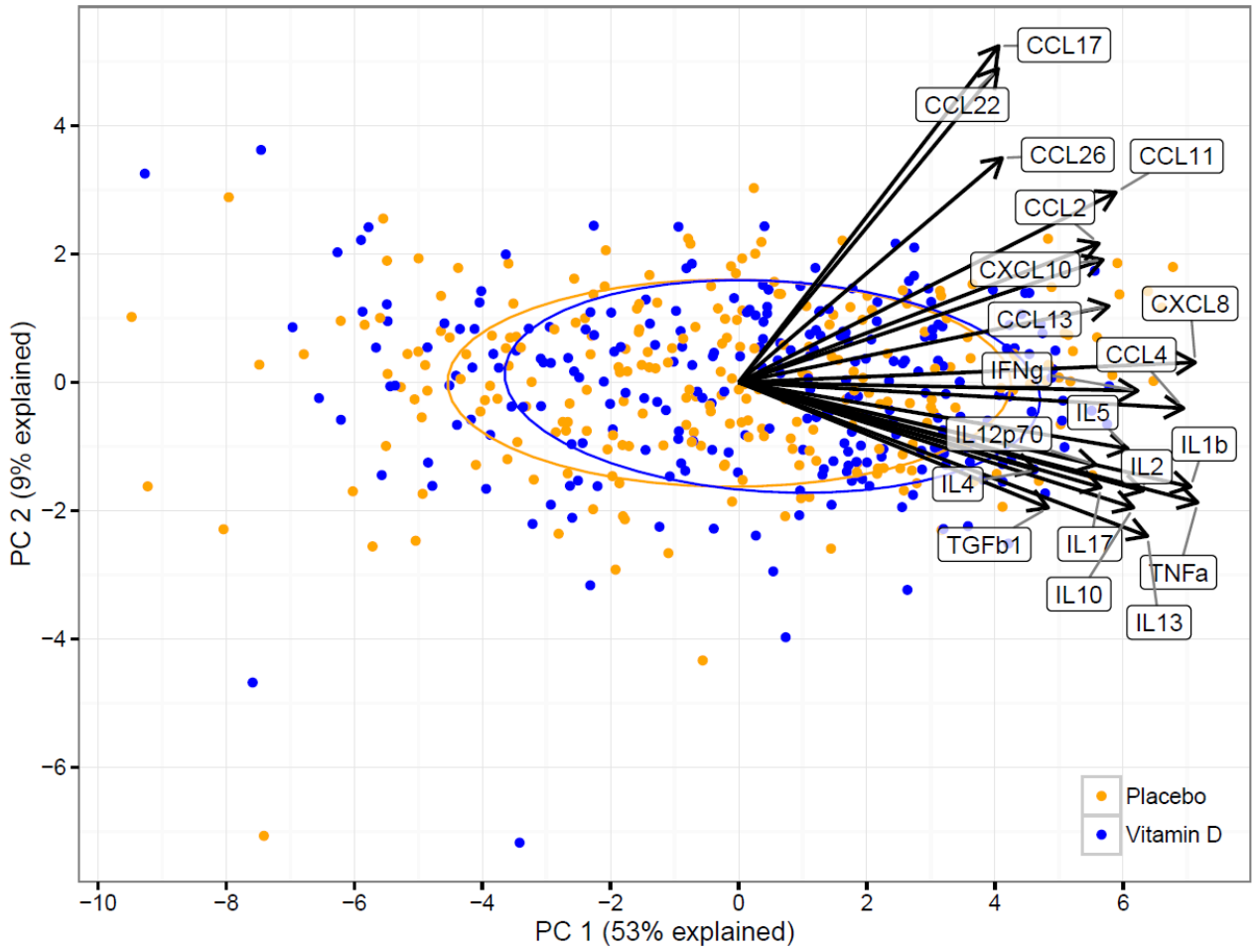


**Figure 6:** Principal component analysis biplot of the effect of the vitamin D intervention in the $COPSAC_{2010}$ cohort on the airway immune mediator level at age 1 month, showing that the intervention resulted in a significantly up-regulated immune profile in PC1.



**Paper II:**

The Journal of Infectious Diseases
MAJOR ARTICLE

# Picornavirus-Induced Airway Mucosa Immune Profile in Asymptomatic Neonates

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***Background.*** Bacterial airway colonization is known to alter the airway mucosa immune response in neonates whereas the impact of viruses is unknown. The objective was therefore to examine the effect of respiratory viruses on the immune signature in the airways of asymptomatic neonates.

***Methods.*** Nasal aspirates from 571 asymptomatic 1-month-old neonates from the Copenhagen Prospective Studies on Asthma in Childhood 2010 birth cohort were investigated for respiratory viruses. Simultaneously, unstimulated airway mucosal lining fluid was obtained and quantified for levels of 20 immune mediators related to type 1, type 2, type 17, and regulatory immune paths. The association between immune mediator levels and viruses was tested by conventional statistics and partial least square discriminant analysis.

***Results.*** Picornaviruses were detected in 58 neonates (10.2%) and other viruses in 10 (1.8%). A general up-regulation of immune mediators was found in the neonates with picornavirus ($P < .0001$; partial least square discriminant analysis). The association was pronounced for type 1– and type 2–related markers and was unaffected by comprehensive confounder adjustment. Detection of picornavirus and bacteria was associated with an additive general up-regulating effect.

***Conclusions.*** Asymptomatic presence of picornavirus in the neonatal airway is a potent activator of the topical immune response. This is relevant to understanding the immune potentiating effect of early life exposure to viruses.

**Keywords.** cytokines; chemokines; children; virus; mucosal lining fluid.

Newborn infants are exposed to microbes from the moment of birth, requiring an immediate ability to mount an appropriate immune response against commensal organisms and invading pathogens. The immune cells of the airway mucosa are the first line of defense against invading microorganisms. When activated, they release an armory of cytokines and chemokines [1], as we demonstrated elsewhere in asymptomatic neonates with bacterial airway colonization [2].

It is well established that the presence of viruses can alter the cytokine response ex vivo in peripheral blood mononuclear cells [3, 4], but it is unknown whether the presence of airway viruses in asymptomatic healthy neonates triggers a topical immune response. The aim of the current study was to investigate the in vivo activity of the immature immune system in the airway mucosa of asymptomatic neonates in response to presence of common respiratory viruses. For that purpose, we quantified the topical immune response in the airway mucosal lining fluid of 1-month-old asymptomatic healthy neonates [5] from the population based Copenhagen Prospective Studies on Asthma in Childhood 2010 ($COPSAC_{2010}$) mother-child cohort in relation to simultaneous detection of airway viruses.

## METHODS

### The $COPSAC_{2010}$ Cohort

The $COPSAC_{2010}$ cohort is an ongoing, prospective, population based clinical mother-child cohort study of 700 children recruited in Zealand, Denmark, during 2009–2010, as described in detail elsewhere [6]. At 1 month of age, the infants were brought to the clinical research unit for sampling of airway mucosal lining fluid and aspirations from nasopharynx and hypopharynx. Each infant was examined by a research pediatrician including assessment of any lower or upper respiratory infection. The assessments were performed at the COPSAC clinical research units (2 clinical research units situated on Zealand, Denmark). In addition, all families kept a day-to-day diary from birth capturing the child's respiratory symptoms between clinic visits.

### Ethics

The study was conducted in accordance with the guiding principles of the Declaration of Helsinki. Approval by the Ethics

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Committee for Copenhagen (H-B-2008–093) and the Danish Data Protection Agency was achieved, and oral and written informed consent was obtained from both parents before enrollment.

#### Airway Inflammatory Mediator Assessment in Nasosorption Samples

Unstimulated airway mucosal lining fluid was sampled at 1 month of age with 3 × 15-mm strips of filter-paper (Accuwik Ultra fibrous hydroxylatedpolyester sheets; catalog No. SPR0730; Pall Life Sciences), as described elsewhere in detail [2, 5]. The filter papers were inserted bilaterally into the anterior part of the inferior turbinate of the nasal cavity. After 2 minutes of absorption, the filter papers were removed and immediately frozen at −80°C. Prior to analyses, the filter papers were thawed and immersed in 300 μL of assay buffer, and subsequently placed in the cup of a tube filter within an Eppendorf tube and centrifuged for 5 minutes in a cooled centrifuge at 16 000*g*.

The samples were analyzed in 2 batches for levels of interleukin 12p70, CXCL10 (interferon γ–induced protein 10), interferon γ, tumor necrosis factor (TNF) α, CCL4 (macrophage inflammatory protein-1β), CCL2 (monocyte chemoattractant protein [MCP]-1), CCL11 (eotaxin-1), CCL13 (MCP-4), interleukin 4 (IL-4), interleukin 5 (IL-5), interleukin 13 (IL-13), CCL26 (eotaxin-3), CCL17 (thymus and activation regulated chemokine), CCL22 (macrophage-derived chemokine), interleukin 17A (IL-17A), interleukin 1β (IL-1β), CXCL8 (interleukin 8), transforming growth factor β1 (TGF-β1), interleukin 10 (IL-10), and interleukin 2 (IL-2). The sensitivities were ≤1 pg/mL for all cytokines and 1–50 pg/mL for chemokines, as described elsewhere [5, 7]. The lower limit of detection was set as the mean signal from blanks plus 3 standard deviations.

Selection of the measured cytokines and chemokines was decided a priori to represent mediators associated with different types of immune responses that we grouped into type 1 (T-helper [Th] 1/$CD8^+$/natural killer /innate lymphoid cell [ILC] 1), type 2 (Th2, eosinophils, ILC2), type 17 (Th17, neutrophils, ILC3), and regulatory type responses [5, 8, 9]. This was based on the present understandings of which cell types mainly produce the given mediators and/or are affected by the mediators.

#### Detection of Airway Viruses in Nasopharyngeal Aspirates

Nasopharyngeal sampling was performed at age 1 month and done after the sampling of mucosal lining fluid. The samples were collected via one of the nostrils and diluted in 1 mL of isotonic saline. Specimens were frozen and stored at −80°C until shipment to Imperial College, London, United Kingdom, for RNA extraction and further analysis with reverse-transcriptase polymerase chain reaction (PCR).

After extraction the RNA was reverse-transcribed to produce complementary DNA representative of all RNA species in the original clinical sample [10]. This complementary DNA was then used in a panel of PCR assays specific for respiratory syncytial viruses (RSVs) A and B [11], influenza A (H1 and H3) and B [12], and picornaviruses [13]. Rhinoviruses were differentiated from enteroviruses by means of restriction enzyme digestion of the PCR product from all picornavirus-positive tests with *Bgl*I [13] and subsequent gel electrophoresis.

#### Statistics

Data were log-transformed before analyses to obtain normally distributed residuals of the mediator levels. Probabilistic principal component analysis of the mediator levels was used to select among a list of candidate covariates (batch of mucosal lining fluid, season of sampling, location of sampling, pathogenic airway bacteria, older siblings, maternal antibiotics consumption, smoking in the third trimester, and influenza virus). Using multiple linear regression analysis with the first principal component as the response variable, significant predictors ($\alpha = 0.05$) were selected among the potential covariates and included as covariates in the analyses. Furthermore, based on our previous studies [3, 8], a maternal history of asthma, allergy, or eczema and detection of any of the pathogenic airway bacteria *Streptococcus pneumoniae, Haemophilus influenzae,* or *Moraxella catarrhalis* were also included as covariates in all statistical models.

The univariate associations between mediator levels and presence of any of the respiratory viruses were analyzed using analysis of variance, with the transformed mediator levels as the outcome variables and presence of viruses as well as possible confounders as the explanatory variables. Results were reported as geometric mean ratios (GMR) of the mean mediator levels, for neonates with a virus detected versus no virus detection with 95% confidence intervals (CIs). For the association between bacteria and viruses, asymptotic CIs were calculated.

In addition to the univariate analysis, partial least square (PLS) discriminant analysis (PLS-DA) was used to unravel the cytokine-to-cytokine covariance structure relevant for differentiating between the children with and those without picornavirus. PLS-DA is a multivariate discrimination method that is especially powerful when the descriptive information is correlated. PLS regression was used to investigate the difference in patterns of mediator levels associated with virus. As a first step, mediator variables were imputed using probabilistic principal component analysis. The first latent PLS component was tested for any association with the viruses detected, using permutation test adjusted for the identified covariates and an analysis of variance with viruses and covariates as explanatory variables and the first latent component as outcome. Analyses were carried out using SAS (version 9.3; SAS Institute) and MATLAB R2013a (version 8.1.0.604; MathWorks) software.

## RESULTS

#### Baseline

Complete information about nasopharyngeal samples for viral detection and immune mediator assessments were available for

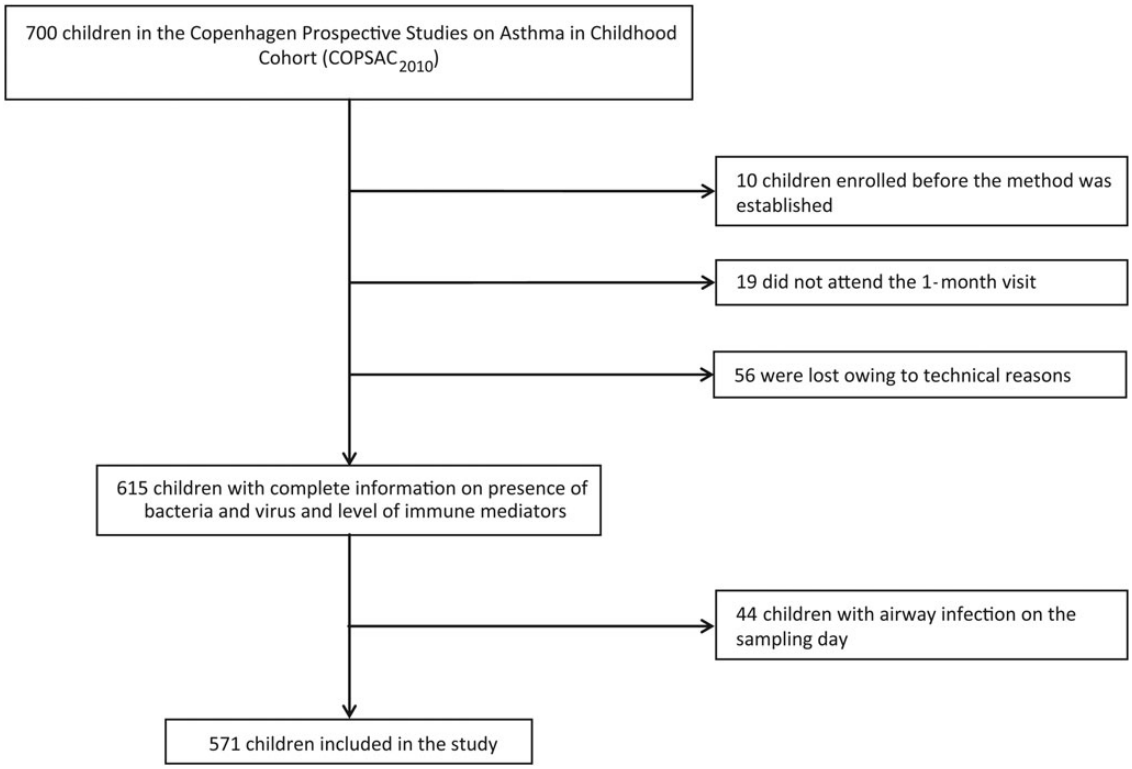


**Figure 1.** Study group flow chart.

82% (n = 571) of the neonates in the cohort after exclusion of neonates with symptoms of airway infection on the day of sampling (see Figure 1 for more details). A dropout analysis of baseline characteristics was performed comparing the 571 children included in the analyses and the 129 excluded children (Supplementary Table 1). The 2 groups were identical except for a higher household income ($P = .02$) and a lower gestational age ($P = .004$) among the excluded children.

Virus was detected in 12% (n = 68) of the 571 included neonates, 85% (n = 58) of these being picornavirus, 4% (n = 3) RSV, and 10% (n = 7) influenza virus. No children had >1 virus detected. Of the 58 picornaviruses, 81% (n = 47) were rhinovirus and 14% (n = 8) were "other picornavirus"; in the remaining 5% (n = 3), no further classification was possible. Because of the very low number of samples positive for influenza virus and RSV, we restricted the analyses to the effect of picornaviruses.

The mean age at sampling in the included children was 32 days (standard deviation, 5.4 days); 51% (292) were boys. Baseline characteristics are depicted in Table 1. The variables significantly associated with having picornavirus (older siblings, maternal smoking in the third trimester, and maternal consumption of antibiotics in the third trimester) were further tested in a multivariable backward selection analysis for association with the immune mediator levels. Older siblings and maternal smoking in the third trimester were found to affect the level of immune mediators and were included as covariates in the final models. Using backward selection with the first principal component as the response variable, sampling site, sampling season,

**Table 1. Baseline Characteristics of Children With Picornavirus Versus Controls**

| Characteristic | Children, % (No.) | | |
|---|---|---|---|
| | With Picornavirus (n = 58) | Controls (n = 513) | *P* Value |
| History of maternal asthma, allergy, or eczema | 47 (27) | 54 (276) | .34 |
| White race | 98 (57) | 96 (491) | .35 |
| High income (>130 000 euro/year) | 12 (7) | 14 (70) | .74 |
| Maternal consumption of antibiotics in 3rd trimester | 29 (17) | 19 (95) | .05[a] |
| Maternal smoking in 3rd trimester[b] | 9 (5) | 3 (16) | .05[a] |
| Young gestational age (<37 wk) | 2 (1) | 4 (19) | .44 |
| Male | 59 (34) | 50 (258) | .23 |
| Low Apgar score (<7 at 1 min) | 7 (4) | 4 (21) | .32 |
| Cesarean delivery | 21 (12) | 21 (110) | .89 |
| Older siblings | 83 (48) | 54 (276) | <.0001[a] |
| Exclusively breastfeeding at 1 month of age | 91 (53) | 93 (472) | .75 |
| Furred pets in the home | 17 (10) | 21 (108) | .5 |

[a] Significant difference ($P \leq .05$). *P* values were determined with $\chi^2$ test, except when otherwise noted.

[b] Fisher exact test.

[c] >1 units/wk (more than 1 beer/1 glass of wine/2 cl of spirits per week).

**Table 2. Median Values of Immune Mediators and Association Between Immune Mediators in Children With Picornavirus and Controls**

| Mediator | Median (IQR), pg/mL | | Crude Ratio (95% CI) | *P* Value | Adjusted[a] Ratio (95% CI) |
|---|---|---|---|---|---|
| | Children With Picornavirus | Controls | | | |
| IL-12p70 | 6.74 (2.82–13.01) | 4.91 (2.07–9.34) | 2.54 (1.88–3.42) | <.0001 | 2.16 (1.61–2.88) |
| CXCL10 | 682.13 (270.8–3664.14) | 487.0 (205.24–1570.18) | 7.22 (4.95–10.52) | <.0001 | 6.52 (4.53–9.40) |
| IFN-γ | 8.37 (3.65–20.5) | 4.76 (1.84–14.09) | 3.86 (2.65–5.62) | <.0001 | 3.47 (2.45–4.91) |
| TNF-α | 60.99 (13.49–182.57) | 27.13 (10.2–79.92) | 6.60 (4.44–9.8) | <.0001 | 4.87 (3.33–7.14) |
| CCL4 | 235.2 (76.04–1337.23) | 149.95 (53.3–497.88) | 5.85 (3.87–8.85) | <.0001 | 4.11 (2.80–6.02) |
| CCL2 | 188.79 (89.44–292.15) | 141.34 (77.65–256.92) | 2.03 (1.55–2.66) | <.0001 | 1.96 (1.52–2.53) |
| CCL13 | 21.66 (12.17–26·58) | 16.52 (11.28–25.17) | 1.42 (1.18–1.72) | <.0001 | 1.35 (1.12–1.61) |
| IL-4 | 2.85 (1.07–6.96) | 2.16 (0.68–4.7) | 2.76 (1.95–3.91) | <.0001 | 2.16 (1.53–3.05) |
| IL-5 | 4.45 (1.82–8.5) | 2.82 (1.01–5.9) | 2.98 (2.11–4.22) | <.0001 | 2.56 (1.83–3.58) |
| IL-13 | 20.5 (9.01–35.07) | 15.8 (7.84–29.73) | 2.20 (1.66–2.92) | <.0001 | 1.89 (1.42–2.51) |
| CCL11 | 114.52 (64.17–196.1) | 83.65 (51.64–138.31) | 2.01 (1.61–2.51) | <.0001 | 1.93 (1.56–2.39) |
| CCL26 | 101.81 (34.08–152.31) | 71.95 (18.53–153.05) | 1.63 (1.17–2.27) | .004 | 1.71 (1.26–2.33) |
| CCL17 | 16.72 (9.23–28.48) | 16.25 (9.84–24.82) | 1.15 (.93–1.42) | .19 | 1.24 (1.03–1.5) |
| CCL22 | 80.48 (47.51–228.63) | 72.6 (43.27–179.52) | 1.59 (1.22–2.06) | .0005 | 1.54 (1.24–1.92) |
| IL-17 | 2.46 (0.78–8.72) | 1.66 (0.43–5.54) | 2.32 (1.38–3.9) | .0015 | 1.87 (1.11–3.15) |
| IL-1β | 332.51 (35.79–1418.34) | 98.88 (22.72–518.16) | 6.71 (4.00–11.27) | <.0001 | 4.66 (2.84–7.65) |
| CXCL8 | 5482.55 (2256.12–17 229.22) | 4202.98 (1426.18–7586.25) | 2.25 (1.58–3.22) | <.0001 | 1.99 (1.46–2.73) |
| TGF-β1 | 61.93 (38.5–86.49) | 51.79 (36.46–69.27) | 1.41 (1.21–1.64) | <.0001 | 1.25 (1.08–1.43) |
| IL-10 | 33.19 (13.14–70.71) | 21.15 (8.95–45.22) | 3.18 (2.20–4.61) | <.0001 | 2.51 (1.76–3.58) |
| IL-2 | 25.66 (9.96–58.88) | 20.18 (9.52–39.75) | 2.13 (1.56–2.92) | <.0001 | 1.82 (1.34–2.47) |

Abbreviations: CI, confidence interval; IFN, interferon; IL-1β, interleukin 1β; IL-2, interleukin 2; IL-4, interleukin 4; IL-5, interleukin 5; IL-10, interleukin 10; IL-12p70, interleukin 12; IL-13, interleukin 13; IL-17, interleukin 17; IQR, interquartile range; TGF, transforming growth factor; TNF, tumor necrosis factor.

[a] Ratio adjusted for maternal history of asthma, allergy, or eczema, older siblings in the home, pathogenic airway bacteria, method of virus sampling, sampling site, season of sampling, batch of immune mediator analyses and maternal smoking in the third trimester.

and batch of immune mediator analysis were found to affect the immune mediator level and were included as covariates in the models, along with a maternal history of asthma, allergy, or eczema and detection of any of the pathogenic airway bacteria *S. pneumoniae, H. influenzae,* or *M. catarrhalis.*

#### Effect of Picornavirus on Airway Immune Mediator Profiles

We found a uniform up-regulation of all immune mediator levels in the children with picornavirus, compared with those without picornavirus. For 19 of 20 immune mediators the GMR was significantly elevated (GMR range, 1.15–7.22; 95% CI, .93–10.52) (Table 2 and Figure 2*A*). The picornavirus-driven immune response was most pronounced for CXCL10, CCL4, and TNF-α, associated with type 1–related responses, and for IL-1β, involved in type 17–associated responses via expansion of Th17 cells when produced by dendritic cells (Table 2). Adjusting the analyses did not modify the associations substantially (Table 2 and Figure 2*B*).

Neonates with bacterial airway colonization had an overall elevated GMR of the measured mediators (Supplementary Figure 1); however, the up-regulation was quantitatively smaller than the effect of picornavirus. Coexistence of picornavirus and bacterial colonization resulted in an additively increased level of all the immune mediators but with no evidence of interaction between bacteria and picornavirus ($P = .91$), solely suggesting an additive and not a synergistic effect (data not shown). The neonates with symptoms of an airway infection on the day mucosal lining fluid was sampled (n = 44) were studied further, and their levels of immune mediators were clearly elevated compared with the neonates with no sign of an airway infection on the day of sampling.

The daily diary cards were further investigated for presence of troublesome lung symptoms. Apart from the 44 with symptomatic airway infection, we found that another 15 neonates had coughing, wheezing, and/or breathlessness 1 week before and/or after the sampling day. Excluding these children from analysis did not modify our findings (data not shown).

The conventional statistical approach was accompanied by a multivariate data-driven PLS-DA to reveal the profiles of coregulated airway immune mediators. In the loading plot (Figure 3*A*), the immune mediators were all clustered in the first component, suggesting a strong intercorrelation between the immune mediator levels. In the score plot (Figure 3*B*), where each dot represented a single child, a separation was found in the first component between picornavirus-positive versus picornavirus-negative children, underscoring that the presence of picornavirus in the nasopharynx of an asymptomatic infant is immune stimulatory. In support of this observation, the PLS regression analysis showed a highly significant up-regulation of the immune mediators in children with picornavirus (confounder adjusted $P < 1 \times 10^{-5}$).

By inspecting the second component, we observed that picornavirus-positive infants generally expressed higher cytokine and chemokine levels (Figure 3*B*), which was primarily driven by

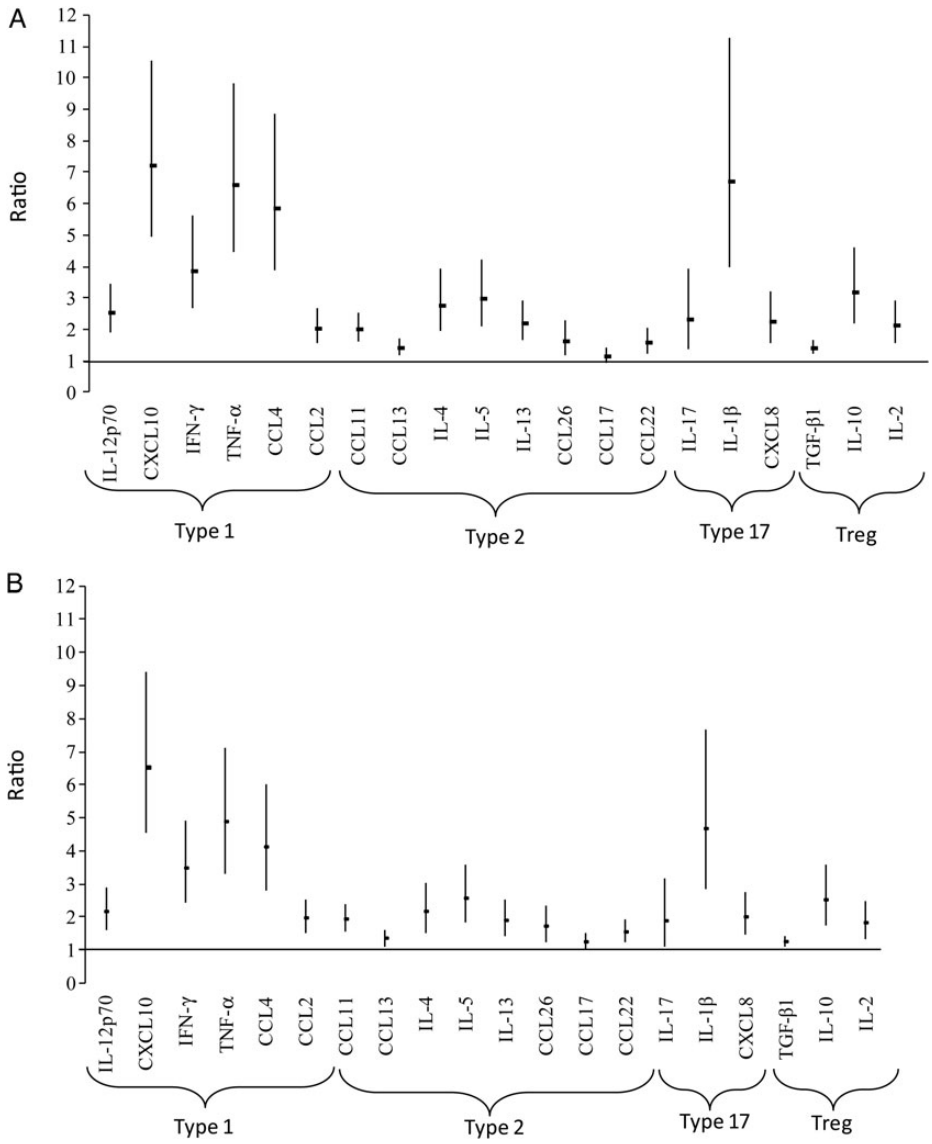


**Figure 2.** Geometric mean ratios with 95% confidence interval of cytokines and chemokines in the airway mucosal lining fluid of healthy 1-month-old neonates with or without picornavirus detected in the airway vs controls. *A*, Crude levels. *B*, Levels adjusted for maternal history of asthma, allergy, or eczema, older siblings in the home, pathogenic airway bacteria, method of virus sampling, sampling site, season of sampling, batch of immune mediator analyses, and maternal smoking in the third trimester. Abbreviations: IFN, interferon; IL-1β, interleukin 1β; IL- 2, interleukin 2; IL- 4, interleukin 4; IL-5, interleukin 5; IL-10, interleukin 10; IL-12p70, interleukin 12p70; IL-13, interleukin 13; IL-17, interleukin 17; TGF, transforming growth factor; TNF, tumor necrosis factor; Treg, regulatory T cells.

the type 1–related immune mediators CXCL10, CCL4, and TNF-α (Figure 3*A*). This is indicative of a particular association between these type 1–associated immune mediators and picornavirus, compared with the remaining investigated mediators, suggesting that picornavirus-positive children have a higher type 1–associated immune response.

## DISCUSSION

### Principal Findings

Presence of picornavirus in the airways of asymptomatic neonates affects the upper airway mucosal immune signature, with an increased release of type 1– and type 2–associated immune mediators but dominated by type 1–associated mediators of importance for clearance of intracellular pathogens. Our findings suggest that presence of picornavirus even in asymptomatic neonates promotes early topical airway immune activation and that the expected type 1–based immune enhancement by viruses is already evident in exposed newborns. The concomitant rise in type 2 immune mediators may be an underlying marker accounting for progression to asthma and allergic sensitization.

### Strengths and Limitations

A major strength of the current study is the mucosal lining fluid sampling method, providing direct biomarker data on levels of immune mediators in vivo in the target organ of respiratory

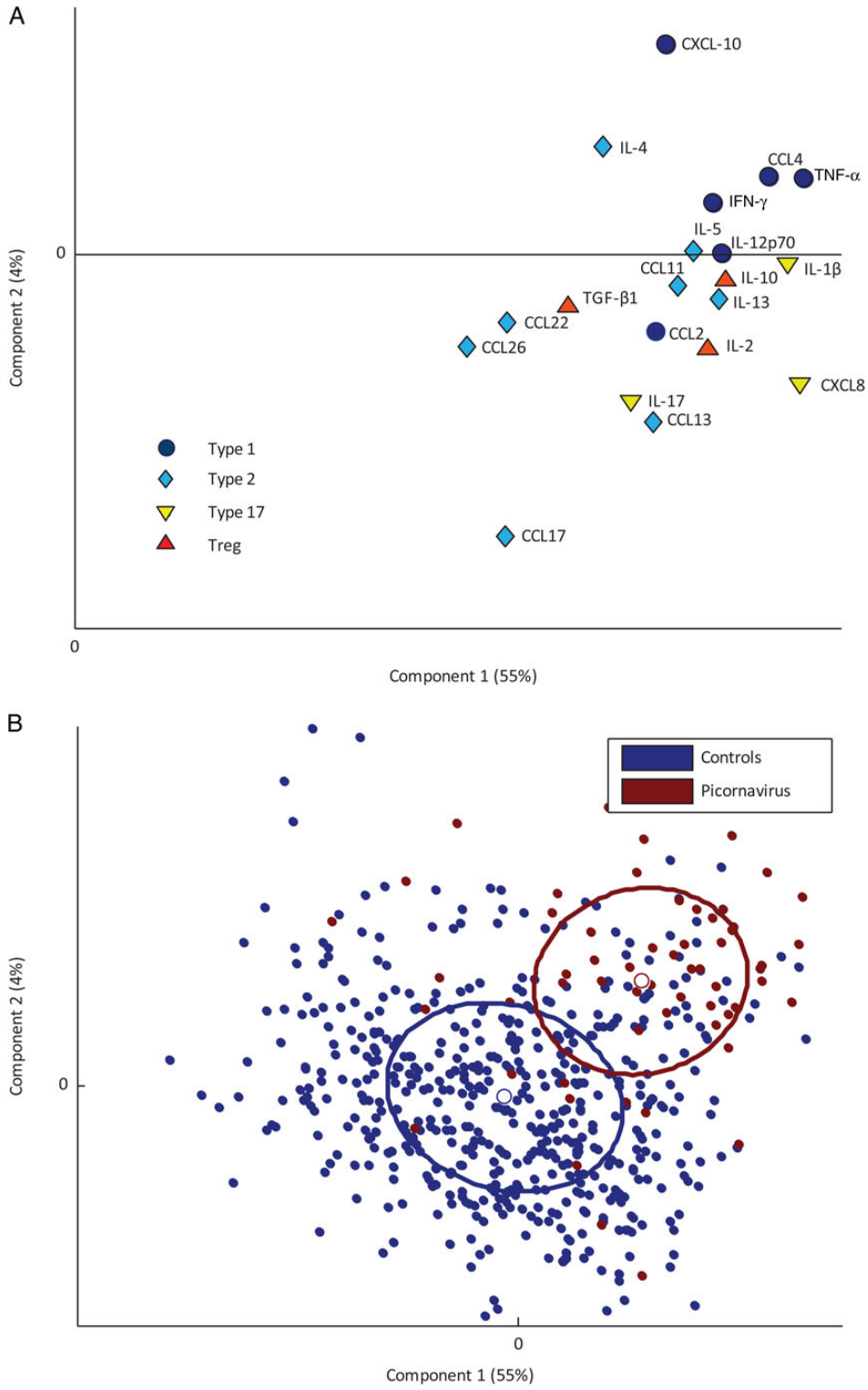


**Figure 3.** Airway mucosal immune profiles. A partial least square discriminate analysis model was used to cluster immune mediators based on presence or absence of picornavirus at time of mediator sampling. *A*, Loading plot showing loadings of the 20 immune mediators. The cytokines are marked and colored according to their function. *B*, Score plot, in which each point corresponds to a single child. The distribution of picornavirus cases versus the rest is shown as ellipses. Differences are registered between the blue circle, representing controls, and the red circle, representing children with picornavirus. The loading plot shows that the children with picornavirus obtain higher values on component 1, which reflect the overall cytokine level. In the second component, the children with picornavirus are skewed toward type 1 cytokines production. Abbreviations: IFN, interferon; IL-1β, interleukin 1β; IL-2, interleukin 2; IL- 4, interleukin 4; IL-5, interleukin 5; IL-10, interleukin 10; IL-12p70, interleukin 12p70; IL-13, interleukin 13; IL-17, interleukin 17; TGF, transforming growth factor; TNF, tumor necrosis factor; Treg, regulatory T cells.

viruses. Viral airway infections are well known to cause a type 1–oriented immune response as assessed in vitro in peripheral blood mononuclear cells [3, 4, 14]. However, it is not fully understood how this extrapolates to the in vivo situation in the airway mucosa, where the signaling between various cell types is in play. Our study is the first to examine the effect of asymptomatic picornavirus presence on the airway mucosal immune signature in healthy neonates.

In this study, respiratory viruses were detected with a PCR technique, and specific virus cultivation and antibody detection was not performed. All PCR assays were conducted with positive and negative controls, and our finding that 12% of the samples were virus positive, with the majority being picornavirus, is comparable to findings of other studies investigating the presence of viruses in asymptomatic children [15, 16].

It is a limitation of our study that we cannot quantify the load of virus based on the applied methodology. Moreover, we tested only for the presence of the most common pathogenic respiratory viruses and not for adenovirus. However, this biases the results toward the null hypotheses, because such false-negatives would have increased the immune mediator levels in the control group. Rhinoviruses were not differentiated into substrains, and use of the most recent primers may have found more positive samples.

Another limitation of our study is that we restricted our mucosal lining fluid analyses to 20 cytokines and chemokines, none of which belonged to the group of type I or III interferons. These particular mediators were carefully chosen a priori to represent both innate and adaptive mediators involving activation of type 1, type 2, type 17, and regulatory type responses, providing a representative view of mediators produced by the different airway immune cells. We use these classification terms to underscore that a variety of leukocytes can be producers of, for example, interferon γ, including natural killer cells, ILC1 cells, Th1 cells, and $CD8^+$ T cells [17, 18]. Along the same lines, type 2 cytokines, such as IL-4, IL-5, and IL-13, can be produced by a variety of cells, including Th2 cells, ILC2 cells (IL-5, IL-13), and eosinophils (IL-4) [19, 20].

The high number of immune mediators raises the concern of multiple testing. To circumvent this problem, we also included a data-driven multivariate approach involving a PLS model. Concordance between results obtained from the conventional statistics and the data-driven approach enhances confidence in the findings.

The combination of pathogenic airway bacteria and picornavirus was associated with an additive effect. Importantly, we found no interaction between the effect of pathogenic airway bacteria and picornavirus and no evidence of a synergistic effect.

#### Interpretation

We found an overall immune-stimulatory effect of picornavirus in the airway mucosa of asymptomatic neonates, with a predominant enhancement of type 1–based inflammatory mediators. This profile was driven primarily by CXCL10 but also by CCL4, and TNF-α.

CXCL10 is a key type 1–related chemokine responsible for the early immune response to viral infection produced in response to both type I and type II interferon stimulation [21]. CXCL10 attracts various $CXCR3^+$ cells, including plasmacytoid dendritic cells, Th1 cells, and $CD8^+$ T cells, important for elimination of viruses. In general, the type 1 immune response is known to be important for intracellular clearance of pathogens such as viruses [22], and the observed type 1–based cytokine signature associated with picornavirus presence is thus biologically meaningful.

The Picornaviridae family includes, among others, the genera rhinovirus and enterovirus, with rhinovirus being the most prevalent [23, 24]. Picornaviruses in general and rhinoviruses in particular are well-known triggers of asthma and asthma exacerbations as well as the causal agents for upper and lower respiratory tract infections [25, 26]. We found a general immune-stimulatory effect of picornavirus on the airway immune profile in asymptomatic neonates, primarily driven by rhinovirus.

Furthermore, picornavirus infections, predominantly rhinovirus in the first year of life, have been proposed to be the main external trigger for wheezing and later asthma development [27, 28]. The effect of early asymptomatic presence of picornavirus has not been studied in this context, and the observed effect of picornavirus in the airways of healthy newborns may play an important role in programming the specific type of memory response to picornavirus during first encounter in early life.

We found an overall elevated level of immune mediators, with predominance of type 1–related mediators, which could simply reflect a normal immune response against virus or represent one early event involved in skewing the Th1-Th2 balance in favor of Th1 in healthy children. Moreover, the type 17–related mediators interleukin 17, IL-1β, and CXCL8 were enhanced by picornavirus, as were regulatory factors such as transforming growth factor β1 and interleukin 10, suggesting that various immune paths are activated by the viral trigger, including both proinflammatory trails as well as those involved in immune cessation.

We also observed an exaggerated type 2 response, which could be explained by the viruses evading type 1–based immunity by inducing a Th2 response [29]. It is well known that early life skewing toward Th2, or type 2 responses has long-term consequences for childhood health, because enhanced production of type 2–related mediators, such as IL-4, IL-5, and IL-13, predispose to development of asthma and allergy [30, 31]. Accordingly, we have shown elsewhere that elevated levels of type 2–associated chemokines within cord blood are associated with increased total immunoglobulin E production in preschool-age children [32].

Presumably, the main external trigger of early life Th1/Th2/Th17 immune development is the composition of the human microbiome [33–35]. Factors influencing the human microbiota and the immunological consequences of such influence is demonstrated in studies of farming communities, where it is evident that being subjected to animals early in life modulates the microbiome and the immunological fingerprint in a type 1– or regulatory-oriented direction, resulting in protection against asthma and allergy development [36, 37]. Similarly, the presence of older siblings has been found elsewhere to inversely relate to the incidence of hay fever [38], and both the presence of older siblings in the home and early daycare attendance have been reported to protect against asthma and wheezy symptoms in later childhood [39]. Because other children in the home or in daycare are the main reservoirs for common respiratory microbes [40], such findings indicate that being subjected to microbes early in life is important for an optimal immune maturation.

In conclusion, the asymptomatic presence of picornavirus in the neonatal airway is a potent activator of the airway mucosal immune system, with predominant enhancement of key proinflammatory mediators of type 1 origin.

## Supplementary Data

Supplementary materials are available at http://jid.oxfordjournals.org. Consisting of data provided by the author to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the author, so questions or comments should be addressed to the author.

## Notes

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***Author contributions.*** The guarantor of the study is H. B., who has been responsible for the integrity of the work as a whole, from conception and design to conduct of the study and acquisition of data, analysis and interpretation of data, and writing of the manuscript. H. M. W., N. V. F., S. Birch, S. Brix, T. T. H., S. L. J., T. K., B. L. C., and K. B. were responsible for data analysis, interpretation, and writing the manuscript. S. Brix was responsible for the laboratory mediator assessments. All coauthors contributed substantially to the analyses and interpretation of the data and provided important intellectual input and approval of the final version of the manuscript.

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***Potential conflicts of interest.*** All authors: No reported conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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**Paper II online supplement:**

## Online data supplement

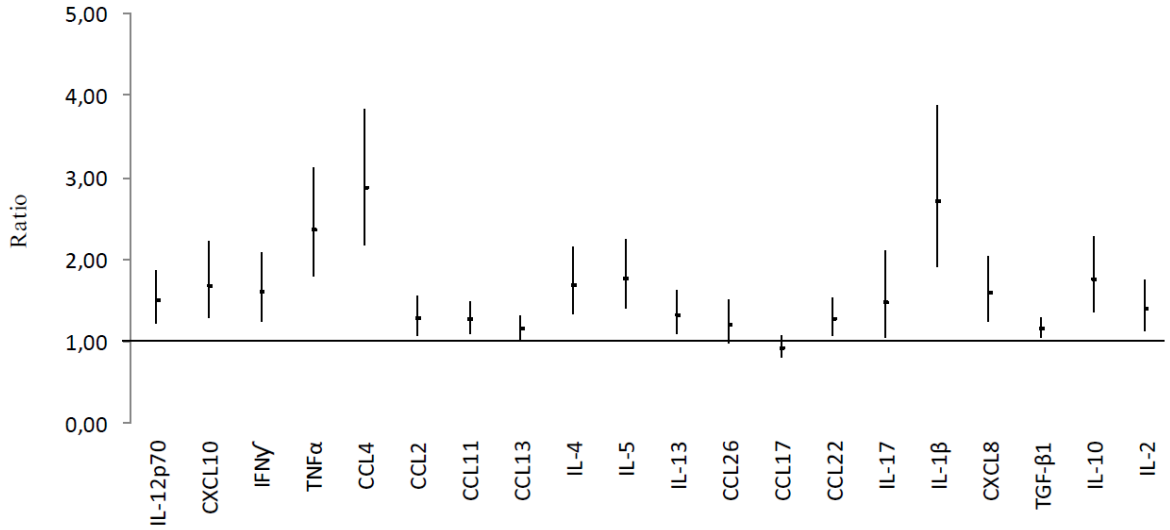
### Picornavirus-induced Airway Mucosa Immune Profile in Asymptomatic Neonates

**Authors:** H. M. Wolsk; N. V. Følsgaard; S. Birch, S. Brix; T. T. Hansel; S. L. Johnston; T. Kebadze; B. L. Chawes; K. Bønnelykke; H. Bisgaard

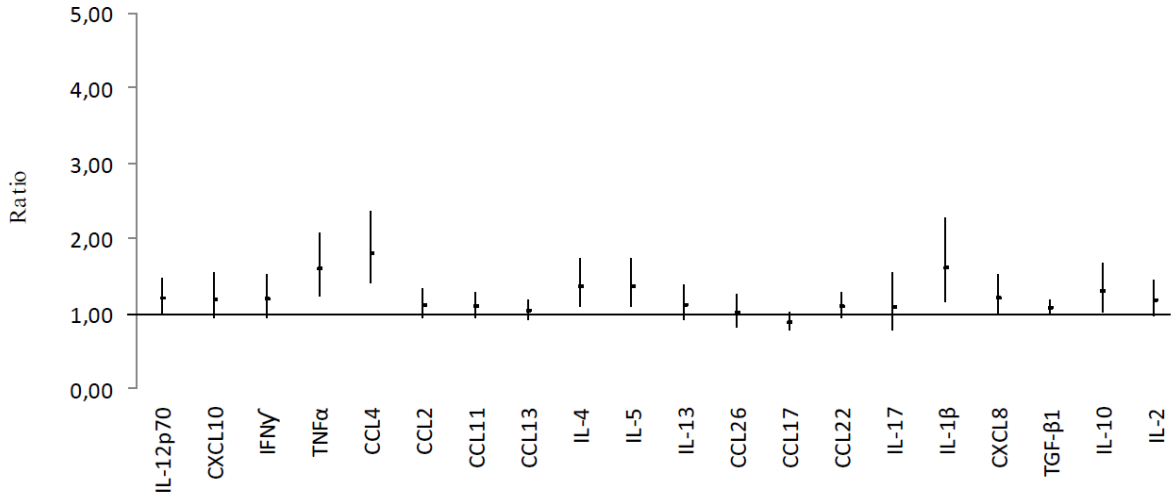
**Online Figure E1:**

Geometric mean ratios with 95% confidence interval of cytokines and chemokines in the airway mucosal lining fluid of healthy one-month-old neonates with pathogenic airway bacteria detected in the airway (n= 159) versus controls (412). a) Crude levels b) Adjusted for maternal history of asthma, allergy, or eczema, older siblings in the home, method of virus sampling, sampling site, season of sampling, batch of immune mediator analyses, maternal consumption of antibiotics in 3rd trimester, and picornavirus.

a)

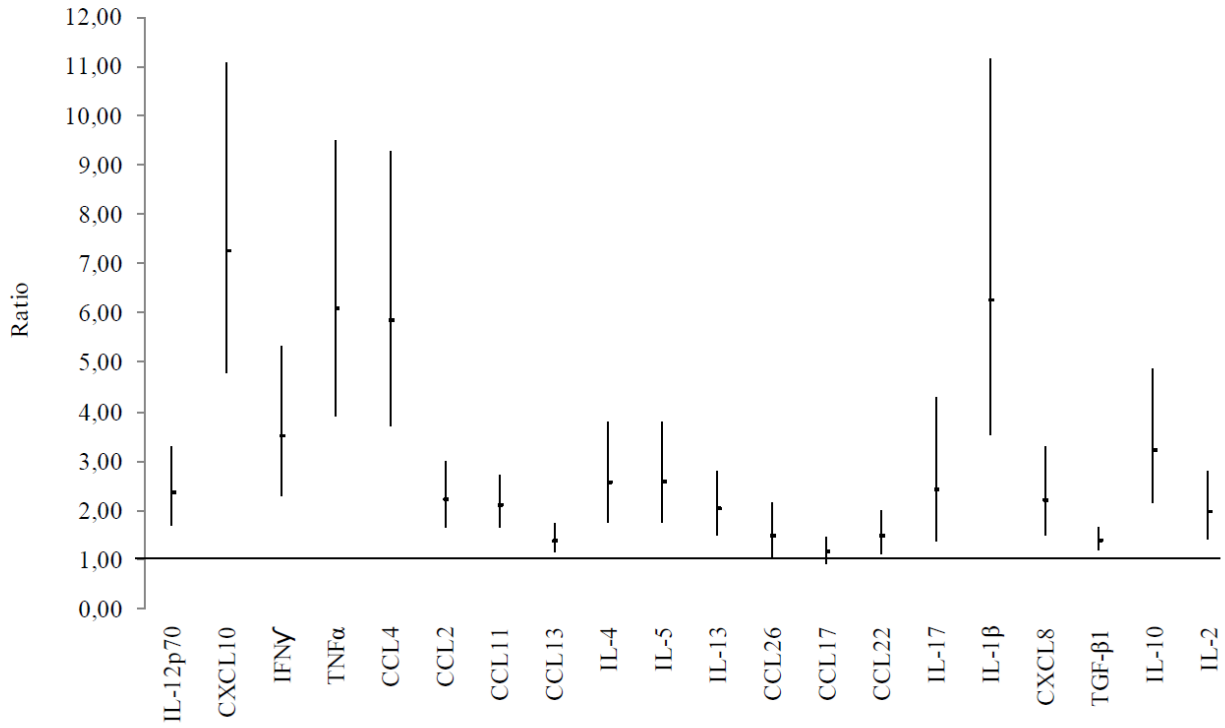



b)



**Online Figure E2:**

Unadjusted geometric mean ratios with 95% confidence interval of cytokines and chemokines in the airway mucosal lining fluid of healthy one-month-old neonates with rhinovirus detected in the airway (n=47) versus controls (n= 524).



# Online data supplement

## Picornavirus-induced Airway Mucosa Immune Profile in Asymptomatic Neonates

**Authors:** H. M. Wolsk; N. V. Følsgaard; S. Birch, S. Brix; T. T. Hansel; S. L. Johnston; T. Kebadze; B. L. Chawes; K. Bønnelykke; H. Bisgaard

**Online Table E1:**

Drop-out table with baseline characteristics of the 571 children included in the study versus the 129 children excluded (chi-squared test,) (see figure 1 for details). P-value ≤ 0.05 is shown in bold.

| | Included % (n=571) | Excluded % (n = 129) | p-value |
|---|---|---|---|
| History of maternal asthma, allergy, or eczema | 53 (303) | 54 (69) | 0.91 |
| Caucasian | 96 (548) | 94 (121) | 0.28 |
| High income (above 130.000 euro) | 13 (77) | 22 (28) | **0.02** |
| Maternal consumption of antibiotics in 3rd trimester | 27 (35) | 20 (112) | 0.06 |
| Maternal smoking in 3rd trimester* | 4 (21) | 3 (4) | 1.00 |
| Maternal alcohol consumption (> 1 u/week in 3rd trimester) | 5 (26) | 6 (8) | 0.42 |
| Low gestational age (< 37 weeks) | 3 (20) | 8 (12) | **0.004** |
| Male | 51 (292) | 52 (68) | 0.75 |
| Low apgar score (< 7 at 1 min.) | 4 (25) | 5 (7) | 0.61 |
| Caesarian section | 21 (122) | 22 (29) | 0.78 |
| Older siblings | 57 (324) | 56 (72) | 0.85 |
| Exclusively breastfeeding at one-month of age | 92 (525) | 95 (109) | 0.37 |
| Furred pets in the home | 21 (118) | 19 (25) | 0.74 |

*Fisher's exact test

**Paper III:**

Allergy EUROPEAN JOURNAL OF ALLERGY AND CLINICAL IMMUNOLOGY

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ORIGINAL ARTICLE EXPERIMENTAL ALLERGY AND IMMUNOLOGY

# Siblings Promote a Type 1/Type 17-oriented immune response in the airways of asymptomatic neonates

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**Keywords**
allergy; asthma; chemokines; children. cytokines; mucosal lining fluid.

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## Abstract

**Background:** Siblings have been shown to reduce the risk of childhood asthma and allergy, but the mechanism driving this association is unknown. The objective was to study whether siblings affect the airway immune response in healthy neonates, which could represent an underlying immune modulatory pathway.

**Methods:** We measured 20 immune mediators related to the Type 1, Type 2, Type 17, or regulatory immune pathways in the airway mucosa of 571 one-month-old asymptomatic neonates from the Copenhagen Prospective Studies on Asthma in $Childhood_{2010}$ birth cohort ($COPSAC_{2010}$). The association between airway mediator levels and presence of siblings was investigated using conventional statistics and principle component analysis (PCA).

**Results:** Neonates with siblings had an upregulated level of airway immune mediators, with predominance of Type 1- and Type 17-related mediators. This was supported by the PCA showing a highly significant difference between children with *vs* without siblings: $P < 10^{-10}$, which persisted after adjustment for potential confounders including pathogenic airway bacteria and viruses: $P < 0.0001$. The immune priming effect was inversely associated with time since last childbirth: $P = 0.0015$.

**Conclusions:** Siblings mediate a Type 1/Type 17-related immune-stimulatory effect in the airways of asymptomatic neonates, also after adjustment for pathogenic bacteria and viruses, indicating that siblings exert a transferable early immune modulatory effect. These findings may represent an *in utero* immune priming effect of the fetal immune system caused by previous pregnancies as the effect was attenuated with time since last childbirth, or it could relate to the presence of unidentified microbes, but further studies are needed to confirm our findings.

The immune system matures in infancy and early childhood. During this period, the composition of the exposome is important for the trajectory toward health or disease, such as asthma and allergic sensitization (1). The presence of siblings in the household has in previous studies been shown to alter the disease risk, although the mechanism is unclear (2–4). The term 'sibling effect' was first proposed from the British Birth Survey showing that exposure to siblings protected against eczema and hay fever at age 5 (5, 6); furthermore, 'the hygiene hypothesis' originally stated that a large household size protected against development of hay fever (2, 7). A possible mechanism is that older siblings convey immune modulation through an increased risk of virus and bacterial

**Abbreviations**
COPSAC, Copenhagen Prospective Studies on Asthma in Childhood; GMR, Geometric Mean Ratio; IL, Interleukin; PCA, Principal Component Analysis.

exposure in very early childhood. In this regard, we have previously shown that the airway immune response is upregulated in asymptomatic neonates with pathogenic airway bacteria and picornaviruses (8, 9). Alternatively or additively, the sibling effect may be caused by *in utero* immune priming related to events induced by previous pregnancies. This could be mediated through a decrease in cord blood IgE with increasing birth order (5) or by decreased anti-inflammatory T-cell activation in first borns (10).

We hypothesized that siblings were associated with a skewing of the neonatal immune response in the airways, as early as 1 month of age. Therefore, we analyzed key immune mediators *in vivo* in the nasal mucosal lining fluid in one-month-old neonates from the unselected Copenhagen Prospective Studies on Asthma in Childhood$_{2010}$ (COPSAC$_{2010}$) birth cohort (8, 11) in relation to presence of siblings while adjusting for coexisting airway viruses and bacteria. A possible *in utero* immune priming effect of siblings was investigated by studying the association between mediator levels and time since last childbirth, for multiparous women.

## Methods

### The COPSAC$_{2010}$ birth cohort

COPSAC$_{2010}$ is an ongoing population-based clinical prospective mother–child cohort with 700 children enrolled at 1 week of age during 2009-2010, which has previously been described in details (12). Exclusion criteria for the pregnant women were any endocrine, kidney, or cardiovascular disease. Children with severe congenital abnormality were excluded. All clinical assessments were performed according to written standard operating procedures including sampling of airway mucosal lining fluid (12). Information on exposures such as siblings was obtained by structured parental interviews conducted at the research clinic.

### Ethics

The study was conducted in accordance with the guiding principles of the Declaration of Helsinki and approved by the Ethics Committee for Copenhagen (H-B-2008-093) and the Danish Data Protection Agency (j.nr. 2015-41-3696). Prior to enrollment of the child, both parents gave their oral and written informed consent.

### Measurements of airway cytokines and chemokines

Unstimulated airway mucosal lining fluid was sampled when the neonates visited the COPSAC clinic at 1 month of age as previously described (8, 11). Strips of filter paper (Accuwik Ultra, fibrous hydroxylated polyester sheets, cat no. SPR0730, Pall Life Sciences, Portsmouth, Hampshire, UK) were inserted bilaterally into the anterior part of the inferior turbinate of the nasal cavity. After 2 min of absorption, the filter papers were removed and immediately frozen at −80°C. The filter papers were thawed and immersed in 300 μl of assay buffer and subsequently placed in the cup of a tube filter within an Eppendorf tube and centrifuged for 5 min in a cooled centrifuge at 16 000 ***g***.

The mucosal lining fluid samples were analyzed for IL(interleukin)-12p70, CXCL10 (IP-10), interferon-gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α), CCL4 (MIP-1β), CCL2 (MCP-1), CCL13 (MCP-4), IL-4, IL-5, IL-13, CCL11 (eotaxin-1), CCL26 (eotaxin-3), CCL17 (TARC), CCL22 (MDC), IL-17, IL-1β, CXCL8 (IL-8), transforming growth factor beta-1 (TGF-β1), IL-10, and IL-2(11, 13) by MesoScale Discovery multiplexed array system (MesoScale Discovery, Gaithersburg, MD, USA) which is a high-sensitivity electrochemiluminescence-based ELISA-type assay. The sensitivities for all cytokines were ≤1 pg/ml and for chemokines 1–50 pg/ml, as previously described (11).

Selection of the measured cytokines and chemokines was decided *a priori* to represent mediators associated with different types of immune responses that we grouped into Type 1 (Th1/CD8+/NK cells/innate lymphoid cells (ILC) 1), Type 2 (Th2, eosinophils, ILC2), Type 17 (Th17, neutrophils, ILC17), and regulatory type (Treg) responses (11, 14, 15). This was based on the present understanding of which cell types mainly produce the given mediators and/or are affected by the mediators.

### Siblings

Information about the presence of siblings in the household was obtained at the visit to the COPSAC clinic at 1 week of age. *Siblings* included biological siblings as well as half-siblings living in the home. Data were analyzed as (i) any siblings (biological and half-siblings), (ii) any biological siblings (excluding half-siblings), (iii) quantitatively as 0 *vs* 1 *vs* >1 sibling, and for multiparous women (iv) time since last childbirth.

### Covariates

*Parental characteristics*

'Maternal asthma, allergy, or eczema' (Yes/No), 'Caucasian' (both parents of Caucasian descent, Yes/No), 'high income' (annual household income >130 000 euro, Yes/No), 'maternal antibiotic consumption in 3rd trimester' (Yes/No), 'maternal smoking in 3rd trimester' (Yes/No), and 'maternal alcohol consumption in 3rd trimester' (>one unit of alcohol per week, Yes/No).

*Characteristics of the child*

'Gestational age <37 weeks' (Yes/No), 'sex' (Male/Female), 'Apgar score <7 at 1 min' (Yes/No), 'Caesarean section' (planned or acute caesarean section, Yes/No), 'exclusively breastfeeding at one month' (Yes/No), 'pets' (cats and/or dogs living in the home of the child, Yes/No),

'Picornavirus' (Yes/No) and 'airway bacteria' (Yes/No). Nasopharyngeal sampling for virus detection was performed after the sampling of mucosal lining fluid. The samples were collected via one of the nostrils and diluted in 1 ml of isotonic saline. Specimens were frozen and stored at −80°C

until shipment to Imperial College, London, UK, for RNA extraction and further analysis with reverse transcriptase–polymerase chain reaction (RT-PCR) (9, 16). Hypopharyngeal aspirates for bacterial culturing were collected with a soft-suction catheter passed through the nose into the hypopharynx. The aspirates were analyzed by bacterial culturing, using standard methods (8, 17). The analyses were adjusted for any presence of pathogenic airway bacteria including Haemophilus influenzae, Streptococcus pneumonia and Moraxella catarrhalis, as previously described (8, 9, 17).

### Statistics

Differences in baseline characteristics between neonates with and without siblings were analyzed with chi-squared test or Fisher's exact test.

To obtain normally distributed residuals of the mediator levels, data were log-transformed prior to analyses. For every immune mediator, a number of reads were below the detection limit. The samples with a level between zero and detection limit have not been changed, while the zero values have been set to half of the minimum of the nonzero values, in order to be able to perform a log transformation, without loss of data.

The univariate associations between presence of siblings in the home and the mediator levels were analyzed using analysis of variance (ANOVA) with log-transformed mediator levels as outcome variables and siblings and possible confounders/covariates as explanatory variables. Results are reported as geometric mean ratios (GMR) of the mean mediator levels for neonates with/without siblings and 95% confidence intervals (CI). Comparison with 'time since last childbirth' is conducted by ANOVA with age as continuous explanatory variable. We explored other representations of this variable, such as log transformation and polynomial as well as non-parametric modeling with no differences in the results.

Based on our previous studies, sampling site, season of sampling, batch of immune mediator analysis, maternal asthma, allergy or eczema, and maternal consumption of antibiotics in 3rd trimester were included as covariates in the models as they all associate with the levels of immune mediators (8, 9, 13).

We also applied a principal component analysis (PCA) to decompose the immune signal from the 20 mediators into a few independent components that capture the overall immunological trends in the data.

Analyses were carried out using SAS version 9.3 (SAS Institute, Cary, NC, USA), and the PCA were conducted in MATLAB R2013a v. 8.1.0.604 (MathWorks Inc, Natick, MA, USA) utilizing the PLStoolbox 7.8.2 (Eigenvector Research Inc., Wenatchee, WA, USA) for building the PCA model and *in-house* algorithms for plotting data.

## Results

### Baseline characteristics

A total of 571 neonates were included in the main study, excluding 10 neonates that were enrolled before the method of airway mucosal lining fluid was established, as well as 19 that did not attend the one-month visit. Furthermore, 44 neonates had an airway infection at the day of sampling, and 56 samples were excluded due to technical reasons (see study flow chart Fig. S1).

The mean age at the time of sampling airway mucosal lining fluid was 32 days (standard deviation (SD) = 5.4 days). A total of 51% ($N$ = 292) were boys, 57% ($N$ = 324) had siblings, whereof 4% ($N$ = 13) were half-siblings, 67% ($N$ = 216) had one sibling, and 33% ($N$ = 108) had >1 sibling. In Table 1, baseline characteristics of the study group are depicted along with a comparison of neonates with and without siblings. Neonates with siblings had mothers with an increased alcohol consumption in the 3rd trimester (7% *vs* 2%, $P$ = 0.01), higher household income (17% *vs* 9%, $P$ = 0.01) and were more often breastfed at age 1 month (94% *vs* 90%, $P$ = 0.04). Furthermore, neonates with siblings had increased presence of pathogenic airway bacteria (40% *vs* 13%, $P < 0.0001$) and picornavirus (15% *vs* 4%, $P < 0.0001$).

Baseline characteristics of the 571 included *vs* the 129 excluded neonates were comparable except for a higher house-hold income (22% *vs* 13%, $P$ = 0.02) and a lower age of gestation (8% *vs* 3%, $P$ = 0.004) (Table S1).

### Siblings and the neonatal airway immune response

The median values of immune mediators in the neonates with siblings compared to the neonates without siblings and the

**Table 1** Baseline characteristics of neonates with siblings and neonates without siblings (chi-squared test). *P*-value ≤0.05 is shown in bold

| | Siblings % ($n$ = 324) | No siblings % ($n$ = 247) | *P*-value |
|---|---|---|---|
| Maternal asthma, allergy, or eczema | 50 (161) | 58 (142) | 0.08 |
| Caucasian | 97 (314) | 95 (234) | 0.19 |
| High Income (above 130 000 euro) | 17 (55) | 9 (22) | **0.01** |
| Maternal antibiotic consumption in 3rd trimester | 22 (70) | 17 (42) | 0.17 |
| Maternal smoking in 3rd trimester | 3 (11) | 4 (10) | 0.68 |
| Maternal alcohol consumption in 3rd trimester* | 7 (21) | 2 (5) | **0.01** |
| Gestational age <37 weeks | 2 (8) | 5 (12) | 0.12 |
| Male | 51 (164) | 52 (128) | 0.78 |
| Apgar score <7 at 1 min | 5 (17) | 3 (8) | 0.25 |
| Caesarean section | 20 (66) | 23 (56) | 0.51 |
| Exclusively breastfeeding at 1 month | 94 (303) | 90 (222) | **0.04** |
| Furred pets | 23 (73) | 18 (45) | 0.21 |
| Picornavirus | 15 (48) | 4 (10) | **<0.0001** |
| Pathogenic airway bacteria | 40 (128) | 13 (31) | **<0.0001** |

*>1 U/week (more than 1 beer/1 glass of wine/2 cl of spirits per week).

number of samples below the detection limit are shown in Table 2. Neonates with siblings had a significant upregulation of all mediators (all *P*-values <0.05), except for CCL17, when compared to neonates with no siblings. This was most pronounced for specific Type 1 (TNF-α, CCL4)- and Type 17 (IL-17, IL-1β)-associated mediators which were 2- to 3.5-fold increased (Fig. 1A).

Adjusting the analysis for maternal asthma, allergy and eczema, sampling site, season of sampling, batch of immune mediator analyses, maternal smoking in 3rd trimester, breastfeeding at 1 month of age, high income and alcohol consumption in 3rd trimester did not significantly modify the results (Fig. 1B). Further adjustment for presence of pathogenic airway bacteria and picornavirus at time for mucosal sampling resulted in a slight decrease in the levels of the immune mediators, but still yielded an overall increased ratio of the 20 immune mediators (Fig. 1B).

The same upregulation of immune mediators was seen when the analyses were restricted to neonates without pathogenic airway bacteria and/or picornavirus (Fig. S2), where all mediators are significantly elevated (all *P*-values <0.05) except for CCL2 and CCL17 ($P > 0.1$). When only the neonates with biological siblings were included ($n = 310$) (Fig. S3), a significant upregulation was found in all immune mediators (all *P*-values <0.05) except for CCL17 and TGF-β1 ($P > 0.07$). Finally, we studied the birth order effect of being second born ($N = 216$) *vs* first born (Fig. S4A and S5) and correspondingly the effect of being third born ($N = 108$) *vs* first born (Fig. S4B and S5) without finding any additional immune-stimulatory or immune-inhibitory effects.

### Multivariate interrelations between siblings and the neonatal airway immune response

The conventional statistics were supported by a principal component analysis (PCA) showing a high intercorrelation between the 20 different immune mediators in the first component of the loading plot (Fig. 2A). In the first component of the score plot (Fig. 2B), a clear distinction was found between neonates with siblings and those without siblings, supporting the findings from the conventional approach. By principal component regression, the difference between the two groups was found to be highly significant ($P < 10^{-10}$). Adjusting for maternal asthma, allergy, or eczema, sampling clinic, season of sampling, batch of immune mediator analyses, maternal smoking in 3rd trimester, breastfeeding at 1 month of age, high income, alcohol consumption in 3rd trimester, pathogenic airway bacteria, and picornavirus did not modify the results substantially ($P < 2 \times 10^{-7}$).

To investigate a possible *in utero* immune priming effect, time since last childbirth was studied in a PCA model (Fig. 3), showing an inverse association between time since last childbirth for the mother and the airway immune response in the neonate ($P = 0.0015$, adjusted $P = 0.02$). Hence, with increasing time since last childbirth, the immune response attenuated toward the level found in neonates with no siblings. No effect of previous miscarriage was found on the immune mediators (data not shown).

The effect of having no siblings *vs* one sibling *vs* more than one sibling was likewise analyzed by PCA (Fig. S4). Testing the first three principal components with respect to birth order revealed no significant effect, underlining that there

**Table 2** Median values and numbers below the detection limit of cytokines and chemokines

| Mediator | Siblings Median (lower–upper quartile) pg/ml | No siblings Median (lower–upper quartile) pg/ml | Below detection limit |
|---|---|---|---|
| IL-12p70 | 5.81 (2.56–10.31) | 3.58 (1.60–6.57) | 120 |
| CXCL10 | 683.3 (245.1–2424) | 372.7 (159.9–919.3) | 8 |
| IFN-γ | 6.04 (2.13–17.17) | 2.98 (1.32–7.16) | 294 |
| TNF-α | 40.55 (15.03–121.43) | 18.70 (6.85–43.04) | 11 |
| CCL4 | 247.9 (80.20–704.4) | 74.08 (33.40–206.7) | 5 |
| CCL2 | 156.3 (81.54–275.2) | 118.9 (63.12–224.8) | 9 |
| CCL13 | 18.91 (12.90–25.89) | 14.26 (9.58–20.82) | 13 |
| IL-4 | 2.52 (1.27–5.62) | 1.26 (0.41–3.12) | 235 |
| IL-5 | 3.48 (1.44–6.92) | 1.89 (0.80–4.08) | 172 |
| IL-13 | 16.19 (9.57–32.71) | 12.28 (5.38–23.15) | 53 |
| CCL11 | 96.59 (57.39–156.60) | 67.56 (44.56–109.6) | 40 |
| CCL26 | 86.63 (24.67–160.89) | 49.59 (14.88–116.1) | 264 |
| CCL17 | 16.29 (9.80–24.90) | 15.04 (9.50–24.14) | 85 |
| CCL22 | 81.27 (49.26–188.37) | 60.27 (36.13–123.39) | 103 |
| IL-17 | 2.56 (0.57–6.22) | 1.00 (0.32–2.96) | 284 |
| IL-1β | 163.48 (35.79–738.09) | 37.60 (14.61–150.51) | 4 |
| CXCL8 | 4967 (1983–9335) | 2535 (901–5292) | 4 |
| TGF-β1 | 54.82 (39.48–75.00) | 47.37 (32.68–65.57) | 60 |
| IL-10 | 22.93 (11.10–43.38) | 13.29 (5.81–26.00) | 19 |
| IL-2 | 26.13 (11.15–54.37) | 13.10 (5.81–29.01) | 22 |

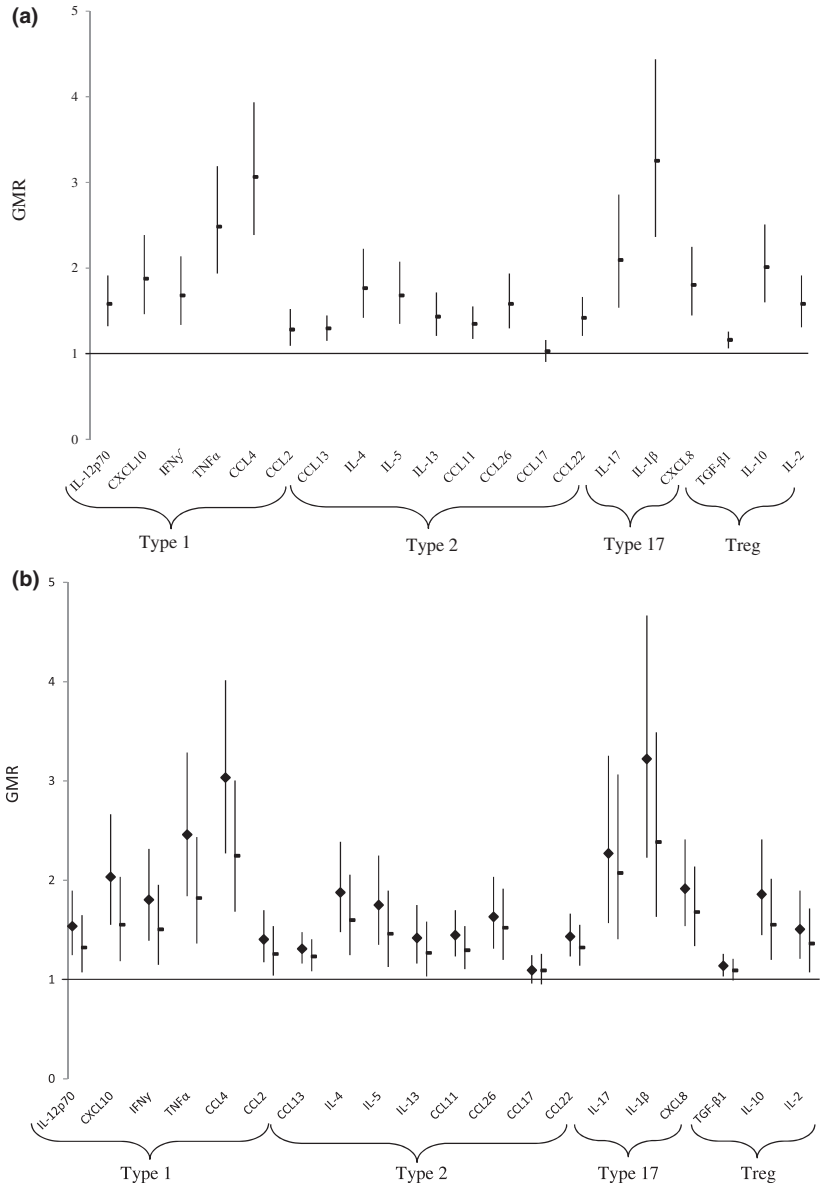


**Figure 1** Geometric mean ratios (GMR) with 95% confidence interval of cytokines and chemokines in the airway mucosal lining fluid of healthy one-month-old neonates with/without siblings in the home. (A) Crude levels. In (B), each mediator is shown as the GMR adjusted (bar with a rhombus shape) for maternal asthma, allergy, or eczema, sampling clinic, season of sampling, batch of immune mediator analyses, maternal smoking in 3rd trimester, breastfeeding at 1 month of age, high income, and maternal alcohol consumption in 3rd trimester, the bar to the right (bar with a horizontal line) represents GMR adjusted for the same parameters as well as bacteria and picornavirus.

was no further immune-regulatory effect on the airway mucosal response profile of having more than one sibling.

## Discussion

### Principle findings

We found an overall upregulated airway immune response in healthy neonates with siblings in the home at birth, independent of the presence of virus and pathogenic airway bacteria at time of sampling. This finding questions the common interpretation that the sibling effect, in relation to risk of asthma and allergy, is mediated solely via spread of microbes. Our data suggest that the sibling effect may additionally represent *in utero* immune priming initiated by events during previous pregnancies as the immune response attenuated with increasing time since last childbirth. However, we cannot exclude that the effect is caused by younger siblings bringing home more pathogens, and further studies are needed to confirm our findings.

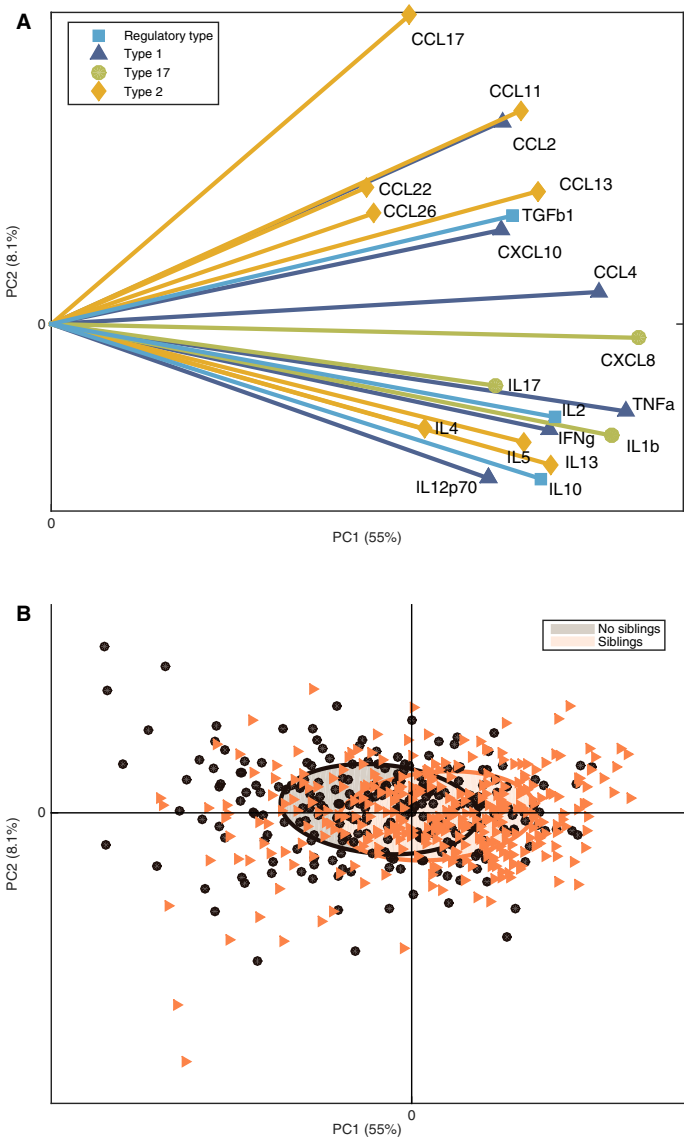


**Figure 2** (A) Loading plot from principal component analysis showing the loadings of the 20 different immune mediators. The cytokines are marked according to their function. (B) Score plot, where each point corresponds to one child. The distribution of neonates with siblings *vs* the rest is shown as ellipses. Differences are registered between the blue circle (neonates without siblings) and the red circle (neonates with one sibling or more). By principal component regression, a highly significant difference was found between neonates with no siblings and neonates with one sibling or more ($P < 10^{-10}$).

### Strength and limitations

It is a strength of the study that the immune response in the airway mucosal lining fluid was assessed undisturbed *in vivo* by a validated method of collecting and measuring cytokines and chemokines (11). The assessment of the immune response was performed in the target organ of airway disease, which is a major advantage compared to measurements of systemic mediators in blood. All baseline information was obtained by interviewing the parents and not from questionnaires.

The data were analyzed with two different statistical methods, yielding similar results, increasing the validity of our findings. The comparable results achieved with the data-driven PCA approach also negates the risk of multiple testing in the conventional approach.

It is a limitation that we only examined 20 cytokines and chemokines, although these 20 immune mediators were carefully chosen *a priori* to represent major phenotypical pathways of the immune system. Furthermore, information about the immune response was only obtained at a single time

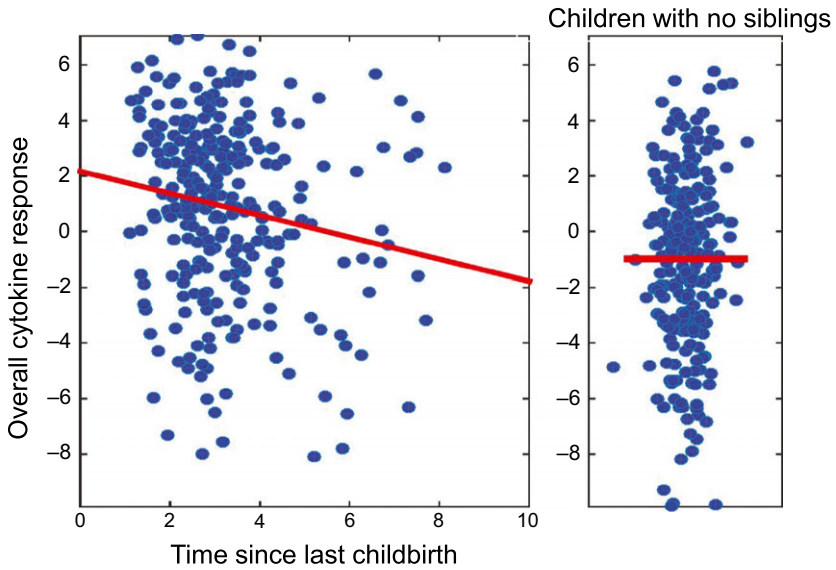


**Figure 3** Principal component analysis of the distribution of the time since last childbirth (*x*-axis) in relation to level of immune mediators (*y*-axis). Significantly higher immune mediator levels are seen with short time since the last pregnancy (unadjusted $P = 0.0016$, adjusted $P = 0.02$).

point, limiting our knowledge about the chronicity of our findings. We only analyzed for the most pathogenic and prevalent viruses and bacteria in childhood, implicating that other viruses and bacteria could be or have been present and represent a residual confounding effect. However, the investigated bacteria and viruses are the main pathogens affecting the immune response and also those predisposing to later development of asthma (8, 9, 17, 18).

### Interpretation

The neonatal exposome may be a very important determinant of later asthma and allergy development. The 'hygiene hypothesis' originally stated that a large household size protected against development of hay fever, supposed to be mediated through increased exposure to infectious agents in early childhood (2, 7). Further, the term 'sibling effect' was proposed from the British Birth Survey showing that exposure to siblings protected against eczema and hay fever at age 5 (5, 6), while the results for asthma are ambiguous with studies both supporting (3, 7, 19–21) and refuting (22) an inverse association between sibship size and asthma.

In support of the above hypotheses, we have previously shown that neonates with siblings had a higher prevalence of carriage of both airway viruses and bacteria than neonates without siblings (8, 9). Moreover, we have demonstrated that asymptomatic presence of picornavirus in the neonatal airway is a potent activator of the immune system in the airways with Type 1 predominance. Independently of this we found that colonization of the neonatal airways with specific pathogenic airway bacteria promoted a general activation of Type 1, Type 2, and Type 17 immune mediators (8, 9). In this article, we found a significant and marked immune-stimulatory effect from having siblings, independently of airway pathogens. Hence, the proposed sibling effect may be mediated by other mechanisms besides carriage of airway pathogenic microbes. This could include an *in utero* programming effect from previous pregnancies, as we observed that increased time since last pregnancy was associated with decreased airway immune activation in the offspring. However, we cannot, with the current data and applied laboratory methodology, exclude that other pathogenic microbes than the ones we searched for are present and possibly affecting our results. A recent study performed in adults described that the distribution of pathogens in relation to respiratory infections, differed according to age (23), which may also apply for children. Hence, the presence of young older siblings could bring a higher risk to transmit those infections to which the neonates are more vulnerable, thereby inducing a greater immune-stimulatory effect. Such relationship could also be an explanation for the inverse relationship between increasing time since last pregnancy and decreasing topical immune mediator release, as increasing age of older siblings may decrease the risk of transmitting infections to the neonate.

We observed that exposure to siblings in the neonatal period was associated with an upregulated immune profile, most pronounced for Type 1- and Type 17-related mediators. Although this effect was unaffected by adjusting for concomitant presence of specific pathogenic bacteria and viruses, the nature of the immune response still indicates that enhanced stimulation with environmental microbes may be the underlying factor for this specific immune signature in neonates with siblings. Also, though still significant, the effect

estimates attenuated after adjustment for bacteria and viruses.

In particular, CCL4 was highly elevated, which we attribute to a Type 1-associated immune function involving responses against intracellular pathogens such as viruses and some bacteria based on its action as a CCR5 ligand. CCR5 is expressed on monocytes, immature dendritic cells, Th1 cells, and CD4+ effector memory T cells, and CCL4 will mediate influx of these cell types upon secretion (24).

Our finding of an exaggerated Type 1 and also Type 17 response in the neonates with siblings may have an important role in shifting the immune response away from Type 2 dominance after birth, thereby supporting the sibling effect and protecting against later development of asthma and allergy. However, we also found elevated levels of Type 2 associated mediators, although not as pronounced as the Type 1 associated mediators. The univocal upregulation of all types of immune responses in the neonatal airway suggests that the immune-stimulatory exposome carried by the siblings convey a direct activation of the neonatal airway immune response.

Alternatively, our findings could be interpreted in support of the siblings effect being caused by *in utero* programming of the fetal immune system due to events occurring during previous pregnancies. Priming of the developing immune system starts *in utero* (4, 5, 25), and differences in the immune activation registered in cord blood mediators and immune cell distribution have been shown in first-born children compared to second or later-born children (5, 10). Kragh et al.(10) found that first-born infants displayed a reduced anti-inflammatory profile in T cells at birth, compared to second and later born, and Karmaus et al.(5) found that cord blood IgE level is dependent on birth order. A correlation between levels of specific cytokines in maternal blood and cord blood has also been documented, indicating that a trans-placental regulation is occurring, which may affect later risk of disease (26). We did not find a 'dose–response-effect' from increasing birth order, but we observed an inverse association between the airway immune response and the time since last childbirth, also when adjusting the analysis for all confounders including pathogenic airway bacteria and picornavirus. This suggests that the immunological effect from siblings may be induced by *in utero* events gradually declining over time. The reported effect could also be mediated by younger siblings having more infections, thereby exposing the vulnerable neonate to more pathogens. A previous study has shown that the attack rate and intervals between airway infections are shorter with younger age (27). Comparing immune signals between the first born children of mothers with or without an earlier miscarriage revealed no significant difference, which also support the hypothesis of pathogenic microbes transmitted by the siblings, as changes in the maternal immune system after a miscarriage should endure similarly to those occurring in a normal pregnancy, unless the miscarriage would occur extremely early in gestation. It should, however, be noted that the study power for this analysis was low.

From our data, an *in utero* effect cannot be excluded, and future studies may need a wider approach to understand the mechanisms in other parts of the early exposome or in maternal influence from previous pregnancies relating siblings and disease risk. Independently of the underlying mechanisms, this study shows that presence of siblings is associated with a clear topical airway immune-stimulatory effect with predominance of Type 1- and Type 17-related mediators. We speculate that this may be an important immune programming event in relation to later development of asthma and allergy.

## Conclusion

The presence of siblings at birth associates with an upregulation of the neonatal airway mucosal immune response, which is not mediated solely through carriage of airway pathogens, but seems related to *in utero* immune priming. These findings indicate that having siblings may exert an early Type 1/Type 17-immune priming effect that may be important for later development of asthma and allergy.

## Acknowledgments

We gratefully express our gratitude to the children and families of the $COPSAC_{2010}$ cohort study for all their support and commitment. We acknowledge and appreciate the unique efforts of the COPSAC research team, and the technical help from Technician Lisbeth Buus Rosholm, DTU Systems Biology for measurement of cytokines and chemokines.

## Source of funding

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## Conflicts of interest statement

The authors declare that they have no conflicts of interest.

## Author contributions

The guarantor of the study is HB who has been responsible for the integrity of the work as a whole, from conception and design to conduct of the study and acquisition of data, analysis, and interpretation of data and writing of the manuscript. HW, NF, SB, MR, and BC were responsible for data analysis, interpretation, and writing the manuscript. SB was responsible for the laboratory mediator assessments. All co-authors have contributed substantially to the analyses and interpretation of the data and have provided important intellectual input and approval of the final version of the manuscript.

## Governance

We are aware of and comply with recognized codes of good research practice, including the Danish Code of Conduct for Research Integrity. We comply with national and international rules on the safety and rights of patients and healthy subjects, including Good Clinical Practice (GCP) as defined in the EU's Directive on Good Clinical Practice, the International Conference on Harmonisation's (ICH) good clinical practice guidelines, and the Helsinki Declaration. We follow national and international rules on the processing of personal data, including the Danish Act on Processing of Personal Data and the practice of the Danish Data Inspectorate.

## Supporting Information

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

**Table S1** Drop-out table: Baseline characteristics of the 571 neonates included in the study *vs* the 129 neonates excluded (chi-squared test) (see Fig. S1 for details). *P*-value ≤0.05 is shown in bold.

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**Paper III- Online supplement:**

**Online data supplement**

# Siblings Promote a Type 1/Type 17-oriented immune response in the airways of asymptomatic neonates

**Authors**: Helene Mygind Wolsk, MD; Bo L Chawes, MD PhD ; Nilofar V Følsgaard, MD PhD;

Morten A Rasmussen, MSci PhD; Susanne Brix, MSci PhD; Hans Bisgaard, MD DMsc.

**Table E1 online:** Drop-out table: Baseline characteristics of the 571 neonates included in the study versus the 129 neonates excluded (chi-squared test) (see online figure E1 for details). P-value ≤ 0.05 is shown in bold.

| | Included % (n=571) | Excluded % (n = 129) | p-value |
|---|---|---|---|
| Maternal asthma, allergy, or eczema | 53 (303) | 54 (69) | 0.91 |
| Caucasian | 96 (548) | 94 (121) | 0.28 |
| Income (above 130,000 euro) | 13 (77) | 22 (28) | **0.02** |
| Maternal antibiotic consumption in 3rd trimester | 27 (35) | 20 (112) | 0.06 |
| Maternal smoking in 3rd trimester* | 4 (21) | 3 (4) | 0.71 |
| Maternal alcohol consumption in 3rd trimester** | 5 (26) | 6 (8) | 0.42 |
| Gestational age < 37 weeks | 3 (20) | 8 (12) | **0.004** |
| Male | 51 (292) | 52 (68) | 0.75 |
| Apgar score < 7 at 1 min | 4 (25) | 5 (7) | 0.61 |
| Caesarean section | 21 (122) | 22 (29) | 0.78 |
| Siblings | 57 (324) | 56 (72) | 0.85 |
| Exclusively breastfeeding at 1-month | 92 (525) | 95 (109) | 0.37 |
| Furred pets | 21 (118) | 19 (25) | 0.74 |

***Fisher's exact test,** ** > 1 U/week

**Online data supplement- Figures**

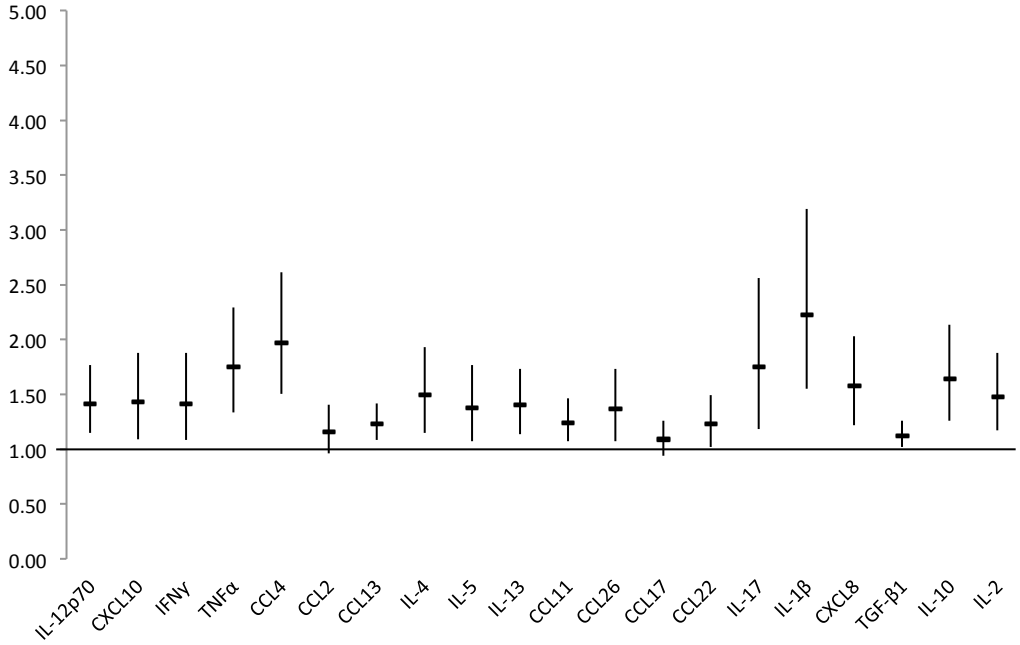
# Siblings Promote a Type 1/Type 17-oriented immune response in the airways of asymptomatic neonates

**Authors**: Helene Mygind Wolsk, MD; Bo L Chawes, MD PhD ; Nilofar V Følsgaard, MD PhD; Morten A Rasmussen, MSci PhD; Susanne Brix, MSci PhD; Hans Bisgaard, MD DMsc

**Online figure E1:** Study group flow-chart

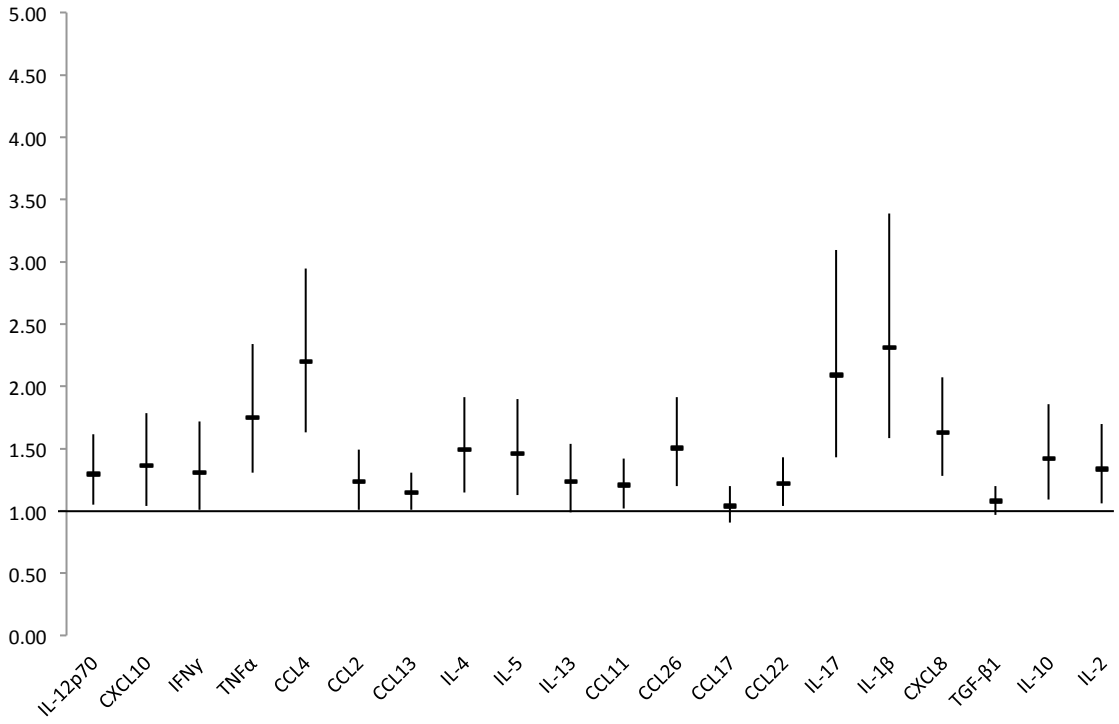
**Online Figure E2:**

Geometric mean ratios with 95% confidence interval of cytokines and chemokines in the airway mucosal lining fluid of healthy one-month-old neonates with/without siblings. Only the neonates without concurrent pathogenic airway bacteria and/or picornavirus are included (n=381).



**Online Figure E3:**

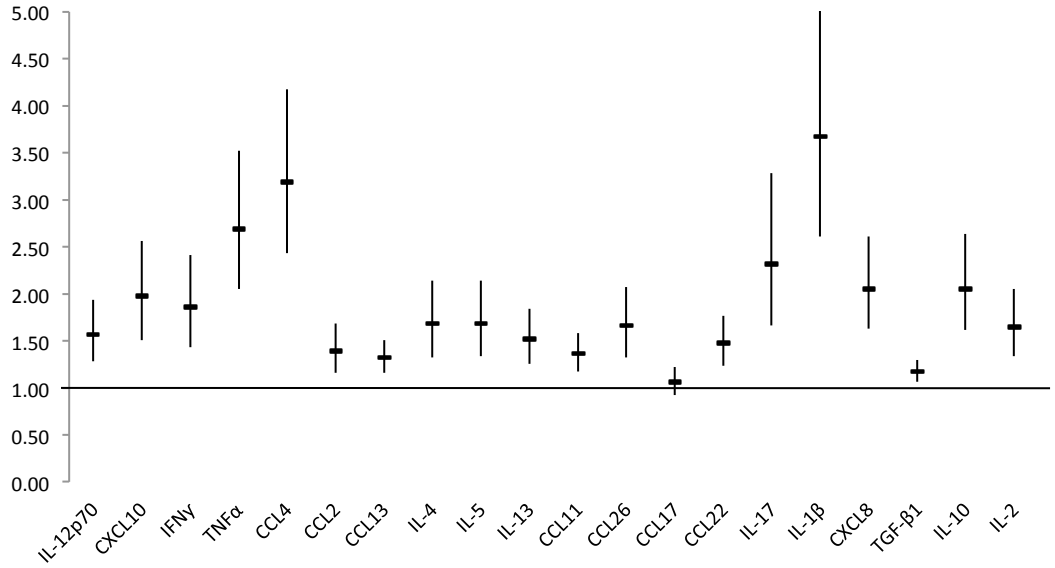
Geometric mean ratios with 95% confidence interval of cytokines and chemokines in the airway mucosal lining fluid of healthy one-month-old neonates with/without biological siblings in the home at birth (n = 310). Adjusted for maternal asthma, allergy, or eczema, sampling clinic, season of sampling, batch of immune mediator analyses, maternal smoking in 3rd trimester, breastfeeding at one-month of age, high income, maternal alcohol consumption in 3rd trimester, bacteria and picornavirus.



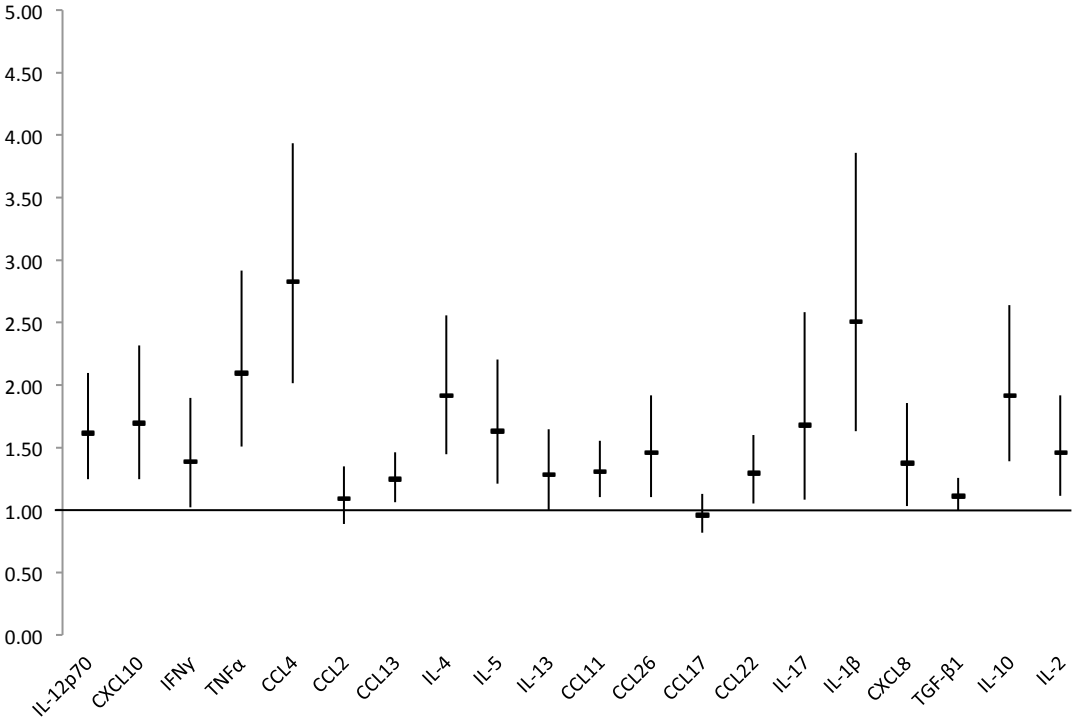
**Online Figure E4: Immune response in second-born and third-born compared to first-born**

Geometric mean ratios with 95% confidence interval of cytokines and chemokines in the airway mucosal lining fluid of healthy one-month-old neonates with/without siblings in the home at birth. A) Second-born (biological and non-biological) (N= 215) compared to first-born (N = 247). B) Third-born (biological and non-biological) (N=108) compared to first-born (N= 247). No confounder adjustment.

**A)**

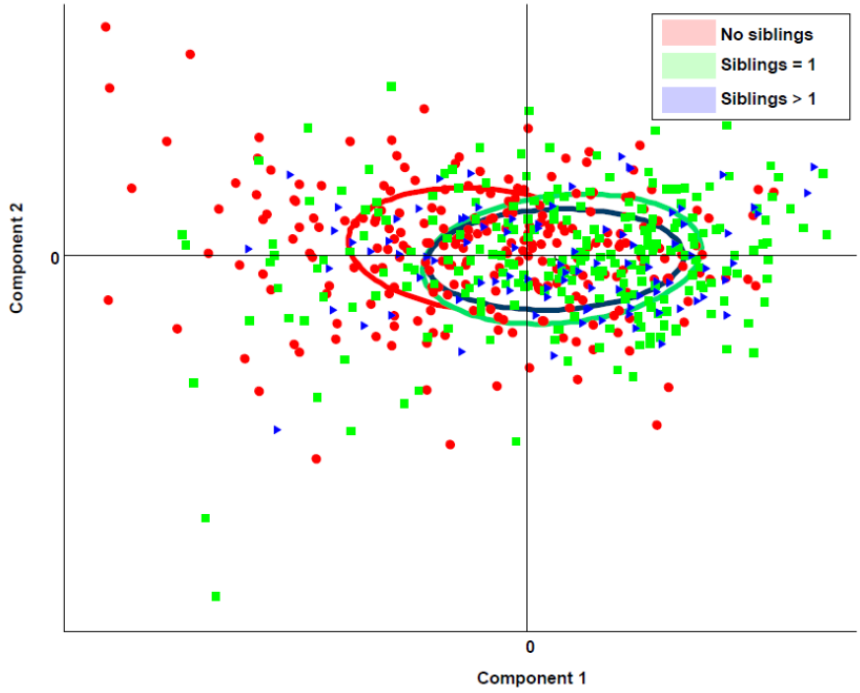



**B)**



**Online Figure E5:**

Principal component analysis of the distribution of the neonates with no siblings (red circle) versus one sibling (green circle) versus more than one sibling (blue circle).



**Paper IV:**

**Neonatal Airway Immune Profiles and Childhood Allergy and Asthma-related Diseases**

**Authors**: Helene Mygind Wolsk, MD[1], Morten A Rasmussen, MSci, PhD[1,2]; Jakob Stokholm, MD, PhD[1]; Klaus Bønnelykke, MD, PhD[1]; Susanne Brix, MSci, PhD[3]; Bo L Chawes, MD, PhD, DMSc[1]; Hans Bisgaard, MD, DMSc[1]

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**Article type:** Original article

**Word count:** 2,902

**Figure count:** 3 + 1 online

**Tables:** 1 + 2 online

**Short Title:** Neonatal airway immunology and childhood asthma and allergy

**Online repository:** Yes

**Abbreviations:** COPSAC – Copenhagen Prospective Studies on Asthma in Childhood; GMR – Geometric Mean Ratio; IL – Interleukin; PCA – Principal Component Analysis.

**Source of funding:** COPSAC is funded by private and public research funds all listed on www.copsac.com. The Lundbeck Foundation (grant no R16-A1694); The Danish Ministry of Health (903516); Danish Council for Strategic Research (0603-00280B) and The Capital Region Research Foundation have provided core support for COPSAC. BC is supported by the European Respiratory Society, Fellowship LTRF 2016.

No pharmaceutical company was involved in the study. The funding agencies did not have any role in design and conduct of the study; collection, management, and interpretation of the data; or preparation, review, or approval of the manuscript.

**Conflict of interest statement:** The authors declare no potential, perceived, or real conflict of interest regarding the content of this manuscript.

**Contributions:** The guarantor of the study is HB who has been responsible for the integrity of the work as a whole, from conception and design to conduct of the study and acquisition of data, analysis and interpretation of data and writing of the manuscript. HW wrote the first draft of the manuscript. HW, JS, KB, BC, SB and MR were responsible for data analysis, interpretation and writing the manuscript. SB was responsible for the laboratory mediator assessments. All co-

authors have contributed substantially to the analyses and interpretation of the data, and have provided important intellectual input and approval of the final version of the manuscript.

**Governance:** We are aware of and comply with recognized codes of good research practice, including the Danish Code of Conduct for Research Integrity. We comply with national and international rules on the safety and rights of patients and healthy subjects, including Good Clinical Practice (GCP) as defined in the EU's Directive on Good Clinical Practice, the International Conference on Harmonisation's (ICH) good clinical practice guidelines and the Helsinki Declaration. We follow national and international rules on the processing of personal data, including the Danish Act on Processing of Personal Data and the practice of the Danish Data Inspectorate.

**Current submission and prior presentations disclosure:** This manuscript has not been previously presented or published and is not under consideration in the same or substantially similar form in any other peer-reviewed media.

**Key-words:** Cytokines; chemokines; children; mucosal lining fluid; asthma; allergy; cohort

## Abstract (243 words)

**Background:** The immune system is thought to play a key role in the pathogenesis of childhood respiratory disorders, but the role of the airway immune composition in early life prior to symptom development is poorly described.

**Objective:** To examine whether the neonatal airway immune composition associates with later development of airway diseases.

**Methods:** We measured 20 immune-mediators related to the Type 1, Type 2, Type 17, or regulatory (Treg) immune pathways in the airway mucosa of 620 one-month-old healthy neonates from the $COPSAC_{2010}$ birth cohort. Asthma, allergy and lower respiratory tract infections (LRTI) until age 5 years were diagnosed by predefined algorithms and objective assessments. Data-driven principal component analyses were utilized to determine airway immune profiles, which were subsequently associated with development of clinical endpoints by logistic- and cox-regression models.

**Results:** A specific neonatal airway immune profile characterized by down-regulated TGF-β1 (Treg type) and CCL22 (Type 2) was associated with later development of elevated specific IgE (p=0.03), total IgE (p=0.005) and allergic rhinitis (p=0.04). Another profile with reduced IL-1β and elevated CCL2 was evident for children developing asthma (p=0.02). Reduced Type 1 and 17 immune mediators (TNF-α, IL-1β and CXCL8) characterized neonates, who later developed wheeze exacerbations (p=0.03) and LRTI (p=0.01).

**Conclusions:** Distinct airway immune profiles of healthy neonates predated development of allergy and asthma-related traits in childhood. This suggests a chronic inflammatory component

of these disorders that originate in pre- or perinatal life and might be a target for prevention of disease.

## Introduction

The immune mediators found in the airway mucosal lining, makes up the first line of defense against airway exposures such as microbes that need clearing by the immune system. The airways also represent the target organ of inhalant allergies and asthma, which are among the most common chronic childhood disorders[1,2], pointing to the airway immune profile as a possible marker of disease already in childhood.

Childhood asthma and allergy-related disorders are thought to arise in a complex interplay between the genetic make-up of the child and early pre- and postnatal environmental encounters, resulting in a disease-promoting skewing of the immune response that lead to development of chronic airway inflammation. The immune system especially matures in early life[3] and exposures in this period seem crucial for the trajectory towards respiratory health or disease[4]. Studies of stimulated and unstimulated cord blood have shown that a specific blood immune profile is evident from birth in children who later develop allergies[5,6] and wheezy disorderes[7]. However, the airways represent the target organ of inhalant allergies and asthma and the role of the neonatal airway immune system in the pathogenesis of these disorders remains to be elucidated.

We have previously shown that asymptomatic presence of pathogenic airway bacteria[8] and viruses[9] in healthy neonates alters their airway immune profiles. Furthermore, asthma and allergy risk factors, including older siblings[10] and maternal asthma and allergy[11] also have an immune-modulatory effect on the neonatal airway immune response, suggesting an *in utero* immune priming of the fetus. The objective of this study was to examine whether the neonatal

airway immune composition is associated with the development of asthma and allergy-related diseases later in childhood. To explore this, we determined levels of 20 key immune mediators after noninvasive collection of airway mucosal lining fluid in one-month-old neonates from the unselected Copenhagen Prospective Studies on Asthma in Childhood$_{2010}$ (COPSAC$_{2010}$) birth cohort[8,12], and studied the association between the airway immune composition and development of allergy and asthma-related traits through age 5 years.

## Methods

### Section 1.01 The $COPSAC_{2010}$ birth cohort

$COPSAC_{2010}$ is an ongoing population-based clinical prospective mother-child cohort with 700 children enrolled at one week of age during 2009-2010, which has previously been described in details[13]. All clinical assessments of the cohort were performed at the COPSAC research unit according to standard operating procedures, including sampling of airway mucosal lining fluid[13]. A randomized clinical trial of vitamin D supplementation during pregnancy (2,800 vs. 400 IU/day) was embedded in the $COPSAC_{2010}$ study and has been found to influence the airway immune profile[14].

### Section 1.02 Ethics

The study was conducted in accordance with the guiding principles of the Declaration of Helsinki and approved by the Ethics Committee for Copenhagen (H-B-2008-093) and the Danish Data Protection Agency (j.nr. 2015-41-3696). Prior to enrollment of the child, both parents gave their oral and written informed consent.

### Section 1.03 Measurements of airway cytokines and chemokines

Unstimulated airway mucosal lining fluid was sampled when the neonates visited the COPSAC clinic at one month of age as previously described[8,12]. Strips of filter paper (Accuwik Ultra, fibrous hydroxylatedpolyester sheets, cat no.SPR0730, Pall Life Sciences, Portsmouth, Hampshire, UK) were inserted bilaterally into the anterior part of the inferior turbinate of the nasal cavity. After 2 minutes of absorption, the filter papers were removed and immediately frozen at -80°C.

The samples were analyzed for IL (interleukin)-12p70, CXCL10 (IP-10), Interferon-gamma (IFN-γ), Tumor necrosis factor-alpha (TNF-α), CCL4 (MIP-1β), CCL2 (MCP-1), CCL13 (MCP-4), IL-4, IL-5, IL-13, CCL11 (eotaxin-1), CCL26 (eotaxin-3), CCL17 (TARC), CCL22 (MDC), IL-17A, IL-1β, CXCL8 (IL-8), Transforming growth factor beta1 (TGF-β1), IL-10, and IL-2[11,12] by MesoScale Discovery multiplexed array system (MesoScale Discovery, Gaithersburg, Md), which is a high-sensitivity electrochemoluminescence-based ELISA-type assay. The sensitivities for all cytokines were ≤1 pg/mL and for chemokines 1 to 50 pg/mL[12].

Selection of the panel of cytokines and chemokines was decided a priori to represent mediators associated with different types of immune responses: Type 1 (Th1/CD8+/NK cells/innate lymphoid cells (ILC) 1), Type 2 (Th2, eosinophils, ILC2), Type 17 (Th17, neutrophils, ILC3), and regulatory type (Treg) responses[12,15,16], based on the present understanding of which cell types that mainly produce the given mediators and/or are affected by the mediators.

*End-points:*

*Allergic sensitization* was determined at 6 and 18 months of age as any skin prick test (SPT) larger than 2 mm (ALK-Abello, Horsholm, Denmark) or specific IgE (sIgE)≥0.35 kUa/L against milk, egg, dog or cat (ImmunoCAP; Thermo Fischer Scientific, Allerod, Denmark)[13]. Children classified as "non-sensitized" where both SPT and specific IgE negative for all tested allergens.

*Total IgE* level was determined at age 6 months using the ImmunoCAP assay (Thermo Fisher Scientific, Uppsala, Sweden)[17].

*Allergic rhinoconjunctivitis (0-5 years of age)* was diagnosed by the COPSAC pediatrician based on significant symptoms during the previous year of 1) sneezing or a runny or blocked nose and/or 2) red, swollen or watery eyes in periods when the child did not have a cold or flu[12,18,19].

*Asthma/persistent wheeze (0-5 years of age)* was diagnosed based on a previously detailed diary-verified quantitative symptom algorithm[20,21].

*Wheeze exacerbations* were defined from either: 1) treatment with inhaled β2-agonist in a pediatric admission ward or during hospitalization, or 2) treatment with oral or high-dose inhaled corticosteroid prescribed by a general practitioner or the COPSAC clinical research unit. *Lower respiratory tract infections (LRTI)* were pneumonia or bronchiolitis from age 0 to 3 years. Pneumonia was defined by troublesome cough accompanied by tachypnea, fever, and abnormal auscultation, whereas bronchiolitis was defined as cough, tachypnea, chest retractions, and auscultative widespread crepitation and/or rhonchi in a child below 1 year of age[22–24].

For more details on the end-points, see the **online supplement**.

*Covariates*

"Maternal asthma, allergy, or eczema" (Yes/No), "Vitamin D intervention" (vitamin D intervention/placebo), "Siblings" (children in the home at birth, Yes/No), "Pathogenic microbes" (*Haemophilus influenzae*, *Streptococcus pneumonia*, *Moraxella catarrhalis*, *Staphylococcus aureus* and/or Picornavirus at one month of age: Yes/No)[8,9,25] and technical "Batch of mucosal lining fluid analysis" (first/second batch)".

*Statistics*

Quality control, preprocessing and normalization of the individual mediator levels are described in the **online supplement**.

We analyzed the airway immune composition by applying a principal component analysis (PCA) to decompose the signal from the 20 mediators into a few independent components that capture the overall immunological trends in the data. These independent principle components (PCs) were used as data-driven surrogate representations of the neonatal airway immune profile.

Subsequently, the association between the neonatal airway immune profile represented in PC1-4 and each of the clinical endpoints asthma/persistent wheeze, wheeze exacerbations, LRTI and allergic rhinoconjunctivitis were analyzed using cox proportional hazard regression of time to event. The associations between PC1-4 and the dichotomized endpoints specific IgE and skin prick test were analyzed by logistic regression. Total IgE was modeled as a continuous outcome as well as a dichotomized outcome using the median as the cut-off.

Based on our previous studies, siblings, airway pathogens, maternal asthma, allergy or eczema, and the vitamin D intervention, were included as covariates in the models as they all associate with the levels of immune mediators[8–11].

Analyses were carried out with R version 3.2.4, using the packages “survival” for cox proportional hazard models and “gglot2” for visualization of data and results.

## Results

*Baseline characteristics*

A total of 620 (89%) of the 700 neonates in $COPSAC_{2010}$ were included in the current study, excluding 10 neonates that were enrolled before the method of airway mucosal lining fluid was established, as well as 19 that did not attend the one month visit. Additionally, 47 samples were excluded because they were extracted and measured in another laboratory used in a pilot study, and 4 samples were lost in transportation (See study flow chart **Online Figure E1**).

Baseline characteristics of the excluded children without any airway immune data and included children were similar except for a lower gestational age in the excluded vs. included children (11% vs 3% with a gestational age below 37 weeks, p=0.001) (**Online Table E1**).

We limited our immune mediator PCA to the first four PCs, which explained a total of 55.9% of the variation in immune mediator data: PC1 explained 25.8%, PC2 11.5%, PC3 10.8% and PC4 7.8% of the variation.

*Neonatal airway immune composition and allergy endpoints*

*Specific IgE:* Elevated sIgE at 6 and/or 18 months were associated with the neonatal airway immune profile represented by PC1: odds ratio (OR) 1.44 ((95% CI 1.03–2.01), p=0.03. Adjusting the model for presence of pathogenic airway microbes, siblings, maternal asthma, allergy or eczema and the vitamin D RCT did not alter this association: adjusted OR (aOR) 1.77 (1.14–2.76), p=0.01 (**Table 1**).

By visual inspection of the PCA biplot (**Figure 2A**) we identified that the profile related to elevated sIgE was characterized by decreased TGF-β1, CCL2, CCL11, CCL13, CCL17, CCL22 and

CCL26. For TGF-β1, CCL22 and CCL26 this was supported by univariate analyses, where significantly lower levels were determined (**Table E2A**).

*Total IgE:* Elevated total IgE (>median value of 4.73 kU/L) was associated with the airway immune profile represented by PC1: OR 1.26 (95% CI 1.07–1.49), p=0.005; aOR 1.36 (1.12–1.87), p=0.002 (**Table 1**). The immune profile was characterized by significantly reduced levels of the key Treg-related mediator TGF-β1, the chemokines CCL2 and CCL17, as well as elevated levels of IL-1β and IL-17A (**Table E2A**)**.** Analyzing total IgE as a continuous variable yielded similar results as seen for the dichotomized data (results not shown).

*Allergic rhinoconjunctivitis*: The immune profile represented by PC1 was also associated with clinical symptoms of allergic rhinoconjunctivitis until age 5 years: hazard ratio (HR) 1.42 (1.01–1.99), p=0.04; aHR 1.71 (1.12 – 2.61), p= 0.01 (**Table 1** and **Figure 2B**). The univariate analyses are outlined in **Table E2B**.

*Neonatal airway immune composition and asthma/wheeze endpoints*

*Asthma/persistent wheeze:* The immune profile represented by PC4 was associated with development of asthma/persistent wheeze until age 5 years: HR 1.21 (1.03–1.45), p=0.02 (**Table 1**). This difference was mainly driven by reduced IL-17A and IL-1β and enhanced CCL2 (**Figure 2**) of which the two latter were significant in the univariate analyses (**Table E2C**). This association did not reach statistical significance in the adjusted model, but the effect estimate was largely unchanged: aHR 1.15 (0.96–1.38), p=0.12 (**Table 1**).

*Wheeze exacerbations:* The airway immune profile in PC2 was associated with subsequent wheeze exacerbations: HR 0.73 (0.55–0.96), p=0.03 and aHR 0.75 (0.58–1.00), p=0.05,

respectively (**Table 1**). The PCA biplot (**Figure 3**) showed that the difference was mainly driven by reduced levels of TNF-α, CXCL8, IL-1β, IL-17A and CCL4 (univariate analyses in **Table E2C**).

*LRTI:* The immune profile in PC2, which associated with wheeze exacerbations was also associated with development of LRTI: HR 0.87, 95% CI 0.78–0.97, p=0.01, and aHR 0.81 (0.68–0.96), p=0.01, respectively (**Table 1**).

## Discussion

*Principle findings*

We explored the role of neonatal airway immune profiles in the pathogenesis of childhood allergy and asthma. Children developing elevated specific IgE, total IgE and allergic rhinoconjunctivitis had a distinct airway immune profile as neonates. This was characterized by reduced CCL22, which mainly recruits Treg and Th2 cells, and the immune suppressive mediator TGF-β1 connected to Treg expansion. Conversely, children developing asthma had a profile with reduced IL-1β, involved in Th17 expansion, and enhanced CCL2, promoting Type 1 and 2 responses. Wheeze exacerbations were associated with reduced TNF-α, IL-1β and CXCL8, representing a profile characterized by reduced immune activation and neutrophilic recruitment. These findings suggest that the composition of the neonatal airway immune response is instrumental for allergy and asthma pathogenesis and that the trajectory towards respiratory diseases is evident already in pre- and perinatal life.

*Strengths and limitations*

It is a strength of the study that the immune response in the airway mucosal lining fluid was assessed undisturbed *in situ* by a validated method of collecting and measuring cytokines and chemokines[12]. The sampling was performed in the upper airways, which is a major advantage compared to measurements of systemic mediators in blood, since the airways represent the target organ of allergic rhinoconjunctivitis and asthma as well as first line of defense against airway exposures.

A priori we decided on a panel of 20 cytokines and chemokines to represent major phenotypical pathways of the immune system; representing both innate and adaptive

mediators involving activation of Type 1, Type 2, Type 17 and regulatory type responses, providing a representative view of mediators produced by the different airway epithelial and immune cells.

We performed a total mediator normalization approach of the cytokine and chemokine mediator levels (sample centering) in order to diminish the individual differences in fluidic excretion dynamics. Thereafter, a multivariate PCA model was applied to the immune mediator data, as the mediators are highly correlated. Such model can be used for unravelling the cytokine to cytokine covariance structure. Furthermore, the multivariate model circumvents the issue of multiple testing raised by the high number of mediators.

The clinical endpoints were established from daily diary cards and acute visits to the research unit at the onset of respiratory or allergic symptoms. This approach provided a much more comprehensive and precise information about the outcomes as compared to questionnaire-based diagnostics. It is also a strength that the objective assessments of specific and total IgE were done repetitively during childhood.

The major limitation of this study is the observational nature. Thus, we cannot establish causality between the neonatal airway immune composition and respiratory health and disease, although such connection is biologically plausible.

*Interpretation*

This study is the first to show that distinct unstimulated airway immune profiles in healthy neonates precede development of allergy- and asthma-related diseases later in childhood. Such findings provide important insight into the pathogenesis of these common respiratory disorders.

Children developing elevated specific and total IgE were characterized by a neonatal airway immune profile dominated by down-regulation of the key Treg related mediator TGF-β1. In healthy individuals, TGF-β1 down-regulates the transcription factors T-bet and GATA-3[26], resulting in a reduced production of Th1 and Th2 related cells from naïve CD4+ T-cells. Thus, reduction of the Treg mediator TGF-β1 will lead to an up-regulation of Type 2 cells, compatible with findings in patients with allergic rhinitis[12,29], and a recent cord blood stimulation study showing that children developing allergic sensitization by age 12 months had a deficient cord blood Treg response[30]. Further supporting the role of Treg responses in the pathogenesis of childhood allergy, we found that neonates developing elevated specific IgE and rhinoconjunctivitis till age 5 years also had reduced levels of CCL22, which is a chemokine mainly attracting Treg cells to the mucosa.

The airway immune profile of children developing elevated specific IgE and rhinoconjunctivitis also included down-regulated levels of the Type 2 immune chemoattractants CCL13 and CCL26, indicative of less recruitment of Type 2 cells to the mucosa. Type 2 cells such as eosinophils, basophils, mast cells and Th2 cells, are considered very important for allergy development and an early skewing towards an overweight of blood-derived Type 2 cells have been associated with an increased risk of allergy[27]. Our data supports the notion of such phenomenon, as less recruitment of Type 2 cells to the developing airway immune system may result in higher blood levels of Type 2 immune cells[28].

Children developing a positive SPT were also characterized by reduced airway CCL22 and CCL26 levels as neonates, but did not show down-regulated TGF-β1, as seen for children with elevated specific IgE. This implies slightly different basic mechanisms of elevated blood specific IgE and

skin reactivity towards allergens, which is in accordance with our previous report showing a poor overlap between a positive SPT and elevated specific IgE in young children[31].

In contrast to the down-regulated Treg and altered Type 2 airway immune profile evident for children developing allergy-related conditions, we observed another specific immune profile in neonates, who developed asthma and experienced wheeze exacerbations and LRTI later in childhood. These immune profiles were characterized by a common down-regulation of IL-1β, which is involved in immune activation and neutrophilic recruitment via Th17 expansion[32]. Also, down-regulated TNF-α and CXCL8 were associated with development of wheeze exacerbations. These mediators are an important part of fighting off respiratory bacterial infections via neutrophilic recruitment and activation[33], and their potential involvement in asthma pathogenesis fits well with studies showing a role of early bacterial airway ecology in the origins of asthma[34].

Along with down-regulated IL-1β, asthma development was also associated with upregulated CCL2, which is involved in recruitment of CCR1- and CCR2-expressing immune cells, including basophils, Th2, Th1, CD8+ T cells, monocytes and immature dendritic cells[35]. The dissimilarities between the immune profiles associated with allergy vs. asthma/wheeze outcomes concur with the fact the most asthma/wheeze during preschool age is non-atopic[36].

We have previously shown that pre- and perinatal exposures such as vitamin D supplementation, presence of siblings, pathogenic airway bacteria and viruses, and maternal atopy are imprinted in the neonatal airway immune profile[8–11,14]. The current study shows that the composition of the early life airway immune profile is also associated with clinical symptoms of allergy and asthma later in childhood with plausible altered immune profiles suggestive of a pathophysiological role in the inception of disease. These findings further

support the hypothesis that childhood asthma and allergy origin in susceptible individuals due to early life environmental exposures, initiating early immune perturbation and a trajectory towards chronic airway inflammation[37], thereby suggesting pre- and perinatal life as an important window of opportunity for prevention of disease.

## Conclusion

We found that distinct airway immune profiles in healthy neonates precede development of allergy- and asthma-related traits in childhood. These findings underline that the chronic airway inflammation characterizing these disorders has its origin already in pre- or perinatal life.

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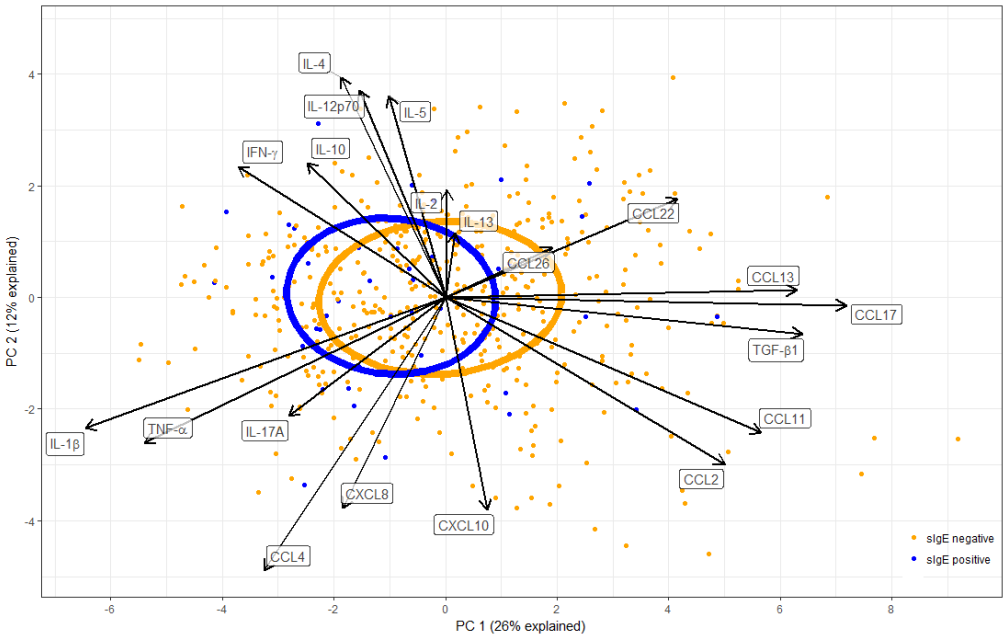
**Table 1:** Associations between clinical parameters and airway immune profiles, analyzed by principal component analyses (PCA). Data are shown as odds ratios and hazard ratios (95% CI) of principal component (PC) 1-4 for every clinical endpoint. Analyses are shown as crude and adjusted for pathogenic airway microbes, siblings, maternal asthma, allergy or eczema and the vitamin D randomized controlled trial. Total IgE, specific IgE and skin prick test are analyzed using a logistic regression model. Allergic rhinitis, asthma, LRTI and exacerbations are analyzed by cox proportional hazard regression of time-to-event. P < 0.05 is shown in bold.

| End-point | PC1 | PC1 adjusted | PC2 | PC2 adjusted | PC3 | PC3 Adjusted | PC4 | PC4 adjusted |
|---|---|---|---|---|---|---|---|---|
| Specific IgE | **1.44 (1.03 – 2.01) p = 0.03** | **1.77 (1.14 – 2.76) p = 0.01** | 0.98 (0.79 – 1.22) p = 0.87 | 0.99 (0.77 – 1.26) p = 0.93 | 0.86 (0.70 – 1.05) p = 0.13 | 0.92 (0.73 – 1.17) p = 0.51 | 1.18 (0.91 – 1.52) p = 0.21 | 1.12 (0.83 – 1.51) p = 0.45 |
| Total IgE | **1.26 (1.07 – 1.49) p = 0.005*** | **1.36 (1.12 – 1.87) p = 0.002** | 0.97 (0.87 – 1.07) p = 0.52 | 0.97 (0.86 – 1.08) p = 0.57 | 0.97 (0.87 – 1.08) p = 0.55 | 0.97 (0.86 – 1.09) p = 0.57 | 0.95 (0.83 – 1.08) p = 0.40 | 0.99 (0.86– 1.14) p = 0.87 |
| Allergic rhinitis | **1.42 (1.01 – 1.99) p = 0.04** | **1.71 (1.12 – 2.61) p = 0.01** | 0.92 (0.74 – 1.13) p = 0.41 | 0.96 (0.76 – 1.21) p = 0.71 | 0.88 (0.72 – 1.07) p = 0.19 | 0.93 (0.73 – 1.18) p = 0.54 | 1.11 (0.87 – 1.43) p = 0.40 | 1.02 (0.77 – 1.35) p = 0.89 |
| Skin prick test | 0.96 (0.83 – 1.13) p = 0.65 | 0.94 (0.78 – 1.13) p = 0.51 | 0.96 (0.75 – 1.21) p = 0.71 | 0.87 (0.67 – 1.13) p = 0.30 | **0.63 (0.46 – 0.87) p = 0.005** | **0.57 (0.40 – 0.82) p = 0.002** | 1.08 (0.81 – 1.45) p = 0.60 | 1.00 (0.72 – 1.37) p = 0.99 |
| Asthma | 0.98 (0.90 – 1.05) p = 0.52 | 0.99 (0.90 – 1.08) p = 0.80 | 0.97 (0.86 – 1.09) p =0.61 | 0.98 (0.87 – 1.11) p =0.75 | 0.95 (0.84 – 1.07) p = 0.40 | 0.96 (0.85 – 1.08) p = 0.49 | **1.21 (1.03 – 1.45) p = 0.02** | 1.15 (0.96 – 1.38) p = 0.12 |
| Wheeze Exacerbation | 0.94 (0.84 – 1.06) p = 0.34 | 0.94 (0.82 – 1.08) p = 0.39 | **0.73 (0.55 – 0.96) p = 0.03** | 0.75 (0.58 – 1.00) p = 0.05 | 1.05 (0.87 – 1.28) p = 0.60 | 1.05 (0.86 – 1.29) p = 0.61 | 1.11 (0.89 – 1.38) p = 0.36 | 1.09 (0.87 – 1.37) p = 0.45 |
| LRTI | 1.06 (0.98 – 1.14) p = 0.12 | 1.05 (0.97 – 1.15) p = 0.19 | **0.81 (0.68 – 0.96) p = 0.02** | **0.79 (0.66 – 0.96) p = 0.01** | 0.99 (0.89 – 1.10) p = 0.88 | 0.96 (0.85 – 1.08) p = 0.48 | 1.08 (0.95 – 1.23) p = 0.24 | 1.09 (0.95 – 1.25) p = 0.22 |

*P = 0.001 as continuous variable in a linear model

**Figure 1:** Neonatal airway immune profiles of healthy and later allergic neonates. The biplots show the principal components analysis of the distribution of children with A) positive specific IgE versus the children with levels below the threshold for specific IgE and a negative SPT, and B) children with allergic rhinitis versus controls; shown as ellipses. Differences are registered between the blue circle (neonates with a positive specific IgE/allergic rhinitis) and the red circle (neonates below the threshold for specific IgE and negative SPT/healthy controls). In A) a separation was found in PC1 between neonates with a positive specific IgE vs. controls (p = 0.005) and in B) a separation was found in PC1 between children with allergic rhinitis vs. controls (p = 0.03).

**A)**




**B)**

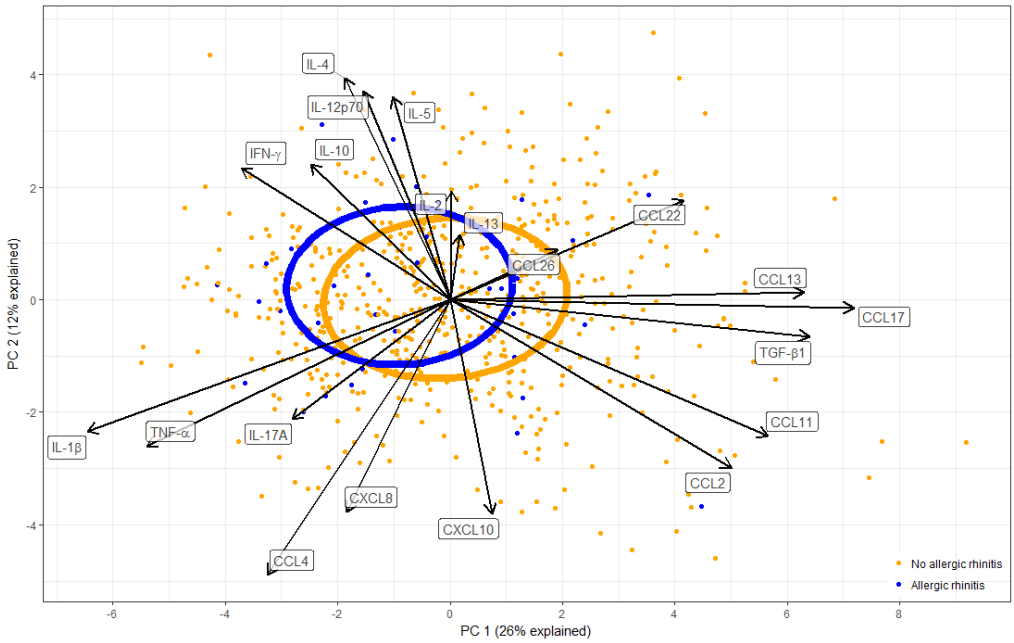


**Figure 2:** Neonatal airway immune profiles of healthy and later asthmatic neonates. The biplot (PC3 vs. PC4) shows the principal components analysis of the distribution of children with asthma versus controls (p = 0.03).

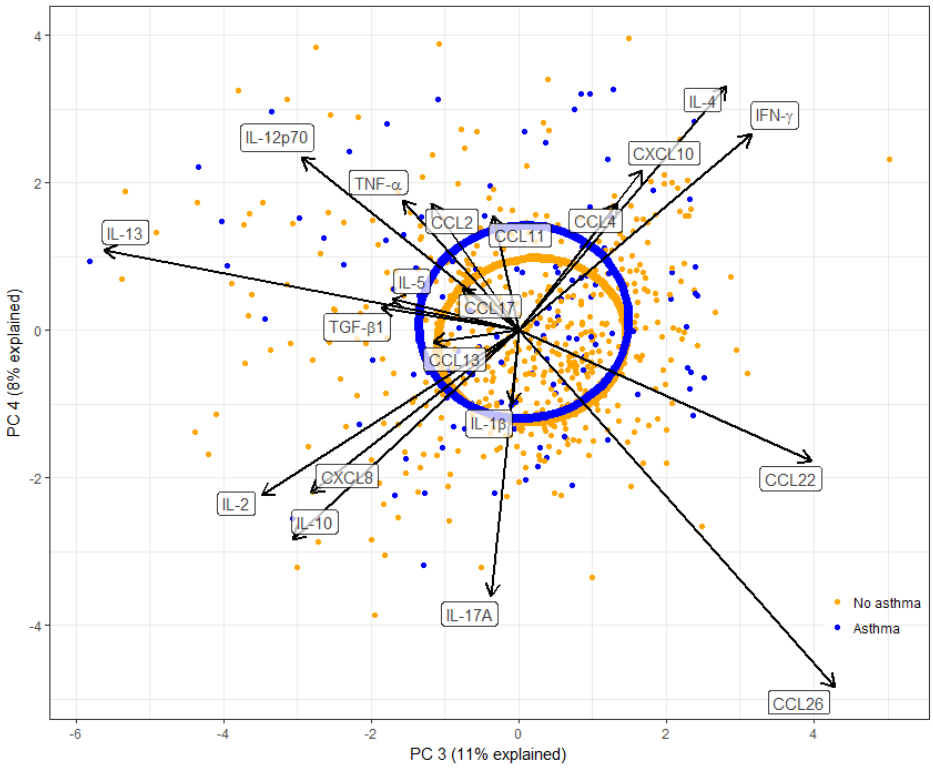


**Figure 3:** Neonatal airway immune profiles of healthy vs neonates with later development of one or more asthma exacerbations from 0-5 years of age. The biplot shows the principal components analysis of the distribution of children with asthma exacerbations (blue circle) versus controls (red circle), showing a significant separation in PC2 between children with asthma exacerbations vs. controls (P = 0.03)

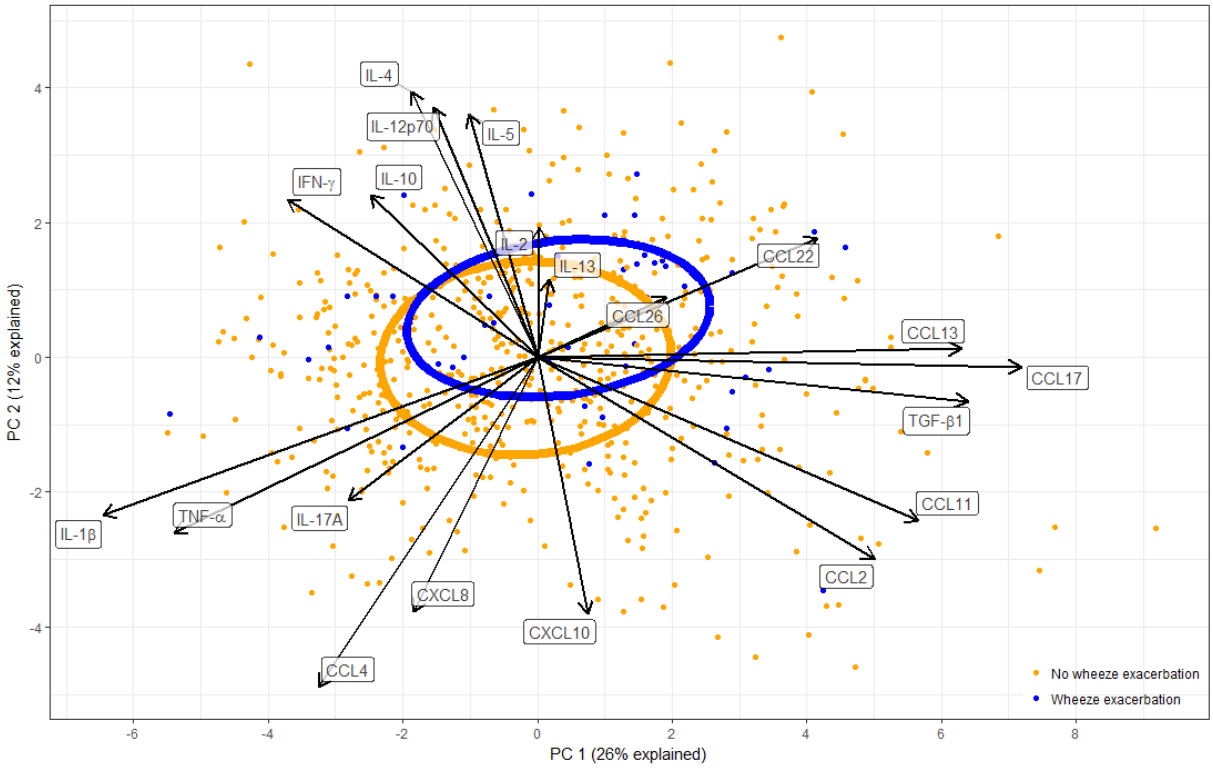


**Table E1:** Drop-out table of the 620 included vs 80 excluded

| | Missing | Excluded (%) | Excluded (N) | Included (%) | Included (N) | p-value |
|---|---|---|---|---|---|---|
| Maternal atopy | 4 | 53 | 42 | 46 | 282 | 0,31 |
| Caucasian | 0 | 3 | 2 | 5 | 29 | 0,55 |
| Caesarian section | 0 | 21 | 17 | 22 | 134 | 1,00 |
| Male | 0 | 49 | 39 | 49 | 301 | 1,00 |
| Maternal antibiotics in 3rd trimester | 1 | 18 | 14 | 18 | 109 | 1,00 |
| Siblings at birth | 0 | 46 | 37 | 58 | 358 | 0,07 |
| Cat and/or dog at birth | 3 | 70 | 56 | 65 | 401 | 0,45 |
| Breast feeding at 1 month | 9 | 5 | 4 | 7 | 46 | 0,66 |
| Maternal smoking in 3rd trimester | 0 | 98 | 78 | 96 | 597 | 0,82 |
| Maternal alcohol consumption in 3rd trim. | 2 | 91 | 73 | 96 | 591 | 0,15 |
| Social circumstances (high) | 0 | 46 | 37 | 50 | 312 | 0,57 |
| Gestational age (< 37w) | 0 | 89 | 71 | 97 | 601 | 0,00 |
| Apgar score | 0 | 95 | 76 | 95 | 591 | 1,00 |

**Table E2: Unadjusted geometric mean ratios (GMR) of A) specific IgE and allergic rhinitis, B) Total IgE and skin prick test and C) Asthma and asthma exacerbations.**

**A)**

| Cytokine | **sIgE** Estimates | CI low | CI high | p-value | **Total IgE estimates*** | **CI low** | **CI high** | **p-value** |
|---|---|---|---|---|---|---|---|---|
| IL-12p70 | 1.35 | 0.92 | 1.96 | 0.12 | 1.02 | 0.88 | 1.18 | 0.79 |
| CXCL10 | 1.08 | 0.80 | 1.46 | 0.60 | 1.01 | 0.86 | 1.17 | 0.94 |
| IFN-ϒ | 1.31 | 0.90 | 1.93 | 0.16 | 1.09 | 0.94 | 1.27 | 0.25 |
| TNF-α | 1.29 | 0.89 | 1.87 | 0.17 | 1.16 | 0.95 | 1.41 | 0.14 |
| CCL4 | 1.14 | 0.82 | 1.59 | 0.42 | 0.95 | 0.80 | 1.12 | 0.52 |
| CCL2 | 0.92 | 0.62 | 1.36 | 0.67 | ***0.79*** | ***0.65*** | ***0.97*** | ***0.02*** |
| CCL13 | 0.68 | 0.42 | 1.08 | 0.10 | 0.81 | 0.64 | 1.02 | 0.08 |
| IL-4 | 1.03 | 0.81 | 1.31 | 0.80 | 1.02 | 0.92 | 1.14 | 0.71 |
| IL-5 | 1.13 | 0.77 | 1.65 | 0.53 | 1.17 | 0.97 | 1.41 | 0.11 |
| IL-13 | 1.31 | 0.85 | 2.03 | 0.22 | 1.05 | 0.84 | 1.32 | 0.65 |
| CCL11 | 0.74 | 0.47 | 1.17 | 0.20 | 0.77 | 0.61 | 0.97 | 0.03 |
| CCL26 | ***0.77*** | ***0.63*** | ***0.94*** | ***0.01*** | 0.95 | 0.84 | 1.06 | 0.35 |
| CCL17 | 0.75 | 0.54 | 1.04 | 0.09 | ***0.82*** | ***0.68*** | ***0.98*** | ***0.03*** |
| CCL22 | ***0.75*** | ***0.57*** | ***0.98*** | ***0.03*** | 0.90 | 0.77 | 1.05 | 0.16 |
| IL-1β | 1.08 | 0.83 | 1.41 | 0.56 | ***1.18*** | ***1.03*** | ***1.35*** | ***0.02*** |
| IL-17A | 1.18 | 0.93 | 1.50 | 0.18 | ***1.13*** | ***1.00*** | ***1.28*** | ***0.05*** |
| CXCL8 | 1.09 | 0.59 | 2.01 | 0.78 | 1.18 | 0.87 | 1.60 | 0.29 |
| TGF-β1 | ***0.58*** | ***0.36*** | ***0.92*** | ***0.02*** | ***0.73*** | ***0.58*** | ***0.91*** | ***0.01*** |
| IL-10 | 1.23 | 0.86 | 1.76 | 0.25 | 1.04 | 0.86 | 1.25 | 0.67 |
| IL-2 | 1.21 | 0.78 | 1.88 | 0.39 | 1.17 | 0.93 | 1.47 | 0.17 |

**B)**

| **Cytokine** | **Allergic rhinitis** Estimate | Cilow | Cihigh | Pvalue | **SPT Estimate** | **CIlow** | **Cihigh** | **pvalue** |
|---|---|---|---|---|---|---|---|---|
| IL-12p70 | 1.20 | 0.84 | 1.72 | 0.32 | 1.16 | 0.80 | 1.68 | 0.44 |
| CXCL10 | 0.93 | 0.68 | 1.27 | 0.63 | 0.88 | 0.62 | 1.25 | 0.48 |
| IFN-ϒ | 1.13 | 0.80 | 1.59 | 0.50 | 0.85 | 0.64 | 1.12 | 0.23 |
| TNF-α | 1.29 | 0.88 | 1.90 | 0.19 | 1.12 | 0.73 | 1.71 | 0.60 |
| CCL4 | 1.05 | 0.75 | 1.48 | 0.77 | 0.76 | 0.50 | 1.14 | 0.18 |
| CCL2 | 0.75 | 0.50 | 1.14 | 0.18 | 1.24 | 0.81 | 1.91 | 0.33 |
| CCL13 | ***0.48*** | ***0.29*** | ***0.79*** | ***0.00*** | 1.31 | 0.79 | 2.18 | 0.30 |
| IL-4 | 1.14 | 0.89 | 1.46 | 0.31 | 0.96 | 0.76 | 1.21 | 0.71 |
| IL-5 | 1.29 | 0.88 | 1.89 | 0.19 | 1.28 | 0.84 | 1.96 | 0.25 |
| IL-13 | 1.24 | 0.77 | 1.99 | 0.37 | 1.56 | 0.93 | 2.59 | 0.09 |
| CCL11 | 0.83 | 0.53 | 1.28 | 0.39 | 1.06 | 0.64 | 1.75 | 0.83 |
| CCL26 | 0.82 | 0.67 | 1.01 | 0.06 | 0.77 | 0.61 | 0.96 | **0.02** |
| CCL17 | 0.84 | 0.59 | 1.19 | 0.32 | 1.24 | 0.83 | 1.85 | 0.30 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| CCL22 | ***0.73*** | ***0.56*** | ***0.96*** | ***0.02*** | 0.84 | 0.62 | 1.14 | 0.25 |
| IL-1β | 1.24 | 0.94 | 1.64 | 0.12 | 0.99 | 0.74 | 1.34 | 0.96 |
| IL-17A | 1.11 | 0.87 | 1.43 | 0.39 | 1.01 | 0.78 | 1.32 | 0.92 |
| CXCL8 | 0.93 | 0.51 | 1.71 | 0.82 | 1.15 | 0.58 | 2.28 | 0.69 |
| TGF-β1 | 0.86 | 0.55 | 1.34 | 0.50 | 1.38 | 0.85 | 2.23 | 0.20 |
| IL-10 | 1.13 | 0.77 | 1.65 | 0.54 | 1.18 | 0.79 | 1.78 | 0.41 |
| IL-2 | 1.43 | 0.93 | 2.21 | 0.10 | 1.36 | 0.82 | 2.26 | 0.23 |

**As binary variable (above median (4.69))*

**C)**

| Cytokine | Asthma estimate | CIlow | CIhigh | pvalue | Exacerbation estimate | CIlow | Cihigh | pvalue |
|---|---|---|---|---|---|---|---|---|
| IL-12p70 | 1.15 | 0.94 | 1.40 | 0.18 | 1.16 | 0.85 | 1.58 | 0.35 |
| CXCL10 | 0.98 | 0.82 | 1.18 | 0.87 | 1.11 | 0.85 | 1.46 | 0.45 |
| IFN-ϒ | 1.09 | 0.90 | 1.33 | 0.38 | 1.07 | 0.80 | 1.44 | 0.64 |
| TNF-α | 0.92 | 0.72 | 1.16 | 0.47 | ***0.65*** | ***0.45*** | ***0.93*** | ***0.02*** |
| CCL4 | 1.03 | 0.84 | 1.26 | 0.78 | 0.76 | 0.55 | 1.05 | 0.10 |
| CCL2 | ***1.28*** | ***1.00*** | ***1.63*** | ***0.05*** | 1.17 | 0.81 | 1.67 | 0.40 |
| CCL13 | 1.07 | 0.81 | 1.43 | 0.63 | 0.97 | 0.64 | 1.49 | 0.90 |
| IL-4 | 1.04 | 0.91 | 1.19 | 0.54 | 1.21 | 0.95 | 1.53 | 0.12 |
| IL-5 | 1.04 | 0.83 | 1.31 | 0.72 | 1.12 | 0.80 | 1.58 | 0.51 |
| IL-13 | 1.02 | 0.77 | 1.34 | 0.90 | 0.90 | 0.60 | 1.36 | 0.62 |
| CCL11 | 1.10 | 0.84 | 1.44 | 0.51 | 1.02 | 0.68 | 1.53 | 0.91 |
| CCL26 | 0.93 | 0.81 | 1.06 | 0.27 | 0.96 | 0.78 | 1.17 | 0.66 |
| CCL17 | 1.14 | 0.92 | 1.42 | 0.24 | 1.07 | 0.78 | 1.48 | 0.67 |
| CCL22 | 0.91 | 0.76 | 1.09 | 0.31 | 1.16 | 0.87 | 1.55 | 0.31 |
| IL-1β | ***0.84*** | ***0.71*** | ***0.99*** | ***0.04*** | ***0.75*** | ***0.58*** | ***0.96*** | ***0.02*** |
| IL-17A | 0.88 | 0.76 | 1.02 | 0.10 | 0.89 | 0.72 | 1.11 | 0.31 |
| CXCL8 | 1.04 | 0.72 | 1.50 | 0.85 | ***0.59*** | ***0.35*** | ***0.98*** | ***0.04*** |
| TGF-β1 | 1.05 | 0.81 | 1.37 | 0.71 | 1.02 | 0.69 | 1.51 | 0.92 |
| IL-10 | 1.00 | 0.80 | 1.26 | 0.99 | 1.15 | 0.81 | 1.62 | 0.43 |
| IL-2 | 0.97 | 0.74 | 1.27 | 0.83 | 1.18 | 0.79 | 1.76 | 0.41 |

**Figure E1**: Flowchart

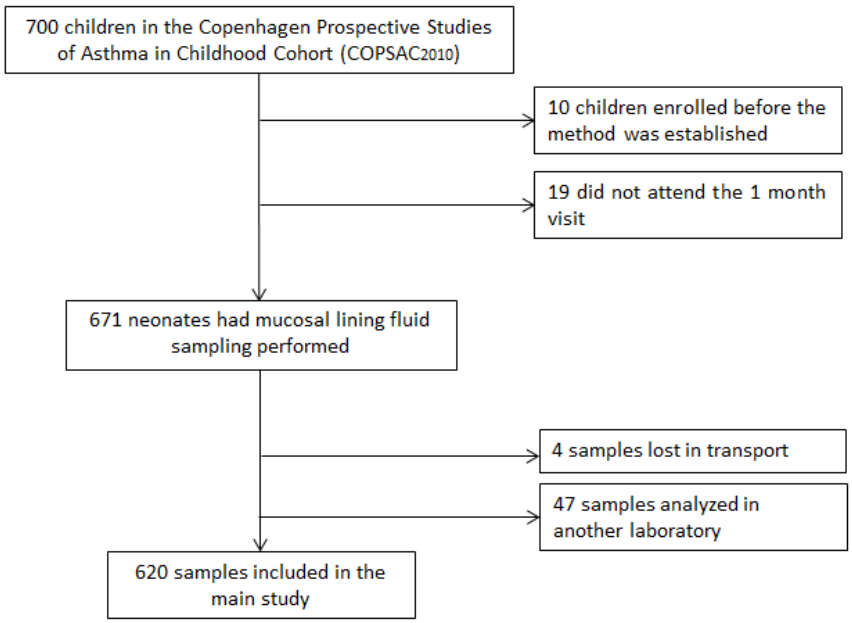


**Figure E2**: Neonatal airway immune profiles of healthy infants with a positive skin prick test (SPT) from 0-5 years of age. The biplot shows the principal components analysis (PC3 vs. PC4) of the distribution of children with a positive SPT (blue circle) versus controls (red circle), showing a significant separation in PC3 between children with a positive SPT vs. controls (P = 0.005).